

The stacking gel solution was prepared by making up a mixture of Acrylamide (0,59ml of 40% acrylamide solution prepared as above); Stacking gel buffer (1,25ml); containing 500mM Tris pH 6,8 and 0,4% SDS, H₂O (3,15ml); Freshly prepared ammonium persulphate solution (0,02ml, 10% w/v) and Temed (0,01ml). This solution was slowly mixed, degassed and then added on top of separating gel once the sample well former was in place above the separating gel. The gel was then left for approximately 1h to set and then either stored in fridge or used immediately for electrophoresis.

Samples were prepared by taking known amounts of protein (3 - 60µg) and adding them to tubes already containing ,05ml of sample buffer. The Sample buffer contained 10ml, 75% (v/v) glycerol, 0,5ml of β-mercaptoethanol and 0,23g SDS dissolved in 10ml of 62,5mM Tris pH 6,8 and 0,1ml of 1% bromophenol-blue. This solution was made up to 100ml with distilled H₂O. The samples were mixed, heated to 90°C for 5 min, cooled and centrifuged. Appropriate dilutions of protein sample and sample buffer were made. Equal volumes of sample buffer and protein sample were added each time electrophoresis was to be run. The individual proteins were then layered into the sample wells in the gel. The gel was placed in position in the buffer tank, overlayed with buffer in the upper vessel and electrophoresed at 11mA until the dye front had reached the separating gel. Electrophoresis was continued for approximately 5h at 17 - 25mA.

Low molecular weight marker proteins, (Laboratory prepared with ranges 6 000 - 43 000 daltons), were treated similarly and were used to determine the molecular weight of protein staining bands. The standards were: ovalbumin (43 000 daltons); trypsin inhibitor (20 100 daltons);

cytochrome c (12 500 daltons) and aprotinin (6 000 daltons). The concentration of each of the standards was 1mg/ml and from 5-8 μ l of each of the standards was loaded at one time. On completion of the electrophoresis, the gel was removed from the plates and placed in a fixing, staining solution containing 10% acetic acid, 25% isopropanol and 0,2% Coomassie Brilliant Blue G-250 for 45 min. The gel was then destained using a 10% acetic acid solution. An R_f value is defined as the quotient of the distance moved by the solute (in this case the different proteins) and the distance moved by the solvent front. A calibration curve for the SDS polyacrylamide gel shows a linear relationship between the migration distance and the $\log_{10}$ of the molecular weight of the proteins of molecular weight 43 000 daltons to 12 500 daltons. The degree of linearity will depend on the composition of the gel. The low molecular weight region beyond 12 500 daltons is non-linear.

2.11 METHOD OF PURIFICATION OF BABOON INTESTINAL CALCIUM BINDING PROTEIN USING HPLC (HIGH PRESSURE LIQUID CHROMATOGRAPHY)

A Waters HPLC Model No. M721 from Millipore (S.A.) was used in the purification and identification of baboon intestinal CaBP. Two I-125 protein columns, capable of separating proteins of molecular weights ranging from 2 000 - 80 000 daltons, were used in series.

The mobile phase was 100mM Tris HCl pH 7,2 containing 100mM Na_2SO_4 . The flow rate of the eluent was 1,0 ml/min. Columns were conditioned for approximately 12 hours before use.

A mixture of molecular weight standards comprising ovalbumin

(43 000 daltons); chymotrypsinogen (23 000 daltons); lysozyme (14 400 daltons) and cytochrome c (12 500 daltons) were used. The concentration of each of the proteins ranged from 5 - 10 mg/ml of protein. Samples (0,125-0,250ml) of above mixture correspondin to 625 μ g - 2,5mg protein were injected into the system and monitored by absorbance at 280nm. Subsequently, protein samples from different stages of the various purification procedures were injected into the system, and eluted and monitored under the same conditions.

The resultant peaks of interest were monitored on the data system and were collected using the integral fraction collecting system. Data collected on the integrator were correlated with samples collected and used for identification of the baboon intestinal CaBP. A graph of log molecular weight vs elution time was plotted for the low molecular weight proteins. Thus the protein peaks eluted from the columns were used to establish the molecular weight of the baboon intestinal CaBP. The calibration curve was extrapolated in order that the molecular of the CaBP be determined.

2.12 EXPERIMENTS TO SHOW THE EFFECTS OF AMMONIUM SULPHATE ON

BOTH THE CHELEX-100 UPTAKE OF $^{45}\text{CaCl}_2$ AND THE BIURET ASSAY

2.12.1 METHOD USED TO INVESTIGATE THE EFFECT OF AMMONIUM

SULPHATE ON THE CHELEX - 100 RESIN UPTAKE OF $^{45}\text{CaCl}_2$

Firstly, a solution of 100% saturated ammonium sulphate was prepared and from this, dilutions of 25%, 50% and 75% saturated ammonium

sulphate solutions were prepared. The volumes of the components used in each of the experiments are shown in Table 3. All tests were carried out in duplicate.

Three experiments were carried out. As per table 3, components in tubes I and II were subjected to the Chelex-100 assay to determine the total and background counts for the experiments. In the first experiment, increasing concentrations of ammonium sulphate replaced H_2O as diluent (Tube III). The aim of this experiment was to show the effect of increasing concentrations of ammonium sulphate on the total counts in the assay system in the absence of both Chelex-resin and CaBP. The resultant counts were expressed as percentages of the total counts and plotted against the increasing concentrations of ammonium sulphate. The second experiment was carried out to determine the effect of increasing concentrations of ammonium sulphate on the total counts in the assay system in the presence of both Chelex-resin and CaBP. In this experiment increasing concentrations of ammonium sulphate replaced H_2O as diluent (Tube IV). The resultant counts were expressed as percentages of the total counts in the assay system and were plotted against the increasing concentrations of ammonium sulphate.

In the third experiment, the ammonium sulphate replaced H_2O as diluent (Tube V). This experiment was designed to show the effect of increasing concentrations of ammonium sulphate on the background counts measured in the assay in the absence of protein. The resultant counts were expressed as percentages of the total counts and plotted against the increasing concentrations of ammonium sulphate.

TABLE 3: THE VOLUMES OF COMPONENTS USED IN THE EXPERIMENT TO INVESTIGATE VARYING CONCENTRATIONS OF AMMONIUM SULPHATE ON CHELEX-100 ASSAY FOR MEASURING (Ca*Pr/Ca*R)

COMPONENTS	T U B E N O.				
	I	II	III	IV	V
$(\text{NH}_4)_2\text{SO}_4$	-	-	0,625ml	*0,500ml	0,500ml
H_2O	0,625ml	0,500ml	-	-	-
$^{45}\text{CaCl}_2$	0,05ml	0,05ml	0,05ml	0,05ml	0,05ml
Chelex -100	-	0,125ml	-	0,125ml	0,125ml
Protein (CaBP)	-	-	-	Appropriate dilution in ,500ml of $(\text{NH}_4)_2\text{SO}_4^*$	-

- TUBE I - Total counts in assay with H_2O used as diluent.
- TUBE II - Background counts in presence of Chelex-100 resin with H_2O used as diluent.
- TUBE III - Total counts in assay containing increasing concentrations of ammonium sulphate which replaced H_2O as diluent.
- TUBE IV - Background tube containing CaBP appropriately diluted in increasing concentrations of ammonium sulphate. Ammonium sulphate replaced H_2O as diluent.
- TUBE V - Tube representing background counts in which ammonium sulphate in increasing concentrations replaced H_2O as diluent. This contained no CaBP.

The $^{45}\text{CaCl}_2$ was diluted to give total counts of approximately 64 000 cpm. The background counts measured in the assay averaged at approximately 500 cpm.

2.12.2 EXPERIMENTS TO SHOW THE EFFECT OF AMMONIUM SULPHATE ON THE BIURET ASSAY

Increasing final concentrations of ammonium sulphate were added to different volumes of a series of standard solutions of BSA (10mg/ml). Three experiments were carried out in duplicate. A solution of 100% saturated ammonium sulphate was prepared and from this, dilutions of 25%, 50% and 75% saturated ammonium sulphate solutions were prepared. The volumes of individual components used were as for the Biuret assay described in section 2.13.1. The ammonium sulphate replaced water as diluent for BSA standard solutions.

2.12.3 PROCEDURE TO SHOW THE EFFECTS OF EDTA AND CaCl_2 ON THE CHELEX-100 UPTAKE OF $^{45}\text{CaCl}_2$

The Chelex-assay was carried out as described in Section 2.4. The effect of EDTA and CaCl_2 on the Chelex-100 resin uptake of CaCl_2 was measured using 15mM Tris, 1mM EDTA pH 8,0 and 15mM Tris, 5mM CaCl_2 pH 8,0 which replaced normal buffer in assay. For the total and background tubes, H_2O replaced all buffer solutions.

2.13 METHOD OF PROTEIN DETERMINATION

Four methods of protein determination were used.

2.13.1 BIURET ASSAY (Gornall, et al, 1949)

A standard protein solution of bovine serum albumin (BSA) (10mg/ml) in saline (0,9%) was made up. Biuret reagent was prepared by dissolving 1,5g $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ and 6,0g of sodium potassium tartrate in 500ml of H_2O . To this was added 300ml of 10% (w/v) NaOH from 50% carbonate free NaOH stock solution. For the measurement of protein content, varying amounts of Standard (0-0,8ml) were added to different test tubes. The standards and likewise unknown samples were all made up to final volume of 0,8ml. 2,0ml of Biuret reagent was added to standards and unknowns to make up a final volume of 2,8ml. Samples were then left for 30 min and read at 540 nm on a spectrophotometer. (See appendix I for a representative standard curve).

