3.2.3 MOLECULAR WEIGHT DETERMINATION OF CaBP BY SDS POLYACRYLAMIDE GEL ELECTROPHORESIS

Proteins and standards were electrophoresed as described in Section 2.10. A calibration curve of R_f versus log molecular weight of the low molecular weight protein standards is depicted in Figure 14. The data were obtained from the experiment shown in Figure 11.

The plot is shown to be curved between cytochrome c and aprotinin and therefore only a crude estimation of the molecular of the CaBP weight can be made. The R_f values of the two doublets are associated with the part of the non-linear curve in Figure 14. The first set of doublets were estimated to have approximate molecular weights of between 11 000 and 12 500 daltons. The molecular weight of the second set of doublets cannot be accurately assessed but are both less than 6 000 daltons.

3.2.4 HIGH PRESSURE LIQUID CHROMATOGRAPHY (HPLC) OF FRACTIONS FROM VARIOUS PURIFICATION PROCEDURES

Standards and unknowns were chromatographed as described in Section 2.11. The elution profile of solutions of standard molecular weight marker proteins is shown in Figure 15. A plot of the log molecular weight versus elution time is shown in Figure 16. In Figure 15, since the 22,03 min peak does not resolve cytochrome c and lysozyme which have molecular weights of 12 500 and 14 400 respectively an average molecular weight of 13 500 was used in the calibration curve. The protein assurred to be CaBP was eluted at an elution time which was outside that for the range of standards used in the calibration curve.

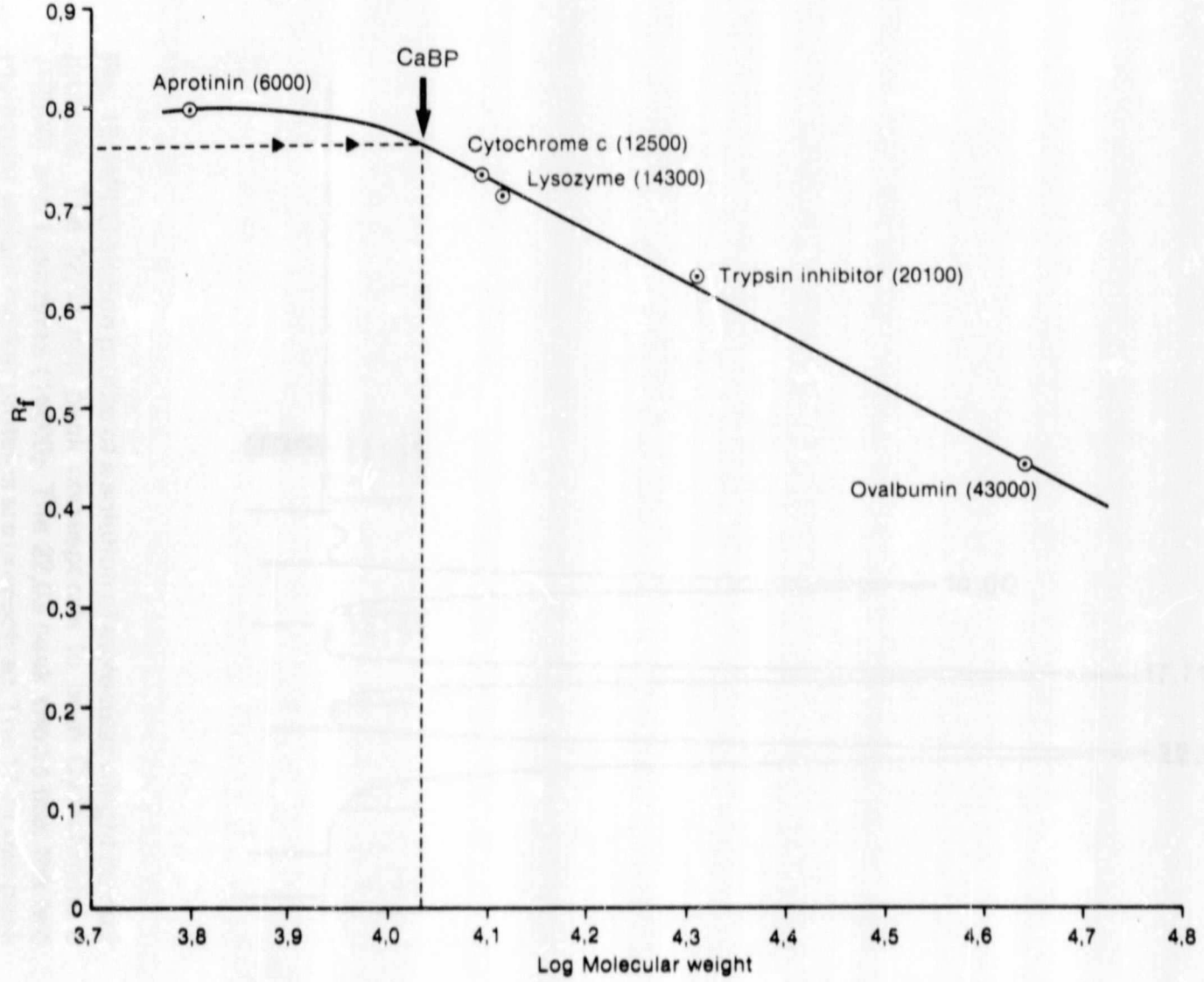


Fig. 14: Calibration curve of molecular weight proteins run on 12,5% SDS gel electrophoresis showing R_f of standard proteins versus their log Molecular weights.

