# MEMO

| From | To |
|---|---|
| Dr. Bidwell | Dr. Maycock |
| Our Reference / Your Reference | 6th December, 1976 |

I enclose a copy of Dr. Smith's report to me for the period Jan - June 1976.

At my suggestion this takes a different form from hitherto. He keeps a full record of individual batch details so has presented his summarised conclusions and selected various memoranda prepared during the relevant period which he thinks worthy of regarding as part of the report. Some of these have already been sent to you - the others are enclosed.

GRO-C

Ethel Bidwell.

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Confidential.

# Bi-annual report on the fractionation of plasma at the Oxford Plasma Fractionation Laboratory

## Period January to June 1976

### 1. *Plasma used and pools fractionated*

A total of 3275 kg FFP was fractionated in 34 pools to recover both factor VIII (8IP or 8IRV) and factor IX (DE(1), 9D) concentrates. In addition, 1568 kg of cryoprecipitate supernatant in 5 ℓ packs was fractionated in 14 pools (19 columns) to DE(1), and DE(1) was also prepared in 25 pools (50 columns) from 4777 kg plasma adsorbed on DEAE-cellulose at BPL and eluted and finished at PFL (Table 1).

No Type C four-factor concentrate was prepared.

All supernatants were transferred to BPL for recovery of IgG and albumin.

Table 1

| | | |
|---|---|---|
| FFP Oxford | 2352.3 kg | |
| FFP Other regions | 922.2 kg | |
| | 3274.5 kg | used for factor VIII production |
| CPS Bristol | 1568.3 kg | |
| CPS adsorbed at BPL | 4777.0 kg | |
| | 9619.8 kg | used for factor IX production |

### 2. *Factor VIII concentrate (8IP and 8IRV)*

#### Fresh frozen plasma sources and quality

Oxford RTC: 5 ℓ Nusac packs were still being frozen on slatted shelves at -30°.

North West Thames (Edgware) RTC: by arrangement with Dr. Ellis, FFP from this RTC (from blood collected in CPD anticoagulant) was used in four pools for 8IP production. Oxford RTC returned the equivalent ACD plasma directly to BPL.

Yorkshire (Leeds) RTC: by arrangement with Dr. Rajah, three pools of routinely collected plasma and two pools collected within 4 h of donation were transported to PFL for 8IP production (two further pools of < 4 h plasma were added in July and August).

Oxford RTC continued to recover about 233 ml of plasma per donation, whereas Edgware appeared to recover about 188 ml and Leeds about 218 ml.

Three pools for factor VIII production (8IP 727, 8IP 733 and 8IRV 754) were composed wholly of packs previously rejected as unsuitable for fractionation use through damage, slight contamination, irregular shape, etc. These pools were fractionated primarily for investigation of processing variables, but were handled in such a way as to produce useful therapeutic material. However, only part of the plasma used would appear in DHSS returns as having been received during the period under review.

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Production methods

8IP continued to be made as in the previous six-month period, with only minor adjustments. Cryoprecipitate was extracted for 30 minutes with 8% plasma volume of 0.02M tris, the extract adsorbed with 5 ml Alhydrogel per kg plasma, the extract diluted with tris to 9.5% plasma volume, and citrate and chloride concentration adjusted using pre-sterilised 50-fold concentrated buffer (also added to the filter wash). After investigating the stability of /5 solution at higher temperatures, from 8IP 772 onwards the solution was warmed to 40° rather than 30° before filtration, in order to increase the mean temperature during sterile filtration. The change appears to have helped to reduce filtration times.

The major innovation in factor VIII production was the extraction of cryoprecipitate in a smaller volume of tris buffer to yield a more potent product called 8 IRV (Intermediate Reduced Volume). Cryoprecipitate was extracted for 30 minutes with 3% plasma volume of 0.02M tris, the extract adsorbed with 5 ml Alhydrogel per kg plasma, no tris diluent added, and the concentrations of citrate and chloride adjusted as for 8IP.

The 8 IRV extract was centrifuged in a single Mistral centrifuge, and took no longer to filter than 8IP. The filtered product was filled in 20-25 ml volumes into factor IX vials, intended to contain approximately 125 iu, or 100 ml was filled into MRC bottles, intended to contain approximately 500 iu. The dissolved product had a potency of 10-15 iu/ml, i.e. 2-3 times that of 8IP, but took longer to redissolve - seldom less than 15 min and occasionally over 30 min. Evacuation of the container did not significantly improve solubility.

Data from 8IP and 8 IRV batches are discussed in the memorandum "Factor VIII Recoveries" dated 23.9.76, and are considered separately below. It should be noted that batches 8IP 727 and 733 were divided at the cryoprecipitate stage, portions extracted with different volumes of tris buffer to derive information leading to 8 IRV production, and finally diluted and finished as 8IP. Batch 764 was extracted as 8 IRV but finished partly as 8IRV and partly as 8IP; data for this batch have been excluded from some analyses of 8IP and 8IRV.

Yields of factor VIII

The results of changing sources of plasma and extraction volumes are compared in the memorandum "Factor VIII Recoveries" dated 23.9.76.

The mean gross yield of factor VIII during this six-month period was calculated from the assay on the redissolved concentrate and from the total volume dispensed and freeze dried, including the volume for sterility tests - this makes a difference of less than 1% from the previous convention of excluding the latter volume. Net yield is based on the volume signed as fit for issue. Percentage yield is based on a notional 0.738 iu/ml in fresh plasma, and "purity" is compared with a notional specific activity of 0.0121 iu/mg in fresh plasma, assuming its protein concentration to be 61 mg/ml. Certain indices of performance are assessed separately for 8IP and 8IRV in Table 2.

The mean gross yield of factor VIII was 237 iu/kg, producing a total gross yield of 775 x $10^3$ iu from 3275 kg plasma. Of this, 750 x $10^3$ iu was the net yield placed at issue after quality control and all other losses, representing a 29% increase over the previous six months. The difference between gross

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and net yields was 5.8%, compared with 8.1% in the previous six months. Approximately 23% of all factor VIII was issued as 8IRV.

The means and standard deviations of gross yield, specific activity and percentage fibrinogen of 8IP were very similar to those of the previous two six-month periods, despite a wide variation in plasma quality. The mean potency was slightly lower than that of 1975, following efforts to standardise the product volume as a percentage of plasma volume. It has since been decided, after consultation with the clinical staff of Oxford Haemophilia Centre, to increase the average potency of 8IP to an intended 5.0 iu/ml by slightly reducing the volume of tris extracting and diluting buffer.

There was no significant difference between OHC's and PFL's assays of 8IP, but OHC assayed 8IRV at a mean 83% of the value found by PFL ($P < 0.02$). This may have been due to under-dilution of 8IRV by OHC where the higher potency of 8IRV was not anticipated.

Patient responses

The mean response of 22 patients to 29 infusions of 13 batches of 8IP was 1.63 (range 0.9 to 3.4), closely comparable with the mean response calculated during 1975.

The mean response of two patients to two infusions of two batches of 8IRV was 1.65, using OHC's lower value for the dose. If this pattern is confirmed in many more infusions, we would have to determine whether:

(1) PFL overestimate the factor VIII content of 8IRV, compared with 8IP.

(2) OHC tend to under-estimate the factor VIII content of 8IRV, PFL assays on 8IRV and 8IP are equally valid, and patients really have a poorer response to 8IRV than 8IP.

(3) OHC tend to under-estimate the factor VIII content of 8IRV, but over-estimate the true volume of this concentrate given to the patient.

The last explanation seems the most likely, warning of an inherent disadvantage of higher potency concentrates which can be partially overcome by improvements in solubility, viscosity and container design.

Unusual losses, delays, etc.

There were no unusual delays in production, large losses of factor VIII or failures of sterility in the six months under review.

