THE FOLLOWING DOCUMENTS ARE SUBJECT
TO RESTRICTIONS ON INSPECTION AND COPYING
AS SET OUT IN THE ORDER OF
MR JUSTICE OGNALL ON 8TH MAY 1990

FACTOR IX SOLUTION HEATING

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23/1/82.

9D, Sorbitol, glucose partitionisation expt.

except for Naptt, values are % of -40°c control, as intact remaining.

			Fix	F x	F II	Naptt. (Sec) %
(ii) pH 7.07	RT	O/N	103	99	92	90.4
(iii) pH 7.07	60°c	10hrs.	34	52	92	258
(i) pH 7.07	-40°c	Contrl	34·3 i0/ml.	-	-	131
(ii) pH 6.5H	RT	O/N	100	102	101	146
(iii) pH 6.5H	60°c	10hrs.	20	47	92	293
(i) 6.5H	-40°c	Contrl.	-	-	-	168

Jyl

90, Sorbitol, glycine partitioning.

TG150 - as requested.

pH 6.54	10hrs	60°c	30' mins.
pH 7.07	10hrs	60°c	30' mins
pH 6.54	-40°c	Control	22mins.
pH 7.07	-40°c	Control	16 mins.
pH 6.54	RT	o/N	22mins
pH 7.07	RT	o/N	-sample not available

[redacted] 30/11/82

Pasteurisation of factor IX concentrate, 9H3, 13.12.82.

Vials of standard 9D were redissolved at the usual potency and 10 ml aliquots treated with sorbitol and glycine added directly as solids. pH of each mixture was readjusted to 7.05 ± 0.01 with dilute sodium hydroxide. Control subsamples were set aside at -40° and the remainder pasteurised within the range 59-60.5° for 10h.

	Addition to 10 ml 9D			% IX	% II	NAPTT sec.
	Glycine g	Sorbitol g	Final Vol. ≈ ml	recovered	recovered	1/10 dil.
A	1.0	7.0	15	6	44	335
B	1.0	13.0	19	44	66	287
C	1.5	7.0	15	8	52	341
D	1.5	13.0	19	34*	73	259
	0	0	10			213 ∿ 324

N.B. 1. Heating was done in glass universals.
2. Most pH were about 6.65 before readjustment.
3. The unheated control for D had a lower IX potency than expected from the dilution factor. The recovery after heating may, therefore, be overestimated.

Factor IX Pasteurization Experiment No. 3 — 9H3 — 15.2.82.

Method:

Factor IX 9D 2231 ; pyrogenic ; 1600 u/vial No HBV/HIV

Redissolved 2 vials in 35 ml each and pooled

samples saved : 9H3/START

4 x 10 ml samples of dissolved IX treated as follows with added glycine and sorbitol

pH			final volume (ml)
6.68 → 7.04	9H3 1007	: + 1 g glycine + 7 g sorbitol	15 ml.
6.64 → 7.05	9H3 1013	: + 1 g glycine + 13 g sorbitol	19 ml
6.64 → 7.05	9H3 1507	: + 1.5 g glycine + 7 g sorbitol	15 ml
6.62 → 7.04	9H3 1513	: + 1.5 g glycine + 13 g sorbitol	19 ml.
6.21	Dummy (no protein).		

pH adjusted as indicated with dil NaOH 5 x 1ml

These four samples were then each split and frozen at -40° as unheated controls.

The remaining parts were heated in plastic glass universals at 60° for 10 hours

Temperature in a dummy sample was monitored and recorded.

T = 59° at 22.57 (see attached recorder chart), and remained within the range 59-60.5° throughout the 10 hour period. All samples remained clear after heating.

Samples removed at 08.57 and passed on to MEH for assay labelled 9H3/1007H etc. . . .

PH3

	For 10 ml 9 Dss dissolved	FIX w/u	FIX %	FII	Naptt Sec's 1/10	1/100	C
PH3/1					213	281	324
1007	1g glycine 7g sorbitol	26.8		41.4	165		
1007 -H			5.6	18.4 44%	335		
1013	1g glycine 13g sorbitol	20.5		27.9	201		
1013 -H			44.3	18.5 66%	287		
1507	1.5g glycine 7g sorbitol	29.2		28.6	160		
1507 -H			8.3	15.0 52%	341		
1513	1.5g glycine 13g sorbitol	17.4		26.6	220		
1513 -H			34.2	19.3 73%	259		

Materials: 9D 2232 Pyrogenic batch dated @ 100 u/bottle.
BDH sorbitol
glycine ?purity Kirkwood Chemicals ancient stock.

Method: A solution of 2M glycine in 40% (w/w) sorbitol was prepared.
Unadjusted pH = 6.25.
½ of buffer was adjusted to pH 7.02 with dilute NaOH.

Bottle A dissolved in 30ml sorbitol/glycine pH 6.25 and the pH of the dissolved 9D was 6.54
Bottle B dissolved in 30ml sorbitol/glycine pH 7.02. The pH of the dissolved 9D was 6.73 and thus was adjusted up to pH 7.07 with dil. NaOH.
All samples were stored at +2° for 6hrs
Then (i) Control sample was frozen (-40°).
(ii) a further control was left at R.T. o/N
(iii) a sample was heated 10hrs @ 60°.

Both A(iii) and B(iii) were clear after pasteurization.
All samples were passed on to HE for assays (see next page)

9H4 Pasteurisation of factor IX concentrate, 24.1.83.

Effects of pH and container type

Vials of 9D 2231 (1000 iu, no heparin, pyrogenic) were redissolved at the usual potency and 10 ml aliquots treated with sorbitol and glycine added directly as solids. pH of each mixture was readjusted to one of three values between 6.5 and 7.1 using dilute HCl or NaOH. At each pH value, two concentrations of glycine but a constant concentration of sorbitol were used. Controls were placed at -40° and thawed only immediately before assay. Six test solutions in plastic universals were pasteurised at 60° for 10h and stored frozen before assay. Half of one test solution was also pasteurised in a new glass universal, as used for 9H1-3. All solutions were clear after pasteurisation.

Addition of 10 ml 9D 2231			Universal	F. IX % recovery	F.II % recovery	NAPTT (1/10 v. 271 sec. C)
Sorbitol	Glycine	pH				
13	1.0	6.52	Plastic	67	ND	197
13	1.0	6.78	"	73	91	171
13	1.0	7.09	"	52	91	172
13	1.5	6.50	"	57	ND	192
13	1.5	6.81	"	67	94	169
13	1.5	7.10	"	66	85	171
13	1.5	6.81	Glass	43	92	189
Unheated				100	100	246
9D before additions						225

Comments

(1) The most dramatic result was the achievement of recoveries similar to PFC's, and much higher than in 9H3, simply by heating in plastic rather than glass containers. The glass-heated control gave a similar result to 9H3.

(2) 1.5g glycine was not demonstrably superior to 1.0g glycine in preserving factor IX yield.

(3) pH 6.8 appears to be optimal for factor IX recovery, although the differences from pH 6.5 to 7.1 are not significant in terms of the factor IX assay.

(4) Since NAPTT times of heated samples were shorter than the controls (even in glass), the earlier conclusion that activated factors are more rapidly inactivated than factor IX is clearly unsafe. The NAPTT level was not alarming, and it might be further modified e.g. by addition of AT III and heparin.

9H4

REQUEST FOR COAGULATION FACTOR ASSAYS

equest from [illegible] Date 26.1.83

Results needed when? _____

mples to be kept? _____ If so, how? _____

Samples provided with this request/available from _____

2 1.5g glycine / 10.ml 9D pH 6.5 pH 6.8 pH 7.1 GLASS pH 6.8
13 g Sorbitol

SAMPLE	CONTROL 9HH 2C		2AH		2BH		2CH		2DH			
INVESTIGATION	EST.	FOUND	EST.	FOUND	EST.	FOUND	EST.	FOUND	EST.	FOUND	EST.	FOUND
Factor VIII, iu/ml 1 st.												
2 st.												
Factor IX, iu/ml 1 st.												
2 st.		—		56.5%		67%		66%		42.9%		
Factor II, u/ml		21.4				20.1 (94%)		18.1 (85%)		19.6 (91.5%)		
Factor X, u/ml												
Factor VII, u/ml (clotting)												
(amidolytic)												
Factor VIII antigen, u/ml												
AT III (Laurell), u/ml												
AT III (Anti-Xa), u/ml												
NAPTT, s. (1/10, 1/100, c̄)	246		192		169		171		189			
	271											
TGt50, min.												
XaGt, min.												
Φ C.T., h.												
Limulus + or -												

Operator [illegible] Date _____

pH 4

REQUEST FOR COAGULATION FACTOR ASSAYS

. quest from LW Date 26.1.83

Results needed when? _____

ıples to be kept? _____ If so, how? _____

Samples provided with this request/available from _____

1: 10g glycine / 10 ml 2D
13.7 Sorbitol

pH 6.5 pH 6.8 pH 7.1

SAMPLE	Control pH 4 IC		1AH		1BH		1CH				START	
INVESTIGATION	EST.	FOUND	EST.	FOUND	EST.	FOUND	EST.	FOUND	EST.	FOUND	EST.	FOUND
Factor VIII, iu/ml 1 st.												
2 st.												
Factor IX, iu/ml 1 st.												
2 st.		16.9		66.6%		73.4%		52.3%				
Factor II, u/ml		22.2		~~20.2~~ (~~91%~~)		20.2 (91%)		20.1 (90.5%)				
Factor X, u/ml												
Factor VII, u/ml (clotting)												
(amidolytic)												
Factor VIII antigen, u/ml												
AT III (Laurell), u/ml												
AT III (Anti-Xa), u/ml												
NAPTT, s. (1/10, 1/100, c̄)		271		197		171		172				225 290
TGt50, min.												
XaGt, min.												
Φ C.T., h.												
Limulus + or -												

Operator GROSH Date _____

EMH | Jan 24th 1985

Constant sorbitol : 13g + 10ml 9D
2 glycine concentrations : 1.0g or 1.5g + 10ml 9D
Variable pH : 6.5 6.8, 7.1 at each glycine concentration.

Plastic tubes in all cases with one parallel glass tube incubation.

Method

9D 2031 (1000 iu/ml) 2 bottles dissolved each in 35ml WFI +3ml more WFI to make sure of having 70ml for expt. sample called 9MH STOCK

1. To each of 3 × 10ml aliquots, added 1.0g glycine and 13.0g sorbitol. Warmed to 37° to dissolve

Adjusted pH as follows

1A. pH 6.68 → 6.52 ē dil HCl — plastic universals.
1B. pH 6.66 → 6.78 ē dil NaOH.
× 1C. pH 6.68 → 7.09

2. To each of 2 × 10ml aliquots, added 1.5g glycine and 13.0g sorbitol warmed to 37° to dissolve.

Adjusted pH as follows.

2A pH 6.62 → 6.50 ē dil HCl — plastic universals
× 2C pH 6.63 → 7.10 ē dil NaOH

To one 20ml aliquot, added 3.0g glycine and 26.0g sorbitol.

Adjusted pH 6.60 → 6.81 ē dil NaOH

Split into 2 fractions

2B into plastic universal
2D into glass universal

9H4 Method (cont.)

Control samples removed from each tube and stored at -40°.

Samples put into 60° bath at 11 40 am.

Samples removed at 4.50 pm + stored frozen. Labelled 9H4 1A H etc.

Appearance: all samples clear

Samples given to MGH for IX and NAPII assay pH 7.1 used as control in each set (1 and 2).

9H5: Pasteurization of 9D 2231

13/14 · 4 83

Aims: 1. To pasteurize 9D in sufficient quantity to attempt removal of sorbitol by ultrafiltration.
2. To check stability of factor IX when frozen in sorbitol solution.
3. To compare again pasteurization behavior in glass vs. plastic containers.

Materials and Method:

9D 2231, 35ml vials, no heparin.

15 bottles of 9D 2231 were reconstituted in 35ml each P.F.W. and pooled = 515g 9H5/1 after sampling.

Glycine added (0.1g/ml 9D) : 51.5g glycine added and stirred briefly to dissolve.

pH = 6.80

Sorbitol (1.3g/ml 9D) added : 669.5g to 515g 9D. and solution was warmed to 30° while stirring to dissolve. Sampled 9H5/S

Approx 30 ml 9H5/S was put into a washed, new 65ml F.D. vial and the remainder was split between two MSE polypropylene centrifuge pots. These samples stood at R.T. for 6 hours and were then heated to 60° for 10 hrs, 12 min.* (See accompanying trace recording factor 13 pasteurization done simultaneously).

After pasteurization both glass and plastic samples were clear. Each was diluted with an equal weight of P.F.W. before sampling and freezing. The following samples were taken:

a. Pasteurized in glass: 9H5/GH 6x1ml } to HC.
b. Pasteurized in plastic: 9H5/SH 6x1ml }
6x20ml

The remainder was frozen in bulk.

* Really ~ 61.5 – 62°

Results:

	Vol. ml.	IX u/ml	IX TOTAL u.	Stage yield	NAPTT	Clotting Time
9H5/1	515					
9H5/S	984 *	15.9			224/270	2hrs
	949 plastic	↓	15,089			
	35 glass	↓	557			
9H5/SH	949 *					
after dilution	2082	3.68	7661	51%	265/270	6+
9H5/GH	35 *					
after dilution	77	3.33	256	46%	219/270	6+

* This assumes 0.7 x sorbitol wt ~~[illegible]~~ increase in volume after addition of sorbitol.

Conclusion: Lower yield of IX than 9H4 etc. ∴ higher effective past.^n temperature

No significant Δ between IX recovery in plastic & glass, but keep doing comparison

All NAPTT times good, Δ plastic-glass perhaps not significant

9H5 13/14 .4.83

~ 500 ml 9D 2231 (~14 vials)

To 9H4, add 0.1 g glycine /ml 9D

Adjust to pH 6.80 ± 0.05

Add 1.3 g glycine /ml 9D

Heat in 2 Polypropylene ? NPE / Buchner pots 10L 60°
[1 sample in small "sterile" i.v. new, washed vial]

a.m. Cool, add 1 vol PF water, sample & freeze bulk
Label 4 'glass sample'

1133 g 9H5 SH
41.8 g 9H5 GH

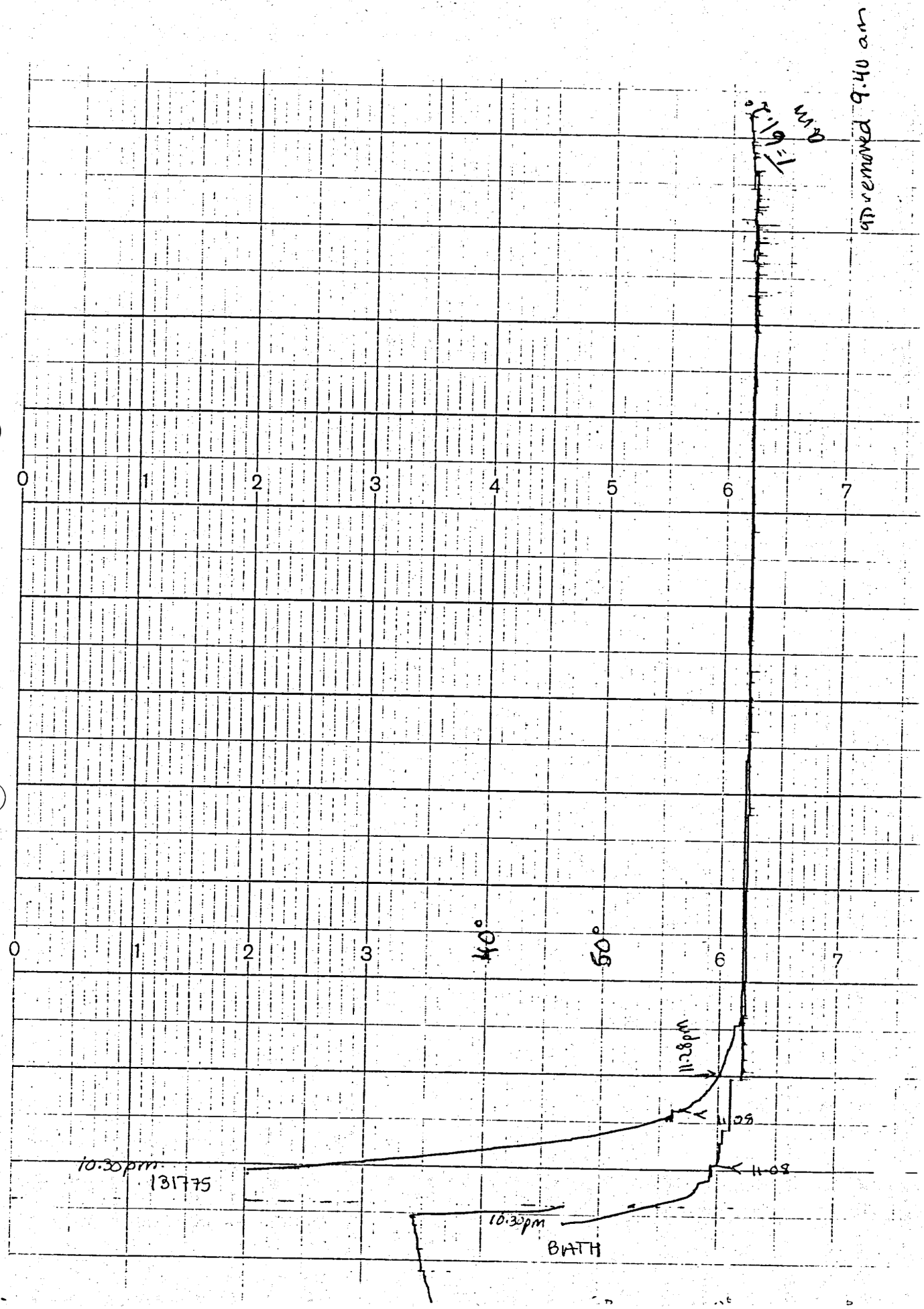
Sample: pre-pasteurised IX, NAPTT; FDA forgotten
6 CL 6 x 1 ml for stability [6 x 1 in glass] not 'glass'
for LW [6 x 20 ml for appt. adsorption & BGAE
Remain, glass [in white box in CR4 bottom right]

13.4.83

3pm 15 bottles x 35 ml resolution in PFW.
517 g 9H5/1 2 x 1 ml LP3
515 g
+51.5 g glycine
pH = 6.80 ∴ No adjustment made.
+ 515 x 1.3 = 669.5 g sorbitol
warmed to dissolve
sampled 9H5/S 4 x 1 ml LP3
~30 ml into 65 ml F.D. vial (clean)
Remainder split between two MSE pots
4.10 pm: left at R.T. to await pasteurisation
4.30 pm: into bath at 50° —> 60°
Reaches T = 59.0 at 11.15 pm.

9H5 ~~SH~~ SH
9H5 GH pm-

9H5/S 15.9 u/ml
SH 3.68 u/ml
GH 3.33 u/ml.


Removed 9.40 am
WAD
0
1
2
3
4
5
6
7
40°
50°
11.28pm
11.25
11.05
10.30pm
131775
10.30pm
BATH

REQUEST FOR COAGULATION FACTOR ASSAYS

Request from [redacted] Date 14.4.83

esults needed when? 15/4

Samples to be kept? YES If so, how? -40

mples provided with this request/available from NEO /LW

: All samples centrifuged, divided & plasma. Note any post freezing

SAMPLE	9115 S		9115 SH chelation III		9115 GH chelation IV							
INVESTIGATION	EST.	FOUND	EST.	FOUND	EST.	FOUND	EST.	FOUND	EST.	FOUND	EST.	FOUND
Factor VIII, iu/ml 1 st.												
2 st.												
Factor IX, iu/ml 1 st.												
2 st.	15	15.9	5	3.68	5	3.33.						
Factor II, u/ml												
Factor X, u/ml												
Factor VII, u/ml (clotting)												
(amidolytic)												
Factor VIII antigen, u/ml												
AT III (Laurell), u/ml												
AT III (Anti-Xa), u/ml												
NAPTT, s. (1/10, 1/100, c)	✓ 224 – 270		✓ 265 – 270		✓ 219 – 270							
TGt$_{50}$, min.												
XaGt, min.												
Φ C.T., h.	✓ 2hrs		✓ 6+		✓ 6+							
Limulus + or -												

Operator [redacted] Date 15/4/83.

REQUEST FOR COAGULATION FACTOR ASSAYS

Request from [illegible] Date 14.4.87

Results needed when? 22/4

Samples to be kept? yes If so, how? -40

Samples provided with this request/available from [illegible]

SAMPLE	9115 S		9115 dil JH		9115 dil GH							
INVESTIGATION	EST.	FOUND	EST.	FOUND	EST.	FOUND	EST.	FOUND	EST.	FOUND	EST.	FOUND
Factor VIII, iu/ml 1 st.												
2 st.												
Factor IX, iu/ml 1 st.												
2 st.	15	16.87	5	3.72	5	3.1	(a repe		4	15/4		
Factor II, u/ml												
Factor X, u/ml												
Factor VII, u/ml (clotting)												
(amidolytic)												
Factor VIII antigen, u/ml												
AT III (Laurell), u/ml												
AT III (Anti-Xa), u/ml												
NAPTT, s. (1/10, 1/100, c̄)	✓ 236		✓ 284		✓ 259							
	←		282		→							
TGt_{50}, min.												
XaGt, min.												
Φ C.T., h.	✓ 2		✓ 6		✓ 6							
Limulus + or -												

Operator [illegible] Date 22/4/87

29.4.83 9H5-DCL-1 PFL

To determine the effect of the sorbitol/glycine mixture used in FIX pasteurization on the binding of the FIX to DEAE cellulose

Materials and methods.

The factor IX was from 9D2231 and had been pasteurized in experiment 9H5 (by L.W. 13.4.83). Some of the pasteurized 9H5 (9H5/SH) was used in experiment P3 (by DC.) to exchange the sorbitol/glycine for Buffer B of the standard FIX preparation.

The DEAE Cellulose had been used for previous FIX absorbtions but it was recycled according to the standard procedure.

In this experiment a constant number of i.u.s of FIX was stirred with a constant amount of DEAE Cellulose in the presence of differing concentration of the sorbitol/glycine mixture for the same length of time.

The buffers used were those of the standard FIX preparation and were either collected or made as shown below:-

Buffer A — collected from 9P1784 [1 litre] was kept at −40°C until thawing for use here.

Buffer B

		in 1 litre
Tri sodium citrate	($Na_3C_6H_5O_7 \cdot 2H_2O$)	2.68g
Sodium Chloride	(NaCl)	14.61g
di Sodium Hydrogen Phosphate	(Na_2HPO_4	1.41g
Citric Acid	($H_3C_6H_5O_7 \cdot 2H_2O$)	0.188g

Buffer C

1M Sodium Chloride	(NaCl)	58.44
0.02M Tri Sodium Citrate	($Na_3C_6H_5O_7 \cdot 2H_2O$)	5.9

29.4.83 9H5-DCL-1

The 9H5/SH was thawed by warming to 20°C in a 30°C water bath. A 2ml sample was taken (9H5/SH 29.4.83) and kept for analysis.

In experiment P3 it was found that the concentration of FIX in 9H5/SH was 3.5 iu./ml. This was used as the basis for the amount of 9H5/SH to add to the gel.

The FIX binding capacity of the gel was taken to be 50iu/g of moist gel at pH 6.95. 5g of the DEAE cellulose (DCL) was used for each dilution of the 9H5/SH (250 iu. FIX binding capacity). 71ml ml (250/3.5) of solution contains 250 iu FIX.

70 ml of 9H5/SH was used in three separate batches and labelled α, β, γ and each was diluted as shown in table 1 using PFW cooled to 20°C.

TABLE 1 — Batches of starting material used.

Batch	Dilution	PH	Sorbitol Conc	Glycine Conc.
α	NONE	6.90	31%	2.37
β	to 140ml (2X)	6.98	15.5%	1.185
γ	to 280ml (4X)	7.07 adjusted to 6.96*	7.75%	0.592

* with 1 drop of 3x diluted 5M Acetic Acid.

5g of moist DCL was added to α β γ at staggered times (α at 12.45pm, β at 1.30pm, γ at 2.35pm) to allow for processing. In each case the mixture was stired for 1 hour and 10 minutes at room temperature (≈ 18°C) and then poured into a 90mm x 15mm chromatography column. Gentle pressure was applied to the column using a 30ml hypodermic syringe. This increased the filtrate flow from the column to a steady 2.5ml/minute. When all of the 9H5/SH — gel mixture was poured into the column and about 2mm of clear solution remained above the gel bed the pressure was released. A 2ml sample of the total filtrate (9H5/SH/SN) was taken. The height of the gel bed was measured and the volume of the gel calculated. Each column was then washed with 2x the bed volume Buffers A B and C. 14 ml of each was used in every case. Slight

9H5 DCL – 1

pressure was applied to the columns using a hypodermic syringe as before and the buffers were changed when about 1mm of the previous buffer was left above the surface of the gel.

The wash from buffer A was collected as one 14ml aliquot and sampled (9H5/SH/A). From Buffer B the wash was collected as aliquots of ≃ ⅓ the gel bed volume [7/3 = 2·33 ; 2·5ml aliquots collected ∴ 5 aliquots] 9H5/SH/B1 to 9H5/SH/B5. Buffer C wash was collected as a 14ml aliquot and sampled (9H5/SH/C).

The filtrates from when the gels were first applied to the columns were discarded, all of the remaining washes, samples and gels in columns were frozen at −40°C.

Summary of samples all prefixed by 9H5/SH /29.4.83

/α Sn , /α B1 , /α B2 , /α B3 , /α B4 , /α B5 , /α C
/β Sn , /β B1 , /β B2 , /β B3 , /β B4 , /β B5 , /β C
/γ Sn , /γ B1 , /γ B2 , /γ B3 , /γ B4 , /γ B5 , /γ C

Results and Discussion

TABLE 2 shows the samples sent for analysis and the results of that analysis.

Sample	FIX iu/ml	NAPTT
9H5/SH/29.4.83	2·5	275
9H5/SH/α Sn	<0·1	289
9H5/SH/β Sn	<0·1	279
9H5/SH/γ Sn	<0·1	268
9H5/SH/α B2	3·7	280
9H5/SH/β B2	17·8	277
9H5/SH/γ B2	15·2	264
9H5/SH/α B3	≃100	269
9H5/SH/β B3	47·7	314
9H5/SH/γ B3	26·5	322

The background NAPTT was 284 for all samples.

29.4.83 9H5-DCL-1 GRO-C: Derrick Cassidy PFL

The FIX concentration in 9H5/SH was found to be 2·5 iu/ml. ∴ 175 iu of FIX were applied to 5g of the moist DCL in each case (35 iu FIX/gram moist DCL) • Almost all of the FIX was absorbed by the gel in α, β and γ (<0·1 iu/ml was left in the column filtrates).

The gel bed height was found to be ≈40mm in each column and hence the volume was ≈ 7·0ml.

The FIX elution had already started by the time the 2nd ⅓ bed volume of Buffer B was washed through the column [In α there was noticeably less than in β or γ — 3·7 iu/ml, 17·8 iu/ml and 15·2 iu/ml respectively]. Most of the FIX was eluted when the 3rd ⅓ bed volume of Buffer B was washed into the column. In α there was a greater concentration of FIX than in β or γ [≈100 iu/ml, 47·7 iu/ml and 26·5 iu/ml FIX respectively.

	Total iu FIX used	Total iu FIX in B2	[% of TOTAL]	Total iu FIX in B3	[% of Total]
α	175	9·25	[5·3]	≈250	[142%]
β	175	44·5	[25·4]	119	[68]
γ	175	38·0	[21·7]	66·25	[37·8]

FIX RECOVERY TABLE. THE >100% recovery of FIX from α is due to measu inaccuracy in the higher FIX concentration.

Although it seems that the Factor IX was eluted earlier from β and γ, in α we had a higher recovery and also a sharper elution profile.

It appears that the higher sorbitol/glycine leads to a greater and more concentrated recovery of Factor IX from the DCL. This observation requires further testing by repeating the α9H5/SH absorption/elution and also using a higher sorbitol/glycine concentration [using the pasteurized mixture before diluting it.

(1)

9H5 2

16·5·83 9H5 – DCL – 2 Dominically Casty PFL

To repeat the α 9H5/SH part of experiment 9H5-DCL-1 in order to look at the elution of FIX from the DCL in detail.

Materials and Methods.

The 9H5/SH was from the same batch which was used in in experiment 9H5-DCL-1. The DCL was also from the same recycled batch which was used in the previous experiment.

It was expected that the FIX concentration would be 2·5 iu/ml (from 9H5-DCL-1). The experiment was scaled up 3× but this time ≈ 40 iu FIX/gram moist gel was used.

250ml of ~~the 9H5~~ thawed 9H5/SH (250 × 2·5 = 625 iu FI were used. 15g of moist gel was added to the 9H5/SH and the mixture stirred at room temperature for 1 hour 10 minute Hence 41·7 iu Factor IX/gram of moist gel were used.

a sample of the thawed 9H5/SH was kept for analysis (2α 9H5/SH)

After stirring the mixture was pumped in a 180mm × 15mm chromatography column. The eluate from the column was passed through a U.V. absorption monitor connected to a chart recorder and then to a fraction collector. The pump was set to give a flow rate of ≈ 10ml/minute; the chart speed was 2mm/minute, chart scale was 50mV and 3·9ml fractions (60 drops) were collected.

The column filtrate was collected as one aliquot and a sample was kept for analysis (A 9H5/SH/Sn)

after filtration the gel bed height was measured and the volume calculated (21·9 ml).

2 × the gel bed volume of Buffer A followed by the same volume of Buffer B and C respectively were pumped through the column while fractions were collected

(2)

9N52

16.5.83 9H5-DCL-2 Derrick Casty PFL.

Materials and Methods cont.

11 fractions and 1 mixed buffer fraction were collected for each buffer. They were numbered as follows :- A1, A2, B1 e

Results and Discussion

The bed height was 12.4 cm hence the volume was 21.9ml. The buffer washes were each 4.3ml.

The recorder trace shows u.v. absorbance profile of the column eluates (the noise and spikes were due to mains electrical interference). NB. The base line of the trace was readjusted during the Buffer B elution.

Buffer B eluted a larger peak which was followed by a smaller one in the tail of the first.

Since the FIX is eluted frontally it was thought that it would be found at the front end of the first Buffer B peak. Hence B3, B4 and B4 were analysed for FIX.

Sample	Vol of Pool	FIX iu/ml	NAPTT	Volume of buffer passed as % of bed volume	Total FIX units in Pool
2α 9H5/SH	250ml	2.7	218	—	675
A 9H5/SH/SA	x 250ml	0.011	248	—	2.75
A6	39ml	3.0	206	106% (A)	11.7
B3	3.9ml	2.9	187	53% (B)	11.31
B4	3.9ml	57.0	195	71% (B)	222.3
B5	3.9ml	52	187	89% (B)	202.8

Background NAPTT was 215

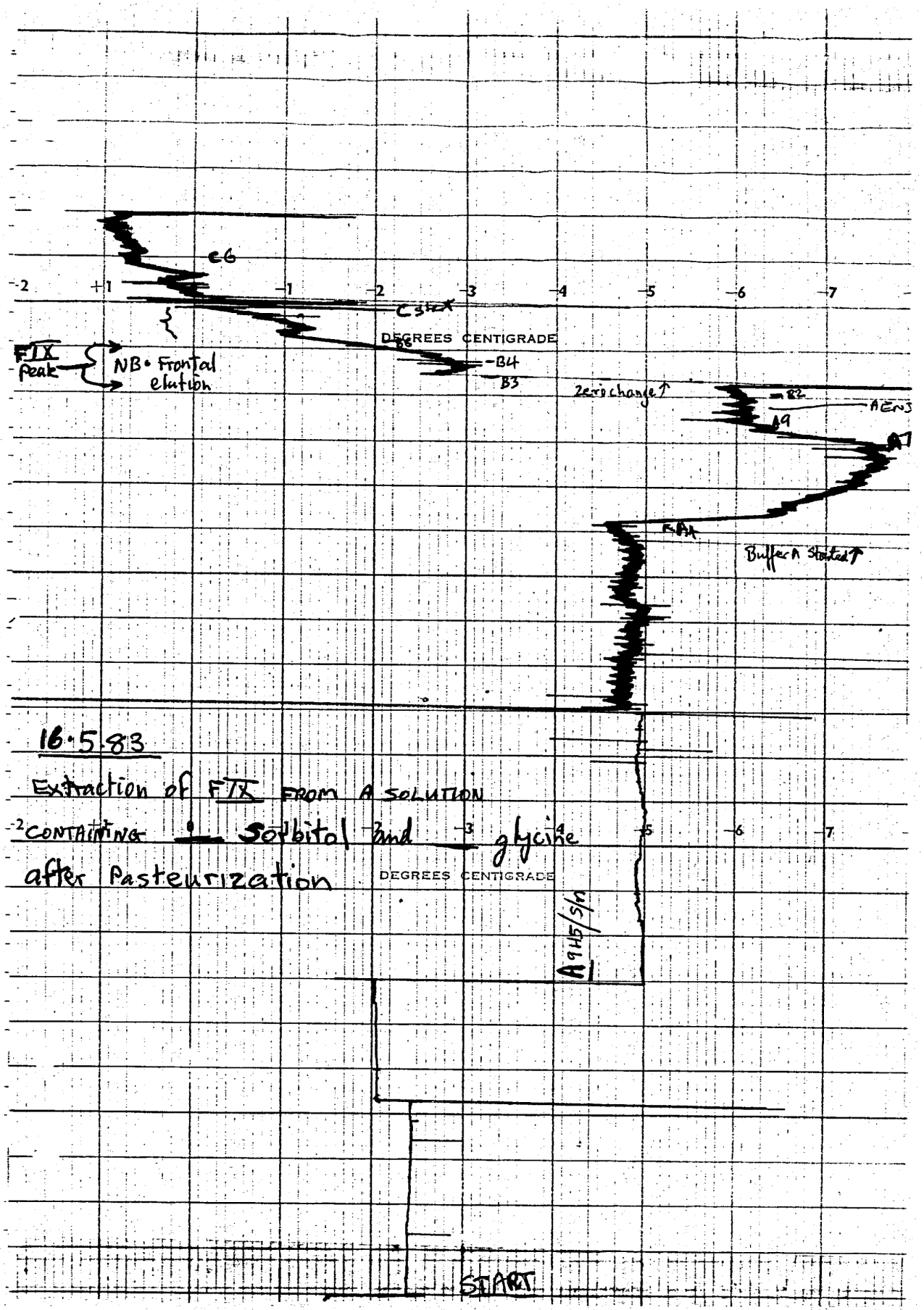
99.9% of the Factor 9 was absorbed by the DCL

B3 to B5 contained 64.6% of the absorbed FIX.

Only 0.89 column volume of Buffer B had passed when Sample B5 was collected. It is expected that sample B6 would have enough FIX in it ~~to~~ to increase the %age of FIX recovered. B6 was not analysed for

(3)

9H5.2



16.5.83

Extraction of FIX FROM A SOLUTION CONTAINING Sorbitol and glycine after Pasteurization

(4)

9H5.2

16.5.83 9H5-DCL-2

Results and Discussion Continued.

FIX but it is clear that using the sorbitol/glycine concentration as in the ∝ part of experiment 9H5-DCL-1 more than 64.6% of the Factor IX may be recoved from a DCL absorption.

Experiment 9H6-01 Dr Derrick Gray PFL

To Pasteurize a reconstituted batch of FIX and to use a sample of the undiluted pasteurized mixture for absorption onto and elution from DEAE Cellulose

Materials and Methods.

Reconstitution and Pasteurization.

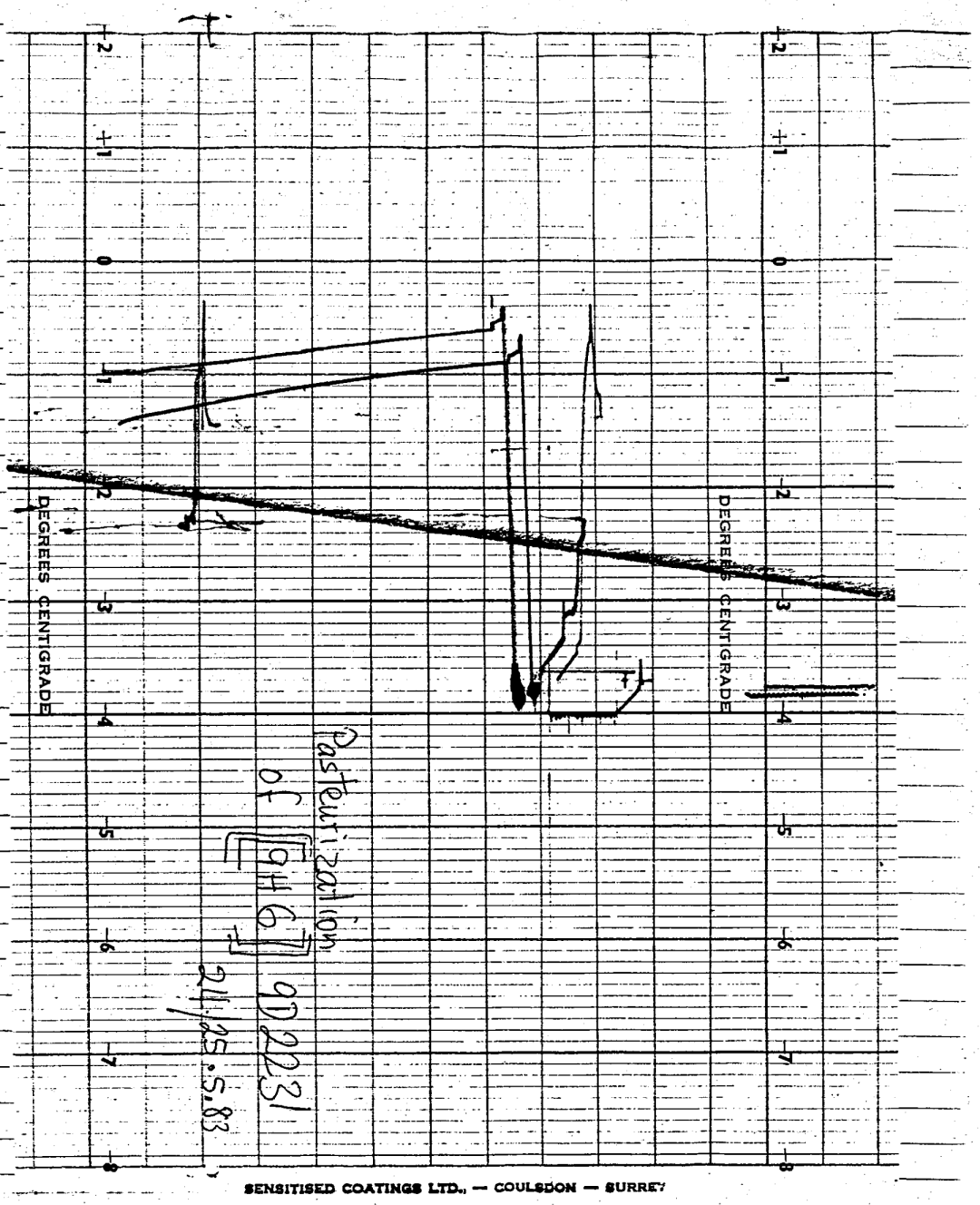
Freeze dried FIX from batch No 9D2231 was reconstituted by adding 35ml of PFW to each of 20 FIX vials Containing ≈ 1000 iu of FIX. The resulting 700ml of solution was sampled [9D2231/PF 70g of glycine and 910g of sorbitol were then added to the FIX solution. The mixture was placed in a 30°C water bath and stired to dissolve the sorbitol

1400ml of a clear solution was obtained. This was sampled [9D2231/S]. Approximately equal portions of the solution were poured into 3 1 litre plastic bottles. The bottles were placed in a waterbath which had its thermostat set to bring the water to 60°C but which at that time contained water a room temperature. Two thermometers connected to a chart recorder were placed one in the FIX/sorbitol/glycine solution, and one in the water of the bath. An electrical timer was set up to switch on the apparatus about 10½ hours before it was due to be inspected the following morning.

The Pasteurized solution was sampled [9H6/25.5.83]

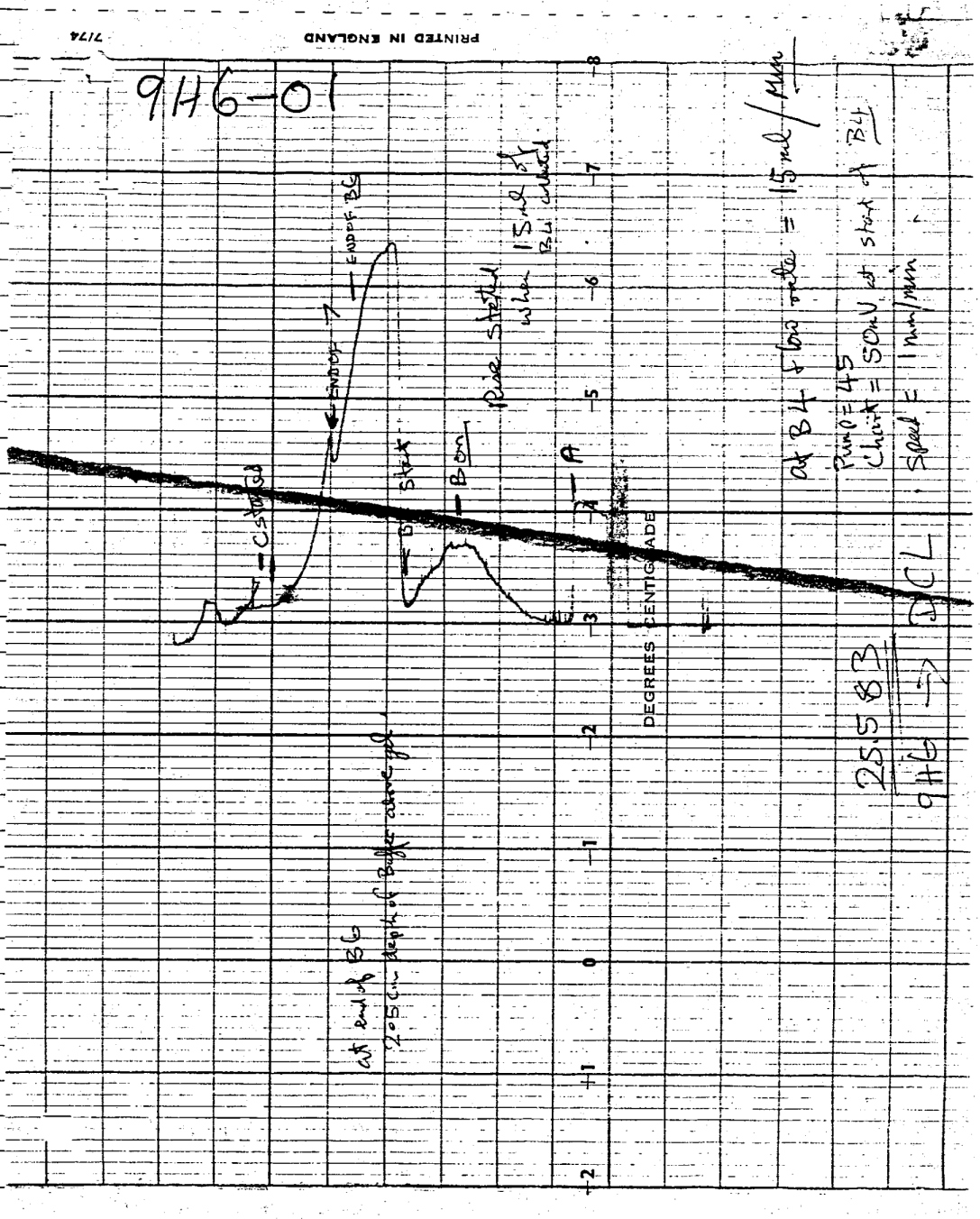
(2)

YH6.01



(3)

9H6 01



(1)

DCL + Pasteurized FIX (high sorbitol/glycine)

9H6 – 01

Summary of Results from Experiment 9H6.

9D2231 PFW

20 000 units of FIX was reconstituted with PFW and made up to 65% sorbitol and 5% glycine in a total volume of 1400 ml. This was pasteurized for ≈ 10 hours at 58.2°C giving 9H6.

420 ml of Pasteurized material absorbed on 50g DCL and eluted in the usual way for FIX. This was to use (14.2 × 420 = 6000 units of FIX and expecting the gel capacity to be 40 iu/g moist gel).

Sample	Pool Volume	iu FIX per ml	Total FIX in pool	NAPTT.
9D2231 in PFW	700 ml	27.1	18970	230
9D2231 + Sorbitol + glycine	1400 ml	14.2	19880	176
9H6/25·5·83	420 ml	8.3	3486 ⊕	147
9H6 Spin supernatant	300 ml	0.01	3	274
9H6 Buffer A resuspension Super.	240 ml	0.08	19.2	295
9H6 Buffer A Wash	255 ml	0.07	17.85	290
9H6/B1	27 ml	0.08	2.16	244
9H6/B4	43 ml	6.0	258	171
9H6/B5	42 ml	24.2	1016.4	163
9H6/B6	43 ml	20.4	877.2	154
9H6/B7	43 ml	8.1	348.3	177

⊕ This was applied to the gel.

% of 9H6/25.5.83 recovered in B4 to B7 = ~~[illegible]~~ 71.7% *

Total iu FIX recovered in B4 – B7 = 2499.9

Column Vol = 274.8 ml

Vol of Buffer B passed at end of collection of 9H6/B7 = 280 ml

∴ ~~[illegible]~~ 71.7% recovery at end of the 3rd 1/3 column bed vol of buffer B.

Perhaps this is not as good as the recovery when the pasteurized FIX was diluted.

* ~~[illegible]~~ 71.7% is not the recovery from the whole of the reconstituted FIX but after it had undergone losses on pasteurization.

In 420 ml of 9H6 we would expect 5964 in total but after Pasteurizat we have 3486 ie 58.4% recovery after pasteurization of the FIX in the Sorbitol/glycine mixture.

9116-01

REQUEST FOR COAGULATION FACTOR ASSAYS

Request from DC Date 25.5.83

Results needed when? ASAP

Samples to be kept? YES If so, how? RETURNED OR FROZEN

Samples provided with this request/available from DC

	7		8		9		10		11			
SAMPLE	9116/B1		9116/B4		9116/B5		9116/B6		9116/B7			
INVESTIGATION	EST.	FOUND	EST.	FOUND	EST.	FOUND	EST.	FOUND	EST.	FOUND	EST.	FOUND
Factor VIII, iu/ml 1 st.												
2 st.												
Factor IX, iu/ml 1 st.	20	0.03	25	6.0	50	24.2	50	20.4	25	8.1		
2 st.												
Factor II, u/ml												
Factor X, u/ml												
Factor VII, u/ml (clotting)												
(amidolytic)												
Factor VIII antigen, u/ml												
AT III (Laurell), u/ml												
AT III (Anti-Xa), u/ml												
NAPTT, s. (1/10, 1/100, c̄)	244		171		163		154		177			
	280											
TGt_{50}, min.												
XaGt, min.												
Φ C.T., h.												
Limulus + or -												

42 42

Operator _____ Date _____

3.6.83 Experiment N° 9H6-02. GROSvenor Cadby PFL

To absorb and eluted pasteurized FIX after diluting the pasteurized mixture with an equal volume of Pyrogen free water (PFW).

Materials and Methods

The DCL was stored below -20°C and was thawed at RT°C overnight before use. It had been used previously but was recycled using the standard procedure.

The buffers were those which had been used in experiment 9H6-01. They were stored below -20°C and thawed before use (Factor IX Buffer A, Buffer B and Buffer C)

The FIX solution was the 9H6 which was pasteurized overnight 24.5.83. This was a 300ml portion which was frozen at ≈ -35°C soon after pasteurization and had not been previously thawed.

A chromatography column (Amicon/Wright MPGA 44) with an internal diameter of 50mm was set up attached to a U.V. Monitor/chart recorder and faction collector

The 9H6 was thawed by immersion in a 30°C water bath and stirring it and bringing it to room temperature. The 300ml of clear solution was sampled [9H6/3.6.83] 300ml of PFW at room temperature was added to the 9H6 and mixed throughly (thus producing ∝9H6/3.6.83 - this was not sampled). The pH of this new solution was found to be 6.93.

107.2g* of DCL was added to the ∝9H6/3.6. and left to stir at room temperature for 1 hour and 10 minutes.

NB after addition of the gel the pH of the mixture was 8.33.

* in 9H6-01 150g DCL was added to 420ml of 9H6. Since this is a direct comparison with that experiment the ratio of 9H6 used to grams of DCL will be kept constant.

3.6.83 9H6-02 PFL

Materials and Methods cont.

After stirring, the DCL was recovered by centrifugation in the Beckman J-6B at 4Krpm; 15minutes. The pellet was tight and the supernatant was clear. A sample of the supernatant was taken [9H6-02/spin Sn.]

The pellet was resuspended in 150ml of buffer A [maiz gel weight x 1.4] and the suspension poured into the chromatography column. Pressure was applied to the top of the column using a pump to give a flow rate from the column of ≈ 8ml/minute. The filtrate from the column was collected and sampled [≪ 9H6-02/Spin

When all of the fluid above the gel had just gone into the surface, the flow from the column was stopped. The gel bed height was measured and the bed volume calculated 251 ml of Buffer A was then pumped through the gel. This was equivalent to the gel bed volume. The filtrate was collected as one aliquot and sampled [≪ 9H6-02/A]. When the last of Buffer A had ~~just~~ gone had just gone into the top of the gel the flow from the column was ~~stopped~~. The bed height was measured again and its volume calculated.

The fraction collector was set to collect enough drops per fraction to give a fraction volume of 1/6 the gel bed volume. In this case 928 drops were collected (it was estimated that each drop volume was ≈ 0.043 ml) — The bed volume was 239.5ml and 40ml fractions were required.

Buffer B was then pumped into the column while the fractions were collected. The flow rate was 12 ml/minute. 400ml of Buffer B was used and aliquots B1 to B8 were sampled [≪ 9H6-02/B1 to B8]

3.6.83 Experiment No 946-02 GRO Emma Patrick Cauty DFL

Result and Discussion.

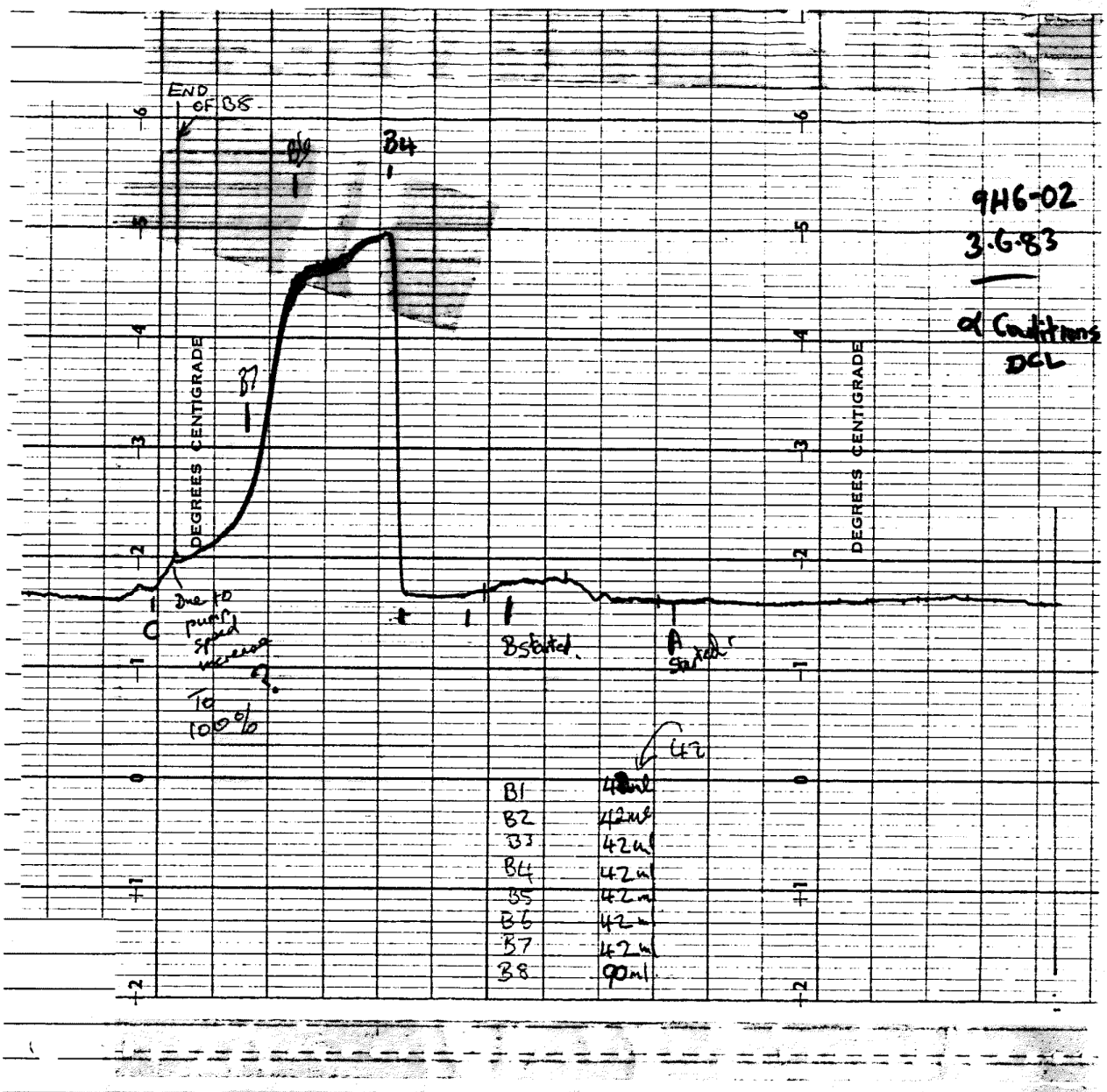
The u.v. absorption of the column eluate is shown on the recorder trace. The peak between B4 and B7 was analysed. A sharper peak would be desired and this may be achieved by preventing too much mixing of the buffer above the gel bed and also compressing the gel bed with the alumn piston as in the standard FIX preparation.

The analysis of samples is summarized in the following table.

SAMPLE	FIX (IU/ml)	VOLUME OF SAMPLE POOL	TOTAL FIX IN POOL (IU)	Vol of BUFFER as % of gel bed vol.	NAPTT
946/3.6.83	9.08	300 ml	2724	—	199
@946-02/Spin Sn	0.01	485 ml	4.85	—	265
@946-02/SpinA	0.01	145 ml	1.45	—	261
@946-02/A	0.01	260 ml	2.60	>100	265
@946-02/B3	0.69	42 ml	28.95	53	296
@946-02/B4	32.3	42 ml	1356.6	70	236
@946-02/B5	20.5	42 ml	861.0	87.6	211
@946-02/B6	13.7	42 ml	575.1	105	224
@946-02/B7	2.57	42 ml	107.9	122.7	230
Background NAPTT = 284.					

④

9H6-02


END OF B5
B4
B7
DEGREES CENTIGRADE
Due to pump speed increase
To 100%
B started
A started
42
B1 42ml
B2 42ml
B3 42ml
B4 42ml
B5 42ml
B6 42ml
B7 42ml
B8 90ml
9H6-02
3.6.83
Conditions
DCL

3.6.83. Experiment 946-02 PFL.

Results and Discussion cont.

The measurements of FIX are accurate to within 10-20%. The higher error on the very low values.

222 μg in FIX* were used against 107g of moist DCL ($\frac{2724}{107}$ = 25.45 μg FIX/g of gel). More than 99% of the FIX was absorbed by the DCL, and Buffer B eluted all of the bound FIX [87.6% column volume of buffer B eluted 82.5% of the bound FIX and 105% column volume eluted "103.6%" of the bound FIX]. The FIX values of greater than 100% are within experimental error. The fraction with the highest amount of eluted FIX contained ≈ 50% of the FIX recovered. Each fraction was 0.175 of the gel bed volume.

* N.B. The FIX inactivated by the pasteurization will be present too and it is possible that this could compete with the still active material for binding sites on the DCL.

Experiment 9H7-1 GRO-sennick Cosy PFL.

To determine the binding capacity of DCL for FIX which had been reconstituted and pasteurized.

Materials + Methods.

The FIX was from batch 9D2232 and had no heparin in it.

The DCL was used previously but had been recycled using the standard procedure.

The PFW, Buffer A and Buffer B were from those used in the standard FIX preparation.

Reconstitution and Pasteurization of FIX

35ml PFW were added to each of 26 vials of freeze dried FIX. Each vial contained ≈ 1000iu F. The FIX solution was pooled (9D2232) and a 10ml sample was removed for analysis. To the remaining 900ml of solution 90g of glycine and 1170g of sorbitol were added. The mixture was heated to ≈ 30°C using a waterbath at 35°C while it was stirred slowly. 1800ml of a clear solution was obtained (9D2232/S) and 5ml of it was removed for analysis. 1750ml of this solution was recovered and pasteurized in 4 equal amounts for 10 hours at 60°C. The solution (9H7) was then cooled to room temperature and a 5ml sample was removed for analysis (9H7/7.7.83).

Experiment 9H7-1 PFL.

Determination of the capacity of DCL to bind the FIX in the Pasteurized solution.

60 ml of the 9H7 solution were diluted to 120ml using PFW (producing α9H7/8·7·83)

The pH of α9H7/8·7·83 was checked to see if it was in the range 6·90 – 7·00.

20ml of the α9H7 were added to each of six plastic 50ml beakers. The appropriate amount of DCL was added to each beaker and the mixture was stirred for 1 hour at room temperature. SEE FOLLOWING TABLE FOR EXPERIMENTAL DETAILS.

Beaker/column N°	1	2	3	4	5	6
N° of units FIX *	142·5	142·5	142·5	142·5	142·5	142·5
Amount of DCL *	4·75g	7·125g	4·75g	3·56g	2·85g	1·425g
Units FIX/g most gel *	10	20	30	40	50	100
Time gel added	11·15am	12·00	13·06	13·36	14·10	15·00
Gel bed height after collection	13·0cm	7·0cm	4·6cm	3·6cm	2·6cm	1·8cm
Gel bed vol. after collection	23ml	12·37ml	8·13ml	6·36ml	4·59ml	3·18ml
Vol of buffer A used	"	"	"	"	"	"
Gel bed height after Buffer A	12·0cm	6·5cm	4·4cm	3·5cm	2·5cm	1·3cm
Gel bed vol. after Buffer A	21·2ml	11·48ml	7·7ml	6·18ml	4·4ml	3·06ml
Vol. of buffer B used.	31·8ml	17·22ml	11·66ml	9·28ml	6·63ml	4·6ml

* In 9D2232/S (ignoring any losses which occur on heating) we have $\frac{1000}{35} \div 2 = 14{\cdot}25$ in FIX/ml.

After stirring the contents of each beaker was poured in a 15mm x 200 mm chromatography column and the filtrate (/S11) collected. The gel bed height was measured and its volume calculated. The bed was then washed with an equal volume of buffer A and afterwards with 1·5x its volume of buffer B. The washes from Buffer A and Buffer B were collected separately as single aliquots.

A hypodermic syringe was used to apply gentle pressure

to give a buffer flow rate of ≈ 5ml/minute.

A 2ml portion of each aliquot and sample was analysed for FIX and NAPTT.

The pH of each supernatant from the column was measure

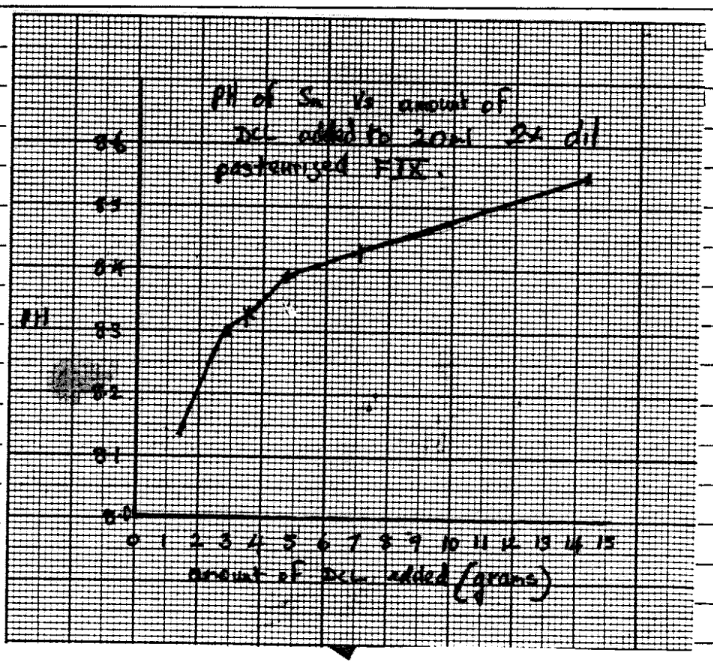
Results and Discussion

The actual pasteurization temperature was 61°C for a duration of 9 3/4 hours.

The recovery of FIX was 40.1 after pasteurization excluding transfer losses. This is lower than in experiment 9H6-1 when the recovery was 58.4%.

The pH of the α9H7/8.7.83 before addition of the DCL was 6.90. This rose when the DCL was added.

Supernatant	1	2	3	4	5	6
pH	8.55	8.43	8.39	8.33	8.30	8.14
Amount of DCL	14.25g	7.125g	4.75g	3.56g	2.85g	1.425g



9H7-1 DC PFL

Results Continued

Sample	Volume (ml)	FIX units/ml	Total FIX units in Sample pool	NAPTT
9D2232	910	33.4	30394	199
9D2232/S	1800	17.2	30960	197
9H7/8.783	1750	6.9	12075	—
∝ 9H7/8.783	120 *	2.7	324	223
/1Sn	10.5	0.07	0.735	/
/1A	24.0	0.04	0.96	/
/1B	32.5	1.60	52	233
/2Sn	15.5	0.02	0.31	/
/2A	13.5	0.02	0.27	/
/2B	18.0	3.70	66.6	200
/3Sn	16.0	0.04	0.64	/
/3A	8.0	0.05	0.5	/
/3B	12.0	4.20	50.4	211
/4Sn	16.5	0.05	0.825	/
/4A	7.0	0.05	0.33	/
/4B	10.0	5.1	51	212
/5Sn	17.5	0.05	0.875	/
/5A	7.0	0.08	0.56	/
/5B	7.25	15.7	113.8	218
/6Sn	18	0.01	0.18	/
/6A	3	0.20	0.6	/
/6B	7	27.1	189.7	248
				Blank = 272

* 20ml of this used in each absorption.

9H7 -1 DC PFL

Results Continued

Column	Amount of DCL	Units FIX/g DCL from 9D23 32/5	ACTUAL TOTAL UNITS FIX / column	ACTUAL FIX UNITS per g moist DCL
1	14.25	12.07	54	3.79
2	7.125	24.14	54	7.58
3	4.75	36.2	54	11.37
4	3.56	48.28	54	15.15
5	2.85	60.35	54	18.95
6	1.425	120.70	54	37.89

Column	TOTAL FIX UNITS RECOVERED	UNITS FIX RECOVERED AS % FIX UNITS APPLIED	RECOVERED FIX AS %age of APPLIED in /SN	RECOVERED FIX AS %age of APPLIED in /A	RECOVERED FIX AS %age of APPLIED in /B
1	53.69	99.4	1.36	1.77	96.2
2	67.18	124.4	0.57	0.5	123.3
3	51.54	95.4	1.18	1.11	93.3
4	52.16	96.5	1.53	0.61	94.4
5	115.23	213.4	1.62	1.03	210.7
6	190.48	352.7	0.33	1.11	351.3

Column	%age of the recovered FIX units in /SN	%age of the recovered FIX units in /A	%age of the recovered FIX units in /B
1	1.36	1.79	96.8
2	0.46	0.40	99.1
3	1.24	0.97	97.8
4	1.6	0.64	99.7
5	0.76	0.48	98.8
6	0.094	0.31	99.59

* % based on results from ∝9H7/8.7.83

All of the FIX applied to the columns was recovered distributed between the supernatant, buffer A and buffer B

Since the percentage of the recovered units eluted by buffer B remained near 100% (estimated error in assays = 10%) over the range it is clear that the maximum gel binding capacity has not been surpassed.

Further experiments with increased FIX : DCL

9H7-1 DC PFL

ratio are required to determine the XL Factor IX binding capacity.

20/7/83 **9H7 . 2 To Determine the optimum capacity of DEAE-Cellulose for heat-treated factor IX concentrate**

This is a continuation of the experiments 9H7.1 which suggested that smaller DE52 : Factor IX ratios improved the selectivity of DE52 for heat-treated factor IX. Using similar methods but lower gel: IX ratios, this present experiment is an attempt to find a limiting, optimum quantity of anion exchanger

Materials

Buffers : factor IX production buffers A and B were used. These have NaCl concentrations of 0.1 M and 0.25 M respectively. pH and conductivity were measured before use :

Buffer A pH : 7.2 Cond.: 4.3 mMh
Buffer B pH : 7.15 Cond.: 9.5 mMh

Factor IX : 450 ml of factor IX concentrate from 9D 2232 (B no heparin) contain 0.1 g glycine 1.3 g sorbitol per ml starting material and heated at 60°c for 10 hours This is the same batch as was used in 9H7.1 and was thawed from frozen in a 37°c water bath.
2 x 2ml samples were taken as "B 9H7

This material was diluted by addition of an equal volume of PFW.
2 x 2ml samples were taken "B9H7α

At this stage, pH : 6.91 and conductivity was 1.3 mMho.

DEAE-Cellulose : Recycled and stored frozen. Thawed at room temperature overnight.

Method

An approximate activity of 3 u/ml was assumed for the 9H7α (on the basis of assay results from 9H7-1) and this value was used to plan six ion-exchange experiments as described over :

	Vol B9H7α (ml)	Theoretical Total factor IX Units	DE-52 (g)	IX Units/g DE-52
1	75	225	5	45
2	100	300	5	60
3	125	375	5	75
4	150	450	5	90
5	200	600	5	120
6	250	750	5	150

The thawed 9H7α was stored at 4°C until needed for each experiment. The equired volume was then added to 5g DE-52 and stirred at room temperature for one hour.

The mixture was then poured into a glass, 1.5 cm ID column and the gel packed under gravity. The effluent supernatant was collected and sampled as "B9H7α – # Sn" where # was the experiment number.

The bed volume was calculated and an equal volume of buffer A then passed through the column, the eluate being collected and sampled as "B9H7α – # A"

The bed volume was re-measured and 1½ volumes of Buffer B passed through. The eluate was collected and sampled as "B9H7α – # B"

Results

	Initial Bed Vol. (ml)	Volume Buffer A (ml)	New Bed Vol (ml)	Volume Buffer B (ml)
1	9.72	9.72	10.07	15.1
2	10.07	10.1	9.72	14.6
3	9.36	9.4	9.2	13.8
4	8.8	8.8	8.66	13.0
5	9.0	9.0	9.0	13.5
6	8.8	8.8	8.8	13.2

Activity of starting material

These were almost double that anticipated; a phenomenon still unexplained. Later assays (see 9H7.3) gave different results yet again.

Pastured factor IX 89H7 13.6 u/ml
Diluted " " 89H7∝ 5.5 u/ml

Using the ∝ value of 5.5 u/ml, the factor IX activity in experiments 1–6 was distributed as below:

Vol 9H7∝ ml	Total IX Units	Sn u/ml	Sn Vol (ml)	Sn Total units	Sn % Units loaded	A u/ml	A Vol. (ml)	A Total Units	A % Units loaded	B u/ml	B Vol (ml)	B Total units	B % Units loaded
75	412	0.05	71	3.55	0.86	0.05	8.5	0.42	0.1	13.4	17.5	234.5	56.9
100	550	0.04	94	3.76	0.68	0.1	10	1.0	0.18	14	17	238	43.3
125	687.5	0.09	122	11	1.6	0.14	9.5	1.33	0.19	20.7	16	331.2	48.2
150	825	0.09	150	13.5	1.64	0.21	9	1.9	0.23	24.1	13	313.3	38
200	1100	0.3	200	60	5.5	0.83	9	7.5	0.68	37.8	14	529.2	48.1
250	1375	0.8	250	200	14.5	1.4	8.5	11.9	0.86	49.3	14	690.2	50.2

In all experiments, the majority of the factor IX activity was recovered from the Buffer B eluate. From this, the units of active factor IX bound per gram of DE52 can be derived.

Vol 9H7∝ (ml)	Total IX Units loaded	Units IX eluted per g DE52	Units IX loaded per g DE52
75	412	46.9	82.4
100	550	47.6	110
125	687.5	66.2	137.5
150	825	62.6	165
200	1100	105.8	220
250	1375	138	275

9H7.2.4

Protein Absorbance

The absorbance at 280 nm was measured for the Sn and B elutes. Assuming an average $\varepsilon^{0.1\%}_{280}$ of 1.0, the specific activities of these samples can be calculated:

Sample	A280 (assume unity)	1X v/ml	Sp. Act (U/mg)	Total "Protein" (mg)
1 Sn	0.31	0.05	0.16	22
2 Sn	0.36	0.04	0.11	33.8
3 Sn	0.54	0.09	0.16	65.9
4 Sn	0.72	0.09	0.12	108
5 Sn	1.07	0.3	0.28	214
6 Sn	1.55	0.8	0.52	387.5
1 B	9.72	13.4	1.38	170.1
2 B	13.71	14	1.02	233.1
3 B	17.49	20.7	1.18	279.8
4 B	23.73	24	1.01	308.5
5 B	24.03	37.8	1.57	336.4
6 B	24.51	49.3	2.01	343.1

Conclusions and comments

Increasing the amounts of factor IX added to a constant amount of gel increases both the total factor IX which binds to the DE-52 and also the specific activity of the subsequently eluted protein.

The accompanying graphs show how this variation occurs with respect to total protein and factor IX activity.

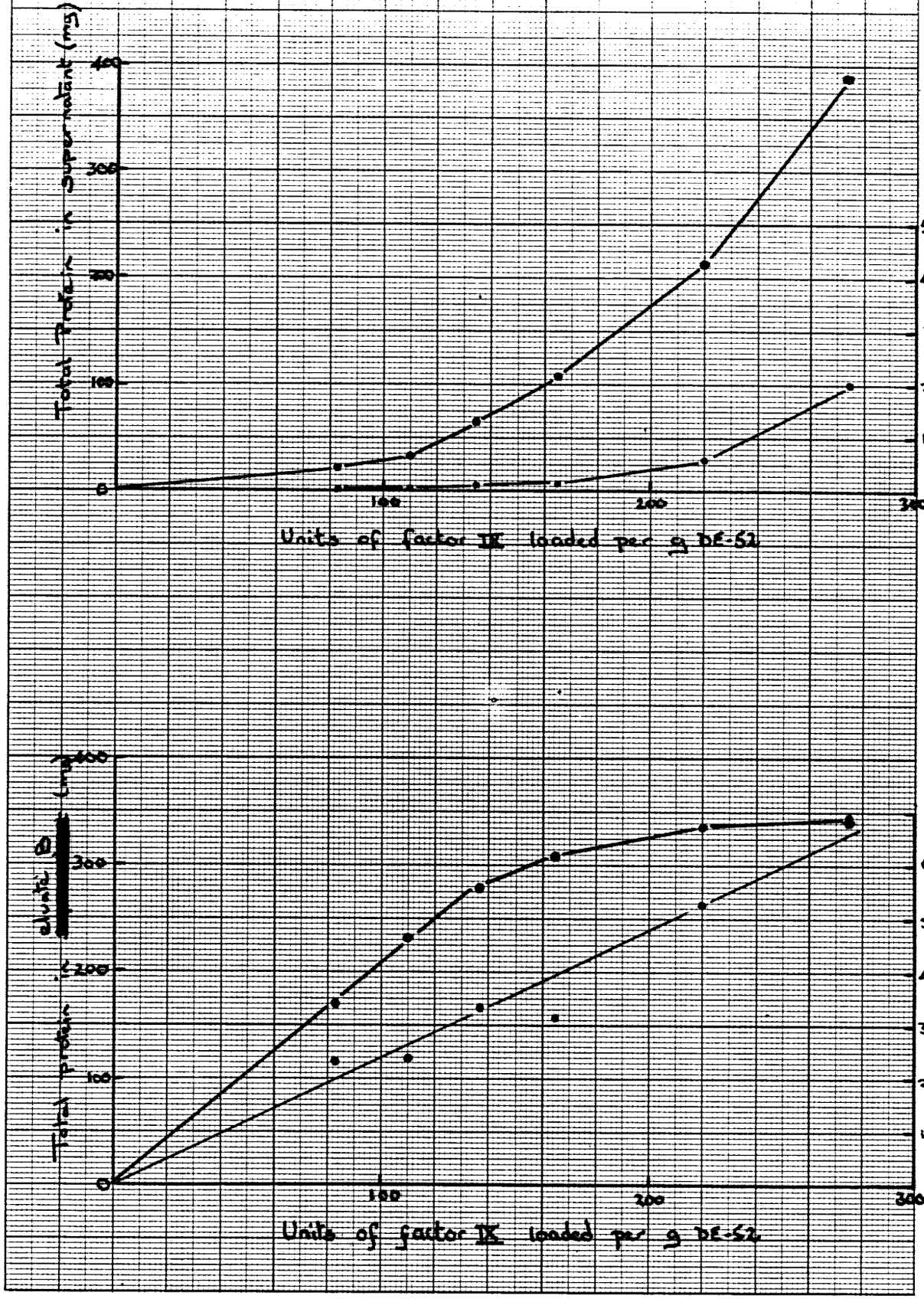
After the total protein eluted in B has stabilised and that in the Sn is increasing, the activity of factor IX in elute B continues to rise. This suggests that

1) DE-52 is totally saturated by protein upon addition of approx. 69 mg protein / g DE 52

2) Factor IX is being preferentially adsorbed, so when over-saturated the binding equilibria favour factor IX over other proteins present. Thus factor IX activity in buffer B elute continues to rise, after the total protein has stabilised.

9H7.2.5

Plots of total protein and total factor IX activity eluted in Sn and B against total units of factor IX loaded per gram of DE-52



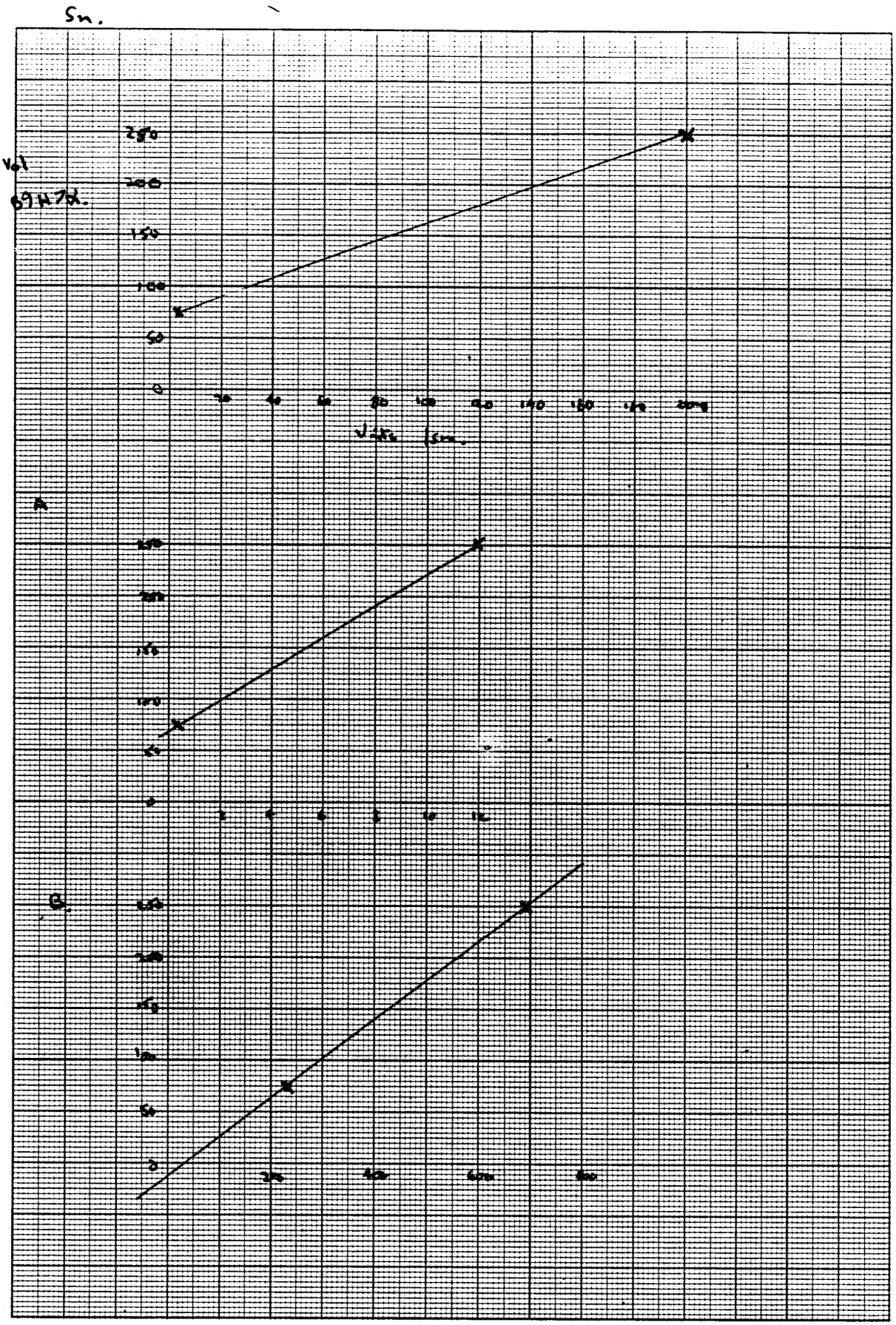
These results suggest an optimum loading of the α-partitioned factor IX on to DE-52 of about 160 units per gram of DE52. At this level there is minimal loss of factor IX in the supernatant (<10%). At higher levels of loading the supernatant loss rises sharply to ~30%, though there is apparent ~2-fold increase in purity of the B eluate.