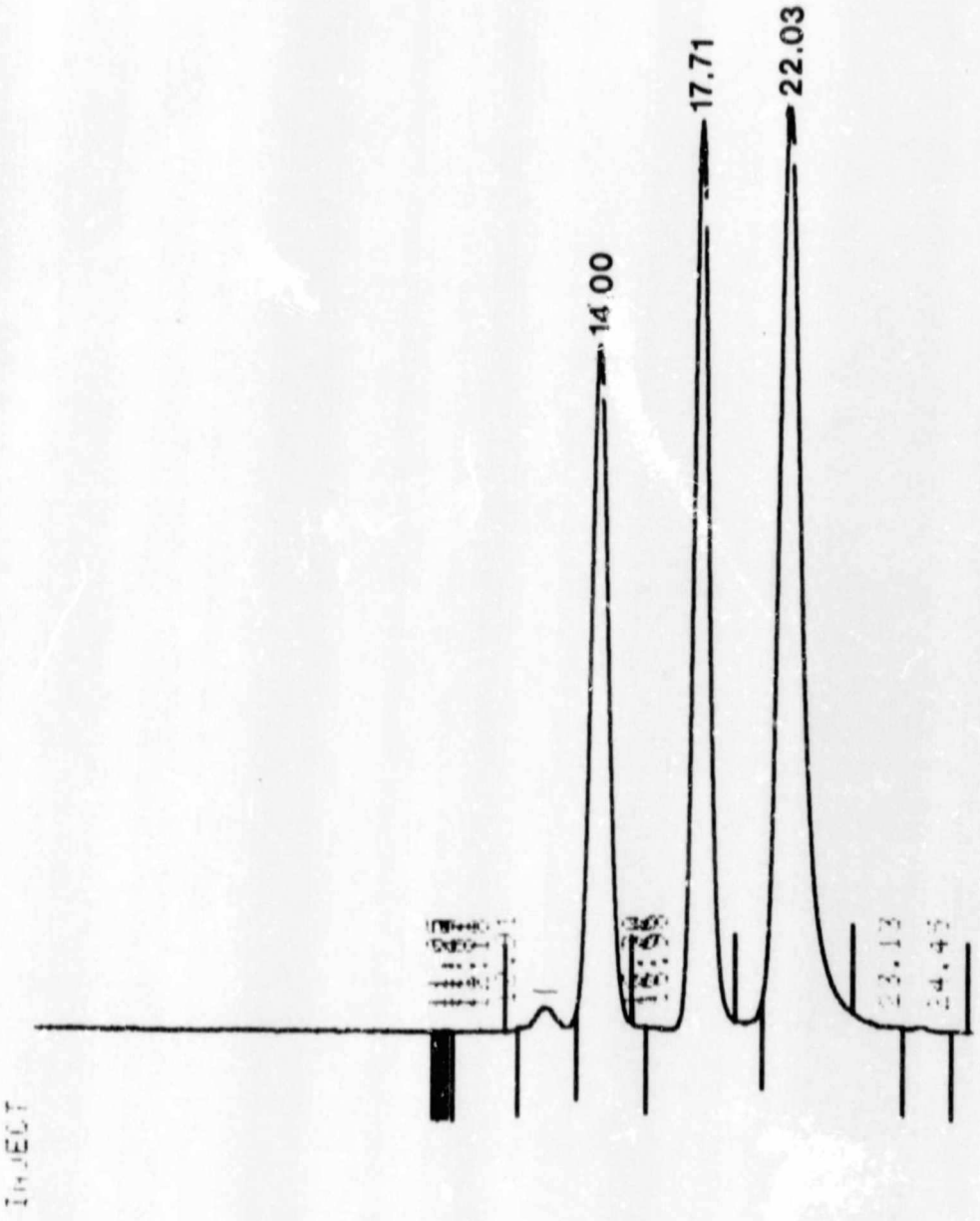


Fig. 15: HPLC elution profile of a solution of molecular weight marker proteins. The 22,03 min peak corresponds to both Cytochrome c (12500) and Lysozyme (14400). The 22,03 peak shows that the two molecular weight marker proteins are unresolved. The 17,71 min peak corresponds to Chymotripsinogen (25000) and the 14,00 min peak corresponds to Ovalbumin (43000).

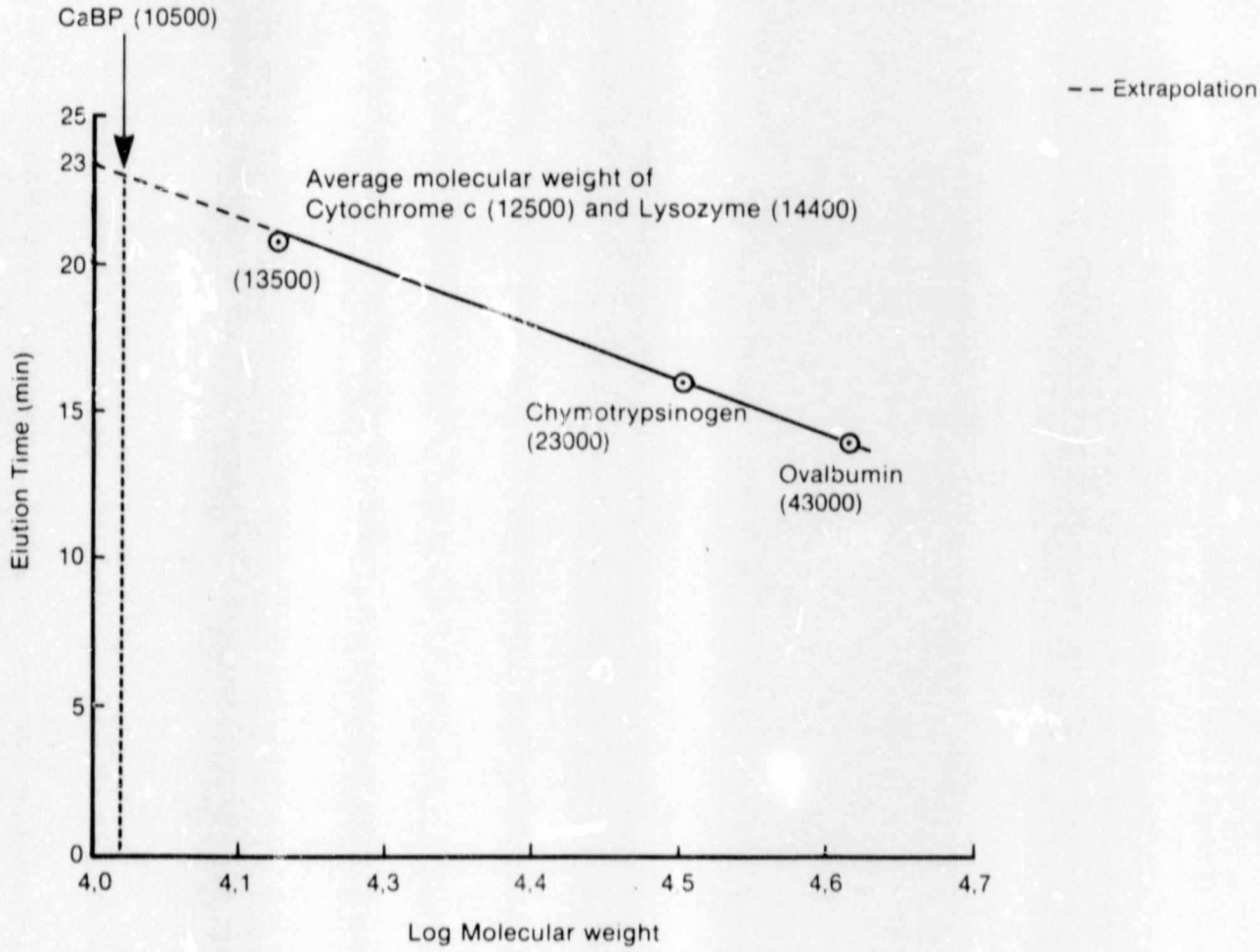


Fig. 16: Plot of elution time (min) versus log molecular weight of proteins eluted by HPLC. Marker proteins are the same as in fig. 15.

Therefore, the line was extrapolated so as to determine the approximate molecular weight of the fraction in question. Assuming again that the extrapolated line was linear (which was not shown experimentally) the protein was found to have a molecuar weight of 10 200 daltons.