2.13.2 LOWRY-FOLIN ASSAY (Lowry et al, 1951)

A range of protein concentrations was made by pipetting 10, 20, 30 and 40 μl of bovine serum albumin (BSA) (1mg/ml) into test tubes. Each of these standards was made up to 0,500ml with H_2O . To each of the test tubes containing either sample or standard was added 2,5ml of a mixture prepared by combining 0,5% CuSO_4 (1,0ml) plus 2% Na_2CO_3 in 0,01N NaOH, 0,1g sodium potassium tartrate (50ml). The samples and standards were then mixed and left to stand at room temperature for 10 min. After 10 min, 0,25ml of 1:1 Lowry Folin Reagent and H_2O was added to each tube and immediately mixed. The tubes were then left in the dark at room temperature for 30 min and read on a Gilford spectrophotometer at 750nm. (See appendix I for representative standard curve).

2.13.3 BIORAD ASSAY (Bradford, 1976)

This protein determination method was carried out as described in the Biorad Booklet (Biorad Instruments). The standard protein solutions (macro method) were made up by preparing dilutions of protein standards containing from 1mg - 5mg/ml (BSA) and 0,01ml of each standard and appropriately diluted samples were used. The colour reagent was firstly filtered and 5ml was then added to all test and standard samples, left to stand for 5 to 10 min and then read on a spectrophotometer at 595nm. (See appendix I for standard curve).

2.13.4 ABSORBANCE METHOD (Murphy and Kies, 1960)

The method involves the estimation of protein content in solution at 280nm. All proteins exhibit an absorption band at 280nm primarily due to the presence of tyrosine and tryptophan residues. Therefore, for qualitative evaluation of the presence of protein in a particular fraction, the absorbance at 280nm was used as a rapid method of protein estimation.

CHAPTER 3

RESULTS

3.1 PURIFICATION PROCEDURES AND EVALUATION OF PURIFICATION

The following results pertain to the various purification procedures employed to isolate the baboon CaBP. An evaluation of the procedures is shown, indicating which was the preferred one.

3.1.1 AMMONIUM SULPHATE FRACTIONATION OF BABOON INTESTINAL MUCOSAL EXTRACT

Mucosal scrapings were prepared and processed for purification as described in Section 2.1 and 2.1.1. Assessment of the purification was carried out by Biuret protein assay, and the calcium binding assay (Chelex method). The results of the purification of the CaBP from the baboon intestinal mucosa by ammonium sulphate fractionation are shown below in Table 4. It is evident from the table that the 0-75% $(\text{NH}_4)_2\text{SO}_4$ supernatant fraction showed the highest specific activity. This fraction was purified approximately 20-fold. The two protein precipitate fractions showed negligible degrees of purification over the crude material. In Table 4, a loss of protein is indicated which could have resulted from non-specific protein interfering in Chelex-100 assay thus showing lower binding.

TABLE 4: PURIFICATION OF CRUDE MUCOSAL EXTRACT BY AMMONIUM SULPHATE FRACTIONATION

FRACTION	VOLUME (ml)	PROTEIN (mg/ml)	UNITS (cpm/ml)	TOTAL UNITS	UNITS (cpm/mg)	YIELD %	PURIFICATION (fold)
Supernatant of crude homogenate	165	8,05	19785	3264525	2457,8	100	1
Supernatant from 0 - 75%(NH ₄) ₂ SO ₄ Fractionation	130	0,28	13925	1810250	49732,1	55,5	20,2
Precipitate after 75%(NH ₄) ₂ SO ₄ Fractionation	110	7,97	11900	1309000	1494,9	40,1	0,61
Supernatant from 75 - 99% (NH ₄) ₂ SO ₄ Fractionation	52	0	-	-	-	-	-
Precipitate after 75 - 99%(NH ₄) ₂ SO ₄ fractionation	15	2,60	10500	157500	4038,5	4,8	1,64

3.1.2 HEAT PRECIPITATION OF AN EXTRACT OF BABOON INTESTINAL MUCOSA

A study to evaluate the effect of time of heating on the CaBP activity of a high speed supernatant of baboon intestinal mucosa was carried out. The method used was as described in Section 2.2.2. Protein concentrations and CaBP activity was determined on all the fractions. Figure 3 shows the effect of heating at 65°C for up to 35 min on protein concentration (mg/ml), CaBP activity (Ca*Pr/Ca*R) and specific activity (Ca*Pr/Ca*R/mg protein). The figure shows that both CaBP activity and specific activity were maximal after heating for 20 min. The specific activity increases gradually from time zero to peak at 20 min. The CaBP activity remains unchanged for the first 10 min of heating and then rises over the next 10 min to peak at 20 min. The phenomenon of the increase in CaBP activity as well as the resulting decrease in activity will be discussed later. The protein content decreases rapidly over the first 5 min and thereafter gradually. A 8,7-fold purification was achieved over the starting material after heat treatment for 20 min at 65°C.

3.1.3 pH PRECIPITATION PROCEDURE ON AN EXTRACT OF BABOON INTESTINAL MUCOSA

A supernatant from the crude homogenate of baboon intestinal mucosa was subjected to the pH precipitation procedure as described in Section 2.2.3. The results obtained were found not to be useful for effecting purification of the baboon CaBP as the specific activity of all the subsequent pH treated fractions was lower than that of the original crude extract.

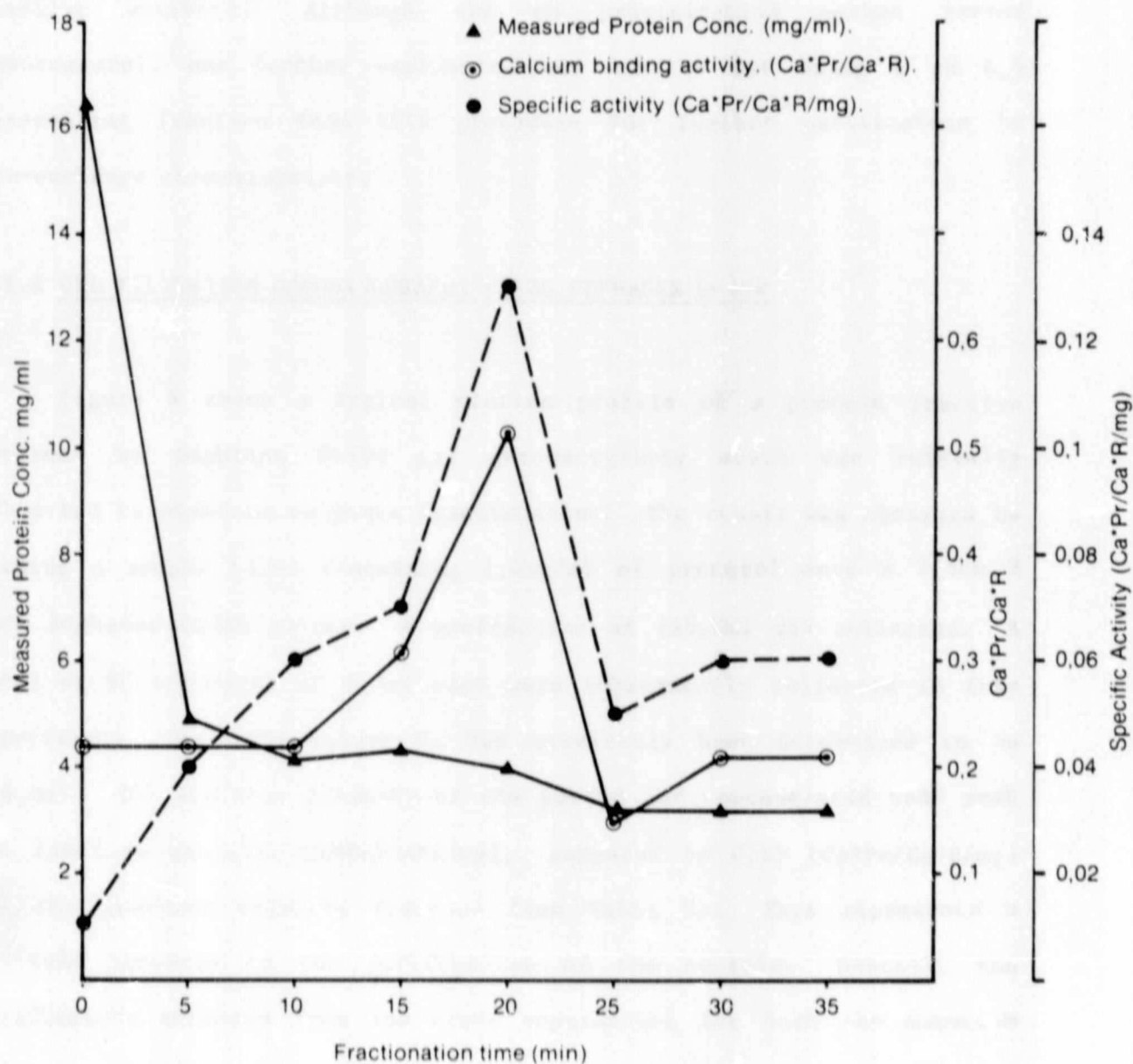


Fig. 3: A time course study to demonstrate the effect of time of heating at 65°C on the CaBP activity of a supernatant fraction of crude homogenate from baboon intestinal mucosa. The plot shows the change in protein concentration (mg/ml), specific activity ($\text{Ca}^*\text{Pr}/\text{Ca}^*\text{R}/\text{mg}$) and the CaBP activity ($\text{Ca}^*\text{Pr}/\text{Ca}^*\text{R}$) following heat treatment for up to 35 min.

Table 5 shows the comparison of the pH precipitation method versus ammonium sulphate fractionation. The results from the table clearly indicate that the ammonium sulphate fractionation was superior to the pH precipitation procedure in that the former yielded a fraction with higher specific activity. Although the pH precipitation method proved unsuccessful, one further experiment was carried out using a pH 6,5 supernatant fraction from this procedure for further purification by ion-exchange chromatography.