Two comments arise; an additional difficulty in applying large volumes to small amounts of gel is a very tedious recovery due to a) the overall bulk and b) slow flow rates (possibly due to accumulation of sorbitol on the DE-52).

A more pressing problem is that the total activities loaded and recovered do not balance. At best 58% of the α-activity loaded is recovered. At worst this value drops to 40%. The question remains as to whether this is a genuine loss of activity by the chosen step alone, or whether it reflects a problem in the assay of the sorbitol-containing solutions. (it may be that initial dilutions by pipette are inaccurate due to the viscosity of 9H7 and 9H7α).

[Using the assumed starting value for 9H7α, from the previous set of experiments, of ~3, the recovery of total units are 106%, 81%, 91.6%, 73%, 99% and 120% for experiments 1, 2, 3, 4, 5 and 6 respectively.]

Method of estimating activities of 2-5.



INV.L

ASSAY REQUEST FORM (1)

Request from: PAF Date: 21/7/83

When results needed: ASAP

Samples to be kept? No. If so, how? _____

Samples provided with form/~~available from~~ _____

SAMPLE INVESTIGATION		B9H7	1 Sn	1A	1B	B9H7X	6 Sn
Factor VIII, iu/ml	1 st.						
	2 st.						
✓Factor IX, iu/ml	1 st.	(6.0)	(.02)	(.04)	(13)	(3.0)	(~~.08~~) (0.8)*
	2 st.	13.6	0.05	0.05	13.4	5.50	0.8
Factor II, u/ml							
Factor X, u/ml							
Factor VII, u/ml (clotting)							
AT III (Anti Xa), u/ml							
AT III (Anti IIa), u/ml							
AT III (Laurell), u/ml							
Factor VIII RAG, u/ml							
Fibrinogen (Laurell), mg/ml							
Prothrombin (Laurell), u/ml							
Factor XIIIA (Laurell), u/ml							
Fibronectin (Laurell), u/ml							
Factor VIII CAG							
✓NAPTT sec. 1/10		240	268	274	259	276	311
TGt50 min.							
FDA hr.							
Limulus + or -							

* May be wild.

Finished 27/7/83.

YNT. 2

ASSAY REQUEST FORM (1)

Request from: PAF Date: 25/7/83

When results needed: ASAP

Samples to be kept? No. If so, how?

Samples provided with form/~~available from~~

SAMPLE B9H70 – INVESTIGATION		2 Sn	2 B	3 Sn	3 B	4 Sn	4 B
Factor VIII, iu/ml	1 st.						
	2 st.		(17)				
Factor IX, iu/ml	1 st.	(0.3)	~~(0.5)~~	(0.5)	(22)	(0.55)	(35)
	2 st.	0.04	14.0	0.09	20.7	0.09	24.1
Factor II, u/ml							
Factor X, u/ml							
Factor VII, u/ml (clotting)							
AT III (Anti Xa), u/ml							
AT III (Anti IIa), u/ml							
AT III (Laurell), u/ml							
Factor VIII RAG, u/ml							
Fibrinogen (Laurell), mg/ml							
Prothrombin (Laurell), u/ml							
Factor XIIIA (Laurell), u/ml							
Fibronectin (Laurell), u/ml							
Factor VIII CAG							
NAPTT sec. 1/10		211	202	251	202	254	178
TGt50 min.							
FDA hr.							
Limulus + or –							

7H7.2

ASAR REQUEST FORM (1)

Request from: PAF Date: 25/7/83

When results needed: ASAR

Samples to be kept? No If so, how?

Samples provided with form/available from

SAMPLE B39H7α— INVESTIGATION		5Sn	5B	2A	3A	4A	5A
Factor VIII, iu/ml	1 st.			of	lesser	importance	(I hope)
	2 st.						
Factor IX, iu/ml	1 st.	(0.6)	(42.8)	(0.6)	(0.7)	(0.8)	(1.0)
	2 st.	0.3	37.8	0.10	0.14	0.21	0.83
Factor II, u/ml							
Factor X, u/ml							
Factor VII, u/ml (clotting)		—					
AT III (Anti Xa), u/ml							
AT III (Anti IIa), u/ml							
AT III (Laurell), u/ml							
Factor VIII RAG, u/ml							
Fibrinogen (Laurell), mg/ml							
Prothrombin (Laurell), u/ml							
Factor XIIIA (Laurell), u/ml							
Fibronectin (Laurell), u/ml							
Factor VIII CAG							
APTT sec. 1/10		221	204	243	217	226	225
TGt50 min.							
FDA hr.							
Limulus + or −							

9N7.

ASSAY REQUEST FORM (1)

Request from: PAF Date: 21/7/83

When results needed: ASAP.

Samples to be kept? No If so, how?

Samples provided with form/~~available from~~

SAMPLE 89N7 α — INVESTIGATION		6A	6B				
Factor VIII, iu/ml	1 st.						
	2 st.						
√Factor IX, iu/ml	1 st.	(0.1)* ~~NDN~~	(35)*				
	2 st.	1·4	49·3				
Factor II, u/ml							
Factor X, u/ml							
Factor VII, u/ml (clotting)							
AT III (Anti Xa), u/ml							
AT III (Anti IIa), u/ml							
AT III (Laurell), u/ml							
Factor VIII RAG, u/ml							
Fibrinogen (Laurell), mg/ml							
Prothrombin (Laurell), u/ml							
Factor XIIIA (Laurell), u/ml							
Fibronectin (Laurell), u/ml							
Factor VIII CAG							
√NAPTT sec. 1/10		280	275		(blank = 296)		
TGt50 min.							
FDA hr.							
Limulus + or −							

* May be wild.

Finished 22/7/83

29.7.83 Experiment 9H7-03 PFL.

Large scale DCL absorption/elution of pasteurized FIX using 150 in FIX per gram of DCL.

Experiment 9H7-02 showed that the binding capacity of DCL for pasteurized FIX was ≈ 160 in FIX/g of moist gel. This experiment is to examine the absorption and elution characteristics of the FIX at this higher FIX : DCL ratio

Materials and Methods.

The FIX solution was pasteurized on 8.7.83 (9H7-01) and was frozen at -40°C immediately afterwards.

Buffer A and B were from the standard FIX preparation and were excess to batch 9P1797. They had been stored at -40°C

The FIX was thawed by bring its temperature up to 30°C in a 35°C water bath.

960ml of solution was obtained (9H7/29.7.83). This was diluted with an equal volume of PFW giving ∝ 9H7/29.7.83 (1920ml). Two 2ml samples were removed for analysis of FIX. 4.45 in/ml of FIX were found and hence there was 8544 in of FIX in total in ∝ 9H7/29.7.83.

The pH of the FIX solution was measured and found to be 6.94 while the conductivity was 2.5mS.

57g of DCL was added to the mixture ($\frac{8544}{150} = 57$ ✓) and stirred at room temperature for 1 hour.

The loaded DCL was collected by centrifugation in the Beckman J.GB (4.2 krpm, 15 minutes, 20°C, brake=3.5)

The supernatant was decanted (9H7-03/Sn); its volume measured, and a 2ml sample was removed for analysis.

9H7-03

9H7-03

Materials and Methods Cont.

The DCL was resuspended in 85.5 ml (57 X 1.5) of buffer A and the suspension poured in a 50mm diameter chromatography column. The U.V. absorption and conductivity of the effluent were monitored and recorded (chart recorder settings :- chart 2mm/min, Conductivity scale 0.5V OD on 20mV). Pressure was applied to the top of the column by a Watson Marlow 501S pump set on 40. The flow rate was found to be 13ml/Minute.

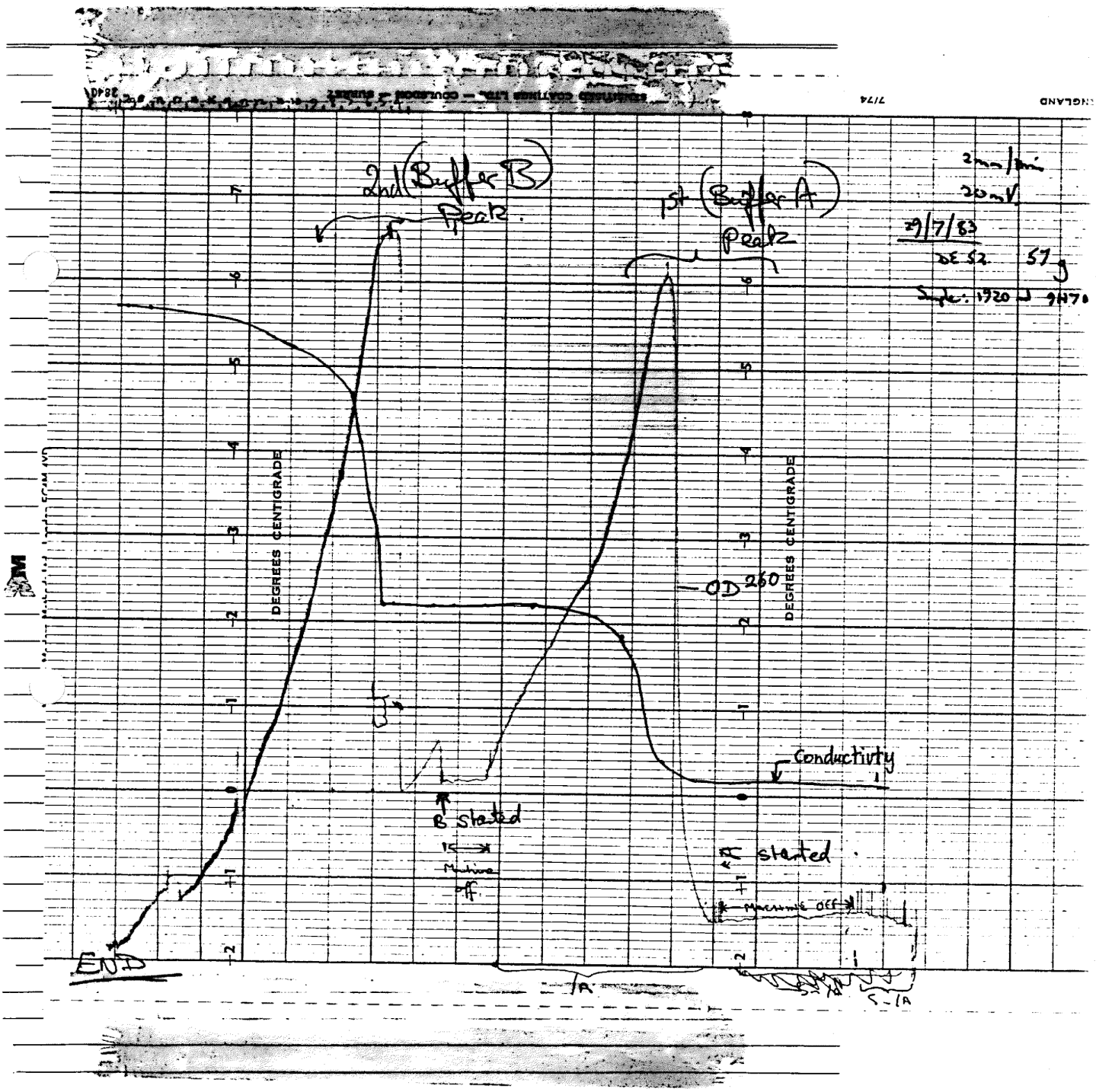
The effluent was collected as a single aliquot (1SnA) of 150ml. The flow was stopped to prevent the gel drying and the bed height was measured (80mm ∴ bed volume = 157ml).

The column was washed at first with 157ml of buffer A but a further 243ml of the buffer was used to bring the OD down to background levels. The effluent during the buffer A was was collected as a single aliquot (1A) its volume was measured (400mls) and it was sampled.

The column was washed with Buffer B until the eluted OD peak was down to background levels. Aliquots of various sizes were taken across the buffer B elution peak. These were sampled after their volumes were measured.

All samples were analysed for for FIX and NAPTT. Some samples were analysed for their total protein content.

9H7-03.3



Elution Profile of protein from DE-52 in 9H7-03.

9H703

Results and Discussion.

A peak in the OD_{280} of the effluent was obtained during the buffer A wash and also during the buffer B elution. There was a departure from the standard use of single column volumes of the buffers because it required more than those volumes to pass through the gel bed before the OD trace returned to background levels.

The Results are shown on the recorder trace included here and in the following table :-

Sample	Volume (ml)	iu FIX/ml	Total FIX in pool (iu)	NAPTT	Total Protein mg/ml	iu FIX per mg/ml Protein
9H7/29.7.83	960	8.4	8064	–	–	–
∝9H7/29.7.83 (1st)	1920	4.45	8544	–	–	–
∝9H7/29.7.83 (2nd)	1920	3.15	6048	255	–	–
/Sn	1760	0.09	158.4	296	–	–
/SnA	105	0.08	8.4	275	–	–
/A	400	3.0	1200	266	2.00	1.5
/B1	68	1.39	94.52	272	–	–
/B2	25	38.4	964	204	17.3	2.2
/B3	100	37.2	3720	214	11.9	3.1
/B4	175	5.8	1015	254	2.24	2.6
/B5	160	0.92	147.2	275	–	–
				control 281		

The values for the amount of FIX/ml in ∝9H7 solution show a large discrepancy (4.45 iu and 3.15 iu). It is not clear why this difference should be so great. The figures obtained for the FIX levels in 9H7 measured on 8.7.83 and ∝9H7/8.7.83 also differ from the results obtained in this experiment (6.9 iu/ml and 2.7 iu/ml respectively). These differences in the assay results may be due to lack of correct mixing of the thawed samples where denser material sinks to the bottom of the sample. This will be investigated in a later experiment.

The recovery of FIX will be expressed in terms of the original 9D2232/F7.83 (allowing for dilutions) and also the 1st and second results obtained for ∝9H7/29.7.83 as well as the result for 9H7/29.7.83.

9H7-03.5

9H7-03

Sample	Total units used/recovered	% used/recovered based on 9D2232	% used/recovered based on 9H7/29.7.83	% used/recovered based on αH7/29.7.83 (1st)	% used/recovered based on αH7/29.7.83 (2nd)
9D2232	16032	100			
9H7/29.7.83	8064	50.3	100	94.3	133
α9H7/29.783 1st	8544	53.3	105.9	100	141
α9H7/29.7.83 2nd	6048	38.0	75	70.7	100
/Sn	158.4	0.99	1.9	1.8	2.6
/SnA	8.4	0.05	0.1	0.08	0.14
/A	1200	7.5	14.8	14.0	19.8
/B1	94.52	0.6	1.2	1.1	1.5
/B2	964	6.0	11.9	11.2	15.9
/B3	3720	23.2	46.1	43.5	61.5
/B4	1015	6.3	12.6	11.8	16.7
/B5	147.2	0.9	1.8	1.7	2.4
Total % in /Sn-/B5		45.54	92.3	85.2	120.5
Total % in /B1-/B5		37.0	73.6	69.3	98

% Recovery of FIX based on the different results of analysing various chosen starting materials.

After allowing for dilution there is good agreement in the % recoveries based on 9D2232, 9H7/29.7.83 α9H7/29.7.83 (1st), α947/29.7.83 (2nd) does not give good agreement with the other analyses. This probably reflects the difficulty of obtaining consistent results from the assays when sorbitol is present.

Generally the results show that approx 45% of the original starting FIX may be accounted for after the column step. The 45% represents 85-92% of the FIX remaining after the heating step. ∴ there is about 10% of the FIX which we cannot account for.

The FIX recovered in /B1-/B5 ie expected usable elution, is 37% of the starting unheating FIX and 69-74% of the FIX applied to the DEAE column

9H7-03

9H7-03

Results and Discussion Cont

About 2% of the FIX applied to the column remained unbound and appeared in the supernatant. This may be due to saturation of the DCL or perhaps to FIX which is still active, but cannot bind to the DCL.

The NAPTT remained above 200 sec in all of the samples analysed. It was longest in the DCL supernatant and in this sample retarded with respect to the "blank" (296 cf 281). The shortest NAPTT is associated with the front part of the FIX eluted with Buffer B (/B2 cf /B3). The NAPTT increased as the amount of FIX decreased at the end of the Buffer B elution. It did not rise above that of the blank.

The significance of the NAPTT variation is not clear at this stage and further experimentation is required to ascertain the general pattern.

Conclusion.

It is possible to bind practically all of the FIX from a solution into DCL when the ratio of FIX to DCL is 150 iu FIX to 1g of DCL. It is possible to recover most of this from the DCL using buffer A and buffer B from the std FIX preparation.

Points for the Future.

It may be possible to reduce the 50% loss of FIX on the heating step by stirring the FIX solution to give a shorter heat up period.

We may be able to reduce the 11% of FIX eluted by buffer A by lowering the salt in the buffer.

The 10% of the FIX which is unaccounted for may be left on the column after the buffer B elution. If this is the case, then we may be able to elute it if the salt in buffer B is increased or by the use of buffer C of the std. FIX preparation.

9H7.03

ASSAY REQUEST FORM (1)

Request from: PAF Date: 1/3/83

When results needed: 4/8/83 **GRADE I**

Samples to be kept? Yes If so, how? Frozen

Samples provided with form/~~available from~~ _____

SAMPLE INVESTIGATION α9H7.3		α9H7	Sn	Sn/A	A	/B1	/B2
Factor VIII, iu/ml	1 st.						
	2 st.						
Factor IX, iu/ml	1 st.	(4.5)	(0.08)	(<0.01)	(0.2)	(<0.01)	(33.4)
	2 st.	3.2 3.1	0.09	0.08	3.0	1.39	38.4
Factor II, u/ml							
Factor X, u/ml							
Factor VII, u/ml (clotting)		--					
AT III (Anti Xa), u/ml							
AT III (Anti IIa), u/ml							
AT III (Laurell), u/ml							
Factor VIII RAG, u/ml							
Fibrinogen (Laurell), mg/ml		--					
Prothrombin (Laurell), u/ml							
Factor XIIIA (Laurell), u/ml							
Fibronectin (Laurell), u/ml							
Factor VIII CAG							
NAPTT sec. 1/10		255	296	275	266	272	204
TGt50 min.							
FDA hr.							
Limulus + or -							

9H7-03

ASSAY REQUEST FORM (1)

Request from: PBF Date: 3/3

When results needed: 4/5/83 (ASAP)

Samples to be kept? Yes If so, how? frozen

Samples provided with form/~~available from~~

SAMPLE / INVESTIGATION		∝9H7.3	/B3	/B4	/B5			
Factor VIII, iu/ml	1 st.							
	2 st.							
Factor IX, iu/ml	1 st.		(26.9)	(8.3)	(0.5)			
	2 st.		37.2	5.8	0.92			
Factor II, u/ml								
Factor X, u/ml								
Factor VII, u/ml (clotting)								
AT III (Anti Xa), u/ml								
AT III (Anti IIa), u/ml								
AT III (Laurell), u/ml								
Factor VIII RAG, u/ml								
Fibrinogen (Laurell), mg/ml								
Prothrombin (Laurell), u/ml								
Factor XIIIA (Laurell), u/ml								
Fibronectin (Laurell), u/ml								
Factor VIII CAG								
NAPTT sec. 1/10			214	254	275		(control)	281
TGt50 min.								
FDA hr.								
Limulus + or -								

9N7-03

ASSAY REQUEST FORM (1)

Request from: PAF Date: 9/7/83

When results needed: YESTERDAY

Samples to be kept? No If so, how?

Samples provided with form/~~available from~~

SAMPLE INVESTIGATION		9N7	X9N7				
Factor VIII, iu/ml	1 st.						
	2 st.						
Factor IX, iu/ml	1 st.	(10)	(4.5)				
	2 st.	8.4	4.45				
Factor II, u/ml							
Factor X, u/ml							
Factor VII, u/ml (clotting)							
AT III (Anti Xa), u/ml							
AT III (Anti IIa), u/ml							
AT III (Laurell), u/ml							
Factor VIII RAG, u/ml							
Fibrinogen (Laurell), mg/ml							
Prothrombin (Laurell), u/ml							
Factor XIIIA (Laurell), u/ml							
Fibronectin (Laurell), u/ml							
Factor VIII CAG							
NAPTT sec. 1/10							
TGt50 min.							
FDP hr.							
Limulus + or -							

m/11 Amg

9H7-0

ASSAY REQUEST FORM (2)

Request from: PAF Date: 10/8/83

When results needed: ASAP

Samples to be kept? No If so, how?

Samples ~~provided with form~~/available from: R+D Freezer.

SAMPLE INVESTIGATION		∝9H7	∝9H7 /A	∝9H7 /B2	∝9H7 /B3	∝9H7 /B4	
Protein	E280	(2.5)	(3)	(15)	(10)	(3)	
✓	biuret g/l		2.00	17.3	11.9	2.24	
Fibrinogen g/l/%							
Sodium mmol/l							
Potassium mmol/l							
Chloride mmol/l							
Citrate mmol/l							
Phosphate mmol/l							
Tris mmol/l							
PEG g/l							
Caeruloplasmin g/l							
Factor XIII u/ml							
PKA % Ref. 2							
AT III (amidolytic) u/ml							
F. VII (amidolytic) u/ml							
XaGT mins.							
pH at 20°C							
Conductivity at 20°C							

For comments contact: JP. Date finished: 10/8/83

9.3.1. HEAT INACTIVATION OF VIRUSES IN FACTOR IX CONCENTRATES

(1) Background

Viral infection has long been recognised as the major risk in the use of human blood plasma concentrates. Though many viruses are destroyed during routine concentrate production, those causing hepatitis (types A, B, and Non-A non-B) are particularly resistant. While antigenic markers have been identified for virus types A and B, thus permitting the screening of potential plasma donors, none are known for NANB hepatitis, which may in fact be due to more than one virus.

Current use of factor IX concentrates results in NANB hepatitis of varying severity in 70%+ of new ("virgin") recipients[1]. The actual level of infection may be even higher, since symptoms of infection can be sub-clinical. This high level of infection may be restraining the clinical use of factor IX concentrates for treatment of a wider range of conditions.

Though hepatitis appears to be the predominant surviving virus from the production scheme, other viruses may also survive the process (as exemplified by the transmission of AIDS to haemophiliacs, via clotting factor concentrates, suggesting that AIDS may have a viral cause[2]).

Work by Scatchard et al (1945)[3] and Gellis et al (1948)[4] on albumin shows that the hepatitis virus can be considerably reduced by heat treatment of 60°C for 10 hours, while the protein retains its properties by the addition of stabilisers. More recently, virucidal agents (e.g. β-propiolactone) have been added and the concentrates irradiated with UV light as an alternative to heat treatment[5].

(2) Aims

This project has the primary aim of reducing the level of viral contamination of factor IX concentrates by heat treatment. This method is initially the most attractive because:

(i) preliminary tests suggest it to be feasible
(ii) such a process can most easily be incorporated into existing production schemes
(iii) alternative processes are more costly (UV irradiation), more hazardous (use of virucides) and technically complex (precipitation followed by protein recovery)[6].

Since virus destruction by heat decreases with time logarithmically, the most realistic aim is to reduce the level to below that which causes infection. This will be attained by the achievement of the following goals:

(a) The establishment of a heat treatment after which the factor IX concentrate will possess a "high level" of its original, untreated activity, and a low degree of activation (i.e. thrombogenicity).

(b) The demonstration that such a heat-treated product has been successfully depleted of measurable virus markers.

(c) The successful transfer of such a product from intermediate scale production to the routine production process.

(d) The successful clinical testing of such a concentrate against previously untreated patients.

(3) Scope

As this project is based upon the principle that heat treatment will destroy viral contaminants, the bulk of the project will be directed towards a variety of methods for improving the yield of active factor IX after the treatment. While this forms the first course of action, should it prove ineffective then re-evaluation of chemical virucidal and precipitation procedures may be necessary.

If successful, there may present the opportunity for manufacturing a wide variety of safer concentrates, as well as improved yields at some stages of the factor IX concentrate production.

This may be a necessary development, since the availability of a much safer factor IX concentrate will increase demand due to more varied use.

(4) Means

Existing expertise in the use of sorbitol/glycine addition to the concentrate to stabilise the factor IX will be developed further. Within this area, the optimal concentrations of stabilisers will be further investigated. However, the stabiliser may be protecting the viruses from heat as much as the protein. Furthermore, the heating conditions may be denaturing an unnecessarily large amount of protein. The project can therefore be divided into two sections, relating to (a) development of a heat-treated, high-yield factor IX concentrate using sorbitol-glycine stabilisers and (b) investigation of alternative methods of heat treatment.

(a) Development of a heat-treated, high-yield concentrate by sorbitol/glycine

This section of work will follow two lines of investigation covering heating and protein recovery after heating.

(i) Heating

The heating conditions currently applied (60^{o}C for 10 hours) are based upon the success of such a process with albumin over 30 years. However, albumin is a more stable protein, so better yields for factor IX may be achieved by altering the temperature and duration of heating.

(ii) Protein Recovery

To render the heated material more manageable, the sorbitol concentration has to be reduced, conventionally by dilution. This also dilutes the factor IX. To recover the factor IX by ion-exchange chromatography a high capacity anion-exchange gel is desirable, to improve the total yield and also to reduce the total gel volume, thus increasing the concentration of eluted factor IX. This serves to compensate for the earlier dilution step, though the eluting salt concentration must be carefully controlled. To this end, a variety of conventional and newly available anion exchangers will be compared.

There exists an alternative, or possibly additional, procedure in membrane filtration as a means to remove sorbitol and concentrate factor IX in one process. However, this technique is generally cumbersome to use with highly viscous media and large amounts of protein are conventionally lost by irreversible binding to the membrane. For these reasons I do not favour this method unless it becomes a necessity.

(b) Alternative Methods of Heat Treatment

Several procedures may be developed and investigated which avoid the problems of handling sorbitol/glycine mixtures at high concentrations. These are set out below.

(i) Dry heating

In the freeze-dried state, proteins are less susceptible to denaturation. While it can be assumed that viruses are similarly stabilised, heat treatment of the dried product is attractive. The treatment of a sealed, freeze-dried material may also permit the temperature or incubation time to be reduced, due to some increase in pressure within the vial.

(ii) Gel heating

An intermediate level of stabilisation may be obtained by heating factor IX while it is bound to anion-exchange resin. Under these conditions the protein will be partially immobilised but still hydrated. The advantages of such a procedure lie in the small volumes of high-capacity resin which would require heating, and the potential for the heat step to run concurrently with the basic factor IX concentrate preparation.

(iii) Gel filtration

Unlike the large molecular sizes of some other clotting factors, factor IX (and II and X) have molecular weights of 50,000-70,000. The separation of these proteins from virus particles (with molecular weights $\approx 10^6$) should therefore be possible by gel filtration. The disadvantages of such a method are dilution and volume as well as the need for a 10^6 reduction in virus concentration - a requirement which may be beyond the limits of gel filtration.

(iv) Alternative specific stabilisers

An unusual property of coagulation factors in factor IX concentrate is their ability to bind divalent metal cations. Various reports of the stabilising efficacy of high calcium concentration and high citrate concentration[7] would appear contradictory. Further investigation should clarify which, if either, additive is most effective. A development of this principle may be to attain added stability by the presence of long chain polymers, bound to the protein through a calcium bridge. The advantage of this method lies in the specificity of calcium ion binding, which may preferentially protect the factor IX over the virus. The disadvantage is the risk of factor IX activation by calcium, which should be carefully monitored.

(5) Resources Required

As described above, conventional existing procedures will be applied to this project. While the outcome of particular experiments may recommend the purchase of new anion-exchange gel, most resources will be reflected as an increase in the use of everyday laboratory consumables.

The major weakness of this and other similar studies is the assumption that viral contamination will be reduced by heat treatment. While all the above studies are aimed at exposing the concentrate to heat and retaining the properties of native material, once such an active concentrate is obtained a study of the viral content will be necessary. To this end the experience and expertise of a virologist is essential, accompanied by the techniques of viral assay. Such does not exist at PFL, may exist at BPL or may require outside advice and/or assistance.

P.A.F.

4.8.83.

References

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3. Scatchard, G., Strong, L.E., Hughes, W.L., Ashworth, J.N. and Sparrow, A.H. (1945) Chemical clinical and immunological studies on the products of human plasma fractionation XXVI. J. Clin. Invest. 24, 671-679.

4. Gellis, S.S., Neefe, J.R., Stokes, J., Strong, L.E., Janeway, C.A. and Scatchard, G. (1948) Chemical clinical and immunological studies on the products of human plasma fractionation XXXVI. J. Clin. Invest. 27, 239-244.

5. Prince, A.M., Stephan, W., Brotman, B., van den Ende, M.C. (1980) Evaluation of the effect of β-propiolactone/ultraviolet irradiation treatment of source plasma on hepatitis transmission by factor IX complex in chimpanzees. Thromb. Haemostas. 45, 138-142.

6. Johnson, A.J., Semar, M., Newman, J., Harris, R.B., Brandt, D., Middleton, S. and Smith, J. (1976) Removal of hepatitis B surface antigen from plasma fractions. J. Lab. Clin. Med., 88, 91-101.

7. Tabor, E., Murano, G., Snoy, P., Gerety, R.J. (1981) Inactivation of hepatitis B virus by heat in AT III stabilised with citrate. Thromb. Res. 22, 233-238.

IRMA 10-8-83

		Protein Aprox. (mg/ml)	FIXC u/ml	FIX Ag u/ml
9H7.03	A	2.9	3.0	5.50
	B_2	16.2	38.4	87.51
	B_3	12.7	37.2	93.59

Sample	FIXC/FIX Ag	% ^{Ag} Units = $A+B_2+B_3$	% C Units = $A+B_2$
A	0.54	2.9	3.8
B_2	0.44	46.9	48.8
B_3	0.80	50.15	47.3

Sample	Vol	Total C units	Total Ag units	% C units	% Ag Units
A	400	1200	3200	20.4	16.0
B_2	25	960	2187	16.3	15.9
B_3	100	3720	9359	63.2	68.1

0.6 I.U. = 1 P.U.

∴ Converting FIXC I.V. to FIXC plasma units.

	FIXC (u/ml)	∴ Total P.U.		Total Ag units	
A	5.0		2000		2200
B_2	64	1986	1600	7800	2187	11,546
B_3	62		6200		9359

9H7 : {6048 / 8594} u (~ 50% yield) ∴ starting IX : 12096 u
∴ ∴ 17088 u.

IgG 1/1000 monoclonal

2 - B
2 - M.C.
12 - Standard
6 - each sample

25
×6
150
16
166

2.6 ml
4°C
washing buffer

plasma dilutions
buffer
0.4 ml
1/2 h. / 23°C

labelled IgG
0.4 ml
12 h / 23°C

Pagam

20 µl

H_2O

count

1/1 1/2 1/4

5 ml

29/7/83 9H8 To test the effect of sorbitol and glycine upon factor IX binding to DE-52, before and after heating

The purpose of this experiment is to determine whether the addition of sorbitol/glycine to factor IX modifies the binding of factor IX to DE-52 in its own right, quite apart from any effect due to heating.

Materials

Buffers: factor IX production buffers A and B were used. These have NaCl concentrations of 0.1 M and 0.25 M respectively.

Factor IX: 1 vial of 9H 3232 was reconstituted in 35 ml PFW. This material is a pyrogen failure and contained no heparin.

DEAE-Cellulose: Recycled and frozen DE-52 was thawed at room temperature overnight

Method

4 x 8 ml aliquots of factor IX concentrate were treated as follows, after designation of A, B, C + D to them.

A: + 0.8 g glycine + 10.4 g sorbitol Final Vol.: 15 ml
B: " " " " " "
C: PFW added to equivalent final volume as A (47 ml) Final Vol.: 15 ml
D: " " " " " " B (47 ml) " " "

All samples were used in plastic universals, and subjected to the following regimen:

A: Heated at 60°C for 10 hours
B: Kept at room temperature for 10 hours
C: Heated at 60°C for 10 hours
D: Kept at room temperature for 10 hours

Heated samples were placed in the water bath at 60°C and the heater automatically switched off after 10 hours. Samples remained in the water bath (now cooling down) for 5 hours. Final temp: 34

All four samples were then diluted ½ by addition of PFW

Based upon an assumed 5.5 u/ml, giving a Total of 165 u,
each sample was added to 1g DE-52 and stirred for 1 hour at room temperature.

The mixture was then poured into a 1.5 cm I.D glass column. After the gel had packed the bed height was measured and 1 column bed volume of Buffer A was washed through it. The eluate was collected in one pool.

The bed volume was re-measured, and 1.5 bed volumes of buffer B were passed through it. The eluate was again collected in one pool.

Samples: Four samples were taken from each run, and were named:

9H8-A, -B, -C, -D : Heated, diluted material before adsorption to DE-52.

/S Material eluted during packing of the column
/A Material eluted during application of Buffer A
/B Material eluted during application of Buffer B

Results

Sample	Vol. (ml)	Factor 1x (u/ml)	Total Units	% starting Material	% Yield from equivalent control
(Normal) -B	30	6.3	189	100	
/Sn	26	0.05	1.3	0.69	
/A	2	3.9	7.8	4.1	
/B	3	37	111	58.7	
(Normal Heated) -A	30	<0.02	<0.6		0
	26	<0.02	<0.52		0
	2	<0.02	<0.04		0
	3	0.05	0.15		0.13
(Sorbitol) - D	30	8.9	267	100	
/Sn	25	0.07	1.75	0.65	
/A	2.2	4.9	10.78	4	
/B	3.3	63.2	208	78.1	
(Sorbitol Heated) -C	30	4.8	144	100	53.9
/Sn	25	0.05	1.25	0.09	71.4
/A	2.2	2.5	55	3.8	51
/B	3.2	17.4	55.7	38.7	26.8

Step Yield

Yield from Unheated

NAPTT's were also measured:

Sample	NAPTT (sec) 1/10
Normal - B	271
Heated Normal - A	267
Sorbitol - D	224
Heated Sorbitol - C	306

Comments :

1) The normal control gives a yield of IB of only 58.7 %. The evidence of experiment 9H7-03 suggests that this is due to insufficient elution buffer B. Using 1 1/2 bed volumes appears to elute only ~ 2/3 of the factor IX activity

2) Heating of factor IX without any stabilisers does indeed cause total loss of factor IX activity

3) The presence of sorbitol and glycine appears to increase factor IX activity prior to any heat treatment by 41 %. This may be genuine or may be due to suppressed factor IX activity in the untreated material (as a result of pH change and loss of buffering capacity following dilution into PFW)

4) The same quantity of buffer B eluted a far greater percentage of the factor IX starting material (78% as compared with 58.7). This does suggest that the sorbitol / glycine itself is modifying the factor IX binding (probably the glycine altering the pH)

5) The sorbitol heated material shows 54% of the unheated material. This recovery is of the same order as obtained in previous experiments.

6) The percentage recovery of heated factor IX in elution B drops to 38.7 % - only half the recovery of the unheated material. This is a genuine drop in capacity, as the percentage is AFTER heat-treatment. This may suggest that heated, denatured factor IX binds 26-52 more tightly than active factor IX, thereby displacing it. However, if this were true, I would expect to see the displaced factor IX eluting in the IS or IA fractions. This is not observed.

Discussion

Addition of sorbitol / glycine appears to modify the assay, probably through a pH effect. This causes problems in assessing the recovery as can be seen below:

Starting 9D	189	Units
+Sorbitol / Glycine	267	Units
After heating	144	Units

Yield upon heating:

From starting material:	76.2 %
From sorbitol / Glycine	53.9 %

Recovery from DE52 55.7 Units

Yield upon recovery:

From heated material	38.7 %
From sorbitol / glycine	20.9 %
From starting material	29.5 %

These results are a cause of concern, suggesting a gross miscalculation in the conditions used, particularly to recover the heated factor IX. This experiment should therefore be repeated, with the following modifications:

1) The normal control should be diluted into buffer for the parallel dilutions to the sorbitol samples.

2) Each sample should be loaded on to DE-52 in two loadings - 75 U/g of gel and 150 U/g of gel

3) Factor IX should be eluted in 2.5 column bed volumes of buffer B.

4) Buffer A should be applied in 2 column bed volumes.

P.A.F.

ASSAY REQUEST FORM (1)

9H8

Request from: PAF Date: 9/8/83

When results needed: ASAP Grade 2

Samples to be kept? No If so, how?

Samples ~~prescribed with form~~/available from R+D Freezer

SAMPLE INVESTIGATION 9H8		A*	A/Sn	A/A	A/B		
Factor VIII, iu/ml	1 st.						
	2 st.						
✓ Factor IX, iu/ml	1 st.	(7.5)	(0.5)	(<0.05)	(33)		
	2 st.	4.8	0.05	2.5	17.4		
Factor II, u/ml							
Factor X, u/ml							
Factor VII, u/ml (clotting)							
AT III (Anti Xa), u/ml							
AT III (Anti IIa), u/ml							
AT III (Laurell), u/ml							
Factor VIII RAG, u/ml							
Fibrinogen (Laurell), mg/ml							
Prothrombin (Laurell), u/ml							
Factor XIIIA (Laurell), u/ml							
Fibronectin (Laurell), mg/ml							
Factor VIII CAG							
→ NAPTT sec. 1/10		✓ 267					
TGt50 min.							
FDA hr.							
Limulus + or -							

* Contains ~ 25% sorbitol

For comments contact Date finished:

ASSAY REQUEST FORM (1)

9 H8

Request from: PAC Date: 9/8/83

When results needed: ASAP GRADE 2

Samples to be kept? No If so, how?

Samples ~~provided with form~~/available from PwD Freeze

SAMPLE INVESTIGATION		9H8 B*	B/Sn	B/A	B/B		
Factor VIII, iu/ml	1 st.						
	2 st.						
✓ Factor IX, iu/ml	1 st.	(7.0)	(3.8)	(3.0)	(35)		
	2 st.	89	0.07	49	63.2		
Factor II, u/ml							
Factor X, u/ml							
Factor VII, u/ml (clotting)							
AT III (Anti Xa), u/ml							
AT III (Anti IIa), u/ml							
AT III (Laurell), u/ml							
Factor VIII RAG, u/ml							
Fibrinogen (Laurell), mg/ml							
Prothrombin (Laurell), u/ml							
Factor XIIIA (Laurell), u/ml							
Fibronectin (Laurell), mg/ml							
Factor VIII CAG							
→ NAPTT sec. 1/10		✓ 271					
TGt50 min.							
FDA hr.							
Limulus + or -							

* Contains Sorbitol ~ 25 %.

For comments contact Date finished:

ASSAY REQUEST FORM (1)

PNF

Request from: PNF Date: 9/8/83

When results needed: ASAP, GRADE 2

Samples to be kept? No If so, how?

Samples ~~provided with form~~/available from R+D Freezer

SAMPLE INVESTIGATION	9H8	C	C/Sn	C/A	C/B		
Factor VIII, iu/ml	1 st.						
	2 st.						
Factor IX, iu/ml	1 st.	(0)	(0)	(0)	(0)		
	2 st.	<0.02	<0.02	<0.02	0.05		
Factor II, u/ml							
Factor X, u/ml							
Factor VII, u/ml (clotting)							
AT III (Anti Xa), u/ml							
AT III (Anti IIa), u/ml							
AT III (Laurell), u/ml							
Factor VIII RAG, u/ml							
Fibrinogen (Laurell), mg/ml							
Prothrombin (Laurell), u/ml							
Factor XIIIA (Laurell), u/ml							
Fibronectin (Laurell), mg/ml							
Factor VIII CAG							
NAPTT sec. 1/10		✓ 306					
TGt50 min.							
FDA hr.							
Limulus + or -							

For comments contact Date finished:

ASSAY REQUEST FORM (1)

9H8

Request from: PAF. Date: 9/8/83

When results needed: ASAP GRADE 2

Samples to be kept? No If so, how?

Samples ~~provided with form~~/available from R+D Freezer

SAMPLE INVESTIGATION 9H8		D	D/Sn	D/A	D/B		
Factor VIII, iu/ml	1 st.						
	2 st.						
✓ Factor IX, iu/ml	1 st.	(7.0)	(3.8)	(3.0)	(35)		
	2 st.	6.3	0.05	3.9	37.0		
Factor II, u/ml							
Factor X, u/ml							
Factor VII, u/ml (clotting)							
AT III (Anti Xa), u/ml							
AT III (Anti IIa), u/ml							
AT III (Laurell), u/ml							
Factor VIII RAG, u/ml							
Fibrinogen (Laurell), mg/ml							
Prothrombin (Laurell), u/ml							
Factor XIIIA (Laurell), u/ml							
Fibronectin (Laurell), mg/ml							
Factor VIII CAG							
→ NAPTT sec. 1/10		✓ 224					
TGt50 min.							
FDA hr.							
Limulus + or -							

For comments contact Date finished:

9H9

Large Scale Absorption/Elution of FIX using 150 iu FIX/g moist DCL.

Materials and Methods.

FIX — 9D2234 containg 3 units of heparin per ml. This was supplied freeze dried in 70 vials. There were 600 iu FIX per vial and 20ml of PFH were used to reconstitute each vial. Therefore it was expected that 1400ml of 9D2234 would be obtained and that this would contain a total of 42000 iu of FIX.

9H9-01 Cont.

Spin 4.2K 15min.

Gel resuspened in 84 ml buffer A.

5100ml of Sn recovered.

Chart - U.V. 10mV. speed 2mm/min.

Cond 0.5V

bed height = 74 cm

∴ Vol = 19.6 × 145.3 ml

A 145ml + 145ml + 145 ml

B 145ml + 145ml + 145

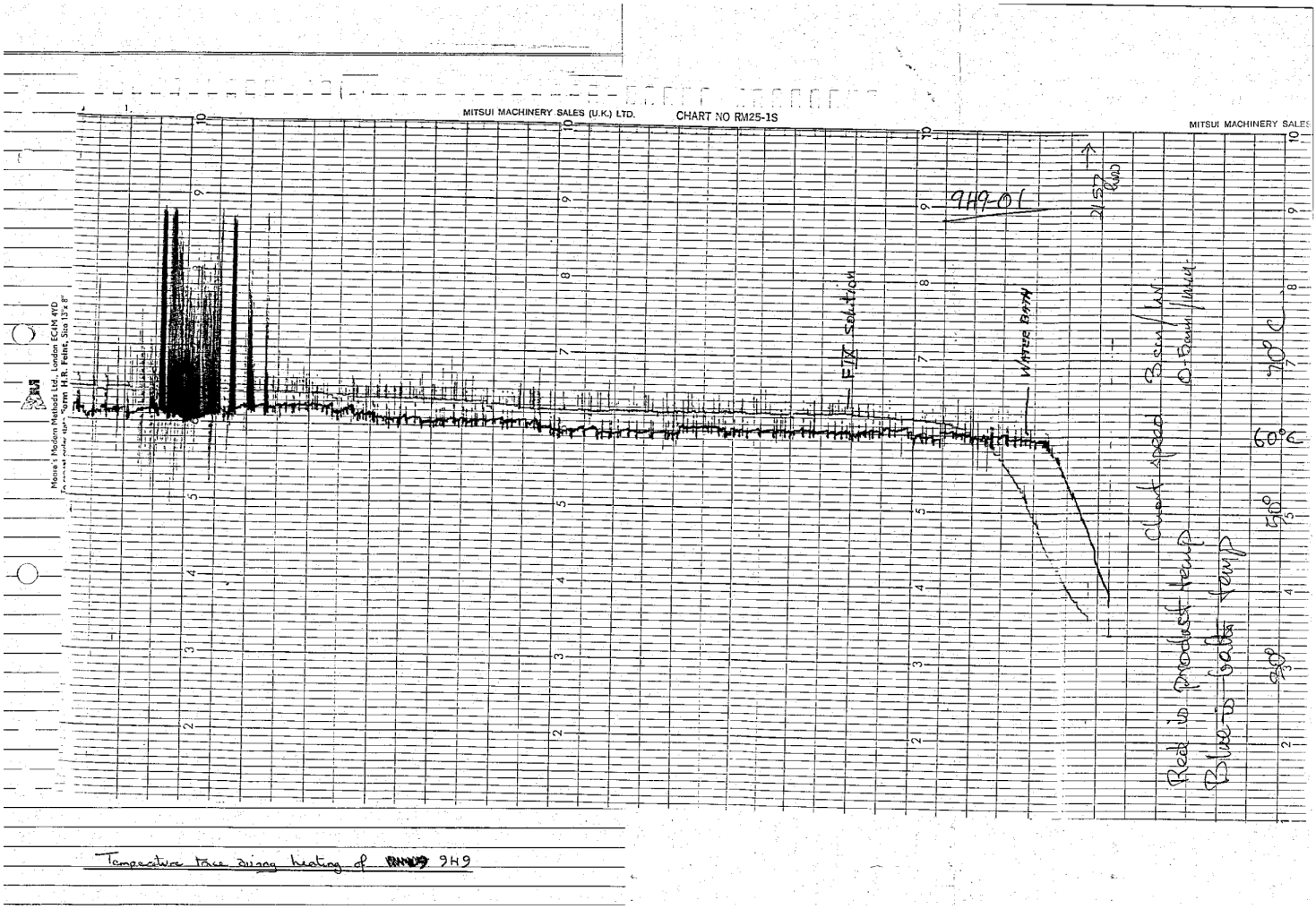
	Vol
9D 2234	1400ml
9D2234/S	2800
9H9/5.8.83	2650
∝9H9 5.8.83	~~5300~~ 5300
/Sn	5100
/SnA	120
A1	200
A2	235
A/B	72ml
B1	185
B2	190

15 → 75

80 → 100

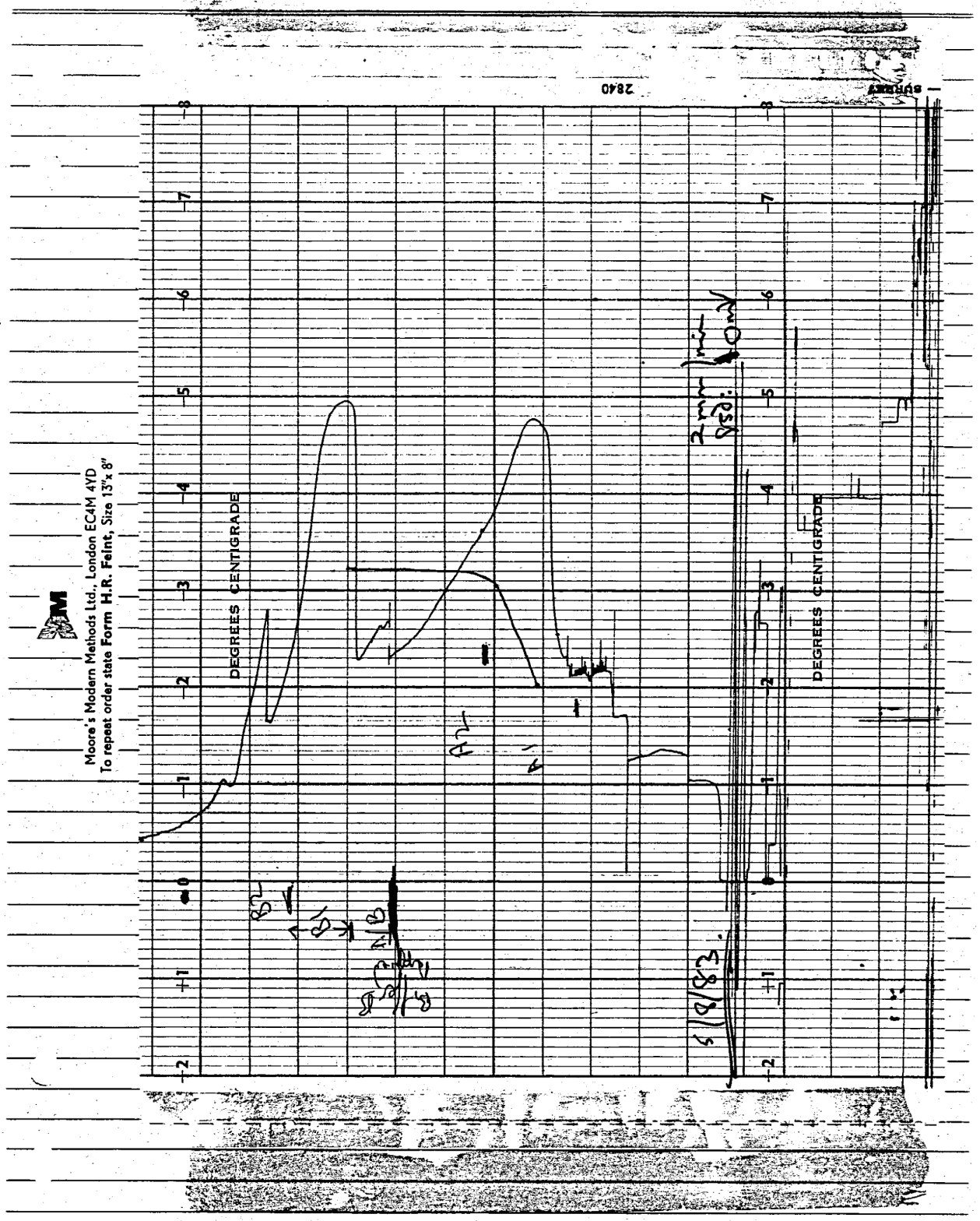
4:20 → 100

42



Temperature trace during heating of 9H9

9H9.4



Elton profile of 9H9 from DE 52.

9H9.5.

Summary of results

Sample	Vol (ml)	[Protein] (mg/ml)	Factor u/ml	Tx total	Sp Act	% Recovery from 9D	from α	NAPTT to (s)
9D2234	1400		35.5	49700		100		230
/S	2800		20.4	57120		114.9		228
9H9	2650		3.1 4.0	94075		18.9		300
~~α9H9~~								
α9H9	5300	1.28	1.7 1.9	9540	1.41	19.2	100	281
/S$_n$	5100		0.42	2142		4.3	22.4	259
/S$_n$A	120		0.5	60		0.1	0.6	251
/A1	200	9.65						267
/A2	235	3.53	2.8	658	0.79	13.2	6.9	262
/AB	72		1.6	115.2		0.2	1.2	261
/B1	158	14.5	14.9	2354	1.03	4.7	24.7	283
/B2.	190	1.14	0.67	127.3	0.59	0.2	1.3	270

Possible reasons for poor yield:

1) Heating temperature too high
2) α9H9 was not pH or conductivity adjusted before adsorption
3) pH of 10 before heating was wrong

ASSAY REQUEST FORM (1)

949

Request from: PAF / DCA Date: 8/8/83.

When results needed: 11/8/83.

Samples to be kept? _____ If so, how? _____

Samples provided with form ~~available from~~ _____

SAMPLE / INVESTIGATION		① 90 2234	② 90 2234 /S	③ 9H9	④ α9H9	⑤ [redacted]	
Factor VIII, iu/ml	1 st.						
	2 st.						
✓ Factor IX, iu/ml	1 st.	(50)	(25)	(5)	(2)		
	2 st.	35.5	20.4	4.0	1.9		
Factor II, u/ml							
Factor X, u/ml							
Factor VII, u/ml (clotting)							
AT III (Anti Xa), u/ml							
AT III (Anti IIa), u/ml							
AT III (Laurell), u/ml							
Factor VIII RAG, u/ml							
Fibrinogen (Laurell), mg/ml							
Prothrombin (Laurell), u/ml							
Factor XIIIA (Laurell), u/ml							
Fibronectin (Laurell), mg/ml							
Factor VIII CAG							
✓ NAPTT sec. 1/10		230	228	300	281		Blank 278
TGt50 min.							
FDA hr.							
Limulus + or -							

① Contains Heparin
② Contains ~ 50% Sorbitol/Glycine (+ ½ heparin in ①)
③ Contains ~ 25% Sorbitol/Glycine (+ ¼ heparin in ①)

For comments contact _____ Date finished: _____

ASSAY REQUEST FORM (1)

Request from: FDA/DC Date: 8/8/93

When results needed: 11/8/93.

Samples to be kept? _____ If so, how? _____

Samples provided with form/available from _____

SAMPLE INVESTIGATION ∝9H9		/Sn	/SnA	/A1	/A2		
Factor VIII, iu/ml	1 st.						
	2 st.						
✓Factor IX, iu/ml	1 st.	(0.04)	(0.1)	(2.6)	(4)		
	2 st.	0.42	0.5	[redacted]	2.8		
Factor II, u/ml							
Factor X, u/ml							
Factor VII, u/ml (clotting)							
AT III (Anti Xa), u/ml							
AT III (Anti IIa), u/ml							
AT III (Laurell), u/ml							
Factor VIII RAG, u/ml							
Fibrinogen (Laurell), mg/ml							
Prothrombin (Laurell), u/ml							
Factor XIIIA (Laurell), u/ml							
Fibronectin (Laurell), mg/ml							
Factor VIII CAG							
✓NAPTT sec. 1/10		259	251	267	262		
TGt50 min.							
FDA hr.							
Limulus + or -							

For comments contact _____ Date finished: _____

9H9

ASSAY REQUEST FORM (1)

Request from: PAF IDC Date: 8/8/83

When results needed: 11/8/83

Samples to be kept? _____ If so, how? _____

Samples provided with form/available from _____

SAMPLE INVESTIGATION ∝9H9		/AB	/B1	/B2.			
Factor VIII, iu/ml	1 st.						
	2 st.						
√Factor IX, iu/ml	1 st.	(1.5)	(40)	(4.2)			
	2 st.	1.6	14.9	0.67			
Factor II, u/ml							
Factor X, u/ml							
Factor VII, u/ml (clotting)							
AT III (Anti Xa), u/ml							
AT III (Anti IIa), u/ml							
AT III (Laurell), u/ml							
Factor VIII RAG, u/ml							
Fibrinogen (Laurell), mg/ml							
Prothrombin (Laurell), u/ml							
Factor XIIIA (Laurell), u/ml							
Fibronectin (Laurell), mg/ml							
Factor VIII CAG							
√NAPTT sec. 1/10		261	283	270			Blank 282
TGt50 min.							
FDA hr.							
Limulus + or -							

For comments contact _____ Date finished: _____

ASSAY REQUEST FORM (1)

9H9

Request from: PAF Date: 5/8/83

When results needed: Today

Samples to be kept? N_2 If so, how?

Samples provided with form/~~available~~ from

SAMPLE INVESTIGATION		&9H9	9H9				
Factor VIII, iu/ml	1 st.						
	2 st.						
√ Factor IX, iu/ml	1 st.	(4.0)	(8.4)				
	2 st.	1.7	3.1				
Factor II, u/ml							
Factor X, u/ml							
Factor VII, u/ml (clotting)							
AT III (Anti Xa), u/ml							
AT III (Anti IIa), u/ml							
AT III (Laurell), u/ml							
Factor VIII RAG, u/ml							
Fibrinogen (Laurell), mg/ml							
Prothrombin (Laurell), u/ml							
Factor XIIIA (Laurell), u/ml							
Fibronectin (Laurell), mg/ml							
Factor VIII CAG							
NAPTT sec. 1/10							
TGt50 min.							
FDA hr.							
Limulus + or -							

For comments contact Date finished:

9H9

ASSAY REQUEST FORM (2)

Request from: PAF Date:

When results needed: ASAR

Samples to be kept? No If so, how?

Samples provided with form/available from:

SAMPLE INVESTIGATION		α9H9	α9H9 /A1	α9H9 /A2	α9H9 /B1	α9H9 /B2	
Protein	E280	(3)	(15)	(4)	(20)	(1.5)	
✓	biuret g/l	1.28	9.65	3.53	14.5	1.14	
Fibrinogen g/l/%							
Sodium mmol/l							
Potassium mmol/l							
Chloride mmol/l							
Citrate mmol/l							
Phosphate mmol/l							
Tris mmol/l							
PEG g/l							
Caeruloplasmin g/l							
Factor XIII u/ml							
PKA % Ref. 2							
AT III (amidolytic) u/ml							
F. VII (amidolytic) u/ml							
XaGT mins.							
pH at 20°C							
Conductivity at 20°C							

For comments contact: Date finished:

18/8/83. 9H10 Heat Treatment and recovery of Factor IX concentrate using sorbitol, glycine and DE-52.

This experiment was intended to demonstrate a process for the successful heating of factor IX in the presence of sorbitol and glycine stabilisers, and then to recover the factor IX by adsorption on DE-52. This is a continuation of 9H9 and 9H7 which gave ambiguous results upon the DE-52 recovery step.

Materials

Buffers: factor IX production buffer A stock solution was diluted 1/6. The conductivity was done that described in standard limits, being only 2 mM. Buffer B was prepared according to the recipe described for factor IX production buffer B.

Factor IX 40 vials of 9D 2231 (35 ml fill, no heparin pyrogen failure) reconstituted with PFW

DEAE-Cellulose: Recycled cake from DE-52 (Batch 6/2) was thawed at room temperature before use

Method

After reconstitution and sampling of 8 ml,
Glycine added
Vol: 1470 ml
147 g glycine added (1 g / 10 ml of 9D)

2x1 ml sample taken, then 1809 g Sorbitol added (1.3 g / ml of 9D)
Dissolved by immersion of stainless steel container in 37°C water bath.
Volume, pH and conductivity were measured.

Material then heated at 60°C for 10 hrs in plastic centrifuge bottles (6). Temperature of water bath and of central bottle monitored over this period (see chart).

After 10 hours, material was removed directly from water bath, volume measured (2660 ml) and an equal volume of PFW added to dilute the sorbitol. Samples taken before and after addition of the PFW.

Assay of this material gave the total units as 22876 and DE-52 was added in the proportion 1g per 150 u (i.e. total of 152.5 g DE-52).
Stirred at room temperature for 1 hour, then centrifuged for 15 mins to collect adsorbed gel - factor IX.

Supernatant collected and sampled.

DE-52 resuspended in Buffer A and packed into Amicon 5 cm I.D. column.

Conductivity and transmission at 259 nm measured continuously.

Column eluted with 1 L Buffer A and 400 ml Buffer B.

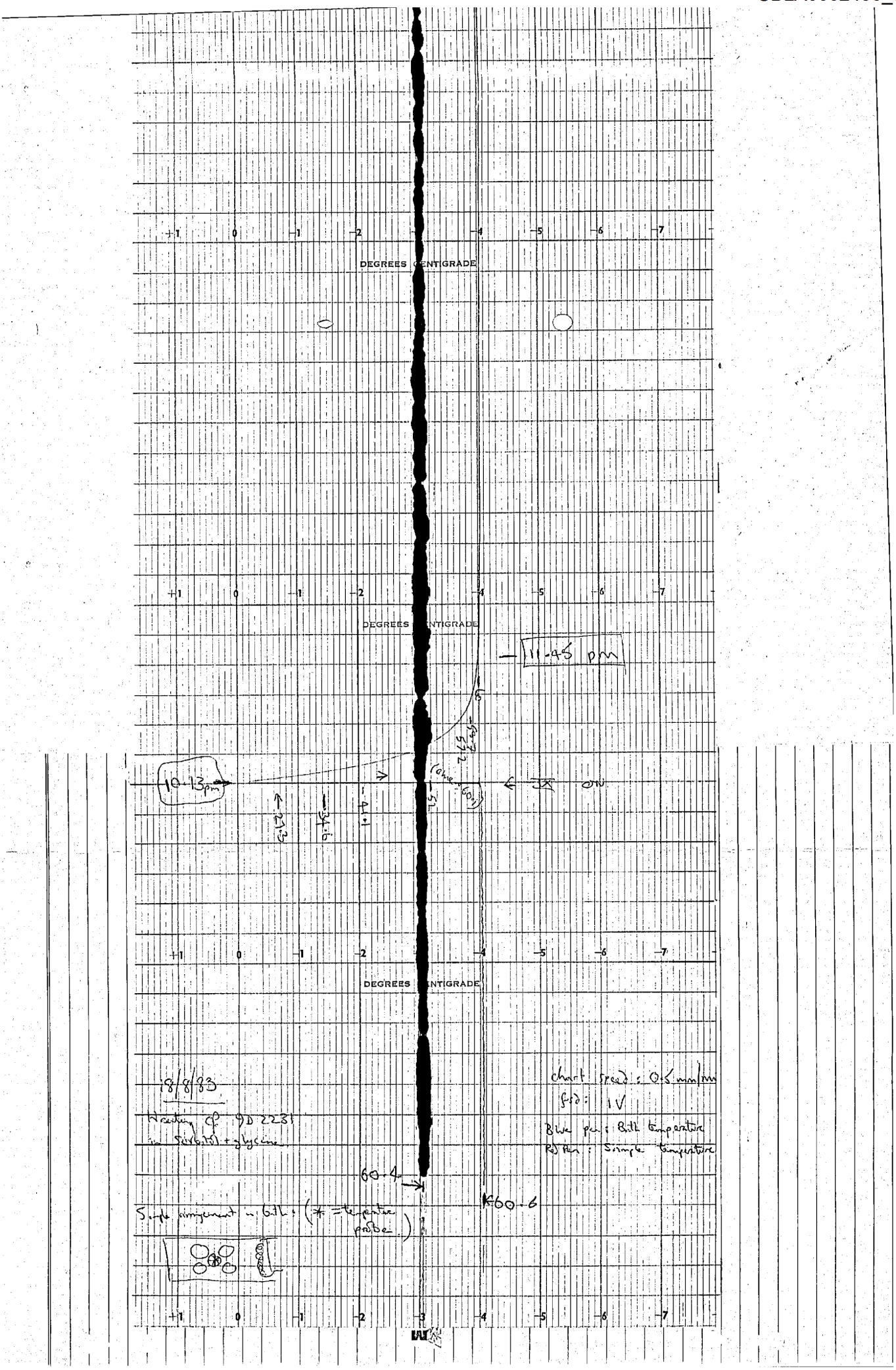
Results

Upon application of Buffer A to the column, a large proportion of the total protein eluted in two peaks which corresponded to jumps in the conductivity. A third, smaller protein peak eluted with buffer B. (See chart recording.

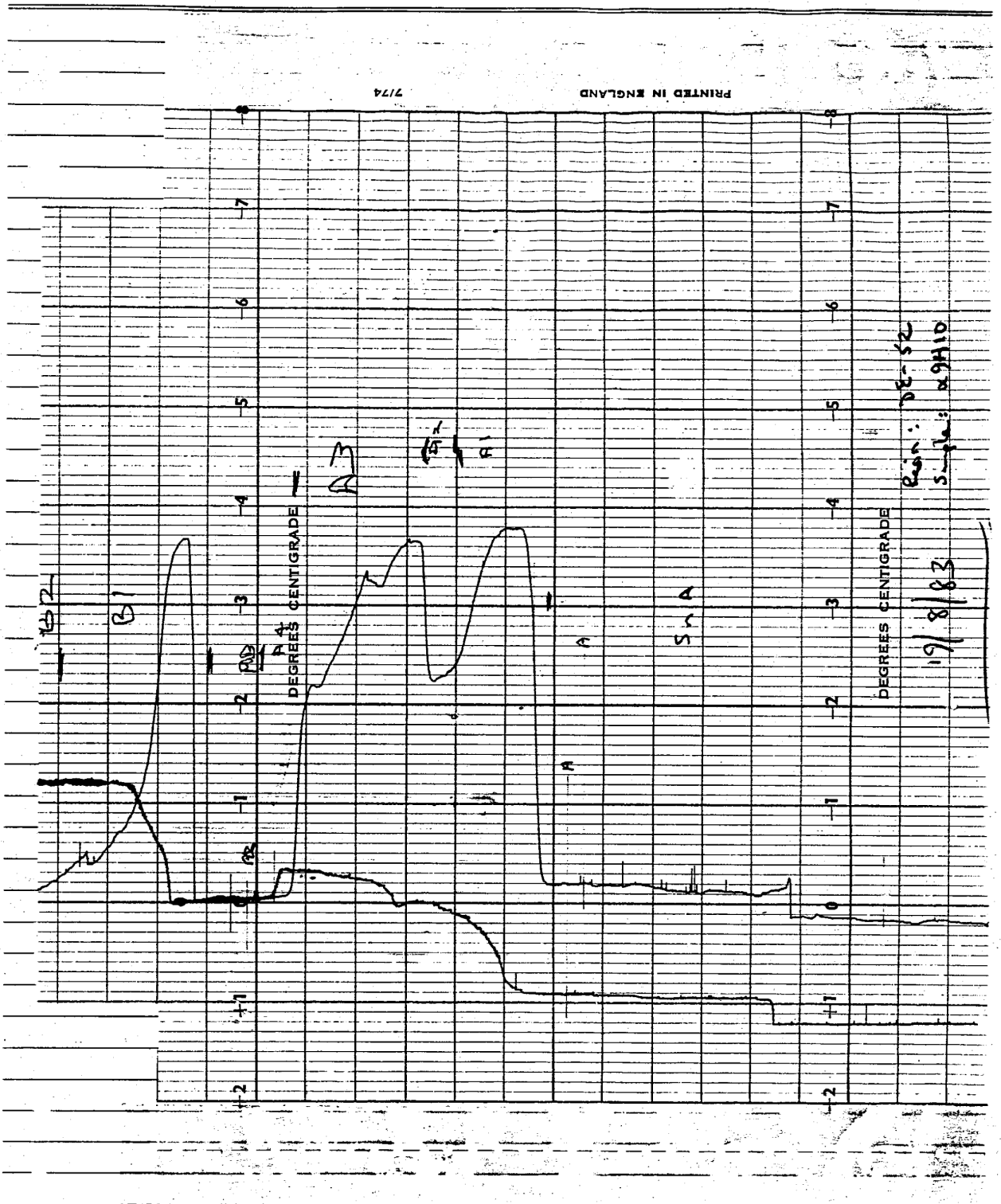
Sample Classification: 9D2231 — Starting material
/Gly — After addition of glycine
/S — After addition of sorbitol
9H10 — After 60° heating for 10 hrs.
∝ 9H10 — After dilution by ½ with PFW
/Sn — Supernatant after DE-52 Adsorpti.
/SnA — Eluate from column during packi
/A1 — First peak of buffer A
/A2 — Trough of buffer A
/A3 — Second peak of buffer A
/B1 — Peak of buffer B

Samples A4, A8 and B2 showed no protein and were discarded.

NB. Preliminary assays on the day for samples 9D2231, /S, 9H10 and ∝ 9H10 were performed. There was some variation (up and down) in the factor IX activities obtained.


DEGREES CENTIGRADE
+1 0 −1 −2 −4 −5 −6 −7
11·45 pm
10·13 pm
−27·3
−34·6
−4·1
−5·2
60·4
60·6
18/8/83
Heating of DD 2231
Sucrose + glycine
chart speed: 0·5 mm/m
fsd: 1V
Blue pen: Both temperature
Red pen: Simple temperature
Simple arrangement — both: (* = temperature probe)

9H10.4



Elution profile off DE-52 column
Red : % Transmission at 259 nm
Blue : conductivity meter reading.

9H10 5-

Initial Assay results (upon which the gel loading was calculated):

Sample	Factor IX u/ml	Factor IX Total Units	% Starting Material
9D 2231	42.6	59640	100
/S	22.1	58123	97.5
9H10	10.1	26866	45
∝9H10	4.3	22876	38.4

Final results based upon frozen samples.:

Sample	Vol (ml)	Factor IX u/ml	Factor IX Total Units	Protein* mg/ml	Sp. Act U/mg	% Starting Material
9D2231	1400	33.7	47180			100
/Cly	1470	35.2	51744			110
/S	2630	25.4	66802			141.6
9H10	2660	8.2	21812			46.2
∝9H10	5320	4.2	22344	1.4	3	47.4
/Sn	4895	0.08	391.6	0.5	0.16	0.8
/SnA	280	0.02	5.6	0.4	0.05	0.0
/A1	440	15.0	6600	10.6	1.41	14
/A2	125	4.5	562.5	4.6	0.98	1.2
/A3	450	23.1	10395	8.4	2.75	22
/B1	500	14.8	7400	5.1	2.9	15.

This division of protein between different eluting buffers shows a worsening in recovery of Factor IX in buffer B compared with expts. 9H7.03 and 9H9. To explain this, the salt concentrations were measured, along with APTT.

* By biuret method.

9H10.6

Sample	[Chloride] (mM)	[Citrate] (mM)	[Phosphate] (mM)	[Total Salt] (mM)
∝9H10	35	7.8	4.7	47.5
/Sn	32	6.1	4.0	42.1
/SnA	38	6.5	4.3	48.8
/A1	53	18.2	8.6	79.8
/A2	87	10.7	10.5	108.2
/A3	85	13.6	13.8	112.4
/B1	205	16.0	13.1	234.1

Sample	NAPTT (s)
9D2231	199
/Gly	163
/S	180
9H10	211
∝9H10	213
/Sn	275
/SnA	262
/A1	257
/A2	230
/A3	201
/B1	148
Control	283

Comments

The loss on heating of factor IX activity is now giving a fairly consistent result, though 46% may be slightly low. The NAPTT suggests that activation is not a problem at this step in the procedure.

Unlike earlier experiments, there is full recovery of factor IX from DCL and the level of loading (150 u /g gel) is satisfactory as judged by the low factor IX levels in the supernatant (reproducably < 1%). This improvement in recovery can be attributed to the continuous monitoring of protein elution and the continuation of buffer washes until the protein peak is fully eluted.

The distribution of the eluted factor IX is disturbing, especially as it becomes broader with successive experiments. In this experiment 37% of the starting factor IX units is eluting in buffer A (representing 79% of the units loaded on to the DE-52). This is not attributable to defective buffers; while there are slight variations in the salt concentrations of Buffer A elutes (A1 - A3) these are not sufficient to account for the large amount of factor IX present. As would be expected the salt concentration of buffer B is still twice that of the highest value for buffer A.

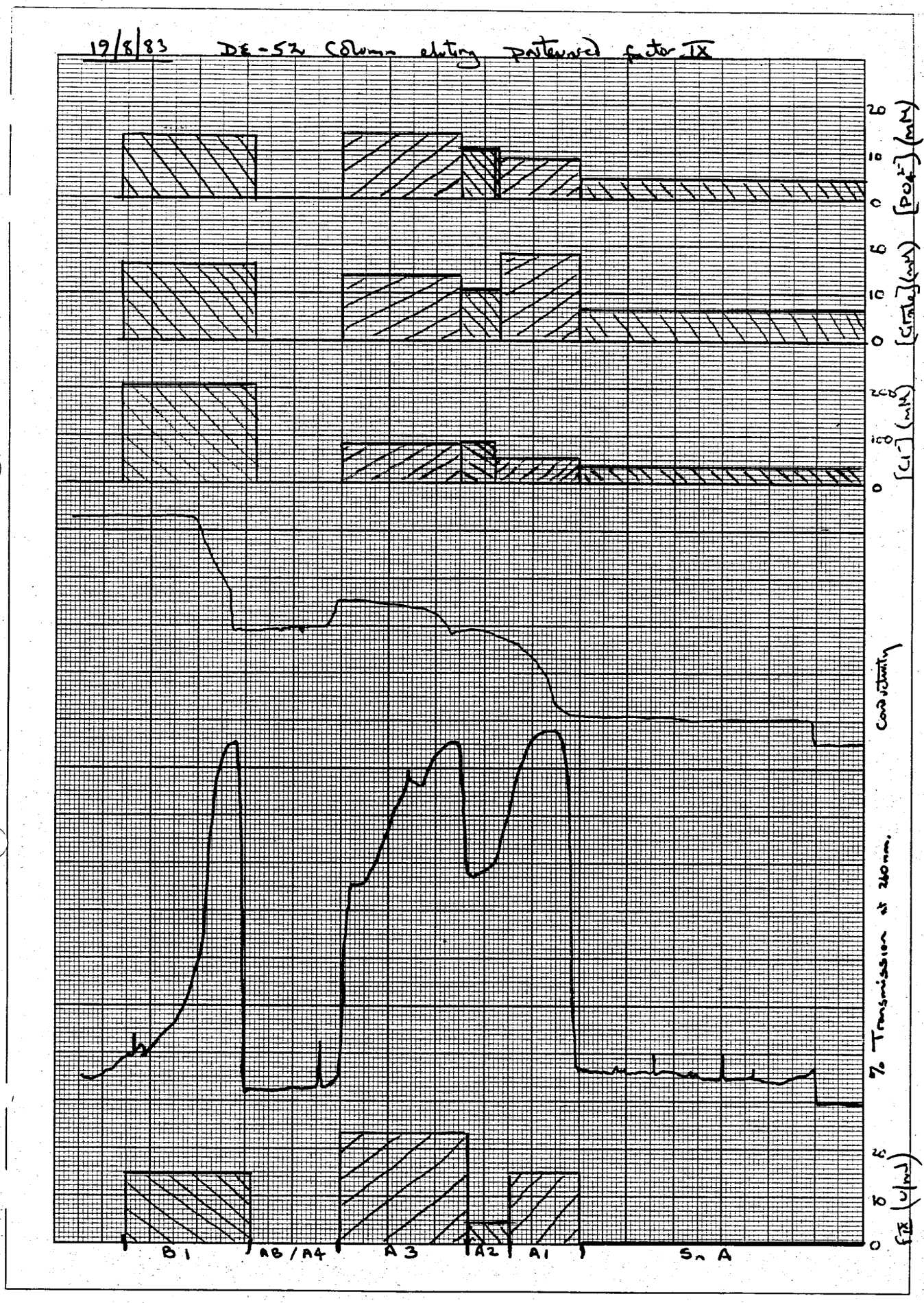
The conclusion that I am drawn to is that there is a defect in the binding capacity of the batch of DCL used which worsens with the aging of the DCL.

Therefore, attempts should be made to re-adsorb the factor IX eluted in buffer A as well as tests on the basic properties of the DCL itself. These are described in the second part of this experiment, 9H10.B

Operators

DC; PAF

9 of 10


19/8/83 DE-52 Column eluting protein of IX
0
10
20
[Cl⁻] (mM)
0
5
10
[SO4²⁻] (mM)
0
5
10
[Cl⁻] (mM)
Conductivity
% Transmission at 280nm.
B1
AB/A4
A3
A2
A1
S. A

ASSAY REQUEST FORM (1)

9H10

Request from: PAG / DC. Date: 19/8/83.

When results needed: TODAY

Samples to be kept? No. If so, how?

Samples provided with form/available from

SAMPLE INVESTIGATION		9D223½	9D223½ */S	9H10 *	∝9H10 ≠		
Factor VIII, iu/ml	1 st.						
	2 st.						
√ Factor IX, iu/ml	1 st.	(30)	(15)	(8)	(4-0)		
	2 st.	42.6	22.1	10.1	4.3.		
Factor II, u/ml							
Factor X, u/ml							
Factor VII, u/ml (clotting)							
AT III (Anti Xa), u/ml							
AT III (Anti IIa), u/ml							
AT III (Laurell), u/ml							
Factor VIII RAG, u/ml							
Fibrinogen (Laurell), mg/ml							
Prothrombin (Laurell), u/ml							
Factor XIIIA (Laurell), u/ml							
Fibronectin (Laurell), mg/ml							
Factor VIII CAG							
NAPTT sec. 1/10							
TGt50 min.							
FDA hr.							
Limulus + or -							

* ~ 40% Sn6.15

≠ ~ 20% Sn6.15.

For comments contact

Date finished:

ASSAY REQUEST FORM (1)

Request from: PAF / ZC. Date: 22/8/83

When results needed: w/e 26/8

Samples to be kept? yes (if left over) If so, how? frozen

Samples ~~provided with form~~/available from (LP3's) R+D freezer.

SAMPLE ∝9H10 INVESTIGATION		/SnA	/A1	/A2	/A3	/B1	
Factor VIII, iu/ml	1 st.						
	2 st.						
✓Factor IX, iu/ml	1 st.	(0.05)	(33)	(19)	(11)	(45)	
	2 st.	0.02	15.0	4.5	23.1	14.8	
Factor II, u/ml							
Factor X, u/ml							
Factor VII, u/ml (clotting)							
AT III (Anti Xa), u/ml							
AT III (Anti IIa), u/ml							
AT III (Laurell), u/ml							
Factor VIII RAG, u/ml							
Fibrinogen (Laurell), mg/ml							
Prothrombin (Laurell), u/ml							
Factor XIIIA (Laurell), u/ml							
Fibronectin (Laurell), mg/ml							
Factor VIII CAG							
✓NAPTT sec. 1/10		262	257	230	201	148	
TGt50 min.							
FDA hr.							
Limulus + or -							

For comments contact _____ Date finished: _____

ASSAY REQUEST FORM (1)

7 H10

Request from: PAF/DC Date: 22/8/83

When results needed: w/c 26/8.