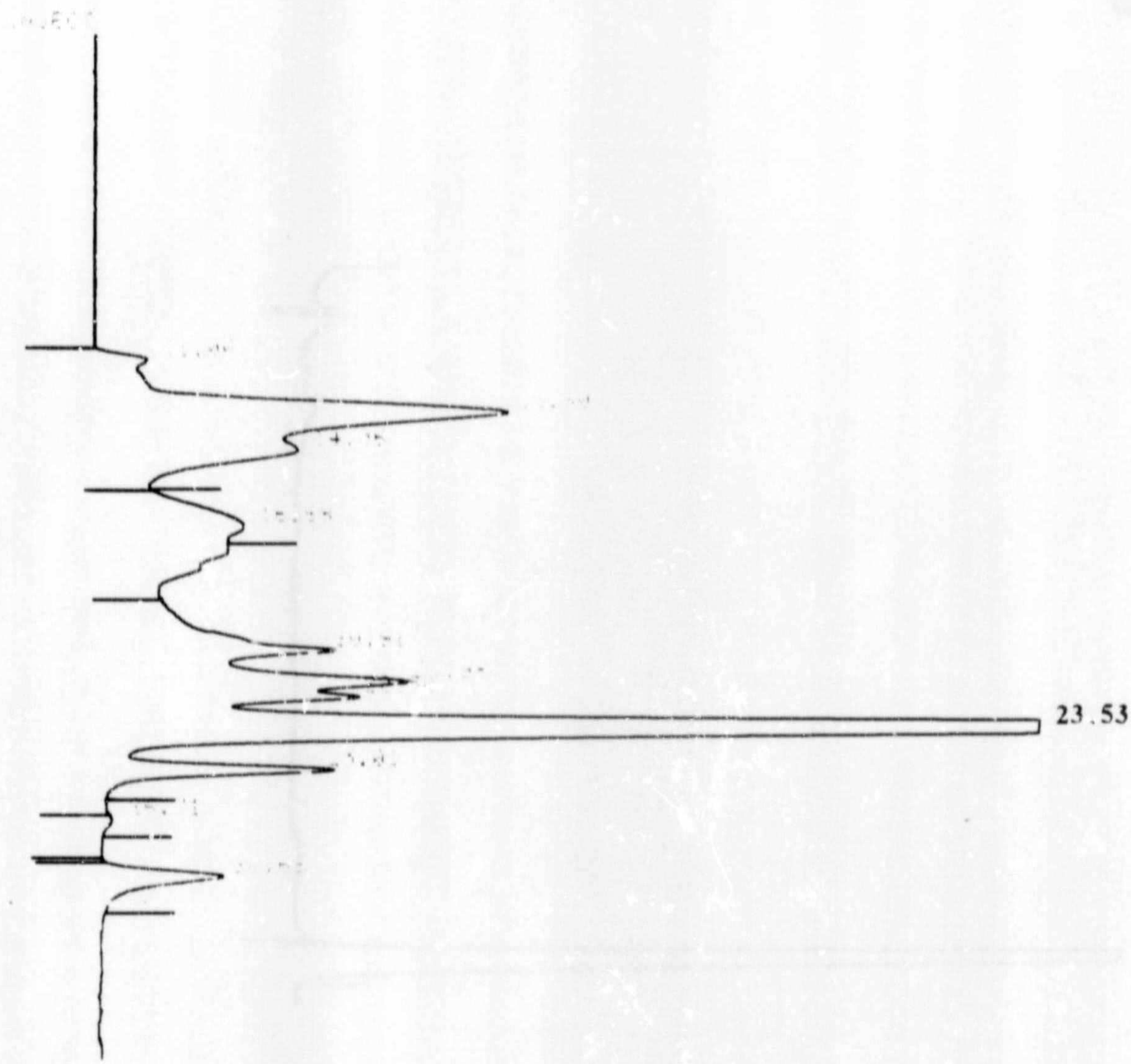
Figure 17 shows the elution profile on HPLC of a fraction previously purified by Sephadex G-75 chromatography. A protein peak was shown to be eluted at 23,53 min which corresponded to 44% of the total protein content. Because of the lack of resolution obtained for the low molecular marker proteins, the molecular weight of the CaBP can only be quoted as being approximately 13 500 daltons.

Figure 18 shows the rechromatography on HPLC of the major peak obtained in the experiment in Figure 17. The profile here shows the elution of a single protein peak with an elution time of 23,40 min. Unfortunately, the protein was found to be biologically inactive as measured by the Chelex assay.

A fraction obtained from DEAE-Sephacel ion-exchange chromatography was also subjected to HPLC. The resulting chromatograph (Figure 19) showed the elution of a protein peak at 23,33 min comprising 78% of the total protein content of the sample. The protein was also shown to have a molecular weight of approximately 10 500 daltons.

3.3.1 ISOLATION AND PARTIAL PURIFICATION OF THE BABOON INTESTINAL CaBP FROM BABOONS ON DIFFERENT DIETS

Figure 20 shows the results of Sephadex G-100 gel filtration chromatography of fractions from the intestinal mucosa of the four groups



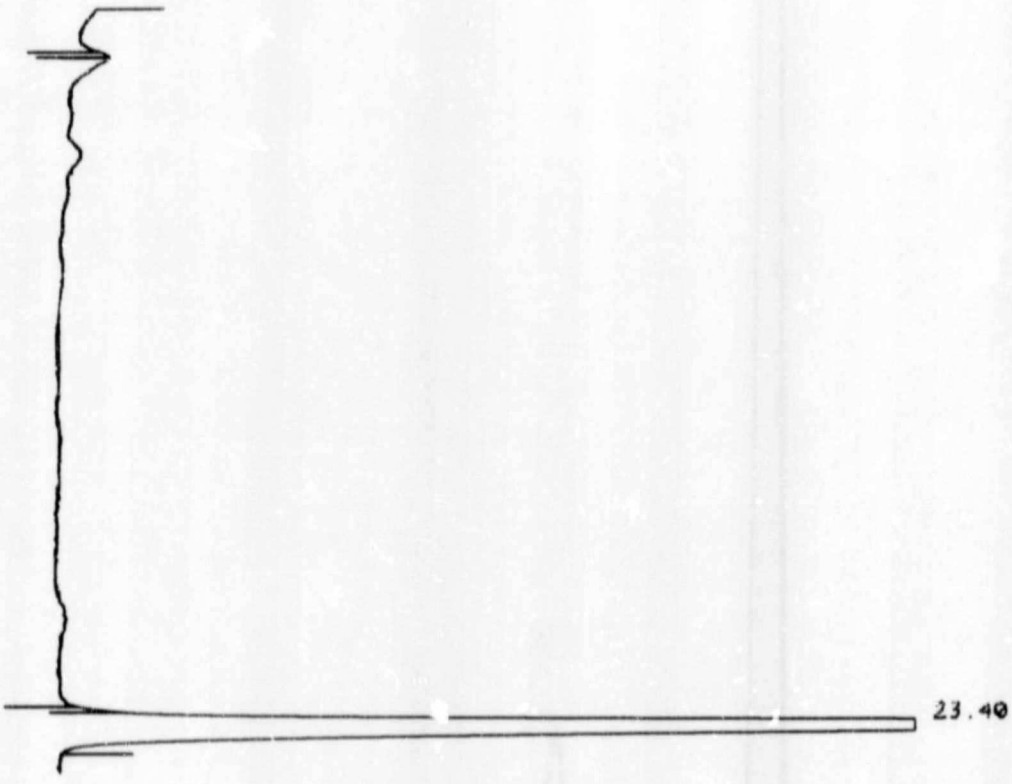
[illegible] DATA REPORT [illegible] AREA PERCENT [illegible]
[illegible] 12/21/83 [illegible]

PROTEIN ANALYSIS
COLUMN: [illegible]PROTEIN [illegible]
MOBILE PHASE : TRIS-SODIUM SULPHATE
FLOW: 1 ML/MIN

SAMPLE NAME: [illegible]
REPORT FILE: NONE PRESSURE: 462 PSI [illegible] 10670
WISP SYSTEM MESSAGES: [illegible] PSI

RT	AREA	PERCENT
11	[illegible]E+06	1.06183
12.5	1.73594[illegible]	19.3558
14.06	4.73305E+07	7.01683
15.58	[illegible].23953E+07	1.83819
20.91	4.2[illegible]32E+07	[illegible]
21.98	4.3[illegible]61E+07	[illegible]
22.5	2.64757E+07	3.92615
23.53	2.97188E+08	44.0711
25.01	3.3311[illegible]E+07	4.93936
25.71	823080	[illegible]
28.[illegible]	1.[illegible]E+07	[illegible].47048

Fig. 17: HPLC elution profile of a protein sample subjected to Sephadex G-75 chromatography after heat fractionation. The profile shows a peak containing 44% of the total protein with a molecular weight ~ 10 500 daltons.