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Table 2. Analysis of 8IP and 8IRV production : January to June 1976

| | Gross Yield iu/kg (/7) | Net Yield iu/kg (/8) | Net Yield % (/7) | Net Yield % (/8) | Potency iu/ml | Protein mg/ml | Sp. Act. iu/mg | Purifn. x | Fibrinogen % |
|---|---|---|---|---|---|---|---|---|---|
| 8IP n | 26* | 26* | | | 27 | 26* | 26* | | 21 |
| Mean | 230 | 220 | 31.2 | 29.8 | 4.72 | 21.6 | 0.222 | 18.3 | 56.3 |
| SD | 25 | 23 | | | 0.50 | 3.4 | 0.034 | | 7.4 |
| 8IRV n | 7* | 7* | | | 8 | 7* | 7* | | 6 |
| Mean | 267 | 238 | 36.2 | | 32.2 | 55.0 | 0.239 | 19.8 | 66.1 |
| SD | 22.9 | 36 | | | 1.84 | 9.2 | 0.042 | | 7.1 |

*excluding batch 8IP/8IRV 764, but including batches 8IP 727 and 733.

Mean gross yield 237 iu/kg

Mean net yield 223 iu/kg

Total gross yield 775 x $10^3$ iu

Total net yield 730 x $10^3$ iu

Total plasma used 3275 kg = 14,627 donations (mean 224g/donation)

*3. Factor IX concentrate, type DE(1)*

A summary of the plasma sources is included in Table 1 above. A major development was the number of batches filled from two or more eluates, the finished product carrying the highest batch number of those pooled. It was decided during this period, after review of /6 and /7 assays, that the factor IX concentration in eluates was reasonably predictable and that replicate assays on the redissolved /7 product would be more valuable than single assays on individual eluates. In principle, it was thought desirable that individual eluates should be tested for HBs Ag and for endotoxin (by limulus test) before pooling, but pressure of demand has made this impracticable. BPL-adsorbed batches were not mixed with other for pooling, but all PFL-adsorbed batches began to be mixed freely during this period.

"Routine" fractionation procedure

(1) Certain changes introduced or predicted during July to December 1975 were incorporated into routine practice; these included:

(a) Collection of inert second-crop cryoprecipitate;

(b) "Edinburgh" recycling with 0.5N acid and alkali, without removal of the DE 52 from the basket centrifuge;

(c) Substitution of freezing for autoclaving recycled DE 52 (from 9D 756).

(2) From batch 9D 760, the diluted cryo-supernatant pool was titrated to pH 6.9 $\pm$ 0.1 with 5N acetic acid before adsorption with DE 52. BPL were not requested to make this change and their batches serve as controls. The aim was to reduce the pH of the dissolved product to nearer physiological pH, avoiding occasional drift above pH 8.

A statistical comparison of five PFL-adsorbed batches before, and five after, this change indicated that the protein concentration was not significantly reduced; pH was reduced (7.64 $\pm$ 0.11 to 7.32 $\pm$ 0.15, $P < 0.01$); citrate concentration and conductivity were not significantly changed; and the potency of the product was slightly increased (423 $\pm$ 28 u/ml to 465 $\pm$ 16 u/ml, $P < 0.05$) - but this must have been the result of other coincidental factors, since the yield of factor IX (u/kg) was not significantly increased.

(3) The citrate wash buffer was omitted from batch 9D 748 onwards. The collected DE 52 was resuspended in 8 ℓ buffer A and the column washed with a further 3 ℓ of buffer A before adding eluting buffer B. Since it was decided not to pursue factor VII recovery by the original method, the citrate wash appeared to be redundant.

A statistical comparison of five PFL-adsorbed plus five BPL-adsorbed batches before, and the same number after, the change indicated that the potency, yield and conductivity of the product remained unchanged; pH was significantly reduced (from pH 7.91 $\pm$ 0.14 to pH 7.60 $\pm$ 0.08, $P < 0.001$); and protein concentration increased (11.8 $\pm$ 1.1 to 14.5 $\pm$ 1.3 mg/ml, $P < 0.001$). This appears to be the change which led to a decrease in purity during the six-month period, compared with previous periods (Table 4).

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(4) Attempts were made to replace the existing pressurised buffer system with peristaltic pumps for buffer addition, but difficulty was experienced in maintaining even flow rates and the innovation was not confirmed.

(5) From batch 9D 737, Sharples backwash was not filtered or washed in the basket centrifuge, but was poured first into the AC 100 column and drained before the addition of the bulk of the DE 52. This saved cleaning and autoclaving of equipment and carried no penalty in overall fractionation time.

(6) During runs 9D 738, 773, 791, fractions of about 100 ml were collected sequentially before and during the elution of the factor IX peak. These experiments are reported more fully in Mr. Snape's memorandum, dated 1.11.76, "Potential thrombogenicity of fractions contributing to type DE(1) factor IX", but the most interesting feature, confirmed in runs 9D 777 onwards, was that the first 100 ml of the "factor IX" peak indicated by continuous refractometry (called fraction /6x) was almost devoid of factor IX activity. This fraction was much more opalescent than the rest of the peak and had an excess of "thrombogenicity" assessed by e.g. the normal recalcification time. From 9D 783, collection of /6x was started at the point where the refractometer trace went through a very sharp minimum, 100 ml of /6x discarded, and collection of the active peak continued up to the total eluate volume relationships defined earlier. The eluate pool therefore contained 100 ml less than before, but at a slightly higher potency. Serial monitoring of refractive index and conductivity showed that the latter could also be used to mark the emergence of the factor IX peak, but that the refractive index and conductivity cues were slightly displaced from each other.

This change was made late in the six-month period and few data on finished batches are available. The expected increase in potency was attained and led to consideration of diluting the eluate to an expected 40 u/ml (approximately equal to 30 iu/ml) to compensate for the loss of product volume; this will be discussed in the next report. A comparison of six matched batches before and after the change revealed no significant change in yield, protein concentration, pH or conductivity of the product.

The change has resulted in PFL issuing a much more consistent concentrate in that opalescence has been virtually eliminated and the normal recalcification time has been greater than 100 seconds since the change. The opalescent fraction now discarded as /6x has evidently been a major cause of membrane filter blockage in the past and it is now standard practice to use a single set of three 142 mm membranes to filter about 3 ℓ of eluate where two or three sets (and possibly centrifugation) would have been required in the past.

See also memorandum "Selection of 9D eluate peak", 1.7.76.

(7) By far the most important development during this period was the abrupt improvement in the quality of factor IX adsorbed at BPL and eluted at PFL. PFL memorandum "Recalcification times of 9D batches from Elstree and other sources", dated 1.3.76, drew attention to the short recalcification times found at /6 stage of BPL-adsorbed batches over the previous eight months.

Batches 9D 769 and 9D 770 were adsorbed at BPL, two 100 ℓ pools being treated in different ways and eluted separately at PFL. The pools were (a) adsorbed overnight and collected by Sharples centrifugation, (b) adsorbed overnight and collected by filtration or (c) adsorbed for 1 h and collected by Sharples centrifugation. These experiments showed unequivocally that excessive

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opalescence of the eluate was associated with filtration of the adsorbed suspension and that a short recalcification time in the product was associated with overnight adsorption. (These experiments were carried out before the opalescent /6x fraction was recognised or dealt with.)

| Batch | Adsorption | Collection of loaded DE 52 | Appearance of /6 | Normal recal. times min/control |
|---|---|---|---|---|
| 769α | Overnight | Filtered | Opalescent | 111/105 |
| 769β | Overnight | Sharples | Clear | 65/110 |
| 770α | 1 h | Sharples | Clear | 157/151 |
| 770β | Overnight | Filtered | Opalescent | 44/151 |

9D 769β and 9D 770β (adsorbed overnight) were not sterilised or dried since the recalcification times were so short.