Samples to be kept? _____ If so, how? _____

Samples ~~provided with form~~/available from (LP3's) R+D freezer

SAMPLE INVESTIGATION		9D2231	9D2231 /Gly	9D2231 /S *	9H10 *	∝9H10 ‡	∝9H10 ‡ /Sn
Factor VIII, iu/ml	1 st.						
	2 st.						
✓ Factor IX, iu/ml	1 st.	(35)	(33)	(19)	(9)	(4.5)	(0.05)
	2 st.	33.7	35.2	25.4	8.2	4.2	0.08
Factor II, u/ml							
Factor X, u/ml							
Factor VII, u/ml (clotting)							
AT III (Anti Xa), u/ml							
AT III (Anti IIa), u/ml							
AT III (Laurell), u/ml							
Factor VIII RAG, u/ml							
Fibrinogen (Laurell), mg/ml							
Prothrombin (Laurell), u/ml							
Factor XIIIA (Laurell), u/ml							
Fibronectin (Laurell), mg/ml							
Factor VIII CAG							
✓ NAPTT sec. 1/10		199	163	180	211	213	275
TGt50 min. NAPTT control :		283 sec.					
FDA hr.							
Limulus + or -							

* About 40% Sorbitol

‡ 10 - 20 % Sorbitol

For comments contact _____ Date finished: _____

9H10

ASSAY REQUEST FORM (2)

Request from: PRF/DC Date: 22/8/83

When results needed: w/e 26/8.

Samples to be kept? No If so, how?

Samples ~~provided with form~~/available from: (C5's) R+D Freezer

SAMPLE INVESTIGATION		∝9H10 *	∝9H10 /Sa	∝9H10 /SaA	∝9H10 /A1	∝9H10 /A2	∝9H10 /A3
Protein	E280	~~(3.1)~~	~~(1.5)~~	~~(1.5)~~	~~(4.8)~~	~~(4.5)~~	~~(9.0)~~
✓	biuret g/l	1.4	0.5	0.4	10.6	4.6	8.4
Fibrinogen g/l/%							
Sodium mmol/l							
Potassium mmol/l							
✓ Chloride mmol/l		(28) 35	(28) 32	(90) 38	(105) 53	(125) 87	(130) 85
✓ Citrate mmol/l		(8) 7.8	(8) 6.1	(10) 6.5	(10) 18.2	(10) 10.7	(15) 13.6
✓ Phosphate mmol/l		(4) 4.7	(4) 4.0	(8) 4.3	(10) 8.6	(10) 10.5	(10) 13.8
Tris mmol/l							
PEG g/l							
Caeruloplasmin g/l							
Factor XIII u/ml							
PKA % Ref. 2							
AT III (amidolytic) u/ml							
F. VII (amidolytic) u/ml							
XaGT mins.							
pH at 20°C							
Conductivity at 20°C							

* Contains ~ 20% sorbitol.

For comments contact: GH Date finished: 24-8-83

9H10

ASSAY REQUEST FORM (2)

Request from: PAF/DC Date: 22/8/83

When results needed: w/e 26/8

Samples to be kept? No If so, how?

Samples ~~provided with form~~/available from: (< 5's) R+D Freezer

SAMPLE INVESTIGATION	∝9H10 /B1					
Protein E280						
✓ biuret g/l	(3) 5.1					
Fibrinogen g/l/%						
Sodium mmol/l						
Potassium mmol/l						
✓ Chloride mmol/l	(200) 205					
✓ Citrate mmol/l	(20) 16.0					
✓ Phosphate mmol/l	(15) 13.1					
Tris mmol/l						
PEG g/l						
Caeruloplasmin g/l						
Factor XIII u/ml						
PKA % Ref. 2						
AT III (amidolytic) u/ml						
F. VII (amidolytic) u/ml						
XaGT mins.						
pH at 20°C						
Conductivity at 20°C						

For comments contact: GH Date finished: 24.8.83

30/8/83 9H10.B To Test the Properties of DE-52 Batch S/2 for factor IX Adsorption

This is a continuation of 9H10. It is designed to show whether material eluted in buffer A from DE52 represents overloading of the original gel or other a different form of factor IX which elutes from DE-52 at lower ionic strength. Some basic tests on the acid-base properties of DE-52 are included.

A Adsorption/Elution of Factor IX

Materials

Protein containing factor IX activity was eluted from DE-52 in expt 9H10, using buffer A. From this material, two distinct protein peaks were obtained, labelled A1 and A3. These were the starting material for this expt.

DE-52 was from the same batch, S/2, used in previous 9H experiments.

Buffers A and B were made up fresh, as stock solutions as described in SOP, then diluted by 1/6 and 1/2 respectively.

2 glass columns ID 1.5 cm were used and elution carried out under gravity.

Method 12 ml of A1 and 9 ml of A3 were thawed at 37°c. As these were in buffer A, they were diluted by 1/2 in PFW to reduce the ionic strength (and thus enable binding to DE-52). Conductivity and pH was measured and pH adjusted to 7.00 ± 0.05 by addition of 5 M HCl

Sample	pH before	Conductivity before (mMho)	pH after	Conductivity after (mMho)
A1	7.85	2.45	7.03	2.5
A3	8.03	5.1	6.96	5.2

The adjusted samples were re-classified: X (A1) and T (A3) and 2 x 2ml samples taken from each.

Each was then absorbed against 2g DE-52. This represented half the normal loading since X contains 150 u and T contained 16½ u factor IX, AS MEASURED IN 9H10 ASSAYS.

Absorption was at room temperature for 1 hour followed by centrifugation in a bench centrifuge.

Supernatant was drawn off and the gel resuspended in a small volume of supernatant and packed into the columns. Eluted supernatant was then pooled with remaining Sn.

Each column was then eluted with 3 column volumes of buffer A and 3 column volumes buffer B. Eluates during each application were collected and labelled "A" and "B".

Absorbance at 280 nm was measured for each sample (or appropriate dilution of sample).

Results

Sample	Vol (ml)	pH	Conductivity (mMho)	A_{280}	Factor IX (u/ml)	(Total)	% Factor IX loaded	Sp.A u/m
X	20	7.03	5	4.8	2.98	59.6	100	0.62
X-Sn	16	8.89	2.3	0.12	0.04	0.64	1	0.33
X-A	16.5	9.0	5.3	2.65	2.3	38	63.7	0.8
X-B	16.5	9.07	12.2	2.35	1.35	22.3	37.4	0.5
T	14	6.94	5.1	80	9.89	138.5	100	1.8
T-Sn	10.5	9.6	4.8	0.63	0.36	3.8	2.7	0.5
T-A	15	9.54	6.5	3.77	7.15	107.2	77.4	1.9
T-B	15	9.63	12.2	0.68	0.99	14.9	10.8	1.4

These results show no consistant trend; the X sample, which originally eluted first in 9H10, shows a distribution between buffers A and B, while the T-sample is primarily eluted in buffer A.

There is however a significant rise in pH. While some increase is to be expected (because the DE-52 is not pre-equilibrated against chloride counter ions) a rise above 9.0 is hazardous to the protein.

These pH values can usefully be compared with the pH of eluates from a production run of factor IX on DE-52:

9P 1809 (1/9/83) A.E./T.K.

Eluate from buffer A wash: pH 7.17
Eluate from buffer B wash: pH 7.23
Eluate from buffer C wash: pH 7.16

The next section describes basic tests on the DE52 used in this experiment.

B Characteristics of DE52.

i) Acid-base titration curves. (28/8/83)

4g DE-52 batch 5/2 suspended in 20ml PFW. SOME SOLID LUMPS WERE OBSERVED

1M HCl was titrated into it, with continuous stirring until pH became constant. This mixture was then titrated up with 1M NaOH to an end-point at high pH.

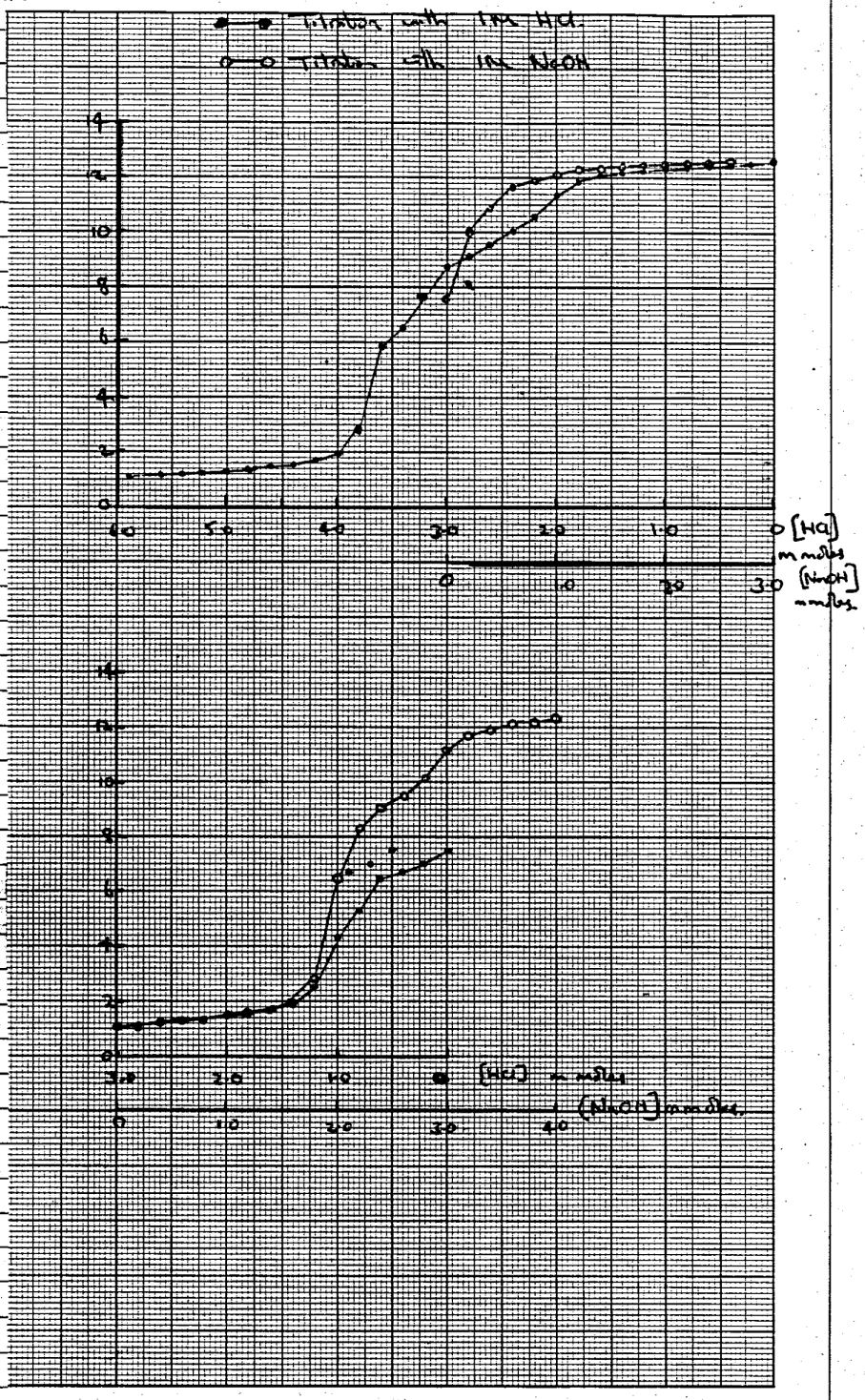
These titrations were repeated with a fresh sample of DE-52, titrating first to high pH, then to low pH.

The results are shown overleaf and give a typical titration curve pattern for an ion-exchanger.

ii) Response to buffers (31/8/83)

2g DE-52 (batch 5/2) and 2g of another, unmarked batch of DE-52 were suspended in PFW and packed into two glass columns, 15cm ID. 3 column volumes each of buffer A and buffer B were passed through the gel and eluates collected.

24/5/23 9410-8 Titration of 4mg DES2 (Batch 5/2) in 20ml PFW



9 HO. 8, 5

Eluate	Before pH	Before Conductivity	After pH	After Conductivity
Batch S/2				
PFW	5.52	0	6.30	0
A	7.02	7.6	9.74	5.2
B	7.05	15	9.91	12
Oil batch				
PFW	5.74	0	5.97	0
A	7.02	7.4	9.08	5.3
B	7.05	14.6	9.24	12.2

Comment

This results indicates that DE-52 batch S/2 is taking a considerable amount of chloride ions out of the buffer, with an increase in pH occuring at the same time as a decrease in conductivity.

It appears therefore that S/2 may have been poorly recycled which may account for the highly variable results obtained when trying to recover factor IX after heating.

The most prudent way to proceed will be to stop using this batch of DE-52 and attempt repeat expts with freshly recycled DE-52.

ASSAY REQUEST FORM (1)

9N10. B

Request from: PAF Date: 31/8/83

When results needed: ASAP (CODE 1)

Samples to be kept? NO If so, how?

Samples ~~provided with form~~/available from PTD freezer.

SAMPLE INVESTIGATION		9H10	1X	1X Sn	1X-A	1X-B		
Factor VIII, iu/ml	1 st.							
	2 st.							
✓ Factor IX, iu/ml	1 st.		(7.5)	(<0.02)	(8.0)	(1.0)		
	2 st.		2.98	0.04	2.3	1.35		
Factor II, u/ml								
Factor X, u/ml								
Factor VII, u/ml (clotting)								
AT III (Anti Xa), u/ml								
AT III (Anti IIa), u/ml								
AT III (Laurell), u/ml								
Factor VIII RAG, u/ml								
Fibrinogen (Laurell), mg/ml								
Prothrombin (Laurell), u/ml								
Factor XIIIA (Laurell), u/ml								
Fibronectin (Laurell), mg/ml								
Factor VIII CAG								
✓ NAPTT sec. 1/10		6.	253	253 → 285	245	237		
TGt50 min.								
FDA hr.								
Limulus + or -								

For comments contact Date finished: 7/9/83

ASSAY REQUEST FORM (1)

9H10-8

Request from: PAF

Date: 31/8/83

When results needed: ASAP (GRADE)

Samples to be kept? No

If so, how?

Samples provided with form/available from R+D freezer.

SAMPLE INVESTIGATION 9 H10		H	H-Sm	H-A	H-B		
Factor VIII, iu/ml	1 st.						
	2 st.						
√ Factor IX, iu/ml	1 st.	(11.5)	(<0.02)	(7)	(2.5)		
	2 st.	9.89	0.36	7.15	0.99		
Factor II, u/ml							
Factor X, u/ml							
Factor VII, u/ml (clotting)							
AT III (Anti Xa), u/ml							
AT III (Anti IIa), u/ml							
AT III (Laurell), u/ml							
Factor VIII RAG, u/ml							
Fibrinogen (Laurell), mg/ml							
Prothrombin (Laurell), u/ml							
Factor XIIIA (Laurell), u/ml							
Fibronectin (Laurell), mg/ml							
Factor VIII CAG							
√ NAPTT sec. 1/10		204	250 ← 285	193 →	245		
TGt50 min.							
FDA hr.							
Limulus + or -							

For comments contact

Date finished: 7/9/83

Experiment 9H11 8·9·83

To Investigate the effect of varying Pasteurigation Time on the FIX

Materials and Methods

12 vials of 9D2231 (each containing ~ 1000 iu FIX and no heparin) were reconstituted using 35ml PFW per vial. 408 ml of the FIX solution were recovered (9D2231/8·9·83). This was sampled by removing 3 x 2ml aliquots leaving 402 ml 40.2g of glycine were added to the FIX solution and dissolved by stirring. 522.6g of sorbitol were added slowly while the mixture was held in a water bath at 30°C and stirred gently.

795ml of FIX/glycine/sorbitol (9D2231/S 8·9·83) were recovered and from this two 2ml samples were taken.

The pH of 9D2231/S 8·9·83 was found to be 6·67

The mixture was divided into two equal parts - "A" and "B". A was set up in the pasteurigation bath with probes and a chart recorder to monitor the FIX and bath temperatures. B was placed in a 4°C fridge overnight.

On the following morning B was removed from the fridge, sampled 4x2ml and then placed in the 60°C water bath with a temperature probe in it.

20ml samples were ~~removed~~ removed from the pasteurized solutions at various times after they had reached 60°C. (see below).

FIX solution	time at 60°C (hours)	Volume of sample.
A	16	20
A	18	20
B	2	20
A	20	20
B	4	20
B	6	20
B	8	335·5
A	24	335·5

9.H11 8.9.83

Materials and Methods Cont.

All the samples were analysed for FIX content and NAPTT.

Results and Discussion

The results are shown in the following table :-

Sample	FIX units/ml	FIX units/ml as % of Pasteurized	NAPTT	NAPTT as % OF BLANK
9D2231/8.9.83	34.7		238	
A9D2231/S8.9.83	20.3	100	226	79
B9D2231/S8.9.83	/	—	—	—
B~ 2 hours	13.3	65.5	205	72
B~ 4 hours	11.5	56.6	212	74
B~ 6 hours	9.6	47.3	241	84
B~ 8 hours	9.3	45.8	243	85.6
A ~ 16 hours	5.6	27.6	291	102
A~ 18 hours	4.9	24.1	283	99.6
A ~ 20 hours	6.0	29.5	331	116.5
A ~ 24 hours	5.6	27.6	307	108
NAPTT BLANK	—	—	284	100

Table 9H11a Variation of FIX content and NAPTT with pasteurization time. [Figures for FIX units subject to up to 10% error.

The amount of active FIX units/ml decreased less sharply than exponentially as pasteurization time increased. This shows that the sorbitol is protecting the FIX making the loss in activity less random.

The FIX decay curve is shown alongside an exponential decay curve based on the 2 and 4 hour pasteurization results.

Fig. 3

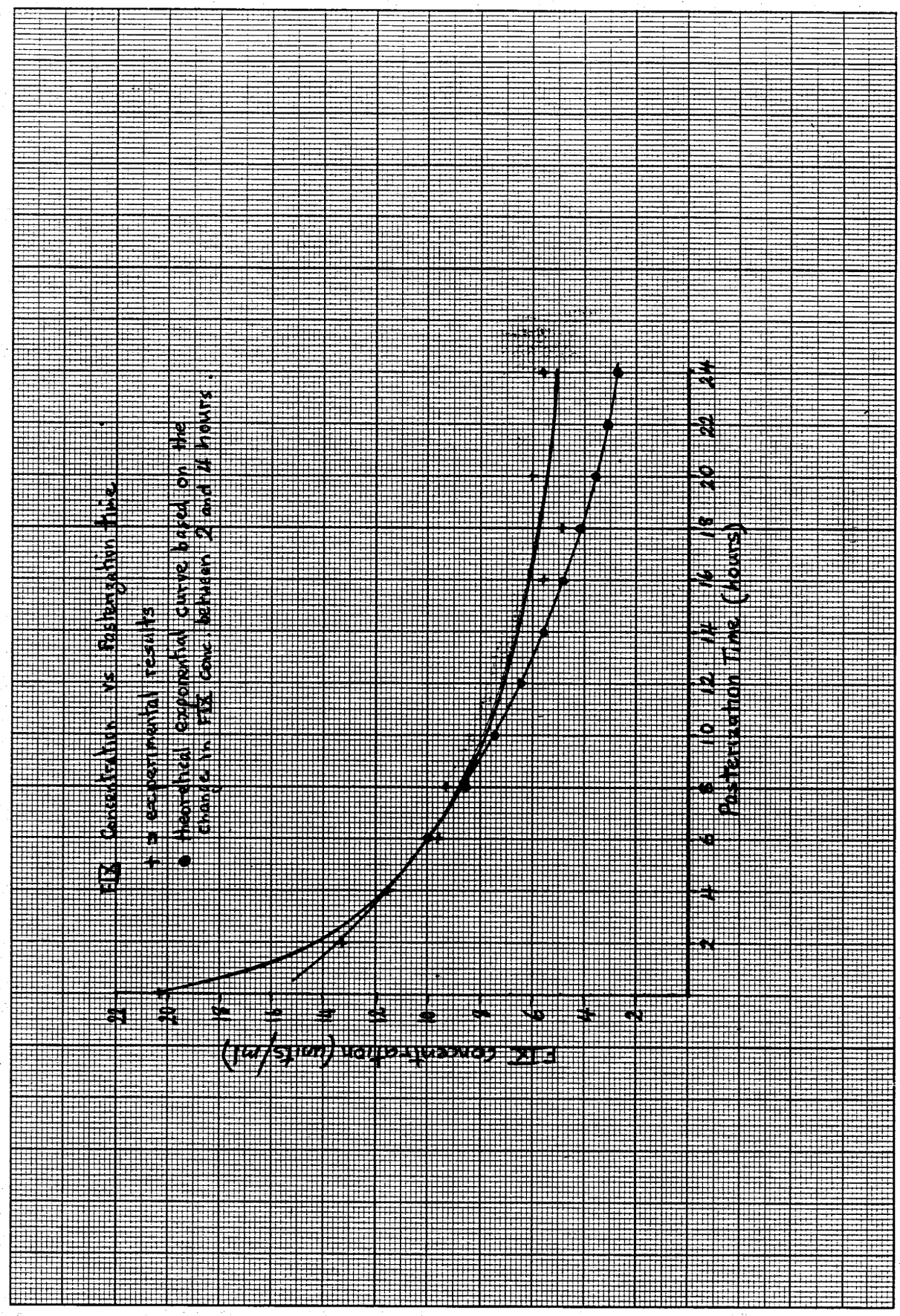


Fig. Concentration vs Polarization time.
+ = experimental results
● = theoretical exponential Curve based on the
change in FDE Conc. between 2 and 24 hours.
FDE concentration (units/ml)
Polarization Time (hours)
24
22
20
18
16
14
12
10
8
6
4
2

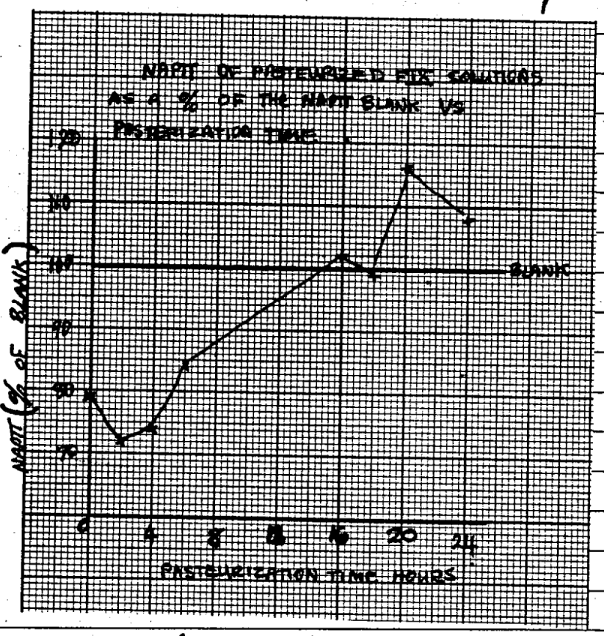
9H 11

Results and Discussion Cont.

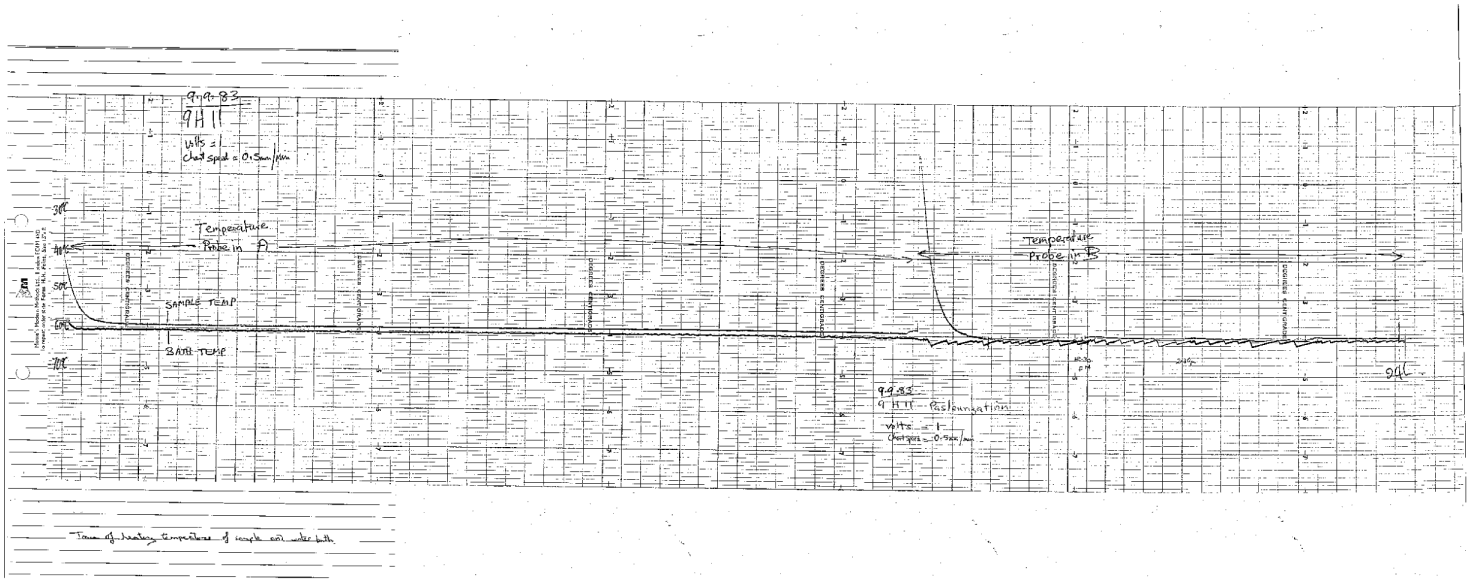
Approximately 50% of the FIX activity is lost after the first 5 hours of pasteurization and a further 11% of the total activity is lost in the second 5 hours.

5 hours is considered too short for the successful destruction of viruses. Shortening the time from 10 hours to between 6 and 10 hours does not save enough of the FIX activity to make the risk of not destroying the viruses acceptable. Sustaining the heating much longer than 10 hours eventually results in an unacceptable loss of FIX. 10 hours is considered to be the best length of pasteurization while an extension to 14 hours may be just acceptable under certain circumstances.

As heating proceeded there was a trend towards increased NAPTT. This is shown in the following graph.



Perhaps Pasteurization would eventually help in salvaging some of the FIX batches which failed their NAPTT tests.


9-9-83
9H11
Volts = 1
Chart Speed = 0.5cm/min
Temperature
Probe in A
SAMPLE TEMP
BATH TEMP
Temperature
Probe in B
9-9-83
9 HTT - Polymerization
Volts = 1
Chart Speed = 0.5cm/min
Time of heating temperature of sample and water bath

ASSAY REQUEST FORM (1)

Request from: DC/RAF Date: 12/9/83

When results needed: ASAP

Samples to be kept? No If so, how?

Samples provided with form/~~available from~~

SAMPLE 9H11 INVESTIGATION		9D2231	1S*	B2*	B4*	B6*	B8*
Factor VIII, iu/ml	1 st.						
	2 st.						
Factor IX, iu/ml *	1 st.	(28.5)	(14)	(11)	(8.5)	(8)	(7.7)
	2 st.	34.7	20.3	13.3	11.5	9.6	9.3
Factor II, u/ml							
Factor X, u/ml							
Factor VII, u/ml (clotting)							
AT III (Anti Xa), u/ml							
AT III (Anti IIa), u/ml							
AT III (Laurell), u/ml							
Factor VIII RAG, u/ml							
Fibrinogen (Laurell), mg/ml							
Prothrombin (Laurell), u/ml							
Factor XIIIA (Laurell), u/ml							
Fibronectin (Laurell), mg/ml							
Factor VIII CAG							
NAPTT sec. 1/10		238	226	205	212	241	243
TGt50 min.							
FDA hr.							
Limulus + or -							

* 90% Sorbin

For comments contact Date finished:

ASSAY REQUEST FORM (1)

Request from: DC / PAF. Date: 12/9/93

When results needed: ASAP

Samples to be kept? No If so, how? _____

Samples provided with form/~~available from~~ _____

SAMPLE INVESTIGATION 9H11/A		16*	18*	20*	24*		
Factor VIII, iu/ml	1 st.						
	2 st.						
✓ Factor IX, iu/ml	1 st.	(6.8)	(6.0)	(5.4)	(5)		
	2 st.	5.6	4.9	6.0	5.6		
Factor II, u/ml							
Factor X, u/ml							
Factor VII, u/ml (clotting)							
AT III (Anti Xa), u/ml							
AT III (Anti IIa), u/ml							
AT III (Laurell), u/ml							
Factor VIII RAG, u/ml							
Fibrinogen (Laurell), mg/ml							
Prothrombin (Laurell), u/ml							
Factor XIIIA (Laurell), u/ml							
Fibronectin (Laurell), mg/ml							
Factor VIII CAG							
✓ NAPTT sec. 1/10		291	283	331	307		blank 284
TGt50 min.							
FDA hr.							
Limulus + or -							

For comments contact _____ Date finished: _____

30.9.83 9H11.02 To establish elution properties of heated factor IX from DE-52 by salt gradient.

The elution of factor IX activity in both buffer A and buffer B in previous recoveries of factor IX suggest that these buffers do not represent optimum salt concentrations for factor IX recovery

The purpose of this experiment is to determine the salt concentrations at which factor IX is eluted. To this end, a salt gradient will be applied to factor IX adsorbed DE-52.

Materials

Factor IX : 250 ml of heated factor IX, containing sorbitol and glycine, was thawed from its frozen storage state. This material was the product of expt 9H11 which was heated at 60° c for 8 hours.

DE-52 : Was old recycled DE.52 - not the s/z batch which has been giving odd results.

Buffers : Buffer "0" 0.05 M trisodium citrate
0.005 M citric acid
0.06 M Na_2HPO_4
pH.7.28. This stock solution was diluted $\frac{1}{6}$ before use

Method 250ml Factor IX (9H11) was thawed and assayed fresh for factor IX activity.

2 x 1ml samples were taken "9H11.02"

As in a conventional recovery, this material was diluted by $\frac{1}{2}$ with PFW

2 x 1ml samples taken "α 9H11.02"

This α - material was stirred with 17.2 g DE52 (representing 150 u / g DE52) for 1 hour at 20°.

The mixture was then spun for 20 minutes in MSE Mistral 6L at 2,000 rpm and the supernatant decanted.

2 x 2ml Sample Taken ∝9H11-02/Sn.

The DE52 was resuspended in Buffer "O" and packed into a 1.5 cm ID glass column, pumping at about 88 ml/hour. The resulting eluate was collected in one pool "/SnO"

2 x 2ml sample Taken ∝9H11-02/SnO.

The packed bed was washed with buffer O (~120 ml).

9.5 ml fractions were collected.

A 2 x 80 ml O – 0.25 M NaCl gradient was then applied. The high salt buffer was the conventional factor IX buffer B.

Due to laboratory fumigation, this experiment had to be stopped at fraction 30, over the weekend.

Subsequent elution in buffer B was performed the following Monday morning (Therefore any results from fraction 31 onwards should be viewed with caution)

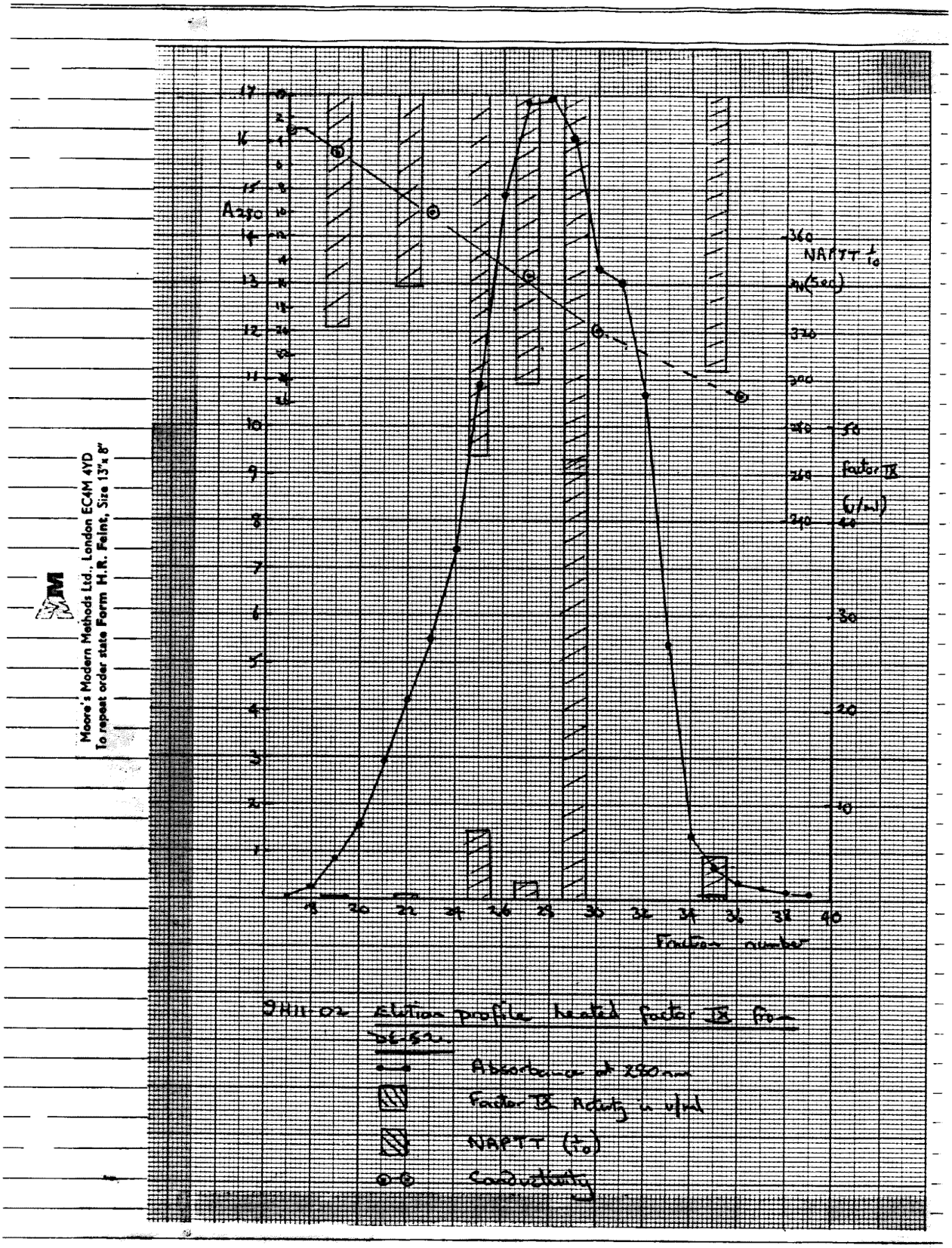
Bed volume during this experiment was 95.3 ml.

Results

Salt Gradient: until fumigation forced the curtailment of the experiment, a good linear gradient was obtained.

Protein elution: A single peak was obtained which began with the gradient and continued throughout. The shoulder (see profile) is more likely due to experimental disruption. The uniformity of the peak

9 Nov. 02.



may be attributable to the steepness of the gradient.

Factor IX Activity: This is positioned asymmetrically under the curve, with the majority of the activity appearing at higher salt concentrations. A small amount of activity elutes at lower salt concentrations (about 10%) and does seem to be discrete.

NAPTT: These fall from ~330 secs to 265 seconds for the first Factor IX peak and 260 seconds for the second. This is well within acceptable limits, though the control time was 300 sec.

It is worthy of note that the NAPTT is reduced almost as much in the early factor IX as in the late, though the activities of these two samples differ by a factor of ~6

If, as is suggested, NAPTT primarily identifies factor IXa, there is an implication that IXa is eluted from DE-52 at lower salt concentrations than IX.

Conductivities: The early eluting factor IX has a conductivity of 13 mMho while the later, main factor IX peak has a conductivity of ~19 mMho.

[Conventional production Buffer A has a conductivity of 11.5 mMho and Buffer B 23.5 mMho. There does appear to be a genuine factor IX species which can elute in buffer A].

Conclusions: Heated factor IX elutes from DE-52 in production buffer B. A small proportion (~10%) of factor IX, which may represent factor IXa, elutes at salt concentrations comparable to production buffer A.

This experiment may benefit from repetition, with an uninterrupted elution, a shallower gradient and a few more assays to identify factor IX more precisely.

ASSAY REQUEST FORM (1)

9H11 a:

Request from: PAF Date: 29/9/83

When results needed: TODAY / TOMORROW PLEASE

Samples to be kept? No If so, how?

Samples provided with form/~~available~~ from

SAMPLE INVESTIGATION		9H11					
Factor VIII, iu/ml	1 st.						
	2 st.						
Factor IX, iu/ml	1 st.	(9.3)					
	2 st.	10.3					
Factor II, u/ml							
Factor X, u/ml							
Factor VII, u/ml (clotting)							
AT III (Anti Xa), u/ml							
AT III (Anti IIa), u/ml							
AT III (Laurell), u/ml							
Factor VIII RAG, u/ml							
Fibrinogen (Laurell), mg/ml							
Prothrombin (Laurell), u/ml							
Factor XIIIA (Laurell), u/ml							
Fibronectin (Laurell), mg/ml							
Factor VIII CAG							
NAPTT sec. 1/10							
TGt50 min.							
FDA hr.							
Limulus + or -							

For comments contact Date finished:

ASSAY REQUEST FORM (1) 9H11.02

Request from: PAF Date: 3/10/83

When results needed: ASAP

Samples to be kept? No If so, how?

Samples ~~provided with form~~/available from R+D Freezer

SAMPLE 9H11.02 INVESTIGATION		α9H11.02	1Sn	119	122	125	127
Factor VIII, iu/ml	1 st.						
	2 st.						
Factor IX, iu/ml	1 st.	(5.1)	(.08)	x (.08)	8x (.11)	16x	20x
	2 st.	4.49	0.24	<0.15	0.53	7.24	1.8
Factor II, u/ml							
Factor X, u/ml							
Factor VII, u/ml (clotting)							
AT III (Anti Xa), u/ml							
AT III (Anti IIa), u/ml							
AT III (Laurell), u/ml							
Factor VIII RAG, u/ml							
Fibrinogen (Laurell), mg/ml							
Prothrombin (Laurell), u/ml							
Factor XIIIA (Laurell), u/ml							
Fibronectin (Laurell), mg/ml							
Factor VIII CAG							
NAPTT sec. 1/10		2		321 ✓	339 ✓	268 ✓	298 ✓
TGt50 min.							
FDA hr.							
Limulus + or -							

For comments contact Date finished:

ASSAY REQUEST FORM (1)

9H11.02

Request from: PAF Date: 3/10/83

When results needed: ASAP

Samples to be kept? No. If so, how?

Samples ~~provided with form~~/available from R+D Freezer

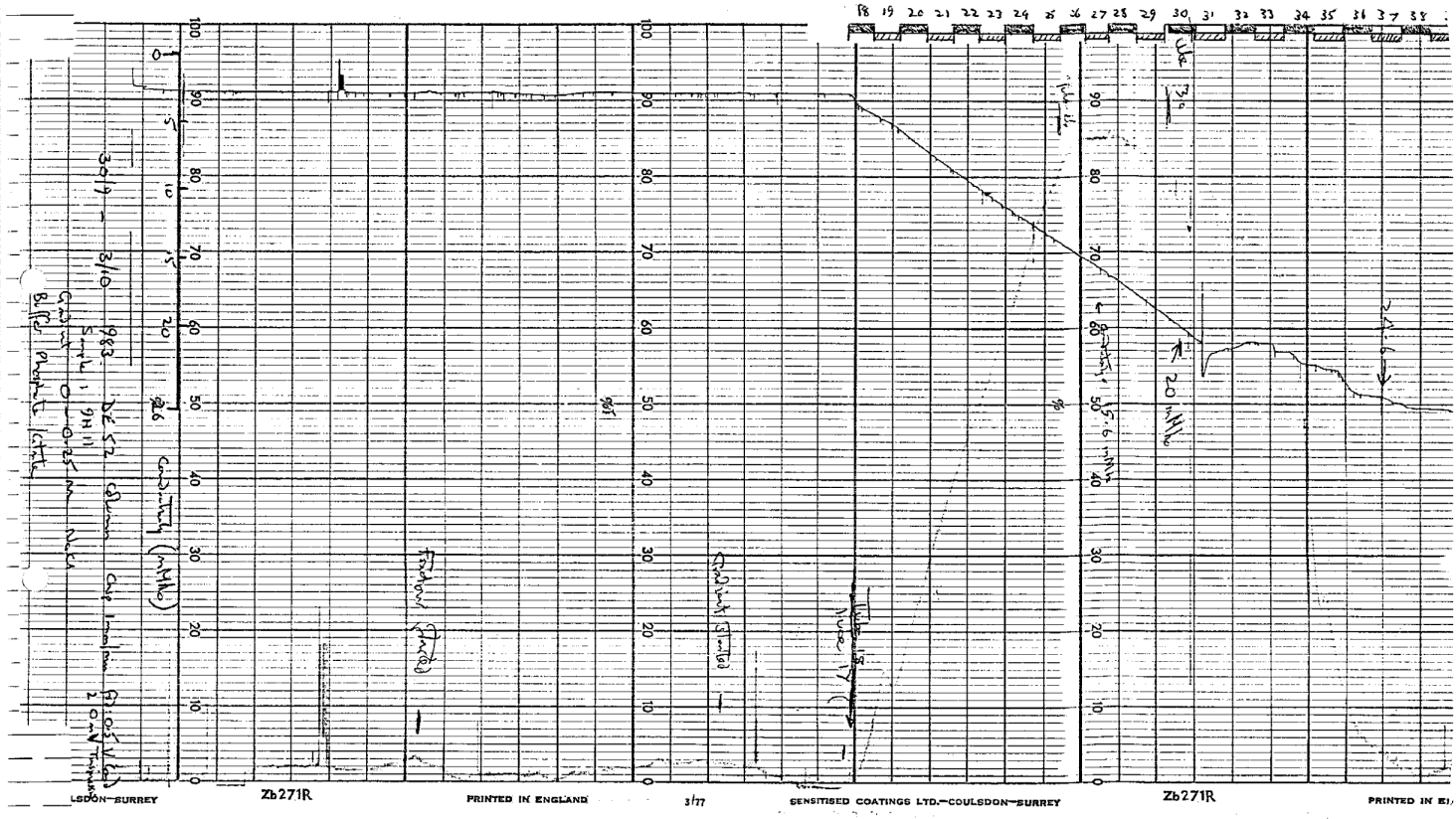
SAMPLE 9H11.02 INVESTIGATION		129	135				
Factor VIII, iu/ml	1 st.						
	2 st.						
✓ Factor IX, iu/ml	1 st.	18x	4x				
	2 st.	46.5	0.48				
Factor II, u/ml							
Factor X, u/ml							
Factor VII, u/ml (clotting)							
AT III (Anti Xa), u/ml							
AT III (Anti IIa), u/ml							
AT III (Laurell), u/ml							
Factor VIII RAG, u/ml							
Fibrinogen (Laurell), mg/ml							
Prothrombin (Laurell), u/ml							
Factor XIIIA (Laurell), u/ml							
Fibronectin (Laurell), mg/ml							
Factor VIII CAG							
✓ NAPTT sec. 1/10		260	30cc	Blank:-	300"		
TGt50 min.							
FDA hr.							
Limulus + or -							

For comments contact Date finished:

9H11.02

9H11-02.

Sample	Volume.	OD280	Factor IX u/ml	Factor IX Total Units.	% Activity Factor IX	fAf
∝9H11.02	495		4.49	22225		
/Sn	500		0.24	120	5.4	
/19	9.5	0.86	<0.15	<1.42	<0.06	
/22	9.5	4.2	0.53	5.03	0.22	0.1
/25	9.5	10.68	7.24	68.8	3.09	0.6
/27	9.5	16.86	1.8	17.1	0.77	0.1
/29	9.5	16.03	46.5	441.7	19.8	2.[illegible]
/35	9.5	0.697	0.48	4.56	0.20	0.[illegible]


LSDON—SURREY
Zb271R
PRINTED IN ENGLAND
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SENSITISED COATINGS LTD.—COULSDON—SURREY
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9H12 15/9/83 Heating and Recovery of factor IX using sorbitol, glycine and DE-52.

The intention of this experiment is to overcome the worsening division and recovery of factor IX, after heating, on DE-52 batch 6/2 which has been observed in 9H7.03, 9H9, 9H10.

It appears that this effect is due to some type of loss on the part of DE-52 and this, coupled with concern in Production over various properties of DE-52, has led to the use of an old, PEL-recycle batch of QC DE-52.

Materials

Buffers: Buffers were made up in R+D lab, according to methods for factor IX Production Buffers A and B. After dilution to the working concentration from a stock solution, the buffers had properties thus:

	pH	Conductivity (mMho)
Buffer A	7.04	11.97
Buffer B.	7.07	23.77

Factor IX: 19 vials of 9D 22 31 (35 ml fill, no heparin, pyrogen pair) were reconstituted with PFW.

DE-52: A recycled batch, approx 2 years old used for QC only. Stored frozen and thawed overnight at room temperature. After taking required amount, remainder was re-frozen.

Method

After reconstitution of 9D, 2 x 1 ml samples were taken for assay.

6.7g glycine was then added (1g/10ml) and dissolved before addition of 8.71g sorbitol. The sample was immersed in hot water (37°) to aid solution.

2 x 1 ml samples were taken /S

The material was then heated at 60° for 10.5 hours in 4 plastic centrifuge bottles. The temperature of heating bath water and samples were monitored, and remained constant over the period. (see chart trace).
The heated factor IX was removed from the water bath immediately.

3 x 1 ml samples were taken 9H12

The 9H12 was diluted by ½ by addition of an equal volume of PFW

3 x 2 ml samples taken @ 9H12

This material was sent for immediate factor IX assay, from which gel loading was calculated.
The pH was adjusted to 6.95 by addition of citrate

2 x 1 ml sample was taken @ 9H12 -H

With a loading of 150 units factor IX per g DE-52 62 g DE-52 were added and the mixture stirred for 1 hour at room temperature (Factor IX assay showing 3.7 u/ml).

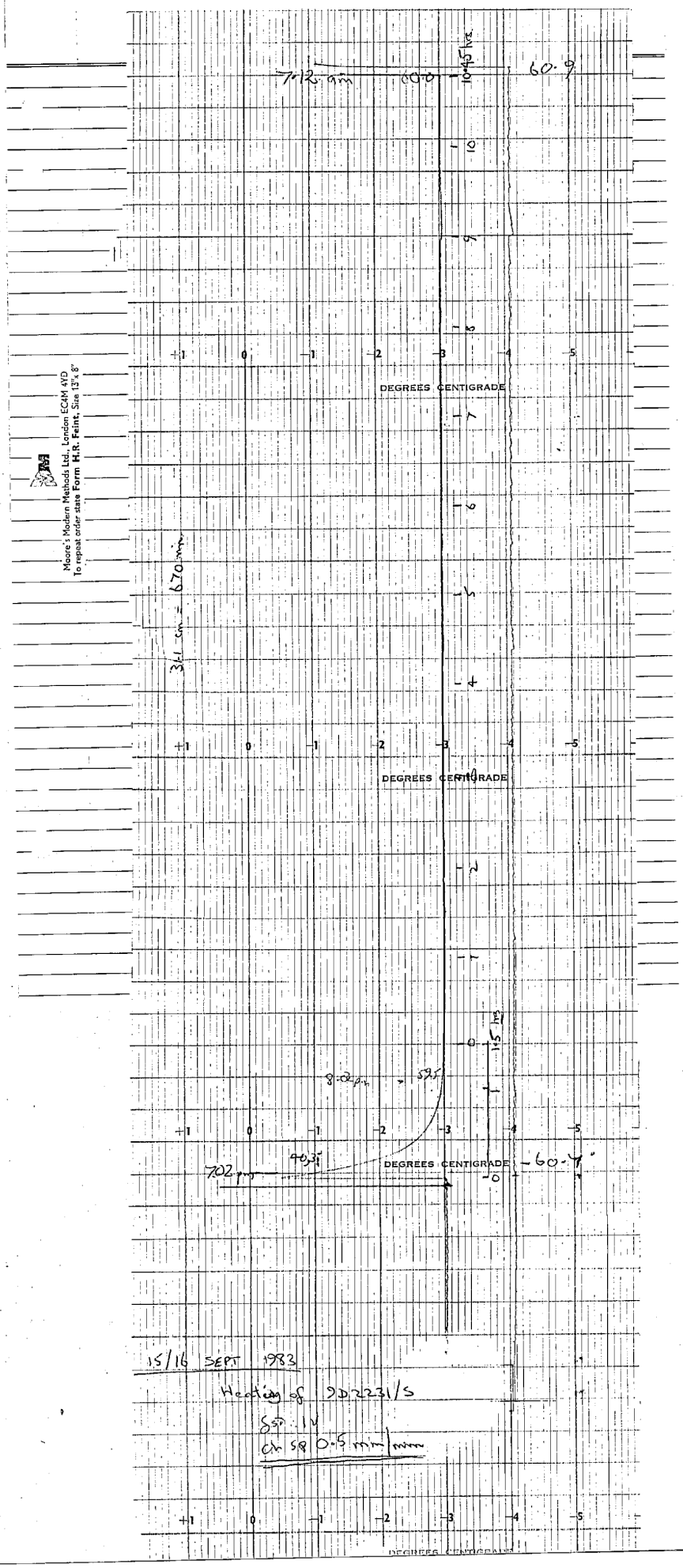
Gel was then spun down and supernatant poured off. Some gel was observed in the supernatant

3 x 2 ml sample taken /Sn

The gel was resuspended in Buffer A and packed into Amicon 5 cm I.D. glass column.
It was then washed with a total volume of 700 ml buffer, pumped on at 20 ml/min.

A total of 840 ml Buffer B then applied.

9H12_3



15/16 SEPT 1983

Heating of DD2231/5

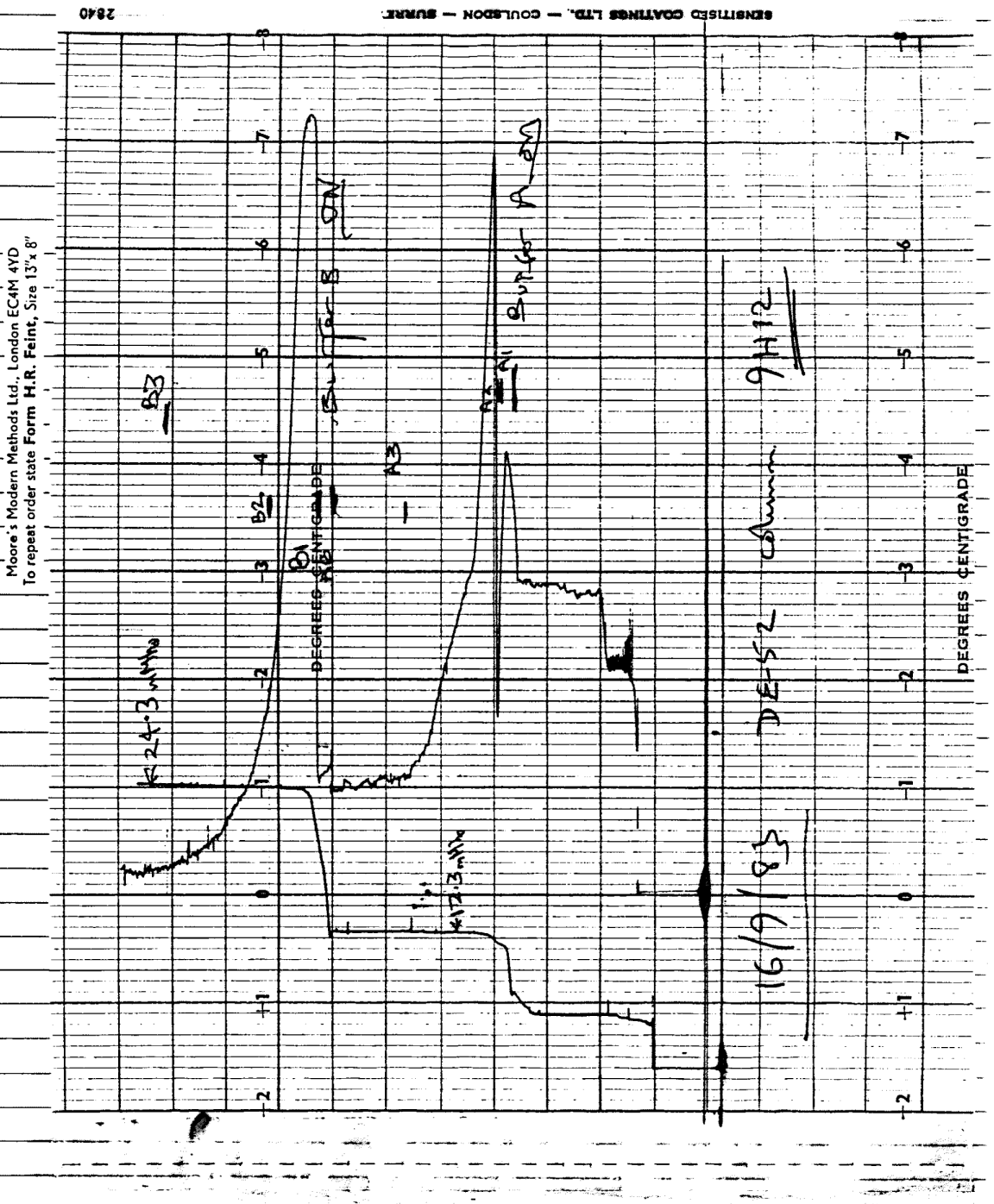
Sat 1N

Ch 58 0.5 min

9H12 4

Results

Below is the recording of transmission at 260 nm and conductivity of eluate from DE-52 column



9H12.5

Compared with 9H10, a far smaller proportion of the total protein eluted came off in buffer A.

Sample	Vol (ml)	pH	cond (mMho)	A_{280}	Factor IX u/ml	Factor IX Total Units	% IX of starting material	Sp Act (u/mg)	FIX lost in sampling	
9D2231	670	7.13	19.67	15.6	30.6	20502	100	1.96	61.2	
/S	1262	6.67			14.5	18299	89.2		29	
9H12	1260	6.72			5.8	7308	35.6		17.4	% I
α9H12	2505	6.87	2.54		2.8	7014	34.2		16.8	Neolog DE52
α9H12-H	2505	6.95		3.95	3.12	7815.6	38.1	0.79	6.2	10c
/Sn	2400	7.77	5.12	1.9	0.37	888	4.3	0.19	2.2	11.4
/A1	79	7.72	6.39	4.0	1.4	110.6	0.54	0.35	5.6	1.4
/A2	375	7.5	11.47	3.74	2.2	825	4.0	0.59	13.2	10.6
/B1	173	7.12	20.33	16.32	26.1	4515.3	22.0	1.6	86.6	57.8
/B2	450	7.08	23.77	0.7	1.1	495	2.4	1.57	6.6	6.3

SDS Polyacrylamide Gel Electrophoresis

Using 12% Acrylamide gel, samples 9D, -H /Sn, /A1, /A2, /B1 and /B2 were compared. This showed the elimination of high molecular weight species (M_r > 200,000) and also loss of minor bands in the M_r region 50,000 - 70,000, for samples B1 and B2. B1 and B2 could not be distinguished As indicated by the specific activity above, B1 and B2 were more highly purified than A1 and A2.

JH12.6

Comments

This experiment does show improved results over preceding ones — a reflection of the change of DE-52.

There are still poor recoveries at both stages of the process. After heating 38% factor IX recovery compares badly with 70% + recoveries for factor VIII concentrates after heating. The final recovery of 22% is not satisfactory, though the gel recovery has been marginally improved to 58% of the activity loaded. Encouraging is the elevation of potency of 81 — almost to the potency of the starting 90 mutant.

Future work shall therefore attempt to improve recoveries at both stages by

1) Increasing the sorbitol concentration used — existing concentrations (13 g/10 ml) are based on improved recovery at that level maximum — no optimum value has yet been found (the factor VIII unit uses the higher value of 18 g/10 ml)

2) Altering the ionic strengths of buffers A and B. Experiments in DC show that sorbitol depresses conductivity. If this is a genuine depression of ionic strength (rather than a machine artefact) it would explain why ionic strength rises and potency is eluted by washing in buffer A after the gel has already been suspended in buffer A. The elution of sorbitol by fresh buffer A would in itself increase the ionic strength.
An improved yield may therefore be obtained using a lower ionic strength buffer A and a higher ionic strength buffer B. This would avoid elution of F IX in A thereby increasing factor IX eluted in buffer B and increasing the concentration due to a sharper step. This would allow for later dilution to reduce the ionic strength to clinically acceptable levels whilst retaining an operating potency in the region of 30 u/ml.

PAF, DC

9H12

ASSAY REQUEST FORM (1)

Request from: PAF/DC Date: 19/9/83

When results needed: ASAP (GRADE 1)

Samples to be kept? _____ If so, how? _____

Samples ~~provided with form~~/available from R+D Freezer

SAMPLE INVESTIGATION		9D2231	9D2231/S	9H12	αH12	αH12 -H	/Sn
Factor VIII, iu/ml	1 st.						
	2 st.						
Factor IX, iu/ml	1 st.	(34)	(20)	(7.5)	(3.7)	(3.7)	(0.08)
	2 st.	30.6	16.5	5.8	2.8	3.12	0.37
Factor II, u/ml							
Factor X, u/ml							
Factor VII, u/ml (clotting)							
AT III (Anti Xa), u/ml							
AT III (Anti IIa), u/ml							
AT III (Laurell), u/ml							
Factor VIII RAG, u/ml							
Fibrinogen (Laurell), mg/ml							
Prothrombin (Laurell), u/ml							
Factor XIIIA (Laurell), u/ml							
Fibronectin (Laurell), mg/ml							
Factor VIII CAG							
NAPTT sec. 1/10							
TGt50 min.							
FDA hr.							
Limulus + or -							

For comments contact _____ Date finished: _____

ASSAY REQUEST FORM (1)

Request from: PNF / DC Date: 9/9/83

When results needed: ASAP

Samples to be kept? If so, how?

Samples ~~provided with form~~/available from R+D freeze

SAMPLE INVESTIGATION ∝9H12		/A1	/A2	/B1	/B2		
Factor VIII, iu/ml	1 st.						
	2 st.						
Factor IX, iu/ml	1 st.	(3.9)	(4.5)	(36)	(2.5)		
	2 st.	1.4	2.2	26.1	1.1		
Factor II, u/ml							
Factor X, u/ml							
Factor VII, u/ml (clotting)							
AT III (Anti Xa), u/ml							
AT III (Anti IIa), u/ml							
AT III (Laurell), u/ml							
Factor VIII RAG, u/ml							
Fibrinogen (Laurell), mg/ml							
Prothrombin (Laurell), u/ml							
Factor XIIIA (Laurell), u/ml							
Fibronectin (Laurell), mg/ml							
Factor VIII CAG							
NAPTT sec. 1/10		235	219	197	228	control 254	
TGt50 min.							
FDA hr.							
Limulus + or -							

For comments contact Date finished:

9H.

ASSAY REQUEST FORM (1)

Request from: PAF/DC Date: 16/9/83

When results needed: THIS VERY MORNING (PLEASE)

Samples to be kept? No If so, how? _____

Samples provided with form/~~available from~~ _____

SAMPLE INVESTIGATION		9D2231	9D2231/S*	9H12*	αH12±		
Factor VIII, iu/ml	1 st.						
	2 st.						
Factor IX, iu/ml	1 st.	(34)	(18)	~~7.45~~ (8.5)	~~3.67~~ (4.2)		
	2 st.	33.9	21.0	7.5	3.7		
Factor II, u/ml							
Factor X, u/ml							
Factor VII, u/ml (clotting)							
AT III (Anti Xa), u/ml							
AT III (Anti IIa), u/ml							
AT III (Laurell), u/ml							
Factor VIII RAG, u/ml							
Fibrinogen (Laurell), mg/ml							
Prothrombin (Laurell), u/ml							
Factor XIIIA (Laurell), u/ml							
Fibronectin (Laurell), mg/ml							
Factor VIII CAG							
NAPTT sec. 1/10							
TGt50 min.							
FDA hr.							
Limulus + or -							

* 40% Sorbitol

± 20% Sorbitol

For comments contact _____ Date finished: _____

19.9.83. 9H13 To Test the effect of different temperature jumps during heating on the loss of factor IX Activity

The previous five heating experiments have placed factor IX / Sorbitol / Glycine mixtures in a preheated 60° water bath directly from standing at room temperature. The evidence of 9H11 suggested that loss of factor IX activity is exponential with time. There may be a coincident loss due to exposing the protein to shock from a high temperature differential (20° → 60°). This experiment is to determine whether loss of factor IX activity over an arbitary period is dependent upon the temperature difference between sample and water bath.

Method 1 vial of 9.b 223 (no heparin, 35 ml fill, pyrogen free) was reconstituted with PFW.

10% w/v glycine added and dissolved, followed by 1.3 g/ml sorbitol.

Once completely dissolved, the solution was divided into 5 ml aliquots and frozen.

Prior to starting the heating experiment, samples were thawed at 20°.

An arbitrary period of 4 hours heating at each of two temperatures was selected. After 4 hours at the lower of the two temperatures, a 2 x 1 ml samples were taken (-/4) before transferring to the higher temperature

Heating was performed on three days (see proto col over).

Tubes A - G on 20.9.83
Tubes H - K on 23.9.83
Tubes L + M on 28.9.83.

Heating Protocol

Tube	Water Bath Temperature 40°	50°	60°	70°
A	8 hrs			
B	4 hrs →	4 hrs		
C	4 hrs →			4 hrs
D		8 hrs		
E		4 hrs →	4 hrs	
F			8 hrs	
G	Control at room temperature (20°) for eight hours			
H	4 hrs →			4 hrs
J			4 hrs →	4 hrs
K				8 hrs
L		4 hrs →	4 hrs	
M		4 hrs →		4 hrs

E and L are the same to compare results on different days.

Assays Factor IX Assays were performed in two blocks A – G on one day and H to M on another.
1 ml samples of 4 hr and 8 hr heating were stored frozen.

Results

		Factor IX u/ml	% Activity of Control
Control Tube G		20.2	100
(A) From Room Temp. 4hrs at			
40°	B4	19.4	96
	C4	19.6	97
	H4	18.4	91
50°	E4	19.5	96
	L4	19.4	96
60°	J4	12.6	62

9 N 13.3

		Factor IX u/ml	% Activity of Control
(B) From room temp 8hrs at			
40°	A	17.6	87
50°	D	18.3	90
60°	F	9.5	47
70°	K	0.3	1

(C) Find % Recovery after 4 hours at each of two temperatures

(Factor IX activity u/ml in brackets)

2nd 4 hours	1st 4 hours 40°	50°	60°	70°
40	(17.6) 87			
50	(14) 69	(18.3) 90		
60	(14.6) 72	(12.7) 63 (11.7) 58	(9.5) 47	
70	(0.6) 3	(3) 15	(1.2) 6	(0.3) 1

(D) % loss of starting Factor IX Activity attributable to 2nd 4hrs heating period (i.e. (C) - (A))

2nd 4 hours	1st 4 hours 40°	50°	60°	70°
40°	8			
50°	26	5		
60°	23	33 38	15	
70°	92	81	56	

9H 13 4

Comments

1. The 4- hour heating has little effect on activity loss below 60°. At 60° there is a dramatic loss of factor IX activity (38%)

2. Under none of the conditions used does factor IX survive heating at 70°. For the purpose of this experiment, heating at that temperature can be ignored.

3. Control experiments E and L gave similar results (60% recovery ± 3%) and F at 60° gave the expected 47% recovery after 8 hours.

4. Preheating at 40° for 4 hours gave recoveries at 50° and 60° of 70% ± 2% while the recoveries at 50° and 60° after 4 hours were 96% and 62% respectively. After 8 hours at 50° and 60° recoveries were 90% and 47% respectively.
Preheating at 40° for 4 hours therefore appears to have no beneficial effect on subsequent heating at 50° (or possibly a deleterious effect) but improves the recovery after heating at 60° by about 10%.

5. Preheating at 50° for 4 hours gave a 60% recovery ± 3% after subsequent 4 hour heating at 60°. Since the "4 hour recovery" at 50° is 96% and the "4 hour recovery" at 60° is 62%, the heating at 50° appears to have no effect on the recovery after 4 hours at 60°. However, it MAY reduce factor IX activity loss in a subsequent heating period at 60° (i.e. the drop from 62% to 47% recovery if an 8-hour heating at 60° is carried out)

6. The loss of factor IX activity at 60° follows the pattern previously observed (9H11); 38% of the activity is lost in the first 4 hours and a further 15% in the second 4 hours. (i.e. exponential loss of activity).

9H13.5

Conclusion

Some improvement in recovery of factor IX activity can be achieved by raising the temperature to 60° from 20° in steps, rather than an immediate jump from one surrounding temperature to the other.

The improvement is, at best, 10% of the starting activity.

The optimum temperature for this improvement is 40°. Whether this is because 40° is the midpoint between the two temperatures, or because a 20° rise is the optimum step, is uncertain (Though to judge from the 70° heating, better recoveries are obtained for the 20° → 50° → 70° than for either of 20° → 40° → 70° or 20° → 60° → 70°. Since loss in the region <50° are minimal, the implication is that greatest loss occurs in the final 20° rise in temperature).

Therefore, in future heating experiments, a more gradual rise in surrounding temperature which improves factor IX recovery by 10% over 4 hours is to be recommended.

P. A. F.

ASSAY REQUEST FORM (1)

Request from: PAF Date: 25/9/83

When results needed: ASAP

Samples to be kept? No If so, how?

Samples ~~provided with form~~/available from R+D freezer

SAMPLE INVESTIGATION 9H13		/A	/B4	/B	/C 4	/C	/D
Factor VIII, iu/ml	1 st.						
	2 st.						
Factor IX, iu/ml	1 st.	(13)	(15)	(10)	(15)	(7.0)	(3.5)
	2 st.	17.6	19.4	14.0	19.6	14.6	18.3
Factor II, u/ml							
Factor X, u/ml							
Factor VII, u/ml (clotting)							
AT III (Anti Xa), u/ml							
AT III (Anti IIa), u/ml							
AT III (Laurell), u/ml							
Factor VIII RAG, u/ml							
Fibrinogen (Laurell), mg/ml							
Prothrombin (Laurell), u/ml							
Factor XIIIA (Laurell), u/ml							
Fibronectin (Laurell), mg/ml							
Factor VIII CAG							
NAPTT sec. 1/10							
TGt50 min.							
FDA hr.							
Limulus + or -							

ALL CONTAIN 40% SORBITOL

For comments contact Date finished:

ASSAY REQUEST FORM (1)

9H13

Request from: PAF Date: 26/9/83

When results needed: ASAP

Samples to be kept? If so, how?

Samples provided with form/available from

SAMPLE INVESTIGATION 9H 13.		1E4	1E	1F	1G		
Factor VIII, iu/ml	1 st.						
	2 st.						
Factor IX, iu/ml	1 st.	(7.0)	(3.5)	(9.5)	(18)		
	2 st.	19.5	12.7	9.5	20.2		
Factor II, u/ml							
Factor X, u/ml							
Factor VII, u/ml (clotting)							
AT III (Anti Xa), u/ml							
AT III (Anti IIa), u/ml							
AT III (Laurell), u/ml							
Factor VIII RAG, u/ml							
Fibrinogen (Laurell), mg/ml							
Prothrombin (Laurell), u/ml							
Factor XIIIA (Laurell), u/ml							
Fibronectin (Laurell), mg/ml							
Factor VIII CAG							
NAPTT sec. 1/10							
TGt50 min.							
FDA hr.							
Limulus + or -							

40% SORBITOL

For comments contact Date finished:

9H13

ASSAY REQUEST FORM (1)

Request from: PAF Date: 29/9/83

When results needed: ASAP

Samples to be kept? No If so, how?

Samples ~~provided with form~~/available from R+D Freeze

SAMPLE INVESTIGATION 9H13		/H4	/H	/J4	/J	/K	/L4
Factor VIII, iu/ml	1 st.						
	2 st.						
✓ Factor IX, iu/ml	1 st.	(19.5)	(11.5)	(15.5)	(10)	(8)	(19.5)
	2 st.	18.4	0.6	12.6	1.2	0.3	19.4
Factor II, u/ml							
Factor X, u/ml							
Factor VII, u/ml (clotting)							
AT III (Anti Xa), u/ml							
AT III (Anti IIa), u/ml							
AT III (Laurell), u/ml							
Factor VIII RAG, u/ml							
Fibrinogen (Laurell), mg/ml							
Prothrombin (Laurell), u/ml							
Factor XIIIA (Laurell), u/ml							
Fibronectin (Laurell), mg/ml							
Factor VIII CAG							
NAPTT sec. 1/10							
TGt50 min.							
FDA hr.							
Limulus + or -							

ALL CONTAIN "40%" SORBITOL

For comments contact Date finished:

ASSAY REQUEST FORM (1)

Request from: RAF Date: 29/9/83

When results needed: ASAP

Samples to be kept? No If so, how?

Samples ~~provided with~~ form/available from R & D Freezer

SAMPLE INVESTIGATION		1L	1M				
Factor VIII, iu/ml	1 st.						
	2 st.						
Factor IX, iu/ml	1 st.	(14.5)	(11)				
	2 st.	11.7	3.0				
Factor II, u/ml							
Factor X, u/ml							
Factor VII, u/ml (clotting)							
AT III (Anti Xa), u/ml							
AT III (Anti IIa), u/ml							
AT III (Laurell), u/ml							
Factor VIII RAG, u/ml							
Fibrinogen (Laurell), mg/ml							
Prothrombin (Laurell), u/ml							
Factor XIIIA (Laurell), u/ml							
Fibronectin (Laurell), mg/ml							
Factor VIII CAG							
NAPTT sec. 1/10							
TGt50 min.							
FDA hr.							
Limulus + or -							

CONTAIN 40 % SORBITOL.

For comments contact Date finished:

26.9.83 9H14 To Determine the effect of raised Stabiliser concentrations on factor IX survival upon heating

9H experiments to date are giving poor yeilds upon heating (35-47% after 10 hours at 60°).
It appears that the amount of sorbitol and glycine added are not optimum concentration but rather maximum concentrations used in test experiments.

The purpose of this experiment therefore, is to see whether an increase in either or both of the sorbitol and glycine added results in an improve factor IX recovery. (cf higher sorbitol levels in factor VIII heating gives ~ 70% yields).

Method

2 vials of factor IX 9D 2231 (no hepain, 35 ml fill, pyrogen failed) reconstituted with PFW. 5 ml were put into each of 9 plastic universal containers.

First glycine was added to each then, once this had dissolved, sorbitol was added, according to the following regime, and the containers labelled:

		Sorbitol (g/10ml) 13	16	20
Glycine (g/10ml)	1.0	A	B	C
	1.5	D	E	F
	2.0	G	H	J
Volume of each (ml):		9.25	11.25	13.3

The containers were then placed in a pre-heated 60° water bath for 12 hours.

Due to volume expansion upon the addition of sorbitol the final sorbitol concentrations were :

Tube	[Sorbitol] g/10ml
A, D, G	6.66
B, E, H	7.11
C, F, J	7.52

These were all diluted to a final sorbitol concentration of 3g/10ml in order to eliminate such variations during assay dilutions (which are harder for smaller volumes and higher sorbitol concentrations). Each was diluted into fresh TX process buffer A.

Sample	Vol (ml)	Add buffer A (ml)
A, D, G	9.75	11.9
B, E, H	11.25	15.4
C, F, J	13.3	20.0

Results

Although not assayed on this occasion, recents assays of 9D2231 give an average (of 3) activity of 34.6 u/ml.

For the comparitive purpose of this experiment it is therefore adequate to assume a total of 173 Units in each vial.

Units recovered.

Starting [glycine] (g/10ml)	Starting [Sorbitol] (g/10ml) 13	16	20
1.0	62.8	80.0	93.24
1.5	60.6	69.3	93.24
2.0	58.5	77.3	109.9

9H 4

% factor IX Recovery after 12 hrs at 60°

Starting [Glycine] g/10ml	Starting [sorbitol] (g/10 ml) 13	16	20
1.0	36.3	46.2	53.9
1.5	35	40.1	53.9
2.0	33.8	44.7	63.5

Conclusion

Increasing the sorbitol content results in improved recoveries of factor IX. An optimum value has still not been attained; the limiting consideration is probably the difficulty of manipulating large concentrations of sorbitol.

Increased glycine does not appear to make a significant difference to the factor IX yield, in the narrow results obtained here.

The use of the higher sorbitol level [20g /10ml) is recommended for future heating experiments.

* There is no evidence of the relative protection afforded by the sorbitol to the protein and to any infecting virus. It should be recognised that viral survival may be similarly improved by higher sorbitol concentrations.

P.A.F.

ASSAY REQUEST FORM (1)

Request from: PAF Date: 27.9.83

When results needed: ASAP

Samples to be kept? No If so, how?

Samples ~~provided with form~~/available from R + D fridge

SAMPLE INVESTIGATION 9H14		/A	/B	/C	/D	/E	/F
Factor VIII, iu/ml	1 st.						
	2 st.						
Factor IX, iu/ml	1 st.	(2.8)	(3.1)	(3.15)	2.8	(3.1)	(3.4)
	2 st.	2.9	3.0	2.3	2.3	2.6	2.8
Factor II, u/ml							
Factor X, u/ml							
Factor VII, u/ml (clotting)							
AT III (Anti Xa), u/ml							
AT III (Anti IIa), u/ml							
AT III (Laurell), u/ml							
Factor VIII RAG, u/ml							
Fibrinogen (Laurell), mg/ml							
Prothrombin (Laurell), u/ml							
Factor XIIIA (Laurell), u/ml							
Fibronectin (Laurell), mg/ml							
Factor VIII CAG							
NAPTT sec. 1/10							
TGt50 min.							
FDA hr.							
Limulus + or -							

SORBITOL AT 0.3g/ml

For comments contact Date finished:

9H14

ASSAY REQUEST FORM (1)

Request from: PAF Date: 27.j.83

When results needed: ASAP

Samples to be kept? No If so, how?

Samples ~~provided with form~~/available from R+D freezer

SAMPLE INVESTIGATION 9H14		/G	/H	/J			
Factor VIII, iu/ml	1 st.						
	2 st.						
✓Factor IX, iu/ml	1 st.	(3.1)	(3.6)	(3.6)			
	2 st.	2.7	2.9	3.3			
Factor II, u/ml							
Factor X, u/ml							
Factor VII, u/ml (clotting)							
AT III (Anti Xa), u/ml							
AT III (Anti IIa), u/ml							
AT III (Laurell), u/ml							
Factor VIII RAG, u/ml							
Fibrinogen (Laurell), mg/ml							
Prothrombin (Laurell), u/ml							
Factor XIIIA (Laurell), u/ml							
Fibronectin (Laurell), mg/ml							
Factor VIII CAG							
NAPTT sec. 1/10							
TGt50 min.							
FDA hr.							
Limulus + or -							

SORBITOL AT 0.3 g/ml

For comments contact Date finished:

9H15.1

7.10.83 9H15 Modified conditions for heating and recovery of factor IX

The results of 9H14 suggest that factor IX survives heating better at higher sorbitol concentrations. This experiment attempts to apply that to a larger scale than before, and also introduces a modified buffer system in an attempt to improve elution from DE-52.

Materials

Factor IX : 18 vials of 9D 2232 (non-heparinised Pyrogen failed) reconstituted in 630 ml PFW

Buffers : *Buffer J : 8.5 mM citrate
9 mM phosphate ($N_{a_2}HPO_4$)
75.6 mM NaCl
} pH 6.9

*Buffer K : 9.2 mM citrate
10.4 mM phosphate
211 mM NaCl
} pH 6.94

* Concentrations according to assay. Concentrations by weight were :

Buffer J : 9.46 mM citrate
9 mM Phosphate
80 mM NaCl

Buffer K : 10.2 mM citrate
10 mM Phosphate
300 mM NaCl

Method

Glycine was dissolved in 9D to the usual concentration of 0.1 g/ml by adding 64 g of solid glycine to the 640 ml of factor IX.

After this had dissolved, sorbitol was added to 2 g/ml, a total addition of 1280 g. The material was dissolved while immersed in a water bath to ensure that the temperature remained

above 20°c.

The heating stage was modified in this experiment instead of immersion of factor IX at 20°c into a water bath at 60°c, the bath temperature was initially 34°c and was subsequently raised in steps, so that the difference between bath temperature and sample temperature was not greater than 10°c. It did appear that heating was more efficient if the temperature difference was greater than 7°c. (See chart).

After the temperature of the sample reached 60°, heating continued for 10 hours and the temperature varied between 60·2° and 60·8°

After 10 hours, samples "9H15" were taken and the factor IX diluted by addition of two volumes of PFW (to compensate for the higher sorbitol concentration. Samples of "α9H15" were taken, and fresh assays performed to determine gel loading.

From the value obtained (2·5 u/ml), 80g recycled DE-52 was added to the factor IX and stirred at room temperature for one hour.

The adsorbed gel was spun down at 3,000 rpm in a Beckman centrifuge and resuspended in 1·5 x dry gel weight (i.e. 120 ml) of buffer J.