[illegible]

PROTEIN ANALYSIS
COLUMN: 22×PROTEIN 125
MOBILE PHASE : TRIS-500[illegible] SOLPHATE
FLOW : 1 ML/MIN

SAMPLE NAME: PETENSATE [illegible]
REPORT FILE: NONE PRESSURE: 222 PSI [illegible]
WISP SYSTEM MESSAGES: 0500-0030 PSI

RT	AREA	PERCENT
23.4	1.2182E+07	**100**

Fig. 18: Rechromatography of the major fraction of protein obtained from the experiment shown in fig. 17.

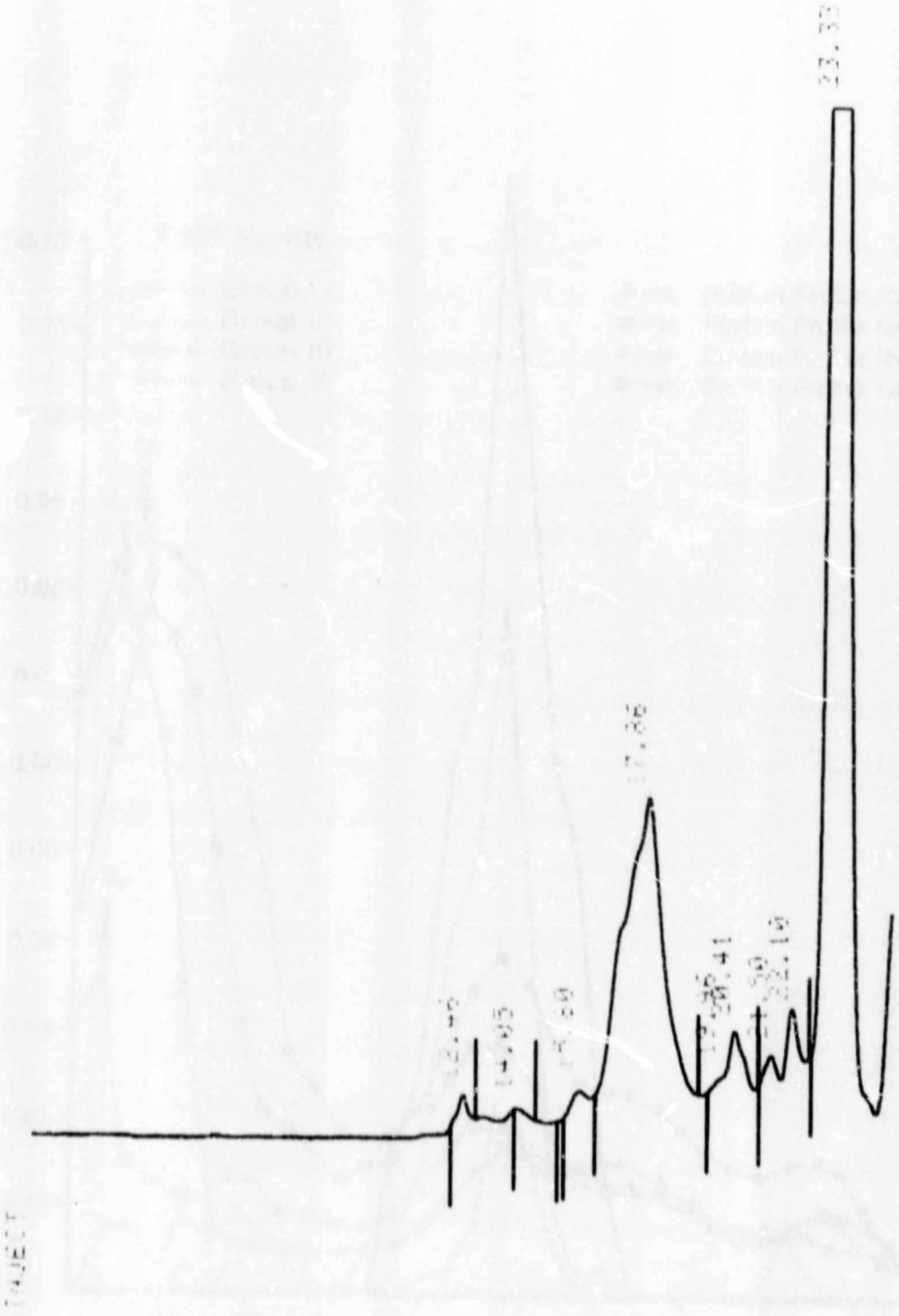


Fig. 19: HPLC elution profile of a protein fraction obtained from DEAE-Sephacel ion-exchange chromatography.