From 9D 773 all pools were adsorbed at BPL for 1 h, DE 52 removed by Sharples centrifuge and the loaded DE 52 brought to PFL within about 4 h of starting adsorption. Between 9D 722 and 762, only four out of fourteen BPL-adsorbed batches had normal recalcification times greater than 90 seconds. Since 9D 773, no batch has had a normal recalcification time shorter than 100 seconds.

Changes in finishing procedure

A permanent change to filling factor IX from a sterile reservoir was made from 9D 768. The option of using a non-sterile valve and syringe with a terminal sterile Twin-90 was reserved for emergencies, but it was considered that the slight reduction in particle count theoretically offered by this technique was outweighed by the risk of increased sub-batch quality control, even when filtrations became more reliable.

Quadrants of filled vials were placed in specially made aluminium boxes replacing the existing "cake stands". Vials so protected were frozen and stored in a shelf-freezer (Lindsell-Dewell) in which air at -40° was supplied by a fan from a remote condenser. Clinbritic overcaps continued to be used, and were removed only just prior to loading vials into the freeze drier.

Comparison of BPL- and PFL-adsorbed batches

During this period the mean potencies of products from batches adsorbed at BPL and PFL were virtually identical. The yield from BPL-adsorbed batches was 541 ± 70 u/kg compared with 578 ± 61 u/kg from PFL-adsorbed batches ($0.05 < P < 0.1$); this difference may be due to BPL's estimation of CPS volume. Protein concentrations of products from the two sources were not significantly different.

After several changes in BPL's procedure at 9D 770 (see above) batches from the two sources became indistinguishable by routine control tests, except pH.

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Deliberate variations in procedure

(1) CPD plasma

Batches 9D 778, 781, 789 and 795 were made from plasma collected at North West Thames RTC in CPD anticoagulant. These eluates were pooled into only two finished batches, 9D 781 and 9D 795, and no statistical analysis would be valid. However, the batches appeared indistinguishable from routine ACD batches, by any characteristic of elution or property of the final product.

(2) Plasma from different RTCs

Finished batches 9D 730, 743 and 749 were made entirely from plasma collected in Leeds RTC. No unusual properties were noted during elution and analysis of these batches and eluates from this source of ACD plasma were thereafter (e.g. 9D 786) mixed with eluates from other sources of ACD plasma, adsorbed at PFL. Differences between PFL- and BPL-adsorbed batches are discussed in detail above.

(3) DEFIX buffers

9D 794 was prepared from plasma packs previously rejected for fractionation use on the grounds of split packs, etc. Some packs were more than two years old. It was decided to clear them from the freezers and attempt a variation in factor IX production which might risk the loss of this "free" batch of plasma. The results are included in Mr. Snape's memorandum dated 1.11.76, "Potential thrombogenicity of fractions contributing to type DE(1) factor IX", and further experiments along the same lines may be discussed in a later report. The major variations were the use of DE 52 in the chloride form, and the use of PFC's wash and elution buffers, containing higher concentrations of citrate and phosphate than the corresponding PFL buffers, in an attempt to obtain a longer $TGt_{50}$ in the eluate. The $TGt_{50}$ was 10 min, slightly longer than the average for DE(1) but not necessarily significant. The high citrate and phosphate content of the eluate placed it outside control limits for DE(1), discouraging further attempts at this modification until the supply of factor IX has improved.

(4) Undiluted CPS or SI

On two occasions, fraction I was removed from CPS before adsorption with DE 52, primarily in order to assess the DE-supernatant as a source of factor VII for adsorption with Sephadex A-50. The supernatant I was not further diluted before adsorption with DE 52. Adsorption time was increased to 2 h for one batch.

With similar motives, two batches of CPS were adsorbed with DE 52 without prior dilution with water.

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Table 3. Adsorptions from undiluted CPS and SI

| Batch 9D | CPS/SI | Diluted | Ads. time h | Ads. pH | Yield u/kg | Potency u/ml | Protein mg/ml | Sp. Act u/mg | $TGt_{50}$ min |
|---|---|---|---|---|---|---|---|---|---|
| 740 | CPS | No | 2 | ≃ 7.5 | 281 | 20.8 | 5.9 | 3.52 | 15 |
| 754 | SI | No | 1 | ≃ 7.3 | 447 | 33.3 | 11.0 | 3.03 | 10 |
| 771 | CPS | No | 2 | 6.9 | | | | | 6 |
| 772 | SI | No | 2 | 6.9 | | | | | 8 |

Data on batches 771 and 772 are incomplete because they were pooled with other eluates for filtration. It appears that adsorption of undiluted CPS or supernatant I incurs some penalty in yield but that the lower protein content of the eluate leaves the purity unchanged. As expected from earlier development work, the very low yield from undiluted CPS makes the option unattractive as a means of recovering useful amounts of factor IX prior e.g. to adsorption with a second ion-exchanger.

The longer $TGt_{50}$ times for batches 9D 740 and 754 were not sustained in 9D 771 and 772, and were in any case related to low potencies of factor IX, i.e. they could be explained by dilution rather than separation of the responsible agent.

Unusual losses

Some losses of issuable material are listed at the start of the next section; these relate mainly to BPL-adsorbed batches with short recalcification times. Batches 9D 747, 756 and 759 also had short recalcification times (45-77 seconds) but were finished, without the addition of heparin, for possible administration to patients with antibodies to factor VIII.

Batches 9D 762 and 764 were refiltered, with 5-10% loss, after premature cap closure in the freeze drier had resulted in only partial drying.

Batch 9D 782 also suffered approximately 10% loss and refiltration after being found contaminated with Gram-negative bacilli.

About 10% of eluate 9D 774 was lost when a bottle broke during thawing.

Since most batches were filled all at 20 ml, without a proportion of 10 ml vials which were once used for sterility testing, repeat sterility testing carried a high penalty in loss of issuable factor IX. However, only 9D 758 had to be sampled a second time.

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Overall yield, etc. of all issued batches

The summary of production data in Table 4 is assembled from 74 9D runs started during the period 1st January - 30th June 1976, namely batches 9D 722 to 795, with the following exceptions:

Eluate 9D 765 (BPL-adsorbed) was unfilterable, possibly because of a high thrombin content (FDA test: 2 h).

Eluates 769β and 770β (BPL-adsorbed) had short recalcification times (65 and 44 seconds respectively) and FDA times (2 h and 1¼ h respectively) and were considered unsuitable for further processing - see the section above on changes in BPL adsorption and filtration conditions.

Eluate 794 was obtained by an approximation to PFC's DEFIX conditions (see above); the concentrations of citrate and phosphate in the product were considered too high for issue as DE(1).

Eluate 9D 792 was held over for finishing with the eluate 9D 800 in the following six-month period.

All these exceptions are included in "work done", but not in the rest of the table which covers 53 issued batches.

After the end of the six-month period, factor IX assays were based on the "provisional International Standard 72/32", prepared from PFL DE(1) and proposed by ICTH to WHO as a standard for factor IX. Adoption of this standard led to a large apparent decrease in the concentration of factor IX in DE(1) concentrate and some data have been expressed in "proposed international units" (piu) to facilitate comparison with earlier reports.

Compared with the second half of 1975, there was an 8% increase in the number of columns run, a 10% increase in the volume of plasma adsorbed at PFL and a 37% increase in the volume of plasma adsorbed at BPL. The increased number of batches was finished in only 53, compared with 69, filtration batches, and issuable factor IX was increased by 20%. The mean factor IX potency and yield/kg were almost unchanged, but purity was reduced as a result of a higher mean protein concentration (see above).