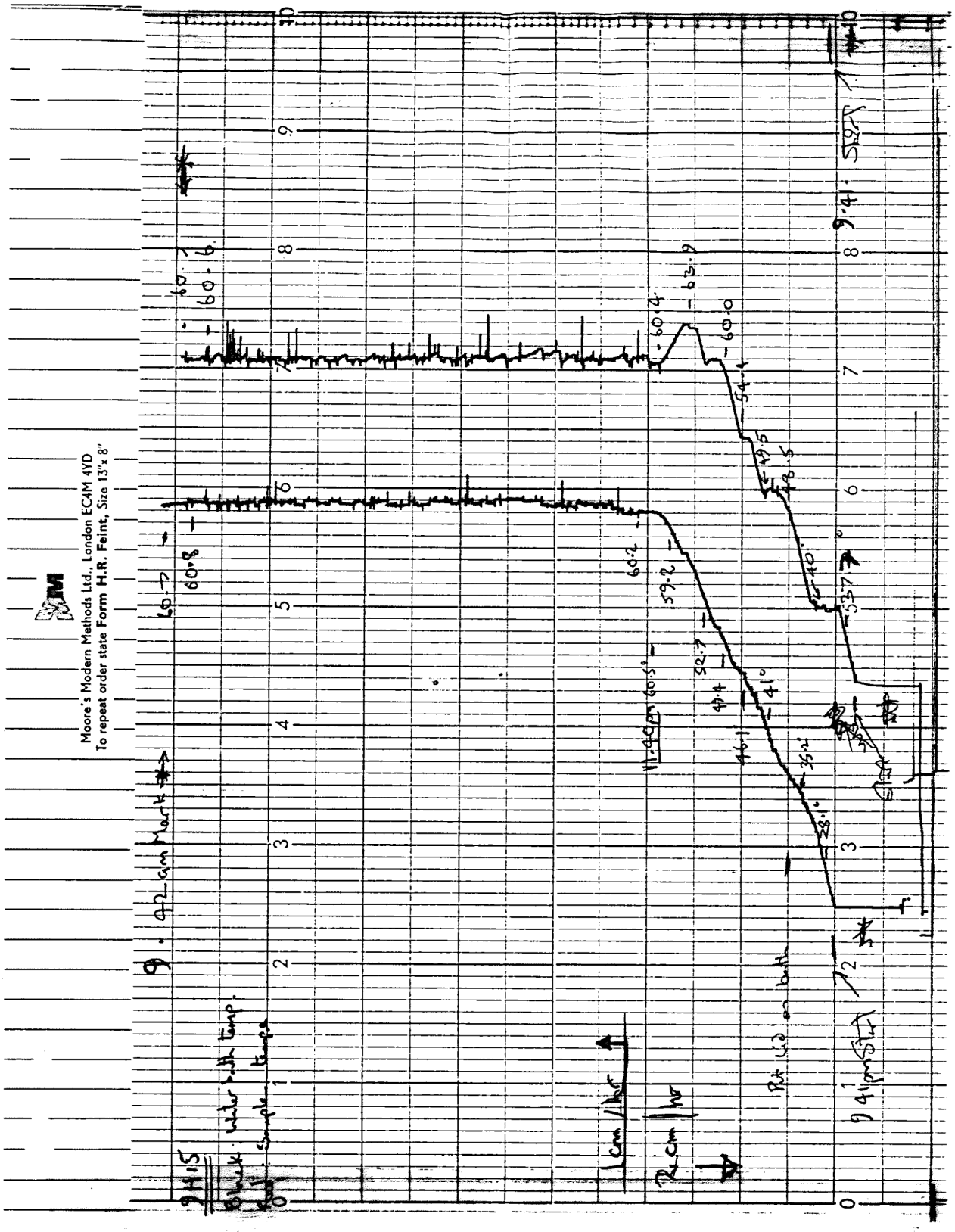
This slurry was packed into a 4 cm ID glass Amicon column.

Once packed, buffer J was pumped through the column at 600 ml/hr until the protein peak had fully eluted (though u.v. Trace did not return to absolute zero).

Buffer K was then applied and a broad protein peak eluted.

Assays were performed on samples for factor IX activity, NAPTT (some samples), chloride, phosphate, citrate (some samples).

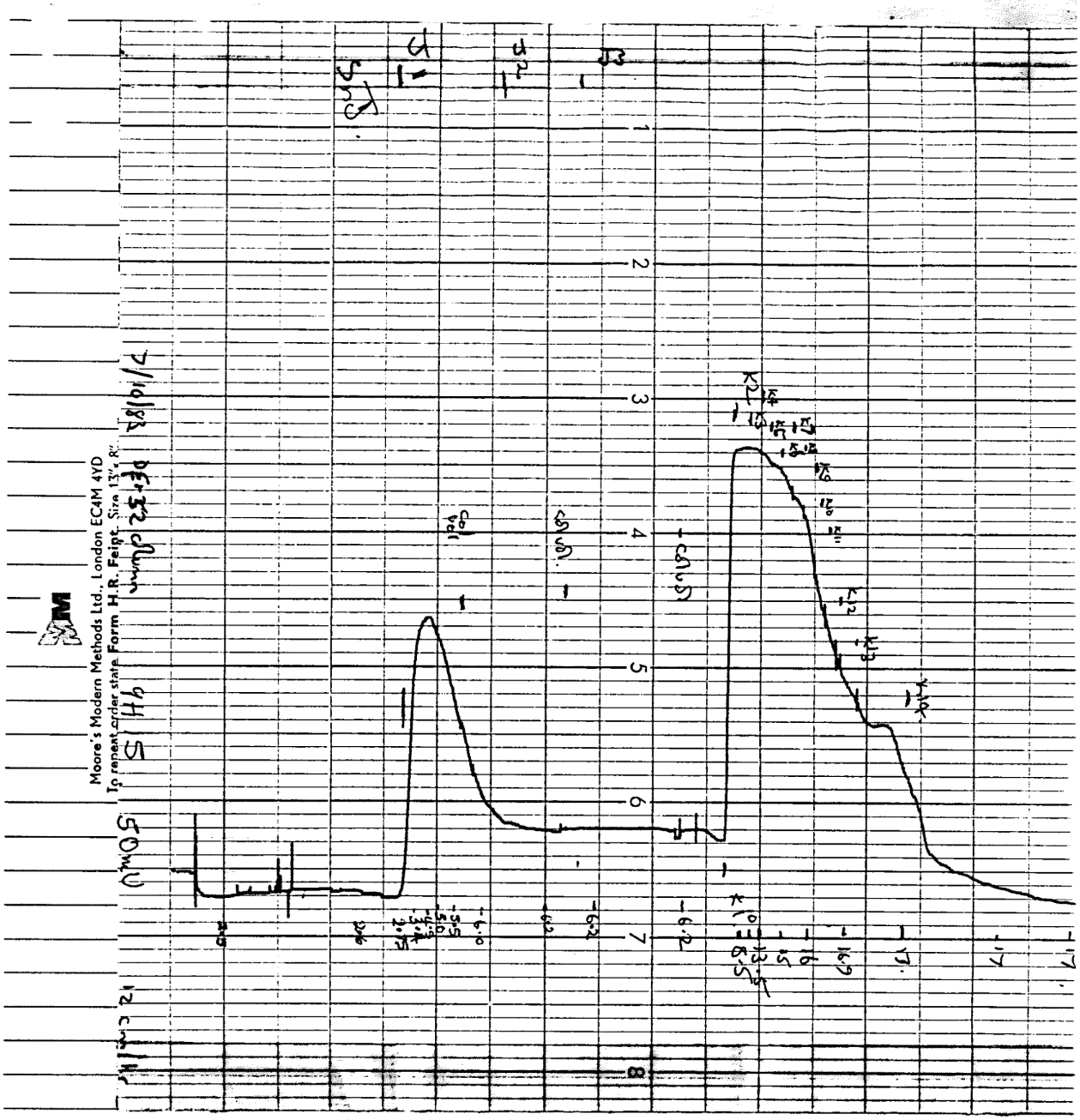
9 N.S 3



Trace of bath and factor IX temperature during heating

Black: Water bath ; Red · factor IX

9H15.4



Trace of U.V. transmission of eluate from DE 52 column

9H15 . 5

Results (sea traces.)

Protein Data

Sample	Vol (ml)	*A280	Factor IX u/ml	Factor IX Total units		Sp. Act u/mg"	% starting factor IX	% gel loade factor IX
9D	640		32·2	(1) 20608	21088		100	
			33·7	21568				
15	1415		14·4	20376	19527		92·6	
			13·2	18678				
9H15	1425		9·1	12967	12682		60·1	
			8·7	12397				
α9H15	4300		2·5	10750	9675		45·9	
			2·0	8600				
(2) 1Sn	4124	0·27	0·12	494·9		0·44	2·3	5·1
1SnJ	120	0·32	0·14	16·8		0·44	0·08	0·17
1J1	100	0·396	0·13	13		0·33	0·06	0·13
1J2	180	3·09	1·7	306		0·55	1·4	3·16
1J3	350	1·18	0·47	1645		0·40	0·8	1·7
1K1	20	20·51	19·5	390		0·95	1·85	4·03
1K2	20	43·2	64·7	1294		1·5	6·14	13·37
1K3	20	32·6	62·4	1248		1·9	5·92	12·9
1K6	20	17	36·4	728		2·1	3·45	7·5
1K9	20	7·6	14·2	107·9		1·9	0·51	1·1
1K13	82	6·5	7·9	6478		1·2	3·07	6·7

(1) First value is from fresh assay, second is from −30° stored sample.
(2) 1Sn : Supernatant after DE 52 adsorption. 1SnJ : column wash during packing.
Other fractions as labelled on trace.
* based on scaled up dilution values

9H15 6

Physical data

Sample	pH	Conductivity mMho	[Citrate] mM	[Phosphate] mM	[Cl⁻] mM	[Combina mM
9D	7.20	18-36				
1Sn	7.9	1.67				
1SJ	7.86	4·1				
1J1	7.88	4.34	7·8	4·74	35	47·5
1J2	7.95	9·01	10.92	6·55	48·4	65·9
1J3	7.77	10.16	7·46	8·74	70·5	86·7
1K1	7.07	8·5	29·9	9·78	67.6	107.3
1K2	7.11	12·6	37·1	12·6	106	155·7
1K3	7.08	16·5				
1K4	7.08	19.2				
1K5	7.05	20	29·6	13·0	210	252.(
1K6	7.04	20·8				
1K7	7.04	21·1				
1K8	7.03	21·5				
1K9	7.03	22·1				
1K10	7.03	22·6	15·7	11·9	251	278.(
1K11	7.01	22·8				
1K12	7.02	22·9				
1K13	7.05	28·7	11·8	10·9	271	293.

Comments

1) There appears to be a drop in factor IX activity after dilution to ∝9H15. However the true extent of this loss is questionable, because there also seems to be assay variation between fresh and stored samples of the same fraction. Observation shows that the high sucktill samples do not freeze as solidly as the normal samples, which may account for a genuine loss of factor IX activity.

2) It also appears that no great value can be placed upon the assays for ions. According to assay the citrate and chloride concentrations are lower than that

9H15

calculated by measured weight in buffers J and K. Indeed, the eluted chloride concentration in the eluate / K13 is higher by assay than the applied buffer concentration.

3) The yield upon heating of factor IX is improved, recommending that this higher concentration of sorbitol is used in future.

4) The recovery from DE-52 is also improved; factor IX potency higher as is the specific activity. The overall factor IX can be accounted for assuming an even distribution between assayed and non-assayed samples:

$$(S_n + S_nJ + J1 + J2 + J3 + K1 + K2 + 2(K3) + 3(K6) + 5(K9) + K13)$$

$$(5.1 + 0.17 + 0.13 + 3.16 + 1.7 + 4.03 + 13.37 + 25.8 + 22.5 + 5.5 + 6.7)$$

= 88.2 % of the factor IX units in α9H15

5) NAPTT values (not shown above)

K1	190"
K2	181"
K3	228"
K6	209"
K9	224"
K13	232"
Blank	279"

These levels appear acceptable over the whole K buffer elution peak.

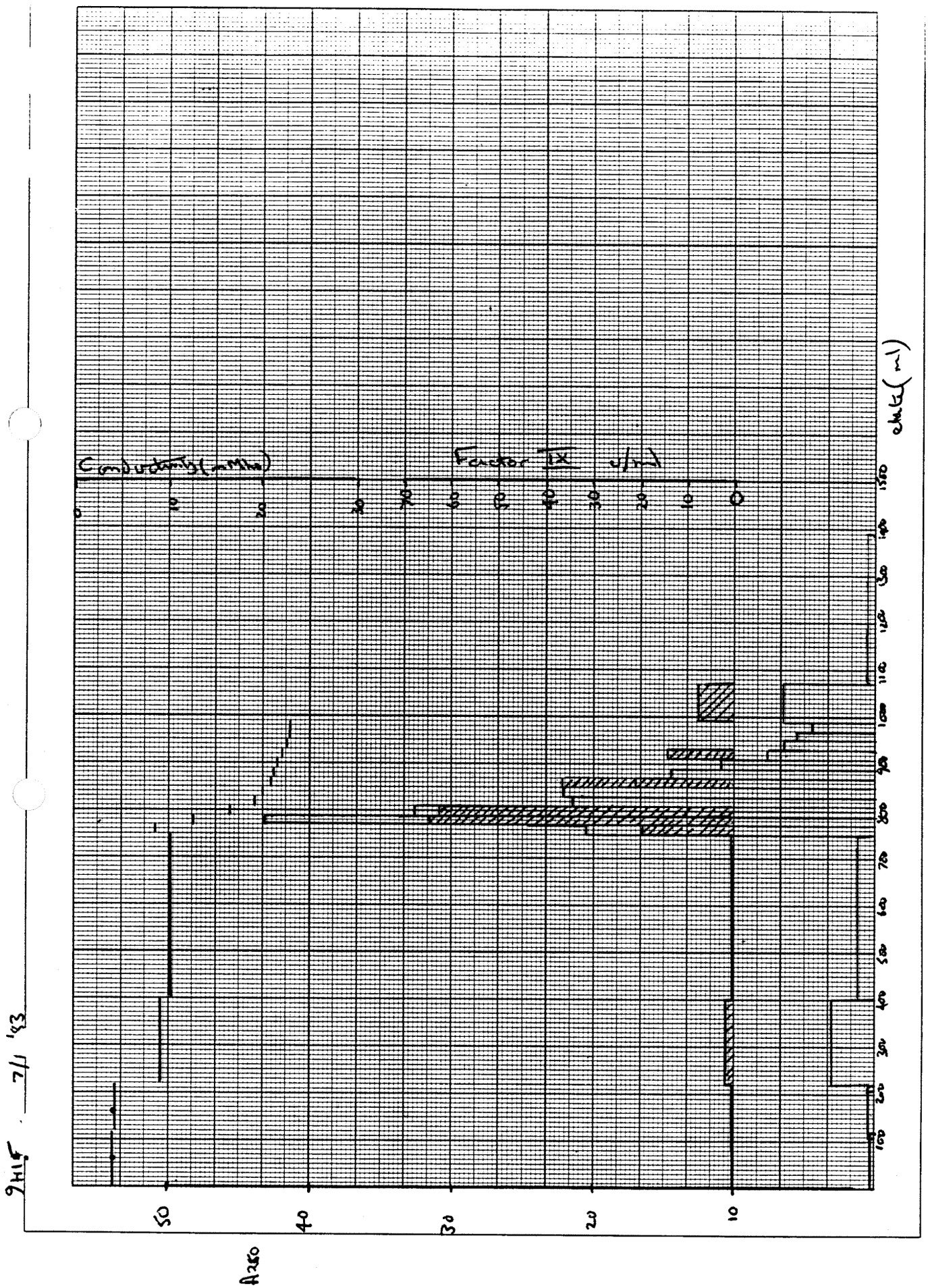
Conclusions

20 g/l sorbitol be used in further factor IX heating experiments.

Buffers J and K be further used to characterise their elution properties.

P.A.F./D.C.

9 HIS


Conductivity (mmho)
Factor 1X
chlor (mg/l)
0
20
30
40
50
60
70
80
90
100
120
140
160
180
200
220
240
260
280
300
320
340
360
380
400
420
440
460
480
500
520
540
560
580
600
620
640
660
680
700
720
740
760
780
800
820
840
860
880
900
920
940
960
980
1000
1020
1040
1060
1080
1100
1120
1140
1160
1180
1200
1220
1240
1260
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1300
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1360
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1400
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1440
1460
1480
1500
1520
1540
1560
1580
1600
50
40
30
20
10
9HIF
7/1 '83
A250

9H15

ASSAY REQUEST FORM (1)

Request from: PAF Date: 7/10/83

When results needed: _____

Samples to be kept? _____ If so, how? _____

Samples provided with form/available from _____

SAMPLE INVESTIGATION		9D	* 15	9H15 *	α 9H15 ‡		
Factor VIII, iu/ml	1 st.						
	2 st.						
Factor IX, iu/ml	1 st.	(30)	(13.5)	(8.8)	(3)		
	2 st.	32.2	14.4	9.1	2.5		
Factor II, u/ml							
Factor X, u/ml							
Factor VII, u/ml (clotting)							
AT III (Anti Xa), u/ml							
AT III (Anti IIa), u/ml							
AT III (Laurell), u/ml							
Factor VIII RAG, u/ml							
Fibrinogen (Laurell), mg/ml							
Prothrombin (Laurell), u/ml							
Factor XIIIA (Laurell), u/ml							
Fibronectin (Laurell), u/ml							
Factor VIII CAG							
NAPTT sec. 1/10							
TGt50 min.							
FDA hr.							
Limulus + or -							

40% Sorbitol

15% Sorbitol

For comments contact _____ Date finished: _____

9H15

Request from: PAF Date: 10/10/83

Which results needed: ASAP GRADE 1

Samples to be kept? YES If so, how: FROZEN

Samples ~~provided with form~~/available from: R+D freezer

SAMPLE / INVESTIGATION 9H15		902232	IS *	9H15 *	∝9H15 ‡	IS_n ‡	IS n J
Factor VIII, iu/ml	1 st.						
	2 st.						
Factor IX, iu/ml	1 st.	(32)	(14)	(9)	(3)	(0·08)	(<0·01)
	2 st.	33.7	13.2	8·7	2·0	0·12	0·14
Factor II, u/ml							
Factor X, u/ml							
Factor VII, u/ml (clotting)							
AT III (Anti Xa), u/ml							
AT III (Anti IIa), u/ml							
AT III (Laurell), u/ml							
Factor VIII RAG, u/ml							
Fibrinogen (Laurell), mg/ml							
Prothrombin (Laurell), u/ml							
Factor XIIIA (Laurell), u/ml							
Fibronectin (Laurell), u/ml							
Factor VIII CAG							
NAPTT sec. 1/10							
TGt50 min.							
FDA hr.							
Limulus + or -							

* 40% Sorbitol

‡ 15% Sorbitol

JHC

Request from: PAF Date: 10 10 93

What results needed: ASAP (Call)

Samples to be kept? Yes If so, how: Frozen

Samples ~~provided with form~~/available from R+D Freezer

SAMPLE INVESTIGATION 9H15		/J1	/J2	/J3	K1	K2	K3
Factor VIII, iu/ml	1 st.						
	2 st.						
Factor IX, iu/ml	1 st.	(0.2)	(1.6)	(0.6)	(35.5)	(75)	(56)
	2 st.	0.13	1.7	0.47	19.5	64.7	62.4
Factor II, u/ml							
Factor X, u/ml							
Factor VII, u/ml (clotting)							
AT III (Anti Xa), u/ml							
AT III (Anti IIa), u/ml							
AT III (Laurell), u/ml							
Factor VIII RAG, u/ml							
Fibrinogen (Laurell), mg/ml							
Prothrombin (Laurell), u/ml							
Factor XIIIA (Laurell), u/ml							
Fibronectin (Laurell), u/ml							
Factor VIII CAG							
NAPTT sec. 1/10					190 ✓	181 ✓	228 ✓
TGt50 min.							
FDA hr.							
Limulus + or -							

Request from: PAF Date: 10.10.93 YHS

When results needed: ASAP (Lvl 1)

Samples to be kept? YES If so, how? FROZEN

Samples ~~provided with form~~/available from R+D freezer

SAMPLE ... INVESTIGATION		K6	K9	K13			
Factor VIII, iu/ml	1 st.						
	2 st.						
Factor IX, iu/ml	1 st.	(29)	(13)	(11.2)			
	2 st.	36.4	14.2	7.9			
Factor II, u/ml							
Factor X, u/ml							
Factor VII, u/ml (clotting)							
AT III (Anti Xa), u/ml							
AT III (Anti IIa), u/ml							
AT III (Laurell), u/ml							
Factor VIII RAG, u/ml							
Fibrinogen (Laurell), mg/ml							
Prothrombin (Laurell), u/ml							
Factor XIIIA (Laurell), u/ml							
Fibronectin (Laurell), ~~mg~~/ml							
Factor VIII CAG		209 ✓	224 ✓	282 ✓		Blank.	279
NAPTT sec. 1/10							
TGt50 min.							
FDA hr.							
Limulus + or -							

For comments contact _____ Date finished: _____

9H15

Request from: PAF Date: 10/10/83

When results needed: ASAP

Are samples to be kept? If so, how?

Samples ~~provided with form~~/available from: R+D Freezer

SAMPLE 9H15 / INVESTIGATION		K1	K2	K5	K10	K13	
Protein	E280						
	biuret g/l						
Fibrinogen g/l/%							
Sodium mmol/l							
Potassium mmol/l							
√ Chloride mmol/l		67.6 (100)	106 (130)	210 (200)	251 (280)	271 (300)	
√ Citrate mmol/l (~10) →		29.9	37.1	29.6	15.7	11.8	
√ Phosphate mmol/l (~10) →		9.78	12.6	13.0	11.9	10.9	
Tris mmol/l							
PEG g/l							
Caeruloplasmin g/l							
Factor XIII u/ml							
% Ref. 2							
AT III (amidolytic) u/ml							
F. VII (amidolytic) u/ml							
XaGT mins.							
pH at 20°C							
Conductivity at 20°C							

For comments contact: JP/HB/GH Date finished:

9H15.

Request from: PAF Date: 10/10/83

When results needed: ASAP

Are samples to be kept? If so, how?

Samples ~~provided with form~~/available from: R+D Freezer (C5's)

SAMPLE 9H15 INVESTIGATION		J	K	~~J1~~	~~J2~~	J3	
Protein	E280						
	biuret g/l						
Fibrinogen g/l/%							
Sodium mmol/l							
Potassium mmol/l							
✓ Chloride mmol/l		(80) 75.6	(300) 211	(50) 35.0	(70) 48.4	(80) 70.5	
✓ Citrate mmol/l		8.51 (9)	9.19 (10)	7.80 (18)	10.92 (10)	7.46 (10)	
✓ Phosphate mmol/l		9.01 (9)	10.4 (10)	4.74 (8)	6.55 (10)	8.74 (10)	
Tris mmol/l							
PEG g/l							
Caeruloplasmin g/l							
Factor XIII u/ml							
% Ref. 2							
AT III (amidolytic) u/ml							
F. VII (amidolytic) u/ml							
XaGT mins.							
pH at 20°C							
Conductivity at 20°C							

For comments contact: JP/TB/GH Date finished:

13.10.83. 9N16. To repeat and quantitate factor IX heat and recovery with high sorbitol and new buffers

This experiment is effectively a repeat of 9H15, though minor changes in heating time and buffer constitution have been made. The effect of diluting into buffer rather than PFW is investigated.

Materials: KB vials of factor IX 9D 2232 (35ml fill, pyrogen fail, no heparin) were reconstituted with PFW.

Buffers: (concentrations by weight, not by assay)

"H"
- 9.01 mM Trisodium citrate
- 1.71 mM citric acid
- 10 mM Na_2HPO_3

} 10.72 mM Citrate

pH 7.00.

"J"
- 10 mM citrate
- 10 mM Phosphate
- 80 mM NaCl

} pH 6.97

"K"
- 10.23 mM Citrate
- 10 mM Phosphate
- 300 mM NaCl

} pH 6.94.

Method: After reconstitution of factor IX, glycine was added at 0.1 g/ml, a total of 64 g in 640 ml factor IX.

Sorbitol was then added to 2 g/ml 9D (a total of 1280 g) by gradual addition, keeping the temperature above 15° C.

3 x 1 ml samples were taken ("/s")

The temperature was then raised to almost 50° (see chart), after which the water bath temperature was raised and the sample heated at 60° C for 12 hours. This time period was

chosen more for reasons of nocturnal habits than for scientific reasons

After removal from the water bath 3x1 ml samples were taken ("9H16") and the heated factor IX divided into two equal portions.

Fraction 1 was diluted with two volumes of PFW (1510 ml) and labelled α1-9H16

Fraction 2 was diluted with two volumes of buffer H (1510 ml) and labelled α2-9H16.

The purpose was to determine whether factor IX loss upon dilution (observed in previous experiments) was due to loss of buffering capacity in the PFW diluent.

The two fractions were then pooled, mixed and a further sample taken ("α 9H16")

After assays for factor IX activity, DE-52 was added at a rate of 150 u/g gel. With a total of 9966 units, this amounted to 66.5 g DE-52. The mixture was stirred for one hour at room temperature.

The gel was brought down by spinning at 4,000 rpm for 20 minutes in a Beckman centrifuge.

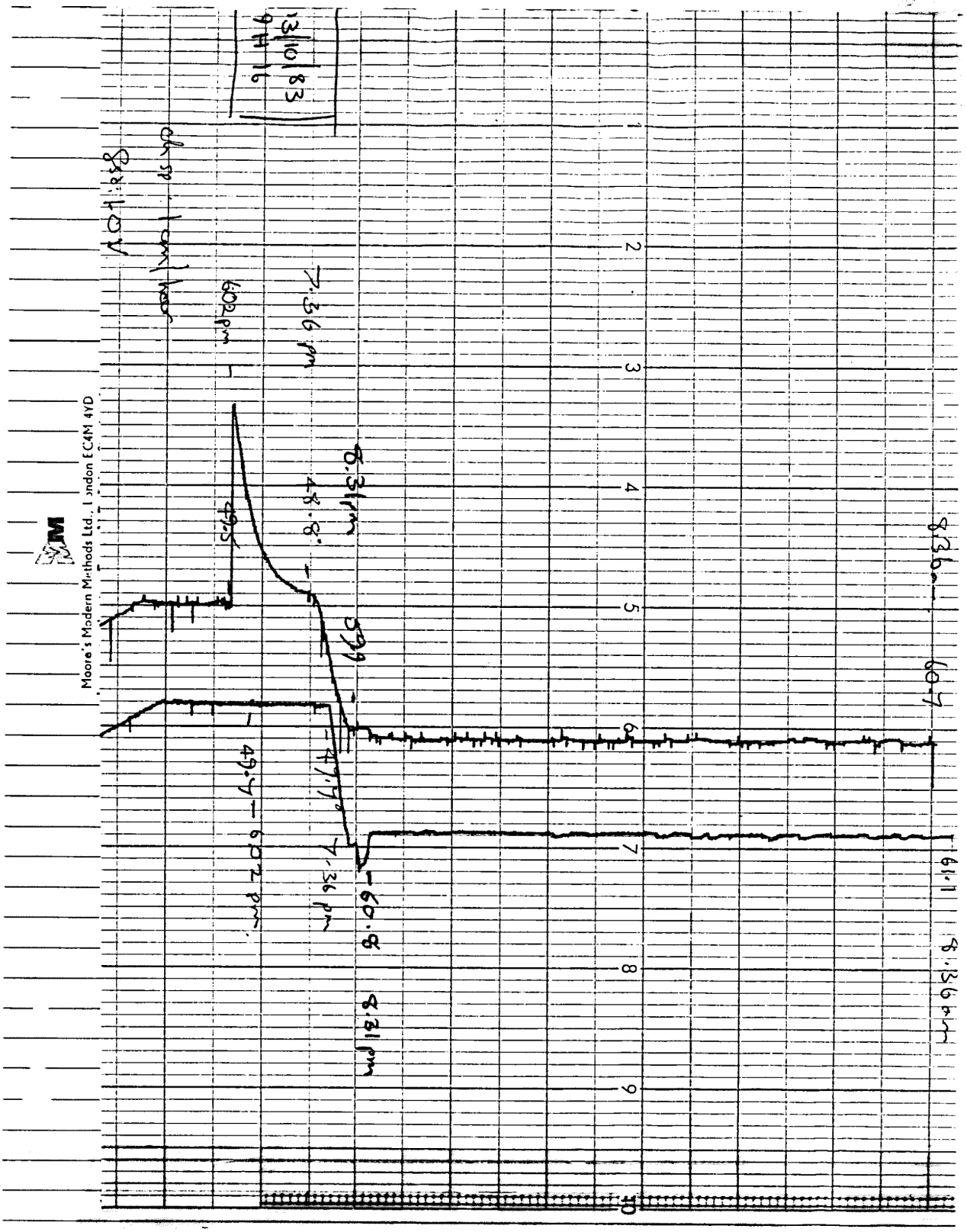
The supernatant was decanted, and the gel resuspended in 1.5 x gel weight of buffer J volume (i.e. 100 ml buffer J).
This slurry was packed into a 4 cm ID glass Amicon column.

The column was pumped at 21 ml/min.

The bed volume of the column was 122 ml.

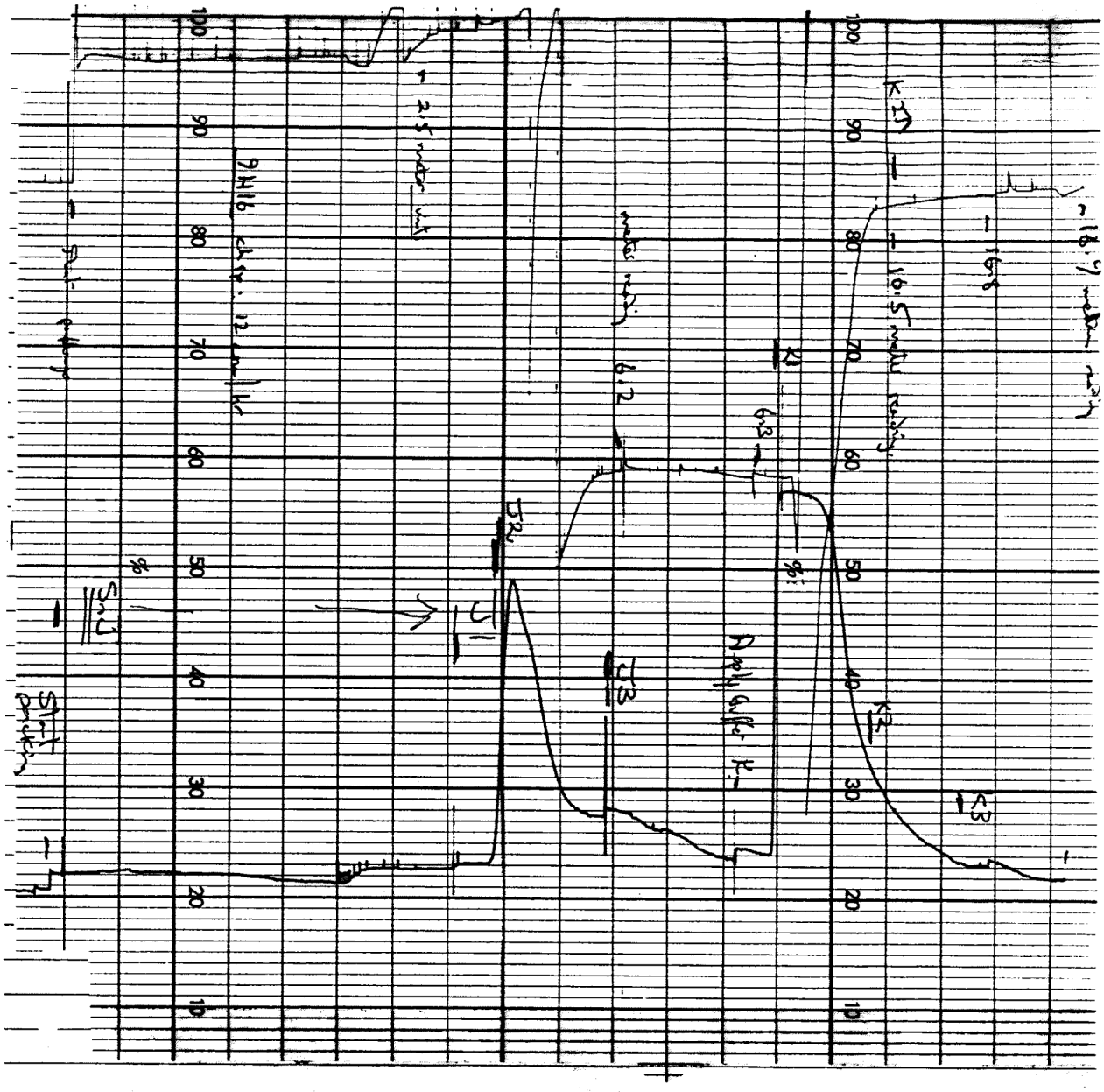
514 ml of buffer J was washed through the column, followed by 600 ml of buffer K. Elution conductivity and transmission at 259 nm were monitored (see trace)

9H16



Trace of heating temperatures for 9H16

9H16.4



Elution profile of 9H16.

Chart speed: 12 cm/hr
Red: 259 nm transmission fsd: 50 mV
Blue: Conductivity fsd: 0.1 V

9H16.5

Results

Effect of diluent

Sample	pH	Conductivity mMho	Factor IX u/ml
α1-9H16	7.00	1.69	2.2
α2-9H16	6.8	2.7	2.2
α 9H16	6.86	2.18	2.2

There is therefore no difference in the factor IX activity between that diluted in PFW and that diluted in buffer.

Protein Parameters

Sample	Vol (ml)	A280	Factor IX u/ml		Total units	Sp.Act u/mg	% of starting IX	% loss IX
9D	640	13.6	35.7	170	22848	2.62	100	
IS	1570		13.5	169	21195		92.7	
9H16	1510		7.0	180	10570	9437	41.3	
			5.5		8305			
α9H16	4530		2.2	194	9966	9287	40.6	10
			1.9		8607			
IS_n	4250	0.435	~0.05	210	~212.5	~0.11	093	2.:
IS1	76	0.581	~0.05	210	~3.8	~0.89	0.01	0.0
IJ_2	196	3.45	2.4	185	470.4	0.69	2.06	5.0
I_{K1}	172	28.42	47.4	138	8152.8	1.67	35.7	87.
I_{K2}	180	1.22	25	156.	450	2.05	2.0	4.8

NAPTT control : 222 s.

9H16·6

Physical parameters

Sample	pH	Conductivity (mMho)
9D	7.23	17.7
α9H16	6.86	2.18
/S1	7.53	1.93
/J1	7.47	3.93
/J2	7.47	8.52
/K1	7.10	21.47
/K2	7.04	26.72

Comments

1. Heating for 12 hours results in greater loss than for 10 hours (~ 10%)

2. Diluting into PFW does not adversely affect factor IX activity so can be continued.

3. Under the condition used, about 100% of the factor IX activity of the α material can be accounted for in column DE-52 elutates.

4. Using buffers J and K > 90% of the factor IX can be eluted in buffer K. Over 85% elutes at high potency (K1-47u/

5. There is a drop in NAPTT which mirrors the rise in factor IX potency. This significance of this $ is questionable since the control time was only 220 seconds and the starting material NAPTT was 170 s (rather low).

6. The specific activity does not attain that of the starting material, though the potency

9 N 16. 7

exceeds it. This probably is due to non-fructose protein now eluting in the second buffer due to the lower ionic strength of buffer J being insufficient to wash it off the DE-52.

7. For a direct comparison ~~between~~ of the two buffer systems a split heating experiment should be performed, half with each set of buffers.

P. A. F.

9H16

Request from: PAF Date: 14/10/83

When results needed: TODAY

Samples to be kept? No. If so, how?

Samples provided with form/~~available from~~

SAMPLE / INVESTIGATION		9H16 *	α1-9H16 ≠	α2-9H16 ≠	α9H16 ≠		
Factor VIII, iu/ml	1 st.						
	2 st.						
✓ Factor IX, iu/ml	1 st.	(8.1)	[2.1)	(2.7)	(2.5)		
	2 st.	7.0	2.2	2.2	2.2		
Factor II, u/ml							
Factor X, u/ml							
Factor VII, u/ml (clotting)							
AT III (Anti Xa), u/ml							
AT III (Anti IIa), u/ml							
AT III (Laurell), u/ml							
Factor VIII RAG, u/ml							
Fibrinogen (Laurell), mg/ml							
Prothrombin (Laurell), u/ml							
Factor XIIIA (Laurell), u/ml							
Fibronectin (Laurell), u/ml							
Factor VIII CAG							
NAPTT sec. 1/10							
TGt50 min.							
FDA hr.							
Limulus + or -							

* ~ 40 % Solution

≠ ~ 15 % Solution

For comments contact

Date finished:

9H16

Request from: PAF Date: 17/10/83

Work results needed: ASAP (Code 1 Super-duper fast)

Samples to be kept? No. If so, how:

Samples ~~provided with form~~/available from R+D Big Freeze

SAMPLE INVESTIGATION		9D2232	/S	9H16	∝9H16	/Sn	/J1
Factor VIII, iu/ml	1 st.						
	2 st.						
Factor IX, iu/ml	1 st.	(32)	(12)	(7.0)	(2.2)	(0.1)	(0.27)
	2 st.	35.7	13.5	5.5	1.9	≤ 0.05	≤ 0.05
Factor II, u/ml							
Factor X, u/ml							
Factor VII, u/ml (clotting)							
AT III (Anti Xa), u/ml							
AT III (Anti IIa), u/ml							
AT III (Laurell), u/ml							
Factor VIII RAG, u/ml							
Fibrinogen (Laurell), mg/ml							
Prothrombin (Laurell), u/ml							
Factor XIIIA (Laurell), u/ml							
Fibronectin (Laurell), u/ml							
Factor VIII CAG							
NAPTT sec. 1/10		170	169	180	154	210	210
TGt50 min.	Control:	222"					
FDA hr.							
Limulus + or -							

For comments contact

Date finished

9N16

Request from: RAF Date: 17/10/83

When results needed:

Samples to be kept? If so, how:

Samples provided with form/available from R+D Big Freezer

SAMPLE / INVESTIGATION		9N16					
		1J2	1K1	1K2			
Factor VIII, iu/ml	1 st.						
	2 st.						
Factor IX, iu/ml	1 st.	(24)	(48)	(7)			
	2 st.	2.4	47.4	2.5			
Factor II, u/ml							
Factor X, u/ml							
Factor VII, u/ml (clotting)							
AT III (Anti Xa), u/ml							
AT III (Anti IIa), u/ml							
AT III (Laurell), u/ml							
Factor VIII RAG, u/ml							
Fibrinogen (Laurell), mg/ml							
Prothrombin (Laurell), u/ml							
Factor XIIIA (Laurell), u/ml							
Fibronectin (Laurell), u/ml							
Factor VIII CAG							
NAPTT sec. 1/10		185 ✓	138 ✓	156 ✓			
TGt50 min.							
FDA hr.							
Limulus + or -							

For comments contact Date finished:

20.10.83 9H17 Comparison of two buffer systems in the recovery of heated factor IX and measurement of adsorption time course.

Purpose: To compare the recoveries of heated factor IX using the conventional "A" and "B" buffers with that from "J" and "K" buffers which have a greater difference of NaCl concentration. As an additional experiment, the time course for adsorption of factor IX on to DE-52 is measured

Materials

Factor IX 36 vials of 90 2360 (pyrogen failed, unheparinised, 35ml fill) were reconstituted with PFW

Buffers:

Buffer A	10 mM citrate 10 mM phosphate 100 mM NaCl	pH 7.08
Buffer B	10 mM citrate 10 mM phosphate 250 mM NaCl	pH 6.97
Buffer J	10 mM citrate 10 mM phosphate 80 mM NaCl	pH 7.01
Buffer K	10 mM citrate 10 mM phosphate 300 mM NaCl	pH 6.95

Method: After reconstitution of factor IX to 1240 ml (1260g) 1 x 5 ml sample and 3 x 2 ml samples were taken. pH and conductivity were measured.

Glycine was added to 0.1 g/ml (total of 126g glycine) and stirred until dissolved.

The solution was then placed in a water bath and sorbitol (2 g/ml) was added (~~250~~ 252 g)

This was stirred until dissolved, the Temperature being maintained between 20°-30° C.

Samples were Taken 1S

The factor IX was then heated at 60°C for ~ 9.5 hours at which time it was removed from the water bath. Volume and weight were measured and "9H17" samples taken.

The heated material was left in a refridgerator until morning when it was diluted by addition of two volumes of PFW. (5920 ml)

The material was then divided into two fractions, α 9H17 and α2 9H17. The α - fraction was used the same day. The α2 - fraction was stored at 4° C for 24 hours before use.

In order to achieve some comparison between the two, several repeat assays were performed thus:

α 9H17 assayed (1) fresh, (2) after freezing with other α - samples, (3) after freezing with other α2 - samples

α2 9H17 assayed after freezing with other α2 - samples

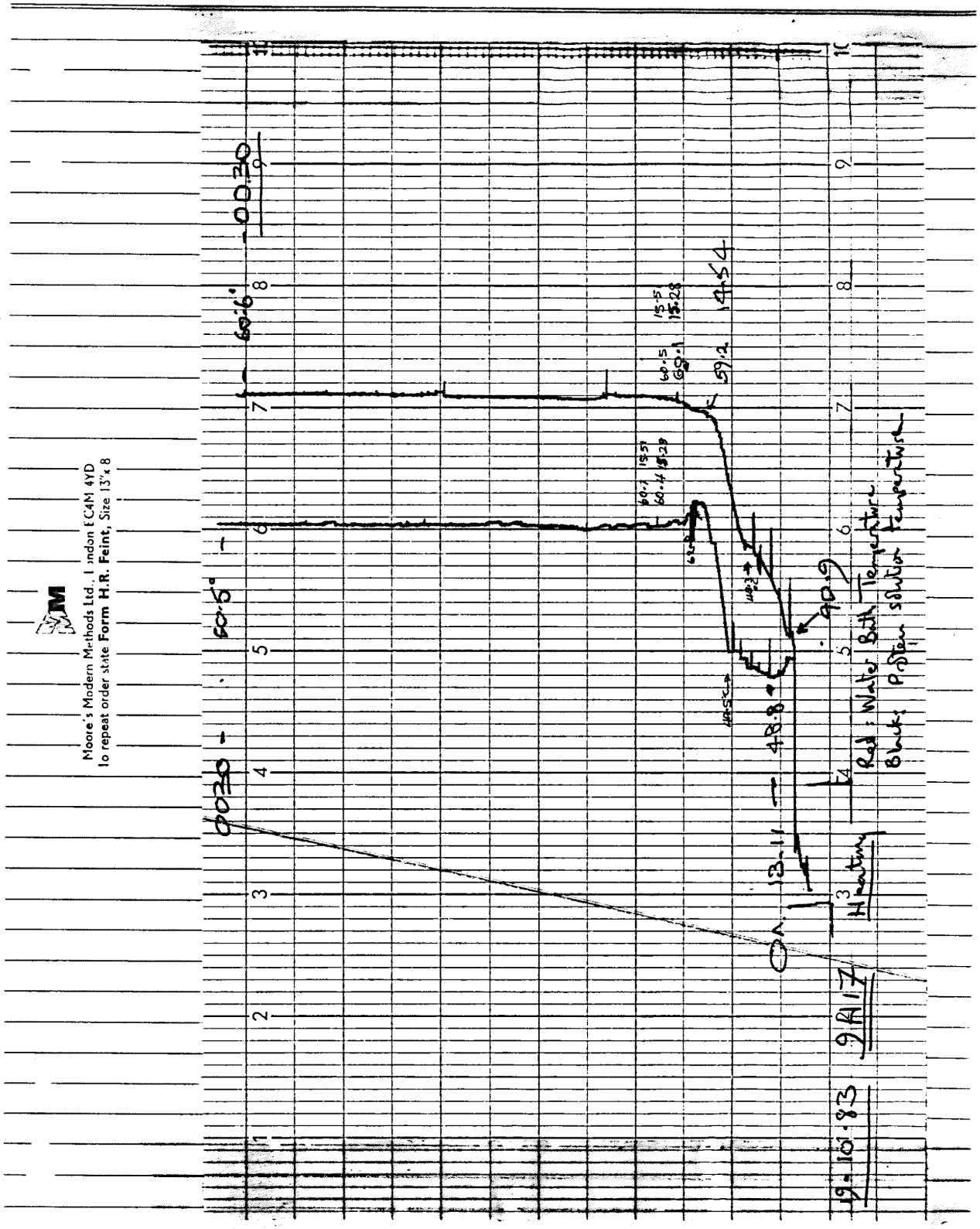
Treatment of α9H17

The fraction α 9H17 was 4355 ml and weighed 4773 g.

After factor IX assay gave an activity of 2.8 u/ml for this material, DE-52 was added at 1g / 150 units, ie. 81.3 g.
This mixture was stirred at room temperature for 1 hour, then spun at 4000 rpm in a Beckman centrifuge for 20 minutes.

The supernatant was decanted and sampled (/Sn) and the gel was resuspended in 1.5 v/w of gel ie. 122 ml of buffer A

9H17.3



Trace of sample and water bath temperature during 9H17 Heating

The slurry was packed into a glass 4 cm I.D. Amicon column.

The gel bed volume was 186 ml

A further four bed volumes of buffer A were pumped through the column at a rate of 24 ml / min.

Three fractions A1 A2 and A3 were collected.

U.V transmission at 259 nm and conductivity of eluate was monitored. Unfortunately, the chart paper stopped during the elution of fraction A (see attached trace), so the optimum fraction cuts may not have been made.

Four column volumes of buffer B were then pumped through, and four fractions B1, B2 B3 and B4 were collected.

In addition to assaying major peak fractions for factor IX, samples were also tested for pH, conductivity, A_{280} and some samples for NAPTT.

Treatment of α2 9H17

This was used after 24 hours storage

The volume was 4290 ml and the weight 4791 g (discrepancy in comparison with α-material most likely due to lack of accuracy of measuring volumes of this material)

After sampling ("α2-9H17") it was assumed that this material had the same activity as the α-, ie. 2.8 u/ml, and 80.1 g DE-52 were added (1 g/150 units) and the mixture stirred for 1 hour at room temperature, with a Vibromixer.

Measurement of binding time-course

During the adsorption, 10 ml samples were removed at 15, 30, 45 and 60 minutes

These were spun in a bench centrifuge and the supernatant sampled. They were labelled "α2 9H17 /Sn15, /Sn 30, /Sn 45, /Sn 60"

The DE-52 was then returned to the mixing vessel.

The supernatants were frozen and later assayed for residual factor IX activity.

After 1 hour's adsorption, the gel was precipitated by spinning for 20 minutes at 4,000 rpm in Beckman centrifuge.

The supernatant was decanted and sampled ("α2 9H17 /Sn")

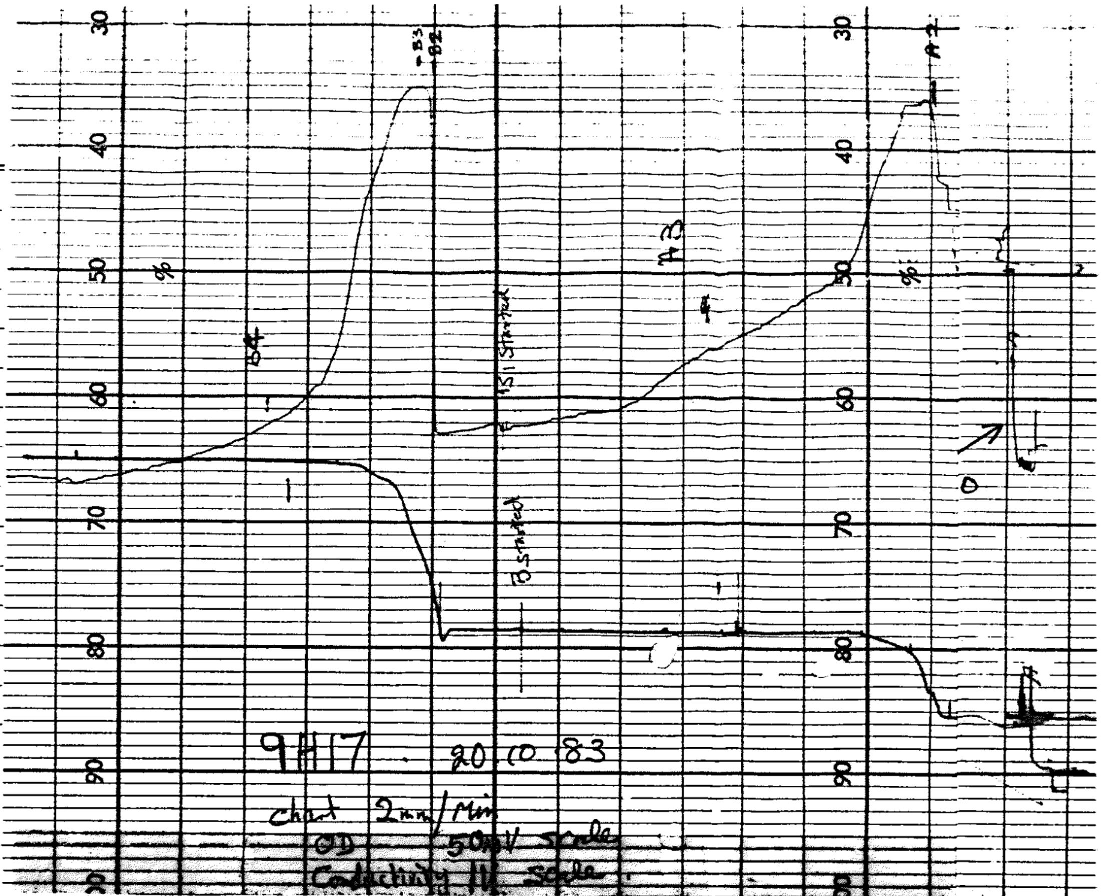
The DE-52 was resuspended in 1.5 v/w of gel Buffer J (i.e. 120 ml) and packed into 4 cm I.D. glass Amicon column.

Bed volume was 133 ml
4 column volumes of buffer J were pumped through the column and two fractions, J1 and J2, collected.
The flow rate was 30.5 ml/min.

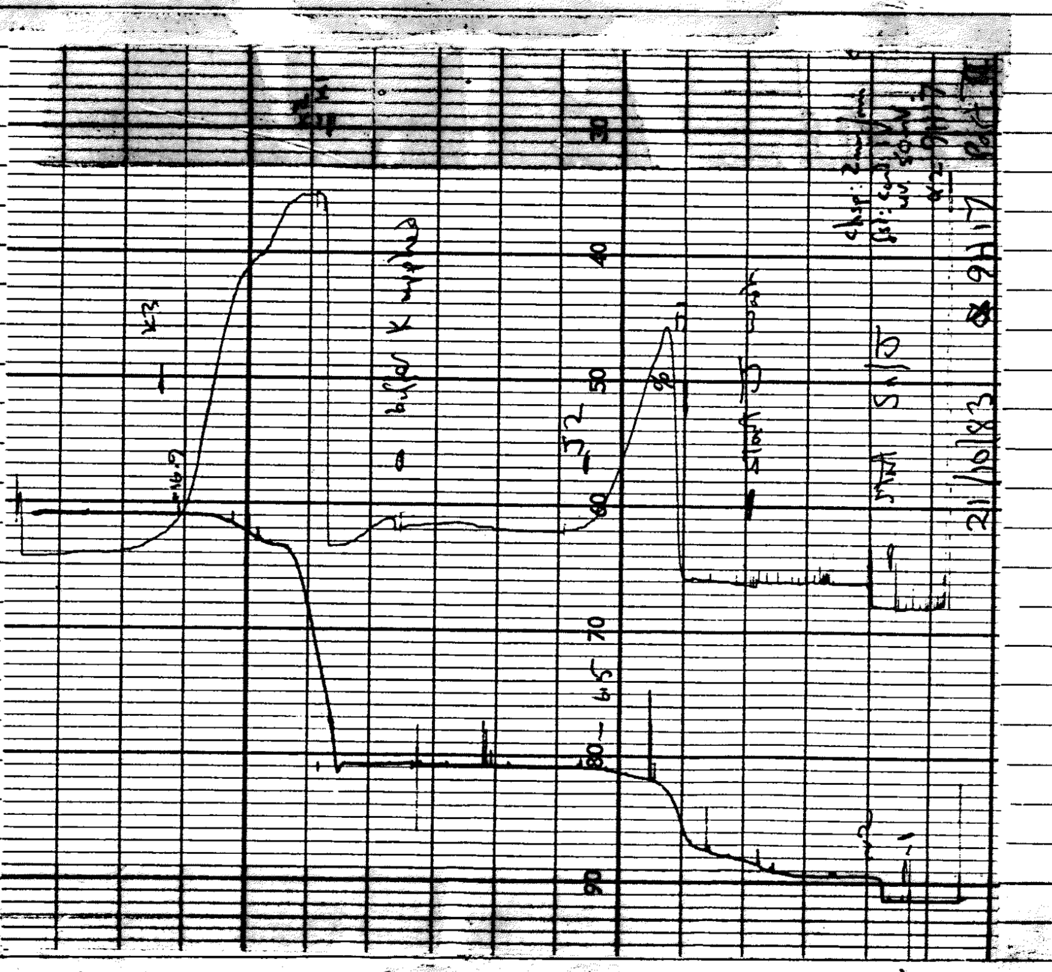
Four column volumes of buffer K were then pumped through the column and three fractions, K1, K2 and K3, were collected (see chart trace).

UV 259 nm and conductivity of eluate was recorded.

Assays for factor IX activity and NAPTT were performed.



u.v. transmission and conductivity of eluate in α9H17 (Buffer A+B) off DE52



u.v. transmission + conductivity of eluate in α2 9H17 (Buffers J + K)

Results

Protein Data

Sample	Volume (ml)	A_{280}	Factor (u/ml)	IX Total Units		Sp. Act (u/mg)	NAPTT (s)	% starting factor IX	% [illegible] factor
9D 2260	1240	16	33·3	‡ 41292	41106	2·07		100	
			33·0	40920					
1S	2960		17	50320				122	
9H17	2960		5·2	15392				37·4	
α9H17	8710		2·8	‡ 24388					
			1·9	16549	20023			49·4	
			2·3	* 20033					
9D	Fractional amount in α9H17			⊹ 20553				100	
α9H17	4355			10161				49·4	10
1Sn	4250	0.532	<0.02	< 85				< 0.4	0
1A1	222	2·1	0·05	11·1		0·02	259	0·05	0
1A2	345	7.27	12·2	4209		1·68	225	20·5	41
[1A3	~~295~~	1.61							
[1B1	97	1.02							
1B2	18	27.7	62·1	1117.8		2·24	154	5·4	11
1B3	220	11.2	33·8	7436		3·02	153	36·2	73
[1B4	407	0·47							

‡ Samples, in order, freshly assayed and assayed after freezing/thaw

* Assayed after freezing along with α2 9H17 samples

⊹ Calculated from (Volume α9H17 / Volume total α9H17) × Total 9D units

Protein Data (cont.)

Sample	Volume (ml)	A280	Factor IX (u/ml)	Total Units	Sp Act (u/mg)	NAPTT (s)	% starting factor IX	% load factor
9D	Fractional amt in X2 expt*			20246			100	
∝29H17	4290		2.6	11154			55.1	100
/Sn	4154	0.67	0.12	498.5	0.18		2.5	4
/SnJ	232	0.41						
/J1	155	4.2	1.46	2263	0.35	257	1.1	2
/J2	392	1.21						
/K1	19.5	36.3	39.3	766.3	1.08	150	3.8	6.
/K2	250	17.6	29.6	7400	1.68	179	36.5	66.
/K3	228	6.44						

*calculated from (Volume ∝29H17/Volume Total ∝9H17) x Total 9D units

Moore's Modern Methods Ltd., London EC4M 4YD
To repeat order state Form H.R. Feint, Size 13"x 8

Physical Data

Sample	pH	Conductivity (mMho)
9D2260	7.16	15.4
∝9H17/Sn	7.88	1.34
/A1	7.93	4.09
/A2	7.99	11.15
/A3	7.29	11.8
/B1	7.06	11.6
/B2	7.08	11.6
/B3	7.15	20.9
/B4	6.99	23.3
Buffer A	7.08	11.6
Buffer B	6.97	23.4

Physical data (cont)

Sample	pH	Conductivity (mMho)
α29H17/Sn	8.01	1.18
/SnJ	7.91	3.43
/J1	7.98	8.20
/J2	7.58	9.51
/K1	7.02	9.67
/K2	7.10	23.77
/K3	6.98	27.05
Buffer J	7.01	10.82
Buffer K	6.95	29.5

Adsorption to DE52, time-course

Sample	Factor IX u/ml	% unbound factor IX
~~α29H17~~		
α29H17	2.6	100
/Sn15	0.74	28.5
/Sn30	0.54	20.8
/Sn45	0.31	11.9
/Sn60	0.16	6.1
/Sn (approx 70 min)	0.12	4.6

9H17 K

Comments

1. The higher sorbitol concentration used in recent experiments gives a better factor IX yield upon heating.

2. The sorbitol-containing samples do not appear to freeze fully, which may account for some assay variations between freshly-assayed and stored samples

3. The adsorption of factor IX to DES does appear to require a full 60 minute to adsorb > 90% of the activity.

4. Buffer J elutes less factor IX activity than Buffer A (1% as against 20%) The NAPTT of each appears the same.

5. Buffer K does not appear any more efficient than buffer B. The overall factor IX recoveries are about the same but the potency and specific activity are less with buffer K than with buffer B. However, this may be due to the buffer K being used on material stored at 4°C for an additional 24 hours. Further comparison of the two buffers is advisable.

P.A.F. / D.C.

44,7

ASSAY REQUEST FORM

Request from: PAF/DC Date: 24/10/83

What results needed: ASAR

Samples to be kept? If so, how?

Samples ~~provided with form~~/available from R+D Big Freezer

SAMPLE INVESTIGATION		902260	9D/S *	9H17 *	αH17	/Sn	/A1
Factor VIII, iu/ml	1 st.						
	2 st.						
Factor IX, iu/ml	1 st.	(33)	(13)	(8)	(2·8)	(0·03)	(1·6)
	2 st.	32.7 33·2	17.0	5·2	1·9	<0·02	0·05
Factor II, u/ml							
Factor X, u/ml							
Factor VII, u/ml (clotting)							
AT III (Anti Xa), u/ml							
AT III (Anti IIa), u/ml							
AT III (Laurell), u/ml							
Factor VIII RAG, u/ml							
Fibrinogen (Laurell), mg/ml							
Prothrombin (Laurell), u/ml							
Factor XIIIA (Laurell), u/ml							
Fibronectin (Laurell), u/ml							
Factor VIII CAG							
NAPTT sec. 1/10							259 ✓
TGt50 min.							
FDA hr.							
Limulus + or -							

* Contain sorbitol at 0·7 g/ml.

For comments contact [illegible] Date finished: 26/10/83

9H17

AT III REQUEST FORM (1)

Request from: PAF/DC Date: 24/10/83

When results needed:

Samples to be kept? If so, how:

Samples ~~provided with form~~/available from R+D Rij Freezer.

SAMPLE INVESTIGATION		9H17	/A2	/B2	/B3			
Factor VIII, iu/ml	1 st.							
	2 st.							
✓ Factor IX, iu/ml	1 st.		(2.5)	(70)	(35)			
	2 st.		12.2	62.1	33.8			
Factor II, u/ml								
Factor X, u/ml								
Factor VII, u/ml (clotting)								
AT III (Anti Xa), u/ml								
AT III (Anti IIa), u/ml								
AT III (Laurell), u/ml								
Factor VIII RAG, u/ml								
Fibrinogen (Laurell), mg/ml								
Prothrombin (Laurell), u/ml								
Factor XIIIA (Laurell), u/ml								
Fibronectin (Laurell), u/ml								
Factor VIII CAG			✓	✓	✓			
✓ NAPTT sec. 1/10			225	154	153			Blank: 269
TGt50 min.								
FDA hr.								
Limulus + or -								

For comments contact: GHC

Date finished: 26/10/83.

9H17

ASSAY REQUEST FORM (1)

Request from: PAF/DC Date: 20/10/83

When results needed: _____

Samples to be kept? _____ If so, how? _____

Samples provided with form/available from _____

SAMPLE INVESTIGATION		9D226O	α9H17*				
Factor VIII, iu/ml	1 st.						
	2 st.						
Factor IX, iu/ml	1 st.	(32)	(2·4)				
	2 st.	33·3	2·8				
Factor II, u/ml							
Factor X, u/ml							
Factor VII, u/ml (clotting)							
AT III (Anti Xa), u/ml							
AT III (Anti IIa), u/ml							
AT III (Laurell), u/ml							
Factor VIII RAG, u/ml							
Fibrinogen (Laurell), mg/ml							
Prothrombin (Laurell), u/ml							
Factor XIIIA (Laurell), u/ml							
Fibronectin (Laurell), u/ml							
Factor VIII CAG							
NAPTT sec. 1/10							
TGt50 min.							
FDA hr.							
Limulus + or -							

* Sorbitol at 3g/10 ml.

For comments contact _____ Date finished: _____

9H17

ASSAY REQUEST FORM (1)

Request from: PAF Date: 24/10/83

What results needed: ASAR

Samples to be kept? No If so, how?

Samples ~~provided with form~~/available from R+D Big Freezer

SAMPLE / INVESTIGATION		5 ∝9H17	6 ∝2% 9H17	∝2 7 /Sn	8 /J1	9 /k1	10 /k2
Factor VIII, iu/ml	1 st.						
	2 st.					(70)	(40)
Factor IX, iu/ml	1 st.	(2·8)	(2·8)	(0.05)	(1·5)	(35)	(20)
	2 st.	2·3	2·6	0·12	1·46	39.3	29·6
Factor II, u/ml							
Factor X, u/ml							
Factor VII, u/ml (clotting)							
AT III (Anti Xa), u/ml							
AT III (Anti IIa), u/ml							
AT III (Laurell), u/ml							
Factor VIII RAG, u/ml							
Fibrinogen (Laurell), mg/ml							
Prothrombin (Laurell), u/ml							
Factor XIIIA (Laurell), u/ml							
Fibronectin (Laurell), u/ml							
Factor VIII CAG							
NAPTT sec. 1/10					257 ✓	150 ✓ Blank: 298	179 ✓
TGt50 min.							
FDA hr.							
Limulus + or -							

For enquiries contact Date finished:

9H17

ASSAY REQUEST FORM

Patient type: PAF Date: 24/10/83.

When results needed: ASAP

Samples to be kept? No If so, how?

Samples ~~provided with form~~/available from R+D Big Freezer

SAMPLE INVESTIGATION ∝2·9H17		1 Sn 15	2 Sn 30	3 Sn 45	4 Sn 60		
Factor VIII, iu/ml	1 st.						
	2 st.						
Factor IX, iu/ml	1 st.	(0.8)	(0.4)	(0.1)	(0.05)		
	2 st.	0.74	0.54	0.31	0.16		
Factor II, u/ml							
Factor X, u/ml							
Factor VII, u/ml (clotting)							
AT III (Anti Xa), u/ml							
AT III (Anti IIa), u/ml							
AT III (Laurell), u/ml							
Factor VIII RAG, u/ml							
Fibrinogen (Laurell), mg/ml							
Prothrombin (Laurell), u/ml							
Factor XIIIA (Laurell), u/ml							
Fibronectin (Laurell), u/ml							
Factor VIII CAG							
NAPTT sec. 1/10							
TGt50 min.							
FDA hr.							
Limulus + or -							

N.B. These are only guesstimates

For comments contact Date faxed: 3.

9.3.1. Heat inactivation of viruses in factor IX concentrates

Progress Report, October 1983

General comments

Since June 1983, the majority of time devoted to this project has been directed at measuring the survival of factor IX after heating it, in solution, in the presence of sorbitol and glycine stabilisers.

Some studies have also been performed on the effect of heat on the freeze-dried material.

Single, unsuccessful attempts at heating factor IX under other conditions are mentioned at the end of this report.

No experiments have been performed to measure the effect of heating on active virus. There is no evidence of whether protein or virus are being protected preferentially from the heat under the selected conditions.

In brackets are shown the relevant experiment numbers.

Heating of factor IX with sorbitol/glycine in solution

A. Heating step

(1) Reconstituted factor IX can be heated for 10 hours at 60°C if sorbitol and glycine are first added at concentrations of 1.3 g/ml and 0.1 g/ml respectively. Under these conditions between 35-50% of the unheated factor IX activity is retained (9H7, 9H9, 9H10, 9H12).

(2) If the temperature is raised to 70°, all activity is lost after 8 hours. If the temperature is maintained at 50° or lower, 90%+ of starting factor IX activity is retained (9H13).

(3) The yield of factor IX can be improved to 50-60% by raising the sorbitol concentration to 2 g/ml of factor IX (9H14, 9H15, 9H17).

B. Recovery of factor IX after heating

(1) Heated factor IX can be separated from the sorbitol and glycine by adsorbing it on DE 52. This adsorption is improved by dilution of the heated material prior to adsorption (9H6.02, 9H7).

(2) The capacity of DE 52 for heated factor IX is much higher than for cryosupernatant (from current 8P method). The most efficient loading was determined to be 1g DE 52 for each 150 units of factor IX in the heated, diluted material (9H10B).

(3) The time for adsorption of 70% of factor IX to DE 52, using the above loading, is 15 minutes. However, to achieve 95% adsorption, the mixing must continue for 60 minutes (9H17).

(4) Considerable amounts of factor IX elute, along with unwanted protein, in an initial 0.1M NaCl buffer wash (production "Buffer A"), while the main factor IX elution in 0.25M NaCl (production "Buffer B") has a long tail with low potencies (9H9, 9H10, 9H12).

(5) Elution yield can be improved by reducing the NaCl in the wash to 0.08M and increasing the NaCl concentration of the second, eluting buffer to 0.3M. The optimum conditions are likely to be a compromise between these two buffer systems (9H15, 9H16, 9H17).

(6) There is a fall in the NAPTT, coincident with the factor IX containing fractions, the value usually lying in the range 150-200 seconds (9H12, 9H15, 9H16, 9H17).

The changes in sorbitol concentration and elution buffers have resulted in an improvement in the overall recovery of factor IX from 24% to 40%.

The quantities of factor IX used so far have been a maximum of 18 vials of 9D, equivalent to about 640 ml of factor IX eluate. The project has now reached a stage where scaling-up experiments will begin, with an objective of handling about 2L, equivalent to a full scale production batch of 9P.

Heating of factor IX dry

Unlike the experience with factor VIII, factor IX appears more stable to dry heating than heating in solution.

(1) Non-heparinised factor IX vials could be heated for 12 hours at 60°, 70° and 80° with resultant losses of factor IX activity of 13%, 20% and 25% respectively. (DH-1)

(2) Vials containing heparin survive heating better than non-heparinised factor IX; at 60°C there is 100% recovery of factor IX, compared with 93% recovery in a parallel experiment on unheparinised factor IX. At 70° these values are 96% and 90% respectively, after heating for 10 hours (DH 2).

(3) Using heparinised factor IX, the temperature and duration of heating can be extended. At 80° for 48 hours, 97% of factor IX activity is retained. This appears to be close to the limit; when heated for 72 hours some activity is lost (14%) and if the temperature is raised to 90°, 21% of activity is lost after only 24 hours (DH 3, DH 4, DH 5).

(4) All samples have NAPTT values of >240 seconds and all samples redissolve after heating in less than 2 minutes, without evidence of any precipitation.

Future experiments will be directed towards measuring the temperature within the factor IX vials during heating, and towards minor alterations of the heating conditions around 80° for 48 hours.

Heating in the presence of citrate

A single experiment to raise the citrate concentration to 2M before heating was unsuccessful due to precipitation of protein. Unless the two heating systems described above develop defects, I do not intend to emphasise this as an alternative method.

Heating of factor IX on DE 52

A preliminary experiment to heat factor IX while it was adsorbed on DE 52 anion exchanger yielded total loss of factor IX activity. Greater control of pH and ionic strength over the temperature range used (20-60°) will be needed in any further attempts.

P.A.F.

20.10.83

24/25 · 11·83. 9H18 Large scale production lab preparation of heated factor IX

The first attempt to heat factor IX in the presence of sorbitol and glycine, on a large scale in the clean conditions of the production lab.

Materials Factor IX - Frozen 9P eluate from BPL batch JD 2982 B/5i
(This had been filed - 1982 for mild pyrogenicity.

DE-52 - R&D recycled batch, as used in 9H15, 9H16, 9H17.

Buffers

Buffer J : 8·5 mM trisodium citrate
1·5 mM citric acid
10 mM Na_2HPO_4
80 mM NaCl
} pH 7·0

Buffer K : 8·9 mM trisodium citrate
1·1 mM citric acid
10 mM Na_2HPO_4
300 mM NaCl
} pH 6·96

Buffer C : 20 mM citrate
1 M NaCl
} pH 7·74.

Method

Throughout this experiment, quantities have been measured by volume and by weight.

6 bottles of JD 2982 B/5i were raised to room temperature for 1 hour from -40°. Complete thawing was achieved in a water bath at 30-40°C.

After sampling ("15") 3 x 2 ml and 1 x 5 ml,

183 g Glycine was added equivalent to 0·1 g/ml eluate or 0·1 g / g eluate.

Once dissolved, sorbitol was added - 3656 g equivalent to 2 g / ml eluate or 1·98 g / g eluate

This was dissolved by immersing the stainless steel container into 35° C water bath and stirring from below with magnetic stirrer (used egg-shaped magnetic bar and the larger bar was too vigorous). The sample temperature was maintained between 30 - 34 °C.

The sample took 3.3hours to dissolve.

The water bath was ~~~~ switched off with the factor IX still being stirred in the bucket. Samples ("/s") were taken.

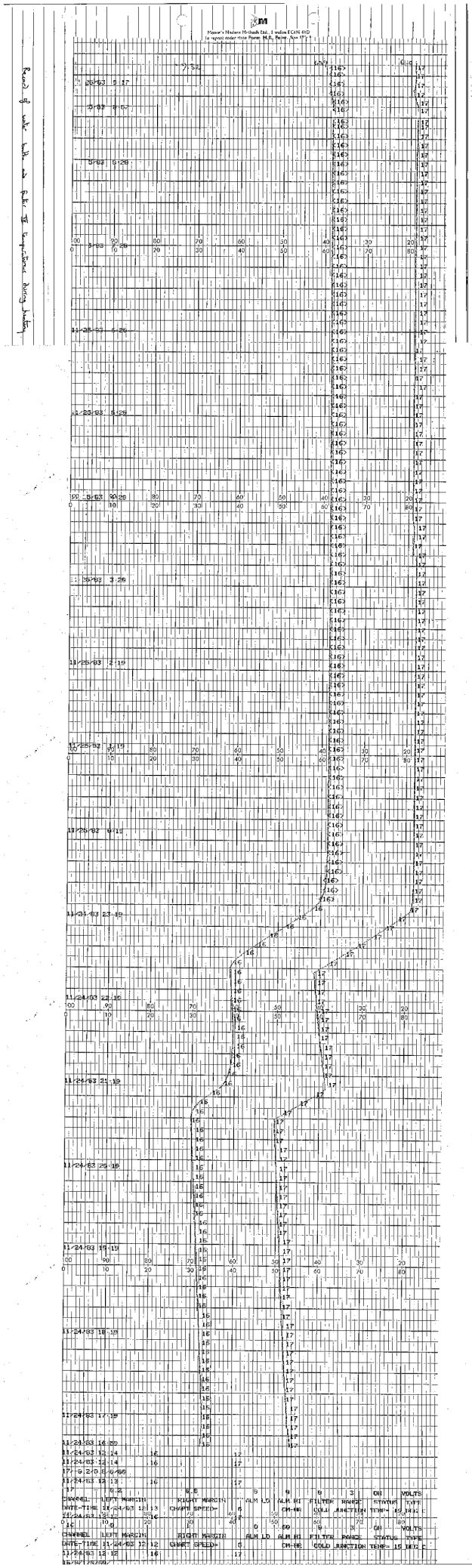
Heating The heating step was slightly disjointed (see trace), due to water bath switching off at 41°. This was corrected but meant that the sample was at 39° for 1.5 hours before being raised to 61°. It was heated at this temperature for 10 hours.

Dilution After 10 hours the factor IX was removed from the water bath, sampled (3 x 2 ml 9H18") and diluted by addition of 2 volumes of PFW.

Volume of 9H10 ~ 4569 ml
Added ~ 9138 ml PFW

Weight of 9H10 : 5575 g

∴ Add 1.64 g PFW per g 9H15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11-25-93 5:26
11-25-93 4:26
11-25-93 3:26
11-25-93 2:19
11-25-93 1:19
11-26-93 0:19
11-24-93 23:19
11-24-93 22:15
11-24-93 21:19
11-24-93 20:15
11-24-93 19:19
11-24-93 18:19
11-24-93 17:19
11-24-93 16:59
11-24-93 12:14
11-24-93 12:14
11-24-93 12:13
CHANNEL LEFT MARGIN RIGHT MARGIN ALM LO ALM HI FILTER RANGE STATUS TYPE
DATE-TIME 11-24-93 12:12 CHART SPEED= 6 CH-NO COLD JUNCTION TEMP= 15 DEG C VOLTS

3 x 2ml and 1 x 5ml samples ("αH18") taken.

The UD starting material and αH18 samples were sent for factor IX clotting assays.

Resulting activity for αH18 was 2.7 u/ml, giving a total of 36242 units factor IX

Adsorption DE 52 was then added - 242g, equivalent to 1g DE 52 per 150 units of factor IX.

This is also equivalent to 1g DE52 per 60g αH18. or 1g DE52 per 7.7g factor IX eluate.

The mixture was then stirred at lab temperature (~18°C) for 1 hour, using the avoid magnetic stirring bar and the Edwards stirrer.

Column Packing The slurry was poured directly into an assembled GAC 110 glass column, to which an extender tube had been attached. The "supernatant" was allowed to flow through, and the gel packed, under gravity.

This procedure took ~ 1½ hours and for the final stages a Watson-Marlow pump was connected to the outlet. This greatly speeded the packing and is to be recommended as routine. The slowness of

packing was due to small head-height and not due to excessive viscosity of the mixture.

The eluate obtained during the packing was labelled and sampled ("/Sn")

Column Elution The gel bed height was ~ 6 cm giving a bed volume of ~ 570 ml

The gel expansion is therefore 2.35 ml/g gel.

The gel was washed with 5 bed volumes of buffer J (2900 ml / 2900 g)

This is equivalent to ~ 12 g buffer J / g DE52 added

The column was run under 5 p.s.i. N_2 giving a flow rate of ~ 300 ml/min.

Elwate was monitored with u.v., conductivity and refraction.

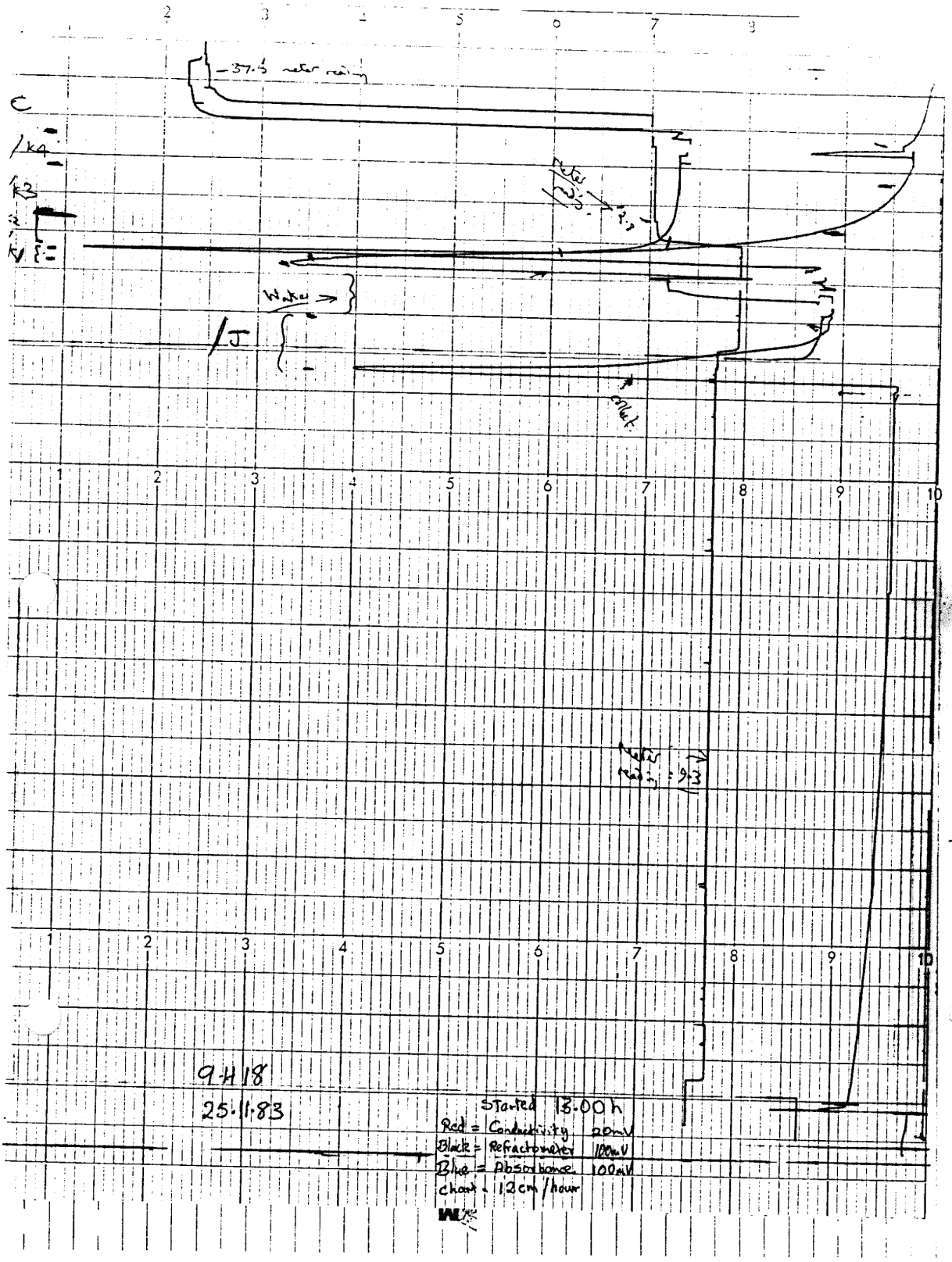
Fractions were collected according to changes in u.v. absorption.