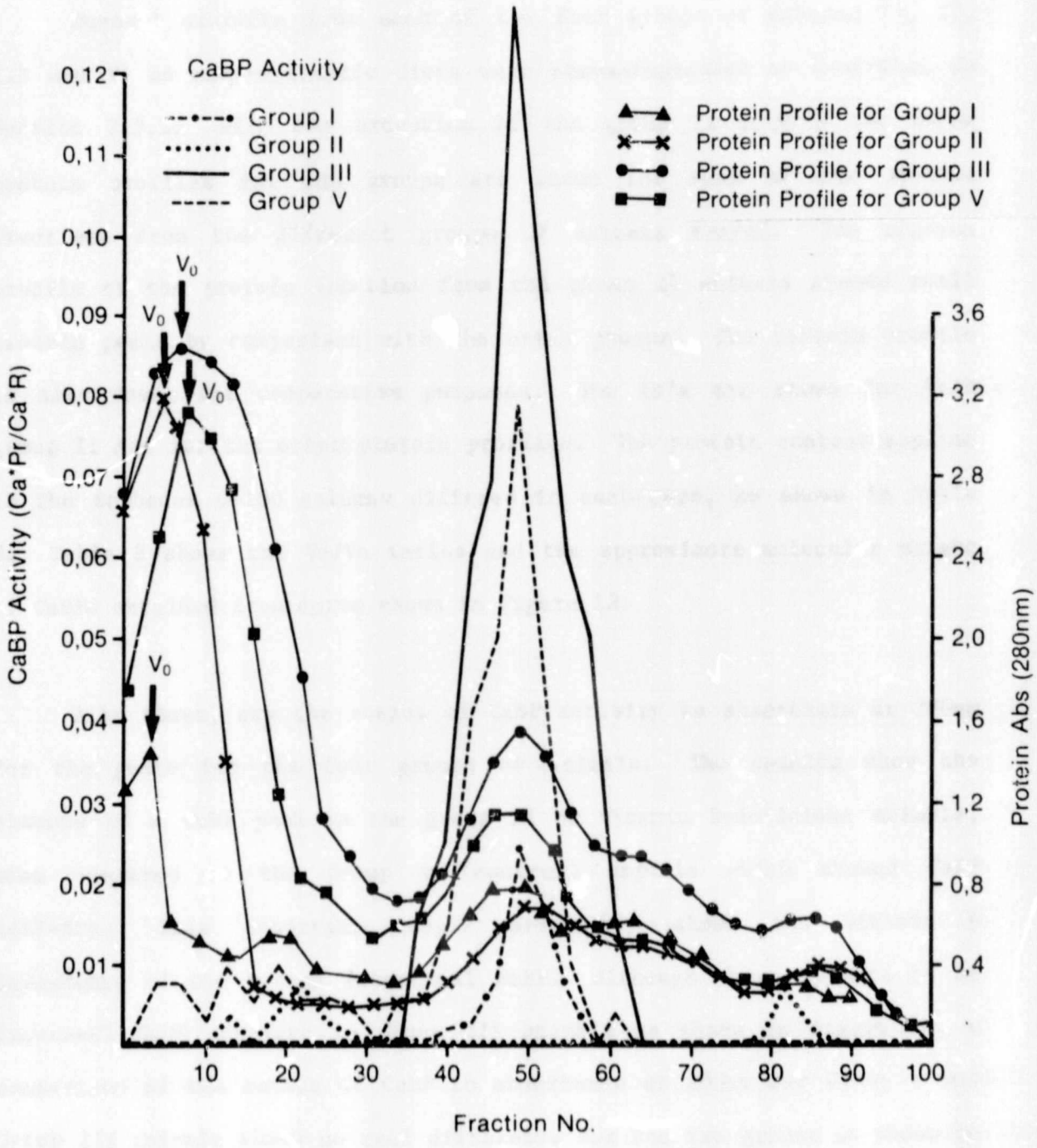


Fig. 20: Sephadex G-100 gel filtration chromatography of fractions obtained from intestinal mucosa from four groups of baboons fed on different semi-synthetic diets. Details of diets are described in text and appendix II.

of baboons fed on semi-synthetic diets.

Mucosal extracts from each of the four groups of baboons (I, II, III and V) on semi-synthetic diets were chromatographed as described in Section 2.3.2. With the exception of the group II animals the 280nm protein profiles for the groups are shown for each of the protein fractions from the different groups of animals tested. The elution profile of the protein fraction from the group II animals showed small protein peaks by comparison with the other groups. The protein profile is also shown for comparative purposes. The Vo's are shown for both group II and for the other protein profiles. The protein content applied to the Sephadex G-100 columns differed in each case, as shown in Table 8. Table 8 shows the Ve/Vo ratios and the approximate molecular weight of CaBPs obtained from curve shown in Figure 12.

Also shown, are the ratios of CaBP activity to absorbance at 280nm for the peaks for the four groups of animals. The results show the absence of a CaBP peak in the group II or vitamin D-deficient animals, when compared to the Group V (control) animals which showed CaBP activity. This important result presumably shows the vitamin D dependency of the baboon intestinal CaBP. Although there appears to be increased CaBP activity in Group III animals as shown in Figure 20, a comparison of the ratios of CaBP to absorbance at 280nm for Group V and Group III animals shows no real difference for the two groups as shown in Table 8. Figure 20 also shows the presence of some calcium binding activity which is evident for the Group I animals. These animals were on a vitamin D-deficient diet containing phosphate. The significance of this and the other results will be discussed later.

TABLE 8: VOLUMES AND PROTEIN CONTENT OF APPLIED FRACTIONS AND APPROXIMATE MOLECULAR WEIGHT OF THE CaBP FRACTIONS FROM THE 4 GROUPS OF BABOONS

GROUP NO.	Amount of Protein applied (mg)	Ve/Vo	Approx. Molecular Weight (daltons)	Peak Fraction CaBP activity/ml per Abs 280 nm
I	38,0	2,01	<12 500	0,09
II	18,6	-	*-	not measurable
III	51,0	2,00	<12 500	0,24
V	30,2	2,00	<12 500	0,21

* No CaBP activity observed.

3.3.2 COUNTER-ION ELECTROPHORESIS OF CaBP FRACTIONS OBTAINED FROM FOUR GROUPS OF BABOONS FED SEMI-SYNTHETIC DIETS

This method was used to confirm the existence of the baboon CaBP in the intestinal mucosa of the 4 groups of baboons discussed in the section above.

Protein samples used were those obtained from tissue extracts of the four groups of baboon following ammonium sulphate precipitation and Sephadex G-100 gel chromatography. Figure 21 shows results which confirm the absence of calcium binding activity measured from intestinal mucosal extracts from Group II animals (vit D-deficient) and varying amounts of CaBP activity for the other three groups. The results show the presence (Groups I, III and V) and absence of a calcium binding peak (Group II) at gel slice number 16.