Even after addition of heparin solution to 2% v/v, the volume of product dispensed for drying was only 94% of that eluted, and the volume recovered in issuable form was only 87% of that eluted. Filtration losses were less than in the previous six months, and should improve further, because of easier filtration and larger pools of eluate. Despite an increased quality control programme, overall control losses were slightly reduced by finishing in larger batches.

Freeze drying conditions were stabilised during this period (see memorandum 1.7.76, "Losses during final stages of DE(1) production"). No significant loss of factor IX is now expected on drying.

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Table 4 . Summary of factor IX (DE(1)) production, January - June 1976

| | |
|---|---|
| No. of CPS pools | 74 |
| No. of columns | 104 |
| Total plasma used | 9620 kg |
| Total plasma used for 53 issued batches | 8966 kg (excludes 9D 765, 769β, 770β, 792, 794) |

Analysis of 53 issued batches (mean ± SD)

| | | |
|---|---|---|
| Total eluate | collected from columns | 120.4 l |
| | dispensed (incl. heparin) | 112.7 l, = 93.6% of eluate |
| | issued (incl. heparin) | 104.5 l, = 86.8% of eluate |
| Factor IX: | Potency of redissolved product | 43.1 ± 5.7 u/ml, = 31.9 piu/ml |
| | Gross yield, dispensed | 550 ± 79 u/kg, = 407 piu/kg |
| | Net yield, issued | 510 u/kg, = 377 piu/kg |
| | Gross recovery, dispensed | 4.89 x $10^6$ u, = 3.62 x $10^6$ piu |
| | Net recovery, issued | 4.54 x $10^6$ u, = 3.36 x $10^6$ piu |
| Protein concentration | | 13.0 ± 2.1 mg/ml |
| Specific activity | | 3.3 ± 0.5 u/mg = 2.5 piu/mg |
| Purification factor | (x 0.01475 u/mg) | 225 times |
| | (x 0.0109 piu/mg) | 225 times |
| Factor II: | Potency of redissolved product | 36.1 ± 5.2 u/ml |
| Factor X: | Potency of redissolved product | 25.7 ± 6.6 u/ml |

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(a) Memoranda referred to in text and filed adjacent to the report

| *Index* | *Date* | *Title* |
|---|---|---|
| III B | 1.3.76 | Recalcification times of 9D batches from Elstree and other sources. |
| III C | 1.7.76 | Selection of 9D eluate peak. |
| III D | 1.7.76 | Losses during final stages of DE(1) production. |
| III E | 23.9.76 | Factor VIII Recoveries. |
| III F | 1.11.76 | Potential Thrombogenicity of Fractions Contributing to Type DE(1) Factor IX. |

(b) Other memoranda relevant to production during the period, but filed separately

| | |
|---|---|
| 13.11.76 | Summary of rate of freezing experiments on type DE(1) factor IX (Ref. CL39 and CL40). |
| 12. 1.76 | Interim report on the situation relating to factor VIII reference preparations in use in PFL on 12.1.76. |
| 1. 3.76 | Dispensing of small volume products. |
| 24. 2.76 | Summary of information on pyrogen testing of Oxford factor VIII and factor IX concentrates. |
| 1. 3.76 | Pyrogen and toxicity testing of plasma fractions. |
| 23. 3.76 | Pyrogen testing of type DE(1) factor IX. |
| 3.76 | Sterility testing of material prepared by PFL, Oxford, for clinical use. |
| 5. 5.76 | Effect of neutralising heparin in samples of type DE(1) factor IX prior to testing for potential thrombogenicity. |
| | Summary of data on two batches of type DE(1) factor IX selected for clinical trial. |
| 17. 9.76 | Progress report on projects related to β-thromboglobulin in plasma. |

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## Selection of 9D eluate peak

In each of the following runs, /6 eluate was collected in two or three fractions suggested by the results of fractional elutions 9D 778 and 9D 773.

9D 777 (Oxford plasma)
9D 778 (Edgware CPD plasma)
9D 781 (Edgware CPD plasma)
9D 782 (Elstree adsorbed)

Eluate from the column was led successively through a conductivity electrode and refractometer cell and these values monitored continuously.

9D 782 was adsorbed at uncontrolled pH, the others at pH 6.9 ± 0.1. All columns were eluted with buffer B after washing with buffer A alone.

Normally the 9D /6 fraction is taken immediately when a sharp dip in the refractive index changes to a sharp rise. In these runs, 100 ml of a fraction (6x) were taken, and elution completed up to the indicated volume, 1200-1400 ml. As figure 1 shows, the steep rise in refractive index coincides with a brief fall in conductivity, the latter rising again after about 100 ml corresponding to /6x.

The first 100 ml eluted was always more opalescent than the remainder of the peak, suggesting a contribution to difficult filtration; it contained not more than 3 u/ml factor IX; and it had a much shorter recal. time than the main peak. The next 100 ml, possibly a little more opalescent than the rest of the pool, contained a valuable concentration of factor IX and did not significantly shorten the recal. time.

The front of the peak could be predicted adequately by the brief dip in conductivity, provided it was continuously recorded, but the warning given by the refractometer is slightly earlier and, at these amplifications, more marked. BPL would not therefore be obliged to invest in a refractometer to select fractions and might concentrate on improving conductivity recording.

From 9D 783, the first 100 ml (6x) fraction was discarded as detrimental to the product and for a period a compensating 100 ml (6z) was sampled after the peak, but not pooled with it. Table 2 indicates that 6z does not contain a useful concentration of factor IX and that the factor IX peak has not simply been transposed. This confirms a few observations made in July - December 1975.

It is therefore expected that the volume of eluate will be reduced by 7-10 per cent as a result of this choice of fractions, but that its potency will be increased. A reliable pre-drying factor IX determination would require at least triplicate assays. It is recommended instead that, if over a period of weeks the mean /7 assay tends to increase beyond 40 u/ml (30 iu/ml), an arbitrary volume of distilled water be added at the filtration stage of every batch, calculated to reduce the potency to 30 iu/ml. This volume might be reviewed monthly or quarterly, or on the basis of a "running mean" of /7, adjusted if necessary for plasma source, anticoagulant or any other recognised variable. It is intended that dilution factors and data supporting them will be suggested in August when the last six months' yields, including recent batches lacking 6x, can be assessed.

M Smith
1.7.76
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Table 1

| Batch 9D | Factor IX, u/ml | | | | Min. recal., secs | | | |
|---|---|---|---|---|---|---|---|---|
| | 1st 100 ml | 2nd 100 ml | Rest | Pool | 1st 100 ml | 2nd 100 ml | Rest | Control |
| 777 | 3.0 | NA | 45.6 | 42.1 | 59 | 137 | 136 | 130 |
| 778 | 0.5 | NA | 61.0 | 46.5 | 80 | 115 | 125 | 116 |
| 781 | 2.9 | 34.9 | 53.5 | 45.7 | 102 (118 @ 1:1) | 105 | 120 | 116 |
| 782 | 1.0 | 15.8 | 47.1 | 39.6 | 78 (117 @ 1:1) | 154 | 139 | 136 |

Table 2. Factor IX content of 6z fractions collected after nominal peak volume

| Batch 9D | Factor IX, iu/ml |
|---|---|
| 783 | 7.2 |
| 784α | 5.8 |
| 784β | 7.5 |
| 787α | 6.4 |
| 787β | 4.7 |