Following the complete elution of the single peak (see trace), buffer K was applied (5,000 g

Four fractions were collected, representing peak front, middle and tailing edge of u.v trace.

3,000 g buffer C was then applied to wash the column.

On the attached record sheet, 2×2 + 1×5 ml samples were taken before volume and weight was recorded. The values given in the tables of results make allowance for this and represent TOTAL yields.



Trace of elution from DE-52, showing UV absorbance at 240 nm, conductivity and refractive

9H18 7

Results

Protein Data

Sample	Volume (ml)	A_{280}	Factor u/ml	TX total units	Sp. Act. (u/mg)	% starting factor TX	% factor TX mixed with gel
JD 2982	1840	14.54	35.6 35.9	65780	2.46	100	
/S	4577		16.9	77351		117.5	
9H18	4571		7.8	35654		54.2	
α9H18	13423		2.7	36242		55.1	100
/Sn	12760	0.37	0.01	127.6	0.03	0.19	0.3
/J	2274	1.35	1.3	2956	0.96	4.5	8.1
/K1	259	36.56	82	21 238	2.24	32.3	58.6
/K2	1639	2.64	4	4327	1.5	6.6	11.9
/K3	2049	0.45	0.09	184	0.2	0.28	0.5
/K4	1009	0.25	0.06	60.54	0.24	0.09	0.1
/C	2249	0.1	—	—	—		

Sample	NAPTT ½ (s)
JD 2982	211
α9H18	259
/Sn	229
/J	211
/K1	235
/K2	225
/K3	220
/K4	250
Blank	

9H18 . 8.

Physical Data

Sample	Volume (ml)	Weight (g)	pH	Conductivity (mMho)	Density (g/ml)
JD2982	1840	1869	7.18	17.27	1.01
/S	4577	5648			1.23
9H18	4571	5575			1.22
∝9H18	13423	14570	7.03	1.73	1.08
/Sn	12760	14035	8.01	3.29	1.09
/S	~~2274~~	2268	7.49	9.82	1.0 ~~0.88~~
* /K1	250	~~247~~	7.08	15.82	0.99
* /K2	1639	1648	7.01	26.36	1.01
/K3	2040	2077	6.96	27.27	1.02
/K4	1000	1009	6.93	27.45	1.0
/C	2240	2439	6.93	71.09	1.09

Comments

1. Assuming a pooling of K1 and K2, an overall factor IX recovery of 39% is achieved.

2. The heating step results in a 45% loss of factor IX activity.

3. Generally the experiment performed much as expected. Possibly the presence of a stirrer during heating resulted in a 5% improvement in yield at that stage.

4. The division of factor IX between different fraction is similar to that obtained in smaller scale experimen

The supernatant showed much less factor IX – possibly due to the gel recovery by packing being total, whereas some (gel + active factor IX) may have been present in supernatants in 9H16 and 9H17 when centrifugation was used.

5. The buffers eluted proteins much faster in this experiment. Previous experiments have taken about 20 minutes to elute 5 volumes of buffer. This was achieved in this experiment in half the time.

6. This appears to relate directly to flow rates.

	4 cm Column	11 cm column
Flow rate ml/min :	20	300
ml/min/cm² :	1.59	3.16
J elution volume ml :	196, 155	2274
column volumes :	1.6, 1.2	4.0
K elution volume ml :	172 270	1880
column volumes :	1.3 2.0	3.3

It appears that the protein peak is eluting in a larger volume relative to the column size, because of the increased relative flow rate. That is the eluting protein is being swamped by advancing buffer.

If this is correct, then a reduction in the flow rate will give the same overall yield in a smaller volume, leading to higher potency of the factor IX. See (7).

7. The theoretical potency of factor IX from pooled K1 + K2 would be 13.5 u/ml. This is far too low to be of clinical use. The improvement predicted in (6) would raise this potency to about 29.5 u/ml – more acceptable.

P.A.F., DC, 4H

JH 18

FACTOR IX WET HEATING 9H18

Starting Material

24/11/83.

1.0 Eluate batch: 9JD2982 B5i.

11.50 am. Eluate volume: 1840 ml

Eluate weight: 1869.0 g

pH : 7.18

Conductivity : 9.5/0.55 17.27 ~~7.1=13.38~~ mMho

Sample: 3 x 2ml (x15)
1 x 5ml
1 x 1 ml f. viable count.
12 ml

OD 280: 20×0.727 = 14.54

12.10 am.

Stabilisers Wt after : 1850 g.
Vol after : 1828 g.

1. Add Glycine 0.1 g per ml of eluate
(equivalent to ~~1.0~~ g per g of eluate)
Weight of glycine added: 183 g

2. Dissolve by stirring with large magnetic stirrer/spoon

3. Place in 37° water bath with magnetic stirrer underneath.

4. Add sorbitol 2g per ml of eluate
(equivalent to ~~2.0~~ g per g of eluate)
Weight of sorbitol added: 3656 g

3. Stir until fully dissolved, monitoring temperature (>20°c)
Temp range 30 - 34°

15.30 4. Sample 3 x 2ml (15)

5. Volume : _____ ml

Weight : 5648 g

9 H 18

Heating

4.30 1. Place mixture in water bath to heat.

2. Set water bath to switch on at 9.00 pm

3. ~~Set chart recorder to switch on at 9.00 pm~~

4. ~~(Set magnetic stirrer to switch on at 9.00 pm.)~~

5. Set water bath to heat up to 60°C

6. Remove from water bath after 10 hours at 60°C

(approx: 8.00 - 8.30 am.)

9.32 am. 7. Sample 3 x 2ml (9H18)

8. Volume: ~4569 ml $14.55 \times 10^2 \times \pi$

9. Weight: 5575 g

Dilution

1. Dilute by 3 by addition of 2×9H volumes of PFW.

2. Volume PFW ~ 13423 ml $(19 \times 15^2 \times \pi)$

9.55 am. Weight PFW 14570 g

3. Sample ✓ 3 x 2ml
✓ 1 x 5ml (α 9H18)

4. pH: ~~7.07~~ 7.03

Conductivity: 1.73 mMho

10.05 am. 5. Dispatch /1 and α 9H18 to factor IX assay.

6. Factor IX activity: 2.7 u/ml

∴ Total factor IX units: ~36,242 (55% yield)

9.H.18

Adsorption

1. Add DE-52 . 1 g dry weight per 150 factor IX units

Wt DE-52 : 242. g

11.35. 2. Stir for 1 hour

using : ovoid magnetic stirrer

Recovery of DE-52

B.05

a) 1. Pour slurry into GAC 110 column with extender tube attached.

2. Fit pressure head . Pack under N_2 pressure or pump ~~from~~ with Watson Marlow pump.

b) 1. Spin down in Beckman centrifuges at 3,000 rpm for 20 minutes.

2. Decant supernatant

3. Resuspend in 1.5 x weight of dry DE 52 volumes of buffer J

Volume of buffer J _____ ml

Weight of buffer J _____ g

4. Pack into GAC 110 ~~column~~.

Volume of supernatant: 12,760 ml ($11^2 \times \pi \times$)

pH: 8.01

(2597- Bucket) Weight of supernatant: 14,035 g conductivity: 3.29.

Sample : 3 x 2 ml
1 x 10 ml

(1 Sn) OD280 : 0.37.

Elution

Bed height : ~6 cm

Bed volume : 570 ml

2551. Wash with 5 bed volumes of buffer J

Volume buffer J : (2900) ml

Weight buffer J : 2900 g

Flow rate ~ 300 ml / min.

Monitor u.v, conductivity, refraction.

1. Collect samples by change in U.V. Transmission

2. Collect first peak in two halves (front peak/F1 and rear peak/F2)

Sample 2 x 2 ml
1 x 5 ml

/J

Volume 2265 ml
Weight 2268 g
pH 7.49
Conductivity 9.82 mMho

OD240 : 1.35.

/F2

Volume _____ ml
Weight _____ g
pH _____
Conductivity _____ mMho.

3. Wash with 5 bed volumes buffer K

Volume buffer K : 5,000 ml

Weight buffer K : _____ g

7N.18

4. Collect eluting peak in three parts:

Protein front /K1
Protein middle /K2
Protein tail /K3

Sample each 2 x 2ml
1 x 5ml

/K1 Volume 250 ml
Weight 247 g
pH 7.08
Conductivity 15.82 mMho OD280: 36.56
(80 x 0.457)

/K2 Volume 1630 ml
Weight 1648 g
pH 7.01
Conductivity 26.36 mMho OD280: 2.64

/K3 Volume 2040 ml
weight 2077 g
pH 6.96
Conductivity 27.27 mMho OD 280: 0.45

/K4 Volume 1000 ml conductivity: 27.45
weight 1009 g pH 6.93
5. Wash ~~Conductivity with 15 bed volumes~~ mMho buffer C OD280: 0.25

Volume buffer c 3000 ml

Weight buffer c _____ g

6. Collect any protein peak that elutes

Sample 2 x 2ml (/c)
1 x 10ml

15.35

/c Volume 2240 ml
Weight 2439 g
pH 6.93
Conductivity 71.09 mMho OD280. 0.10

All fractions stored in cold room 3.

ASSAY REQUEST FORM (1)

Request from: PAF / DC Date: 28/11/83

When results needed: 5/12/83

Samples to be kept? No If so, how?

Samples ~~provided with form~~/available from R+D freezer

SAMPLE 9H18 INVESTIGATION		/K1	/K2	/K3	/K4	/C.▲	
Factor VIII, iu/ml	1 st.						
	2 st.						
✓ Factor IX, iu/ml	1 st.	(10)	(15)	(1.5)	(0.3)	(<0.05)	
	2 st.	≈82.0	≈4	0.09	0.06		
Factor II, u/ml							
Factor X, u/ml							
Factor VII, u/ml (clotting)							
AT III (Anti Xa), u/ml							
AT III (Anti IIa), u/ml							
AT III (Laurell), u/ml							
Factor VIII RAG, u/ml							
Fibrinogen (Laurell), mg/ml							
Prothrombin (Laurell), u/ml							
Factor XIIIA (Laurell), u/ml							
Fibronectin (Laurell), mg/ml							
Factor VIII CAG							
✓ NAPTT sec. 1/10		211	235	225	220		Blank: 250
TGt50 min.							
FDA hr.							
Limulus + or -							

▲ 2M NaCl.

For comments contact Date finished:

ASSAY REQUEST FORM (1)

Request from: PAF

Date: 25/11/83

When results needed: TODAY

Samples to be kept? No

If so, how?

Samples provided with form/~~available from~~

SAMPLE INVESTIGATION		JD 2982 15	9H18				
Factor VIII, iu/ml	1 st.						
	2 st.						
Factor IX, iu/ml	1 st.	(35)	(2.6)				
	2 st.	35.6	2.7				
Factor II, u/ml							
Factor X, u/ml							
Factor VII, u/ml (clotting)							
AT III (Anti Xa), u/ml							
AT III (Anti IIa), u/ml							
AT III (Laurell), u/ml							
Factor VIII RAG, u/ml							
Fibrinogen (Laurell), mg/ml							
Prothrombin (Laurell), u/ml							
Factor XIIIA (Laurell), u/ml							
Fibronectin (Laurell), mg/ml							
Factor VIII CAG							
NAPTT sec. 1/10							
TGt50 min.							
FDA hr.							
Limulus + or -							

1840
1423. ~ 52 Soleth
131

For comments contact _____ Date finished: _____

ASSAY REQUEST FORM (1)

Request from: PAF / DC Date: 28/11/83

When results needed: 5/12/83

Samples to be kept? No If so, how?

Samples ~~provided with form~~/available from R+D Frozen

SAMPLE INVESTIGATION		JD 2982 /5	JD 2982 /5 ⊕	9H18 ⊕	αH18 ‡	/Sn ‡	/J
Factor VIII, iu/ml	1 st.						
	2 st.						
Factor IX, iu/ml	1 st.	(35)	(14)	7.7	(2.7)	(0.13)	(0.3)
	2 st.	35.9	16.9	7.8	2.7	≈0.01	1.3
Factor II, u/ml							
Factor X, u/ml							
Factor VII, u/ml (clotting)							
AT III (Anti Xa), u/ml							
AT III (Anti IIa), u/ml							
AT III (Laurell), u/ml							
Factor VIII RAG, u/ml							
Fibrinogen (Laurell), mg/ml							
Prothrombin (Laurell), u/ml							
Factor XIIIA (Laurell), u/ml							
Fibronectin (Laurell), mg/ml							
Factor VIII CAG							
NAPTT sec. 1/10 ·····->		211 *				259 *	229 *
TGt50 min.					Blank	261	
FDA hr.							
Limulus + or -							

⊕ Sorbitol 2g/ml

‡ Sorbitol 0.5g/ml

For comments contact _____ Date finished: _____

Buffer K

8.9 mM	trisodium citrate	(2.62 g/L)
1.1 mM	citric acid	(0.23 g/L)
10 mM	Na_2HPO_4	(1.42 g/L)
300 mM	NaCl	(17.53 g/L)

pH 7.00 ± 0.05 6.96

conductivity 29.5 ± 1.00 mMho 28.7

To make up 5 L of

13.1 g	trisodium citrate
1.15 g	citric acid
7.1 g	Na_2HPO_4
87.65 g	NaCl

Make up to 5L (5 kg) with PFW.

Buffer J

8.5 mM	trisodium citrate	(2.5 g/L)
1.5 mM	citric acid	(0.31 g/L)
10 mM	Na_2HPO_4	(1.42 g/L)
80 mM	NaCl	(4.67 g/L.)

pH	7.00	± 0.05		7.02
Conductivity	11.00	± 0.5	mMho	10.2

~~[redacted]~~

To Make up 5 L.:

12.5 g	trisodium citrate
1.55 ~~5.7~~ g	citric acid
7.1 g	Na_2HPO_4
23.35 g	NaCl.

Make up to 5 L (5 Kg) with PFW

REAGENT "BUFFER C" 9P 33

File Reference: P39P3302
Authorised by: [signature]
Date Effective: 3.8.83

Product: DRIED FACTOR IX FRACTION
Processing Run: 9P _____
This page started: _____

Reagent Title: Buffer C

Short title for label etc.: Buffer C

Use described in Production Records 9P 13, 9P 14.

Standard weight: 6.00 kg for GAC 180 column.
3.00 kg for GAC 110 column.

If non-standard weight prepared, insert calculated quantities in pencil under "Qty. non-std." and have calculations checked by a second operator, who should sign here: _____

☑ Standard container 4-10L bucket ☐ Used here

Special instructions for this reagent: Make up fresh.

Discard reagent: } 5h after collection of PFW.

PFW collected 11.20 h, 25.11.83 date.

Substance or PFL concentrate	Control No.	Qty. (GAC 180)	Qty. (GAC 110)	Qty. used	Molar
Trisodium citrate	9203700D	35.3 ± 0.1g	17.7 ± 0.1g	17.7g	0.02M citrate
Sodium chloride	9289870	350.6 ± 0.1g	175.3 ± 0.1g	175.3g	1.0M chloride
PFW		5,614 ± 10g	2,807 ± 10g	2808g	

pH limits 8 ± 1. Found pH * 7.74

Adjustment of pH

Conductivity limits 80 ± 10 mS. Found * 74.9 mS.

Adjustment of conductivity

*Consult Chief Technician or Senior Technician if outside limits.

Remarks on preparation:

Labelled " for batch 9P _____, _____" (date).

**Reagent prepared and labelled by LH. **Controlled by NeO.
**These must be different people.

Co-ordinating Technician _____ Checked by _____

8.12.83 9H19 Large Scale heating and recovery of Factor IX in solution.

To further test the efficacy of this heating method on a large, production scale.

Materials Factor IX - frozen 9P eluate from BPL, JD 2982 x 5i (filled in A82 for mild pyrogenicity)

DE-52 - a mixture of that recycled and used as in 9H15 - 9H18 plus newly recycled batch GC/RD/1

Buffers

Buffer J: 8.5 mM tri sodium citate, 1.5 mM citric acid, 10 mM Na_2HPO_4, 80 mM NaCl } pH 6.95

Buffer K: 8.9 mM trisodium citate, 1.1 mM citric acid, 10 mM Na_2HPO_4, 300 mM NaCl } pH 6.95

Buffer C 20 mM trisodium citate, 1.0 M NaCl } pH 7.45

Method

Thawing: 2155 ml JD 2982 where softened at room temperature for 1 hour then thawed in water at 30° - 40° C

3x2ml, 1x5ml and 1x1ml (viable count) samples were taken (1x5i)

Stabilisers: In this experiment, stabilisers were added w/w instead of w/v.

217.1g Glycine dissolved (equivalent to 0.1g glycine/g JD) stirring with large Edmunds magnetic stirrer.

4323g sorbitol then added gradually (2g sorbitol/g JD) with magnetic stirring.

Material was held in poly ~~propylene~~ propylene bottle to be used for heating. Due to narrow neck, there was some difficulty in sorbitol adding/mixing and observation.

3 x 2ml samples were Taken (/s)

Heating: Sample placed in water bath, set to switch on and heat to 60°c at 9.00 p.m.
The mixture was stirred by magnetic stirrer underneath.

The material reached 60°c at 11.21 pm and was then heated for 10 hours, during which bath and sample temperatures were steady.

After heating, 3 x 2ml samples were Taken (/9H19)

Weight and volume were measured and material diluted by 3 with addition of 11691 ml PFW (2x 9H19 volumes)

3x2ml, 1x5ml sample taken (α9H19)

Samples of /5i and α9H19 sent for immediate factor IX assay.
Conductivity checked to be below buffer J conductivity (10 mMh
Found to be 1.58 mMho.

Adsorption: Factor IX activity: 3.5 u/ml

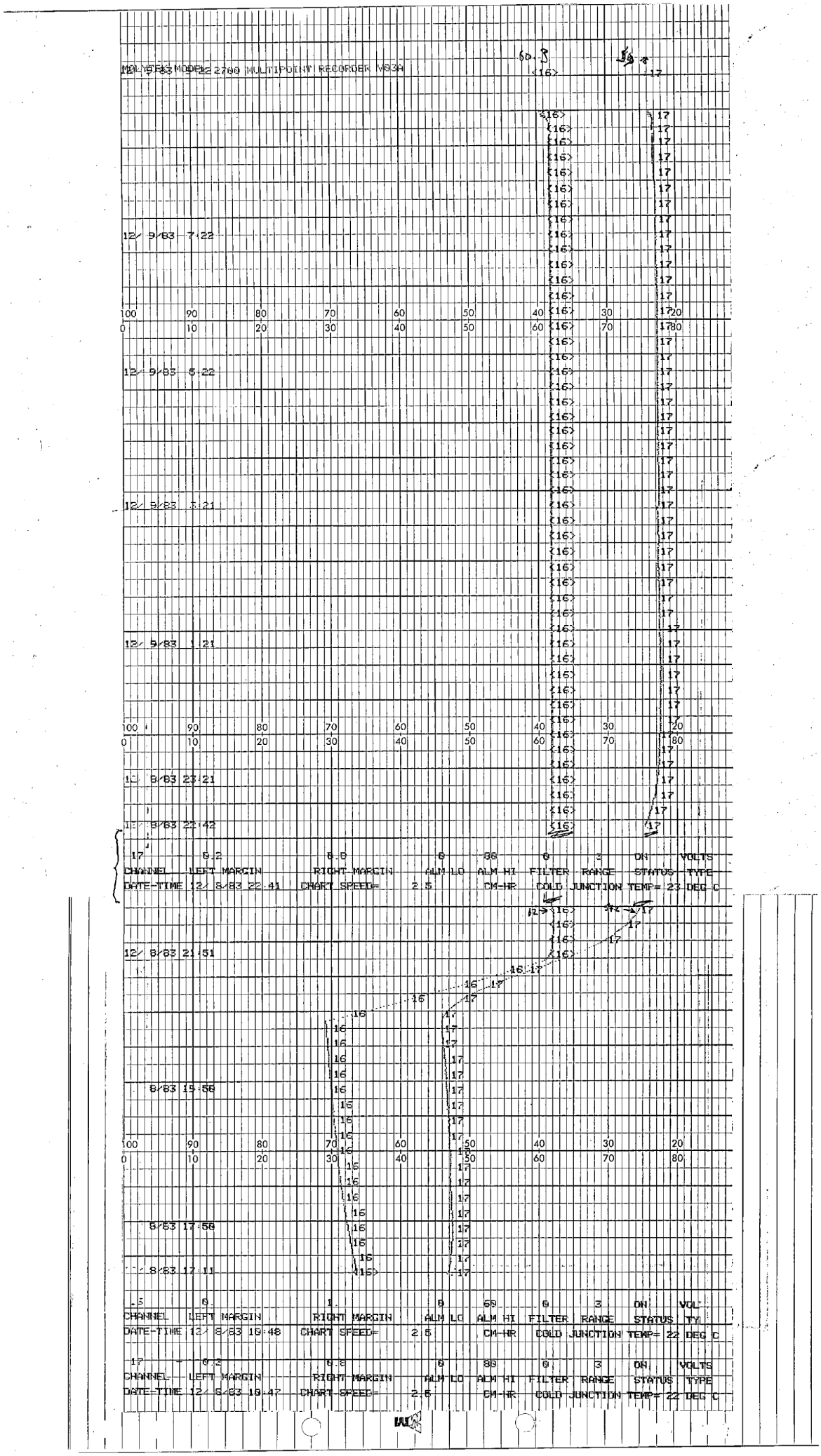
DE-52 added at 1g /150 units factor IX

This was actually an approximation, as insufficient DE52 was available.
Actually added 415.6g DE52 (≈ 1g per 157/150? units IX)

Mixture stirred for 1 hour at lab. temp, with magnetic stirrer.

Gel then recovered by pouring slurry into GAC 110 column (with extension) and pumping through the supernatant with a Watson-Marlow peristaltic pump.

Collect supernatant and sample 3x2ml, 1x5ml (/sn)


MODEL 2700 MULTIPOINT RECORDER V03A
12/ 9/83 7:22
12/ 9/83 6:22
12/ 9/83 5:21
12/ 9/83 4:21
12/ 8/83 23:21
12/ 8/83 22:42
CHANNEL LEFT MARGIN RIGHT MARGIN ALM LO ALM HI FILTER RANGE STATUS TYPE
DATE-TIME 12/ 8/83 22:41 CHART SPEED= 2.5 CM-HR COLD JUNCTION TEMP= 23 DEG C
12/ 8/83 21:51
8/83 18:58
8/83 17:58
8/83 17:11
CHANNEL LEFT MARGIN RIGHT MARGIN ALM LO ALM HI FILTER RANGE STATUS TYPE
DATE-TIME 12/ 8/83 10:48 CHART SPEED= 2.5 CM-HR COLD JUNCTION TEMP= 22 DEG C
CHANNEL LEFT MARGIN RIGHT MARGIN ALM LO ALM HI FILTER RANGE STATUS TYPE
DATE-TIME 12/ 8/83 10:47 CHART SPEED= 2.5 CM-HR COLD JUNCTION TEMP= 22 DEG C

Monitoring: In theory, intended to monitor u.v. absorbance, u.v. transmission, conductivity, refractometry. However, due to dislocated uvicord cell, no u.v. trace was obtained (only discovered half way through, so fraction collecting was hurriedly re-aligned to refractometry changes.

After packing, the bed height was 12 cm, giving a bed volume of 1140.5 ml

Elution: Buffer elution was effected by N_2 pressure through the buffer reservoir.

1) ~~4~~ 4 bed volumes buffer J applied (4995 ml)

Peak collected as refractometer signal dropped (chart recorder uncalibrated.)

2) 6010 ml buffer K then applied.

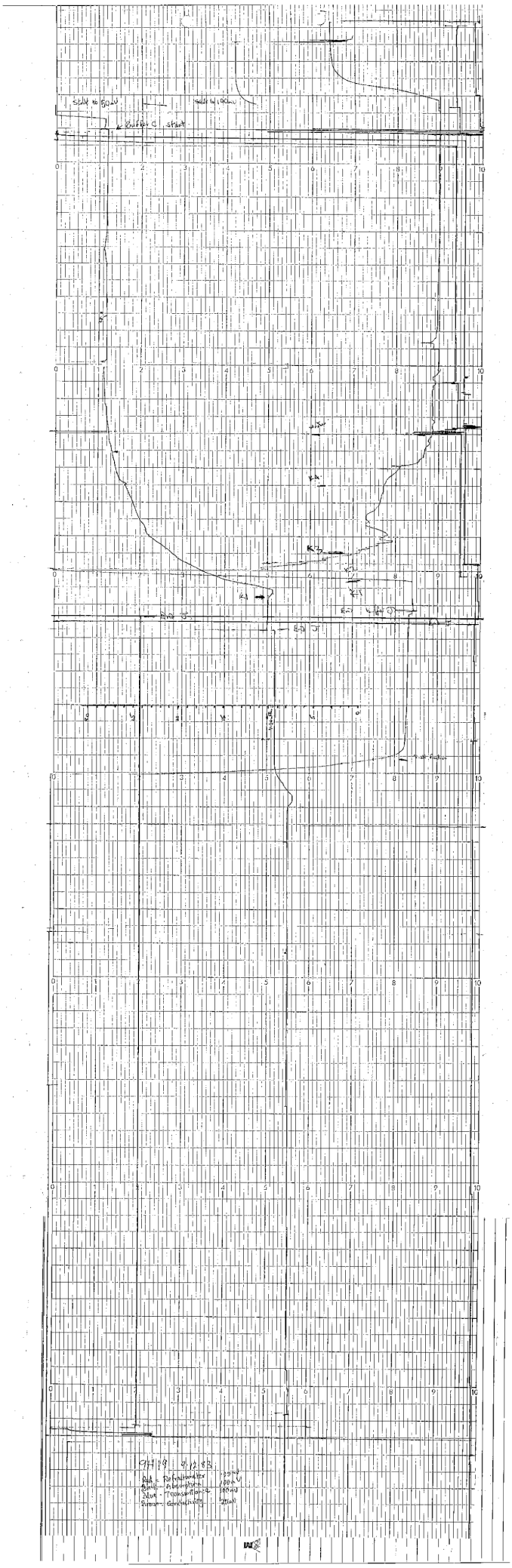
Collected four fractions corresponding to 2 peaks in refraction. K1 – K4.

Each fraction was sampled for factor IX, NAPTT assay and each fraction tested for pH and conductivity.

3) 3000 ml buffer C then applied, eluate peak collected in one fraction (1C).

Assays performed on selected samples:

Factor IX
NAPTT
Protein (biuret)
Chloride concentration
pH
Conductivity
Viable counts.



9H19.6

Results Protein Data

Sample	Volume (ml)	Protein (mg/ml)	Factor IX (u/ml)	Factor IX Total units	Sp. Act (u/mg)	% starting factor IX	% factor mixed with
γ 5i	2155	10.8	37.4 37.6	80812	3.46	100	
/5	5846		20.7	121012		150	
9H19	5846		10.5	61383		76	
α9H19	18633		3.5 3.2	62420		77	100
/Sn	~15,000	0.38	0.01	150	0.02	0.18	0.24
/J	3420	0.62	0.58	1983	0.93	2.45	3.18
/K1	98	4.27	2.0	196	0.47	0.24	0.31
/K2	200	40.2	128.8	25760	3.2	31.9	41.27
/K3	540	11.3	24.5	13230	2.17	16.4	21.19
/K4	244	2.39	5.2	1268	2.17	1.56	2.0

Sample	NAPTT to (s)
γ/5i	223
α9H19/Sn	246
/J	183
/K1	209
/K2	228
/K3	196
/K4	188

Viable Counts

γ/5i 350 cfu/ml

α9H19/K2 6 cfu/ml.

9NA-7

Physical Data

Sample	pH	Conductivity mMho	[Chloride] mM
γ/5c	7.17	17.7	139
α 9H19		1.58	
/Sn	8.18	1.62	17.9
/J	8.02	9.34	62
/K1	7.00	9.18	72.6
/K2	7.08	13.28	90.7
/K3	7.07	23.44	223
/K4	7.06	26.23	253
Buffer J	6.95	10.0	80 (by weight)
Buffer K	6.95	28.2	300 (by weight)

Comments

1. Noticable is the dramatic improvement of TK survival after heating, from 55% (9H18) to 77%. The only major change in the heating is the use of a polypropylene container instead of stainless steel.

2. There is known to be an improvement if plastic is substituted for glass. It now appears that this is also true if plastic is substituted for stainless steel.

3. The only other alteration at this stage is the addition of stabiliser on a weight-for-weight basis. This leads to a slight increase in stabiliser concentration.

9H19.9

	w/w	wt/vol	
Glycine	0.1 g/g	0.1 g/ml	
	1.34 M	1.33 M	ie. 0.75 % difference
Sorbitol	2 g/g	2 g/ml	
	11.06	10.98 M	ie. 0.73 % difference

4. After recovery on DE52 the sum of all buffer K elutes is also increased from 39% (9H18) to 49%. This reflects on drop in the recovery off the column.

5. There are two observable reasons for poor column recovery; lack of suitable monitor may have resulted in our not catching the full protein peak. More important column pressure was too high, particularly during buffer K application.

This generated a large depression in the centre of the gel which may have led to smeared elution

6. It is possibly this effect which generated the hither to unobserved double peak eluting with buffer K (K1 + K2), (K3 + K4).
Further investigation was carried out to see whether any difference existed between them.

Specific activity: The spec. act. of K2 is 3.2 u/mg very close to the starting value 3.46 u/mg. K3 and K4 have the same sp. act. at 2.17 u/mg. At the same time they show a shortening of NAPTT. This in itself is unusual, since short NAPTT is conventionally associated with the front of any elution peak.
To see whether K3 and K4 represent different molecular species from K2, SDS PAGE was performed. This also characterised changes which may occur upon heating. In all, three gels were run with acrylamide at 5% (to look for molecular aggregates) 10% and 15% (to look for breakdown products, etc.).
The 5% gel, run on a faster VIII experiment

Piggy-back can be found in record of 8H31. It showed no significant high molecular weight aggregate after heating (K2), not present in the starting material (5).

The 10% gel shows some high molecular weight material which does not even get into the stacking gel.
All the major bands are the same before and after heating though minor bands do show some differences.

K2 shows reduced l.m.w. bands ("A") while band 'A' is enhanced in K1, relative to the major staining bands. A faint "B" band disappears in K2, K3 and K4, though band C is enhanced in K3.
The other difference is in the grouping of bands "D" in the factor II, factor I region. These are more spread out in K1, closing together in K2 and broadening again in K3 and K4. It is possible that it is in this region that NAPTT shortening is reflected by band broadening. K1 NAPTT is shorter than K2 which is shorter than K3 and K4.

Apart from these minor modifications, there are no differences between fractions. It seems more likely therefore that the double elution peak was due to discontinuities in the gel with resulting delayed elution from part of it.

Future Experiments should be directed towards improving the column recovery, which should be about 90% +

Aims: 1) To repeat the >7% recovery after heating factor II

2) To improve the column monitoring facility

3) To reduce flow rates and buffer hydrodynamics to generate an even gel bed, with regard to the small gel volumes used.

4) To eliminate the time-consuming on-the-spot assay at the ∝9H stage, and thereby reduce the experiment time which is presently too long.

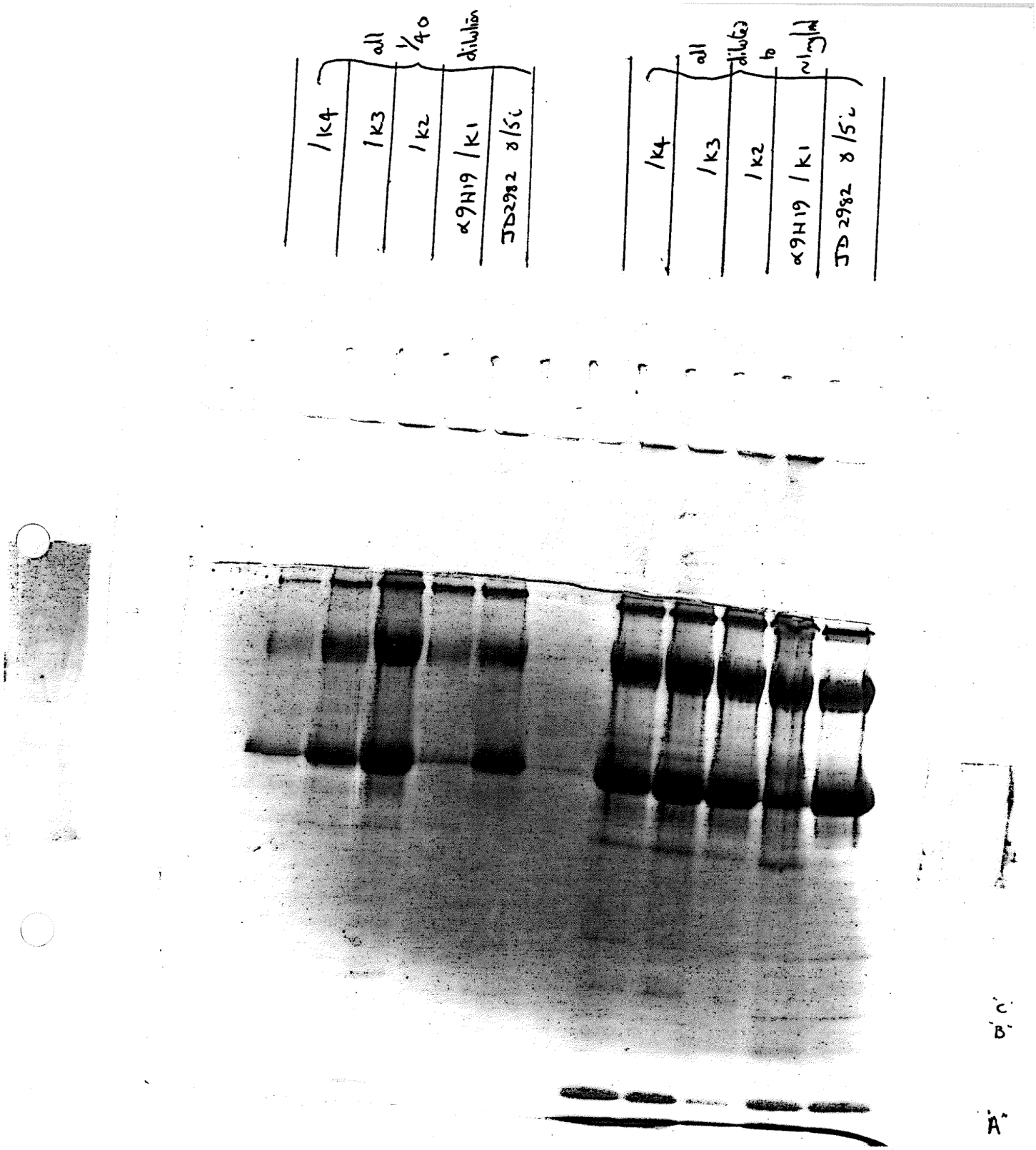
5) In view of the heating-up time being longer than expected, to set the water bath to switch on earlier.

9 H 19.10

Time Table

Stage	Day	Time Complete	Expt Hours
Find Elute	1	9.30	0
Soften/Thaw	1	11.30	2
Dissolve Glycine	1	12.23	3
Dissolve Sorbitol	1	~ 4.00	6 ½
Start Water bath	1	9.00	18 ½
Reach 60°	1	11.[redacted]22	14
Heat	2	9.22	24
Dilute	2	10.17	24 ¾
Assay	2	11.25	26
1 hour DEAE Adsorption	2	12.45	27 ¼
Pour slurry/pack	2	14.10	28 ¾
Apply Buffer J	2	15.00	29 ½
Apply buffer K	2	15.40	30
Apply buffer C	2	16.00	30 ½

PAF, DC, WH.


all 1/40 dilution
1K4
1K3
1K2
αGH19 1K1
JD2982 δ15i
all dilutes to 1mg/ml
1K4
1K3
1K2
αGH19 1K1
JD2982 δ15i
C
B
A

10% SDS PAGE.

all dilution by 1/40	/K4	/K3	/K2	α9H19 / K1	JD 2982 δ/Sc
all dilution to neutralize	/K4	/K3	/K2	α9H19 / K1	JD 2982 δ/Sc
all dilution to neutralize	/K4	/K3	/K2	α9H19 /K1	JD2982 δ/Sc

15 % SDS PAGE.

8/12/83

FACTOR IX WET HEATING

9H19

Starting Material

Time

9.30 Eluate batch: JD2982 γ5i

Eluate volume: 2155 ml

Thurs 11.30. Eluate weight: 2171 g

wt of heating pot + lid : 542 g

pH : 7.17

Conductivity : 17.7 mMho

wt of stirring bar : 118 g

Sample: 3 x 2ml (γ5i)
1 x 5ml
+ 1ml Viable count

Stabilisers (Batch: 9256760D)

12.04. 1. Add Glycine ~~0.1~~ g per ml of eluate

(equivalent to 0.1 g per g of eluate)

Weight of glycine added: 217.1 g

2. Dissolve by stirring with large magnetic stirrer.

3. Place in 30° water bath with magnetic stirrer underneath.

12.23 4. Add sorbitol ~~2g per ml of eluate~~

(equivalent to 2 g per g of eluate)

Weight of sorbitol added: 4342 g

3. Stir until fully dissolved, monitoring temperature (>20°c)

✓ 4. Sample 3 x 2ml (1S)

5. Volume: _____ ml

Weight: _____ g

9H19

T in

Heating

1. Place mixture in water bath to heat.
2. Set water bath to switch on at 9.00 pm
3. Set chart recorder to switch on at 9.00 pm
4. (Set magnetic stirrer to switch on at 9.00 pm.)
5. Set water bath to heat up to 60°C

9.22

6. Remove from water bath after 10 hours at 60°C
(approx: 8.00 - 8.30 am.)
7. Sample 3 x 2ml (9H19)
8. Volume: 5845.7 ml ← $\left(\frac{30.5}{2}\right)^2 \times \pi \times 8$
9. Weight: 6793.2 g

7335.2
542
6793.2

Dilution Pot: 30L effet. Weight of bucket: 4478.4.

10.17

1. Dilute by 3 by addition of 2 (2x9H) volumes of PFW.
2. Volume PFW 11691 ml
Weight PFW 11691.1 g
3. Sample 3 x 2ml
1 x 5ml (α 9H19)
4. ~~pH~~ Weight 22258g / Volume pH6.98: $\left(\frac{30.5}{2}\right)^2 \times \pi \times 26.5 = 18633$ ml
Conductivity: 1.58 mMho

22800
542
22258

5. Dispatch 1 and α 9H19 to factor IX assay.
6. Factor IX activity: 3.5 u/ml
∴ Total factor IX units: 85215.5

9HJ9

Adsorption

(Mixture of DE52 from same batch as 9N 15, 16, 17, 18 + DE52 batch #RD/1)

1. Add DE-52 , 1 g dry weight per 150 factor IX units

Should use Wt DE-52 : 4348 g

In fact, not enough thawed DE-52 so used 415.6 g instead. (Loading ~ 157 v 1g DE52).

11.25 2. Stir for 1 hour using Magnetic stirrer.

Recovery of DE-52

12-45 a) 1. Pour slurry into GAC 110 column with extender tube attached. Pack with Watson-Marlow pump.

2. Fit pressure head. Pack under N_2 pressure or pump ~~from~~ with Watson Marlow pump.

b). 1. Spin down in Beckman centrifuges at 3,000 rpm for 20 minutes.

2. Decant supernatant

3. Resuspend in 1.5 x weight of dry DE 52 volumes of buffer J

Volume of buffer J _____ ml

Weight of buffer J _____ g

4. Pack into GAC 110 column.

Collect ~ 30 L bulk: 4297 g

Pa 2 at 14:10

Volume of supernatant: _____ ml
Weight of supernatant 17303 g
pH 8.18
Conductivity 1.62 mMho
Sample : 3 x 2 ml (1 Sn)
1 x 5 ml

9A 4
9N19

Elution

Bed height : 12 cm

Bed volume : 1140.5 ml

14.28. 1. Wash with 5 bed volumes of buffer J

Volume buffer J : ~~4995~~ ml (5 ml sample taken)

Weight buffer J : ~~4995~~ g

2. Measure flow rate : 155 ml/min.

Monitor u.v, conductivity, refraction.

3. Collect samples by change in U.V. Transmission

3. Collect first peak in two halves (front peak J1 and rear peak J2)

Sample 2 x 2 ml
1 x 5 ml

J1 Volume 3420 ml
Weight 3423.2 g
pH 8.02
Conductivity 9.34 mMho

J2 Volume _____ ml
Weight _____ g
pH _____
Conductivity _____ mMho

4. Wash with 5 bed volumes buffer K

Volume buffer K : 6 0~~4~~6 ml

Weight buffer K : 6,0~~4~~6 g

7N.5
9N9

5. Collect eluting peak in three parts:

Protein front /K1
Protein middle /K2
Protein tail /K3

Sample each 2 x 2ml
1 x 5ml

/K1
Volume 98. ml
Weight 103.7 g
pH 7.00 ?
Conductivity 9.18 ? mMho

Visible cont

/K2
Volume 200 ml
Weight 200.9 g
pH 7.08
Conductivity 13.28 mMho
1ml taken for visible count.

/K3
Volume 540 ml
Weight 543.3 g
pH 7.07
Conductivity 23.44 mMho

/K4 Volume: 244ml
Weight 245.4 g.
pH 7.06
Conductivity: 26.23

6. Wash with 3 bed volumes buffer C.

Volume buffer C 3000 ml
Weight buffer C 3000 g

7. Collect any protein peak that elutes

Sample 2 x 2ml (/C)
1 x 5ml

/C
Volume 2560 ml
Weight 2656.7 g
pH 7.08
Conductivity 67.21 mMho

9NA

BACTERIAL CONTAMINATION : VIABLE COUNTS

File Reference: _____ Product: JD 2982

Authorised by: [signature] Processing Run: _____

Date effective: 21.4.83 This page started: 8.12.83

PRODUCTION STAFF			QUALITY CONTROL STAFF			
Sample Stage	Volume to be taken	Sample taken by:	SAL	Day 1 at °C	Day 4 at °C	.'. CFU/ml
185i	1ml			35	350	350
			Control	0	0	0

EXTRA SAMPLES as requested by Scientist i/c Production or QC staff:

			Control			

COMMENTS by sampling, testing or reading staff:

INTERPRETATION by Scientist i/c Production, and any action taken:

COMMENTS by Quality Controller:

9H19

BACTERIAL CONTAMINATION : VIABLE COUNTS

File Reference: _____ Product: _____

Authorised by: [signature] Processing Run: x9H19B

Date effective: 21.4.83 This page started: 13.12.83

PRODUCTION STAFF			QUALITY CONTROL STAFF			
Sample Stage	Volume to be taken	Sample taken by:	SAL	Day 1 at °C	Day 2 at °C	.'. CFU/ ml
1K2	1ml				5+1 spreader	6
			Control		0	0

EXTRA SAMPLES as requested by Scientist i/c Production or QC staff:

			Control			

COMMENTS by sampling, testing or reading staff:

INTERPRETATION by Scientist i/c Production, and any action taken:

COMMENTS by Quality Controller:

ASSAY REQUEST FORM (1)

9N19

Request from: PAF Date: 9/12/85

When results needed: RIGHT NOW HONEYCHILE

Samples to be kept? → If so, how? ~

Samples provided with form/~~available from~~

SAMPLE INVESTIGATION		JD2982 8/5;	9H19.				
Factor VIII, iu/ml	1 st.						
	2 st.						
✓ Factor IX, iu/ml	1 st.	(33)	(3).				
	2 st.	37.4	3.5				
Factor II, u/ml							
Factor X, u/ml							
Factor VII, u/ml (clotting)							
AT III (Anti Xa), u/ml							
AT III (Anti IIa), u/ml							
AT III (Laurell), u/ml							
Factor VIII RAG, u/ml							
Fibrinogen (Laurell), mg/ml							
Prothrombin (Laurell), u/ml							
Factor XIIIA (Laurell), u/ml							
Fibronectin (Laurell), mg/ml							
Factor VIII CAG							
NAPTT sec. 1/10							
TGt50 min.							
FDA hr.							
Limulus + or -							

For comments contact _____ Date finished: _____

ASSAY REQUEST FORM (1)

9H19

Request from: PAF Date: 14/12/83

When results needed: ASAP

Samples to be kept? No If so, how?

Samples ~~provided with form~~/available from R+D Big Freezer

SAMPLE INVESTIGATION		JD2982 8/5i	IS *	9H19 *	K9H19 ‡	K9H19/Sn ‡	K9H19/J
Factor VIII, iu/ml	1 st.						
	2 st.						
✓ Factor IX, iu/ml	1 st.	(35)	(13)	(8·0)	(3·5)	(~0·01)	(1·6)
	2 st.	37·6	20·7	10·5	3·2	0·07	0·58
Factor II, u/ml							
Factor X, u/ml							
Factor VII, u/ml (clotting)							
AT III (Anti Xa), u/ml							
AT III (Anti IIa), u/ml							
AT III (Laurell), u/ml							
Factor VIII RAG, u/ml							
Fibrinogen (Laurell), mg/ml							
Prothrombin (Laurell), u/ml							
Factor XIIIA (Laurell), u/ml							
Fibronectin (Laurell), mg/ml							
Factor VIII CAG							
✓ NAPTT sec. 1/10 where marked •		223 *	X	X	X	246 •	183 •
TGt50 min.							
FDA hr.							
Limulus + or -							

* Sorbitol at 2

For comments contact Date finished:

ASSAY REQUEST FORM (1)

9H19

Request from: PAF Date: 14/12/83

When results needed: ASAP

Samples to be kept? No If so, how?

Samples ~~provided with form~~/available from Rob Big freezer

SAMPLE 9H19 / INVESTIGATION		/K1 +	/K2 +	/K3 +	/K4 +		
Factor VIII, iu/ml	1 st.						
	2 st.						
Factor IX, iu/ml	1 st.	(80)	(130)	(18)	(4)		
	2 st.	2.0	128.8	24.5	5.2		
Factor II, u/ml							
Factor X, u/ml							
Factor VII, u/ml (clotting)							
AT III (Anti Xa), u/ml							
AT III (Anti IIa), u/ml							
AT III (Laurell), u/ml							
Factor VIII RAG, u/ml							
Fibrinogen (Laurell), mg/ml							
Prothrombin (Laurell), u/ml							
Factor XIIIA (Laurell), u/ml							
Fibronectin (Laurell), mg/ml							
Factor VIII CAG							
NAPTT sec. 1/10		209	228	196	188		
TGt50 min.							
FDA hr.							
Limulus + or -							

+ Guesstimates only.

For comments contact Date finished:

9H19

ASSAY REQUEST FORM (2)

Request from: PAF Date: 14/12/83

When results needed: 21/12/83

Samples to be kept? _____ If so, how? _____

Samples ~~provided with form~~/available from: R+D Big Freezer (in C5's)

SAMPLE INVESTIGATION		JD 2982 γ/5i	α9H19 /Sn	α9H19 /J	α9H19 /K1	α9H19 /K2	α9H19 /K3
Protein	E280	~~20~~	~~10~~	(20)	(15)	(15)	(8)
	biuret g/l	10.8	0.38	0.62	4.27	40.2	11.3
Fibrinogen g/l/%							
Sodium mmol/l							
Potassium mmol/l		~~5~~ (150)	(40)	(70)	(90)	(180)	(250)
Chloride mmol/l		139	(17.9)	62	72.6	(90.7)	223
Citrate mmol/l							
Phosphate mmol/l							
Tris mmol/l							
PEG g/l							
Caeruloplasmin g/l							
Factor XIII u/ml							
PKA % Ref. 2							
AT III (amidolytic) u/ml							
F. VII (amidolytic) u/ml							
XaGT mins.							
pH at 20°C							
Conductivity at 20°C							

For comments contact: _____ Date finished: _____

ASSAY REQUEST FORM (2)

9H19

Request from: PAF Date: 14/12/83

When results needed: 21/12/83

Samples to be kept? _____ If so, how? _____

Samples ~~provided with form~~/available from: R+D Bj Bearer

SAMPLE INVESTIGATION	9H19 1kg					
Protein E280						
✓ biuret g/l	2.39					
Fibrinogen g/l/%						
Sodium mmol/l						
Potassium mmol/l	300					
✓ Chloride mmol/l	253					
Citrate mmol/l						
Phosphate mmol/l						
Tris mmol/l						
PEG g/l						
Caeruloplasmin g/l						
Factor XIII u/ml						
PKA % Ref. 2						
AT III (amidolytic) u/ml						
F. VII (amidolytic) u/ml						
XaGT mins.						
pH at 20°C						
Conductivity at 20°C						

For comments contact: _____ Date finished: _____

9H19

Date 9.12.83 Buffer J for 9H19

Find concentrations

8.5 mM trisodium citrate
1.5 mM citric acid
10 mM Na_2HPO_4
80 mM NaCl

To Make up 5 L (5kg)

Weight		Batch No.	Quantity used
12.5g	trisodium citrate	9293700D	12.5g
1.55g	citric acid	5816300B	1.55g
7.1g	Na_2HPO_4	9260374D	7.1g
23.35g	NaCl	9289873D	23.34g

Make up to 5 L (5kg) with PFW

pH limit: 7.00 ± 0.05 pH found 6.95

Adjust as necessary

Conductivity limit: 11.0 ± 1.0 Conductivity found 10.0

9H19

Date 9.12.83 Buffer K for 9H 19

Find concentrations

8.9 mM trisodium citrate
1.1 mM citric acid
10 mM Na_2HPO_4
300 mM NaCl

To Make up 8 L (8 Kg)

Weight		Batch No	Quantity used
20.96g	trisodium citrate	92937000	21g
1.84g	citric acid	58163008	1.84g
11.36g	Na_2HPO_4	92603740	11.4g
140.2g	NaCl.	9289873	140.2g

Make up to 8 L (8 Kg) with PFW

pH limit: 7.00 ± 0.05 pH found 6.95

Adjust as necessary

Conductivity limit: 29.5 ± 1.0 Conductivity found 28.2

9HA

Date 9.12.83 Buffer C for 9H 19

Final concentrations

20 mM trisodium citrate

1.0 M NaCl

To make up 3 L (3 Kg)

Weight		Batch No	Quantity used
17.7 g	trisodium citrate	9293700D	17.7g
175.3 g	NaCl	9289873D	175.4g

Make up to 3L (3 kg) with PFW

pH limit: 8.0 ± 1.0 pH found 7.45

Adjust if necessary

Conductivity limit: 80 ± 10 Conductivity found 76.4

LH DC

19.1.84 9H20 Large scale heating and recovery of wet factor IX

This experiment is mainly to check reproducibility with the 9H19 results. There are some minor differences
1) polycarbonate bottle used for heating instead of polythene
2) factor IX assayed before treatment and this result used to determine gel loading
3) plastic filter used on top of column to diffuse buffer stream
4) buffers applied by pumping instead of N_2 pressure.

Materials

Factor IX - frozen 9? eluate from BPL, UD2982 α 5i (failed at BPL due to mild pyrogenicity)

DE52 - recycled batch QC/RD/1, stored frozen and thawed overnight.

Buffers

Buffer J	8.5 mM trisodium citrate 1.5 mM citric acid 10 mM Na_2HPO_4 80 mM NaCl	pH 6.99
Buffer K	8.9 mM trisodium citrate 1.1 mM citric acid 10 mM Na_2HPO_4 300 mM NaCl	pH 6.94
Buffer C	20 mM Trisodium citrate 1.0 M NaCl	pH 7.65

Method

2330 ml of UD2982 α 5i were softened at room temperature then thawed in water.

3×2ml, 1×5 ml, 1×1 ml (viable count), 2×1ml immediate factor IX assay samples were taken (α/5i)

Stabilisers: 232.7 g glycine was added to factor IX in the polycarbonate heating bottle. This is equivalent to 0.1 g glycine per g factor IX, i.e. added on a

w/w basis. This was dissolved by stirring with large Edwards magnetic stirrer.

The bottle was placed in a 30°c water bath and 4654 g sorbitol (equivalent to 2g sorbitol / g factor IX was added.
This was a very time consuming process. Handing of sorbitol is a clumsy procedure, especially through the narrow neck of the heating bottle.
Attempts to stir with the magnetic stirrer were not successful, probably due to a) the liquid being too viscous b) the oval stirring bar being too small.

Once dissolved, 3 x 2ml samples were taken (1s)

Heating: The bottle was left in the water bath which was set to switch on at 9.00 pm. Bath and sample temperatures were recorded on Multitek machine.

The following morning, the sample was removed when it had been at 60°c for 10 hours.
The stirrer did not appear to be working.

3 x 2ml samples were taken, and 4 x 50 ml samples for 9H20.02 experiment (9H20)

The factor IX was then diluted by 1/3 by the addition of >760 ml (2 volumes) of PFW.

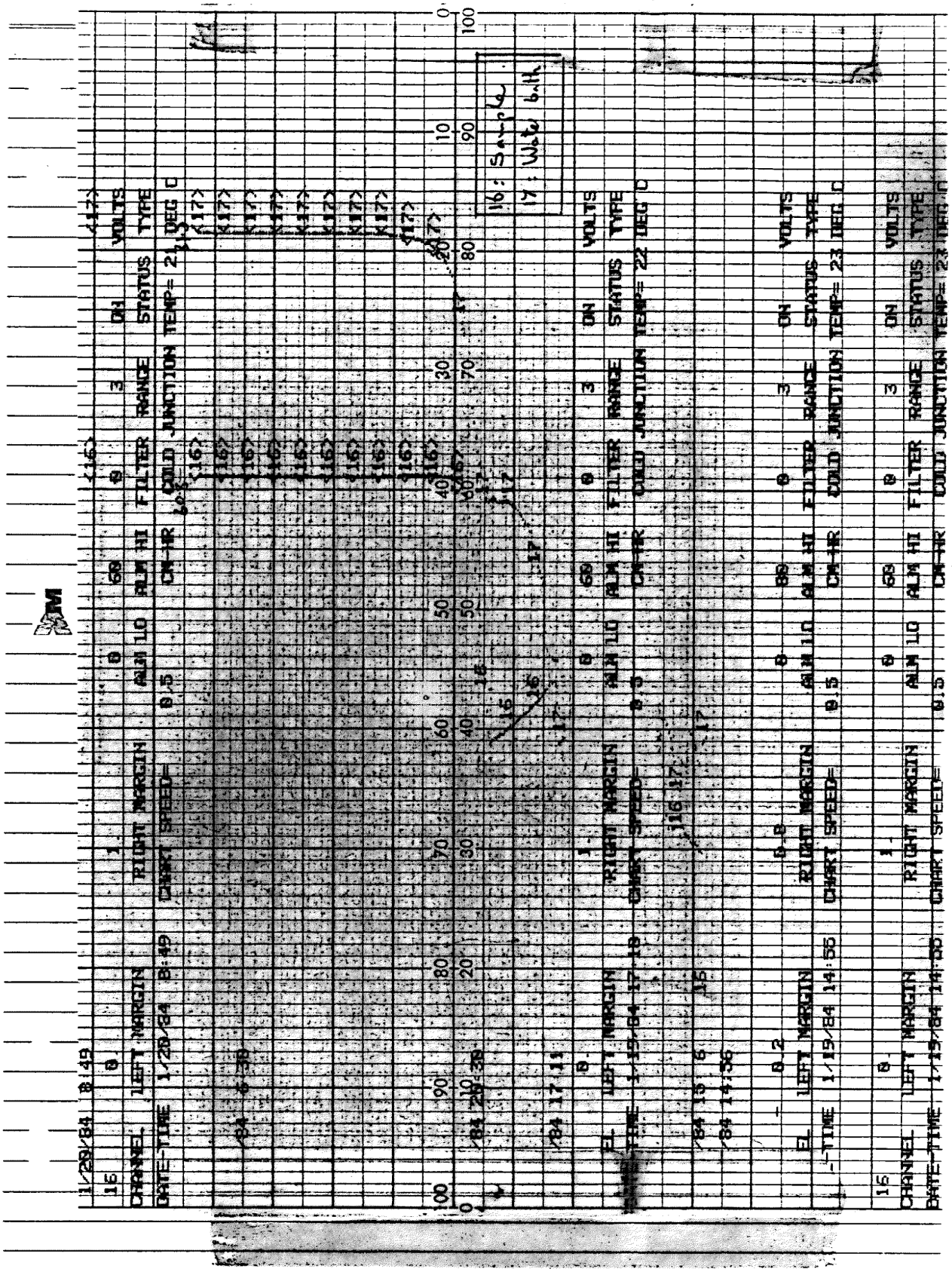
3 x 2 ml 1 x 5 ml samples were taken (x9H20)

Adsorption:

The amount of gel used was based upon the following assumptions:
1) Starting a/s i contained 31.5 u/ml factor IX
2) Heating recovery yield was 77.0%
3) Haven previously loaded at 1g DE52 / 160 units factor IX
4) The equivalent loading is 1g DE52/207 units of factor IX starting material.

Therefore rounded the value up to a loading of 1g DE52 for each 210 units factor IX in the starting material.

9H20.3



Recording of heating conditions of 9H20

Initial factor IX units: 73395

∴ Added 349.5 g DE 52 and stirred with magnetic stirrer for 1 hour at lab temperature.

Column packing + elution:

Slurry poured into GAC 110 glass column and packed by pumping from below with a Watson-Marlow pump at ~ 210 ml/min.
Elwate collected as supernatant.

Bed height of ~ 8 cm gave a bed volume of 760 ml.

The plunger (with plastic filter insert fitted) was placed in the column and 5,000 ml buffer J applied by pumping from above with W-M pump.

Flow rate: 100 ml/min

Elwate monitored by u.v. absorbance, u.v. transmission and conductivity.

The protein peak eluted by buffer J was collected as one fraction (IJ).

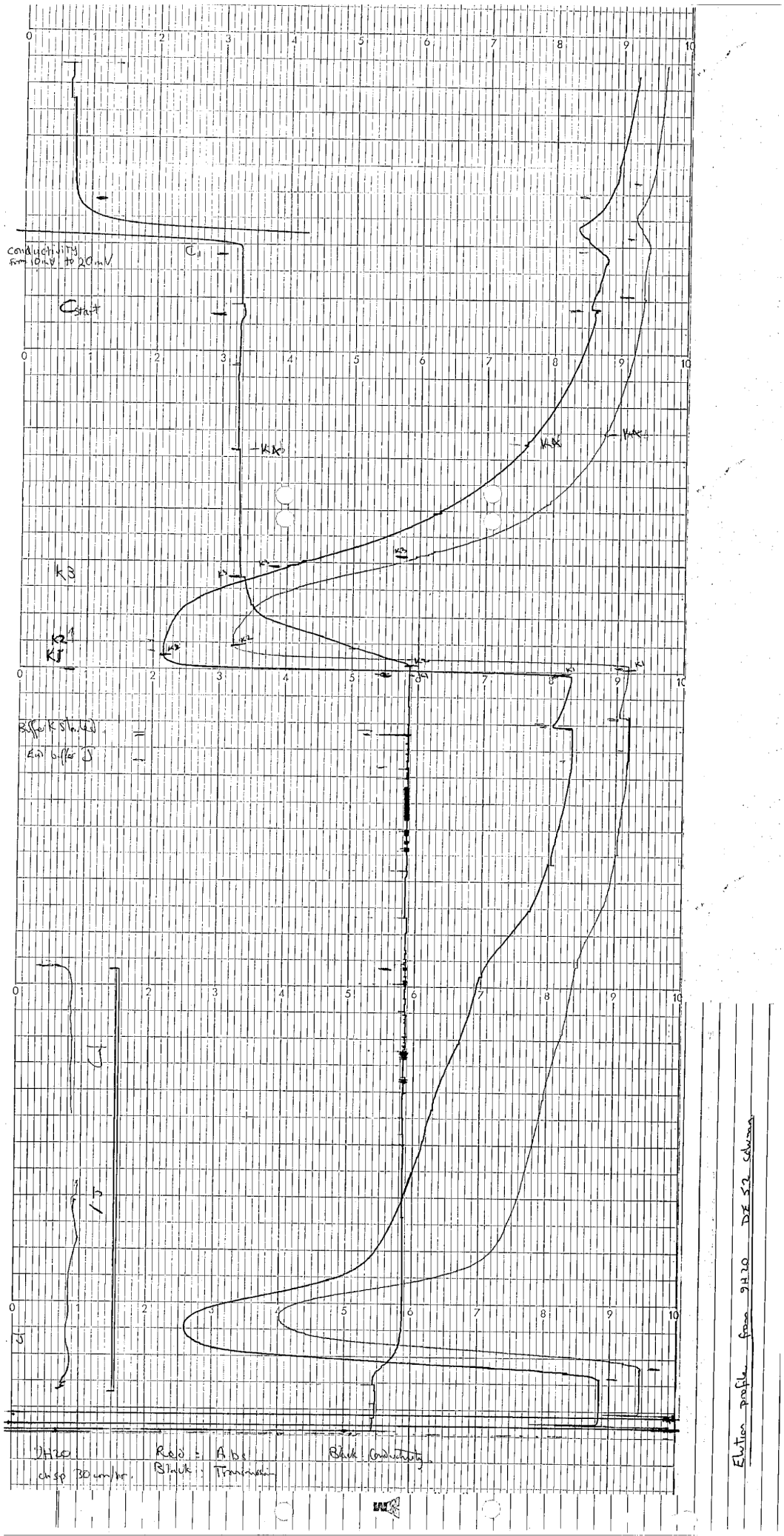
Sampling: All elwates were sampled 3 x 2ml and 1 x 5ml

Buffer K (~4,000 ml) applied and fractions IK1 — IK4 collected. (See trace).

Column was finally washed with buffer C and then dried out.

Assays performed on selected samples:

Factor IX
NAP TT
Protein A_{280}
pH
Conductivity
Viable counts.


conductivity
from 10mV to 20mV
Elution profile from 9H20 DE 52 column

Results Protein Data

Sample	Volume (ml)	Protein (A280)	Factor IX (u/ml)	IX (total units)	Sp Act (u/mg)	% starting factor IX	% factor IX missing
2982 α/Si	2330	13·88	32.6	75958	2.35	100	
9H20	5678		9.3	52806		69.5	
After sampling:							
9H20	5472	6.2	9.3	50889.6	1.5	67	
α9H20	13685	2.67	3.5	47897.5	1.31	63	100
/Sn	15285	0.33	0.2	3057	0.60	4	6.4
/J	3010	2.08	1.4	4214	0.67	5.5	8.8
/K1	135	31.96	67.6	9126	2.11	12	19.0
/K2	645	20.28	47.9	30895	2.36	40.7	64.5
/K3	970	1.48	2.0	1940	1.35	2.5	4.0
/C	410	0.36	0.4	164	1.11	0.2	0.3

Sample	NAPTT (s)
α/Si	213
9H20	215
α9H20	229
/Sn	241
/J	200
/K1	172
/K2	142
/K3	171
/C	248

Viable counts In contrast to previous results, samples of α/Si and /K2 both showed extremely heavy contamination. Cannot be explained in comparison with 9H19 where the /K2 sample had almost no contamination. Suggest poor handling technique.

Physical Data

Sample	pH	Conductivity (mMho)	Vol ml	Wt g	Density
α/Si	7.18	21.36	2330	2327	0.99
α9H2O	6.91	1.71	13685	14705	1.07
/Sn	8.16	1.57	15285	13767	0.90
/J	8.25	9.87	3010	3039	1.00
/K1	7.04	9.18	135	132.3	0.98
/K2	7.14	20.65	645	655.4	1.0
/K3	7.13	25.24	970	976.2	1.0
/K4	7.01	25.41	950	967.1	1.0
/C	7.00	45.9	410	418.7	1.0

Comments

1. The factor IX survival of heating is higher than 9H18 but lower than 9H19. This drop may be within variation limits or may be due to the failure of the stirrer to work properly during the heating step.

2. Efforts shall be made to improve the sorbital addition step.

3. The combination of filter and slower running flow rate appears successful; there is no double peak observed in 9H19, nor was there any sign of distortion of the gel surface, once the piston had been removed after the experiment.

4. The drop in NAPTT in parallel with factor IX elution is disturbing. It does not appear in the frontal material but across the whole /K protein peak.

5. The proportion of factor IX activity in /sn and /J is also higher than in previous experiments. This (and possibly the NAPTT distribution) may be due to inappropriate gel loading. If so, it may be moderated by variation of the gel: factor IX loading ratio.

6. For the first time, 100% of the loaded factor IX has been accounted for in the eluate. Also the proportion of factor IX eluted in buffer K has risen to 87.5% — a far more acceptable recovery.

7. Combination of fractions K1, K2 and K3 gives an overall yield for the whole procedure of 55%. Such a pooled product would have an approximate potency of 24 u/ml. The NAPTT may be between 150 - 160 seconds

8. Future experiments should use the slower flow rates and column buffer filter / spreader to overcome the double peak observed in 9H19 (probably due to poor flow properties). Apparently much greater care is needed to maintain clean conditions.
Work also needs to be done on the buffers - see attached buffer sheets. The pH is on target but in this experiment and 9H19, the conductivities appear slightly too low.

9. The low conductivity of buffer J may account for the shift of NAPTT towards a tighter binding component.

9H20.9

Time table

		Cumulative
Softening / Thawing	2 hours	2
Glycine addition	1½ hours	3½
Sorbitol addition	2+hours	5½
Heating	10 hours	15½
Adsorption	1½ hours	17
Packing	1¼ hours	18¼
Apply buffer K	2 hours	20¼
Finish	1 hour	21¼

9H.1 9H2O 9H2O

19/1/82.

FACTOR IX WET HEATING

Starting Material

T = Eluate batch: JD 2982 ∝ 5i Softer at 12.27

Eluate volume: 2330 ml Thawed . 14.10

Eluate weight: 2327 g

pH : 7.18

Conductivity: 21.36 mMho

Sample: 3 x 2ml (X15)
1 x 5ml
2 x 1ml sent for immediate factor IX assay
1ml viable count.

Stabilisers

13.45 1. Add Glycine ~~0.1 g per ml of eluate~~

(equivalent to 0.1 g per g of eluate)

Weight of glycine added: 232.7 g

2. Dissolve by stirring with large magnetic stirrer.

3. Place in 30° water bath with magnetic stirrer underneath.

1500 4. Add sorbitol ~~2 g per ml of eluate~~

(equivalent to 2 g per g of eluate)

Weight of sorbitol added: 4654 g

NB Stirring is pathetic must improve solution Time + effort

3. Stir until fully dissolved, monitoring temperature (>20°c)

— . 4. Sample 3 x 2ml (1S)

5. Volume: 5869 ml 20.7 cm high

Weight: 7297 g

Polycarbonate bottle : polycarbonate

Diameter : ~~16cm~~ 19 ?

∴ Volume of H_2O : $64 \times \pi \times 19.3$

$\approx$ ~~3880~~ ml. 5472.1 ml

~~5925~~ ?

9H2C
9H.2

Heating

1. Place mixture in water bath to heat.
2. Set water bath to switch on at 8.30 pm
3. ~~Set chart recorder to switch on at 9.00 pm~~
4. ~~(Set magnetic stirrer to switch on at 9.00 pm.)~~
5. Set water bath to heat up to 60°C
6. Remove from water bath after 10 hours at 60°C (approx: ~~8.00 - 8.30~~ 8.30 - 9.00 am.)

8-53
NB Timer alarm on Molyteb didn't work,

7. Sample 3 x 2ml (9H20)
   and 4 x 50 ml into plastic 60ml pots.
8. Volume: _____ ml
9. Weight: ~~72965~~ g

Bottle weight = 20cm.
Bottle weight after sampling 19.3 cm.
Weight before samples: 72965
Weight after sampling: 7017 g.

Dilution

1. Dilute by ~~2~~ 3 by addition of 2x9H volumes of PFW.
2. Volume PFW 7760. ml
   Weight PFW 7760. g
3. Sample 3 x 2ml
   1 x 5ml (∝ 9H: ___)
4. ~~Volume~~ Volume 13685 ml Approx: (36 x 11 x 11 x ~~11~~)n
   Weight 14,705 g
   Conductivity: 1.71 mMho 17,~~2~~00 g.
   pH: 6.91
5. ~~Dispatch 7l and ∝ 9H to factor IX assay~~
6. Factor IX activity: 31.5 u/ml in 15l

∴ Total factor IX units: 73395

17 200
2 595
14,~~8~~05

Result of 9H19 suggested no difference in capacity between 150 u/g DE52 and 160 u/g DE52.

∴ Use an effective loading of 160 u/g DE-52.

Assuming 77% survival of factor IX after heating, this can be related back to the activity of the starting material (15i)

Amount of DE52 to add is therefor

$$\left(\frac{\text{Total IX units in 15i}}{160 \times \frac{100}{77}}\right)$$

$$\equiv \frac{\text{15i factor IX activity} \times \text{volume 15i} \times 77}{160 \times 100}$$

$$\equiv (\text{15i activity in u/ml}) \times (\text{15i volume}) \times 0.0048125$$

Sn vol: 2010
+2000
+2000
+2015
+1960
+2000
+300
15285

9Hcc
9H.3

Adsorption

10.50 1. Add DE-52, 1 g ~~dry~~ weight ~~per 150 factor IX unit~~ 210 ~~207.8~~ units factor IX in 15 c

Wt DE-52 : 349.5 g

2. Stir for 1 hour

using Edmonds magnetic stirrer and oval flea at 250 rpm.

Recovery of DE-52

12.15

a) 1. Pour slurry into GAC110 column with extender tube attached.

~~2. Fit pressure head. Pack under N2 pressure~~ at pump ~~from~~ with Watson Marlow pump.

Flow rate ~ 210 ml/min.

b). 1. Spin down in Beckman centrifuges at 3,000 rpm for 20 minutes.

2. Decant supernatant

3. Resuspend in 1.5 x weight of dry DE 52 volumes of buffer J

Volume of buffer J _____ ml

Weight of buffer J _____ g

4. Pack into GAC 110 column.

Packed by 1.30

Volume of supernatant: 15,285 ml

Weight of supernatant 13,767 ~~13,767~~ g

PH 8.16

Conductivity 1.57 mMho (1 Sn)

Sample : 3 x 2 ml
1 x 5 ml

9H.4 7H20

Elution

Bed height : ~8 cm

GAC 110 Bed volume : ~760 ml

Insert AP25 No 1 between metal filter & plugs.

1. ~~Wash with 5 bed volumes of buffer J~~

Volume buffer J : 5000 ml

Weight buffer J : 5000 g

Slope on A and T parallels elution of various solution into bucket.

2. Measure flow rate : 100 ml/min.

Monitor u.v, ~~refration~~ conductivity.

3. Collect samples by change in u.v. Transmission

3. Collect first peak ~~in two tubes (front peak J1 and rear peak J2)~~

Sample 2 x 2 ml
1 x 5 ml

/J1 Volume 3010 ml
Weight 3039 g
pH 8.25
Conductivity 9.87 mMho

/J2 Volume _____ ml
Weight _____ g
pH _____
Conductivity _____ mMho.

4. Wash with 5 bed volumes buffer K

.5e3 Volume buffer K : _____ ml

Weight buffer K : _____ g

9H7H2

5. Collect eluting peak in three parts:

Protein front /K1
Protein middle /K2
Protein tail /K3

Sample each 2 x 2ml
1 x 5 ml

/K1
Volume 13.5 ml
Weight 132.3 g
pH 7.04
Conductivity 9.18 mMho

/K2
Volume 645 ml
Weight 655.4 g
pH 7.14
Conductivity 20.65 mMho

/K4 Vol: 950 ml
Wt: 967.1 g
pH 7.01
Cond 25.41
(300 stored first aliquot)

/K3
Volume 970 ml
Weight 976.2 g
pH 7.13
Conductivity 25.24 mMho

6. Wash with 3 bed volumes buffer C

Volume buffer C _____ ml

Weight buffer C _____ g

7. Collect any protein peak that elutes

Sample 2 x 2ml (/C)
1 x 5ml

Dilute by 1/10 for IX assay.

/C
Volume 410 ml
Weight 418.7 g
pH 7.00
Conductivity 45.9 mMho

Date _____ **Buffer J for 9H20**

9H2e

Find concentrations

8.5 mM trisodium citrate
1.5 mM citric acid
10 mM Na_2HPO_4
80 mM NaCl

To Make up 5 L (5kg)

Weight		Batch No.	Quantity used
12.5g	trisodium citrate	92937001751	12.5
1.55g	citric acid	5816300B	1.55
7.1g	Na_2HPO_4	9210374061-5	7.1
23.35g	NaCl	9357687-1489	23.3

Make up to 5 L (5kg) with PFW (5008g).

pH limit: 7.00 ± 0.05 pH found 6.99

Adjust as necessary

Conductivity limit: 11.0 ± 1.0 Conductivity found 9.09

OK PAF

YH2e

Date _____ Buffer K for 9H20

Final concentrations

8.9 mM trisodium citrate
1.1 mM citric acid
10 mM Na_2HPO_4
300 mM NaCl

To Make up ~~8~~ 7 L (~~8~~ 7 kg)

Weight		Batch No	Quantity used
18.34 ~~20.96~~ g	trisodium citrate	92937007-36	18.34
1.61 ~~1.84~~ g	citric acid	5916300 B	1.62
9.94 ~~11.36~~ g	Na_2HPO_4	9260374 D	9.94
122.7 ~~140.2~~ g	NaCl.	9357687 D	122.71

Make up to ~~8~~ L (~~8~~ kg) with PFW 7002 g

pH limit: 7.00 ± 0.05 pH found 6.94

Adjust as necessary

Conductivity limit: 29.5 ± 1.0 Conductivity found 25.8

OK PNF

9H20

Date 20/1/84 Buffer C for 9H 20.

Final concentrations

20 mM trisodium citrate

1.0 M NaCl

To make up 3 L (3 Kg)

Weight		Batch No	Quantity used
17.7 g	trisodium citrate		
175.3 g	NaCl		

Make up to 3L (3 kg) with PFW 3028.9 g

pH limit: 8.0 ± 1.0 pH found 7.05

Adjust if necessary

Conductivity limit: 80 ± 10 Conductivity found

9A20

BACTERIAL CONTAMINATION : VIABLE COUNTS

File Reference: _____ Product: _____

Authorised by: [signature] Processing Run: α 9A 20 / [illegible] R+D

Date effective: 21.4.83 This page started: 25.1.84

PRODUCTION STAFF			QUALITY CONTROL STAFF			
Sample Stage	Volume to be taken	Sample taken by:	SAL	Day 1 at °C	Day 2 at °C	∴ CFU/ml
				21	21 spreaders	Heavy contamination
			Control	0		0

EXTRA SAMPLES as requested by Scientist i/c Production or QC staff:

			Control			

COMMENTS by sampling, testing or reading staff:

INTERPRETATION by Scientist i/c Production, and any action taken:

COMMENTS by Quality Controller:

YH2c

BACTERIAL CONTAMINATION : VIABLE COUNTS

File Reference: _____ Product: R+D 9D2982

Authorised by: [signature] Processing Run: 9D 2982/5'

Date effective: 21.4.83 This page started: 20·1·84

PRODUCTION STAFF			QUALITY CONTROL STAFF			
Sample Stage	Volume to be taken	Sample taken by:	SAL	Day 1 at °C	Day 2 at °C	.'. CFU/ ml
5'	1ml					Heavy Growth
			Control			

EXTRA SAMPLES as requested by Scientist i/c Production or QC staff:

			Control			

COMMENTS by sampling, testing or reading staff:

INTERPRETATION by Scientist i/c Production, and any action taken:

COMMENTS by Quality Controller:

9N20

ASSAY REQUEST FORM (1)

Request from: PAF/DC Date: 19/1/84

When results needed: By 9.00 am Friday.

Samples to be kept? No If so, how?

Samples provided with form/~~available from~~

SAMPLE INVESTIGATION		UD2982 α/5i					
Factor VIII, iu/ml	1 st.						
	2 st.						
Factor IX, iu/ml	1 st.	(35)					
	2 st.	31.5					
Factor II, u/ml							
Factor X, u/ml							
Factor VII, u/ml (clotting)							
AT III (Anti Xa), u/ml							
AT III (Anti IIa), u/ml							
AT III (Laurell), u/ml							
Factor VIII RAG, u/ml							
Fibrinogen (Laurell), mg/ml							
Prothrombin (Laurell), u/ml							
Factor XIIIA (Laurell), u/ml							
Fibronectin (Laurell), mg/ml							
Factor VIII CAG							
NAPTT sec. 1/10							
TGt50 min.							
A hr.							
Limulus + or -							

9 Nec

ASSAY REQUEST FORM (1)

Request from: PAF Date: 24/1/14

When results needed: ASAP

Samples to be kept? No If so, how?

Samples ~~provided with form~~/available from R+D Big Freezer

SAMPLE INVESTIGATION		JD29S2X 15i	9H20	X9H20	1Sn	1J	1K1
Factor VIII, iu/ml	1 st.						
	2 st.						
✓ Factor IX, iu/ml	1 st.	(31.5)	8.7	(2.9)	(<0.05)	(0.5)	(22)
	2 st.	326	9.3	3.5	0.2	1.4	67.6
Factor II, u/ml							
Factor X, u/ml							
Factor VII, u/ml (clotting)							
AT III (Anti Xa), u/ml							
AT III (Anti IIa), u/ml							
AT III (Laurell), u/ml							
Factor VIII RAG, u/ml							
Fibrinogen (Laurell), mg/ml							
Prothrombin (Laurell), u/ml							
Factor XIIIA (Laurell), u/ml							
Fibronectin (Laurell), mg/ml							
Factor VIII CAG							
✓ NAPTT sec. 1/10		213	215	229	241	200	172
TGt50 min.							
)A hr.							
Limulus + or -							

9H20

ASSAY REQUEST FORM (1)

Request from: PAF

Date: 24.1.84

When results needed: ASAP

Samples to be kept? No

If so, how?

mples ~~provided with form~~/available from K+D Big Freeze.