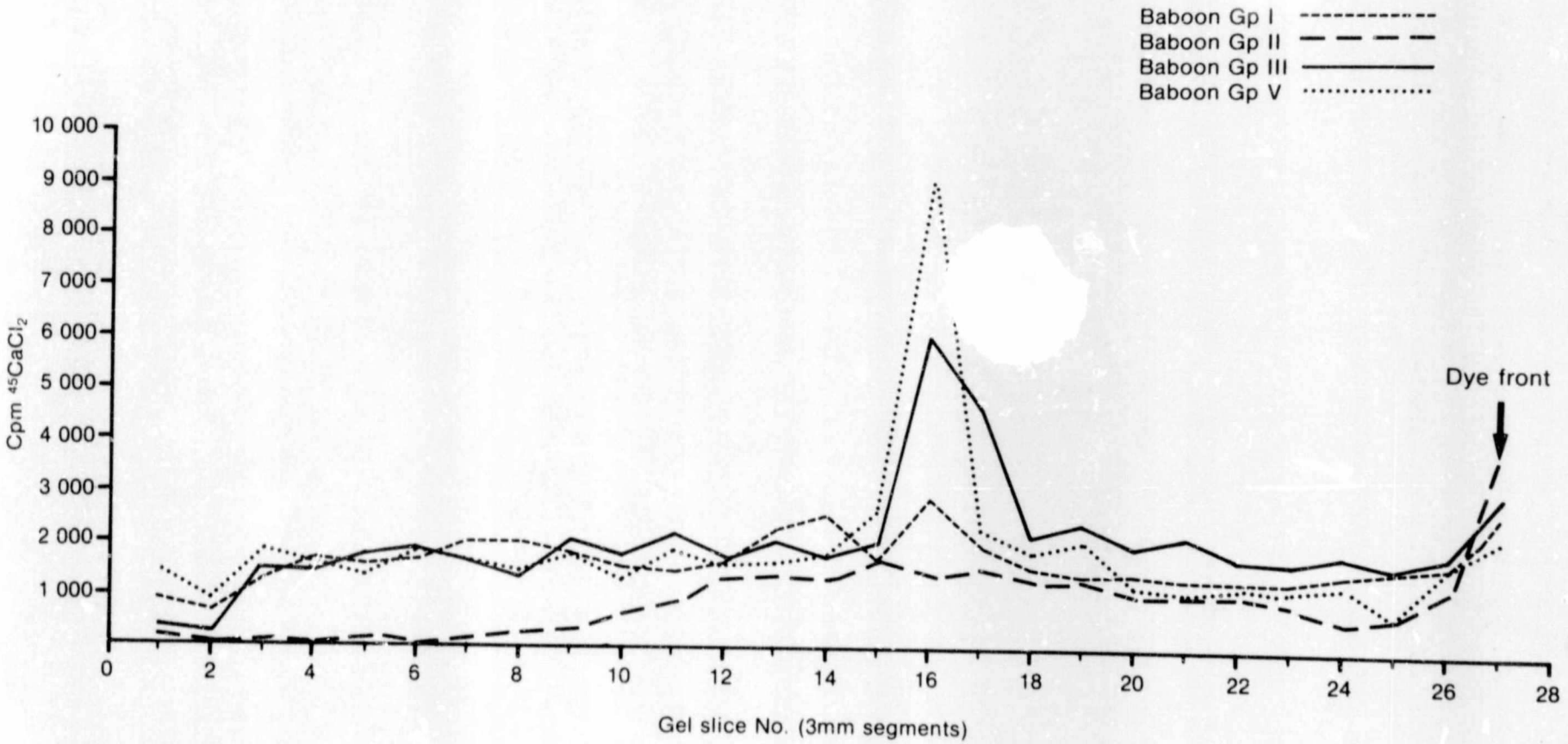


Fig. 21: Counter-ion electrophoresis of partially purified CaBP from four different groups of baboons fed on semi-synthetic diets.

3.3.3 COMPARISON OF PURIFIED CaBP FRACTIONS SUBJECTED TO COUNTER-ION ELECTROPHORESIS AND COOMASSIE-BLUE STAINING

Duplicate gels to those described in 3.3.2 were stained for protein and are shown in Figure 22. The presence of a protein staining band at the position of CaBP activity is evident in group I and group V. The gels show clearly the absence of this band in group II. The result confirmed the vitamin D-dependency of the CaBP as equal amounts of protein (120 μg) were applied to each gel.

3.4.0 THE EFFECT OF CONCENTRATION OF AMMONIUM SULPHATE ON BOTH THE CHELEX ASSAY AND THE BIURET ASSAY

3.4.1 THE EFFECTS OF AMMONIUM SULPHATE ON THE CHELEX-100 UPTAKE OF $^{45}CaCl_2$

Table 9 shows the effect of increasing concentrations of ammonium sulphate on the Chelex-100 uptake of $^{45}CaCl_2$. Three different experiments were carried out as described in Section 2.12.1. The results are illustrated in Figures 23-25. Figure 23 shows the effect of increasing concentrations of ammonium sulphate on the Chelex-assay in absence of CaBP. The result shows a decrease in percentage counts in the assay which is maximal at a final ammonium sulphate concentration of 49%. Hereafter there is a steady decrease in the counts measured. In Figure 24 the assay system was tested in the presence of CaBP. Here, the protein competition for binding of isotope is decreased in solutions of increasing concentrations of ammonium sulphate. The results again show a steady decrease in the percentage counts measured in the assay.

Table 9: EFFECTS OF INCREASING CONCENTRATIONS OF AMMONIUM SULPHATE ON THE CHELEX-100 UPTAKE OF $^{45}CaCl_2$

The total counts tube contained 66 550 cpm which was calculated as the average over two different sets of experiments carried out. The background counts tube contained 429 cpm which was calculated as the average of the two experiments carried out.

Tubes Containing	Final % of Ammonium Sulphate in Assay	Counts in Assay(cpm)	
Total Counts (H_2O)	0	66 550	
Blank Counts (H_2O)	0	429	
		EXP. I	EXP II
1) Total counts plus ammonium sulphate to a final concentration of: (see Figure 23)	0	66 550	66 550
	24,8	24 624	23 292
	49,6	8 652	7 321
	74,4	2 795	2 596
	99,2	2 462	2 063
2) Total counts plus CaBP and ammonium sulphate to a final concentration of: (see Figure 24)	0	37 934	37 934
	19,8	35 937	33 275
	39,6	17 503	15 839
	59,5	11 713	10 582
	79,4	5 058	4 924
3) Background counts plus ammonium sulphate to a final concentration of: (see Figure 25)	0	429	429
	19,8	30 495	25 740
	39,6	13 084	11 797
	59,5	11 154	8 794
	79,4	5 491	5 877

N.B. The data shown in Table 9 above are expressed as percentages of the total counts in the assay system versus increasing concentration of ammonium sulphate in Figures 23 - 25.

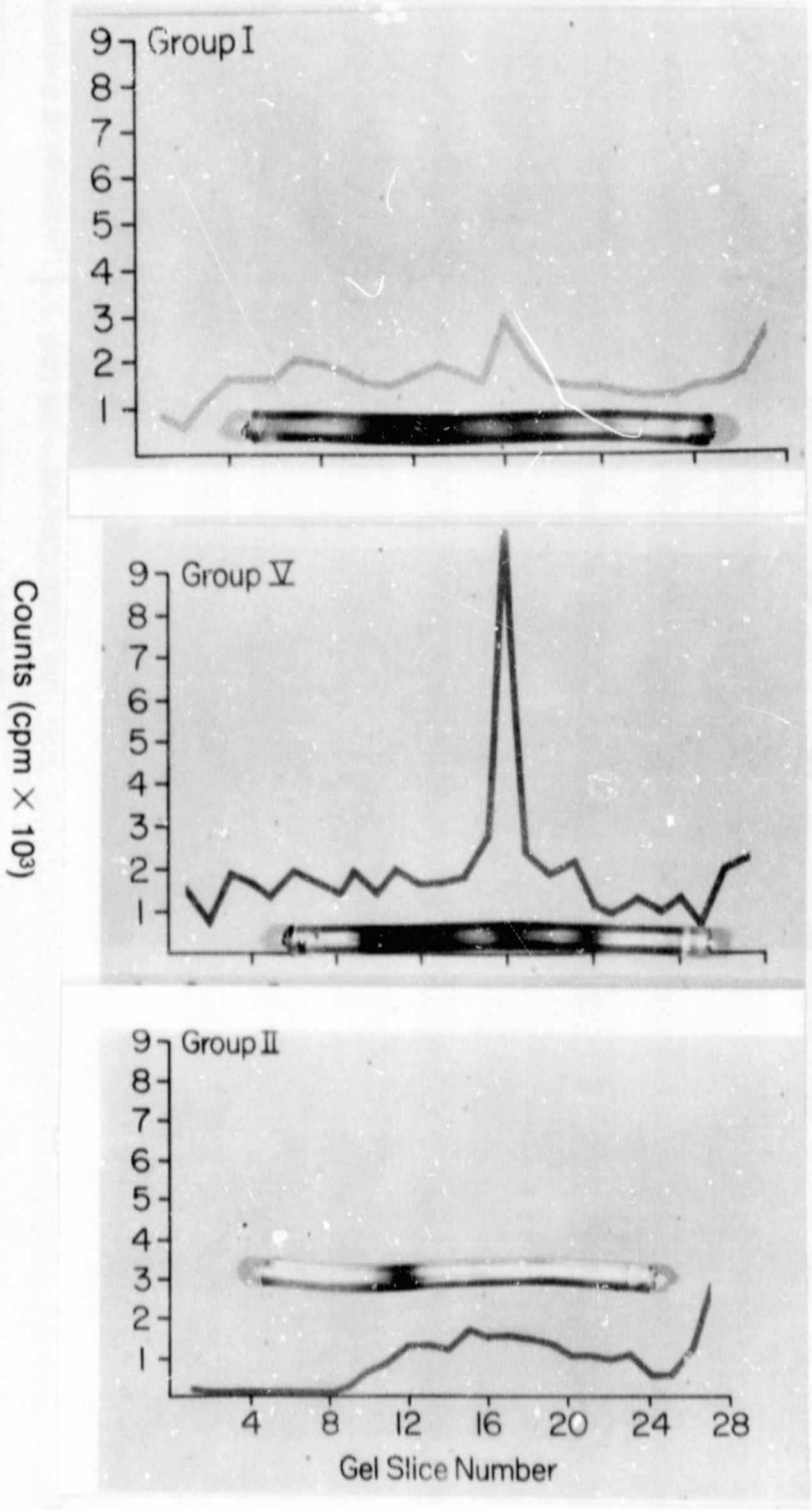


Fig. 22: Comparison of purified CaBP fractions subjected to counter-ion electrophoresis and Coomassie-blue staining. Results are shown here for 3 of the 4 different groups of baboons.