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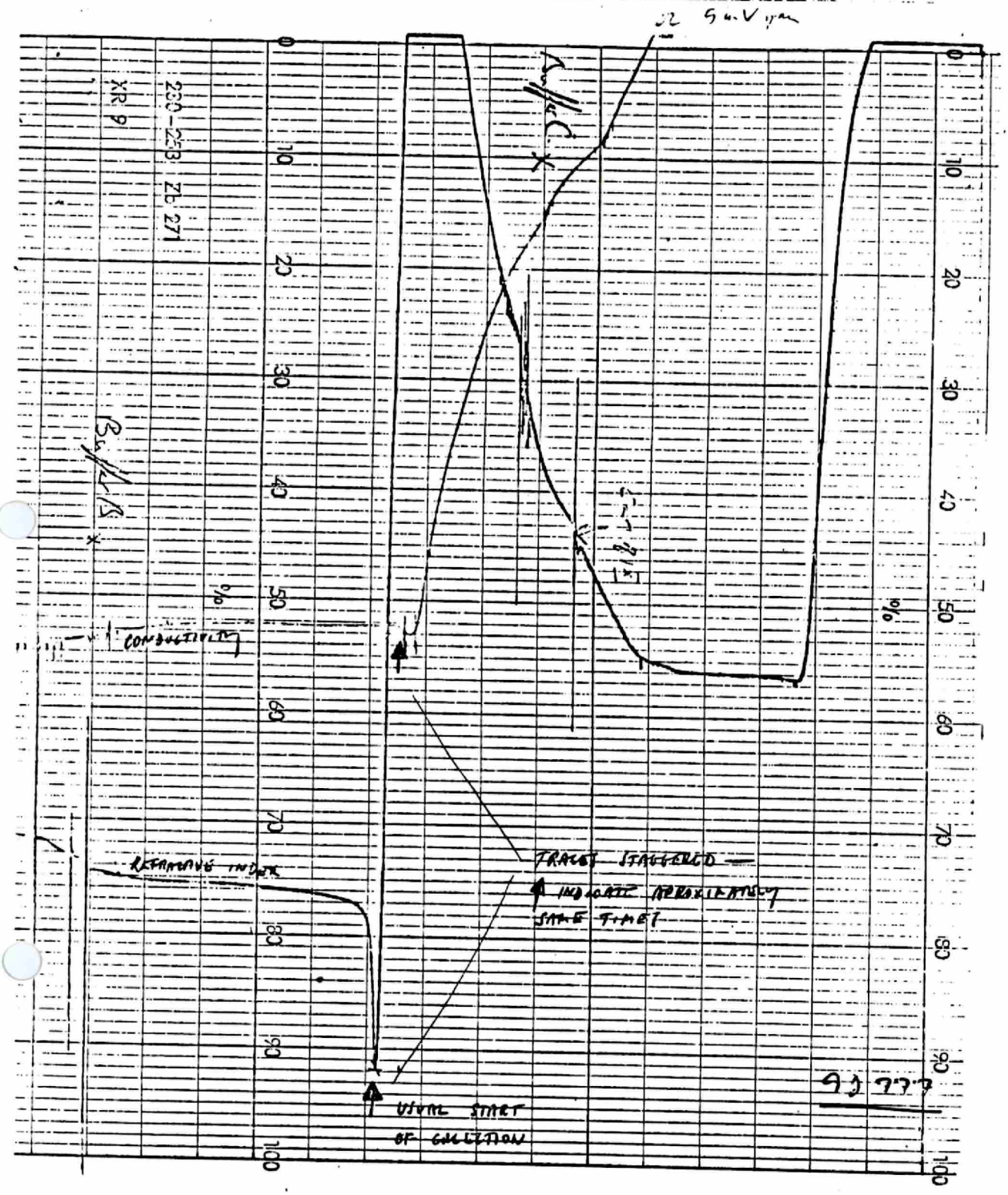


Fig. 1 Simultaneous recording of conductivity and refractive index during collapse of 90777.

74120

## Losses during final stages of DE(1) production

During July - December 1975 many batches of DE(1) were assayed for factor IX content at the eluate (/6) and dried (/7) stages, normally by a single two-stage assay. These data suggested that factor IX was being lost between these two stages, perhaps as a result of routine freezing of eluate prior to pairing and finishing, or to losses during sterile filtration and drying. No firm conclusion could be reached on the available data.

For a series of 15 finished batches of DE(1), factor IX was assayed at the eluate stage (and a mean factor IX content estimated where two eluates were pooled); immediately after thawing, heparinising and sterilisation (/6c); and after freeze drying (/7). The former were single assays, while /7 was assayed in the form STTS.

Of 15 fully consecutive sets of data, only the last three were from batches lacking the 6x fraction and the latter did not present a different pattern. During the entire period, the most important parameter of freeze drying, i.e. the primary drying temperature, was specified at-36° and this value was achieved ± 2°. No major changes were made in established routines of eluate finishing or filtration and all batches were heparinised. All batches were prepared by adsorption at pH 6.9 ± 0.1 except those adsorbed at Elstree, with collection of backwash on the column, and without citrate wash prior to buffer A. The usual range of plasma sources was included.

Two-tailed t tests were carried out on pairs of data.

(a) Comparison of 6 and 6c: effect of freezing eluate, addition of heparin and sterile filtration.

On 12 pairs of data, the mean change was a rise of 1.85 u/ml, not significant at p = 0.05. This apparent rise contrasts with an expected 8 per cent reduction of apparent activity expected simply on the addition of heparin (CL 44 ) without protamine sulphate neutralisation.

(b) Comparison of 6c and 7: effect of freeze drying.

On 12 pairs of data, the mean change was a rise of 2.29 u/ml, not significant at p = 0.05. It was concluded that drying temperature of-36° could be confirmed as carrying no significant loss of factor IX activity.

(c) Comparison of 6 and 7: effect of all operations between elution and drying.

On 15 pairs of data, the overall change was a rise of 4.39 u/ml, which appeared to be significant at p = 0.01. In the absence of a possible physical explanation, it is suggested that the result might arise from the large SD of single factor IX assays on /6 and /6c samples.

JM Lick
1.7.76

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Table 1

| Batch 9D | Factor IX u/ml at stage /6 | /6c | /7 |
|---|---|---|---|
| 762 | 27.2 | NA | 41.6 |
| 764 | 43.8 calculated | 39.3 | 49.0 |
| 768 | 41.5 calculated | 45.3 | 45.8 |
| 767 | 53.3 | 45.2 | 45.7 |
| 770α | 34.7 calculated | NA | 37.3 |
| 773 | 40.6 | 33.3 | 37.9 |
| 774 | 33.5 calculated | 31.7 | 33.2 |
| 776 | 37.0 | 40.1 | 46.8 |
| 777 | 32.3 calculated | 49.1 | 40.0 |
| 780 | 40.0 calculated | 36.0 | 46.8 |
| 781 | 46.1 calculated | 44.5 | 51.2 |
| 779 | 35.1 | 43.0 | 43.3 |
| 782 | 39.6 | 42.8 | 45.7 |
| 785 | 47.9 calculated | NA | 47.1 |
| 784 | 37.2 | 51.9 | 44.3 |

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# POTENTIAL THROMBOGENICITY OF FRACTIONS CONTRIBUTING TO TYPE DE(1) FACTOR IX

1.11.76.

## Batches considered

9D 773 - was prepared from DE 52/Protein complex adsorbed at Elstree using DEAE cellulose in the hydroxyl form and transported to Oxford for elution. The cellulose was suspended in 8 litres of buffer A and successively eluted with 3 litres of each of buffers A, B and C. Formulation of these buffers was conventional.

9D 794 - was prepared from DE 52/protein complex adsorbed at Oxford using DEAE cellulose in the chloride form. Volumes of eluting buffer were as usual but PFC wash buffer was used instead of buffer A and PFC eluant replaced buffer B.
NB Source plasma was haemolysed and very old.

9D 801 - was prepared from DE 52/protein complex adsorbed at Oxford using DEAE cellulose in the chloride form. Buffers were as for 9D 794 except that the DEAE cellulose was resuspended in only 3 litres of the PFC type wash buffer, washed with 2 litres of the same buffer and then eluted with 3 litres of PFC eluant followed by 3 litres of buffer C. In other words, conditions were very similar to those used routinely at PFC.