3.1.4 GEL FILTRATION CHROMATOGRAPHY USING SEPHADEX G-100

Figure 4 shows a typical elution profile of a protein fraction obtained by Sephadex G-100 gel chromatography which was initially subjected to ammonium sulphate fractionation. The result was obtained by loading a sample (13ml containing 1,4mg/ml of protein) onto a 2,5cm X 94cm Sephadex G-100 column. A prefraction of 155,0ml was collected. A total of 80 fractions of 3,3ml each were subsequently collected in this experiment. The void volume V_0 had previously been determined to be 168,2ml. The specific activity of the pooled and concentrated CaBP peak was found to be 0,55 (Ca*Pr/Ca*R/mg), compared to 0,15 (Ca*Pr/Ca*R/mg) for the ammonium sulphate fraction (see Table 5). This represents a 3,7-fold increase in the purification of the protein. Overall, the purification obtained from the crude supernatant for both the ammonium sulphate fractionation and Sephadex G-100 gel chromatography was approximately 37-fold.

TABLE 5:

COMPARISON OF THE PURIFICATION OF HIGH SPEED SUPERNATANTS OF BABOON INTESTINAL
MUCOSA BY AMMONIUM SULPHATE FRACTIONATION AND THE pH PRECIPITATION METHODS

FRACTION	Ca*Pr/Ca*R	PROTEIN CONC (mg/ml)	SPECIFIC ACTIVITY	FRACTION	Ca*Pr/ Ca*R	PROTEIN CONC mg/ml	SPECIFIC ACTIVITY
Supernatant of crude homogenate	0,12	8,05	0,015	Supernatant of crude homogenate	0,115	7,45	0,015
0-75% (NH ₄) ₂ SO ₄ Supernatant	0,042	0,28	0,15	pH 4 Supernatant	0,03	4,7	0,006
0-75% (NH ₄) ₂ SO ₄ Precipitate	0,002	7,96	0,0003	pH 4 Precipitate	0	8,0	0
75 - 99% (NH ₄) ₂ SO ₄ Supernatant	0	0	0	pH 6,5 Supernatant	0,025	3,35	0,007
75 - 99% (NH ₄) ₂ SO ₄ Precipitate	0,025	2,6	0,0096	pH 6,5 Precipitate	0,043	4,70	0,009

Specific Activity is defined as (Ca*Pr/Ca*R/mg protein)

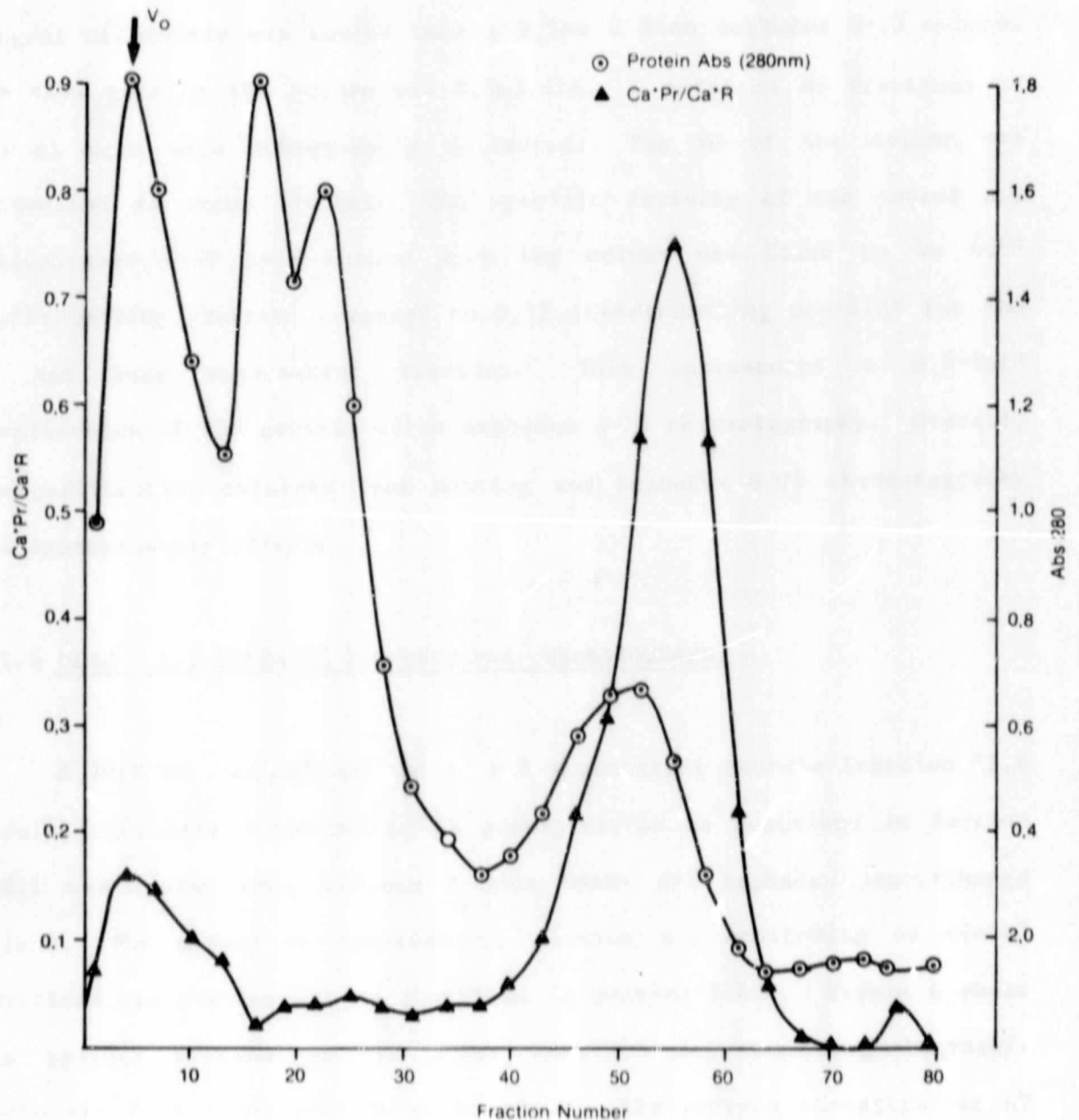


Fig. 4: Sephadex G-100 gel filtration chromatography of a dialysed, concentrated supernatant fraction after 0-75% ammonium sulphate precipitation of a high speed supernatant of baboon intestinal mucosa. The plot shows the protein content measured by absorbance at 280nm and CaBP activity (Ca*Pr/Ca*R) versus fraction number. The V_e of the eluted CaBP peak = 349,7ml (V_e/V_0 ratio = 2,08).

3.1.5 GEL FILTRATION CHROMATOGRAPHY USING SEPHADEX G-75

Figure 5 shows the elution profile obtained from Sephadex G-75 of a protein fraction which had been previously subjected to heat precipitation for a maximum period of 20 min. A 20ml sample containing 15mg/ml of protein was loaded onto a 2,5cm X 94cm Sephadex G-75 column. The flow rate of the column was 0,5ml/min. A total of 80 fractions of 5,0 ml each were subsequently collected. The V_0 of the column was determined as being 170,6ml. The specific activity of the pooled and concentrated CaBP peak eluted from the column was found to be 0,89 (Ca*Pr/Ca*R/mg protein) compared to 0,13 (Ca*Pr/Ca*R/mg protein) for the 20 min heat supernatant fraction. This represented a 6,8-fold purification of the protein after Sephadex G-75 chromatography. Overall, the purification obtained from heating and Sephadex G-75 chromatography was approximately 59-fold.

3.1.6 DEAE - A25 SEPHADEX ION-EXCHANGE CHROMATOGRAPHY

A 10,0 ml aliquot of the pH 6,5 supernatant protein fraction (1,6 mg/ml) previously subjected to pH precipitation as described in Section 2.2.3 was loaded onto a 2,6cm X 35cm DEAE- A25 Sephadex ion-exchange column. The method of application, elution and monitoring of eluted fractions was carried out as described in Section 2.6.1. Figure 6 shows the protein elution profile after DEAE-A25 Sephadex chromatography. There are four major protein peaks shown. The protein concentration of the protein peak fraction (5ml) eluted with 15mM Tris pH 8,0 containing 5mM CaCl_2 pH 8,0 was 0,3 mg/ml. The CaBP activity could not be determined because of the presence of EDTA but this fraction was later examined by non-denaturing and SDS polyacrylamide electrophoresis.