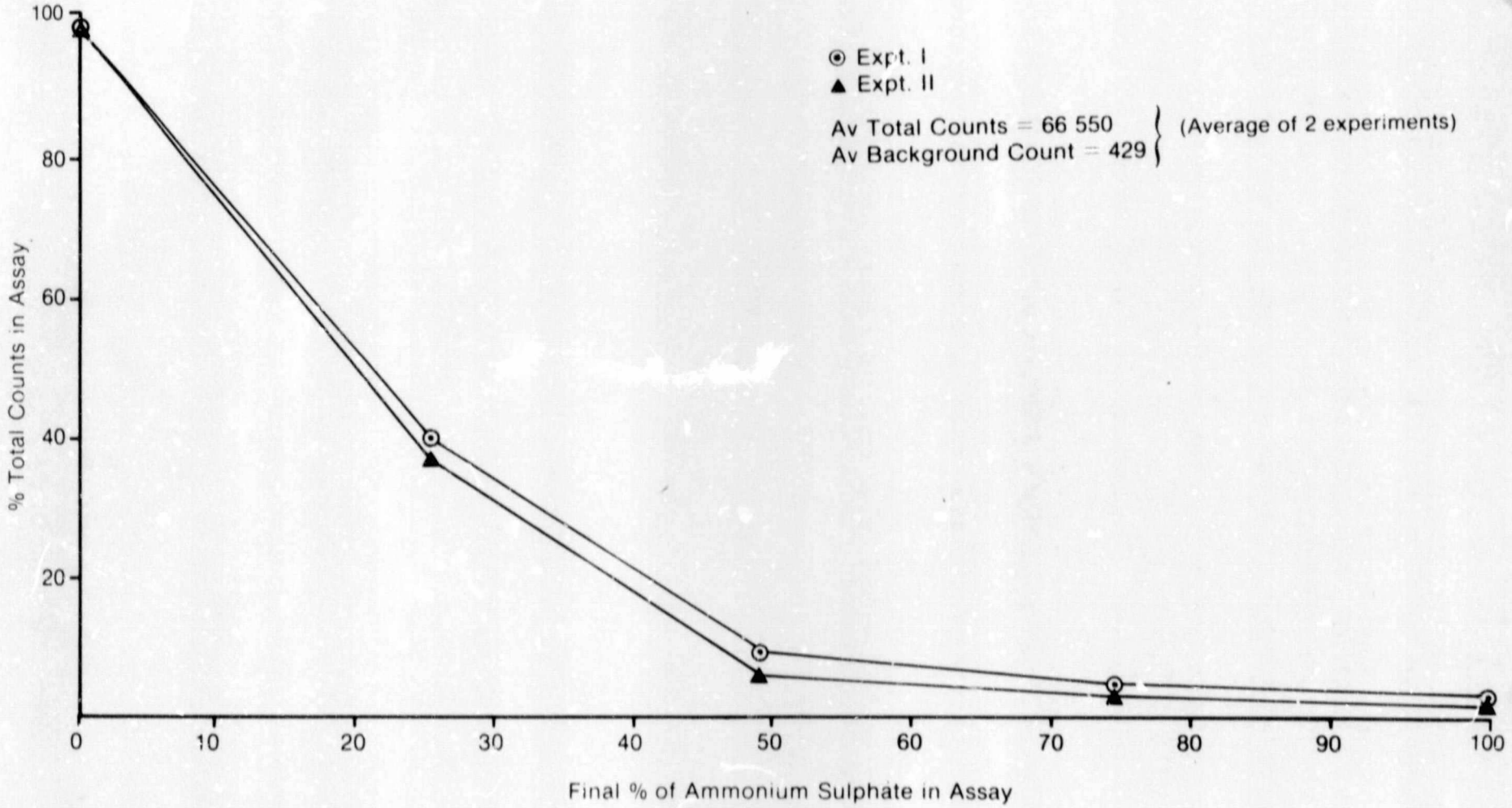


Fig. 23: The effect of increasing concentrations of ammonium sulphate on Chelex-100 resin uptake of $^{45}CaCl_2$ in the absence of CaBP in the assay system.