SAMPLE INVESTIGATION		/K2	/K3	/C			
Factor VIII, iu/ml	1 st.						
	2 st.						
Factor IX, iu/ml	1 st.	(35)	(7)	(<1)			
	2 st.	47.9	2.0	0.4			
Factor II, u/ml							
Factor X, u/ml							
Factor VII, u/ml (clotting)							
AT III (Anti Xa), u/ml							
AT III (Anti IIa), u/ml							
AT III (Laurell), u/ml							
Factor VIII RAG, u/ml							
Fibrinogen (Laurell), mg/ml							
Prothrombin (Laurell), u/ml							
Factor XIIIA (Laurell), u/ml							
Fibronectin (Laurell), mg/ml							
Factor VIII CAG							
NAPTT sec. 1/10		142	171	~248	mean	control 237	
TGt50 min.							
FDP hr.							
Limulus + or -							

23.1.84 9H20.02 To determine essential dilution of 9H to enable binding to DE-52.

Preliminary experiments (9H6) showed that dilution of sorbitol-containing, heated factor IX resulted in better adsorption characteristics to DE-52. However these experiments involved lower sorbitol concentrations than are now used. So far, the dilution has been extrapolated, so that the diluted (x9H) material has the same sorbitol concentration as in 9H6. This requires a three-fold dilution with the complications of talk handling.

This dilution factor may not be necessary, since the adsorption characteristics are more likely to be due to overall viscosity and ease of mixing than to absolute sorbitol concentration.

This experiment seeks to clarify the requirement for dilution of these high sorbitol-containing solutions.

Materials

Heated factor IX: 200 ml (4 x 50 ml) of 9H2x heated factor IX were extracted from that experiment. The source material was JD2982 x/5i and the heat treatment was 10 hrs at 60°c. The sorbitol had been added at 2g / 1g factor IX (2g / 1.99 ml factor IX).

Buffers: Buffer J
8.5 mM trisodium citrate
1.5 mM citric acid
10 mM Na_2HPO_4
80 mM NaCl
pH 6.95

Buffer K
8.9 mM trisodium citrate
1.1 mM citric acid
10 mM Na_2HPO_4
300 mM NaCl
pH 6.94

Method The four 9H20 samples were thawed, pooled and sampled (9H20.02)

This was then divided into 4 x 50 aliquots and diluted to varying amounts in PFW

Sample	Ratio of 9H20 : PFW	Vol 9H20 (ml)	Vol PFW (ml)
A	1 : 0.5	50	25
B	1 : 1.0	50	50
C	1 : 1.5	50	75
D	1 : 2	50	100

The aliquots were stored at 4°c until mixed with DE 52.

Each was sampled (2×0.5 ml)

Loading of DE52

This was calculated by the following principles:

1. 200ml 9H20 weighs 258g
2. Assume total volume of bulk 9H20 ~ 5500 ml
3. Equivalent units of α/5i starting factor IX in the 200 ml sample = $73395 \times \frac{200}{5500} = 2668.9$ units

(based on assay result for α/5i)

4. 50 ml sample contains 667.2 units
5. Load at 210 equivalent IX units / g DE 52
   ie 3.2g DE 52 per sample.

Each sample was mixed with 3.2g DE-52 for 1 hour at room temperature.

The gel was spun down at 2,000 rpm in MSE Mistral centrifuge for 15 minutes at 15°c.

The supernatant was decanted and sampled "/Sn"

The gel was resuspended in 7 column volumes of buffer J and packed into 1.4 cm I.D. glass column, under gravity.

The eluate was collected and sampled "/J"

In each case the bed volume was 6 ml ± 0.1 so 42 ml of buffer were used.

42 ml of buffer K were then applied and the eluate collected and sampled (/K)

The flow in each case, under gravity, increased as the initial dilution increased i.e. A → D.

In between each sample, the column was emptied and thoroughly washed with water, PFW and more PFW.

Assays To economise ~~of~~ on assays only the starting 9H20 and the /K samples were assayed, for factor IX activity. The remaining samples were stored, just in case no activity at all could be identified in the /K samples.

Results

Sample	Volume (ml)	Factor IX u/ml	Factor IX total units	% factor IX of starting material
9H20-02	49*	8.7	426.3	100
A/K	42	7.6	319.2	74.9
B/K	41.5	8.5	352.7	82.7
C/K	42	8.0	336	78.8
D/K	42	8.2	344~~4~~	80.8

Conclusions

1. There is little difference in the adsorption/elution properties of 9H20 between 1:1 and 1:2 dilution with PFW.

2. Below 1:1 dilution, some loss of factor IX from /K does appear

* corrected for each sample + 1 ml removed for assay.

9H20-02.4

3. The approximate 80% yield is directly comparable to the combined yield of /K1 - /K3 in 9H20 on the large scale. This is an encouraging correlation, though in both cases the assumed loading of 210 u/g DE 52 gives an absolute loading for the α 9H20 sample of 133 u/g DE 52 rather than 160 u/g DE 52.

4. It therefore seems unnecessary to continue with the (1+2) dilution in PFW during future 9H experiments.
It appears that a 1:1 dilution is sufficient not to impede adsorption to DE 52.

5. A further experiment to determine optimum loading of such a 1:1 diluted 9H fraction is advisable.

6. This result does support viscosity as the limiting property during adsorption, rather than absolute sorbitol concentration. This is because the volume change is not linearly dependent upon sorbitol concentration, therefore neither is the viscosity.

P. A. F.

ASSAY REQUEST FORM (1)

Request from: PAF Date: 23 1 84

When results needed: if poss 27 1 84

Samples to be kept? no If so, how?

Samples ~~provided with~~ form/available from R+D Big Freezer

SAMPLE 9H20.02 'ESTIGATION		9H20-02 *	A/K	B/K	C/K	D/K	
Factor VIII, iu/ml	1 st.						
	2 st.						
✓ Factor IX, iu/ml	1 st.	(8.5)	(3.5)	(4.5)	(6)	(7)	
	2 st.	8.7	7.6	8.5	8.0	8.2	
Factor II, u/ml							
ctor X, u/ml							
Factor VII, u/ml (clotting)							
AT III (Anti Xa), u/ml							
AT III (Anti IIa), u/ml							
AT III (Laurell), u/ml							
Factor VIII RAG, u/ml							
Fibrinogen (Laurell), mg/ml							
Prothrombin (Laurell), u/ml							
ctor XIIIA (Laurell), u/ml							
Fibronectin (Laurell), mg/ml							
Factor VIII CAG							
NAPTT sec. 1/10							
TGt50 min.							
F?? hr.							
Limulus + or -							

* High Sxbtn.

2.2.84. 9H20-03 The Effect of mixing long- and short-NAPTT fractions from 9H20

The NAPTT from 9H20 fractions showed a distribution around that of the starting material. Immediately after heating there was no change but the supernatant time was lengthened and the factor IX peak was shortened.

The purpose of this experiment is to determine whether there is a component which is removed which effectively lengthens an otherwise short NAPTT.

Method

Samples taken from 9H20 fractions were thawed from storage at −40°c. These sampled had not been thawed previously.

To each of /K1, /K2 and /K3 was added /Sn and /J.

On the principle that all the parts should make up the whole, these were mixed in the same ratio as the total fraction volume.

To check whether any result was due to dilution and not "inhibition", controls using equivalent volume of PFW in place of /Sn and /J were included.

K1 : Sn	1 : 113
K1 : PFW	1 : 113
K2 : Sn	1 : 24
K2 : PFW	1 : 24
K3 : Sn	1 : 16
K3 : PFW	1 : 16
K1 : J	1 : 22
K2 : J	1 : 5
K2 : PFW	1 : 5
K3 : J	1 : 3
K3 : PFW	1 : 3

Results

Sample	NAPTT t_{10} (s)	Δ NAPTT
K1 + Sn	181	− 42
K1 + PFW	223	
K2 + Sn	227	+ 13
K2 + PFW	214	
K3 + Sn	221	+ 27
K3 + PFW	194	
K1 + J	201	
K2 + J	183	+ 39
K2 + PFW	144	
K3 + J	193	− 30
K3 + PFW	223	
Sn	213	
J	206	
Mean Blank	233	
From 9H20		
α Si	213	
/Sn	241	
/J	200	
/K1	172	
/K2	142	
/K3	171	
Mean blank	237	

Comments

These results are not very significant. The variation in the J and Sn values since the original 9H20 assays throw doubt on any reproducability.

There is a temptation to put greater weight on

9N20-03.3

changes of shorter NAPTT (ie. K2 +J over K2 + PFW), but there is not sufficient data to support such an attitude.

All that can be said of this experiment is that it exemplifies the need for sorting out the whole question of NAPTT, their validity and their reliability.

In spite of the /K fractions looking much like the starting material on SDS PAGE (9H19) it does seem safe to say that it is being modified in some way to shorten the NAPTT, whatever that is a reflection of.

P.A.F.

9N20-03

ASSAY REQUEST FORM (1)

Request from: PAF Date: 2/2/84

When results needed: 16/2/84

Samples to be kept? No If so, how?

Samples ~~provided with form~~/available from R+D Big Freeze

SAMPLE 9N20-03 INVESTIGATION		K1+J	K2+J	K3+J	K2+PFW B	K3+PFW B	Sn
Factor VIII, iu/ml	1 st.						
	2 st.						
Factor IX, iu/ml	1 st.						
	2 st.						
Factor II, u/ml							
Factor X, u/ml							
Factor VII, u/ml (clotting)							
AT III (Anti Xa), u/ml							
AT III (Anti IIa), u/ml							
AT III (Laurell), u/ml							
Factor VIII RAG, u/ml							
Fibrinogen (Laurell), mg/ml							
Prothrombin (Laurell), u/ml							
Factor XIIIA (Laurell), u/ml							
Fibronectin (Laurell), mg/ml							
Factor VIII CAG							
√ NAPTT sec. 1/10		201	183	193	144	223	213
TGt50 min.							
A hr.							
Limulus + or -							

ASSAY REQUEST FORM (1)

Request from: PAF Date: 2/2/84

When results needed: 16/2/84

Samples to be kept? No If so, how?

Samples ~~provided with form~~/available from R+D Big Freezer

SAMPLE 9H20-03 INVESTIGATION		K1+PFW A	K2+PFW A	K3+PFW A	K1+Sn	K2+Sn	K3+Sn
Factor VIII, iu/ml	1 st.						
	2 st.						
Factor IX, iu/ml	1 st.						
	2 st.						
Factor II, u/ml							
Factor X, u/ml							
Factor VII, u/ml (clotting)							
AT III (Anti Xa), u/ml							
AT III (Anti IIa), u/ml							
AT III (Laurell), u/ml							
Factor VIII RAG, u/ml							
Fibrinogen (Laurell), mg/ml							
Prothrombin (Laurell), u/ml							
Factor XIIIA (Laurell), u/ml							
Fibronectin (Laurell), mg/ml							
Factor VIII CAG							
NAPTT sec. 1/10		223	214	194	181	227	221
TGt50 min.							
4A hr.							
Limulus + or -							

7 NCC-6

ASSAY REQUEST FORM (1)

Request from: PAF Date: 2/2/84

When results needed: 16/2/84

Samples to be kept? No If so, how?

Samples provided with form/available from

SAMPLE 9N20-03 INVESTIGATION		J					
Factor VIII, iu/ml	1 st.						
	2 st.						
Factor IX, iu/ml	1 st.						
	2 st.						
Factor II, u/ml							
Factor X, u/ml							
Factor VII, u/ml (clotting)							
AT III (Anti Xa), u/ml							
AT III (Anti IIa), u/ml							
AT III (Laurell), u/ml							
Factor VIII RAG, u/ml							
Fibrinogen (Laurell), mg/ml							
Prothrombin (Laurell), u/ml							
Factor XIIIA (Laurell), u/ml							
Fibronectin (Laurell), mg/ml							
Factor VIII CAG							
√NAPTT sec. 1/10		206		mean	Blank	233	
TGt50 min.							
A hr.							
Limulus + or -							

9H21 26.1.84

Large Scale Pasteurization of FIX and its recovery by chromatography using DE52 (DCL).

To test the reproducibility of our methods and results, and to examine the effect of our methods on FIX with a short NAPTT.

Materials

Two FIX batches produced in PFL and which had short NAPTT were pooled as follows :-

9P1814 /5iA – 5iC (920ml) } 9P1814-15/5iPool(1735ml)
9P1815 /5iA – 5iC (815ml) }

The FIX batches were stored frozen at –40°C immediately after their preparation.

The buffers J, K, and C were made up fresh for use. Their contents and parameters are recorded on the three buffer sheets following this page.

The sorbitol was from BDH and was completely dry and free flowing when used. The Glycine was also from BDH and it was checked by Q.C. for production use.

Methods.

Thawing – The /5iA to /5iC portions of the FIX batches 9P1814 and 9P1815 were removed from the –40°C freezer and left at room temperature (≈18°C) for approximately 1 hour. After this softening step, they were thawed in water at 30–40°C. The portions in each separate batch were mixed and the individual /5 batch was sampled (2×2ml from each). It was noted that each batch was cloudy.
The batches were mixed to produce

Date 27.1.84 Buffer J for 9H 2

Find concentrations

8.5 mM trisodium citrate
1.5 mM citric acid
10 mM Na_2HPO_4
80 mM NaCl

To Make up 5 L (5 kg)

Weight		Batch No.	Quantity used
12.5 g	trisodium citrate	9293708-1737	12.5 g
1.55 g	citric acid	5816300B	1.55 g
7.1 g	Na_2HPO_4	9260374D61-S	7.1 g
23.35 g	NaCl	9357687-H.89	23.35 g

Weighed into 1 pot 26/1/84
Make up to 5 L (5 kg) with PFW

pH limit: 7.00 ± 0.05 pH found 6.93
Adjust as necessary
Conductivity limit: 11.0 ± 1.0 Conductivity found 9.09

16

Date 27.1.84 Buffer K for 9H 2-1

Final concentrations

8.9 mM trisodium citrate
1.1 mM citric acid
10 mM Na_2HPO_4
300 mM NaCl

To Make up 8 L (8 Kg)

Weight		Batch No	Quantity used
20.96g	trisodium citrate	9293700D	20.96
1.84g	citric acid	~~5~~816300B	1.84
11.36g	Na_2HPO_4	026037-061S ~~5870300B~~	11.37
140.2g	NaCl.	935768749	2x70.1g

Weighed into 3 pots 26/1/84
Make up to 8 L (8 Kg) with PFW

pH limit: 7.00 ± 0.05 pH found 6.94

Adjust as necessary

Conductivity limit: 29.5 ± 1.0 Conductivity found 27.82

Date 27.1.84 Buffer C for 9H 21 IC

Final concentrations

20 mM trisodium citrate

1.0 M NaCl

To make up 3 L (3 Kg)

Weight		Batch No	Quantity used
17.7 g	trisodium citrate	293700-17-37	17.7 g
175.3 g	NaCl	9357687-14-89	175.3 g

Made up in 1 400 ml beaker.

Make up to 3L (3 Kg) with PFW

pH limit: 8.0 ± 1.0 pH found 7.80

Adjust if necessary

Conductivity limit: 80 ± 10 Conductivity found 70.54

9H21

1735ml of 9P1814-15/5pool . This was sampled as shown below, and the PH and conductivity wee measured. The weight of the 15pool solution was found to be 1742g.

Sampling of 9P1814-15 .

2 x 1ml (sent for immediate analysis) .
3 x 2ml
1 x 5ml

Addition of Glycine and Sorbitol

The FIX solution was poured into a 10L polycarbonate bottle and glycine was added to give 0.1g of glycine to 1g of the FIX solution. 174.2g of glycine was added slowly and dissolved.

The solution was transfered to a 35°C waterbath and warmed to 20.0°C while being stired with a magnetic stirrer. 3484g sorbitol (2g sorbitol/1g of 15pool solution) were added slowly over 1hour and dissolved producing "~/S".

Pasteurization 10hours at 60°C

The ~/s solution was left stirring in the waterbath. The waterbath heater was set to be switched on automatically so the the end of the pasteurization would be at approx 9.00am. The temperature control setting was on 61°C.

2 temperature probes and a chart recorder were set up to monitor the temperature of the water bath and the sample. The bath and contents were then covered with Al-foil and left overnight.

The pasteurization did not occur as planned. The FIX solute took 3¼ hours to reach 60°C (only 1½ hours were allowed for this); the chart paper jammed after about 3¼ hours of heating and the temperature of the bath dropped to 56.9°C while that of the FIX solution dropped to 56.5°C. The length of time

which the FIX solution spend at 56·5°C could not be determined because of the jammed recorder paper. The temperature of the bath was reset to 61°C at 8·48am and when the temperature of the FIX solution reached 60° (at 9.00am) the pasteurization was continued for a further 1½hours. Hence the sample was kept at 56·5°C to 60°C for 11½ hours.

The heating record is included following this page.

DE 52 Absorption and Elution.

The pasteurized FIX soution 9H21 was diluted with and equal volume of cooled PFW (4454 ml were used instead of the calculated 4423 ml). The resulting ∝9H21 was sampled (3×2ml and 1×5ml) weighed and its volume pH and conductivity measured.

Volume	=	
Weight	=	9836g
Conductivity	=	1·53mMho
pH	=	6·98

The results of the FIX analysis in 9P1814-15/5i pool gave 46·4 iu FIX/ml. Hence we had a total of 80504 iu FIX in the pool.

383·1g of DE52 were added to the ∝9H21 (1g per 210 iu FIX in the original 5i Pool) and the mixture stirred for 1 hour at room temperature using a magnetic stirer. N.B. The mixture became very frothy during the stirring period.

After stirring the mixture was poured in the GAC110 column with extension piece. The supernatant was pumped from the bottom of the column using a Watson Marlow 501S pump set on 60%. When the gel was packed and ≈3cm of supernatant were left above its surface, the column effluent tube was sealed; the pump removed, the extension piece removed, and the column was connected in the standard way to monitor and record refractive index,

2a
9H2 Heating record.
Water bath
Fix solution
32.9
33.1
9.30
21.8 cm
3 cm/hr
7.3 hr
9.5 hrs
3.16 hrs
12.40
Moore's Modern Methods Ltd., London EC4M 4YD
To repeat order state Form H.R. Feint, Size 13"x 8"

9H21

absorbance, transmittance and conductivity of the eluent.
The supernatant was collected, and its parameters were measured (see following list).

Vol	=	8743 ml
Weight	=	9180 g
Conductivity	=	1·34 mMHo
pH	=	8·30

3 × 2ml and 1 × 5ml samples were removed for analysis and a 300ml portion retained.

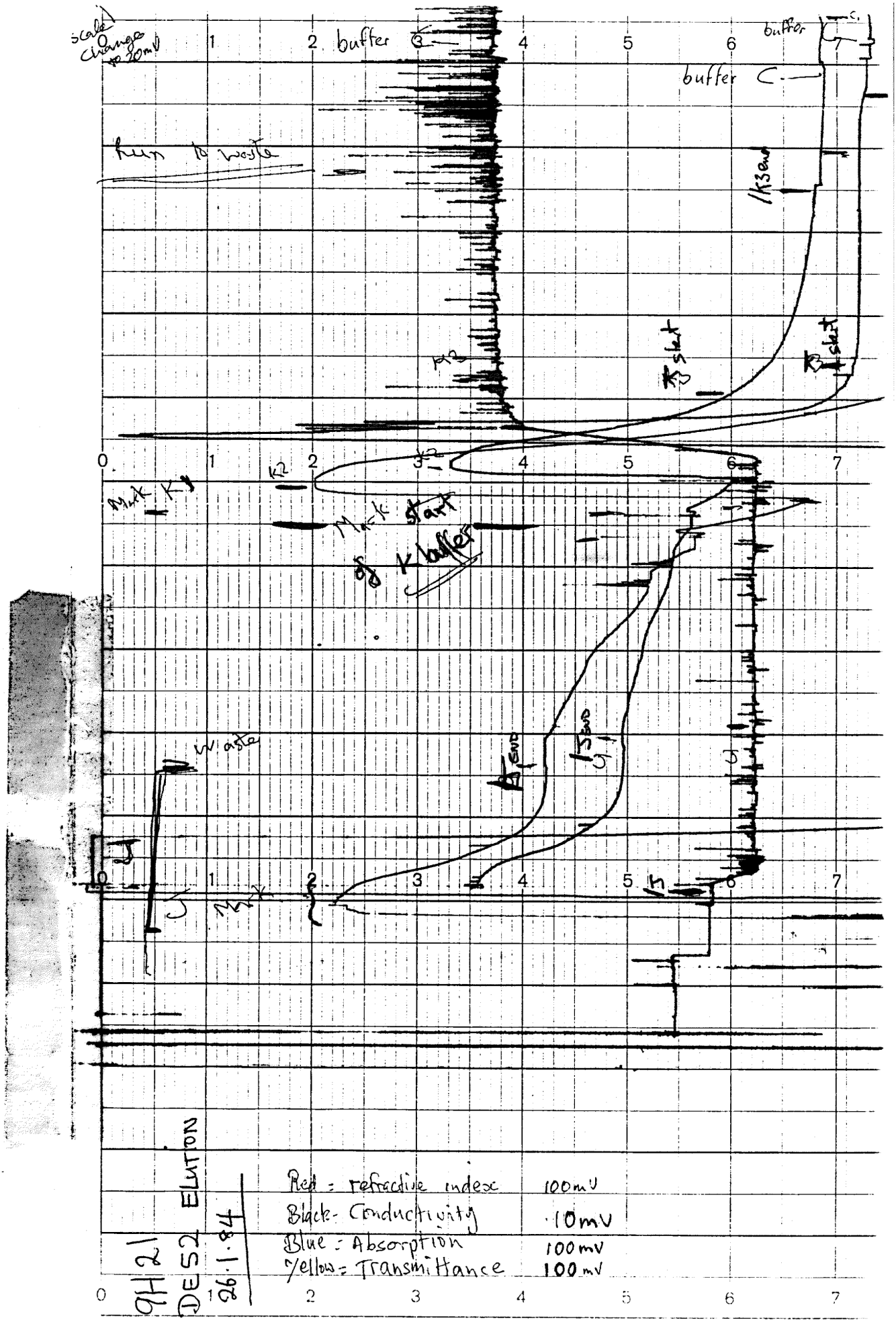
The piston was added to the column in the usual way and the gel was washed with buffer J using Nitrogen gas pressure at 1 lb/sq in. On application of buffer J, the gel bed height was 6 cm and hence its volume was 570 ml. The flowrate was 130ml/minute and 5 × the bed volume of buffer J were used (2851 ml). One aliquot of the buffer J wash (J1) was collected its parameters were measured and it was sampled (2 × 2ml, 1 × 5ml and a 300ml portion of it was retained)

After buffer J the column was washed in a similar way with buffer K. 5940g ie ≈ 10·5 × the gel bed volume of buffer K were used and three aliquots of the eluate were collected. The parameters of these aliquots were measured and they were sampled (2 × 2ml and 1 × 5ml). All of the first two aliquots (K_1 and K_2) were retained but only 600ml of the third (K_3)

Following the buffer J wash buffer C was applied to the column in the same way. One 550ml aliquot was collected

The points of collection of the eluents and aliquots of them are indicated on the elution profile included after this page.

The column was allowed to flow dry and the DES retained to be recycled.



9H21
DES2 ELUTION
26.1.84

Red = refractive index 100mV
Black = Conductivity 10mV
Blue = Absorption 100mV
Yellow = Transmittance 100mV

(5)

9H21

9H21

Parameters of aliquots collected During Buffers J, K and C washes

Buffer/aliquot N°	Vol(ml)	Weight(g)	PH	Conductivity (mMHo)
J_1	2475	2516	8.38	6.56
K1	144	145	7.03	9.51
K2	1020	1053	7.07	21.31
K3	2955	2980	7.02	22.62
C1	550	567	7.03	55.74

9H21 Results and Discussion

Methodology

The rate of addition of the sorbital was regulated so that the temperature of the mixture did not fall below 19°C and that no large lumps formed. It took 1 hour to add and dissolve the sorbital with moderate stirring and this probably represents the shortest time in which 3.0kg of sorbital may be dealt with.

The failure to bring the temperature of the FIX to 60°C within 1½ hours and the fall in temperature to 56.5°C were due to there being too little water in the waterbath and inadequate covering of the bath. The very slow rise in the temperature of the FIX solution may also be due to inefficient stirring and consequent slow transfer of heat into the mixture

The use of a diffusor and careful control of the Nitrogen pressure prevented the formation of a hole in the top of the gel bed.

Buffer K elution produced a yellowish-brown band which remained well defined until it had moved about 4½ cm through the gel bed. After this it quickly diffused.

Analysis of the Samples.

There is a significant discrepancy between the results of the first and second FIX analysis in 9P1814-15/5ip (68.2 and 46.4). The loading of the gel was based on the lower figure. This would give 1g of DCL to 210 ml FIX in the 5i pool; however if the higher figure is used, we would have 1g of DCL to 309 ml FIX in the 5i pool. Our choice of 1g DCL/210 ml FIX reflected the loading capacity of the gel which was found in previous experiments (9H20). 1g DCL/309 ml FIX would probably lead to overloading of the gel which would in turn lead to high FIX levels in the supernatant. We do not have a

9H21

Results and Discussion Cont.

significant amount of FIX, in the DE52 supernatant (< 0·01 iu/ml (< 87 units total). The results have been worked out in terms of the higher and the lower figures (SEE THE FOLLOWING TABLE)

Sample summary Data

	Sample	Vol (ml)	PROTEIN A280 (≈ mg/ml)	FIX units /ml	FIX units Total	Sp Act. FIX units / A_{280} units	% of STARTING FIX	NAPTT (sec)	PH	Cond. MS
2nd	9P1814-15/5.P	1735	15·6	68·2	118327	4·37	100	120	7·38	15·0
1st	9P1814-15/5.P	1735	15·6	46·4	80504	2·97	68 (100)	–		
	9H21	4423	–	10·8	47768		40·36 (59·3)	132		
	α9H21	9836	6·37	5·5	54098	0·86	45·72 (67·2)	106	6·98	1·53
	/Sn	8743	1·21	<0·01	<87	0·008	0·07 (<0·11)	220	8·30	1·34
	/J	2475	6·3	5·1	12622	0·80	10·66 (15·6)	>420	8·38	6·56
	/K1	144	34·8	63·9	9202	1·83	7·78 (11·4)	88	7·03	9·51
	/K2	1020	11·3	27·3	27046	2·37	23·53 (33·6)	64	7·07	21·31
	/K3	2955	0·41	0·2	591	0·48	0·49 (0·73)	128	7·02	22·62
	/C		0·14					–		
	NAPTT BLANK		.					233		

Moore's Modern Methods Ltd., London EC4M 4YD
To repeat order state Form, H.B. Faint, Size 13"x 8"

Figures in brackets are derived by using 46·4 iu FIX/ml as the starting value 9P1814-15/5.P.

Total starting units of FIX = 118327 (80504)
Total units of FIX in /Sn to K3 = 49548

∴ % of FIX units recovered in Sn to K3 = 41·9% (61·5%)

≈ 50933 units FIX were in "9H21/α9H21" after pasteurization. This is 43·04% (63·2%) of the starting material and it is close to the total FIX units recovered in /Sn to K3.

The column step allows 97% of the units from the heating step to be accounted for.

Generally the overall pattern of the procedure and results are similar to those of our previous 9H large scale experiments. However, the recovery of the FIX after the heating is significantly lower than the highest obtained in previous experiments.

9H21

Results and Discussion Cont.

The present figures for post heating recovery are 41·9% or 61·5% compared with 73% to 77% obtained in 9H19, 9H20. A low yield on the heating stage gave rise to the low recovery of FIX after the column step. Better control of the heating stage is required.

The distribution of the FIX in the column eluates shows that we had not exceeded the binding capacity of the column. Buffer K was effective in eluting all the bound FIX, but some of the FIX (10·66% or 15·6%) was eluted by buffer J. This is the usual pattern in the column elution, but the ≈10% of the FIX in the buffer J elution is significant and steps (eg a reduction in the salt in J) should be taken to avoid it.

The specific activity of the FIX in the eluates does not rise above that of the starting material. Hence the FIX has not been purified by the overall process, but since the sp. act in K2 approaches that of the starting material there must be an enrichment of FIX in this fraction. This becomes clear when we consider that FIX activity was lost on heating.

The A280 reading for 9H21 does not fit in with the known dilution of the 9P1814-15/5ip. It is either the reading for 9H21 or the sorbitol has given the sample too high an absorbance.

The NAPTT profile suggests that buffer J eluted an inhibitor of NAPTT which gave rise to a NAPTT of >1420 sec in /J. However this NAPTT prolonging effect does not appear to "tail off" in /K1 which has a NAPTT of 88 sec. Neither sorbitol or salts concentration appear to prolong the NAPTT.

Low NAPTT is associated with the FIX in the K eluates. The more FIX we have the lower the NAPTT (/K1 - 88 sec, /K2 - 64 /K3 - 128 sec), but in /J we have more FIX iu/ml than in /K3 (5·1 vs 0·2) and yet it has the longest NAPTT. Hence the NAPTT does not follow the pattern of iu/ml of FIX.

The NAPTT profile in this experiment should be

9H21

Results and Discussion cont.

Compared with those of previous 9H experiments in order to come to a general conclusion about its association with our intended product (We do know that after heating and DE52 recovery the FIX in the K eluates has a lower NAPTT than our starting material):

This experiment was carried out on material which was cloudy and had been rejected because of low NAPTT, so our results may not be typical of those which we may get from a "good" starting batch.

Points for further experimentation.

① control the heating.

② Do the pasteurisation with a usable batch of FIX. ie not a pyrogen or NAPTT failed batch.

③ Investigate the difference if any between the FIX eluted in buffer J and that eluted in K. Does re-absorbing that in J mean that it will all come off the column before buffer K is used?

④ Reduce the monitoring to give a clearer elution profile Trace.

Use conductivity and U.V. absorption or refractive inde

D.C.

ASSAY REQUEST FORM (1)

Request from: DC/PAS Date: 26/1/84

When results needed: TOMORROW MORNING.

Samples to be kept? NO If so, how?

Samples provided with form/~~available from~~

SAMPLE INVESTIGATION		14/15/SP					
Factor VIII, iu/ml	1 st.						
	2 st.						
Factor IX, iu/ml	1 st.	(55)					
	2 st.	46·4					
Factor II, u/ml							
Factor X, u/ml							
Factor VII, u/ml (clotting)							
AT III (Anti Xa), u/ml							
AT III (Anti IIa), u/ml							
AT III (Laurell), u/ml							
Factor VIII RAG, u/ml							
Fibrinogen (Laurell), mg/ml							
Prothrombin (Laurell), u/ml							
Factor XIIIA (Laurell), u/ml							
Fibronectin (Laurell), mg/ml							
Factor VIII CAG							
NAPTT sec. 1/10							
TGt50 min.							
F hr.							
Limulus + or -							

ASSAY REQUEST FORM (1)

Request from: DC/PAF Date: 30/1/84

When results needed: ASAP

Samples to be kept? No If so, how?

Samples ~~provided with form~~/available from R+D Big Freezer

SAMPLE INVESTIGATION		14-15 15P	9H2J	∝9H2I	1Sn	1J	1K1
Factor VIII, iu/ml	1 st.						
	2 st.						
Factor IX, iu/ml	1 st.	(46.5)	(12.8)	(6.4)	(0.26)	(1.2)	(47)
	2 st.	68.2	10.8	5.5	<0.01	5.1 Haem	63.9
Factor II, u/ml							
Factor X, u/ml							
Factor VII, u/ml (clotting)							
AT III (Anti Xa), u/ml							
AT III (Anti IIa), u/ml							
AT III (Laurell), u/ml							
Factor VIII RAG, u/ml							
Fibrinogen (Laurell), mg/ml							
Prothrombin (Laurell), u/ml							
Factor XIIIA (Laurell), u/ml							
Fibronectin (Laurell), mg/ml							
Factor VIII CAG							
NAPTT sec. 1/10		120	132	106 233	220	420+	88
TGt50 min.							
FDA hr.							
Limulus + or -							

For comments contact Date finished:

ASSAY REQUEST FORM (1)

Request from: DC/PAF Date: 30/1/34

When results needed: ASAP

Samples to be kept? No If so, how?

Samples ~~provided with form~~/available from R+D Big Freeze.

SAMPLE INVESTIGATION		/K2	/K3				
Factor VIII, iu/ml	1 st.						
	2 st.						
Factor IX, iu/ml	1 st.	(25)	(0.6)				
	2 st.	27.3	0.2				
Factor II, u/ml							
Factor X, u/ml							
Factor VII, u/ml (clotting)							
AT III (Anti Xa), u/ml							
AT III (Anti IIa), u/ml							
AT III (Laurell), u/ml							
Factor VIII RAG, u/ml							
Fibrinogen (Laurell), mg/ml							
Prothrombin (Laurell), u/ml							
Factor XIIIA (Laurell), u/ml							
Fibronectin (Laurell), mg/ml							
Factor VIII CAG							
NAPTT sec. 1/10		64 & 233	128 —				
TGt50 min.							
FDA hr.							
Limulus + or -							

For comments contact Date finished:

16.2.84 9H22 Large-scale preparation, heating and recovery of factor IX in solution.

This has three objectives

i) To test the reproducibility of the basic procedure

ii) To provide material for comparitive, unheated recovery by chromatography (9H22-02)

iii) To provide material for gel-loading experiments (9H22-03).

Materials

Factor IX: Dried factor IX, 9D 2260, was used in this experiment. This material was unhepained, 35 ml fill and failed upon pyrogen testing

DE-52: Recycled batch QC/RD/2 (recycled on 10/2/84) was thawed overnight from storage at −20°C.

Buffers:

Buffer J	8.5 mM trisodium citrate 1.5 mM citric acid 10 mM Na_2HPO_4 80 mM NaCl	pH 7.03 Conductivity: 10 mMho
Buffer K	8.9 mM trisodium citrate 1.1 mM citric acid 10 mM Na_2HPO_4 300 mM NaCl	pH 6.95 Conductivity: 27.27 mM
Buffer C	20 mM trisodium citrate 1 M NaCl	pH 7.69 Conductivity 76.0

Method

Reconstitution 100 vials of 9D 2260 were swabbed down, opened and reconstituted in PFW (35 ml approx. to each vial).

Weight, volume, pH and conductivity were measured.

Samples were taken: 3 x 2 ml, 1 x 5 ml, 1 x 1 ml (viable count) 2 x 1 ml (immediate factor IX assay).

Stabilisers 3639 g glycine was dissolved (0.1 g/g eluate), the mixture transferred to a polycarbonate bottle and placed in a water bath set at 35°C.

Mixing with a magnetic stirrer, 7278 g sorbitol

were added. Because the vessel was not big enough, the material had to be poured into a stainless steel bucket for complete addition of sorbitol (2g/g eluate)

Samples were taken: 3 x 2ml, 2x 300 ml (1s)

The remaining 1s was then returned, mostly to the 10L polycarbonate heating pot and the remainder (~2000ml) to a polythene heating bottle. The large container was placed over the stirrer, the contents of the small container were not stirred.

Heating

The water-bath switched on automatically at 8.15 in the evening and the factor IX heated up to 60°C in 1.9 hours. This temperature was then maintained for 4½ hours at which point the chart recording paper stopped running. After 10 hours however, the temperature of sample and bath were 61.5° and 61.6° respectively so we assume the Temperature was maintained over the entire 10 hr heating period.

Samples were then taken

3 x 2 ml large stirred bottle 9H22 A

3 x 2 ml small unstirred bottle 9H22 B

The weights and volumes of each bottle were measured then the contents combined.

3 x 2 ml samples 9H22- were taken, along with 4 x 50 ml and 4 x 100 ml samples.

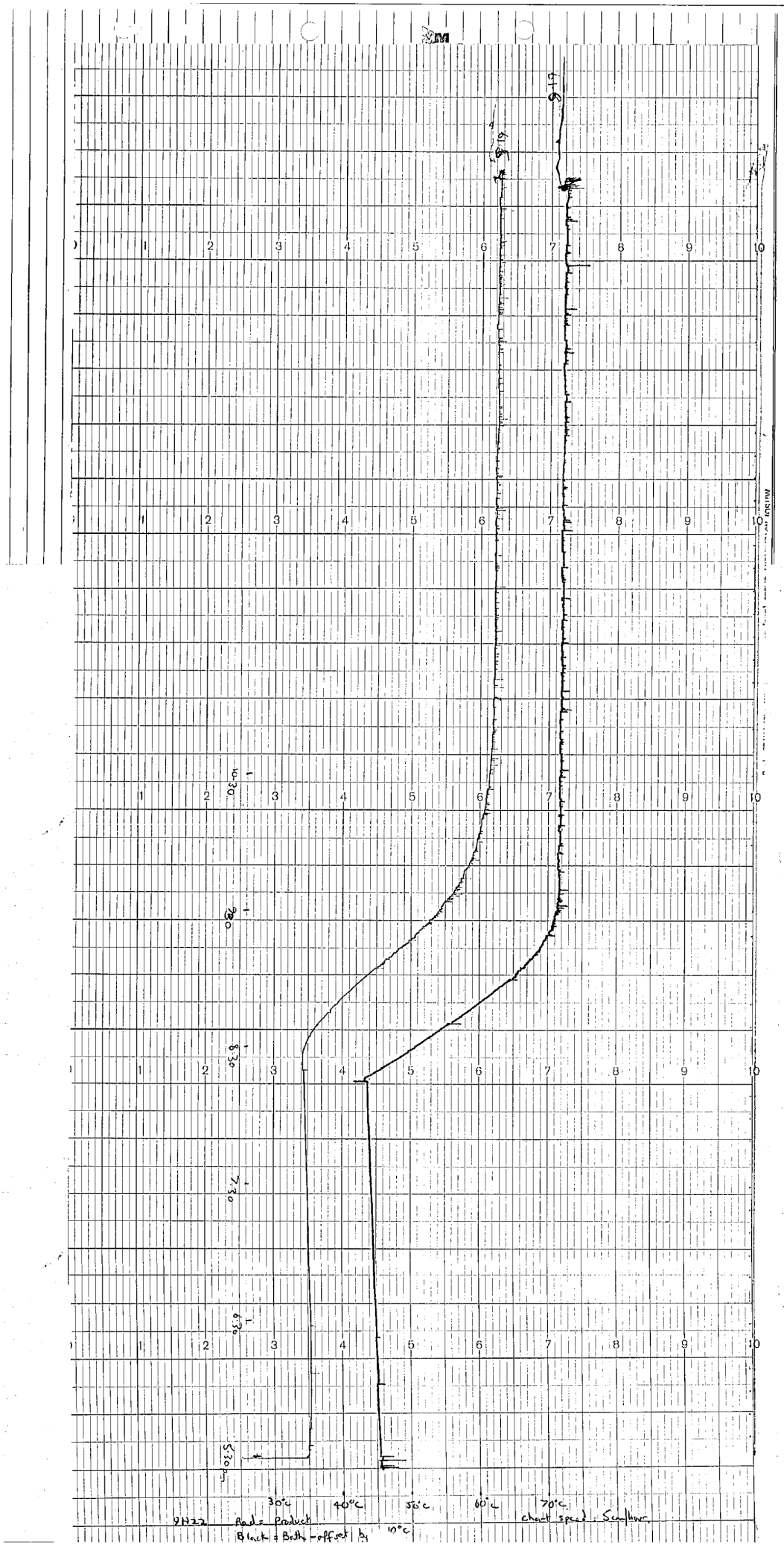
Absorption

The 9H22 was diluted ½ by addition of an equal volume (7686 ml) of PFW.

Samples were taken 3x2ml (of 9H22)
1x5 ml

The assay result for the reconstituted 9D showed 32.8 u/ml, giving a total of 124,410 units of factor IX.

Because of the removal of considerable quantities of factor IX for future work then gel loading per equivalent factor IX units was dropped from the 210 units/g calculated for 9H21 to 190 units/g DE ≤ 2.


9H22 Red = Product
Black = Bath offset by 10°C
30°C
40°C
50°C
60°C
70°C
chart speed: 5cm/hr

This value was a very rough approximation and in retrospect was a total miscalculation on my part, since a reduction in the actual units present should be compensated by increasing the u/g DE 52, not decreasing it

[As is shown in the results this loading used is equivalent to 49.3 units of ∝9H22 / g DE 52. Relative to the established value 9H17 – 9H20 of 150 u of ∝9H / g DE 52, it appears we have underloaded the gel threefold.]

Adsorption was effected by stirring for 1 hour at lab. temperatures, with a magnetic stirrer.

Elution

The slurry was then packed into a GAC 110 glass column, pumping from below with a Watson-Marlo pump.

The supernatant was collected and, as with all further fractions 3 x 2 ml and 1 x 5 ml samples were taken

With a bed volume of 135.9 ml, 7 L of buffer J were applied, pumped from a pressure vessel with ~ 1 lb/sq in N_2. This generated a flow of about 30 ml / hour.

The elute was monitored by u.v. transmission and absorbance, conductivity and refraction.

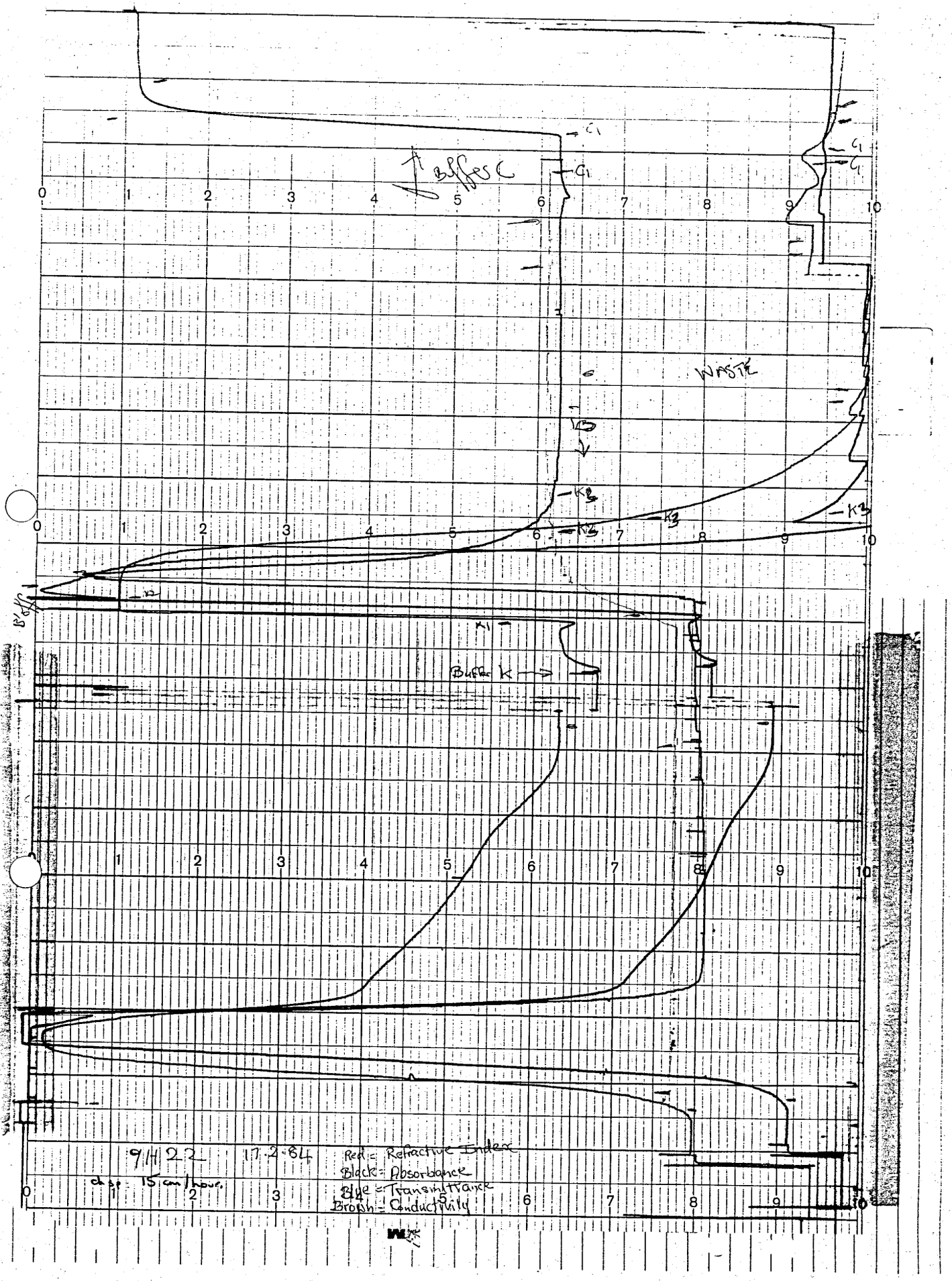
The protein peak eluted by buffer J was collected in one fraction according to changes in the u.v measurements.

8 L buffer K were then applied and fractions collected according to the criteria:

/K1 to peak light of u.v change
/K2 to the leveling off of conductivity
/K3 to the tail of the u.v peak.

Additional 1 ml sample of /K2 was taken for viable count.

Finally the column was washed with 3 L buffer C then blown dry.


Buffer C
C1
WASTE
K3
Buffer K
9H22
17.2.84
Red = Refractive Index
Black = Absorbance
Blue = Transmittance
Brown = Conductivity
0
1
2
3
4
5
6
7
8
9
10

Results Protein Data

Sample	Volume (ml)	A_{280}	Total A_{280}	Factor IX u/ml	Factor IX total	Sp Act u/mg	% Yield Starting 9D	% Yield DE52
9D2260	3793	14·7	55751	32·8	124410	121515	2·23	
				31·3	118721		2·13	

The equivalent of 255 ml 9D is removed as 600 ml of IS. Equivalent to 8173 units.
∴ Assume : 52008 113392 100

9H22A	6152			6·8	41833	50% proportionally (37% total)
9H22B	2138			4·2	8979	31% proportionally (8% total)
α9H22	15372	3·03	46577	2·1	32281	0·69	100

+ Equivalent of 1200 ml α9H22 is removed as 600 ml 9H22. This is equivalent to 2520 units. To compare heated IS with starting material:

Total α9H22	50213	34801		30·7 %
/Sn	15345	0·381	5846	0·1	1534	0·26	4·7
/J	6050	1·6	9680	0·81	4900	0·51	15·2
/K1	240	14·6	3504	34·6	8304	2·37	25·7
/K2	1537	10·1	15524	15·4	23670	1·52	73·3

Sample	NAPTT ½	% NAPTT blank
9D2260	201	80.7
9H22A	203	81.5
9H22B	179	71.9
α9H22	201	80.7
/Sn	239	95.9
/J	229	91.9
/K1	175	70.3
/K2	183	>3.5

* See note under "Comments"

9H22.7

Physical Data

Sample	Volume (ml)	pH	Conductivity (mMho)	Weight (g)	Density (g/ml)
9D2260	3793	7.12	13.82	3639	0.96
/S	8290			10619	1.28
9H22	7684			9390	1.22
α9H22	15372	6.91	1.12	(17076) [illegible]	1.11
/Sn	15345	8.28	2.18	15660	1.02
/5	6050	8.4	7.91	6249	1.03
/K1	240	7.23	12.54	243	1.01
/K2	1537	7.05	22.91	1568.7	1.02
/K3	2075	6.96	26.55	2098	1.01
/6	650	6.98	62.73	656	1.01

Viable Counts

9D2260 — 0 CFU/ml
α9H22/K2 — 0 CFU/ml

Suggesting that not only did the material start uncontaminated but it remained so throughout the experiment. This is probably a reflection of the greater care taken throughout the experiment to maintain a clean environment, compared with previous experiments.

Experimental time after heating

9H22 Dilution	1.5 hours
Adsorption	1 hour
Column Packing	1 hour
Elution	3.25 hours.

Comments

1) Sorbitol addition is still time consuming and difficult to control.

2) The accidental need to use two heating bottles, one stirred, the other not stirred does confirm the evidence from 9H18, and exceeds it, that yield is much improved by continuous stirring. In proportion to their volumes, the stirred material survives 60% better than the unstirred portion.

3) Stirring also seems to protect against shortened NAPTT. The 24 second difference between A and B, in that time range, is significant.

4) One of the problems in this experiment is that removal of substantial volumes for further experimental use has made the calculation of yields complicated and approximate. But there is no doubt that the recovery after heating is very poor, in common with 9H21. This is disappointing after the ~70% survival of factor IX in 9H19 and 9H20.

5) The differences which may in some unknown way affect this poor recovery are: i) addition of sorbitol in bigger aliquots
ii) stirring in the heating vessel
iii) stirring with a large magnetic follower
iv) heating in a smaller, older, less well-controlled water bath (even so, 60°c is maintained ±1°c)
v) 9H is diluted ½ rather than ⅓.
This last change may be significant in some way

6) The recovery on DE-52 now appears to be giving good (~80% +) recovery of factor IX in Buffer K compared with ∝9H material.

7) The /K eluate still has a shorter NAPTT than either the starting factor IX or the ∝9H, while the /Sm and /J NAPTT's are longer. This merits further examination.
NB Subsequent assays of 9D/S and ∝9H22 (in expts 9H22-02 and 9H22-03) give factor IX assay values of 8.2 u/ml and 3.23 u/ml respectively. Allowing for dilution, this gives a yield on heating of ~~NB~~ 78%, but implies loss upon sorbitol addition.

PAF, DC, EB

JH

9N22

FACTOR IX WET HEATING

Starting Material

Time ~~Elute~~ batch: 9D 2260 (100 vials)
Reconstitute in PFW.
Elvate volume: ~ 3793 ml Height 12.7cm | Bucket vessel diameter 19.5c
Elvate weight: 3639 g
Wt bucket + stirrer : 4688.5 − 1049.5 = 3639.0
pH : 7.12
Conductivity: 13.82 mMho

Sample: ✓3 x 2ml (X/5)
✓1 x 5ml
Send ✓2x 1ml for immediate factor IX assay.
✓ 1x1 ml → Viable count.

Stabilisers

1. Add Glycine : 0.1 g per g of elvate

Weight of glycine added: 363.9 g

2. Dissolve by stirring with large magnetic stirrer.

3. Place in 30° water bath with magnetic stirrer underneath.

4 (14-15) Add sorbitol 2g per g of elvate

Weight of sorbitol added: 7278 g

3. Stir until fully dissolved, monitoring temperature (>20°c)

4. Sample 3 x 2ml (/5)
2x 300 ml. → store in cold room 3.

5. Volume _____ ml ~ 8230 ml.
Wt. (31718-382) + (8478.7-1049.5) Weight : 10 619 g

Diameter small bottle: 15 cm

Hight of wine in small bottle: 12.1 cm

Volume in small bottle: 2138

Diam large bottle 19.5 cm.

Hight large bottle 20.6 cm.

∴ Volume large bottle: 6152 ml

Total volume: 8290 ml

before sampling:

- Weight large bottle: 8596.9 − 1049.5 = 7547.4 g
- Weight small bottle: 3171.5 − 382 = 2789.5
- Total weight: 10336.9 g

Weight of bucket 4209.7 g.

Diameter bucket, 30.3 cm

1360
4209.7

9990.3

14,400
4209.7

10,190.3

7h.2
9H22

1 ml

Heating

1. Place mixture in water bath to heat.
2. Set water bath to switch on at ~~9.00~~ 8.30 (20.30) pm
3. Set chart recorder to switch on at 9.00 pm
4. (Set magnetic stirrer to switch on at 9.00 pm.)

~~Cool~~ up to 60° at ~ 10.30

5. Set water bath to heat up to 60°C

8.44 6. Remove from water bath after 10 hours at 60°C

(approx : 8.00 - 8.30 am.)

7. Sample 3 x 2ml 4 x 50ml ~~4~~ x 100ml. (9H___)
8. Volume : _____ ml 7684 ml (calculated volume)
9. Weight : ~~10190~~ 3. g 9390.3 g.

Big bottle stored (9H22A)
Small bottle not stored (9H22B) Post Sample: 9H22

Dilution

1. Dilute by 2 by addition of 1x9H volume of PFW.
2. Volume PFW ~~7684~~ 7686 ml
   Weight PFW 7686 g
3. Sample 3 x 2ml 1 x 5ml (∝ 9H___)
4. ~~Volume~~ Volume _____ ml
   Weight _____ g
   Conductivity : 1.12 mMho
   pH : 6.91
5.
6. Factor IX activity : 32.8 u/ml IN ELUATE

∴ Total factor IX units : 124,410. IN ELUATE

Sn bucket : diameter 26.9 cm.
" depth : 27 cm.
" weight 3460

weight Sn + bucket : 19120

7A.3
9H22

T1 – Adsorption

1. Add DE-52 . 1 g recycled weight per ~~810~~ factor IX 190 units in elute

Wt DE-52 : 655. g

10-17. 2. Stir for 1 hour using magnetic stirrer.

Recovery of DE-52

11.30 Packed by 12.30

a) 1. Pour slurry into GAC110 column with extender tube attached.

2. Fit pressure head . Pack under N_2 pressure or pump ~~from~~ with Watson Marlow pump.

b) 1. Spin down in Beckman centrifuges at 3,000 rpm for 20 minutes.

2. Decant supernatant

3. Resuspend in 1.5 × weight of dry DE 52 volumes of buffer J

Volume of buffer J _____ ml

Weight of buffer J _____ g

4. Pack into GAC 110 column.

15345

Volume of supernatant: ~~5500~~ ml

Weight of supernatant 15660 g

pH 8.28

Conductivity 2.18 mMhc

Sample : 3 × 2 ml (1 Sn)
1 × 5 ml

7N 4

9N22

I. Elution

Bed height: 14.3 cm

Bed volume: 1359 ml

Insert filter in piston head before fitting piston into column.

1. Wash with 5 bed volumes of buffer J

Volume buffer J: [redacted] ml 6795

Weight buffer J: 6.80 kg

2. Measure flow rate: ~130 ml/min.

Monitor u.v, conductivity, refraction.

3. Collect samples by change in U.V. Transmission

3. Collect first peak in two halves (front peak/J1 and rear peak/J2)

Sample 3 x 2 ml
1 x 5 ml

/J1

Volume 6050 ml
Weight 6249 g
pH 8.4
Conductivity 7.91 mMho

/J2

Volume _____ ml
Weight _____ g
pH _____
Conductivity _____ mMho

4. Wash with ~~5~~ bed volumes buffer K

Volume buffer K: 8000 ml

Weight buffer K: _____ g

9N22

5. Collect eluting peak in three parts:

Protein front /K1
Protein middle /K2
Protein tail /K3

Sample each 3 x 2ml
1 x 5ml

/K1
Volume 240 ml
Weight 243 g
pH 7.23
Conductivity 12.54 mMho

/K2
Volume 1537 ml
Weight 1568.7 g
pH ~~7.05~~
Conductivity 22.91 mMho
→ Sample 1 ml viable count.

/K3
Volume 2075 ml
Weight 2098 g
pH ~~7.05~~ 6.96
Conductivity ~~22.91~~ mMho 26.55

3.35

6. Wash with 3 bed volumes buffer C.

Volume buffer C 30200 ml
Weight buffer C 30200 g

7. Collect any protein peak that elutes

Sample ~~2ml~~ (/C)
1 x 5ml

/C
Volume 650 ml
Weight 656 g
pH 6.98
Conductivity 62.73 mMh

9H22

Date 16/2/84 Buffer J for 9H

Find concentrations

8.5 mM trisodium citrate
1.5 mM citric acid
10 mM Na_2HPO_4
80 mM NaCl

To Make up ~~8~~ L (~~8~~ kg)

Weight		Batch No.	Quantity used
20g ~~12.5~~ g	trisodium citrate	9293700-17-39	20.1
2.48 ~~g~~	citric acid	91481500-83-6	2.47
11.36 7.1 g	Na_2HPO_4	9260374-06-5	12.2
37.36 ~~g~~	NaCl	9397545-14-90	37.4

Make up to ~~8~~ L (~~8~~ kg) with PFW (7930g)

pH limit: 7.00 ± 0.05 pH found 7.03

Adjust as necessary

Conductivity limit: 10.0 ± 1.0 Conductivity found 10.0

9H22

date 16/2/84 Buffer K for 9H

Final concentrations

8.9 mM trisodium citrate
1.1 mM citric acid
10 mM Na_2HPO_4
300 mM NaCl

To Make up ~~10~~ 10 L (~~11~~ Kg)

Weight		Batch No	Quantity used
26.2 ~~20.96~~ g	trisodium citrate	9293700-17-39	26.2
2.3 ~~1.84~~ g	citric acid	91481500-03-6.	2.3
14.2 ~~11.36~~ g	Na_2HPO_4	9260374-061-5.	14.2
175.25 ~~140.2~~ g	NaCl.	9397545-14-98	175.5.

Make up to 10 ~~8~~ L (10 ~~8~~ Kg) with PFW (~~2010~~ 7782g) 9792 g

pH limit: 7.00 ± 0.05 pH found 6.95

Adjust as necessary

Conductivity limit: 29.5 ± 1.0 Conductivity found 27.27 ~~33.4~~

D.t 16/2/84 Buffer C for 9H ____ 9H<2

Final concentrations

20 mM trisodium citrate

1.0 M NaCl

To make up 3 L (3 Kg)

Weight		Batch No	Quantity used
17.7 g	trisodium citrate	9293700-17-39.	17.7 g.
175.3 g	NaCl	9397545-14-90.	175.3

Make up to 3L (3 kg) with PFW (2808g).

pH limit: 8.0 ± 1.0 pH found 7.69

Adjust if necessary

Conductivity limit: 80 ± 10 Conductivity found 76.0

Request from: PAF DC Date: 10/2/84 9H22

When results needed: ASAP FRIDAY

Samples to be kept? No If so, how? _____

Samples provided with form/~~available from~~ _____

SAMPLE INVESTIGATION		9D L260					
Factor VIII, iu/ml	1 st.						
	2 st.						
Factor IX, iu/ml	1 st.	(33)					
	2 st.	32.8					
Factor II, u/ml							
Factor X, u/ml							
Factor VII, u/ml (clotting)							
AT III (Anti Xa), u/ml							
AT III (Anti IIa), u/ml							
AT III (Laurell), u/ml							
Factor VIII RAG, u/ml							
Fibrinogen (Laurell), mg/ml							
Prothrombin (Laurell), u/ml							
Factor XIIIA (Laurell), u/ml							
Fibronectin (Laurell), mg/ml							
Factor VIII CAG							
NAPTT sec. 1/10							
TGt50 min.							
FDA hr.							
Limulus + or -							

For comments contact _____ Date finished: _____

7H22

Request from: PAF Date: 1/2/84

When results needed: ASAP

Samples to be kept? No If so, how?

Samples ~~provided with form~~/available from R&D G. Furer

SAMPLE INVESTIGATION		9D2260	9H22 A	9H22 B	9H22	1 Sn	1 J
Factor VIII, iu/ml	1 st.						
	2 st.						
Factor IX, iu/ml	1 st.	(33)	(10.5)	(9)	(5)	(0.3)	(1.3)
	2 st.	31.3	6.8	4.2	2.1	0.10	0.81
Factor II, u/ml							
Factor X, u/ml							
Factor VII, u/ml (clotting)							
AT III (Anti Xa), u/ml							
AT III (Anti IIa), u/ml							
AT III (Laurell), u/ml							
Factor VIII RAG, u/ml							
Fibrinogen (Laurell), mg/ml							
Prothrombin (Laurell), u/ml							
Factor XIIIA (Laurell), u/ml							
Fibronectin (Laurell), mg/ml							
Factor VIII CAG							
NAPTT sec. 1/10		201	203	179	201	239	229
TGt50 min.							
FDA hr.							
Limulus + or -							

For comments contact _____ Date finished: _____

Request from: PAF

Date: 17/2/94 THL2

When results needed: ASAP

Samples to be kept? IV

If so, how?

Samples ~~provided with form~~/available from R+D By Fuzzs

SAMPLE INVESTIGATION		/K1	/K2				
Factor VIII, iu/ml	1 st.						
	2 st.						
✓ Factor IX, iu/ml	1 st.	(64)	(35)				
	2 st.	34.6	15.4				
Factor II, u/ml							
Factor X, u/ml							
Factor VII, u/ml (clotting)							
AT III (Anti Xa), u/ml							
AT III (Anti IIa), u/ml							
AT III (Laurell), u/ml							
Factor VIII RAG, u/ml							
Fibrinogen (Laurell), mg/ml							
Prothrombin (Laurell), u/ml							
Factor XIIIA (Laurell), u/ml							
Fibronectin (Laurell), mg/ml							
Factor VIII CAG							
✓ NAPTT sec. 1/10		175	183	(control 249)			
TGt50 min.							
FDA hr.							
Limulus + or -							

For comments contact _____ Date finished: _____

MEMO

To

19.....

From

Your Reference

Our Reference

9H22 A280's

9D ½₀ 0.735

29H22 ½₀ 0.306

15ᵃ ½₀ 0.033 Nett 0.381

1J ½₀ 0.080

1K1 ½₀ ~~0.758~~ 1.404 ½₀ 0.729

1K2 ½₀ 0.506

1K3 ½₀ 0.041

1L ~~0.~~ Nett 0.117

9H22

BACTERIAL CONTAMINATION : VIABLE COUNTS

File Reference: _____ Product: R+D

Authorised by: [signature] Processing Run: _____

Date effective: 21.4.83 This page started: 22.2.84

PRODUCTION STAFF			QUALITY CONTROL STAFF			
Sample Stage	Volume to be taken	Sample taken by:	SAL	Day 1 at °C	Day 2 at °C	.'. CFU/ ml
x9H22 /K2				0	0	0
9D2260				0	0	0
			Control	0	0	0

EXTRA SAMPLES as requested by Scientist i/c Production or QC staff:

			Control			

COMMENTS by sampling, testing or reading staff:

INTERPRETATION by Scientist i/c Production, and any action taken:

COMMENTS by Quality Controller:

21.2.84 9H22.02 To examine the recovery on DE52 of factor IX in sorbitol, without heating it.

This experiment is intended as a parallel for 9H22, to find out if the elution characteristics of factor IX after heating are attributable to the effect of heating, or the effect of sorbitol or neither.

Materials Factor IX : 9D 2260/5 taken from 9H22 experiment and frozen. 585 ml of this material were thawed at 37°c.

Buffers : Buffer J 8.5 mM trisodium citrate, 1.5 mM citric acid, 10 mM Na_2HPO_4, 80 mM NaCl } pH 6.98, Conductivity 9.2 mMh

Buffer K 8.9 mM trisodium citrate, 1.1 mM citric acid, 10 mM Na_2HPO_4, 300 mM NaCl } pH 6.96, Conductivity 26.72

DE52 : Batch QC/RD 2 stored frozen and thawed over night at room temperature. This is the same batch as used in 9H22.

Method.

585 ml of 9D 2260/5 were thawed.

2 x 2ml samples were taken "9D 2260/5 - 02"

Diluted ½ by addition of an equal volume of PFW

2 x 2ml samples were taken "∝9D/5-02"

Addition of DE 52 was at a rate of 115 u/g DE 52. This value was based upon 70% survival of heated factor IX, in which a loading of 160 units of heated factor IX were added per g of gel. The lower loading is designed to take account of the additional 30% of activity which should be present in unheated factor IX.

Assume that IS has a potency of ~ 14 u/ml of factor II
This is derived as follows:

9D starting material (3793 ml) has 32.8 u/ml
i.e. total of 124410 units
14 mls were removed, leaving 123951 units

Volume of 9D/S = 8890 ml
∴ Potency of 9D/S = $\frac{3793}{8890} \times 32.8 = 14$ u/ml.

Accordingly 71.2 g DE 52 were added to ~ 9D/S-02 and the mixture stirred for 1 hour at room temperature.

The slurry was then packed into a 4.4 cm I.D. glass Wright/Amicon and pumped from below.

Pump rate of flow was calculated to be the same per cross-sectional area as in 9H22.
In that case, the column cross-sectional area (CAC110) was 95 cm^2, giving a flow of 1.37 ml min^{-1} cm^{-2}

In the present column the cross-sectional area is 15.9 cm^2
∴ require a flow rate of 22 ml/min.

In fact, the flow rate was 20 ml/min.

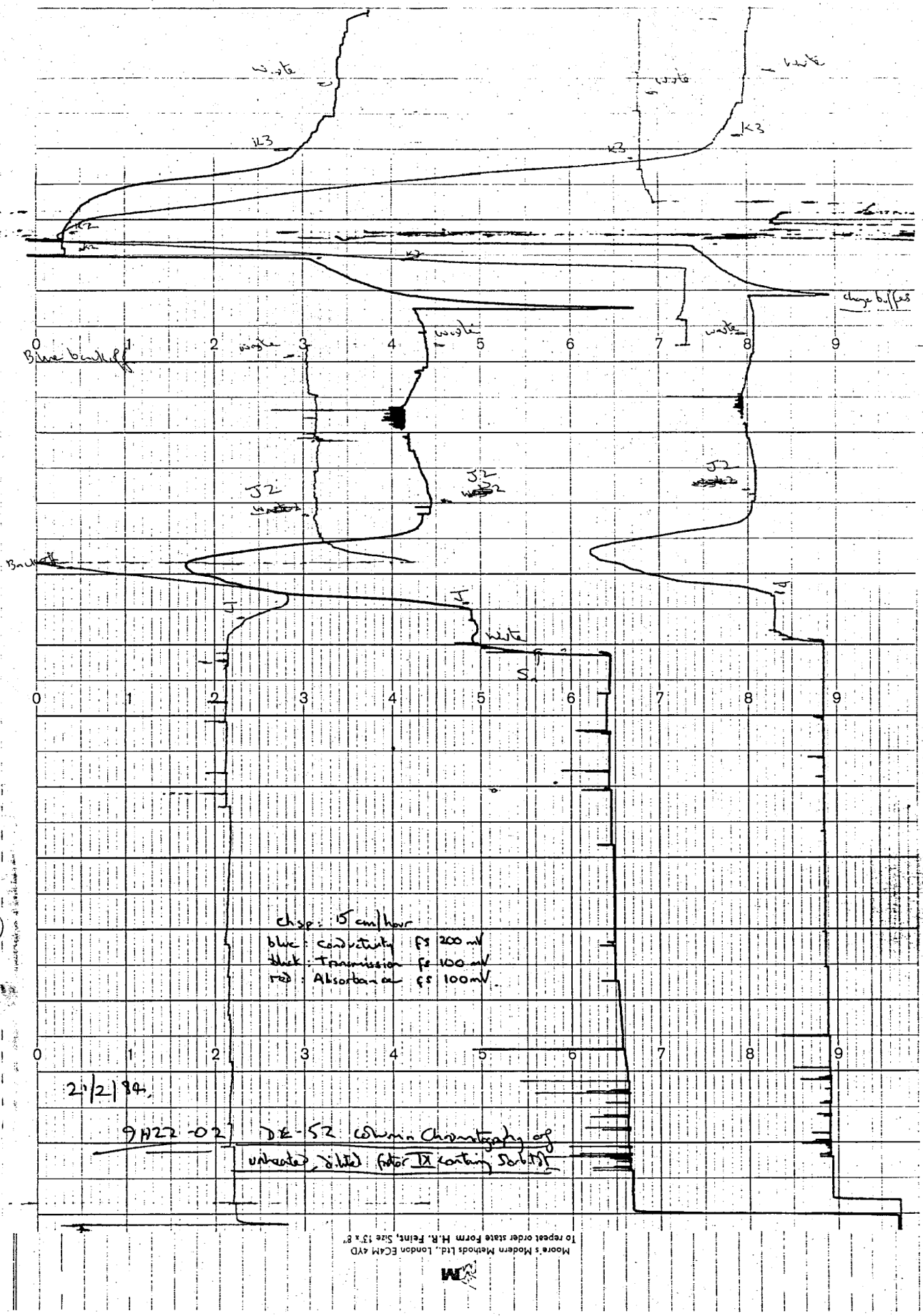
The eluate during packing was collected as one sample "/Sn".

Elution The bed volume was 146.3 ml.

5 bed volumes (731 ml) of buffer J were washed through the column, and two fractions, J1 and J2, were collected.

Similarly, 731 ml buffer K were applied and three fractions, K1 K2 and K3 collected.

Monitoring The column was monitored by u.v. transmission and absorption and by conductivity.



9H22.024

The /k fractions were collected as in 9H22, namely
K1 is the absorbance rise to a maximum
K2 is the bulk of the protein peak as conductivity rises to a maximum.
K3 is the protein tail at constant conductivity.

Results

Sample	Vol (ml)	A280	Factor 1x u/ml	Factor 1x total	Sp Act u/mg	% of 9H/5	NAPTT	% of NAPT blank
9D 2260		14.7	32.8		2.23		(6.249) 201	80.7
α9D/5	1140		8.2	9348		100	234	86.95
/Sn	1030	0.298	0.06	61.8	0.2	0.66	310	115.2
/J1	214	1.18	0.88	188.3	0.74	2.01	250	92.9
/J2	332	0.54	0.31	102.9	0.57	1.1	248	92.2
/K1	20	25.98	68.5	1370	2.64	14.6	117	43.5
/K2	216	7.7	21.3	4600	2.77	49.21	129	47.9
/K3	132	0.83	0.98	129.4	1.17	1.38	252	93.6

Sample	Volume (ml)	Weight	pH	Conductivity (mMho)
α9D/5	1140	1296		
/Sn	1030	~~1157~~	8.49	1.18
/J1	214	~~219~~	8.6	6.72
/J2	332	~~332~~	8.61	9.34
/K1	20	198	8.59	11.88
/K2	216	216	7.3	24.92
/K3	132	132	7.07	27.54

Comments

1. The drop in NAPTT is even more marked than with heated material in /K material. Similarly the /Sn NAPTT is substantially lengthened. This suggests that the separation of NAPTT activity is not a function of the heating step but rather due to either the presence of sorbitol or the level of loading of the gel.

2. Because the shortest NAPTT's are longer with the heated material (9H22), both in time and as a percentage of the blank, it appears that heating the factor IX does kill some of the activity which causes short NAPTT (whatever that may be).

3. The specific activities of unheated factor IX are very slightly higher than for the heated material in both /J and /K eluates. However the percentage recovery from DE 52 is lower with the unheated material. This may be due merely to less inclusive sampling of the unheated eluate in the /K buffer.

4. The amount of factor IX activity in J is much reduced in the unheated sample (3% ; cf 15% in 9H22). A similar reduction occurs in the /Sn (0.66% vs 4.7%). This reductions, of 79% and 86% respectively, suggest that the differences are due to the heating step. It is possibly due to the change in gel loading, but as this change is only a 29% reduction in available capacity such a cause seems unlikely. It should be remembered that this high content of heated factor IX in /J is a fairly new phenomena starting with 9H20. It may therefore be due to gel loading in as much as the assay upon which loading was determined was first related back to the starting material in this experiment

5 The conductivities of the various fractions are very similar to those for the heated material eluate.

The pH of these, in particular /K1, is much higher in this experiment compared to the heated factor IX (8.6 vs. 7.2). While the potency has doubled, the specific activity is the same in both cases. It would seem that the factor IX elutes before the pH drops and that this was not picked up in 9H22 when a larger volume was collected for /K1, thus including the pH drop of the buffer front. It should be noted, however, that the /K1 fraction in each experiment forms about the same proportion of the total volume of the /K protein peak (~5-6%), which does not support such a notion.

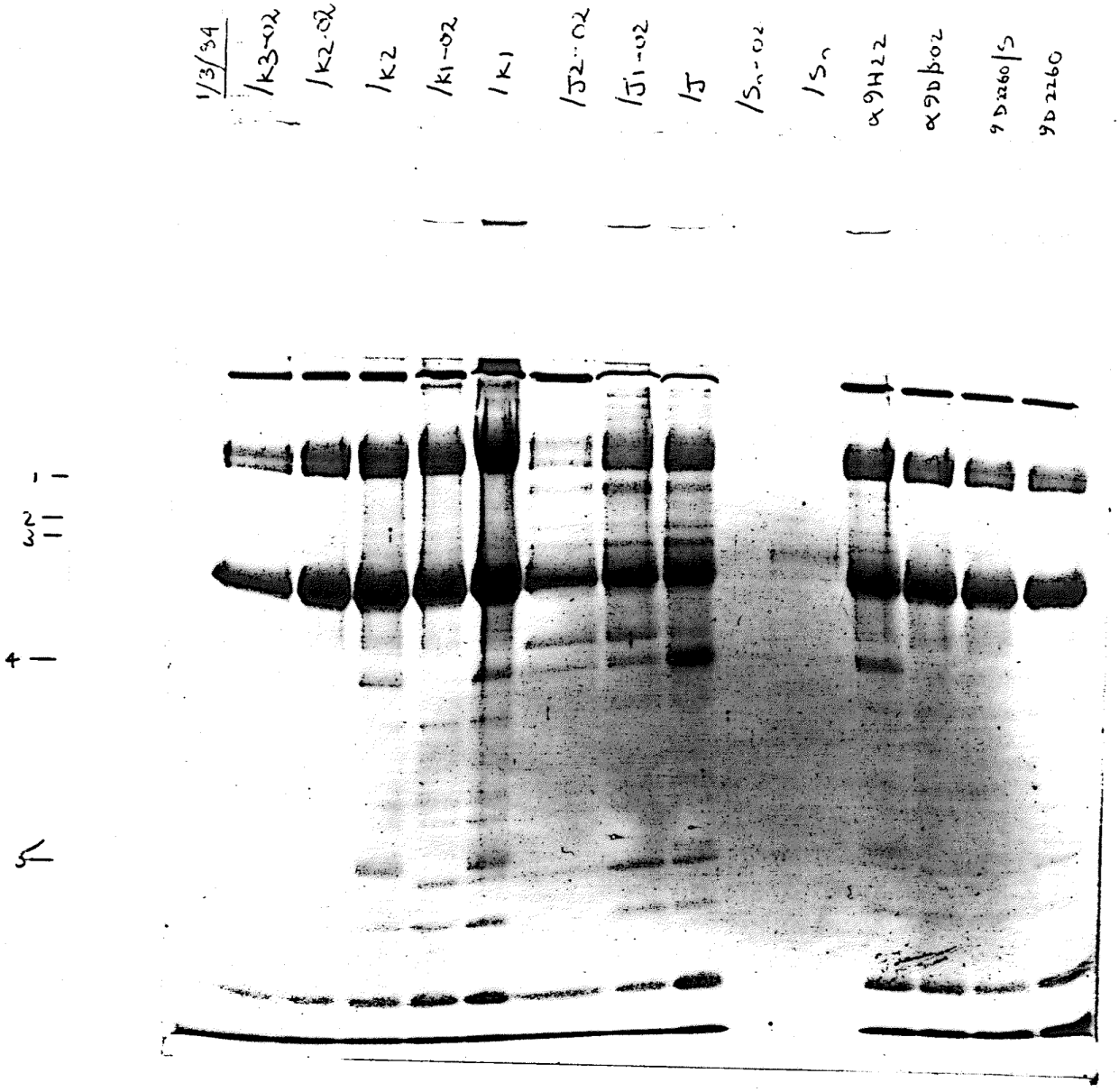
6. It is disappointing that the overall percentage yields are not more conclusive. However the distribution of protein absorbing material is

	Non heated		Heated	
Σ	10.1 %	(0.9%)	16.9 %	(4.0%)
Σ J	14.2 %	(4.5%)	28 %	(12.7%)
Σ K	75.6 %	(94.5%)	55 %	(83.2%)

In brackets is the distribution of factor IX activity

It does seem fairly conclusive that heating alters the distribution of Total protein and factor IX activity. Therefore the material which elutes in J, from a source material derived from production buffer B elute, is not the carried over Tail from buffer A (as this value would not change with heating). It must, in part at least, represent protein which is modified ~~for~~ as a result of the heating step.

P.A.F.



10% SDS PAGE. Samples are from heated 9H22 and unheated 9H22.02 from before and after DE52 chromatography.

Request from: PAF Date: 23/2/94

When results needed:

Samples to be kept? NO If so, how?

Samples ~~provided with form~~/available from V+D freezer

SAMPLE α90S/S-02 INVESTIGATION		1K3					
Factor VIII, iu/ml	1 st.						
	2 st.						
✓ Factor IX, iu/ml	1 st.	(2.8)					
	2 st.	0.98					
Factor II, u/ml							
Factor X, u/ml							
Factor VII, u/ml (clotting)							
AT III (Anti Xa), u/ml							
AT III (Anti IIa), u/ml							
AT III (Laurell), u/ml							
Factor VIII RAG, u/ml							
Fibrinogen (Laurell), mg/ml							
Prothrombin (Laurell), u/ml							
Factor XIIIA (Laurell), u/ml							
Fibronectin (Laurell), mg/ml							
Factor VIII CAG							
✓ NAPTT sec. 1/10 b 269		252					
TGt50 min.							
FDA hr.							
Limulus + or -							

For comments contact Date finished:

SDS PAGE of heated and unheated samples shows primarily the generation of thrombin during the heat Treatment. This is characterised by the appearance of bands 4 and 5 (prothrombin and thrombin respectively)

The other difference is the appearance and size of bands 1, 2 and 3, band 2 being particularly strong in heated / J. The band which appears in Sn does not seem traceable to any other band in the α9H22.