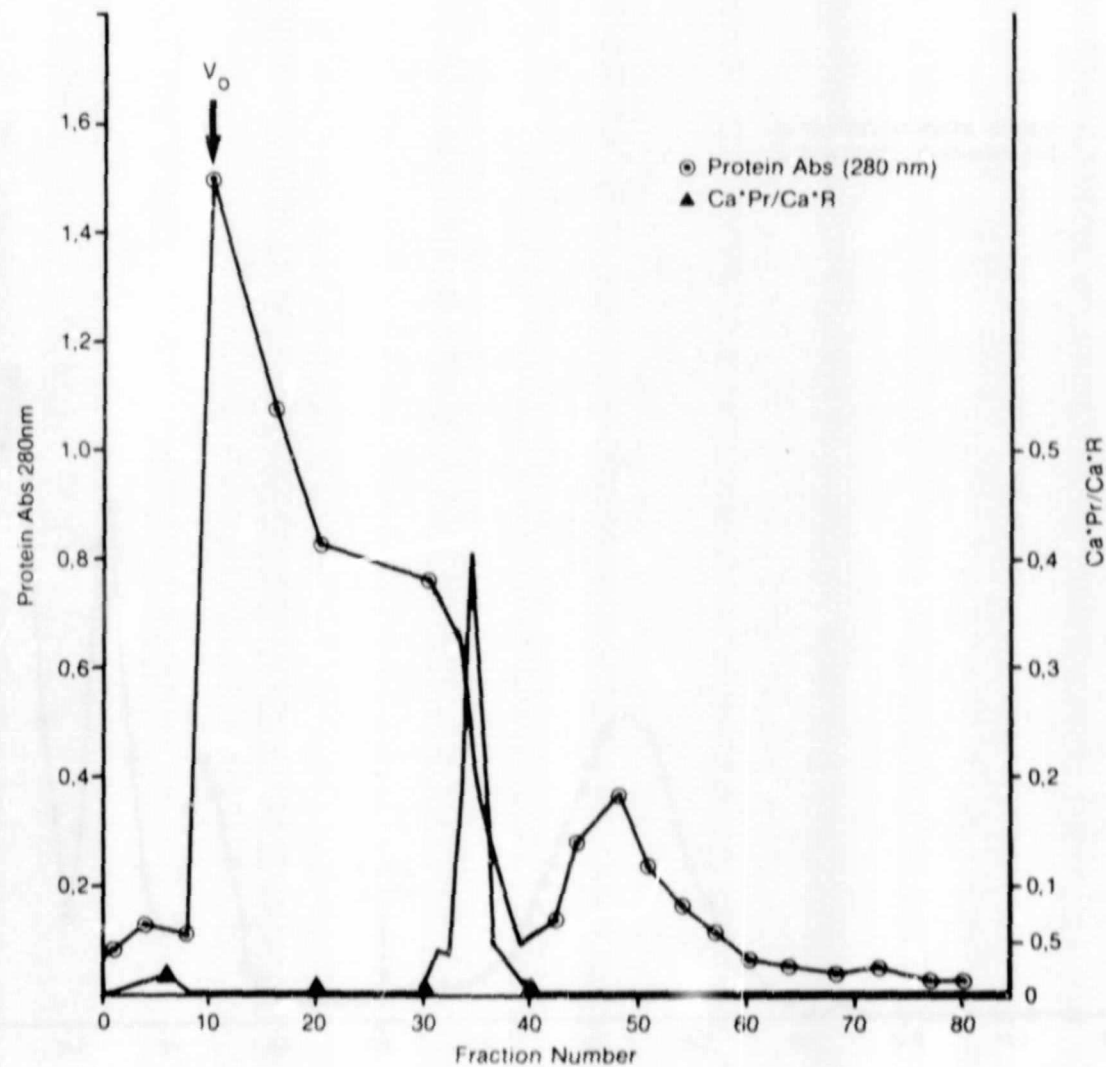


Fig. 5: Elution profile of a heat precipitated supernatant fraction (20 min) subjected to Sephadex G-75 gel filtration chromatography. The plot shows the protein content measured by absorbance at 280nm and CaBP activity ($\text{Ca}^*\text{Pr}/\text{Ca}^*\text{R}$) versus fraction number. The V_e of the eluted CaBP peak = 355ml (V_e/V_0 ratio = 2.08).

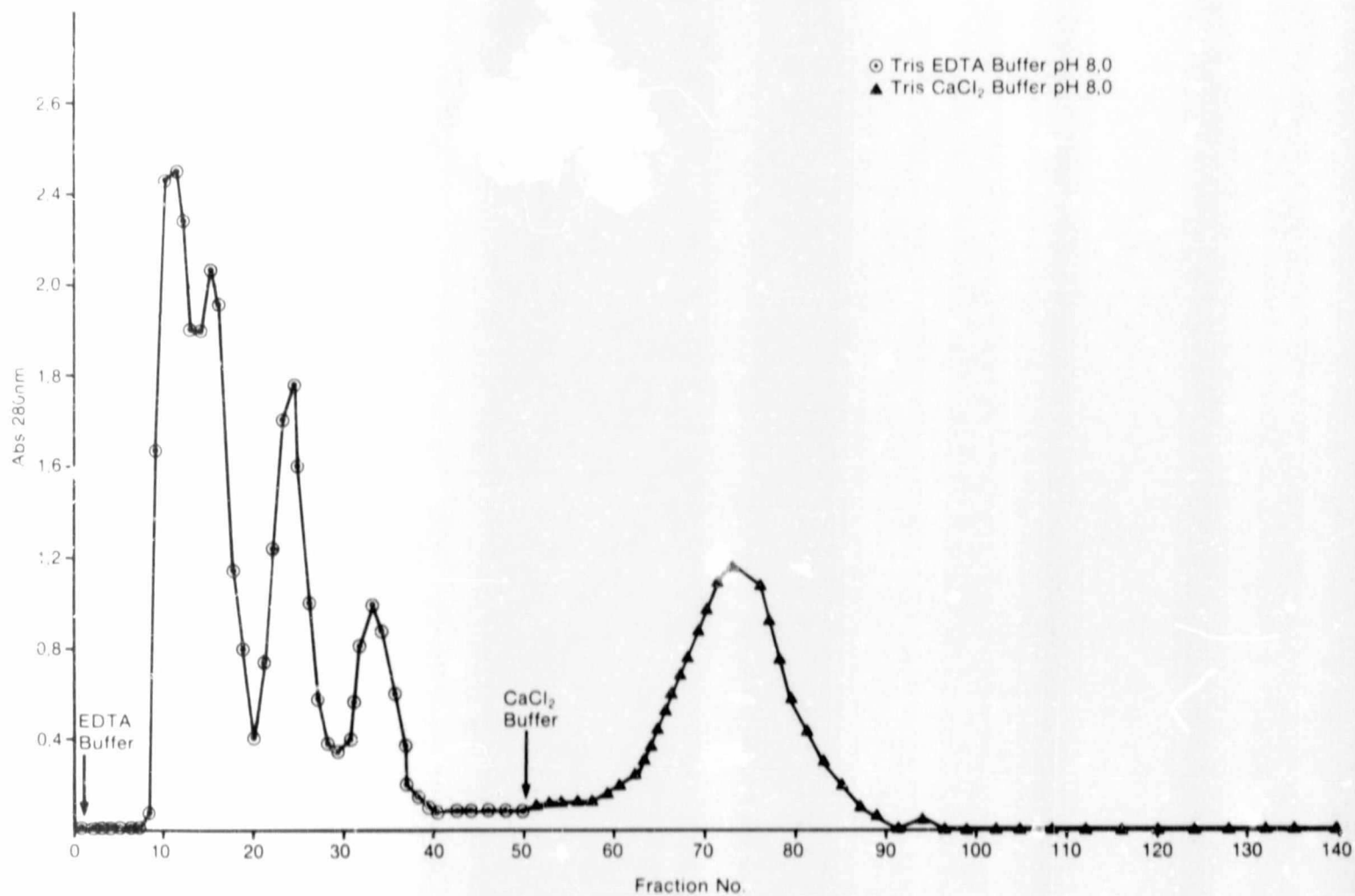


Fig. 6: DEAE-A25 Sephadex ion-exchange chromatography on a pH6,5 supernatant from pH precipitation procedure. Elution of the protein was firstly with 15mM Tris,1mM EDTA pH8,0 and then with 15mM Tris,5mM CaCl₂ pH8,0.

3.1.7 DEAE-SEPHACEL ION-EXCHANGE CHROMATOGRAPHY

A 3,0ml sample (3,2 mg/ml) of protein sequentially subjected to heat purification and to Sephadex G-100 gel chromatography in 20mM Tris pH 7,5 containing 30mM NaCl was loaded onto a 1cm X 10cm DEAE-Sephacel column and eluted as described in Section 2.6.2. Figure 7 displays the protein elution profile as measured by absorbance at 280nm following stepwise elution firstly with 20mM Tris pH 7,5, 30mM NaCl and then the same buffer containing 150mM and finally 2M NaCl.

The CaBP activity of the fractions eluted with starting buffer is shown. The CaBP activity of the pooled fractions eluted with the buffer containing 150mM and 2M NaCl could not be established because of the high ionic strength. All three protein peak fractions were later pooled dialysed and assessed by SDS polyacrylamide gel electrophoresis, but the only results seen on SDS gels were of the fraction eluted in 20mM Tris pH 8,0, 30mM NaCl. The peak CaBP fractions eluted in 20mM Tris pH 8,0, 30mM NaCl were pooled as one fraction and not kept separate.

3.1.8 EVALUATION OF PURIFICATION PROCEDURES USING A PARTICULAR PURIFICATION PROTOCOL

On the basis of the various purification procedures attempted, a protocol was chosen in which aliquots of a supernatant fraction of crude homogenate of baboon intestinal mucosa (260ml) were subjected to ammonium sulphate and heat fractionation. The protocol was carried out as described in Section 2.7. An aliquot (16ml) from the concentrated heat supernatant fraction was also subjected to Sephadex G-100 gel chromatography and the resulting active fraction was then pooled and