9D 804 - was prepared from DE 52/protein complex adsorbed at Oxford using DEAE cellulose in the chloride form. The usual volumes of Oxford buffers A, B and C were used.
NB Source plasma was cryoprecipitate supernatant from North West Thames CPD plasma.

Despite the variations in source plasma, anion-form of the DE 52 and volumes and types of buffers, the elution patterns for these four column runs were remarkably similar.

Figure 1 attached shows the optical density at 280 nm, refractive index and conductivity for the fractions of batch 9D 794. The five 50 ml fractions at the beginning of the elution allow detailed study of the early part of the peak. Optical density at 280 nm and refractive index follow a very similar pattern, beginning to rise in fraction 0 and reaching a peak in fraction 5. The pattern for conductivity is reproducibly different. A sharp drop in conductivity from fraction 0 to 1 is followed by a steady rise. Histograms similar to figure 1 are not shown for the other column runs though the pattern is very similar. In the other three runs fractions 0 and 1 are combined to give a fraction 'X'. Succeeding fractions are labelled 1, 2, 3, etc. regardless of volume.

Figures 2 to 5 are histograms of factor IX (by either 1-stage or 2-stage assay), FDA thrombin-fibrinogen time, $TGt_{50}$, NAPTT and recalcification time for the four column runs.

Factor IX: measured by either the 1-stage or the 2-stage assay peaks at approximately 4 to 500 ml eluate in all four columns and then falls off rather more gradually. This peak is invariably 100 ml or more after the protein peak as measured by either refractive index or $E_{280}$. The first 100 ml (as indicated by a rise in refractive index or $E_{280}$) contains little or no factor IX and on this criterion at least could well be omitted. For the elution of 9D 794 (figure 3) the factor IX levels were determined by both 1-stage and 2-stage assay.

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Much the same pattern was observed with the two techniques though the 1-stage assay gave values about 2 x the values suggested by the 2-stage procedure.

FDA thrombin-fibrinogen time: generally prolonged throughout the elution though in the case of 9D 773 (figure 2) the last fraction had an FDA time of less than two hours. The same fraction had lengthened $TGt_{50}$ and normal NAPTT and recalcification time. It is not difficult to reconcile the level of thrombin ($\simeq 10^{-2}$ NIH u/ml)(see memo TS to EB 21.5.76 on 'Detection of thrombin in prothrombin complex concentrates using the FDA thrombin/fibrinogen test system') suggested by the FDA test for this fraction with the result of the other tests.

$TGt_{50}$: generally short in earlier fractions becoming more prolonged towards the end of the elution. Only in the case of 9D 794 and 9D 801 do the $TGt_{50}$s for the later fractions exceed 20 mins.

NAPTT: the times shown were obtained using 1/10 dilutions of the test samples. The test shows little discrimination between the fractions in any of the batches except for fraction 0 from 9D 773 (figure 2).

Recalcification time: the times shown were obtained using a 1/1 dilution of the test sample - they are not necessarily the shortest times obtained. The most important feature of every run is the shortened recalcification time for the earlier fractions and in particular for the fraction labelled X. Given the very low factor IX assay of this fraction for all of the batches investigated and its apparently major contribution to potential thrombogenicity as measured by the recalcification time test, the possibility of excluding this fraction from the pool for future batches was investigated. Dr. Smith reports on the success of this investigation in his six-monthly report January to June 1976.

As always the interpretation of the recalcification time test and the NAPTT is complicated by the variation in control times obtained using the two test systems. Times obtained using dilutions of test preparations do not appear to be strictly dependent on the control times obtained on any one occasion. It is probably better to take satisfactory control times as indicating proper performance of the test system and then consider the clotting time for a dilution of the preparation under test. On two occasions (recalcification time for 9D 773 in figure 2 and NAPTT for 9D 804 in figure 5) one might conclude that the relevant test systems were not functioning properly in view of the unsatisfactory control times and that results obtained on the test dilutions were suspect.