It appears that thrombin generation does take place though it is surprising that it is stable under these heating conditions.

ASSAY REQUEST FORM (...)

YN22-02

Request from: CAF Date: 23/2/84

When results needed:

Samples to be kept? No If so, how?

Samples ~~provided with form~~/available from R+D By Freeze

SAMPLE αYD/S-02 INVESTIGATION		αYD/S-02	/S$_n$	/J1	/J2	/K1	/K2
Factor VIII, iu/ml	1 st.						
	2 st.						
Factor IX, iu/ml	1 st.	(14)	(0.4)	(2.0)	(0.9)	(70)	(20)
	2 st.	8.2	0.06	0.88	0.31 ~~0.3~~	68.5	21.3
Factor II, u/ml							
Factor X, u/ml							
Factor VII, u/ml (clotting)							
AT III (Anti Xa), u/ml							
AT III (Anti IIa), u/ml							
AT III (Laurell), u/ml							
Factor VIII RAG, u/ml							
Fibrinogen (Laurell), mg/ml							
Prothrombin (Laurell), u/ml							
Factor XIIIA (Laurell), u/ml							
Fibronectin (Laurell), mg/ml							
Factor VIII CAG							
NAPTT sec. 1/10 b. 269		234	310	250	248	117	129
TGt50 min.							
FDA hr.							
Limulus + or -							

For comments contact Date finished:

22.2.84 9H22-03 To compare recovery of factor IX from DE-52 at different levels of loading of the heated material.

In the light of problems when relating gel loading back to the starting factor IX material this experiment is intended to determine the optimum amount of DE52 for successful recovery of factor IX While a similar experiment was performed in 9H7 we are now using higher ~~factor~~ sorbitol concentrations. Furthermore it is hoped that this experiment will demonstrate the margins for variation of the factor IX : gel ratio which may occur due to varying survival of factor IX during th heating step.

Materials

Factor IX : Samples of 9H22, stored frozen and thawed at 37°c.

Buffers : Buffers J and K prepared in 9H22-02.

DE 52 : Batch QC/RD/2 as used in 9H22 and 9H22-02.

Method

7 x 50 ml samples of 9H22 (labelled A-G were diluted each with 50 ml PFW.

Volume starting 9D = 3779 ml
Volume 9H22 = 8896 ml
∴ 50ml 9H22 ≡ $\frac{3779}{8896}$ x 50 = 21.24 ml of 9D

9D contained 32.8 u/ml
∴ 21.24 ml contains 696.7 units of factor

∴ Each sample contains the heated equivalent of 696.7 units of starting factor IX

DE-52 was added to each sample to generate, per g of DE52, 100, 135, 170, 200, 230, 260, 290.

Sample	units of 9D per g of DE52	Wt DE 52 added (g)
A	100	6.97
B	135	5.16
C	170	4.10
D	200	3.48
E	230	3.03
F	260	2.68
G	290	2.40

Each sample was stirred for 1 hour at room temperature then spun at 2000 rpm for 20 mintes in MSE Mistral 2L centrifuge.

The supernatant was decanted and the DE 52 resuspended in 40 ml of buffer J. This slurry was poured into 1.4 cm I.D. glass columns and packed under gravity.

Sample	Bed height (cm)	Bed volume (ml)
A	8.1	12.5
B	6.4	9.8
C	5.2	8.0
D	4.3	6.6
E	3.3	5.08
F	2.9	4.46
G	2.9	4.46

The packed columns were washed with a further 27 ml buffer J. This allowed even the largest bed volume to be washed with 5 volumes of buffer, sufficient to remove maximum protein at that ionic strength.

The eluate was collected as one pool from each sample, "1J"

62 ml Buffer K were then washed through each column and the eluate collected as "1K"

Assays Factor IX clotting assays and A280 measurements were made on all the samples. NAPTT were measured on starting material and /J and /K fractions.

Results

Sample	Volume (ml)	A280 /ml	A280 total	Factor IX u/ml	Factor IX total	Sp Act (u/mg)	% starting mater. A280	% starting mater. Factor IX
∝9422-03	100	3.26	326	3.23	323	0.99	100	100
A/Sn	84	0.43	36.12	0.04	3.36	0.09	11.1	1.0
B/Sn	86	0.38	32.68	0.04	3.44	0.10	10.0	1.1
C/Sn	89	0.46	40.94	0.04	3.56	0.09	12.6	1.
D/Sn	92	0.58	53.36	0.12	11.04	0.21	16.4	3.
E/Sn	91	0.69	62.79	0.14	12.74	0.20	19.3	3.
F/Sn	93	0.92	85.56	0.21	19.53	0.23	26.2	6.
G/Sn	93	1.03	95.79	0.38	35.34	0.37	29.4	10.
A/J	67	0.30	20.1	0.09	6.03	0.3	6.2	1.
B/J	66	0.44	29.04	0.11	7.26	0.25	8.9	2.
C/J	63	0.61	38.43	0.15	9.45	0.25	11.8	2.
D/J	62	0.75	46.5	0.23	14.26	0.31	14.3	4.
E/J	63	0.75	47.25	0.32	20.16	0.43	14.5	6.
F/J	64	0.72	4608	0.38	24.32	0.53	14.1	7.5
G/J	62	0.68	42.16	0.28	17.36	0.41	12.9	5.4
A/K	53	4.14	219.42	5.06	268.18	1.22	67.3	83
B/K	60	3.58	214.8	5.05	303	1.41	65.9	93
C/K	64	3.22	206.08	4.52	289.28	1.40	63.2	89.
D/K	63	3.0	189	4.21	265.23	1.40	58.0	82
E/K	64	2.86	183.04	3.73	238.72	1.30	56.1	73.
F/K	62	2.58	159.96	3.94	244.28	1.53	49.1	75.
G/K	62	2.48	153.76	3.61	223.82	1.46	47.2	69.

9H22-03

Sample	NAPTT (½)	% of NAPTT control (257 s)
∝ 9H22-03	218	84.8
A/J	264	102.7
B/J	257	100
C/J	310	120.6
D/J	292	113.6
E/J	313	121.8
F/J	293	114
G/J	230	89.5
A/K	253	98.4
B/K	231	89.9
C/K	244	94.9
D/K	267	103.9
E/K	225	87.5
F/K	211	82.1
G/K	205	79.8

Sample	pH	Conductivity
∝ 9H22-03	6.88	1.02
A/Sn	8.53	1.03
B/Sn	8.42	1.02
C/Sn	8.32	1.0
D/Sn	8.22	1.0
E/Sn	8.18	1.0
F/Sn	8.11	1.0
G/Sn	8.08	1.0
A/J	8.31	5.98
B/J	7.98	6.69
C/J	7.68	7.13
D/J	7.46	7.54
E/J	7.3	7.7
F/J	7.22	7.7
G/J	7.2	7.87
A/K	8.03	22.46
B/K	7.28	23.77
C/K	7.08	23.93
D/K	7.02	24.59
E/K	7.03	24.59
F/K	7.01	24.59
G/K	7.01	25.08

9H22-03.5

As an additional test the supernatants were assayed for NAPTT, to test whether there is any separation of long- and short-NAPTT material into /Sn and /K fractions. (as has been apparent in recent large scale 9H experiments).

Sample 9H22-03/Sn:	NAPTT ½ (s)	% of NAPTT control (290s)
A	268	92.4
C	258	88.9
D	277	95.5
E	247	85.2
F	252	86.9
G	250	86.2

Comments

1. The graph best shows the overall dependence on gel quantity.

The gel is saturated when 4.2 g are added as shown by /K protein content. This is roughly mirrored by total factor IX units

2. An intensity point occurs at 3.45 g DE 52 Although the gel is slightly overloaded at this point (approx 12% loss of factor IX), the NAPTT of /K lengthens while at the same time the NAPTT of /J shortens. This suggests that at this loading an activated form of factor IX is preferentially being displaced. At higher amounts of gel, NAPTT drops as activity increases, suggesting that the two form are running together.

3. At even lower amounts of gel, this effect is lost as more factor IX is eluted in /Sn and /J. At the same time the NAPTT of /K is drastically shortened along with that of /J. Curiously the NAPTT

of the /Sn at this level is constant. This might indicate a specific form of binding domain on the DE52 which, when binding factor IX, is responsible for the generation of short NAPTT

4. However, to judge from the specific activities of IX samples, there is little difference in the total protein adsorbed at the diff ent loadings.

5. Still there is scope for a more detailed study of the "NAPTT Effect".

6. From these results, the best loading of DE52 is 3.45 g DE 52. For the 9H22 heated material used, this is equivalent to 1g DE 52 for each 200 units of factor IX in the unheated starting material.

7. This heating experiment has shown up a potential activity loss during sorbitol addition. 9H22-02 shows 9D/S to have 8.2 u/ml factor IX — a substantial loss over 9D. 9H22-03 shows ∝9H22 to contain 3.23 u/ml factor IX. If these values are correct, then the recovery on heating is about 78%, not 30%.

Assuming a 78% recovery, the above gel loading, of 1g DE52 per 200 units factor IX 9D, is equivalent to 1 g DE 52 per 156 units of heated factor IX.
This is in excellent agreement with earlier loading experiments related to activity of heated material.

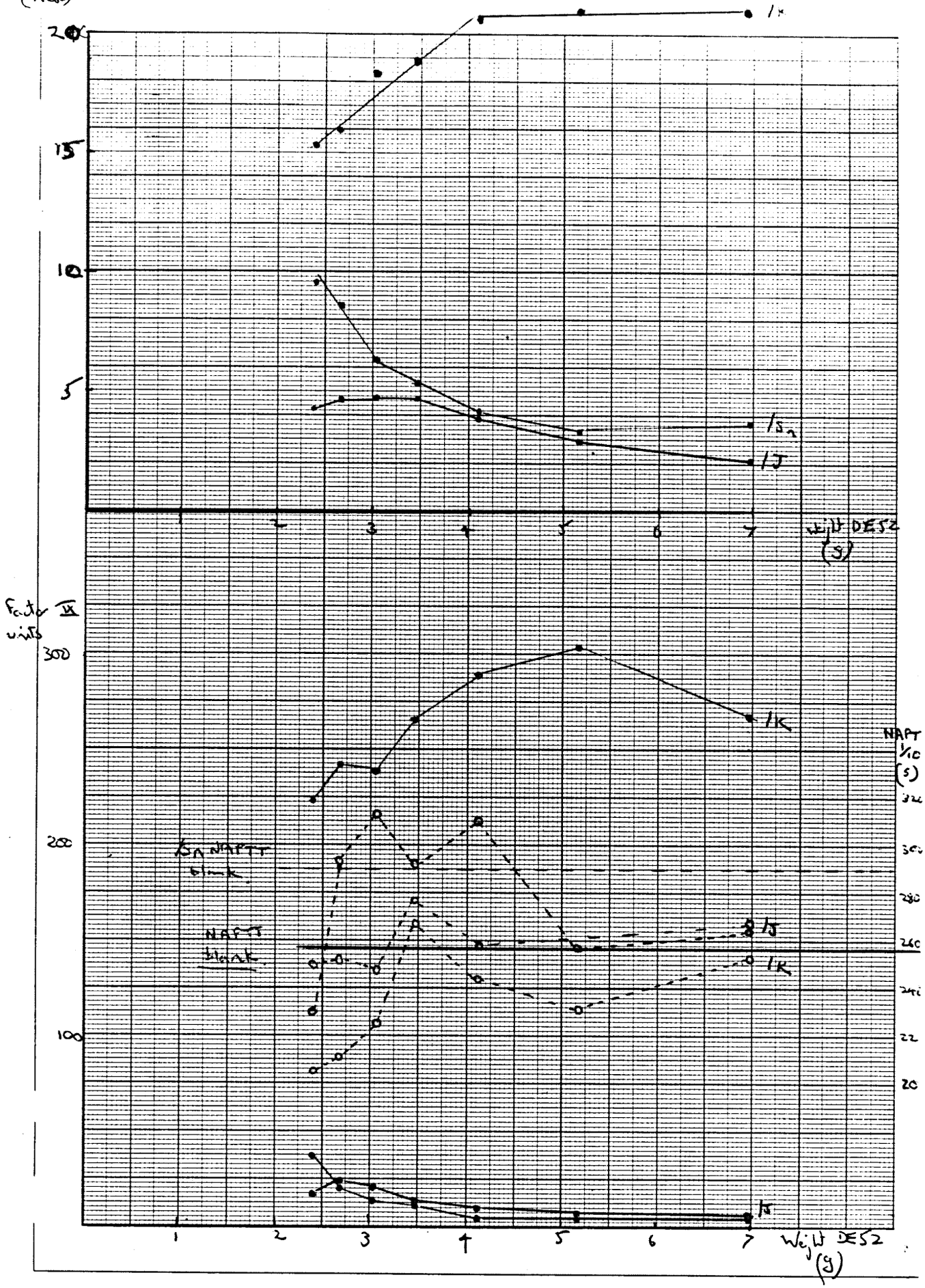
8. The problem/possibility of factor IX loss during sorbitol addition must therefore be investigated with top priority. Once solved, the indications of this set of 9H22 experiments are that
i) the heating yield of factor IX is 70-80% as originally thought in 9HA, 9H20
ii) the γ separation pattern on DE52 is not a function of heating, but may be a function of sorbitol content
iii) the DE 52 can be loaded at 1 g per 200 units 9D (starting material) ≡ 156 units heated factor IX

P.A.F.

7H22-03 >

Tow Pos
(Amps)

7H22-03



9H22-03

Request from: PAF Date: 16/3/84

When results needed: ASAP

Samples to be kept? No If so, how?

Samples ~~provided with form~~/available from R+D Big Freeze

SAMPLE 9H22-03 INVESTIGATION /Sn		A	C	D	E	F	G
Factor VIII, iu/ml	1 st.						
	2 st.						
Factor IX, iu/ml	1 st.						
	2 st.						
Factor II, u/ml							
Factor X, u/ml							
Factor VII, u/ml (clotting)							
AT III (Anti Xa), u/ml							
AT III (Anti IIa), u/ml							
AT III (Laurell), u/ml							
Factor VIII RAG, u/ml							
Fibrinogen (Laurell), mg/ml							
Prothrombin (Laurell), u/ml							
Factor XIIIA (Laurell), u/ml							
Fibronectin (Laurell), mg/ml							
Factor VIII CAG							
√ NAPTT sec. 1/10		268	258	277	247	252	250
TGt50 min.		Contr	290				
FDA hr.							
Limulus + or -							

For comments contact _____ Date finished: _____

ASSAY REQUEST FORM

Request from: DAF Date: 23/2/84 9H22-03

When results needed: 4 sheets, Friday 1

Samples to be kept? No If so, how?

Samples ~~provided with form~~/available from R+D Big Freezer

SAMPLE 9H22-03 INVESTIGATION		X 9H22-03	A/Sn	B/Sn	C/Sn	D/Sn	E/Sn
Factor VIII, iu/ml	1 st.						
	2 st.	(2.5)	(0.01)	(0.01)	(0.02)	(0.03)	(0.05)
Factor IX, iu/ml	1 st.	~~(2.5)~~	~~(0.01)~~	~~(0.01)~~	~~(0.1)~~	~~(0.15)~~	~~(0.3)~~
	2 st.	3.23	0.04	0.04	0.04	0.12	0.14
Factor II, u/ml							
Factor X, u/ml							
Factor VII, u/ml (clotting)							
AT III (Anti Xa), u/ml							
AT III (Anti IIa), u/ml							
AT III (Laurell), u/ml							
Factor VIII RAG, u/ml							
Fibrinogen (Laurell), mg/ml							
Prothrombin (Laurell), u/ml							
Factor XIIIA (Laurell), u/ml							
Fibronectin (Laurell), mg/ml							
Factor VIII CAG							
NAPTT sec. 1/10 b 257		218					
TGt50 min.							
FDA hr.							
Limulus + or -							

For comments contact Date finished: 2/3/84

9H22-03

Request from: PAF Date: 23/2/84

When results needed: Priority 2

Samples to be kept? No If so, how?

Samples ~~provided with form~~/available from R+D freezer

SAMPLE 9H 22-03 INVESTIGATION		F/Sn	G/Sn	A/K	B/K	C/K	D/K
Factor VIII, iu/ml	1 st.						
	2 st.	(0.08)	(0.1)	(4.0)	(3.5)	(3.2)	(3)
Factor IX, iu/ml	1 st.	~~(0.4)~~	~~(0.5)~~	~~(24)~~	~~(21)~~	~~(20)~~	~~(18)~~
	2 st.	0.21	0.38	5.06	5.05	4.52	4.21
Factor II, u/ml							
Factor X, u/ml							
Factor VII, u/ml (clotting)							
AT III (Anti Xa), u/ml							
AT III (Anti IIa), u/ml							
AT III (Laurell), u/ml							
Factor VIII RAG, u/ml							
Fibrinogen (Laurell), mg/ml							
Prothrombin (Laurell), u/ml							
Factor XIIIA (Laurell), u/ml							
Fibronectin (Laurell), mg/ml							
Factor VIII CAG							
NAPTT sec. 1/10 b257				253	231	244	267
TGt50 min.							
FDA hr.							
Limulus + or -							

For comments contact Date finished: 2/3/84

Request from: PHF Date: 23/2/84

When results needed: Priority 3

Samples to be kept? No If so, how?

Samples ~~provided with form~~/available from R+D Freeze

SAMPLE / INVESTIGATION		9H22-03 E/K	F/K	G/K	G/J		
Factor VIII, iu/ml	1 st.						
	2 st.	(2.8)	(2.5)	(2.3)	(0.15)		
Factor IX, iu/ml	1 st.	~~(17)~~	~~(15)~~	~~(19)~~	~~(0.3)~~		
	2 st.	3.73	3.94	3.61	0.28		
Factor II, u/ml							
Factor X, u/ml							
Factor VII, u/ml (clotting)							
AT III (Anti Xa), u/ml							
AT III (Anti IIa), u/ml							
AT III (Laurell), u/ml							
Factor VIII RAG, u/ml							
Fibrinogen (Laurell), mg/ml							
Prothrombin (Laurell), u/ml							
Factor XIIIA (Laurell), u/ml							
Fibronectin (Laurell), mg/ml							
Factor VIII CAG							
NAPTT sec. 1/10	b 257	225	211	205	230		
TGt50 min.							
FDA hr.							
Limulus + or -							

For comments contact Date finished: 2/3/84

Request from: AHF Date: 23/2/84

When results needed: Priority 4

Samples to be kept? No If so, how?

Samples ~~provided with~~ form/available from R+D Freezer

SAMPLE αYH22-03 INVESTIGATION		A/J	B/J	C/J	D/J	E/J	F/J
Factor VIII, iu/ml	1 st.						
	2 st.	0.03	(0.05)	(0.12)	(0.13)	(0.15)	(0.15)
Factor IX, iu/ml	1 st.	~~(0.2) (0.3)~~	(0.3)	(0.7)	0.8	(0.9)	(0.9)
	2 st.	0.09	0.11	0.15	0.23	0.32	0.38
Factor II, u/ml							
Factor X, u/ml							
Factor VII, u/ml (clotting)							
AT III (Anti Xa), u/ml							
AT III (Anti IIa), u/ml							
AT III (Laurell), u/ml							
Factor VIII RAG, u/ml							
Fibrinogen (Laurell), mg/ml							
Prothrombin (Laurell), u/ml							
Factor XIIIA (Laurell), u/ml							
Fibronectin (Laurell), mg/ml							
Factor VIII CAG							
NAPTT sec. 1/10 b292		264	257	310	292	313	283
TGt50 min.							
FDA hr.							
Limulus + or -							

For comments contact Date finished: 2/3/84

5.4.84. 9H23 Large Scale heating and recovery of factor IX using different methods of sorbitol addition

The main purpose of this experiment is to test for possible losses during the sorbitol addition, and also to find out whether a different method of dissolving the sorbitol ~~after~~ affects the yield.

Materials

Factor IX : 9D 2260, 35 ml fill, no heparin, pyrogen failed material

Buffers,

Buffer J	8.5 mM trisodium citrate 1.5 mM citric acid 10 mM Na_2HPO_4 80 mM NaCl	pH 6.95 Conductivity 10.0 m
Buffer K	8.9 mM trisodium citrate 1.1 mM citric acid 10 mM Na_2HPO_4 300 mM NaCl	pH 6.95 Conductivity 27.6 mS
Buffer C	20 mM trisodium citrate 1.0 M NaCl	pH 7.75 Conductivity 63 mS

DE-52 Batch QC/RD/2, thawed overnight from -40°

Method

After reconstitution in PFW and sampling of 3 x 2 ml ; 1 x 5 ml ; 2 x 1 ml ; 253.2 g glycine were added (0.1 g per g 9D) and stirred until dissolved. The batch was then divided into two fractions, "A" and "B"

Fraction A was placed in a 30°c water bath and 2g sorbitol added per g starting factor IX.

Fraction B was gradually added to sorbitol (a total of 2g per g factor IX), making a thick ~~paste~~ which gradually diluted slightly.

Both samples were left in a 40° c water bath.

Volumes and weight were measured after sampling 3 x 1 ml of each.

Leaving each in a four litre polythene beaker the fractions were lined up over Edwards magnetic stirrers.

Temperature probes were connected to each and into the water bath to monitor temperatures.

Using a timer switch, the water bath was set to heat up to 60° c.

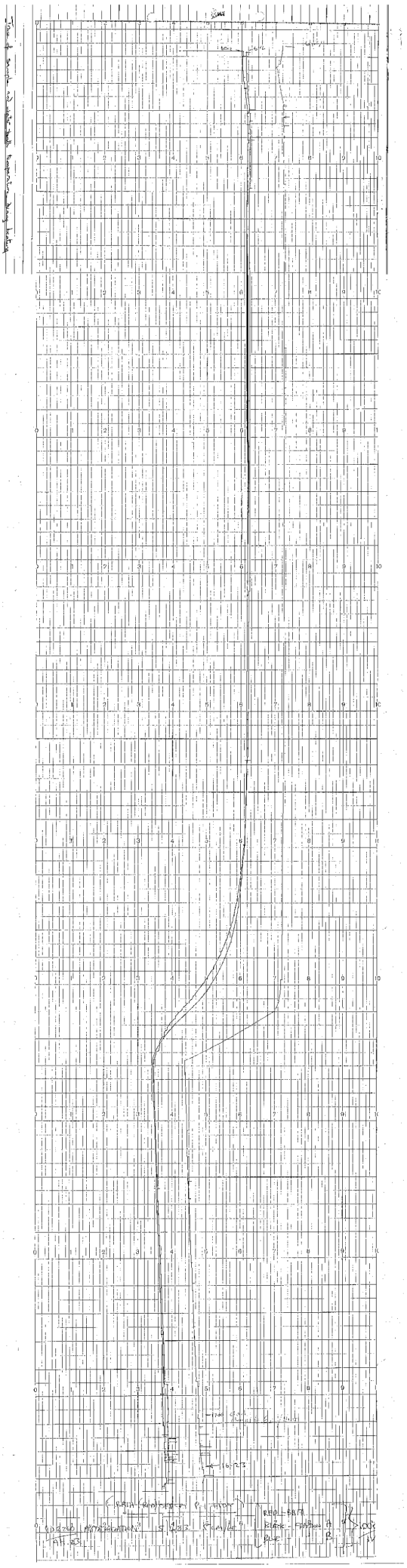
After 10 hours at 60°c the two fractions were removed from the water bath, 3 x 2ml samples of each taken, the ~~samples~~ fractions combined and mixed, then a further 3 x 2 ml samples taken.

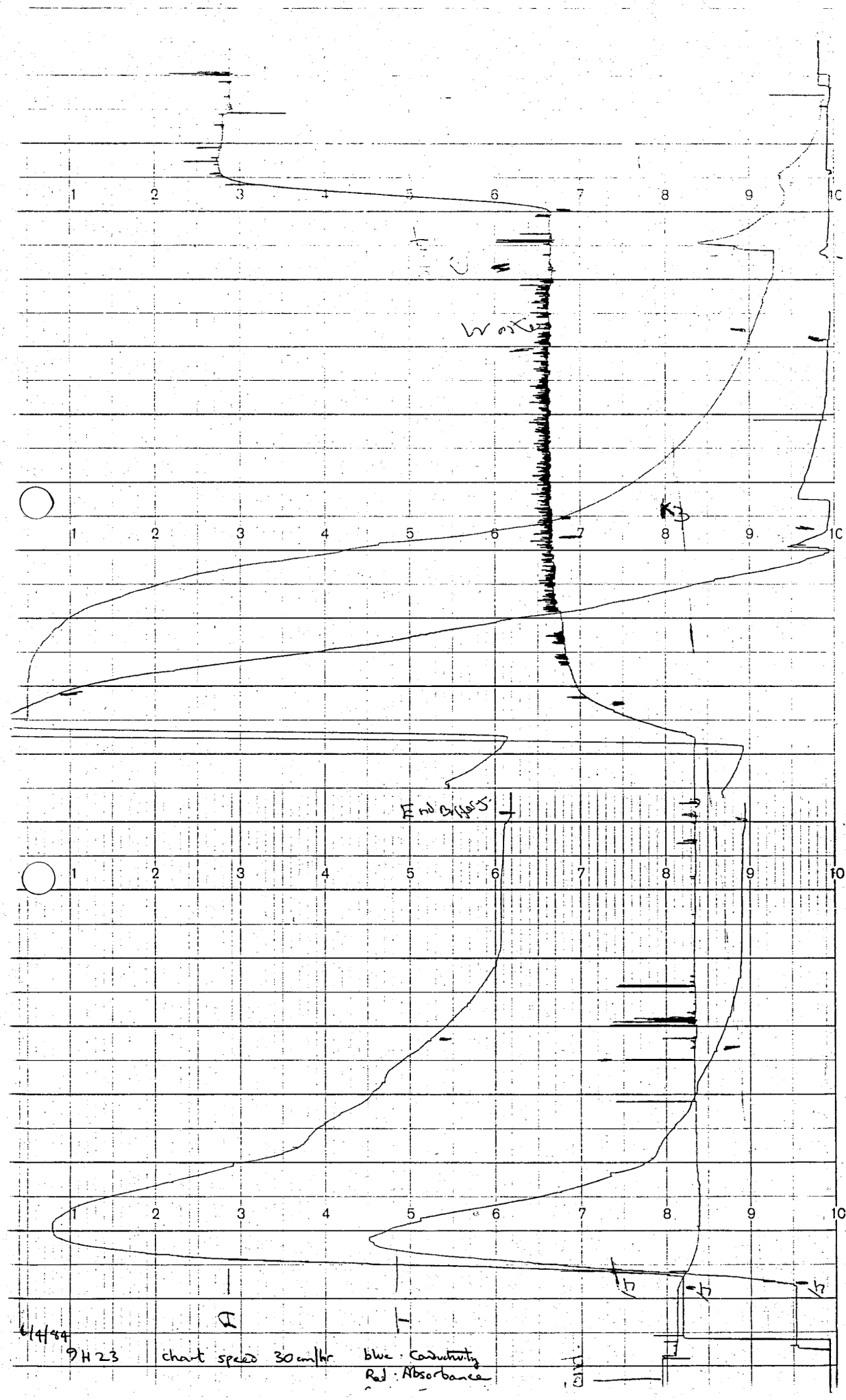
The procedure then followed the pattern described in 9H23 and in the accompanying schedule. This comprises:

Dilution in equal volume of PFW
Adsorption for 1 hour at 20° c to DE52 at a rate of 1 g DE52 per 200 units factor IX in the starting material
Elution, after packing into SAC 110 columns, with buffers J, K and C.
Flow rate: 80 ml / min.

Results

See attached traces of the heating temperatures and of the elution profile from the column.




1 2 3 4 5 6 7 8 9 10
Water
1 2 3 4 5 6 7 8 9 10
End Buffers
1 2 3 4 5 6 7 8 9 10
A
6/4/84
9H23
chart speed 30 cm/hr
blue · Conductivity
Red · Absorbance

JH 5

Protein Data

Sample	Volume (ml)	A280	Factor IX u/ml	Factor IX total	Sp Act u/mg	% starting factor IX	% applied factor IX	NAPTT	% NAPT blank (275)
9D2260	2510	1.48	i) 29.4 ii) 29.2	73794 73292	2.02	100		180	65
/AS	3200		i) 15.0 ii) 15.4	48640		66.4			
/BS	3110		i) 12.3 ii) 16.7	45095		61.5			
A9H23	3200		8.3	26560		36.2			
B9H23	3110		8.5	26435		36.1			
9H23	6300		8.5	53550		73.1			
α9H23	12600		3.2	40320		55.0	100	229	83
Sn	13428	0.41	0.06	806.7	0.15	1.1	2.0	272	99
J	2000	2.37	1.2	2400	0.51	3.3	6.0	294	107
K1	310	36.96	60.9	18879	1.65	25.8	46.8	146	53
K2	420	6.82	11.7	16614	1.72	22.7	41.2	197	72
K3	1570	0.61	0.38	596.6	0.62	0.8	1.5	232	84

Physical Data

Sample	Volume	pH	Conductivity
9D2260	2510	7.15	4.91
α9H23	12600	6.86	0.96
Sn	13428	8.16	1.21
J	2000	8.23	7.38
K1	310	7.18	15.08
K2	1420	7.14	25.74
K3	1570	7.03	26.72

Comments

1. For some reason, sorbitol elevates the apparent factor IX activity. This is seen in AS, BS A9H and B9H samples when the sample is diluted to X, then factor IX activity gives a yield of 55% on heating and this can be wholly accounted for in the column elutates.

2. The methods of sorbitol addition used make no difference to the survival upon heating. However, the final solution of each fraction A and B were little different both contained considerable amounts of trapped air.

3. Sorbitol addition in itself does not seem to cause a loss of factor IX activity.

4. Pooling of K1 and K2 gives an overall yield of 48.5%, a mixture containing 20 units/ml factor IX with a specific activity of 1.64 u/mg.

5. Again there is a significant drop in NAPTT with elution of K1. The possibility that the short NAPTT material is being pushed into the /K elutate due to the low salt in buffer J should be investigated by raising the salt concentration in the first buffer in an attempt to elute the short NAPTT material preferentially.

PAF.

AHF/DC/ES 9/4/84. 9H23

Samples to be kept? If so, how?

Samples ~~provided with form~~/available from R+D Big Fever

SAMPLE INVESTIGATION		9D2260	/AS*	/BS*	A 9H23*	B 9H23*	9H23*
actor VIII, iu/ml	1 st.						
	2 st.						
Factor IX, iu/ml	1 st.	(29.4)	(15)	(12.3)	(10.5)	(8.6)	(9.5)
	2 st.	29.2	15.4	16.7	8.3	8.5	8.5
Factor II, u/ml							
Factor X, u/ml							
Factor VII, u/ml (clotting)							
AT III (Anti Xa), u/ml							
AT III (Anti IIa), u/ml							
AT III (Laurell), u/ml							
Factor VIII RAG, u/ml							
Fibrinogen (Laurell), mg/ml							
Prothrombin (Laurell), u/ml							
Factor XIIIA (Laurell), u/ml							
Fibronectin (Laurell), mg/ml							
Factor VIII CAG							
NAPTT sec. 1/10		180					
TGt50 min.							
FDA hr.							
Limulus + or -							

* 200 µl (w/v) Solub. to

PAF/DC/EB 9/4/89 9H23

Samples to be kept? No If so, how?

Samples provided with form/available from Rx D By Freeze

SAMPLE INVESTIGATION		αγH23*	/Sn	/J	/K1	/K2	/K3
actor VIII, iu/ml	1 st.						
	2 st.						
Factor IX, iu/ml	1 st.	(4.8)	(0.15)	(1.4.)	(75)	(13)	(0.8)
	2 st.	3.2	0.06	1.2	60.9	11.7	0.38
Factor II, u/ml							
Factor X, u/ml							
Factor VII, u/ml (clotting)							
AT III (Anti Xa), u/ml							
AT III (Anti IIa), u/ml							
AT III (Laurell), u/ml							
Factor VIII RAG, u/ml							
Fibrinogen (Laurell), mg/ml							
Prothrombin (Laurell), u/ml							
Factor XIIIA (Laurell), u/ml							
Fibronectin (Laurell), mg/ml							
Factor VIII CAG		✓	✓	✓	✓	✓	✓
NAPTT sec. 1/10		229	272	294	146	197	232
~~TGT50~~ min. Naptt (control)		275	→				
FDA hr.							
Limulus + or -							

* 100 % (w/v) solution

-NF/DHEB 5/4/84 9N23

Samples to be kept? No If so, how?

Samples provided with form/~~available from~~

SAMPLE INVESTIGATION		9D2260	1AS*	1BS*			
actor VIII, iu/ml	1 st.						
	2 st.						
Factor IX, iu/ml	1 st.	(30)	(28)	(28)			
	2 st.	29.2 30.6	19.2 10.8	12.3 12.7			
Factor II, u/ml							
Factor X, u/ml							
Factor VII, u/ml (clotting)							
AT III (Anti Xa), u/ml							
AT III (Anti IIa), u/ml							
AT III (Laurell), u/ml							
Factor VIII RAG, u/ml							
Fibrinogen (Laurell), mg/ml							
Prothrombin (Laurell), u/ml							
Factor XIIIA (Laurell), u/ml							
Fibronectin (Laurell), mg/ml							
Factor VIII CAG							
NAPTT sec. 1/10							
TGt50 min.							
FDA hr.							
Limulus + or -							

* 200 % w/v Sorbitol

9H23

Factor IX Wet Heating Procedure - Provisional, Version 1.

Time

Starting Material

Eluate Batch: 9D2260.

Eluate Volume: 2510 ml

Eluate Weight: 2532.9 g

pH: 7.15

Conductivity: 14.91 mS

Fib-kit 4.104 g

Sample: 3 x 2ml
1 x 5ml
Send 2 x 1ml for immediate factor IX assay.

Stabilisers

10.30 1. Add glycine, 0.1g per g starting eluate

Weight of glycine added: 253.2 g

2. Dissolve by stirring ~~with~~ MANUALLY

3. Divide batch into 2 equal fractions, labelled "fraction A" and "fraction B".

4. Place fraction A in water bath set at 30o C.

½ to each fraction

_____ 5. Add sorbitol, slowly, with stirring, to fraction A at 1g of ~~sorbitol per g starting eluate (equivalent to 2g sorbitol per g of material).~~

Weight of sorbitol added: ~~1266~~ 2532g g

6. Weigh out sorbitol for fraction B, 1 g sorbitol per g eluate starting material (2g per g eluate in fraction B).

Weight of sorbitol: ~~1266~~ 2532g g.

Total sorbitol: 5065g

Sorbitol in each fraction: 2532.

9H23

Time

____ 7. Gradually add fraction B to the sorbitol, making first a thick paste, then diluting it by addition of more fraction B (cf Cambell's Condensed Soup). It will probably be necessary to do this step at 30° C.

Actually taken up to 40° C water temperature.

8. Sample 3 x 1ml fraction A/sorbitol (/AS)

3 x 1ml fraction B/sorbitol (/BS)

9. Volume fraction A/S: 3200(±20) ml

Weight fraction A/S: 3850 g

10. Volume fraction B/S: ~3110 (±10) ml

Weight fraction B/S: 3818 g

Heating

1. Transfer fraction A and fraction B to individual heating bottles and place in water bath over Edwards magnetic stirrers.

2. Connect temperature probes from each bottle and from the water bath to Rikadenki chart recorder.

3. Set water bath to switch on at 20.30 hrs, to heat up to 60o C. Ensure auto shut-off on water heater will accommodate this temperature.

9.45. 4. Remove bottles from water bath after 10 hours at 60o C.

5. Sample 3 x 2ml fraction A (A9H23)

3 x 2ml fraction B (B9H23)

6. Volume fraction A: _____ ml

9N23

Time

Weight fraction A: _____ g

7. Volume fraction B: _____ ml

Weight fraction B: _____ g

____ 8. Combine fractions A and B

Sample 3 x 2ml (9H23)

Volume: 6300 ml approx sum of (A+B) − 15 ml.

Weight: 7610 g

Dilution

10-20 1. Dilute 1/2 by addition of 1 9H volume of PFW. Mix.

Volume (or weight) PFW added: 6.37 ~~kg~~ g)

2. Sample 3 x 2ml

1 x 5ml (∝9H23)

3. Volume: ~~12600~~ 12600 ml

Diameter 27 cm, Height 22.5 cm. → 12882 ml

Weight: 13.96 ~~k~~ g

pH: 6.86

Conductivity: 0.96 mS

Adsorption

1. Factor IX activity (from day 1 assays)

Starting eluate: 29.4 u/ml

A/S : 15 u/ml mean of 2 (±4.2)

B/S : 12.25 u/ml mean of 2 (±0.05)

2. Total factor IX units before heating: ~~Average of~~ 48 000

73 412 38 097 +

86097

9N23

Time

_____ 3. Add DE 52, 1 g recycled DE52 weight for every 200 factor IX units before heating.

Weight DE 52 added : 430 g

4. Stir for 1 hour at lab. temperature, using Edwards magnetic stirrer. START 10-47.

Recovery of DE 52

11.50 1. Pour slurry into GAC 110 column with extender tube attached. Pump through with Watson Marlow pump, attached to bottom of column.

2. Collect wash-through in one container. (/Sn)

Elution

1. Disconnect pump and fix pressure head set-up.

2. Bed height : 9 cm

Bed volume : 95.03 x Bed height = 855 ml

_____ 3. Fill pressure vessel with 5 x bed volumes of Buffer J

Volume Buffer J: 4275 ml — CALCULATED

Weight Buffer J: 4275 g — MEASURED.

4. Measure flow rate: 80 ml/min

Nitrogen pressure : ½ 16

5. Monitor : u.v. transmission ✓
u.v. absorbance ✓
conductivity ✓
refraction ✗

9N 23

Time

6. Collect material which elutes as indicated by change in transmission (/J).

_____ 7. When buffer J has finished, fill pressure vessel with 5 bed volumes of Buffer K and elute.

8. Volume Buffer K: _____ ml

Weight Buffer K: 4275 g

9. Collect fractions:

/K1 - from beginning of transmission rise to peak maximum.

/K2 - from end of /K1 to stabilisation of eluate conductivity.

/K3 - remainder of transmission peak and tail.

_____ 10. Wash with 3 bed volumes of Buffer C.

Volume Buffer C: _____ ml

Weight Buffer C: _____ g

11. Collect any protein transmission peak that elutes. (/C).

12. Blow column dry.

9H23

Date _____ Buffer K for 9H23

Final concentrations

8.9 mM trisodium citrate

1.1 mM citric acid

10 mM Na_2HPO_4

300 mM NaCl

To make up 8L (8Kg)

Weight		Batch no.	Quantity used
20.96 g	Trisodium citrate	9293700-17-38.	21.0g
1.84 g	citric acid	91481500	1.85g
11.36 g	Na_2HPO_4	9260374-061-5.	11.35g.
140.2 g	NaCl	9397545-14-90.	140.2g

Make up to 8L (8Kg) with PFW

pH limit: 7.00 +- 0.05 pH found: 6.95

Adjust as necessary

Conductivity limit: 28.0 +- 1.0 Conductivity found: 27.6 mS

7H23

Date _____ Buffer J for 9H23

Final concentrations

8.5 mM trisodium citrate

1.5 mM citric acid

10 mM Na_2HPO_4

80 mM NaCl

To make up 5L (5Kg)

Weight		Batch no.	Quantity used
12.5 g	Trisodium citrate	9293700-17-38	12.5g
1.55 g	citric acid	91481500	1.55g
7.1 g	Na_2HPO_4	9260374-061-5	7.1g
23.35 g	NaCl	9397545-14-90	23.4g

Make up to 5L (5Kg) with PFW

pH limit: 7.00 +_ 0.05 pH found: 6.95

Adjust as necessary

Conductivity limit: 10.0 +_ 1.0 Conductivity found: 10.0 mS

9H23

Date _____ - Buffer C for 9H 23

Final concentrations

20 mM trisodium citrate

1.0 mM NaCl

To make up 3L (3Kg)

Weight		Batch no.	Quantity used
17.7 g	Trisodium citrate	92937001 17-38	17.7g
140.2 g	NaCl	93975451 14-90	140.3g

Make up to 3L (3Kg) with PFW

pH limit: 8.00 +- 1.0 pH found: 7.75

Adjust as necessary

Conductivity limit: 80.0 +- 10 Conductivity found: 63 mS

9N23

Record of samples from 9H 23

From each collect fraction of eluate (/sn - /C) take: 3 x 2ml samples

1 x 5ml sample.

1×5 ml syringe.

Sample

/Sn

Volume 13428 ml

Weight 13650 g

pH 8.16.

Conductivity 1.21 mS

Diameter 30.4 cm
Height 18.5 cm

A280: 0.41

retain 300 ml specimen only.

Sample

/J

Volume 2000 ml

Weight 2071 g

pH 8.23

Conductivity ~~2.94~~ mS 7.38

A280: 2.37

Sample

/K1

Volume 310 ml

Weight 317.7 g

pH ~~7.88~~ 7.18

Conductivity 15.08 mS

A280: 36.96

Sample

/K2

Volume 1420 ml

Weight 1437.3 g

pH 7.14

Conductivity 25.74 mS

A280: 6.82

9N23

Sample

/ K3.

Volume 1570 ml

Weight 1592 g

pH 7.03

Conductivity 26.72 mS

Avg: 0.61

Sample

/_____

Volume _____ ml

Weight _____ g

pH _____

Conductivity _____ mS

Sample

/_____

Volume _____ ml

Weight _____ g

pH _____

Conductivity _____ mS

Sample

/_____

Volume _____ ml

Weight _____ g

pH _____

Conductivity _____ mS

Sample

/_____

Volume _____ ml

Weight _____ g

pH _____

Conductivity _____ mS

9.3.1. Heat inactivation of viruses in factor IX concentrates

Progress Report, April 1984

General comments

Over the last five months experiments on the heating of factor IX have concentrated upon only two procedures: heating of factor IX, in solution, in the presence of sorbitol and glycine, and the heating of factor IX in the dried state.

The heating experiments on factor IX in solution have involved the scaling-up of procedures previously reported to a size which is comparable with a single PFL production batch of 9P 1/5 eluate.

The dry-heating experiments have involved lengthening of the heating period and increasing the heating temperature, both for heparinised and non-heparinised factor IX.

The relevant experiment numbers are indicated in brackets.

Heating of factor IX with sorbitol/glycine in solution

A. Heating Step

(1) There is little difference in the behaviour of factor IX starting materials from different sources such as finished, dried vials, unfinished eluate, finished eluate, pyrogen failed material or short NAPTT material (9H18 - 9H23).

(2) Sorbitol addition (2g per ml starting material) when the starting volume is 2 litres presents problems both in the physical addition, which is time-consuming and tedious, and in the solution itself during which a large amount of air is trapped in the viscous fluid.

(3) Recoveries of factor IX after 10 hours' heating at 60°C ranges from 45% (9H21) to 77% (9H19) with an average of 54%. The wide variation may be due to:

(i) differences in stirring efficiency during heating
(ii) differences in trapped air in solution
(iii) difficulties in accurately measuring the bulk volumes of material
(iv) improved yield using polythene or polycarbonate heating vessels instead of stainless steel (9H19, 9H20).

B. Recovery of factor IX after heating

(1) To facilitate adsorption to DE 52, after heating, the solution needs to be diluted by half in its own volume of PFW. Adding less PFW impairs the adsorption and adding more PFW does not improve it further (9H20-02).

(2) The optimum amount of gel is 1g per 156 units of heated factor IX (9H22-03). Attempts to relate this value back to a starting potency for factor IX (in order to eliminate in-process assays) have been complicated by the variation in yield after heating.

(3) 100% of factor IX applied to DE 52 can now be accounted for in the eluate fractions. This is attributable to the application of larger buffer volumes (5-6 column bed volumes) and to a modification of the factor IX production buffers, comprising a lower salt first wash with a higher salt second wash.

(4) The elution characteristics are highly dependent upon flow rate of buffer because the height of the gel is small. The flow rate should be kept down to about 1-3 ml min^{-1} cm^{-2}.

(5) Of the heated factor IX loaded onto the DE 52, 65-70% can be recovered in the second salt wash as a usable fraction.

(6) The overall yield of the procedure is therefore 35-38%.

(7) Regardless of the NAPTT of the starting material, there is a shortening of the NAPTT of factor IX in the front of the second elution buffer. This is matched by a slight lengthening of NAPTT in the earlier fractions.

Future experiments will concentrate on this problem by slightly raising the salt in the first buffer wash and also to test for possible interactions between components in the different fractions which may normally cause a composite NAPTT when not separated.

(8) SDS PAGE indicates that the proteins are little changed by the heating, though a small amount of thrombin may be generated (then inactivated) (9H19).

The overall recovery on the large scale therefore appears similar to that on the smaller scale. The heating step yields are now a fairly consistent 50-55%. This is disappointing after early yields using plastic containers of 65-77% (9H19, 9H20). The method and care with which the sorbitol is added does not affect the factor IX potency (9H23) so the loss is genuinely incurred during heating. The presence of trapped air may be significant in this process.

Heating of factor IX dry

1. Factor IX is very stable to heating in the dry state and, under all the conditions used, reconstituted to give a normal solution.

2. Contrary to early indications, there is little difference in the behaviour of factor IX upon heating in the presence or absence of heparin.

3. At 60°C there is 100% factor IX survival after 72 hours. This is the longest period tested to date and not the maximum which yields 100% survival (DH8).

4. At 70° 100% factor IX remains after 48 hours, 90-95% after 72 hours.

5. At 80° 90-100% factor IX activity is retained after 48 hours, dropping to 85-95% after 72 hours.

6. A few experiments at 90° suggest that 80-90% factor IX remains after 24 hours. This is the longest period so far tested.

7. The NAPTT values are always within 70% of the NAPTT blank. In absolute terms this means times of between 170-240 seconds.

8. One experiment has now been carried out using the production laboratory oven to heat the dry factor IX vials, instead of a water bath (DH9). After 48 hours at 80° there is a 75% recovery of factor IX activity, with unchanged NAPTT. This follows the experience with factor VIII that changing the means of heating may alter the recovery, and may be due to differences in the distribution of heat in the container. More experiments are necessary to confirm this.

In view of the poor recovery and time-consuming work involved in heating factor IX in solution, the dry-heating may well provide an easier and more efficient alternative.

Experiments are now in progress in collaboration with BPL to assess the effect of dry-heating factor IX on viruses added before drying. The outcome of these experiments may help in the selection of a particular procedure for further development.

P.A.F.

16.4.84.

9H24 25.4.84 DC, EB, PA

To examine the effect of increasing the "salt" in the first DE52 buffer on the NAPTT of the FIX solution eluted by buffer K.

In order to reduce the amount of FIX washed from the DE52 column by the 1st buffer, we had decreased the salt from the level in buffer A (100mM NaCl) to 80mM NaCl in buffer J (9H17). The citrate and phosphate concentrations were kept constant (10mM in each case).

We have observed a sharp dip in the NAPTT of the FIX eluate on going from the ~/J eluate to the ~/K1 eluate. This was most marked in 9H21 where we had the following :-

Sample	NAPTT	NAPTT as % of CONTROL
Control	233	100
starting FIX	120	51
/J	>420	180
/K1	88	37
/K2	64	27
/K3	128	54

but in general the results from 9H23 are more typical.

From 9H23

Sample	NAPTT	NAPTT as % of control
Control	275	100
starting FIX	180	65
/J	294	106
/K1	146	53
/K2	197	71
/K3	232	84

It was suggested (via PAF) that the low salt in the 1st buffer may have given rise to the short NAPTT in the K eluates this experiment (9H24) is so designed to see the effect of changing buffer J for a new one (buffer L) containing 125mM NaCl.

25.4.84 - 9H24 DC

Materials

FIX : This was from the batch 9D2231.9t contained no heparin and was presented in 35ml filled vials which had been freeze dried.

GLYCINE : FROM BDH Batch NO 9413380D

SORBITOL : FROM BDH

PFW : This was collected at ≈ 16:00h on the day before use and cooled to 4°C in CR2.

BUFFER L : 8.9mM Trisodium Citrate
1.1mM Citric Acid
10mM Na_2HPO_4
125mM NaCl
} pH 7.0 ± 0.0

BUFFER K : 8.9mM Trisodium Citrate
1.1mM Citric Acid
10mM Na_2HPO_4
300mM NaCl
} pH 7.0 ± 0.0

BUFFER C : 20mM Trisodium Citrate
1.0M NaCl
} pH 8 ± 1

DE52 : This was from the recycled batch R+D/3

Methods

FIX RECONSTITUTION : Each vial of dried FIX was reconstituted with 35ml of PFW at room temperature. The resulting FIX solution of all the vials was pooled and mixed thoroughly in a 5l plastic beaker. The pH and conductivity as well as volume and weight were measured. The solution was sampled as follows: 2x1ml (sent for immediate FIX assay) 3x2ml and 1x5ml.

25.4.84 - 9H24 X

Method Continued.

ADDITION OF GLYCINE AND SORBITOL : 170.2g of glycine were added to the FIX solution (10% weight of FIX solution). This was dissolved by manual stirring.

The FIX / glycine mixture was placed in a waterbath at 30°C. 3404g of sorbitol (2g per 1g FIX solution) were added over 2 3/4 hours while the mixture was stired by an Edwards 80" magnetic stirer placed under the waterbath.

The temperature of the mixture was not allowed to fall below 19°C during the sorbitol addition.

When all the sorbitol had dissolved (producing 9D 2231/S 25.4.84) the mixture was sampled - 3x1ml.

HEATING : a ss bucket lid which fitted the lip of the beaker tightly and left a small (≈ 1 cm) air gap between it and the surface of the FIX solution was placed on the beaker.

Two temperature probes were set up : one to measure the temperature of the FIX solution and the other to measure the temperature of the water near the beaker of FIX. The probes were connected to individual electronic thermometers which were connected to the 6-pen Rickadenki chart recorder.

A time switch was set to switch on the waterbath heater at 21.00h and the heater regulator reset to 60°C.

The surface of the bath water was covered with plastic spheres and the whole set up was covered with Al foil.

The mixture was left stirring over night.

DAY 2

The FIX solution was removed from the heating bath at 10.00h. It had been at ≈ 60°C for 10 3/4 hours. Hence 9H24 had been produced. It was weighed and its volume was measured.

DILUTING OF 9H24 :

4080g of cold PFW (4°C) were added to the 4080ml of 9H2 producing ≈ 9H24. This was then weighed and its volume, pH and conductivity measured. The ≈ 9H24 was sampled (3x2ml and 1x5ml.

PU

25·4·84 9H24 DC

Methods continued

Addition of DES2 and Absorption of FIX

The Day 1 FIX assay of 9D2231/25·4·84 showed that we had 31·6 u FIX/ml. Therefore in the 1670 ml of the starting material we had a total of 52772 u FIX.

263·9g of recycled DES2 were added to ∝ 9H24 giving 1g DES2 to 200 u FIX in the starting 9D2231.

The DES2/FIX mixture were stirred for 1 hour at room temperature (≈18°C) using an Edwards 80 magnetic stirrer

Packing the column

A 4Cm GAC110 column was set up during the DES2 absorption step. It was arranged so that its effluent would pass through a 'Uvicord' transmittance/absorbance monitor and conductivity meter. The outputs from the monitoring instruments were connected to the 6-pen Rikadenki chart recorder which was adjusted as follows:-

— Transmittance: 100mV, Absorbance: 100mV, Conductivity: 20mV chart speed 12cm/hour.

The column was extended using a 60cm GAC110 barrel

After the DES2 absorption step the slurry was poured into the column and the outlet connected to a Watson-Marlow 501S pump. The pump speed setting was 70%. The supernatant was pumped from the bottom of the column and collected → 9H24/Sn. When ≈ 5cm of supernatant were left above the DES2 bed the pump was stopped, the column outlet clamped off and the pump removed. The Uvicord and Conductivity meter were then connected to the column. A 50cm packing piece (piston), bubble trap and pressure vessel were set up in the usual way to pump buffer through the DES2 using N_2 pressure.

The DES2 bed height was measured and its volume calculated. The bed was washed with 5x its volume of buffer L (2110g = 2138ml) using ½lb/sq in N_2 pressure with a flow rate of 54ml/minute.

The diluted material associated with the transmittance was collected as a single aliquot /L.

P5

25.4.84 9H24 DC

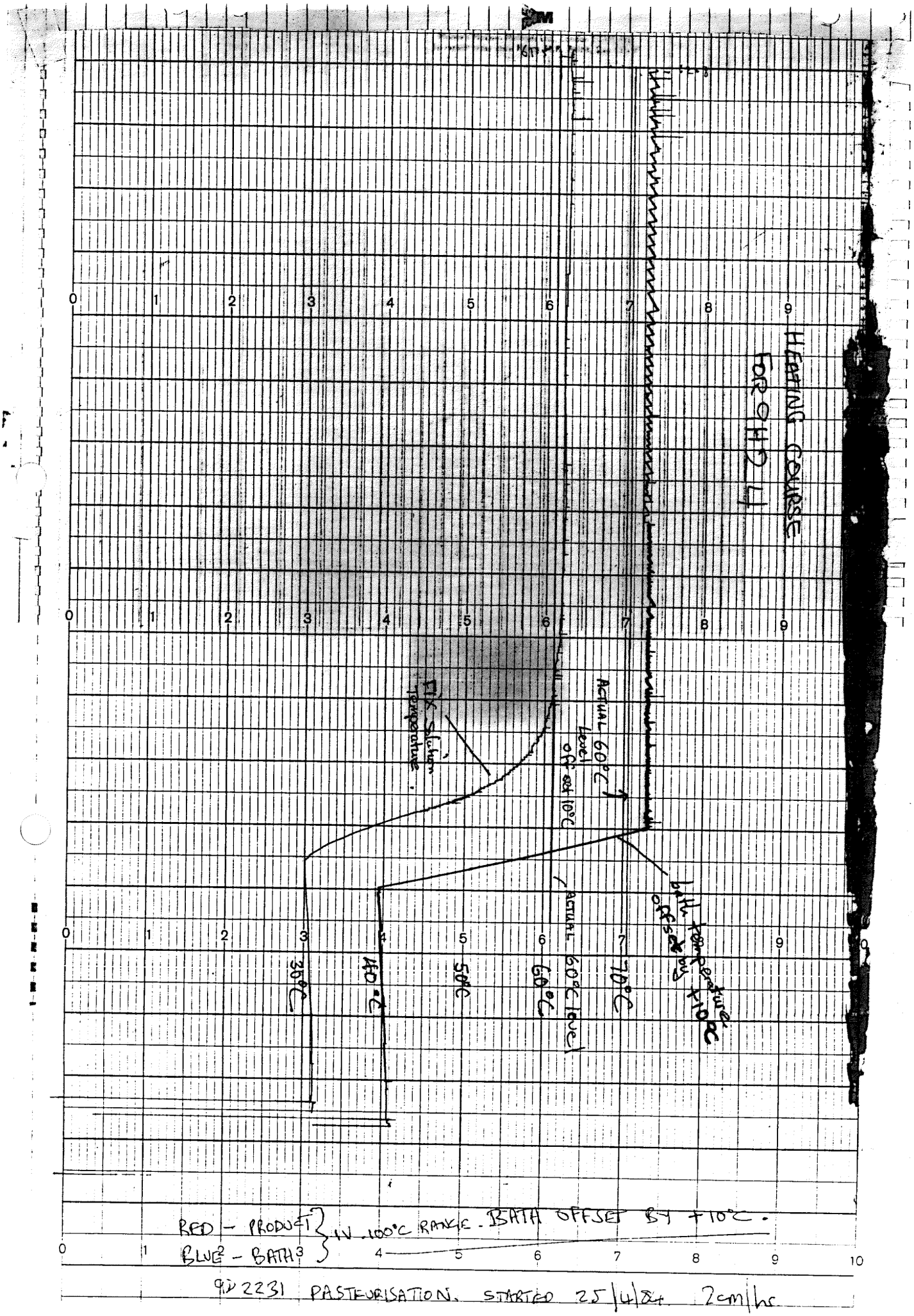
Method continued

When buffer L was finished the column was washed with ≈6x the bed volume of buffer K (2580g). The eluted material associated with a transmittance/absorbance peak was collected in 3 aliquots determined as follows:-

~/K1 from beginning of transmittance rise to its peak
~/K2 from end of /K1 to stabilization of eluate's conductivity
~/K3 remainder of transmittance peak and tail.

Time = 15.10 → Once buffer K was finished the column was washed with 3x the bed volume of buffer C and allowed to blow dry. The DE52 was collected for recycling:
a 540 ml aliquot which was associated with the change in conductivity between buffer K and buffer C was collected (~/C).

The parameters of each collected aliquot as shown in the results table were measured. They were sampled (3x2) and analysed for FIX, NAPTT and Protein content.


HEATING COURSE FOR OHD 4
0 1 2 3 4 5 6 7 8 9 10
FIX Solution temperature.
ACTUAL 60°C Level off set 10°C
ACTUAL 60°C level
bath temperature offset by +10°C
35°C
40°C
50°C
60°C
70°C
RED - PRODUCT
BLUE - BATH
+V .100°C RANGE - BATH OFFSET BY +10°C.
9D 2231 PASTEURISATION. STARTED 25/4/84 2cm/hr

25.4.84 - 9H24 DC

Results

On reconstitution the 9D2231 dissolved easily and produced a transparent slightly yellow solution.

HEATING

The recording of the heating course is shown on the preceeding page.

It took approximately 3 hours for the temperature of the FIX solution to rise from 30°C to 60°C after the bath was switched on. The water temperature rose from 30°C to 61.5°C in 1 hour.

The FIX temperature remained between 60°C and 61.6°C for 10 hours before it was removed from the waterbath. Up to 8 hours of this time were spent with the FIX temperature between 61°C and 61.6°C.

When the FIX was removed from the waterbath lines of refractive index discontinuities were seen in it. This shows that despite the stirring action of the magnetic stirrer there was incomplete mixing during the pasteurization.

The pasteurized FIX solution was free of particles and air bubbles.

ABSORPTION AND RECOVERY OF THE FIX FROM DE52

The elution profile (transmittance, absorbance and conductivity) is shown on the following page. The points at which fractions were collected are also shown.

CBLA0002456_0367

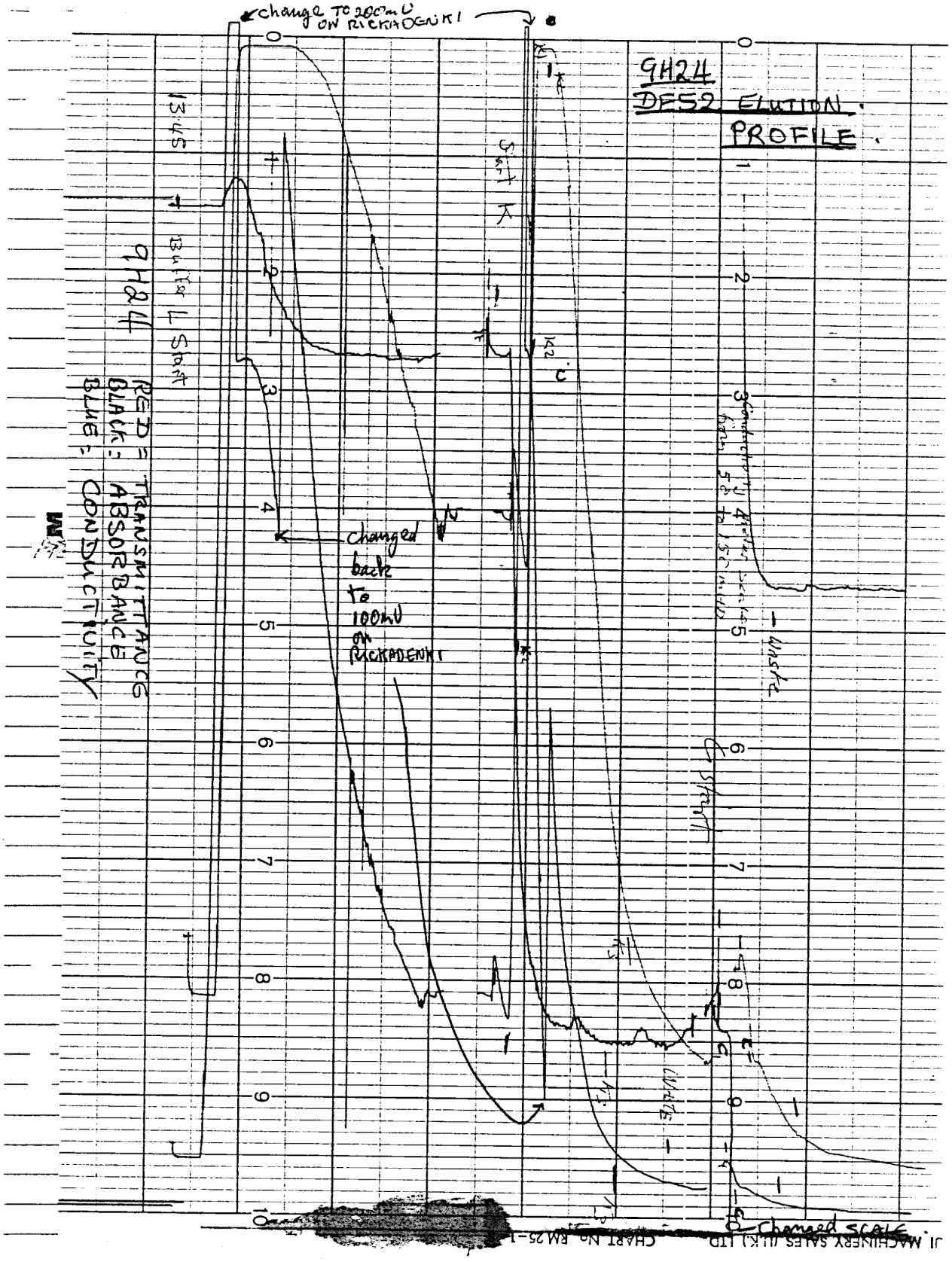
9H24

P66

25.4.84

9H24

DX



25-4-84 9H24

Results Continued.

ABSORPTION AND ELUTION OF THE FIX FROM THE DE52

The general parameters of starting materials and collected fractions are shown in the following table.

MATERIAL/FRACTION	Vol (ml)	Weight (g)	pH	Conductivity
9D2231	1670	1701.9	7.10	17.82
9H24	4080	5217	—	—
α9H24	8230#	9204	6.85	1.64
—/Sn	8686*	8830	8.06	1.46
/L	2150	2186	8.12	11.47
/K1	160	144	7.52	15.74
/K2	1210	1227	7.31	26.23
/K3	685	701	7.07	27.54
/C	540	561	7.01	54.10

calculated using Formula for volume of a cylinder
* Calculated assuming a density of 1.016g/ml.

The results of the analysis of the samples are shown below:-

Sample	Vol(ml)	FIX						NAPTT	
		14/ml	14 TOTAL	14 % of 9D2231	14 % of α9H24	A280	Specific Activity		% of blank
9D2231	1670	39.4	65798	100	—	15.38	2.56	198	66.9
α9H24	8230	2.85	23455	35.6	100	—	—	270	91.2
/Sn	8686	0.15	1303	1.98	5.5	0.582	0.26	278	93.9
/L	2150	4.56	9804	14.9	41.7	5.9	0.77	237	80.1
/K1	160	30.4	4864	7.3	20.7	14.0	2.17	207	69.9
/K2	1210	4.8	5808	8.8	24.7	3.22	1.49	248	83.8
/K3	685	0.39	267	0.4	1.1	0.388	1.00	279	94.2
/C	540	0.21	113	0.17	0.48	0.183	1.15	300	101
								blank 296	100

9N4
P8

25.4.84 9H24

Conclusions and Discussion

35.6% of the FIX survived the heating step in ∝9H24. This is based on the 2nd assay results for 9D2231, but if the results of the 1st Assay were used then the figure would be 44.4% (25% up). Even if the survival was 44.4% it would still be lower than our aim of 50% minium. The survival of FIX in 9H23 was 55% (measured in ∝9H23).

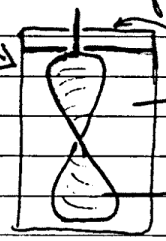
It may be possible to increase the FIX survival in the following ways:-

① By using a vessel which allows better heat penetration than the polystyrene beaker used here. This will give a shorter heat up time.

② Manual stirring of the FIX while heating it to 60°C at the start of the heating procedure and allowing the pasteurization to be stopped by a timeswitch.

This would ensure even heat distribution in the mixture from a known time and also the reduction of the viscosity at the higher temperature would allow air bubbles to escape quickly.

③ The mixture should be stirred more efficiently. This may be done from overhead using a spiral paddle. eg



volume of solution is stirred and also any evaporated water which recondenses back on the surface may be mixed in quickly.

94.2% of the FIX applied to the DE52 may be accounted for. Its distribution is as follows:-

Sn	5.5%
L	41.7%
K1	20.7%
K2	24.7%
K3	1.1%
C	0.48%

PS 9H24

25.4.84 9H24 DC-

Conclusions and Discussion

This contrasts with 9H23 where we were able to account for 97.5 % of the FIX applied to the DE52 and where we had the following distribution

/Sn	2%
/J	6%
/K1	46.8 %
/K2	41.2 %
/K3	1.5 %

It seems that the high amount of FIX eluted by buffer L is due to the higher salt in that buffer compared to buffer J. The salt in buffer L should therefore be reduced towards that of buffer J.

The relatively high amount of FIX in /Sn may be due to the front of buffer L mixing with the tail of the /Sn and not to poor absorption.

Despite the higher amount of FIX in /L than /K1 the specific activity is much lower in /L (0.77 cf. 2.17).

In 9H23 the specific activity in /J, /K1 and /K2 were 0.51, 1.65 and 1.72 respectively. It seems therefore that the higher salt in buffer L not only brings off more contaminating proteins but a greater proportion of FIX. Hence though the buffer L salt concentration should be dropped it should still remain higher than in buffer J.

There is no long NAPTT associated with the 1st buffer wash as in 9H21. However this does not appear to be closely related to the 1st buffer salt concentration. Although the pattern we have in this experiment is "unique" it is still similar to that obtained when buffer J was used in 9H23. The similarity of these patterns lies in the fact that the NAPTT lengths follow

25·4·54 9H24 JC

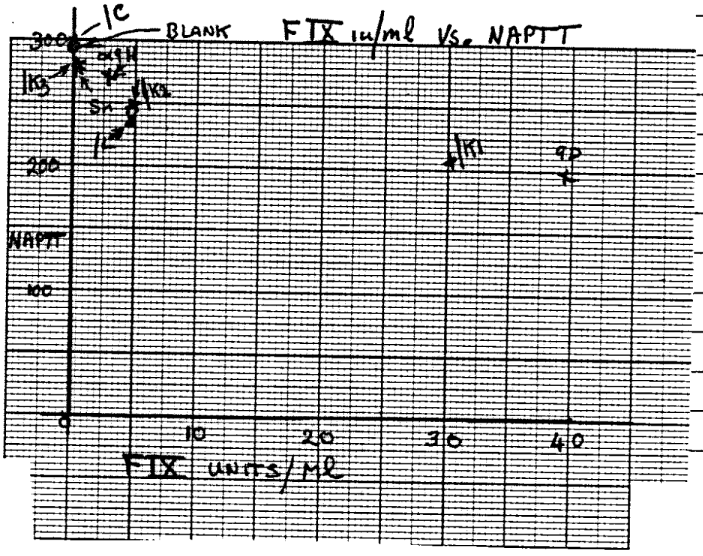
the pattern of iu FIX per ml. The lower the amount of FIX the closer the NAPTT to the blank. It seems therefore that the NAPTT observed in these experiments is not independent of the FIX concentration, and that it may be varied with simple dilution of the FIX solution. The "α" dilution usually has a longer NAPTT than the stating "9D"

More data on NAPTT and FIX concentration is required before general conclusions can be made, but the graph included here shows the trend.

Could the unusual NAPTT pattern seen in 9H21 have been due to there being as much as 68 iu FIX/ml in one of the stating batches?

It would be interesting to see the NAPTTs carried out as usual, and also diluted to give the same No of iu FIX/ml in each fraction.

9H24



25.4.84 9H24 DC

Overall Points:

① Be aware of the variability of the FIX analysis in the 1st and 2nd assays.

② Only 16.1% of the starting (9D2231) was recovered in the potential usuable eluates (K_1 and K_2). This is a very poor yield.

To increase yield in the K eluates use a new buffer L with lower NaCl concentration but still more than in buffer J.

To improve the FIX survival on heating – heat to 60°C early on; use a more heat transparent vessel and stir to keep all parts of the solution moving.

③ Within the limits of our experiments the NAPTTs appear to follow the dilution of the FIX and not the salt concentration.

DC

PAF

25/4/94 9H27

Samples to be kept? No If so, how?

Samples provided with form/~~available from~~

SAMPLE INVESTIGATION		9D 2231					
actor VIII, iu/ml	1 st.						
	2 st.						
✓Factor IX, iu/ml	1 st.	(29)					
	2 st.	31.6					
Factor II, u/ml							
Factor X, u/ml							
Factor VII, u/ml (clotting)							
AT III (Anti Xa), u/ml							
AT III (Anti IIa), u/ml							
AT III (Laurell), u/ml							
Factor VIII RAG, u/ml							
Fibrinogen (Laurell), mg/ml							
Prothrombin (Laurell), u/ml							
Factor XIIIA (Laurell), u/ml							
Fibronectin (Laurell), mg/ml							
Factor VIII CAG							
NAPTT sec. 1/10							
TGt50 min.							
FDA hr.							
Limulus + or -							

C/PAF/EB 27/4/84 9H24

Assay results sent to: ASAP

Samples to be kept? No If so, how?

Samples ~~provided with form~~/available from R+D Big Frozen

SAMPLE INVESTIGATION		9D2231 ‡	∝9H24 *	1Sn	1L	1K1	1K2
actor VIII, iu/ml	1 st.						
	2 st.						
Factor IX, iu/ml	1 st.	(30)	(4·5)	(<0·1)	(5·5)	(30)	(6·0)
	2 st.	39·4	2·85 ~~3·0~~	0·15	4·56	30·4	4·8
Factor II, u/ml							
Factor X, u/ml							
Factor VII, u/ml (clotting)							
AT III (Anti Xa), u/ml							
AT III (Anti IIa), u/ml							
AT III (Laurell), u/ml							
Factor VIII RAG, u/ml							
Fibrinogen (Laurell), mg/ml							
Prothrombin (Laurell), u/ml							
Factor XIIIA (Laurell), u/ml							
Fibronectin (Laurell), mg/ml							
Factor VIII CAG							
NAPTT sec. 1/10		198	270	278	237	207	248
TGt50 min.							
FDA hr.		3	6+		3	3	5½
Limulus + or -							

* 1g/ml Sorbitol

‡ No heparin.

PAF 2/14/14 9H24

Samples to be kept? No If so, how?

Samples ~~provided with form~~/available from R&D Big freeze.

SAMPLE INVESTIGATION ✓9H24		/k3	/C				
actor VIII, iu/ml	1 st.						
	2 st.						
✓ Factor IX, iu/ml	1 st.	(0.6)	(0.1)				
	2 st.	0.39	0.21				
Factor II, u/ml							
Factor X, u/ml							
Factor VII, u/ml (clotting)							
AT III (Anti Xa), u/ml							
AT III (Anti IIa), u/ml							
AT III (Laurell), u/ml							
Factor VIII RAG, u/ml							
Fibrinogen (Laurell), mg/ml							
Prothrombin (Laurell), u/ml							
Factor XIIIA (Laurell), u/ml							
Fibronectin (Laurell), mg/ml							
Factor VIII CAG							
✓ NAPTT sec. 1/10		279	300		control 296		
TGt50 min.							
FDA hr.							
Limulus + or -							

YH24

Date 25.4.84

Factor IX Wet Heating Procedure - Provisional, Version 2.

Time

Starting Material

Eluate Batch: 9D2231

Eluate Volume: 1670 ml

Eluate Weight: 1701.9 g

2112.4
-410.5
1701.9

pH: 7.10

Conductivity: 17.82 mS

Sample: 3 x 2ml
1 x 5ml
Send 2 x 1ml for immediate factor IX assay.

Stabilisers

14.25 1. Add glycine, 0.1g per g starting eluate

Weight of glycine added: 170.2 g 9413380 D

2. Dissolve by stirring (with) MANUALLY

3. Place in water bath set at 30° C.

14.30 4. Add sorbitol, slowly, with stirring, equivalent to 2g sorbitol per g of material.

Weight of sorbitol added: 3404 g

17.15. 5. Sample 3 x 1ml (/S)

6. Volume /S: _____ ml

Weight /S: _____ g

9#24

Time

Heating

1. Transfer /S to heating bottle and place in water bath over Edwards magnetic stirrer.

2. Connect temperature probes from bottle and from the water bath to Rikadenki chart recorder.

3. Set water bath to switch on at 20.30 hrs, to heat up to 60° C. Ensure auto shut-off on water heater will accommodate this temperature.

10.00. 4. Remove bottle from water bath after 10 hours at 60° C.
(Though stirring, the mixture, now clear, showed refractive patterns suggesting that the sorbitol was not evenly distributed through the soluble volume.).

5. Sample 3 x 2ml (9H___)

6. Volume 9H24 : 4080 ml

Weight 9H24 : 5217·2 g 5627.7 − 410.5 = 5217.2

7. ~~Sample 3 x 2ml (9H___)~~

~~Volume: _____ ml~~

~~Weight: _____ g~~

Dilution

10.26 1. Dilute 1/2 by addition of 1×9H volume of PFW. Mix.

~~Volume~~ (or weight) PFW added: 4080 ~~ml~~ (g)

2. Sample 3 x 2ml

1 x 5ml (∝9H24)

3. Volume: 8230·2 (±90) ml $\left(\frac{23.8}{2}\right)^2 \times \pi \times 18.5 \ (\pm 0.2)$

444·88

9 N24

Time

Weight: 9204.4 g

11360.3
2155.9-
9204.4

pH: 6.85

Conductivity: 1.64 mS

Adsorption

1. Factor IX activity (from day 1 assays)

   Starting eluate: 31.6 u/ml

2. Total factor IX units before heating: 52772 unit

10.30 3. Add DE 52, 1 g recycled DE52 weight for every 200 factor IX units before heating.

   Weight DE 52 added : 263.9 g

4. Stir for 1 hour at lab. temperature, using Edwards magnetic stirrer.

Recovery of DE 52

_____ 1. Pour slurry into GAC 110 column with extender tube attached. Pump through with Watson Marlow pump, attached to bottom of column.

2. Collect wash-through in one container. (/Sn)

Elution

1. Disconnect pump and fix pressure head set-up.

2. Bed height : 4.5 cm

9 H24

Time

Bed volume : 95.03 x Bed height = 428 ml

13.40 3. Fill pressure vessel with 5 x bed volumes of Buffer L

Volume Buffer L: 2135 ml

Weight Buffer L: 2110 g

4. Measure flow rate: 54 ml/min

Nitrogen pressure : ≈ ½16/sq in

5. Monitor : u.v. transmission ✓
u.v. absorbance ✓
conductivity ✓
refraction ✗

6. Collect material which elutes as indicated by change in transmission (/L).

_____ 7. When buffer has finished, fill pressure vessel with 6 bed volumes of Buffer K and elute.

8. Volume Buffer K: _____ ml

Weight Buffer K: ~~2566~~ 2580 g.

9. Collect fractions:

/K1 - from beginning of transmission rise to peak maximum.

/K2 - from end of /K1 to stabilisation of eluate conductivity.

/K3 - remainder of transmission peak and tail.

14.40 10. Wash with 3 bed volumes of Buffer C.

Volume Buffer C: _____ ml

9424

Time

Weight Buffer C: _____ g

11. Collect any protein transmission peak that elutes. (/C).

15.10 12. Blow column dry.

9424

Record of samples from 9H

From each collect fraction of eluate (/sn - /C) take: 3 x 2ml samples

1 x 5ml sample.

A_{280}

Sample

/ Sn

Volume 8686* ml

Weight 8.83 kg

pH 8.06

Conductivity 1.46 mS

Neat: 0.582

A_{280}

(* calculated assuming a density of 1.016 g/ml.)

Sample

/ L

Volume 2150 = (2130 (+20ml -SAMPLE)) ml

Weight 2186 g

pH 8.12

Conductivity 11.47 mS

2x300 ml kept remainder discarded.