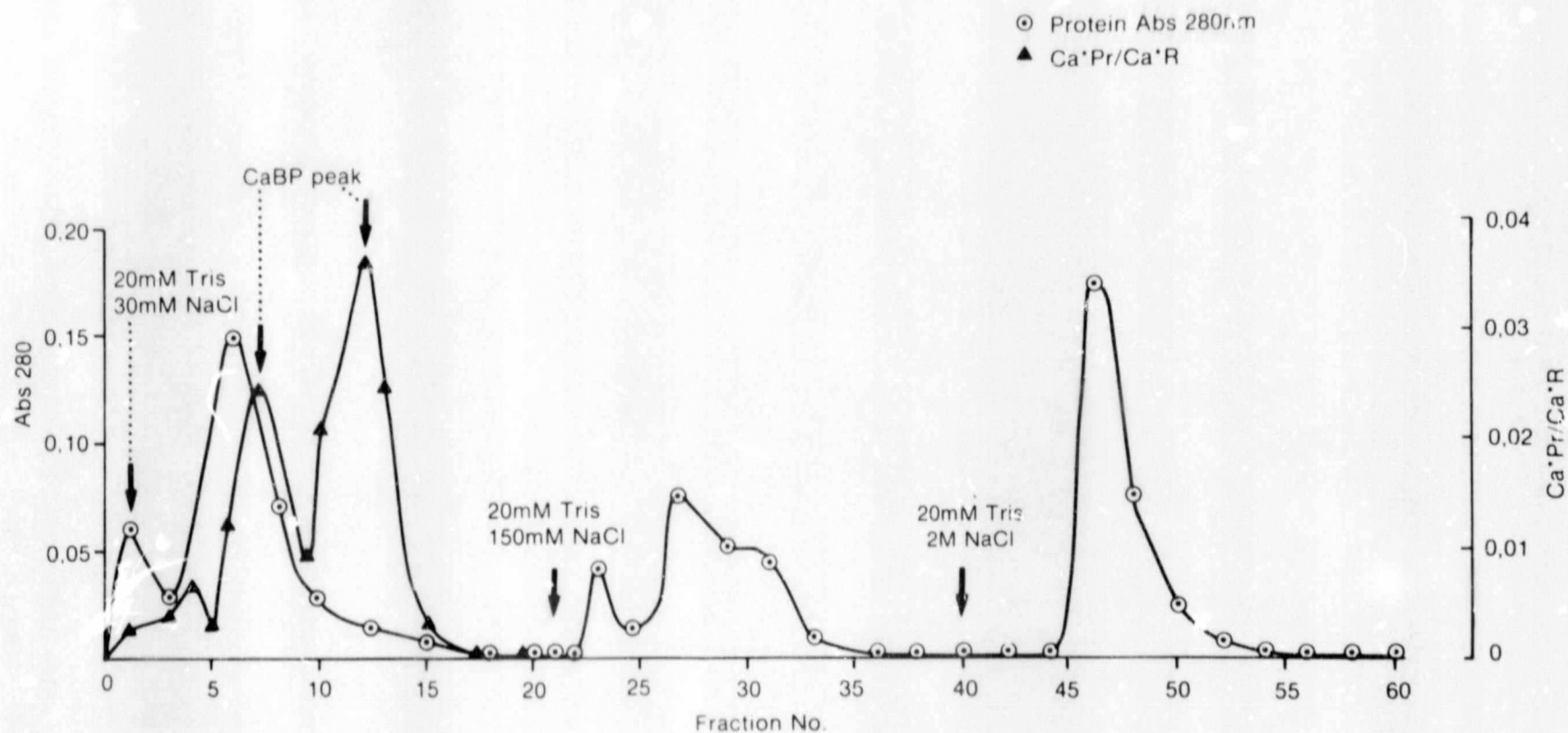


Fig. 7: DEAE-Sephacel ion-exchange chromatography of a fraction of heat precipitated protein already chromatographed on Sephadex G-100. Elution of the protein was firstly carried out with 20mM Tris pH 7,5 containing 30mM NaCl. A step wise addition of 150mM NaCl and finally 2M NaCl were also added in initial starting buffer of 20mM Tris pH 7,5. The protein was monitored at Abs 280nm.

TABLE 6: A SUMMARY OF THE OVERALL PURIFICATION OF THE BABOON INTESTINAL
CALCIUM BINDING PROTEIN USING THREE SEQUENTIAL STEPS,
SHOWING THE PURIFICATION AND SPECIFIC ACTIVITY OF THE PROTEIN

FRACTION	PROTEIN CONC. (mg/ml)	CALCIUM BINDING ACTIVITY (Ca*Pr/Ca*R)	SPECIFIC ACTIVITY (Ca*Pr/Ca*R/mg)	(PURIFICATION) (fold)
Supernatant crude Homogenate	16,4	0,2	0,01	1
* 0 - 75% (NH ₄) ₂ SO ₄ fraction (supernatant)	0,4		0,20	16,6
20 min Heat precipitation supernatant fraction	8,6	0,97	0,11	9,1
Sephadex G-100 concentrated protein fraction	3,2	1,08	0,34	28,3
DEAE-Sephacel Ion-exchange fraction	0,1	0,04	0,40	33,3

* This represents a 5ml aliquot from the original crude supernatant. This step is distinct from the following three steps given below. All calculations in this step are based on this 5ml aliquot. (see text 3.1.8.)

TABLE 7: SUMMARY OF THE OVERALL PURIFICATION OF THE CaBP USING 3 SEQUENTIAL STEPS,

SHOWING THE PURIFICATION AND YIELD OF THE PROTEIN

FRACTION	FINAL Volume(ml)	PROTEIN CONC. (mg/ml)	UNITS/ ml	TOTAL Units	UNITS/ mg protein	YIELD %	PURIFICATION (fold)
Supernatant of crude homogenate	260 o	16,4	15 667	4 073 420	955	100	1
0-75% (NH ₄) ₂ SO ₄ fractionation	5 +	0,4	7 000	35 000	17 500	43,8	17,6
20 min heat preci- pitation supernatant fraction	80 **	8,6	25 000	2 000 000	2 906	49,1	3,0
Sephadex G-100 Concentrated protein fraction	16 +	0,8	13 750	220 000	17 187		17,9
DEAE-Sephacel Ion-exchange Fraction	3 *	0,1	3 000	9 000	30 000	4,4	31,3

o This represents the original crude supernatant fraction.

+ This fraction represents 5ml of the original crude supernatant and is distinct from the remaining 3 steps. Results quoted for this step are calculated on the 5ml aliquot only. The remaining 255ml fraction was subjected to heat fractionation.

** The supernatant after heat treatment was pooled and concentrated to 80ml. A 16ml aliquot of this was subjected to Sephadex G-100 gel chromatography.

* A 3ml aliquot of the 16ml pooled and concentrated active fraction eluted off Sephadex G-100 was subjected to DEAE-Sephadex ion-exchange chromatography.

+ and * Both these results have been calculated for the 16ml and 3ml aliquots which were subjected to Sephadex G-100 gel chromatography and DEAE-Sephacel ion-exchange chromatography respectively.

In both cases, the aliquots applied were approximately one fifth of the original fraction concerned. The yields quoted in each case must therefore be multiplied by 5 and 5,3 respectively to get what the total yield for the fractions would be (55% and 21,7% respectively).

x A unit is defined as cpm/ml of supernatant fluid.

concentrated and a 3ml aliquot from this was subjected to DEAE-Sephacel ion-exchange chromatography. The results are shown in Tables 6 and 7 and are expressed in two different ways. Table 6 shows changes in specific activity and purification of the fractions as measured by the Chelex-100 assay. Table 7 shows the yield and purification of the fractions containing CaBP. It is evident from the two tables, that the magnitude of the results obtained in the purification procedure are not quite similar even though they are expressed in the two different units.

In both Table 6 and Table 7, the ammonium sulphate result is present for comparative purposes and is not part of the overall purification. In Table 6 the ammonium sulphate fraction showed that a high degree of purification had been achieved (about 16-fold). By comparison the heat treated supernatant fraction demonstrated a lower specific activity that is the purification was only 9,1-fold. The heat purified fraction after Sephadex G-100 gel chromatography showed a 3,1-fold increase in specific activity and DEAE-Sephacel ion-exchange chromatography achieved a further 1,2-fold purification over the Sephadex G-100 fraction, thus giving an overall purification of 33,3-fold over the crude mucosal extract.

As with Table 6, overall purification of CaBP shown in Table 7 was achieved through Sephadex G-100 gel chromatography with a purification of 5,9-fold over the heat precipitation fraction. A 1,7-fold purification was achieved through ion-exchange chromatography over the fraction chromatographed on Sephadex G-100 chromatography. Overall, the purification achieved was shown to be 31,3-fold. These are the differences.

3.1.9 ASSESSMENT OF PURIFICATION PROCEDURES AS ANALYSED BY ANALYTICAL (NON-DENATURING) POLYACRYLAMIDE GEL ELECTROPHORESIS

Polyacrylamide gel electrophoresis was carried out as described in Section 2.8. Figure 8 shows the electrophoretic separation on non-denaturing electrophoresis using a 10% slab gel of protein samples purified by Sephadex G-100 gel chromatography, a pH 6,5 precipitate fraction, a concentrated pH 6,5 supernatant fraction and a fraction of protein after DEAE-A25 ion-exchange chromatography. The results indicated a single band of protein in the fraction after off the DEAE-A25 Sephadex ion-exchange resin. The single band shown in wells 4 to 6 does not seem to correspond to any bands seen in wells 1 - 3. The appearance of multiple bands seen in wells 1 - 3 indicated that the fractions in question were impure. Furthermore the appearance of a single band seen in wells 4 - 6 would imply the possibility of a pure protein. SDS electrophoresis of this fraction (not shown), however, revealed a number of bands. The latter result will be discussed later.

3.1.10 DENSITOMETRIC SCANS OF 10% NON-DENATURING POLYACRYLAMIDE ROD GELS

In a separate experiment varying amounts (5 - 80 μg) of the protein fractions obtained from DEAE-A25 ion-exchange chromatography were also subjected to discontinuous, non-denaturing 10% rod polyacrylamide gel electrophoresis. The stained gels were scanned on a gel scanner and the scans can be seen in Figure 9. The results showed a single protein band in all cases with the exception of the 3 gels which were loaded with 40, 60 and 80 μg of protein. These scans showed the presence of one additional protein band. In each case, the top of the gel is on the R.H.S. and the bottom of the gel on the L.H.S. The patterns below each of the scans are the integration patterns.