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Fig 1

Optical density, refractive index and conductivity of fraction no 9773


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Fig 5

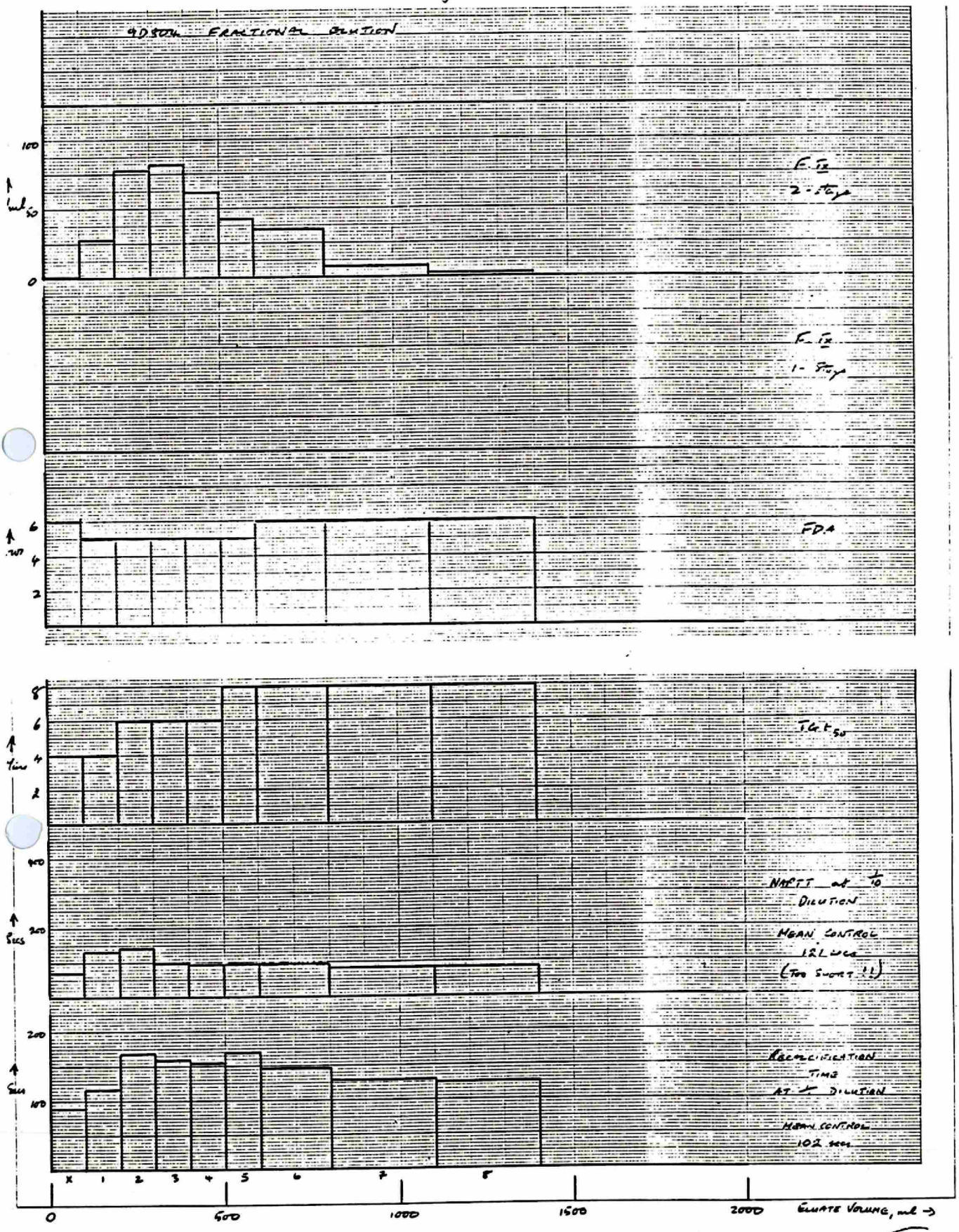


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Fig 2