1/10 : 0.590

TotN: 5.9

Sample

/ K1

Volume 160 = (140 (+20 ml -SAMPLE)) ml

Weight 144 g

pH 7.52

Conductivity 15.74 mS

1/40 : 0.350

TotN: 14.0

Sample

/ K2

Volume 1210 ml

Weight 1227 g

pH 7.31

Conductivity 26.23 mS

1/20 : 0.161

TotN : 3.22

9D

A280 1/20 dil: 0.769

TotN : 15.38

9424

A280

Sample

/ K3

Volume 685 ml

Weight 701 g

pH 7.02

Conductivity 27.54 mS

Net: 0.388

Sample

/ C

Volume 540 ml

Weight 561 g

pH 7.01

Conductivity 54.10 mS

Net: 0.183

Sample

/_____

Volume _____ ml

Weight _____ g

pH _____

Conductivity _____ mS

Sample

/_____

Volume _____ ml

Weight _____ g

pH _____

Conductivity _____ mS

Sample

/_____

Volume _____ ml

Weight _____ g

pH _____

Conductivity _____ mS

9H24

Date 26/4/84. Buffer L for 9H24

Final concentrations

8.9 mM trisodium citrate

1.1 mM citric acid

10 mM Na_2HPO_4

125 mM NaCl

To make up 5L (5Kg)

Weight		Batch no.	Quantity used
13.1 g	Trisodium citrate	9293700-17-38	13.2g
1.14 g	citric acid	91481500-03-6	1.14
7.1 g	Na_2HPO_4	9260374-061-5	7.1g
36.52 g	NaCl	9397545-14-90	36.6g

Make up to 5L (5Kg) with PFW 4943g;

pH limit: 7.00 +_ 0.05 pH found: 7.09

Adjust as necessary

Conductivity limit: 13.0 +_ 1.0 Conductivity found: 13.7 mS

9H24

Date 26/4/84 Buffer K for 9H24

Final concentrations

8.9 mM trisodium citrate

1.1 mM citric acid

10 mM Na_2HPO_4

300 mM NaCl

To make up 8L (8Kg)

Weight		Batch no.	Quantity used
20.96 g	Trisodium citrate	9293700-17-38.	20.9g
1.84 g	citric acid	9148150-03-6.	1.84g
11.36 g	Na_2HPO_4	9260374-061-5.	11.3g
140.2 g	NaCl	9397545-14-90.	140.3

Make up to 8L (8Kg) with PFW (7831 g).

pH limit: 7.00 +_ 0.05 pH found: ~~6.92~~ 6.93 FB

Adjust as necessary

Conductivity limit: 28.0 +_ 1.0 Conductivity found: 27.6 mS

9H24

Date 26/4/84 Buffer C for 9H24.

Final concentrations

20 mM trisodium citrate

1.0 mM NaCl

To make up 3L (3Kg)

Weight		Batch no.	Quantity used
17.7 g	Trisodium citrate	9293700-17-38.	17.7g
140.2 g	NaCl	9397545-14-90.	140.4

Make up to 3L (3Kg) with PFW (2844g)

pH limit: 8.00 +_ 1.0 pH found: 7.63

Adjust as necessary

Conductivity limit: 80.0 +_ 10 Conductivity found: 63.3 mS

10.5.84 9H25 Large Scale factor IX heating – a new method of sorbitol addition.

This is a conventional preparation, using high salt in both eluting buffers, L and K. A new approach of sorbitol addition is tried – increasing the water bath temperature as more sorbitol is added. This is an attempt to speed up the sorbitol addition step and also to generate a less viscous solution which allows air bubbles to escape more readily. As a consequence, the heating step is started immediately with automatic switching off of the heater, rather than automatic switching on.

Method

This is as set out in the accompanying sheets.

Sorbitol addition

1. The first 35% of the sorbitol (1211 g) was added with the water bath at 30°c. Previous experience suggests that this proportion dissolves with moderate ease.

2. The remainder of the sorbitol was divided into 6 equal fractions. Prior to the addition of each fraction, the water bath temperature was raised by 5°c

3. Though not recorded, the sample temperature did not rise in parallel due to the competing cooling effect of sorbitol solution

4. Again not recorded, but the total solution time was faster than in previous experiments and the final solution was clearer, with fewer air bubbles.

Heating

After addition of the final sorbitol fraction, the temperature of the sample was allowed to rise to 60° and the heating step commenced.

A auto-timer switch then switched the water heater off after 10 hours. As can be seen in the temperature trace, there followed about 4 hours before the sample was removed from the water bath the following morning. During this period, the temperature dropped approximately 13°c in the water, and 10°c in the factor IX material.

Column Step

Dilution and adsorption to DE 52 were carried out normally. However, the total amount of gel was small, and the column being used was not suitable. The result was a very small bed volume, with the column piston unable to make contact. This resulted, during application of buffer L, in a large buffer volume above the gel and a distorted bed cross-section. Between buffer L and buffer K the piston was removed, the gel resuspended to pack flat and the column run "open", pumping from below with a Watson Marlow 501 pump.

These problems made fraction collecting a fairly pointless task. Accordingly the eluate from each of buffer L and K were collected as one large fraction /L and /K respectively

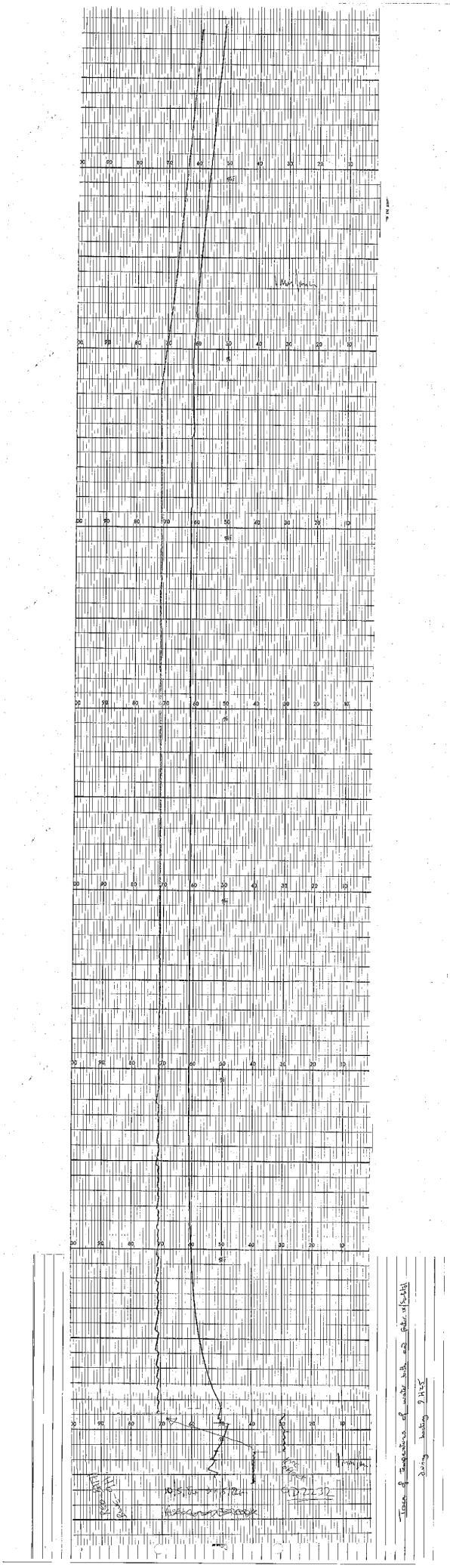
Assays

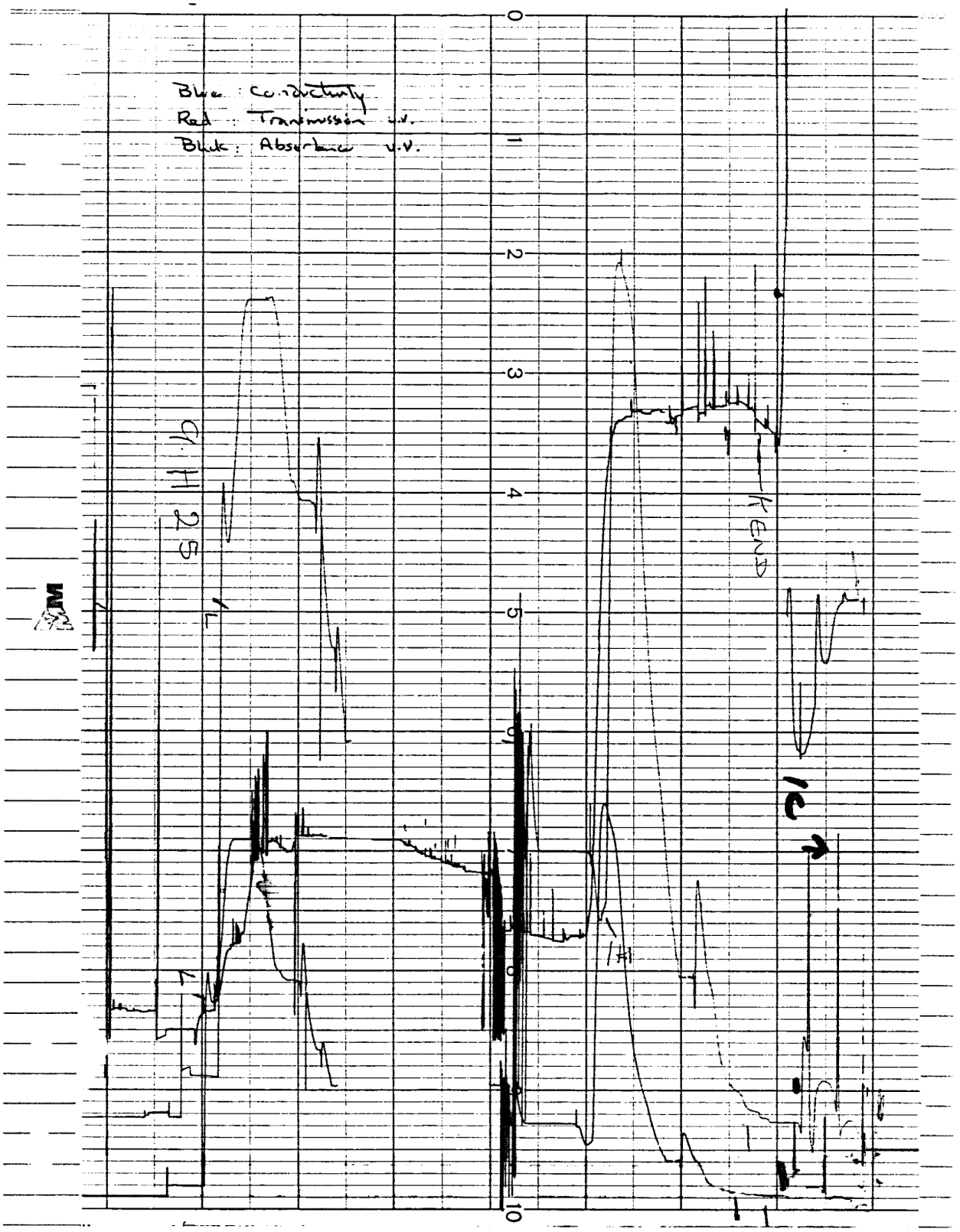
Fraction volumes and weights were measured before sampling.

pH and conductivity were measured

Absorbance at 280 nm of appropriately diluted samples was also measured. In the table of results they are corrected for this dilution.

Factor IX activity and NAPTT were also measured.


Trace of Temperature of water both and Gelatin solution
During heating 9 H 55'



Elution "profile" of heated factor IX, 9H25, from DE 52

9H25 5

Results

Sample	Volume (ml)	A280	Factor IX u/ml	Factor IX total	SpAct u/mg	NAPTT $t_{1/2}$ (s)	NAPTT % control	% factor IX starting 9D	% factor IX α9H
9D2232	1700		31.1	52870	57375			100	
			36.4	61880		203	60		
1S	4180		15.9	66462				116	
9H25	4180		6.9	28842				50	
α9H25	8360		4.1	34276		254	75	60	100
$1S_n$	7540	0.37	0.1	754	0.27	339	100	1.3	2.2
1L	1970	5.24	6.8	13396	1.3	227	67	23.3	39
1K	1690	4.36	7.5	12675	1.7	167	49	22.1	37

Sample	Volume (ml)	Weight (g)	Density (g/ml)	pH	Conductivity (mS)
9D2232	1700	1730	1.02	7.16	17.2
9H25	4180	5442	1.30		
α9H25	8360	9433	1.13	6.89	1.31
$1S_n$	7540	8782	1.16	8.06	1.43
1L	1970	2022	1.03	7.72	10.49
1K	1690	1717	1.02	7.25	24.59

9H25 (

Comments

1. The step addition of Sorbitol as the water bath temperature was raised led to a quicker dissolving solution with fewer air bubbles. There is no evidence that any activity was lost during the procedure.

2. The yield after heating is no worse than in previous experiments. The process of heating immediately and switching off automatically should therefore be adopted as it renders the material ready for an early start on day 2, even allowing for up to 5 hours leeway.

3. Buffer L (125 mM NaCl) serves no purpose other than elute almost 25 % of the factor IX in the wrong fraction. There is no preferential elution of material with a short NAPTT.

4. No real conclusion can be drawn about the total recovery. Small bed volume and poor flow characteristics led to the collection of IK in one large pool. Even with this larger than normal volume, there is a shortening of NAPTT.

P.AF

9N2Y

Factor IX Wet Heating Procedure - Provisional, Version 3.

Time -

Starting Material

Eluate Batch: 902232

Eluate Volume: 1700 ml

Eluate Weight: 1730 g

pH: 7.16

Conductivity: 17.2 mS

Sample: ✓ 3 x 2ml
✓ 1 x 5ml
✓ Send 2 x 1ml for immediate factor IX assay.

Stabilisers

1. Add glycine, 0.1g per g starting eluate

   Weight of glycine added: 173 g 9256760 D

2. Dissolve by stirring with MANUAL.

3. Place in water bath set at 30° C.

4. Weigh out sorbitol, equivalent to 2g sorbitol per g of material.

   Weight of sorbitol: 3460 g

5. Divide into 7 aliquots, A - G, where A contains 35% total sorbitol weight and B - G each contain 11% of the total sorbitol weight.

   Weight sorbitol A: 1211 g

   Weight of each of B - G : 380.6 g

6. Add A, with stirring, to the factor IX.

7. When dissolved, raise the water bath temperature to 35° C

9 N25

Time

and add sorbitol B. ✓

8. Repeat for C - G, each time raising the water bath temperature by 5° C. The water bath should therefore be heating up to 60° C while sorbitol G is being added.

C ✓ D ✓ E ✓ F ✓ G ✓

40 45 50 55 60

9. When dissolved, sample 3 x 1ml (/S)

10. Volume /S: _____ ml

Weight /S: _____ g

Heating

1. Locate in water bath over Edwards magnetic stirrer.

2. Connect temperature probes from bottle and from the water bath to Rikadenki chart recorder.

3. Ensure that water bath and sample are both at 60° C, and that the mixture is being stirred. Set the water bath to switch off after 10 hours.

4. Remove bottle from water bath after 10 hours at 60° C.

5. Sample 3 x 2ml (9H____)

6. Volume 9H____ : 4180 ml

Weight 9H____ : 5442 g

Dilution

1. Dilute 1/2 by addition of 1 9H volume of PFW. Mix.

Volume (or weight) PFW added: 4180 g

2. Sample 3 x 2ml

1 x 5ml (∝9H____)

9H25

Time

3. Volume: 8180 ml

Weight: ~~9433~~ 9433 g.

pH: 6.89

Conductivity: 1.31 mS

Adsorption

1. Factor IX activity (from day 1 assays)

Starting eluate: 31.1 u/ml

2. Total factor IX units before heating: 52870

3. Add DE 52, 1 g recycled DE52 weight for every 200 factor IX units before heating.

Weight DE 52 added : 265 g

10.15

4. Stir for 1 hour at lab. temperature, using Edwards magnetic stirrer.

Recovery of DE 52

1. Pour slurry into GAC 110 column with extender tube attached. Pump through with Watson Marlow pump, attached to bottom of column.

2. Collect wash-through in one container. (/Sn)

Elution

1. Disconnect pump and fix pressure head set-up.

2. Bed height : 4.75 cm

4 hrs

Time

4.75 m

Bed volume : 95.03 x Bed height = 451 ml

3. Fill pressure vessel with 5 x bed volumes of Buffer L

Volume Buffer L: _____ ml

Weight Buffer L: 2255 g

4. Measure flow rate: _____ ml/min

Nitrogen pressure : _____

5. Monitor : u.v. transmission
u.v. absorbance
conductivity
refraction

6. Collect material which elutes as indicated by change in transmission (/L).

NB. Bed too small. Formed hole in middle, Remove piston, resiroad + pack gel, attach pump again and run "open" column, pump, from below.

2

7. When buffer L has finished, fill pressure vessel with 5 bed volumes of Buffer K and elute.

8. Volume Buffer K: _____ ml

Weight Buffer K: _____ g

9. Collect fractions:

/K1 - from beginning of transmission rise to peak maximum.

/K2 - from end of /K1 to stabilisation of eluate conductivity.

/K3 - remainder of transmission peak and tail.

10. Wash with 3 bed volumes of Buffer C.

Volume Buffer C: _____ ml

9A25

Time

Weight Buffer C: _____ g

11. Collect any protein transmission peak that elutes. (/C).

12. Blow column dry.

9H25

Record of samples from 9H

From each collect fraction of eluate (/sn - /C) take: 3 x 2ml samples

1 x 5ml sample.

Sample

/Sn

Volume 7540 ml

Weight 8782 g

pH 8.06

Conductivity 1.43 mS

A_{280} Neat: 0.373

Sample

/L

Volume 1970 ml

Weight 2022.5 g

pH 7.72.

Conductivity 10.49 mS

A_{280}

$\frac{1}{10}$: 0.524

Sample

/K

Volume 1690 ml

Weight 1717.4 g

pH 7.25.

Conductivity 24.59 mS

A_{280}

$\frac{1}{10}$: 0.436

Sample

/C

Volume 1500 ml

Weight _____ g

pH _____

Conductivity _____ mS

9N25

Sample

/_____ Volume _____ ml

Weight _____ g

pH _____

Conductivity _____ mS

Sample

/_____ Volume _____ ml

Weight _____ g

pH _____

Conductivity _____ mS

Sample

/_____ Volume _____ ml

Weight _____ g

pH _____

Conductivity _____ mS

Sample

/_____ Volume _____ ml

Weight _____ g

pH _____

Conductivity _____ mS

Sample

/_____ Volume _____ ml

Weight _____ g

pH _____

Conductivity _____ mS

9N25

Date 11/5/84 Buffer L for 9H25

Final concentrations

8.9 mM trisodium citrate

1.1 mM citric acid

10 mM Na_2HPO_4

125 mM NaCl

To make up 5L (5Kg)

Weight		Batch no.	Quantity used
13.1 g	Trisodium citrate	312293-17-40	13.1g
1.14 g	citric acid	91481500-03-6	1.13g
7.1 g	Na_2HPO_4	9260374-061-5	7.1g
36.52 g	NaCl	94427 40-14/93	36.4g

Make up to 5L (5Kg) with PFW (4975g [redacted]).

pH limit: 7.00 +- 0.05 pH found: 7.11

Adjust as necessary

Conductivity limit: 13.0 +- 1.0 Conductivity found: 13.8 mS

9H25

Date 11/5/84 . Buffer K for 9H25

Final concentrations

8.9 mM trisodium citrate

1.1 mM citric acid

10 mM Na_2HPO_4

300 mM NaCl

To make up 8L (8Kg)

Weight		Batch no.	Quantity used
20.96 g	Trisodium citrate	312293-17-40	20.9 g
1.84 g	citric acid	91481500-03-6	1.85 g.
11.36 g	Na_2HPO_4	9260374-061-5.	11.4 g
140.2 g	NaCl	9442740-14/93.	140.2

Make up to 8L (8Kg) with PFW (7852 g)

pH limit: 7.00 +– 0.05 pH found: 6.95 .

Adjust as necessary

Conductivity limit: 28.0 +– 1.0 Conductivity found: 28 mS

9H25

Date 4/5/84 Buffer C for 9H 25.

Final concentrations

20 mM trisodium citrate

1.0 mM NaCl

To make up 3L (3Kg)

Weight		Batch no.	Quantity used
17.7 g	Trisodium citrate	312293-17-40.	17.7g
140.2 g	NaCl	94427 40 - 14/93.	140.1g

Make up to 3L (3Kg) with PFW (2848g)

pH limit: 8.00 +- 1.0 pH found: ~~7.9~~ 8.14

Adjust as necessary

Conductivity limit: 80.0 +- 10 Conductivity found: 62 mS

9H25

amples to be kept? No If so, how

amples provided with form/available from Rate By Freezer

SAMPLE INVESTIGATION		9D2232	IS #	9H25 #	X9H25 *	IS	IL
Factor VIII, iu/ml	1 st.						
	2 st.						
Factor IX, iu/ml	1 st.	(30)	(15)	(8.5)	(4.2)	(0.05)	(6.5)
	2 st.	36.4	15.9	6.9	4.1	0.10	6.8
Factor II, u/ml							
Factor X, u/ml							
Factor VII, u/ml (clotting)							
AT III (Anti Xa), u/ml							
AT III (Anti IIa), u/ml							
AT III (Laurell), u/ml							
Factor VIII RAG, u/ml							
Fibrinogen (Laurell), mg/ml							
Prothrombin (Laurell), u/ml							
Factor XIIIA (Laurell), u/ml							
Fibronectin (Laurell), mg/ml							
Factor VIII CAG							
APTT sec. 1/10		203			254	339	227
TGt50 min.		CONTROL	340				
FDA hr.							
Limulus + or -							

2g/ml Sorbitol

* 1g/ml sorbitol

9H25

amples to be kept? NO If so, how

amples provided ~~with form~~/available from R+D freeze

SAMPLE 9H25 INVESTIGATION		1k					
tor VIII, iu/ml	1 st.						
	2 st.						
Factor IX, iu/ml	1 st.	(7.1)					
	2 st.	7.5					
Factor II, u/ml							
Factor X, u/ml							
Factor VII, u/ml (clotting)							
AT III (Anti Xa), u/ml							
AT III (Anti IIa), u/ml							
AT III (Laurell), u/ml							
Factor VIII RAG, u/ml							
Fibrinogen (Laurell), mg/ml							
Prothrombin (Laurell), u/ml							
Factor XIIIA (Laurell), u/ml							
Fibronectin (Laurell), mg/ml							
Factor VIII CAG							
NAPTT sec. 1/10		167					
TGt50 min.		Conca 340					
FDA hr.							
Limulus + or -							

24/5/84 9H26.

To standardize the large scale pasterization and absorption and elution of FIX from DES2
— use of a new buffer L with lower salt than in 9H24
— use of a thinner walled vessel for the pasteurization

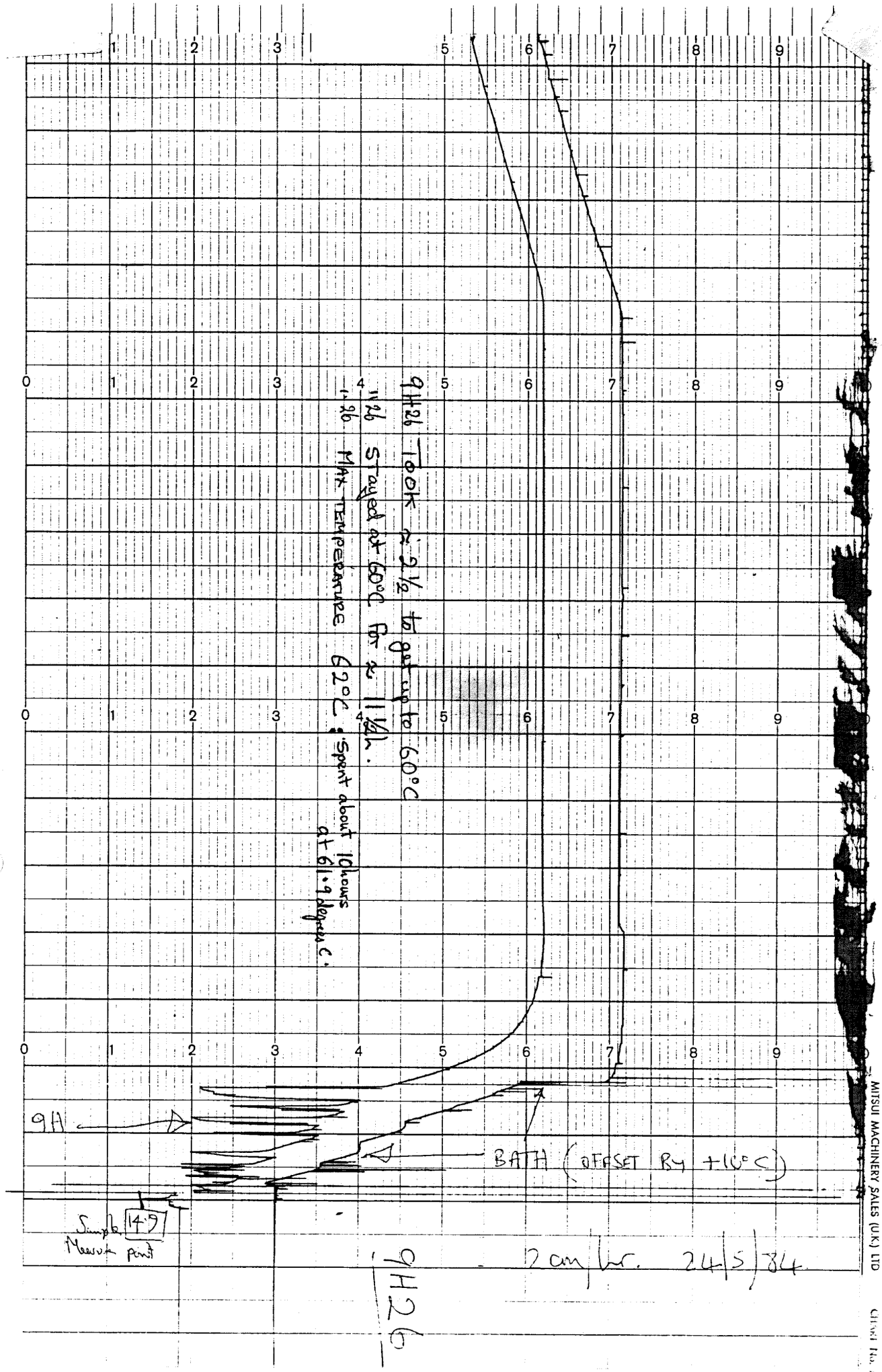
This was carried out in a similar way to 9H25 but this time the buffer L (L2) contained 110mM NaCl and much more care was applied in avoiding the poor flow characteristics of 9H25.

Materials and Methods.

The details are given on the printed work sheets included here.
There were no problems or unusual occurrences in carrying out the procedures.

Results

The results of the heating are shown on the next page.


9H26 100K ≈ 2½ to get up to 60°C
1½ stayed at 60°C for ≈ 1½h.
1.26 MAX TEMPERATURE 62°C
Spent about 10 hours at 61.9 degrees C.
9H
BATH (OFFSET BY +15°C)
Simple Measure point
14.9
12 am/hr. 24/5/84
9H26
MITSUI MACHINERY SALES (U.K.) LTD

24.5.84 9H26

Results

General Parameters of the Fractions

FRACTION	Volume (ml)	Weight (g)	PH	Conductivity (mS)
9D2231-2/24.5.84	2240	2261	7.13	18.1
9H26	5480	6933	6.90	1.42
∝9H26	10960	12320	—	—
" /Sn	11691	12240	8.02	1.7
/L2	3400	3453	7.63	11.6
/K1	325	334	7.19	19.6
/K2	840	860	7.17	27.8
/K3	1750	1771	6.97	29.09

Analysis of Fractions

FRACTION	Vol (ml)	iu/ml FIX	Total iu	% of 9D	A^{280}	Sp Act.	NRPTT(s)	% of NRPTT BLANK
9D2231-2	2240	33	73920	100	13.78	2.39	198	63
9H26	5480	5.4	29592	40.15	—	—	—	—
∝9H26	10960	3.0	32880	44.48	—	—	265	84
/Sn	11691	0.06	701	0.95	0.46	0.13	318	101
/L	3400	3.5	11900	16.15	3.47	1.01	269	85.6
/K1	325	62.5	20215	27.43	30	2.07	170	54
/K2	840	2.8	2352	3.19	2.09	1.34	250	79.6
/K3	1750	0.41	717	0.97	0.38	1.08	314	100
							BLANK 314	

Approximately 40% of the FIX survived the heating step (9H26 This is lower than the 50% after heating in 9H25 but is higher than the 35% measured in ∝9H25 (NB measurement of FIX in "∝9H s" usually gives a "higher" recovery figure)

There was a clear less dense layer of liquid on the surface of the 9H26. This shows that the mixing was incomplete and the FIX survival may be low for this reason. A more effective stirring arrangement should be used in the next 9H heating run.*

% recovery

The A of the FIX which was added to the DE52 is

* This is a retrospective recommendation; this report being written after completion of 9H27 experiment. RAE.

9H26

shown in the following table :-

Fraction	% of starting 9D	% of ∝9H26	
∝9H26	44.48	100	
/Sn	0.95	2.13	109%
/L	16.15	36.29	
/K1	27.43	61.65	
/K2	3.19	7.15	
/K3	0.97	2.18	

We can account for all of the FIX applied to the DE52. The total recovery is above 100% (109%). This is within the normal experiment error of the assays.

The % distribution of the FIX in the column eluates has changed from the 50/50 L/K observed in 9H24 and 9H25 to 37/63. This shift of FIX from /L to /K may be due to the lower salt in the L buffer. Hence a still lower NaCl concentration in buffer L may enable us to recovered an even greater proportion of the FIX in the buffer K elution.

The NAPTT of Fraction /K1 is lower than that of the starting 9D2231-2 (170s cf 198s), but it is still above 150s.

As it was noted in 9H24 the NAPTT appears to follow roughly the dilution of the FIX units. The greater the iu/ml of FIX the lower the NAPTT. This is shown on the graph on the following page.

If the NAPTT is a determining factor as to whether a batch of Pasteurized FIX is to be used, it may be that the potency of the FIX must be kept below a certain level.

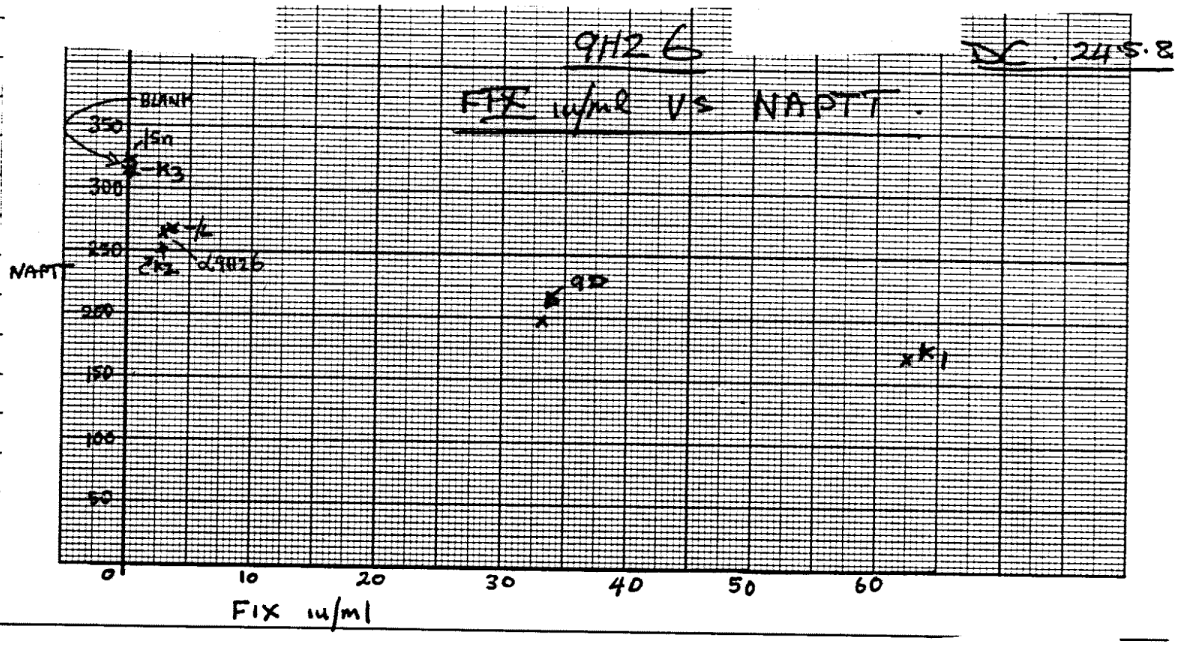
For a particular number of FIX iu/ml in the K eluate the NAPTT of that eluate may depend on the NAPTT of the original 9D material. Hence better NAPTT in 9D may lead to better NAPTT longer in our high potency K eluates.

9H26.5

9H26

9H26

FIX iu/ml VS NAPTT.



Moore's Mode
To repeat order

Viable Counts

Viable count of bacteria in /K2 was made, yielding a value of 7 CFU /ml. This is rather high, suggesting that we are handling this material rather poorly. This may be rectified by more diligent vigilance against contaminants.

9H26

9H26

Factor IX Wet Heating Procedure - Provisional, Version 3.

Time

Starting Material

Eluate Batch: 9D2232 x52 VIALS, PWS 9D2231 x13 VIALS.

Eluate Volume: 2240 ml

Eluate Weight: 2261.1 g

pH: 7.13

Conductivity: 18.1 mS

Sample: 3 x 2ml
1 x 5ml
Send 2 x 1ml for immediate factor IX assay. ✓

Stabilisers

14.45 1. Add glycine, 0.1g per g starting eluate

Weight of glycine added: 226.1 g

2. Dissolve by stirring with MANUALLY.

3. Place in water bath set at 30° C.

4. Weigh out sorbitol, equivalent to 2g sorbitol per g of material.

Weight of sorbitol: 4522 g

5. Divide into 7 aliquots, A - G, where A contains 35% total sorbitol weight and B - G each contain 11% of the total sorbitol weight.

Weight sorbitol A: 1583 g ✓

Weight of each of B - G : 497 g

15.00 6. Add A, with stirring, to the factor IX.

15.15 7. After addition is complete, ~~When dissolved~~ raise the water bath temperature to 35° C

Add sorb at	35 B	40 C	45 D	50 E	55 F	60 G
Added	✓	✓	✓	✓	✓	

9H28

Time

and add sorbitol B. Time. 1530 1550 16-10

8. Repeat for C - G, each time raising the water bath temperature by 5° C. The water bath should therefore be heating up to 60° C while sorbitol G is being added.

9. When dissolved, sample 3 x 1ml (/S)

10. Volume /S: _____ ml

Weight /S: _____ g

Heating

1. Locate in water bath over Edwards magnetic stirrer.

2. Connect temperature probes from bottle and from the water bath to Rikadenki chart recorder.

3. Ensure that water bath and sample are both at 60° C, and that the mixture is being stirred. Set the water bath to switch off after 10 hours.

9-10 4. Remove bottle from water bath as soon as is convenient after 10 hours at 60° C.

5. Sample 3 x 2ml (9H26) ✓

6. Volume 9H26 : 5480 ml

Weight 9H___ : 6933 g

PH = 6.90

Conductivity = 1.42 MS

Dilution

9-27 1. Dilute 1/2 by addition of 1 9H volume of PFW. Mix.

Volume (or ~~weight~~) PFW added: 5480 ml ~~(g)~~

2. Sample 3 x 2ml

1 x 5ml (∝9H26) ✓

9A26

Time

3. Volume: ~~10,000~~ ~~5,000~~ 55 ml

LOT'S OF FROTH ON POURING - DIFFICULT TO MEASURE VOLUME ACCURATELY.

10960 DC

Weight: 12.32 kg

pH: _____

Conductivity: _____ mS

Adsorption

1. Factor IX activity (from day 1 assays)

Starting eluate: 33.0 u/ml

2. Total factor IX units before heating: 73920

10-00 3. Add DE 52, 1 g recycled DE52 weight for every 200 factor IX units before heating.

Weight DE 52 added : 370 (371.8) g

4. Stir for 1 hour at lab. temperature, using Edwards magnetic stirrer.

Recovery of DE 52

11-22 1. Pour slurry into GAC 110 column with extender tube attached. Pump through with Watson Marlow pump, attached to bottom of column.

2. Collect wash-through in one container. (/Sn)

Elution

1. Disconnect pump and fix pressure head set-up.

2. Bed height : 7.0 cm

9N26

Time

Bed volume : 95.03 x Bed height = 665 ml

3. Fill pressure vessel with 5 x bed volumes of Buffer L

Volume Buffer L: 3325. ml

Weight Buffer L: 3325. g

4. Measure flow rate: 100 ml/min

Nitrogen pressure : _____

5. Monitor : u.v. transmission ✓
u.v. absorbance ✓
conductivity ✓
refraction ✓

4 Pen RIKADENKI RECORDER USE.

6. Collect material which elutes as indicated by change in transmission (/L).

7. When buffer L has finished, fill pressure vessel with 6 bed volumes of Buffer K and elute.

8. Volume Buffer K: _____ ml

Weight Buffer K: 3390 g

9. Collect fractions:

/K1 - from beginning of transmission rise to peak maximum.

/K2 - from end of /K1 to stabilisation of eluate conductivity.

/K3 - remainder of transmission peak and tail.

10. Wash with 3 bed volumes of Buffer C.

Volume Buffer C: _____ ml

9426

Time

Weight Buffer C: _____ g

11. Collect any protein transmission peak that elutes. (/C).

NONE.

12. Blow column dry.

9H26

Record of samples from 9H

From each collect fraction of eluate (/sn - /C) take: 3 x 2ml samples

1 x 5ml sample.

Sample

/ Sn

Volume 11.691 ml

Weight 12.240 g

pH 8.02

Conductivity 1.7 mS

Sample

/ L2

Volume 3400 ml

Weight 3453 g

pH 7.63

Conductivity 11.6 mS

Sample

/ K1

Volume 325 ml

Weight 334 g

pH 7.19

Conductivity 19.6 mS

Sample

/ K2

Volume 840 ml

Weight 860.1 g

pH 7.17

Conductivity 27.8 mS

9R26

Sample

/ K3

Volume 1750 ml

Weight 1771 g

pH 6.97

Conductivity 29.09 mS

Sample

/ C

Volume NONE ml

Weight _____ g

pH _____

Conductivity _____ mS

Sample

/_____

Volume _____ ml

Weight _____ g

pH _____

Conductivity _____ mS

Sample

/_____

Volume _____ ml

Weight _____ g

pH _____

Conductivity _____ mS

Sample

/_____

Volume _____ ml

Weight _____ g

pH _____

Conductivity _____ mS

YH26

Date 25.5.84 Buffer LZ for 9H26

Final concentrations

8.9 mM trisodium citrate

1.1 mM citric acid

10 mM Na_2HPO_4

110 ~~135~~ mM NaCl

To make up 5L (5Kg)

Weight		Batch no.	Quantity used
13.1 g	Trisodium citrate	312293	13.1g
1.14 g	citric acid	91481500	1.14
7.1 g	Na_2HPO_4	9260374 D	7.1
32.12 ~~35.82~~ g	NaCl	9442740	32.12

Make up to 5L (5Kg) with PFW ✓

pH limit: 7.00 +_ 0.05 pH found: 7.05

Adjust as necessary

Conductivity limit: 12.4 ~~13.5~~ +_ 1.0 Conductivity found: 13.1 mS

9H26

Date 25.5.84 Buffer K for 9H26

Final concentrations

8.9 mM trisodium citrate

1.1 mM citric acid

10 mM Na_2HPO_4

300 mM NaCl

To make up 8L (8Kg)

Weight		Batch no.	Quantity used
20.96 g	Trisodium citrate	312293	20.96g
1.84 g	citric acid	91481500	1.84g
11.36 g	Na_2HPO_4	92603740	11.36
140.2 g	NaCl	94427466	140.29

Make up to 8L (8Kg) with PFW ✓

pH limit: 7.00 +– 0.05 pH found: 6.95

Adjust as necessary

Conductivity limit: 28.0 +– 1.0 Conductivity found: 29.0 mS

9H26

Date 25.5.84 Buffer C for 9H26

Final concentrations

20 mM trisodium citrate

1.0 mM NaCl

To make up 3L (3Kg)

Weight		Batch no.	Quantity used
17.7 g	Trisodium citrate	312293	$\left(\frac{17.7g}{2}\right)$: 8.85
140.2 g	NaCl	9442746	$\left(\frac{140.2}{2}\right)$: 70.1

Make up to ~~3L (3Kg)~~ with PFW 1.5 kg

pH limit: 8.00 +- 1.0 pH found: 7.64

Adjust as necessary

Conductivity limit: 80.0 +- 10 Conductivity found: 63.8 mS

9H26

BACTERIAL CONTAMINATION : VIABLE COUNTS

File Reference: _____ Product: FIX (9H)

Authorised by: [signature] Processing Run: 9H26

Date effective: 21.4.83 This page started: 25.5.84

PRODUCTION STAFF			QUALITY CONTROL STAFF			
Sample Stage	Volume to be taken	Sample taken by:	SAL	Day 4 at °C	Day 2 at °C	.'. CFU/ ml
K2	1ml			7		7
			Control	0		0

EXTRA SAMPLES as requested by Scientist i/c Production or QC staff:

			Control			

COMMENTS by sampling, testing or reading staff:

INTERPRETATION by Scientist i/c Production, and any action taken:

COMMENTS by Quality Controller:

9826

ASSAY REQUEST FORM (1)

Request from: PAF Date: 24 5.84

When results needed: ASAP

Samples to be kept? _____ If so, how? _____

Samples provided with form/available from _____

SAMPLE INVESTIGATION		PAF's 9D.	WET CWE				
Factor VIII, iu/ml	1 st.						
	2 st.						
Factor IX, iu/ml	1 st.						
	2 st.	33.0					
Factor II, u/ml							
Factor X, u/ml							
Factor VII, u/ml (clotting)							
AT III (Anti Xa), u/ml							
AT III (Anti IIa), u/ml							
AT III (Laurell), u/ml							
Factor VIII RAG, u/ml							
Fibrinogen (Laurell), mg/ml							
Prothrombin (Laurell), u/ml							
Factor XIIIA (Laurell), u/ml							
Fibronectin (Laurell), mg/ml							
Factor VIII CAG							
NAPTT sec. 1/10							
TGt50 min.							
FDA hr.							
Limulus + or -							

For comments contact _____ Date finished: _____

MAF 1DC4EB 25/5/84 9H26

ASAR

amples to be kept? No If so, how

amples ~~provided with form~~/available from R&D Bio Factor

SAMPLE 9H26 / INVESTIGATION		9D 24/5/84	9H26	α9H26	1Sn	1L(2)	1K1
Factor VIII, iu/ml	1 st.						
	2 st.						
Factor IX, iu/ml	1 st.	(33)	(8)	(4)	(0.1)	(3.5)	(63)
	2 st.	32.8	5.40	3.0	0.06	3.5	62.2
Factor II, u/ml							
Factor X, u/ml							
Factor VII, u/ml (clotting)							
AT III (Anti Xa), u/ml							
AT III (Anti IIa), u/ml							
AT III (Laurell), u/ml							
Factor VIII RAG, u/ml							
Fibrinogen (Laurell), mg/ml							
Prothrombin (Laurell), u/ml							
Factor XIIIA (Laurell), u/ml							
Fibronectin (Laurell), mg/ml							
Factor VIII CAG							
APTT sec. 1/10 b 3.4		198		265	318	269	170
TGt50 min.							
FDA hr.							
ulus + or -							

PHT 2/5/84 9H26

...applies to this case? No If so, ref# ...

amples provided with form/available from R+D Bjfreezer

SAMPLE INVESTIGATION 9H26		1k2	1k3				
Factor VIII, iu/ml	1 st.						
	2 st.						
Factor IX, iu/ml	1 st.	(4.5)	(0.65)				
	2 st.	2.8	0.41				
Factor II, u/ml							
Factor X, u/ml							
Factor VII, u/ml (clotting)							
AT III (Anti Xa), u/ml							
AT III (Anti IIa), u/ml							
AT III (Laurell), u/ml							
Factor VIII RAG, u/ml							
Fibrinogen (Laurell), mg/ml							
Prothrombin (Laurell), u/ml							
Factor XIIIA (Laurell), u/ml							
Fibronectin (Laurell), mg/ml							
Factor VIII CAG		✓	✓				
NAPTT sec. 1/10		250	314				
TGt50 min.							
FDA hr.							
...ulus + or -							

7.6.84 9H27 Large Scale factor IX heating: effect of new mixing system and low salt wash

Objectives 1) To test new heating vessel with added holes for overhead mixer and temperature probes.

2) To test effect of increased glycine concentration

3) To test effect of starting with hepainised factor IX

4) To test effect of lowering NaCl concentration in the first DEAE wash upon factor IX recovery, NAPTT and protein absorbance.

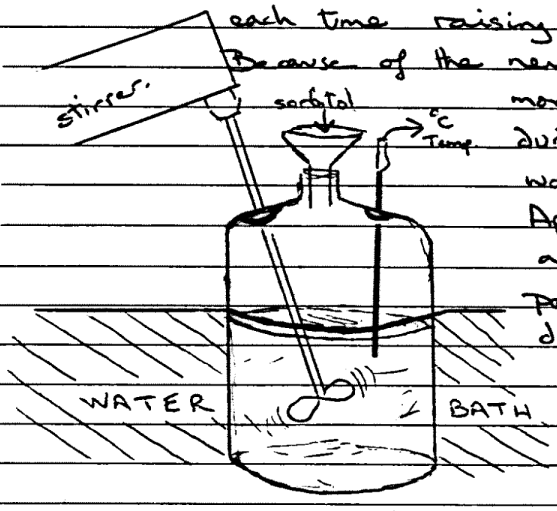
5) To assay for thrombin (some evidence in previous experiments of both thrombin activity and fibrin formation).

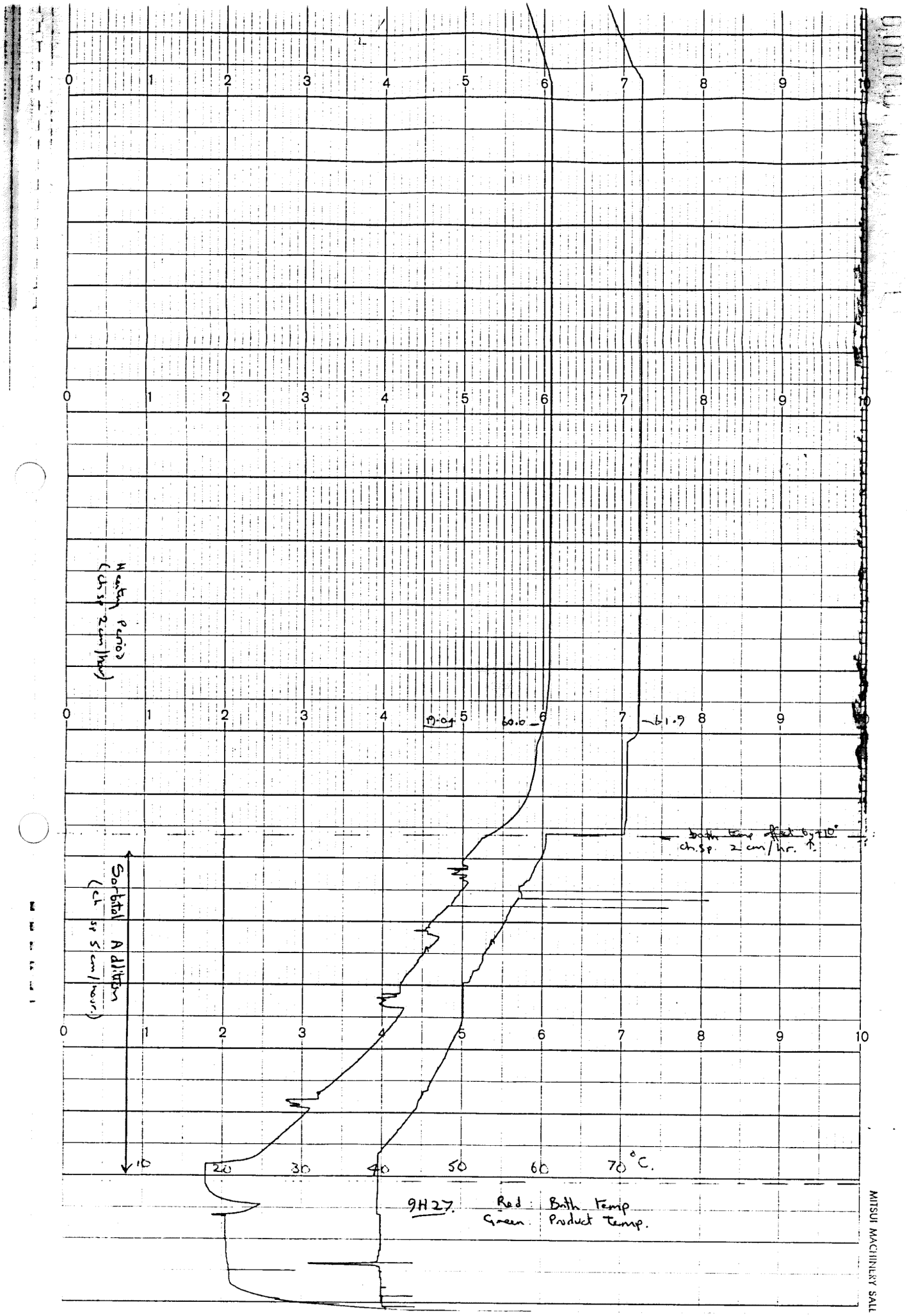
Notes on method

1. As no unheparinised factor IX was available a batch containing 60 units heparin, 9D 2253 was used as starting material. These vials were 20ml fills.

2. Glycine addition was increased from 0.1g to 0.15g per g starting material solution. Although 9H15 indicated no increased protection by this, the scale is so different that a second test seemed worthwhile.

3. Following solution, the heating bottle was placed in a 40° water bath and 46% of the sorbitol was added. The remainder was added in 11% lots, each time raising the temperature of the bath by 5°C. Because of the new bottle set-up, it was possible to monitor the temperature of the factor IX during sorbitol addition, as well as the water bath temperature (see trace). Apart from an initial temperature drop as sorbitol is added, the sample temp. parallels the bath temperature with a difference of 7 - 10 °C.





4. When the sample attained 60°c, an auto-timer was set to switch off the bath after 10 hours.

5. Subsequent handling was as described in accompanying sheets. Small amount of precipitate material observed on 9H surface.

6. DE 52 Batch RD/3 was used.

7. During elution with buffer L, the uvicord trace had not returned to zero after application of 5 bed volumes, so a further 1 kg buffer L ~ 2 bed volumes was applied.

8. Factor IX was eluted with 9 bed volumes of buffer K

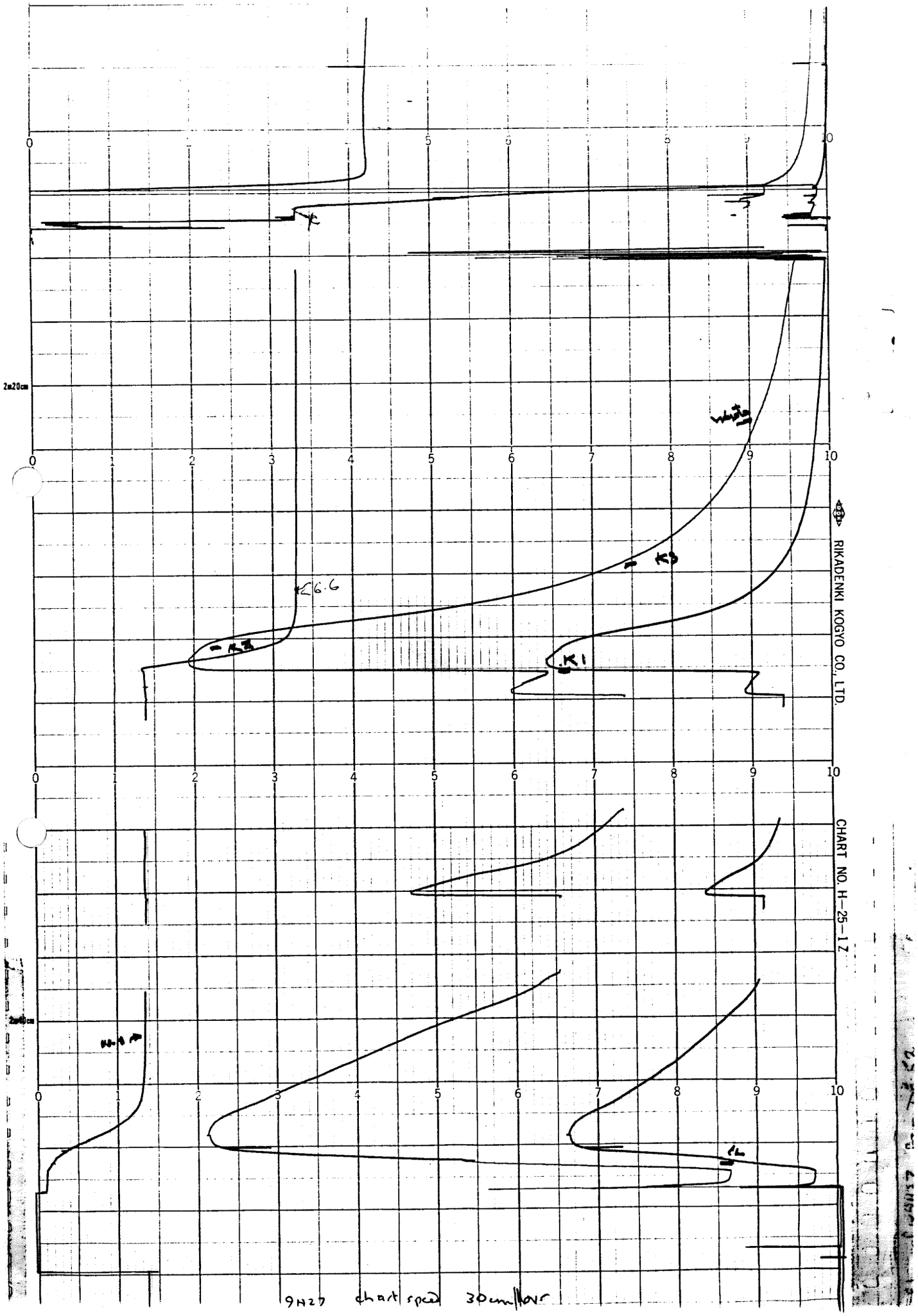
9. Buffer C was mixed to 2M NaCl, hopefully to elute any bound heparin.

Samples: 1 x 5 ml and 3 x 2 ml samples were taken

Assays: Factor IX, NAPTT, FDA (Thrombin), pH, conductivity, heparin, A280.

Results - Physical data

Sample	Volume (ml)	pH	Conductivity (ms)	Weight (g)	Density (g/ml)
9D2253	1940	7.18	15.45	1967	1.01
9H27	4730			5694	1.20
α 9H27	9460	6.83	1.18	10897	1.15
/Sn	8716	7.91	1.42	10371	1.19
/L	3425	7.30	12.0	3507	1.02
/K1	280	7.14	18.0	296	1.06
/K2	855	7.19	26.06	859	1.00
/K3	1530	7.03	27.05	1549	1.01
/C	1130	6.94	114.75	1215	1.07


RIKADENKI KOGYO CO., LTD.
CHART NO. H-25-1Z
9H27 chart speed 30cm/hour

9N27.5

Protein Data

Sample	Volume	A_{280}	Factor IX u/ml	Factor IX total	Sp. Act u/mg	NAPTT t_{10} (s)	FDA (hr)	% of IX 9D	% of IX 9N
9D2253	1940	15.38	34.4 35.4	67706	2.27	291	4	100	
9D/S	4730		14.7	69531				102.7	
9H27	4730		8.5	40205				59.4	
α9H27	9460	3.31	3.7	35002	1.11	221	1	51.7	1C
/Sn	8716	0.75	0.17	1481.7	0.23	252	2	2.2	4
/L	3425	3.05	4.7	16097	1.54	233	1	23.8	4(
/K1	280	24.64	57.8	16184	2.34	194	2	23.9	4(
/K2	855	2.17	4.0	3420	1.84	206	4	5.0	9
/K3	1530	0.35	0.3	459	0.86	231	6+	0.7	1
/C	1130	0.08				420†			

Heparin

Sample	Volume (ml)	Heparin u/ml	Heparin total
9D2253	1940	2.02	3919
9N27/K1	280	0.008	2.2
/K2	855	—	—
/K3	1530	—	—
/C	1130	3.06	3458

Comments

1.) The new system of stirring can be seen to be more efficient. The effect on sorbitol addition is to make it easier, if not quicker.

2. The sorbitol addition does not cause loss of factor IX. The temperature of the sample suggests that sorbitol addition could be speeded up by addition of larger amounts at 40 - 45 °C water temperature, instead of the step wise addition currently used.

3. There is a 58% survival of factor IX on heating. This value is both the average of 9H27 and 9H27 assays and the sum of recovered factor IX off DE-52.

4. Factor IX is still distributed between the two buffer elutes. This drastically reduces the recoverable units. It is worth examining 1L fraction on a second DE-52 adsorption step to determine the elution properties of factor IX contained within it.

5. NAPTT have improved considerably. There is still a shortening upon heat treatment but all values are considerably > 150 s (the limit). This effect may be due to the heparin present in this batch.

6. The FDA thrombin times show dramatic generation of thrombin upon heating. A time of 1 hour is equivalent to ~ 0.02 u/ml thrombin, much of which appears to elute in the first buffer. If NAPTT reflects fIX a, it is surprising that there should be such a discrepancy between the two assays. Obviously FDA times must be closely followed in future, as none of these fractions 1Sa - 1K1 will pass QC tests.

7. Heparin binds DE52 tightly as seen by the recovery of 58% of the starting amount in 1C, and also the NAPTT (unless of course, trace amounts of heparin in 1L - 1K are artificially lengthening NAPTT).

P.A.F.

9H27

Factor IX Wet Heating Procedure - Provisional, Version 3.

Time

Starting Material

98 VIALS.

Eluate Batch: 9D2253 Reconstitute in 20 ml.

Eluate Volume: 1940 ml

Eluate Weight: 1967 g

pH: 7-18

Conductivity: 15.45 mS

Sample: 3 x 2ml
1 x 5ml
Send 2 x 1ml for immediate factor IX assay.

Stabilisers

1. Add glycine, 0.15 ~~g~~ per g starting eluate

   Weight of glycine added: 295.05. g

2. Dissolve by stirring with _____

3. Place in water bath set at 30° C, set up temperature probes and stirrer. 1 temp. probe to bottle, 1 temp. probe to water bath Connect to chart recorder.

4. Weigh out sorbitol, equivalent to 2g sorbitol per g of material.

   Batch No: 93259300

   Weight of sorbitol: 3934 g

5. Divide into 7 aliquots, A - G, where A contains 35% total sorbitol weight and B - G each contain 11% of the total sorbitol weight.

   Weight sorbitol A: 1377 g

   Weight of each of B - G : 433 g

2-45. 6. Add A, with stirring, to the factor IX.

7. When ~~dissolved~~ Added, raise the water bath temperature to 35° C

RAISE TO BATH TEMP. 40°C → 45

1 ONCE ADDED TEMP 35 40 45 50 55 60

A B C D E F

✓ ✓ ✓ ✓ ✓ ✓

YH L7

Time

and add sorbitol B.

8. Repeat for C - G, each time raising the water bath temperature by 5° C. The water bath should therefore be heating up to 60° C while sorbitol G is being added.

17.20 9. When dissolved, sample 3 x 1ml (/S)

10. Volume /S: _____ ml

Weight /S: _____ g

Heating

~~1. Locate in water bath over Edwards magnetic stirrer.~~

2. ~~Connect temperature probes from bottle and from the water bath to Rikadenki chart recorder.~~

19.04 3. Ensure that water bath and sample are both at 60° C, and that the mixture is being stirred. Set the water bath to switch off after 10 hours.

8.50. 4. Remove bottle from water bath as soon as is convenient after 10 hours at 60° C.

5. Sample 3 x 2ml (9H27)

6. Volume 9H___ : 4730 ml

Weight 9H___ : 5694 g (Before sampling)

Dilution

9-35 1. Dilute 1/2 by addition of 1 9H volume of PFW. Mix.

Volume (or weight) PFW added: 4730 ml (g)

2. Sample 3 x 2ml

1 x 5ml (∝9H27)

YHC7

Time

3. Volume: 9460 ml — BY ADDITION OF 473C + 473C. (NOT BY MEASURING.)

Weight: 10897 g

4. Measure pH and conductivity.

pH: 6.83

Conductivity: 1.18 mS } MARKED ☑.

Adsorption

1. Factor IX activity (from day 1 assays)

Starting eluate: 34.4 u/ml

2. Total factor IX units before heating: 66736

10-20 3. Add DE 52, 1 g recycled DE52 weight for every 200 factor IX units before heating. 333.7

Weight DE 52 added : _____ g

BATCH: RD13

4. Stir for 1 hour at lab. temperature, using Edwards magnetic stirrer.

Recovery of DE 52

1. Pour slurry into GAC 110 column with extender tube attached. Pump through with Watson Marlow pump, attached to bottom of column.

2. Collect wash-through in one container. (/Sn)

Elution

1. Disconnect pump and fix pressure head set-up.

2. Bed height : _____ cm

9HJ

Time

Bed volume : 95.03 x Bed height = 523 ml
5½ cm

3. Fill pressure vessel with 5 x bed volumes of Buffer L

Volume Buffer L: 2615 ml

Weight Buffer L: 2615. g WEIGHT USED.

ADD a further 1000 g buffer L.

4. Measure flow rate: 240 ml/min

Nitrogen pressure : _____

5. Monitor : u.v. transmission
u.v. absorbance
conductivity
~~refraction~~

6. Collect material which elutes as indicated by change in transmission (/L).

7. When buffer ~~K~~ L has finished, fill pressure vessel with 9 bed volumes of Buffer K and elute.

8. Volume Buffer K: _____ ml

Weight Buffer K: ~~4700~~ 4.776 kg

9. Collect fractions:

/K1 - from beginning of transmission rise to peak maximum.

/K2 - from end of /K1 to stabilisation of eluate conductivity.

/K3 - remainder of transmission peak and tail.

14.30 10. Wash with 3 bed volumes of Buffer C.

Volume Buffer C: _____ ml

9427

Time

Weight Buffer C: _____ g

11. Collect any protein transmission peak that elutes. (/C).

45 12. Blow column dry.

9H27

Record of samples from 9H

From each collect fraction of eluate (/sn - /C) take: 3 x 2ml samples

1 x 5ml sample. A210

Sample

/ Sn

Volume 876 ml

Weight 10371 g

pH 7.91

Conductivity 1.42 mS

5900
316
8716

Net: 0.745

Sample

/ L

Volume 3425 ml

Weight 3507 g

pH 7.30

Conductivity 12.0 mS

$\frac{1}{10}$: 0.305

Sample

/ K1

Volume 280 ml

Weight 296 g

pH 7.14

Conductivity 18 mS

$\frac{1}{40}$: 0.616.

Sample

/ K2

Volume 855 ml

Weight 859 g

pH 7.19

Conductivity 26.06 mS

$\frac{1}{10}$ 0.217

9H27

A280

Sample

/ K3 Volume 1530 ml Neut. 0.347

Weight 1549 g

pH 7.03

Conductivity 27.05 mS

Sample

/ C Volume 1130 ml

Weight 1215 g $\frac{1}{10}$: 0.008

pH 6.97

Conductivity 114.75 mS

9D $\frac{1}{20}$: 0769

Sample

/_____ Volume _____ ml

Weight _____ g

pH _____

Conductivity _____ mS

α 9H23 $\frac{1}{5}$: 0.322

Sample

/_____ Volume _____ ml 22/6/84

Weight _____ g

pH _____ 9 9H26.

Conductivity _____ mS $\frac{1}{9}$ 0.821

$\frac{1}{5}$ 0.671

Sample

/_____ Volume _____ ml $\frac{1}{3}$ 1.112

Weight _____ g $\frac{1}{10}$ 0.333

pH _____

Conductivity _____ mS

NaCl → 1 K. P P

2 NaCl → P 2 K.

9N27

Date 8/6/84 Buffer L^3 for 9H 27

Final concentrations

8.9 mM trisodium citrate

1.1 mM citric acid

10 mM Na_2HPO_4

100 ~~135~~ mM NaCl

To make up 5L (5Kg)

Weight		Batch no.	Quantity used
13.1 g	Trisodium citrate	312293	13.1 g.
1.14 g	citric acid	91481500	1.16 g.
7.1 g	Na_2HPO_4	9260374D.	7.12 g
29.22 ~~36.52~~ g	NaCl	9487526 11/84	29.23

Make up to 5L (5Kg) with PFW 5 kg.

pH limit: 7.00 +– 0.05 pH found: 7.03

Adjust as necessary: with 0.2 g citric acid solid.

Conductivity limit: 12.5 ~~[redacted]~~ +– 1.0 Conductivity found: 11.5. mS

9H27

Date 8/6/89 Buffer K for 9H27

Final concentrations

8.9 mM trisodium citrate

1.1 mM citric acid

10 mM Na_2HPO_4

300 mM NaCl

To make up 8L (8Kg)

Weight		Batch no.	Quantity used
20.96 g	Trisodium citrate	312293.	20.62
1.84 g	citric acid	91481500.	1.86
11.36 g	Na_2HPO_4	92603740	11.36
140.2 g	NaCl	94875261 94.	140.24.

Make up to 8L (8Kg) with PFW 8059 g.

pH limit: 7.00 +_ 0.05 pH found: 6.95

Adjust as necessary

Conductivity limit: 28.0 +_ 1.0 Conductivity found: 27.27 mS

9H27

Date 8/6/84 Buffer C for 9H 27

Final concentrations

20 mM trisodium citrate

2.0 M ~~140 mM~~ NaCl

To make up 3L (3Kg)

Weight		Batch no.	Quantity used
17.7 g	Trisodium citrate	312293	17.7
350.6 ~~350.2~~ g	NaCl	9487528-114/9	350.7

Make up to 3L (3Kg) with PFW 3195 g.

pH limit: 8.00 +- 1.0 pH found: 7.80

Adjust as necessary

Conductivity limit: 140 ± 20 ~~140 ± 10~~ Conductivity found: 120 mS

9N27

ASSAY REQUEST FORM (1)

Request from: PAF | DC | EB Date: 7/6/84.

When results needed: _____

Samples to be kept? _____ If so, how? _____

Samples provided with form/available from _____

SAMPLE INVESTIGATION		9D2253					
Factor VIII, iu/ml	1 st.						
	2 st.						
Factor IX, iu/ml	1 st.	(30)					
	2 st.	34.4					
Factor II, u/ml							
Factor X, u/ml							
Factor VII, u/ml (clotting)							
AT III (Anti Xa), u/ml							
AT III (Anti IIa), u/ml							
AT III (Laurell), u/ml							
Factor VIII RAG, u/ml							
Fibrinogen (Laurell), mg/ml							
Prothrombin (Laurell), u/ml							
Factor XIIIA (Laurell), u/ml							
Fibronectin (Laurell), mg/ml							
Factor VIII CAG							
NAPTT sec. 1/10							
TGt50 min.							
FDA hr.							
Limulus + or -							

Heparin at 3 u/ml.

YNZ

ASSAY REQUEST FORM (2)

Request from: PAF Date: 12/6/84

When results needed: ASAP

Samples to be kept? _____ If so, how? _____

Samples ~~provided with form~~/available from: R+D Big Freezer

SAMPLE INVESTIGATION		902253	/k1	/k2	/k3	/c	
Protein	E280						
	biuret g/l						
Fibrinogen g/l/%							
Sodium mmol/l							
Potassium mmol/l							
Chloride mmol/l							
Citrate mmol/l							
Phosphate mmol/l							
Tris mmol/l							
PEG g/l							
Caeruloplasmin g/l							
Factor XIII u/ml							
PKA % Ref. 2							
AT III (amidolytic) u/ml							
F. VII (amidolytic) u/ml							
XaGT mins.							
pH at 20°C							
Conductivity at 20°C		(3)	(0.06)	(0.19)	(0.4)	(4.5)	
✓ Heparin		2.02	0.008	N/D	N/D	3.06	

For comments contact: _____ Date finished: _____

9N27

ASSAY REQUEST FORM (1)

Request from: PAF/DZ/EB Date: 11/6/84

When results needed: ASAP

Samples to be kept? _____ If so, how? _____

Samples ~~provided with form~~/available ~~from R+D little freezer~~ R+D little freezer.

SAMPLE / INVESTIGATION		9D2253	IS *	9H27 *	9H27 ≠	IS	L
Factor VIII, iu/ml	1 st.						
	2 st.						
Factor IX, iu/ml	1 st.	(34)	(14)	(9.5)	(4.5)	(0.05)	(2.0)
	2 st.	35.4	14.7	8.5	3.7	0.17	4.7
Factor II, u/ml							
Factor X, u/ml							
Factor VII, u/ml (clotting)							
AT III (Anti Xa), u/ml							
AT III (Anti IIa), u/ml							
AT III (Laurell), u/ml							
Factor VIII RAG, u/ml							
Fibrinogen (Laurell), mg/ml							
Prothrombin (Laurell), u/ml							
Factor XIIIA (Laurell), u/ml							
Fibronectin (Laurell), mg/ml							
Factor VIII CAG							
NAPTT sec. 1/10		29.1			22.1	.252	233
TGt50 min.							
FDA hr.		4			1	2	1
Limulus + or -							

* Sorbitol 20 g/ml

≠ Sorbitol 1.0 g/ml.

9H27.

ASSAY REQUEST FORM (1)

Request from: PAF Date: 11/6/84

When results needed: _____

Samples to be kept? _____ If so, how? _____

Samples provided with form/a[redacted] from _____

SAMPLE 9H27. INVESTIGATION		1K1	1K2	1K3	1C		
Factor VIII, iu/ml	1 st.						
	2 st.						
Factor IX, iu/ml	1 st.	(60)	(10)	(0.5)	(<0.05)		
	2 st.	57.8	4.0	0.3			
Factor II, u/ml							
Factor X, u/ml							
Factor VII, u/ml (clotting)							
AT III (Anti Xa), u/ml							
AT III (Anti IIa), u/ml							
AT III (Laurell), u/ml							
Factor VIII RAG, u/ml							
Fibrinogen (Laurell), mg/ml							
Prothrombin (Laurell), u/ml							
Factor XIIIA (Laurell), u/ml							
Fibronectin (Laurell), mg/ml							
Factor VIII CAG							
NAPTT sec. 1/10 control 279		194	206	231	420+		
TGt50 min.							
FDA hr.		2	4	6+			
Limulus + or -							

14.6.84 9H27-02 To examine the DE 52 elution properties of re-chromatographed 9H27/L

Purpose

Due to the presence of 46% of bound factor IX in 9H27 eluting from DE52 in buffer K, this experiment is to test whether the factor IX in /L is chromatographically different from that in /K.

Materials

Buffer L
8.9 mM trisodium citrate
1.3 mM citric acid
10 mM Na_2HPO_4
100 mM NaCl
pH 7.05
Conductivity 11.97 mS

Buffer K
8.9 mM trisodium citrate
1.1 mM citric acid
10 mM Na_2HPO_4
300 mM NaCl
pH 7.03
Conductivity 27.54

DE52 Use batch RD/3, as used for 9H27

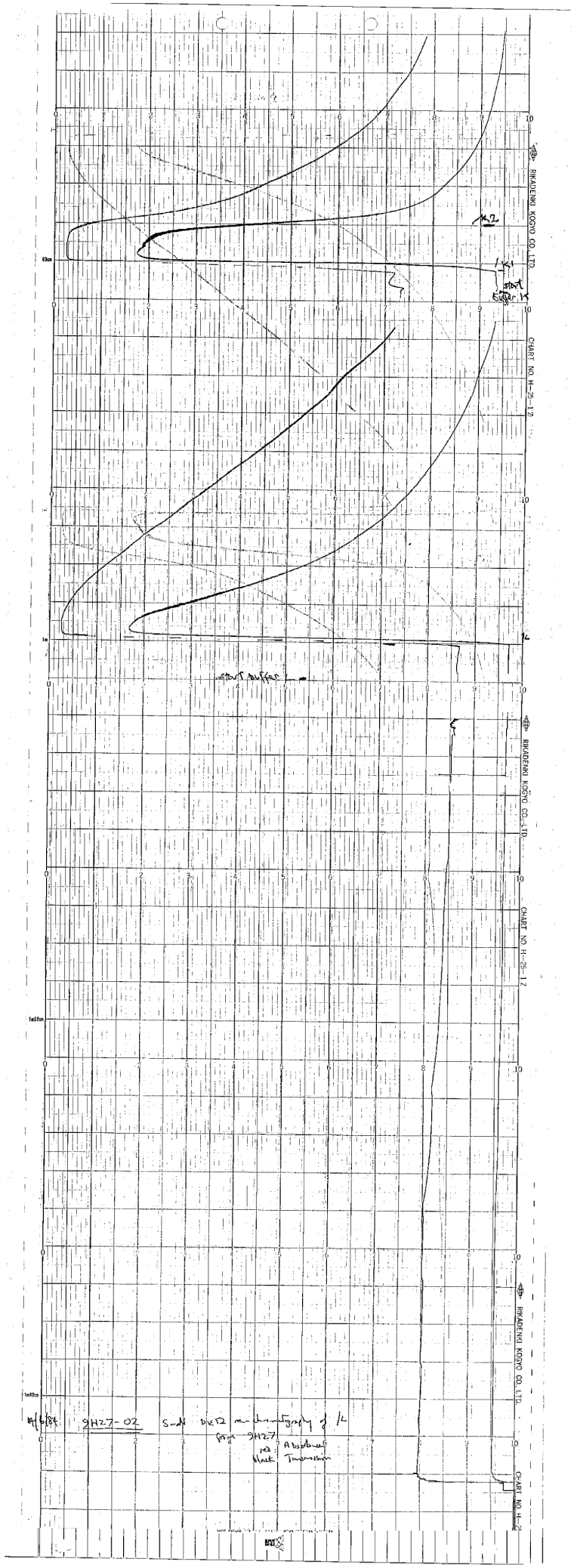
Factor IX A sample of 9H27/L was removed from cold room 3 and softened at room temperature.

Method

1. Once thawed the 9H27/L was diluted with PFW to reduce the ionic strength so that adsorption to DE 52 could occur. The 300 ml sample was diluted by ½ by addition of 300 ml PFW. pH and conductivity were measured and 3x1ml and 1x3 ml samples was taken. ("/XL")

2. Loading of DE-52. The previous factor IX assay for /L → 4.7 u/ml, so 300 ml contained 1410 units fIX.

Gel was added at 1 g per 150 units factor IX (heat
This loading is slightly higher than that used in 9H27
[1 g per 200 units of 9D ≡ 1 g per 110 units fIX heated.]
i.e. 9.4 g DE-52 added.


RIKADENKI KOGYO CO.,LTD.
CHART NO. H-25-1Z
start
start
RIKADENKI KOGYO CO.,LTD.
CHART NO. H-25-1Z
RIKADENKI KOGYO CO.,LTD.
CHART NO. H-25-1Z
9H27-02
9H27
red
black
Absorbance
Transmission

3. The gel was mixed for 1½ hours at room temperature, then poured into a 3.3 cm I.D. glass Wight column and allowed to pack under gravity.

4. Once packed, the bed volume was 19.7 ml. The gel was washed with 7 bed volumes buffer L (136 ml) followed by 5 bed volumes buffer K (100 ml).
Flow rate ~ 3.5 ml/min (i.e. 0.18 bed volumes/min)
(cf. 9H27 flow rate 200 ml/min = 0.38 bed volumes/min).

5. Elute was monitored by u.v transmission and absorption with Uvicord S monitor and fractions were collected accordingly, as shown on accompanying trace.

Assays

Samples were assayed for factor IX, NAPTT, A280, pH and conductivity.

Results

Sample	Vol (ml)	A280	Factor IX u/ml	Factor IX total	Sp Act u/mg	pH	Conductivity (ms)	NAPTT $\frac{1}{10}$ (s)	% FIX loaded
αL	600	1.53	1.8	1080	1.18	7.67	5.7	251	100
/Sn	600	0.29	0.19	114	0.65	8.78	5.65	260	10.5
/L	128	3.37	4.4	563	1.31	8.26	11.80	291	53
/K1	16	10.5	23.8	380	2.27	7.21	20.16	230	35
/K2	75	1.07	1.5	112.5	1.40	7.10	27.54	257	10.4

Comments

1. There is obviously factor IX in /L which could, under certain conditions, be eluted later in buffer K. The distribution between /L and /K may depend upon the initial gel loading or upon a limiting value which will always be eluted in buffer L. The implication is that an equilibrium exists:

$$\text{FIX} + \text{DEAE} \rightleftharpoons \text{FIX}\cdots\text{DEAE}$$

in which the limiting effect is [DEAE]. Then, at a certain loading, there will be a certain proportion of factor IX which can be eluted by buffer L.

9H27-02.1

2. Some further studies on [redacted] /L and rechromatography with varying amounts of DE52 would therefore be worthwhile, as would a revision of current heating recoveries to give a loading related to starting material.

3. There is still a slight shortening of NAPTT in /K1 compared to αL, but /L is longer.

4. The specific activities indicate that there is no intrinsic difference between the factor IX which elutes (on the first chromatography) in buffer L and buffer K

P.A.F

ASSAY REQUEST FORM (1)

Request from: PAF Date: 14/6/84

When results needed: ASAP

Samples to be kept? No If so, how?

Samples provided with form/available from

27.

SAMPLE 9H25-02 / INVESTIGATION		αL	/Sn	/L	/K1	/K2	
Factor VIII, iu/ml	1 st.						
	2 st.						
Factor IX, iu/ml	1 st.	(2.3)	(0.06)	(5.2)	(23)	(1.6)	
	2 st.	1.8	0.19	4.4	28.8	1.5	
Factor II, u/ml							
Factor X, u/ml							
Factor VII, u/ml (clotting)							
AT III (Anti Xa), u/ml							
AT III (Anti IIa), u/ml							
AT III (Laurell), u/ml							
Factor VIII RAG, u/ml							
Fibrinogen (Laurell), mg/ml							
Prothrombin (Laurell), u/ml							
Factor XIIIA (Laurell), u/ml							
Fibronectin (Laurell), mg/ml							
Factor VIII CAG							
NAPTT sec. 1/10 blank 285		251	260	291	230	257	
TGt50 min.							
FDA hr.				1			
Limulus + or -							