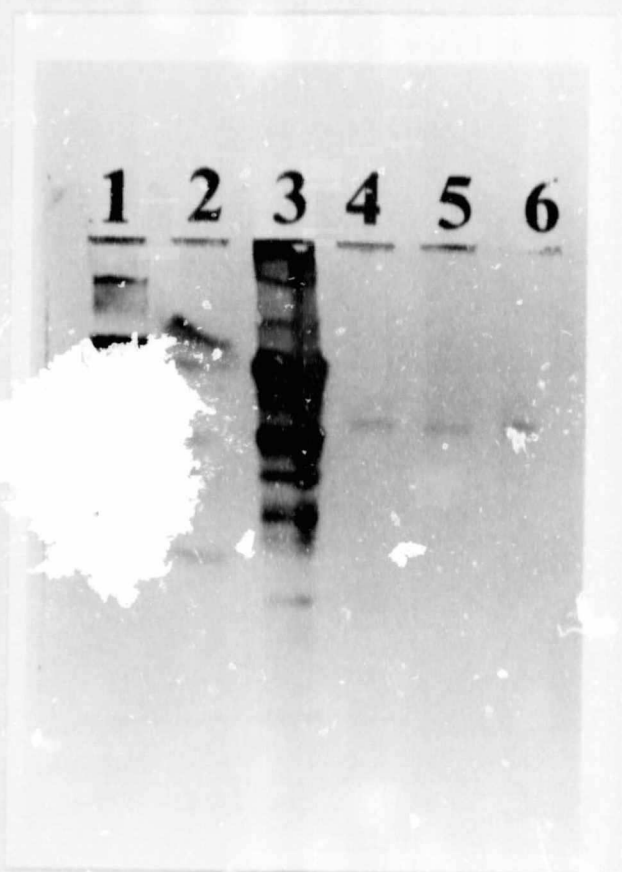


Fig. 8: Non-denaturing polyacrylamide gel electrophoresis on a 10% slab gel. Protein samples were purified by Sephadex G-100 gel chromatography (Well 1), pH6.5 precipitate (Well 2), pH6.5 supernatant (Well 3), and DEAE-A25 Sephadex ion-exchange chromatography (Wells 4, 5 and 6).

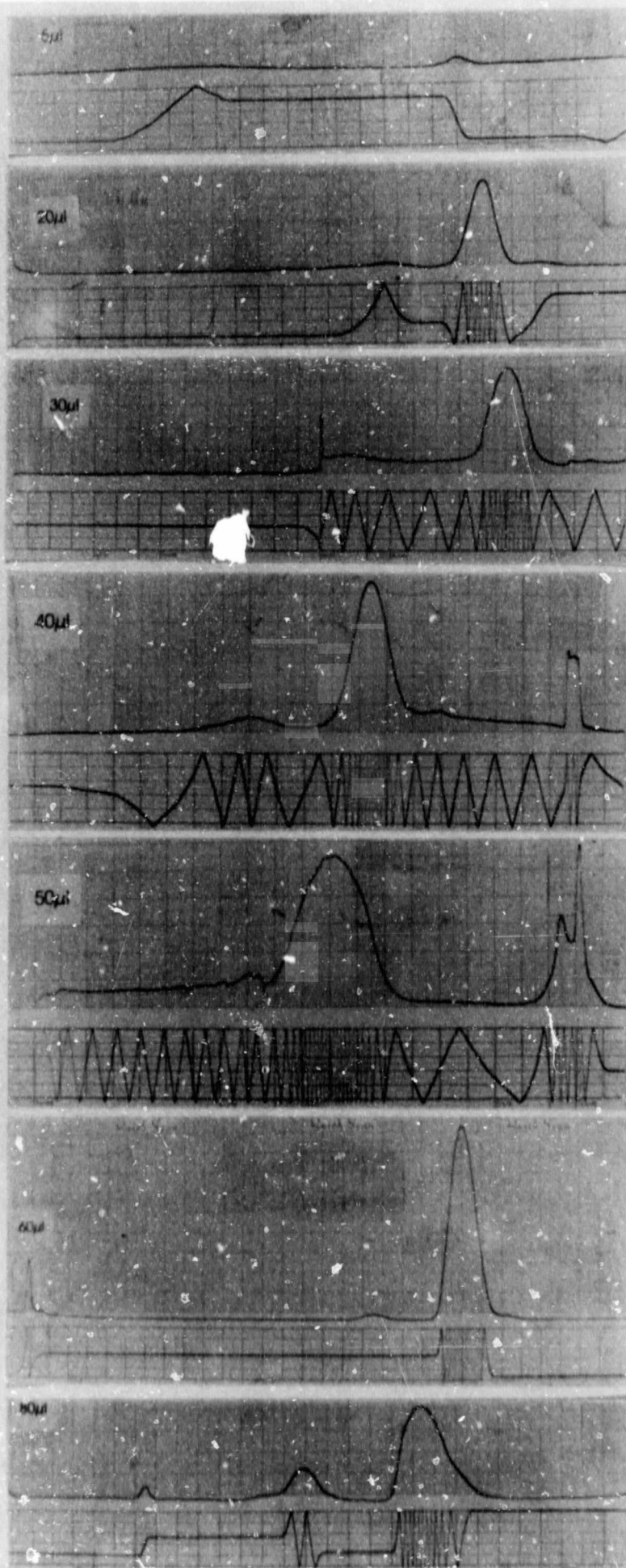


Fig. 9: Densitometric scans of protein stained discontinuous polyacrylamide rod gels. The rod gels (10%) were run under non-denaturing conditions. The amount of protein applied to each of the gels scanned ranged from 5μg — 80μg. Protein concentration (1μg/μl).

3.1.11 ASSESSMENT BY COUNTER-ION ELECTROPHORESIS OF CaBP FRACTIONS AFTER SEPHADEX G-100 GEL CHROMATOGRAPHY

Using the method described in Section 2.9 samples of protein collected after Sephadex G-100 gel chromatography were subjected to counter-ion electrophoresis. Figure 10 depicts graphically a CaBP peak which was detected 48mm from the origin on 7,5% non-denaturing polyacrylamide gels in two separate experiments. CaBP activity correlated with a specific protein staining band on gels run in duplicate but not shown here. The graph also depicts a radioactive peak which corresponds to the bromophenol-blue dye front. In one run (11), there is also a large peak of Ca^{2+} binding at the top of the gel.

3.1.12 EVALUATION BY SDS POLYACRYLAMIDE GEL ELECTROPHORESIS OF A 3 STEP SEQUENTIAL PURIFICATION PROTOCOL

The protein fractions obtained from the various purification procedures are described in Section 2.7. The purified protein fraction from each of the procedures was electrophoresed on a 12,5% SDS polyacrylamide slab gel containing 6,4% glycerol in the separating gel.

Between 20 to 60 μg of protein was loaded onto the gel. The results are shown in Figure 11. The results show that after the purification with DEAE-Sephacel ion-exchange chromatography two sets of doublet protein staining bands are evident. These two sets of doublets are also evident in the Sephadex G-100 fraction. The first doublet of protein was found to be resolved between cytochrome c and aprotinin. The second protein doublet seen in the DEAE-Sephacel fraction, was resolved below aprotinin.

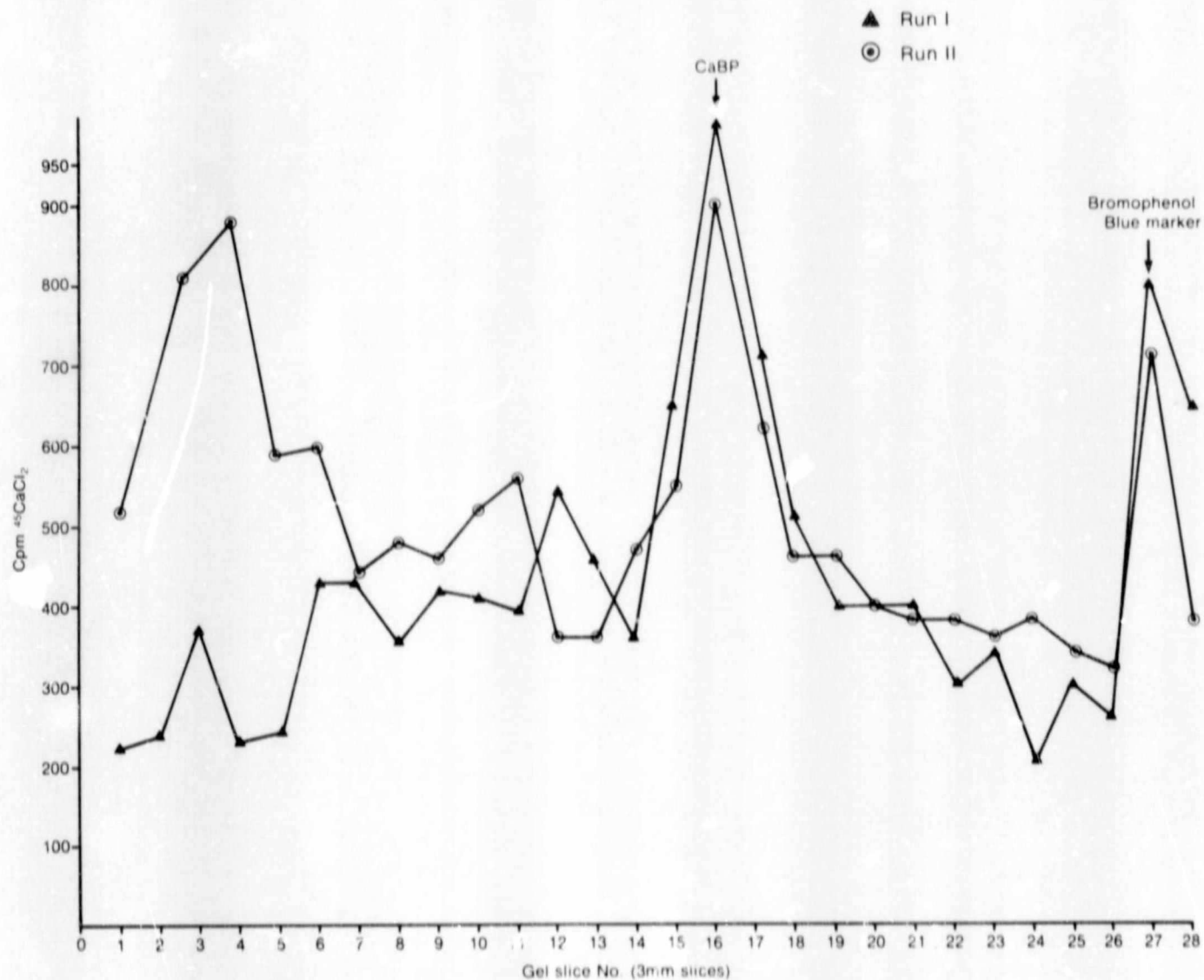


Fig. 10: Counter-ion electrophoresis on pooled and active protein samples off Sephadex G-100 gel chromatography showing the detection of calcium binding peaks at 48mm from the point of application. The plot represents two experiments run on different protein fractions.