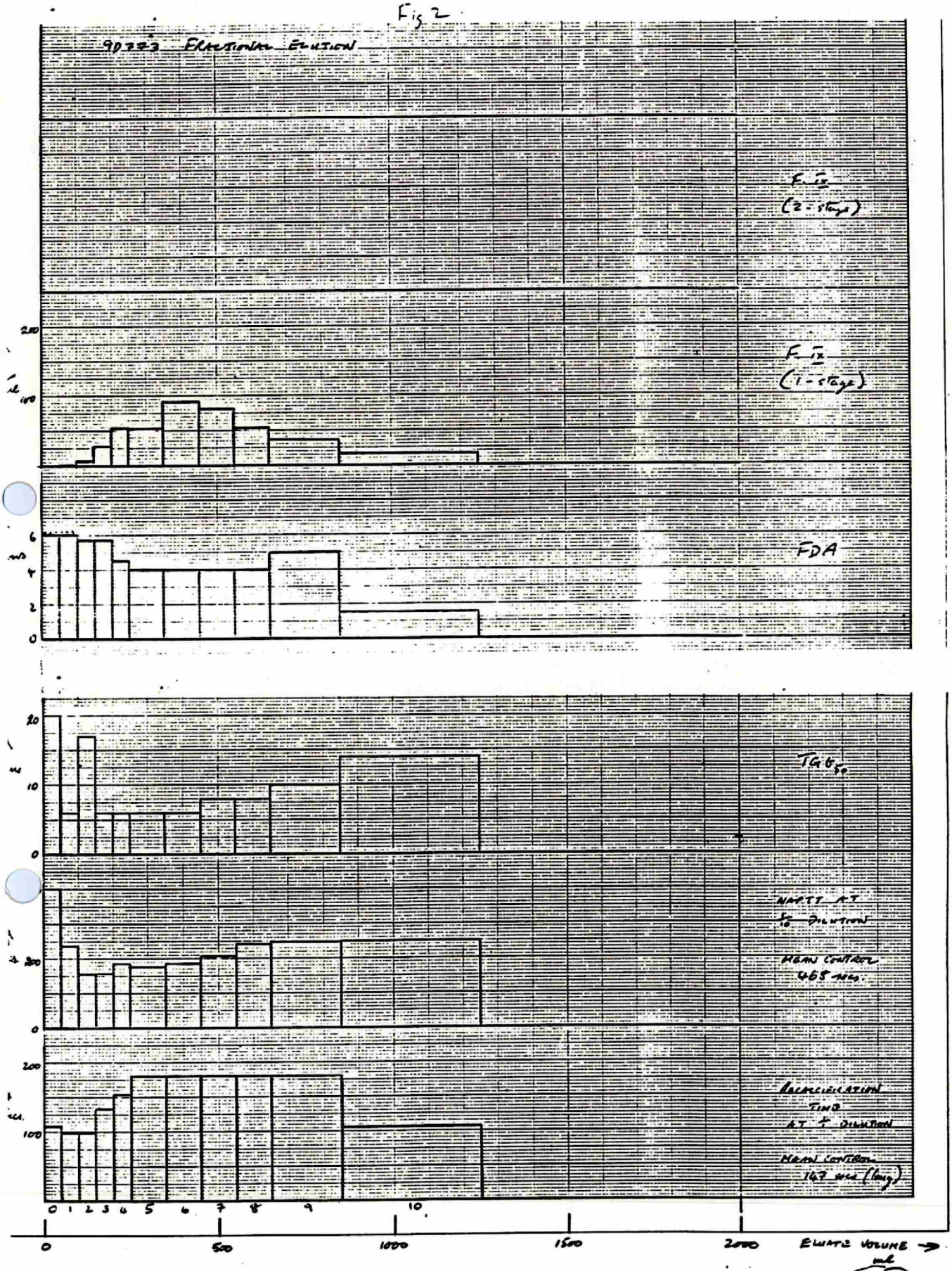

Fig 3

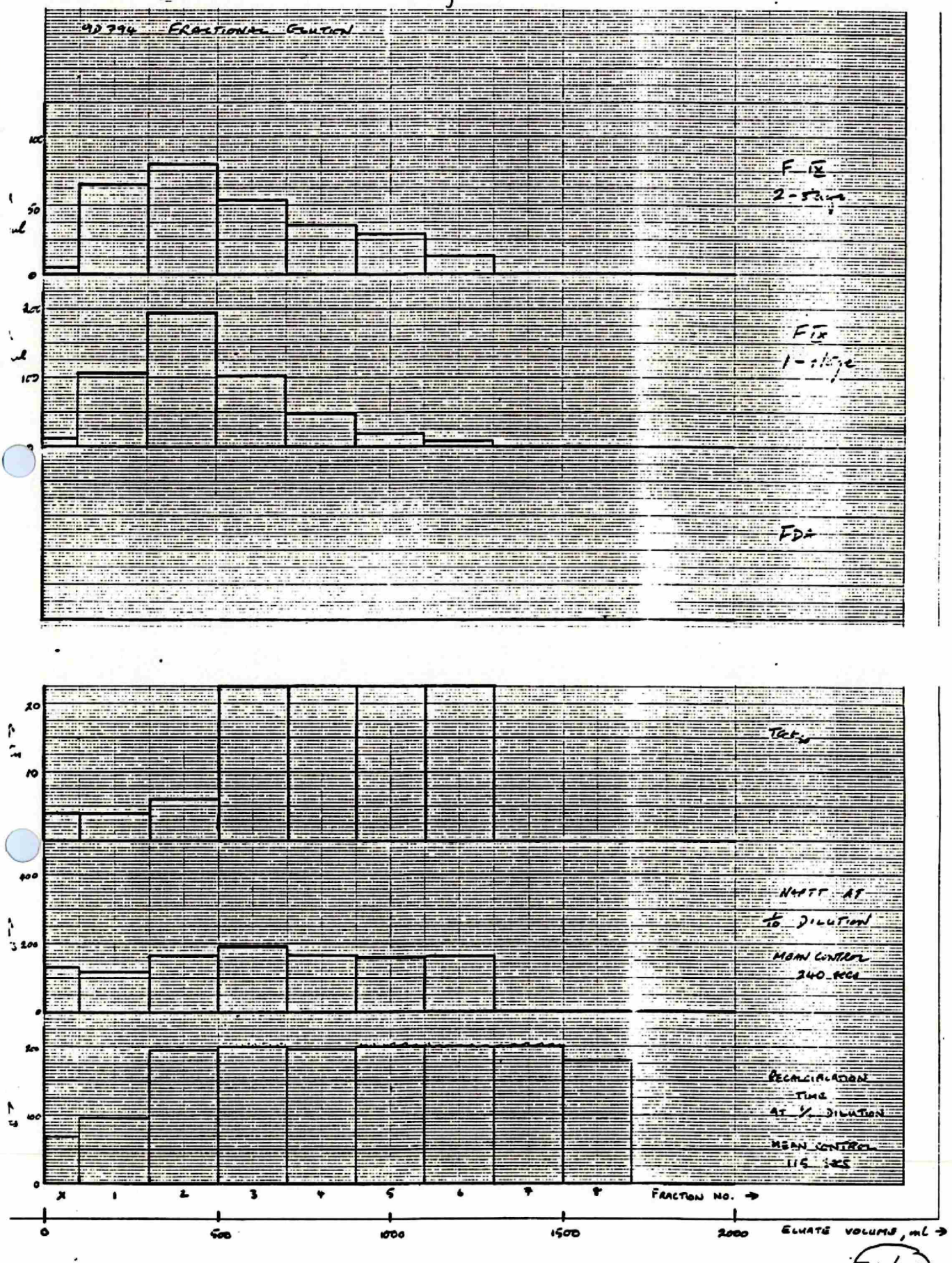


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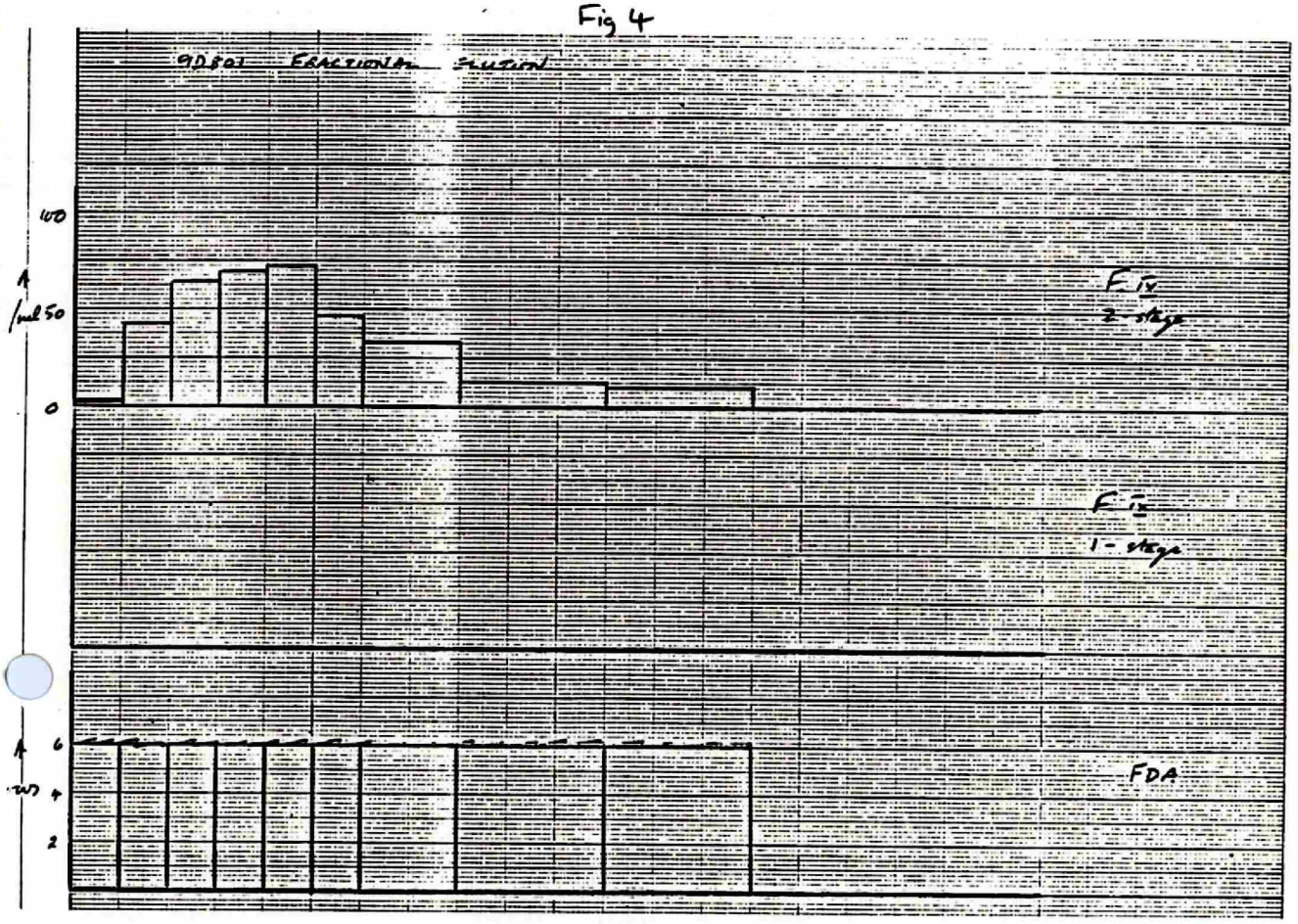
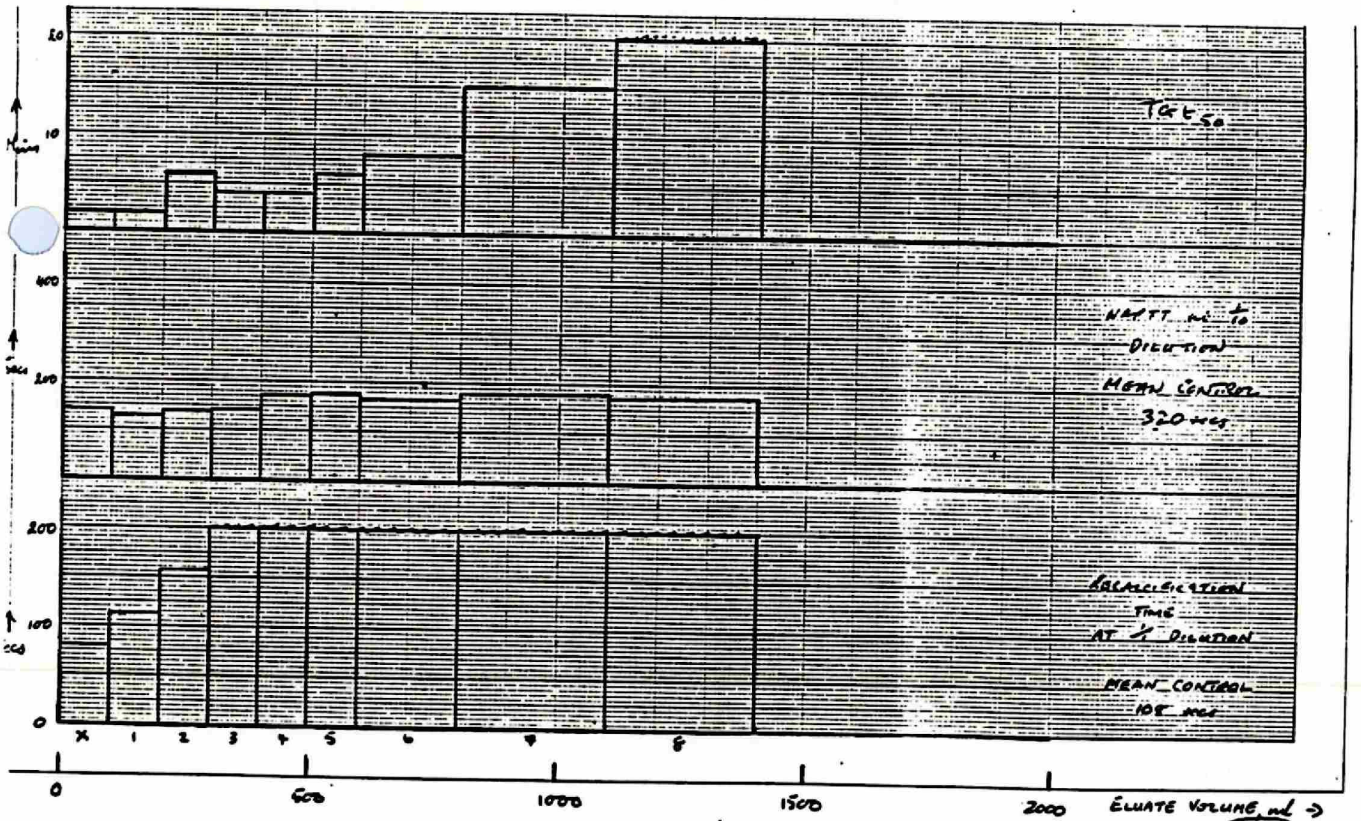
Fig 4

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