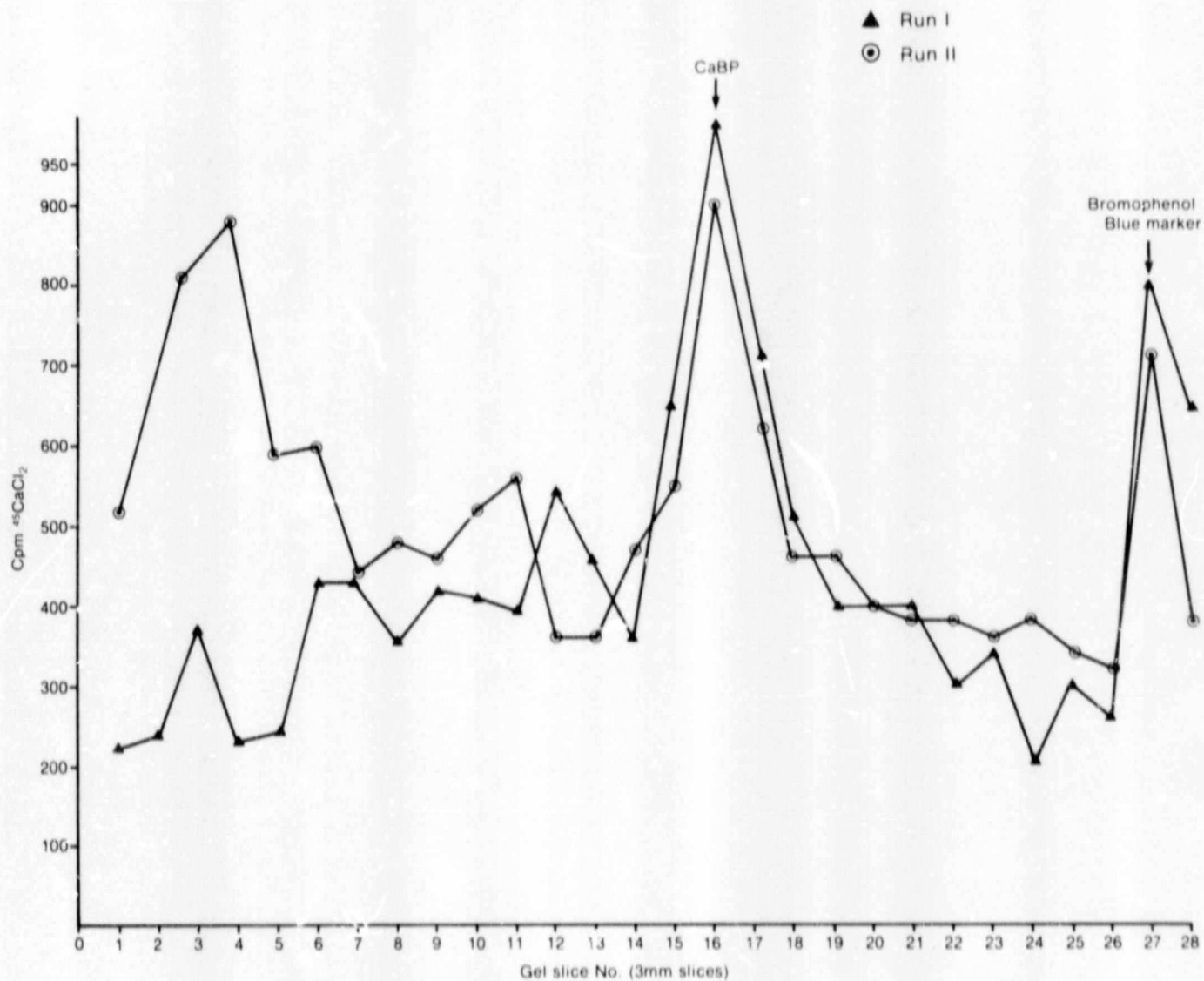


Fig. 10: Counter-ion electrophoresis on pooled and active protein samples off Sephadex G-100 gel chromatography showing the detection of calcium binding peaks at 48mm from the point of application. The plot represents two experiments run on different protein fractions.

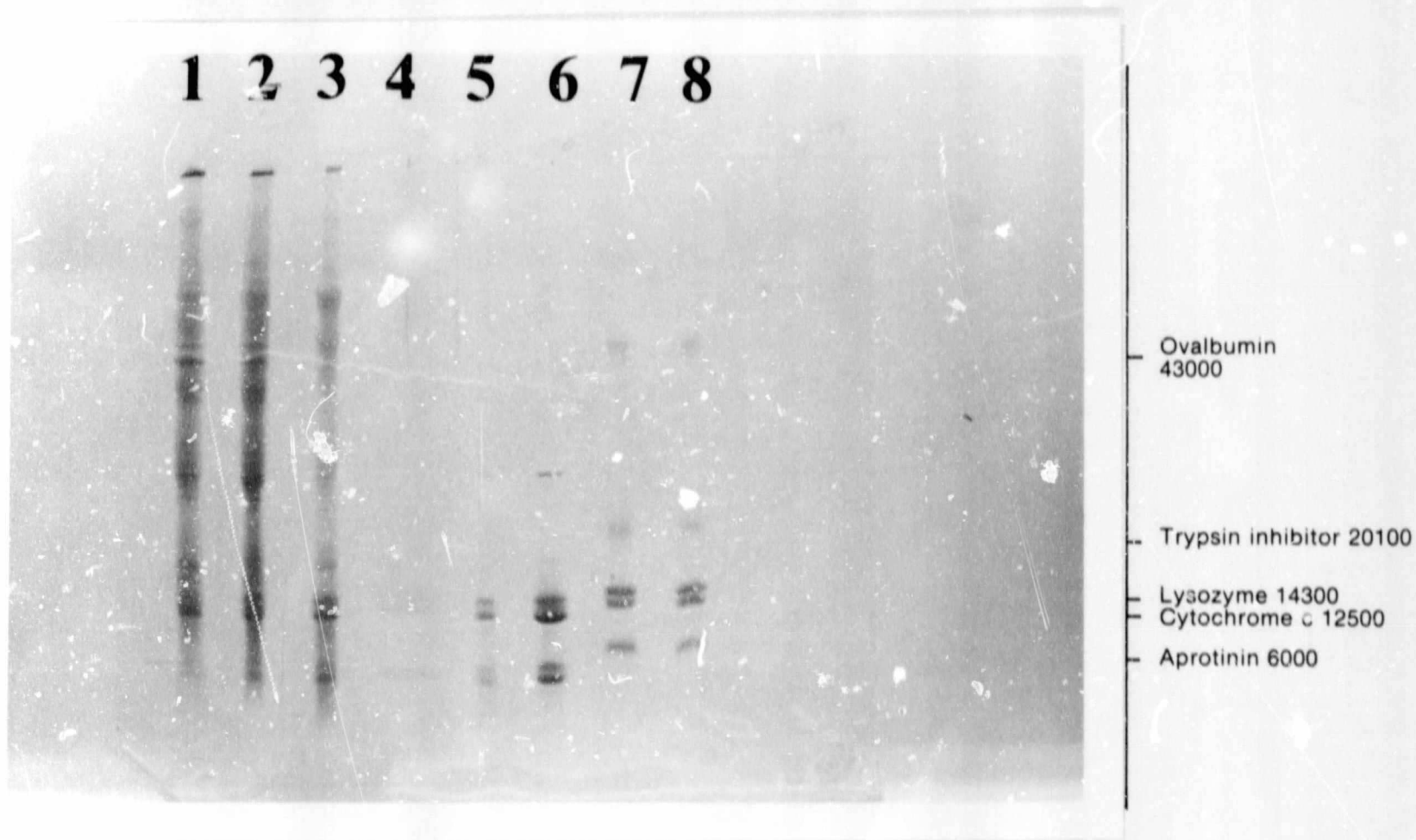


Fig. 11: SDS polyacrylamide gel electrophoresis on 12,5% gel of different protein fractions, as described in section 2.10. These were: crude homogenate (Well 1), supernatant of crude homogenate (Well 2), heat precipitation supernatant fraction (Well 3), $(\text{NH}_4)_2 \text{SO}_4$ supernatant fraction (0-75%) (Well 4), Sephadex G-100 gel chromatography (Well 5), DEAE-Sephacel ion exchange chromatography (Well 6), low molecular weight marker proteins (6000-43000 daltons) (Wells 7 and 8).

The latter result is also seen in Well 3. Well 4 has a singlet of low molecular weight. The purification achieved from the crude material is evident by the disappearance of the larger molecular weight proteins after Sephadex G-100 gel chromatography. The $(\text{NH}_4)_2\text{SO}_4$ fraction as seen in the gel is very pure. The standards have resolved themselves clearly especially the low molecular standards, namely, (cytochrome c and aprotinin).

3.2.0 DETERMINATION OF THE MOLECULAR WEIGHT OF THE INTESTINAL CaBP

In addition to the use of the Sephadex gel filtration chromatography for protein purification the results obtained from the calibrated columns were also used to estimate the molecular weight of the CaBP. Furthermore, the techniques of SDS PAGE and HPLC were also used to estimate the molecular weight of the CaBP.

3.2.1 CALIBRATION OF SEPHADEX G-100 AND SEPHADEX G-75 CHROMATOGRAPHY COLUMNS

Both columns were calibrated as described in Section 2.3.1. The log molecular weight versus the V_e/V_o ratios for each of the molecular weight protein standards was plotted for each column. Figure 12 shows the resultant calibration curves for both Sephadex G-100 and Sephadex G-75. The calibration curves were both linear, although not in the range to determine the molecular weight of CaBP accurately. The lines were thus extrapolated to their origins.

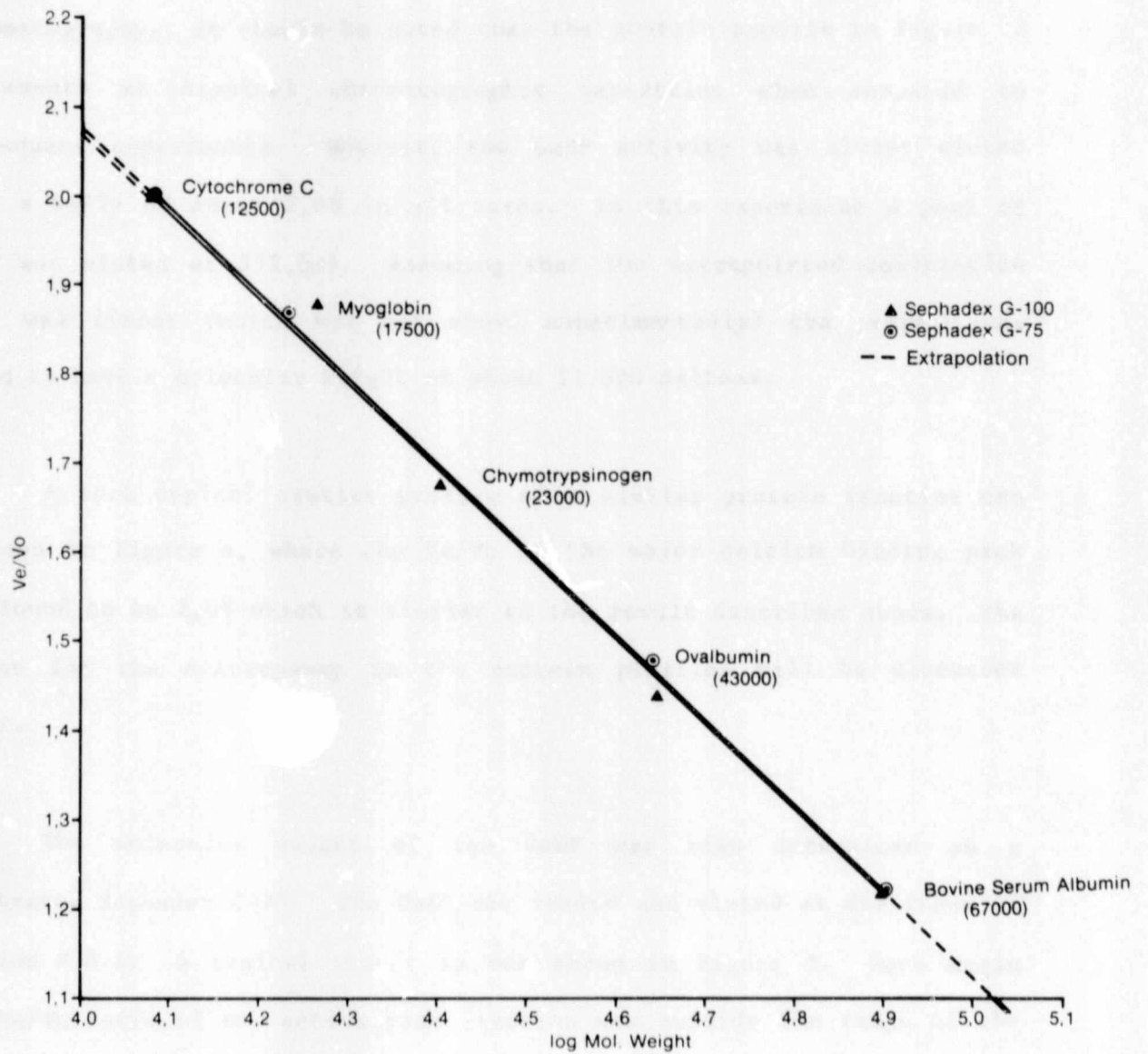


Fig. 12: The plot of V_e/V_o versus $\log$ Molecular weight for standard molecular weight marker proteins chromatographed on Sephadex G-100 and Sephadex G-75 columns. The calibration curves are both shown to be linear. Molecular weight markers used as described in section 2.3.1 are shown in the figure.

3.2.2 MOLECULAR WEIGHT OF CaBP DETERMINED FROM SEPHADEX G-100/G-75 GEL CHROMATOGRAPHY COLUMNS

A 22,0ml protein sample (0,4mg/ml) was loaded onto a calibrated Sephadex G-100 column as determined in Section 2.3.1. Figure 13 shows the resultant elution profile of this fraction after Sephadex G-100 gel chromatography. It should be noted that the protein profile in Figure 13 represents an atypical chromatographic separation when compared to subsequent experiments. However, the CaBP activity was always eluted with a V_e/V_o of about 2,09 in all cases. In this experiment a peak of CaBP was eluted at 352,5ml. Assuming that the extrapolated calibration line was linear (which was not shown experimentally) the protein was found to have a molecular weight of about 11 900 daltons.

A more typical elution profile of a similar protein fraction can be seen in Figure 4, where the V_e/V_o of the major calcium binding peak was found to be 2,08 which is similar to the result described above. The reason for the discrepancy in the protein profiles will be discussed later.

The molecular weight of the CaBP was also determined on a calibrated Sephadex G-75. The CaBP was loaded and eluted as described in Section 2.3.1. A typical result is one shown in Figure 5. Once again the V_e/V_o ratio of the active peak fraction was outside the range of the proven linear curve. Assuming that the extrapolated line was linear, (which was not shown experimentally) the protein was found to have a molecular weight of 11 220 daltons.

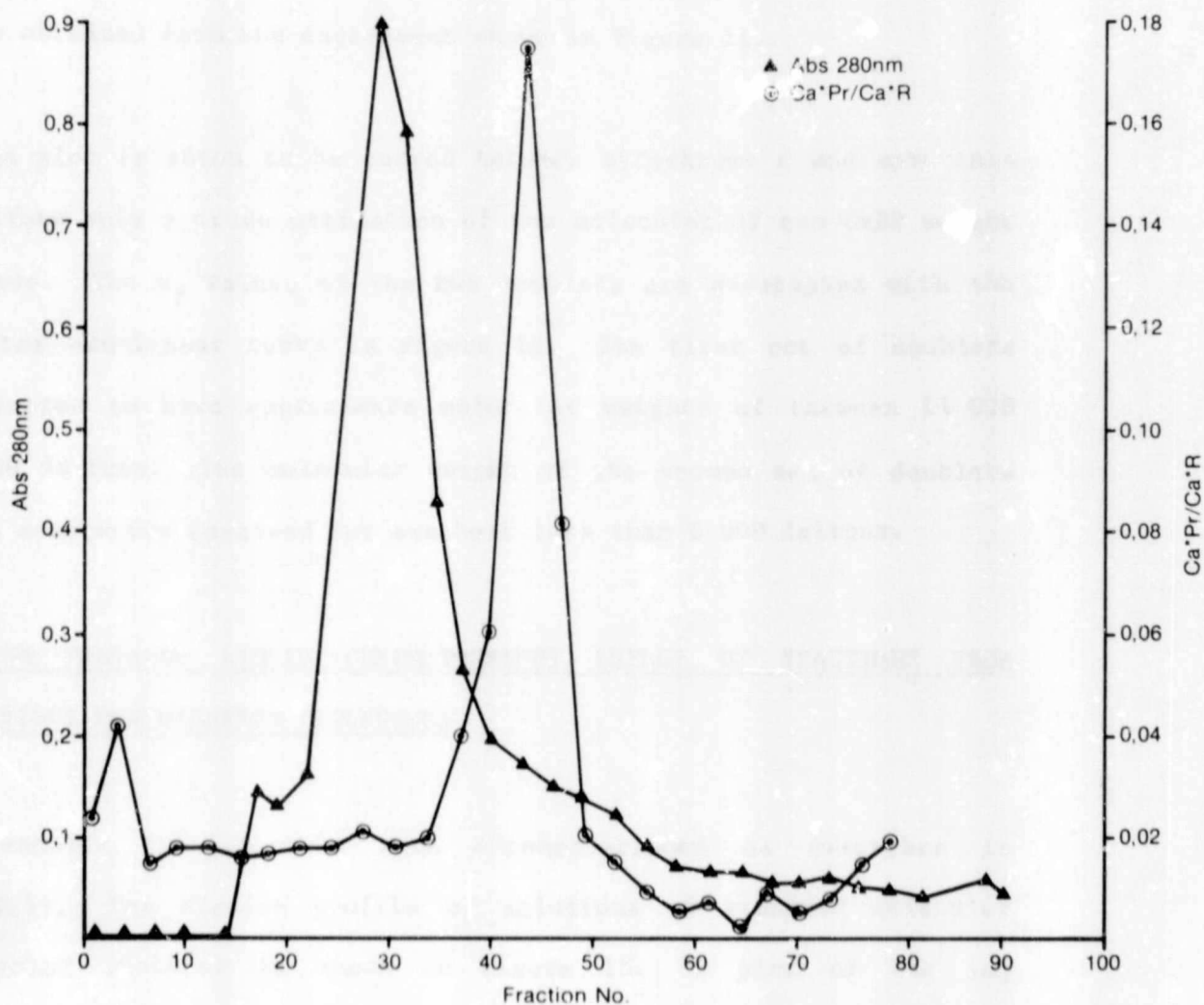


Fig. 13: Sephadex G-100 gel filtration chromatography of a 0-75% ammonium sulphate supernatant fraction. The plot shows protein content measured by absorbance at 280nm and CaBP activity ($\text{Ca}^*\text{Pr}/\text{Ca}^*\text{R}$) versus fraction number. The V_e of eluted peak = 352,5ml ($V_e/V_o = 2,09$).

Author Bloch Jeffrey Max

Name of thesis The Purification And Properties Of An Intestinal Calcium Binding Protein From The Baboon. 1985

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