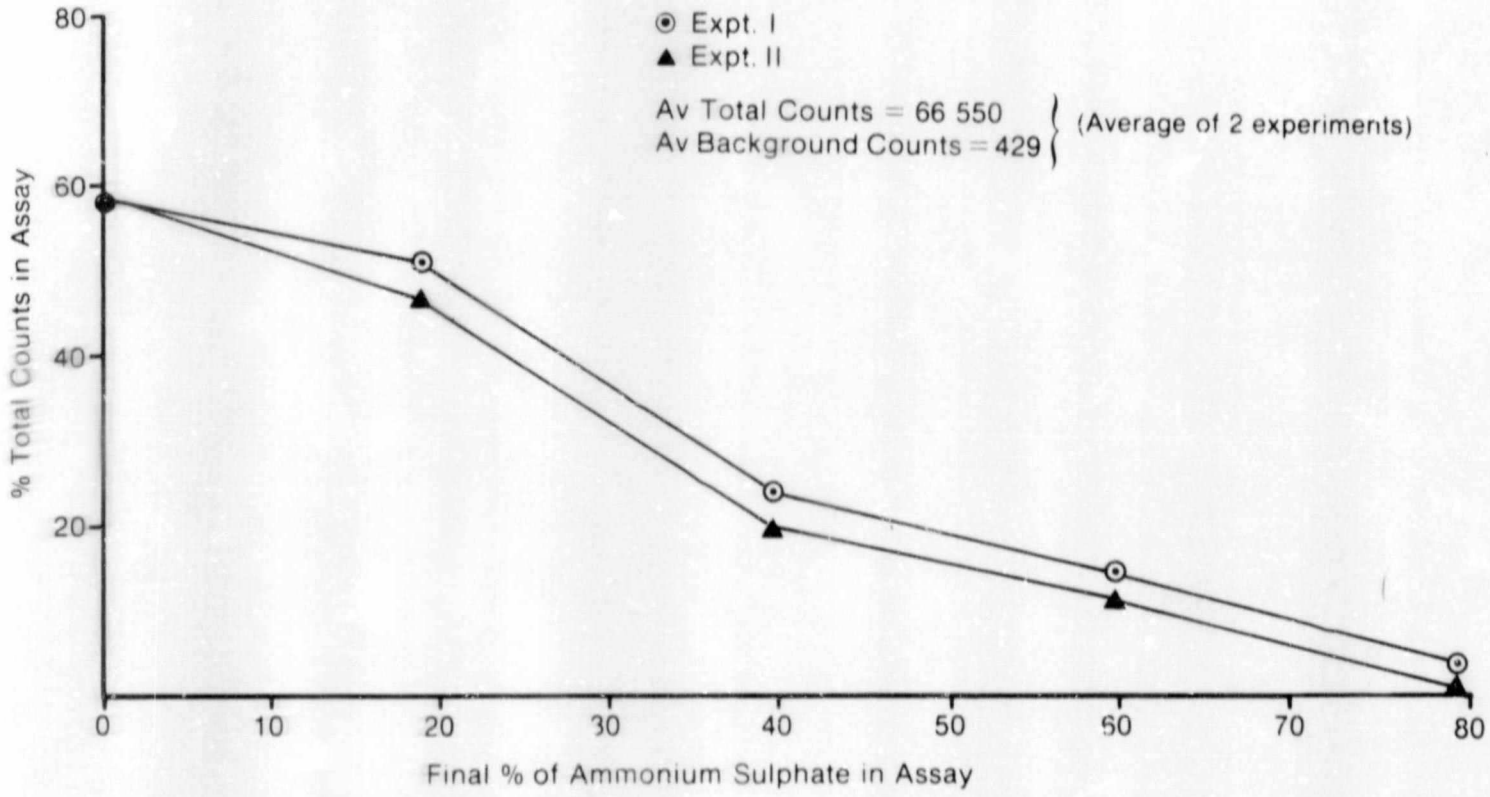


Fig. 24: The effect of increasing concentrations of ammonium sulphate on Chelex-100 resin uptake of $^{45}CaCl_2$ in the presence of CaBP in the assay system.

In Figure 25, the plot shows the effect of increasing concentrations of ammonium sulphate on the background counts in the absence of protein in the assay. The result shows a large increase in background counts up to an ammonium sulphate concentration of 20% and then a steady decrease.

3.4.2 THE EFFECT OF AMMONIUM SULPHATE ON BIURET ASSAY

Table 10 shows the effects of increasing amounts of ammonium sulphate solutions on the Biuret Assay as described in Section 2.12.2. Figures 26 - 28 show graphical representations of the effects.

Results from Figure 26 show that when using 0,6ml of standard protein plus 0,2ml of ammonium sulphate in increasing concentrations, an approximate 10% drop in the protein concentration from 6 mg/ml to 5,55 mg/ml was observed. This drop only occurred at a final ammonium sulphate concentration of 2,5%. When adding equal volumes of protein (0,4ml) and 0,4ml of ammonium sulphate of increasing concentration, the result showed a 50% decrease in the measured protein concentration i.e. from 4,0 mg/ml to 2 mg/ml. This decrease occurred at final ammonium sulphate concentrations of between 37,5 - 50% (Figure 27). Addition of 0,6ml of increasing concentrations of ammonium sulphate solution to 0,2ml of protein showed no significant change in the measured protein concentration. (Figure 28)

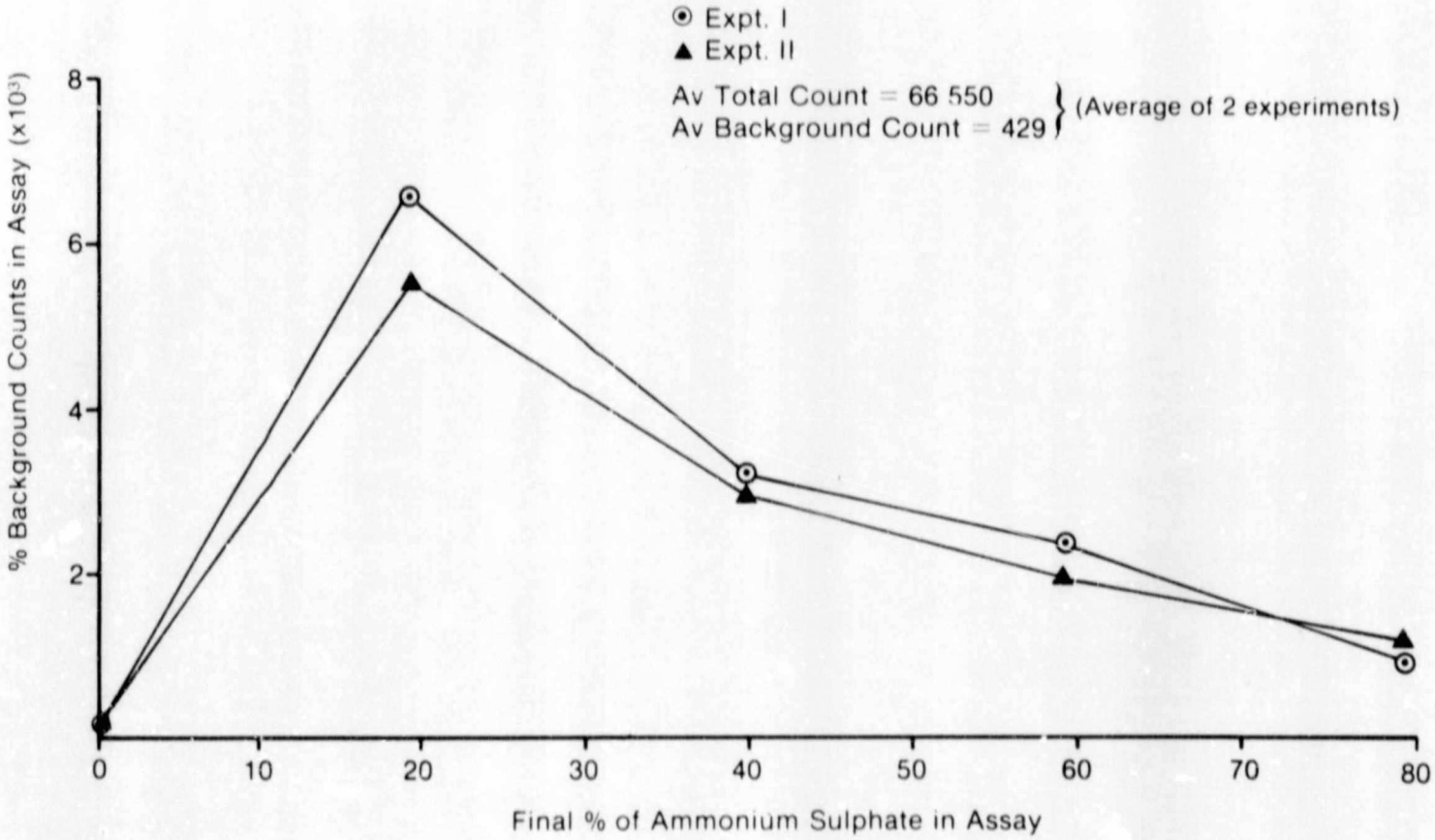


Fig. 25: The effect of increasing concentrations of ammonium sulphate on the blank counts on the Chelex-100 resin uptake of $^{45}CaCl_2$.

Table 10: EFFECTS OF AMMONIUM SULPHATE ON BIURET ASSAY

Three experiments were carried out to show the effects of varying amounts of increasing concentrations of ammonium sulphate on specified amounts of protein in the Biuret assay.

Sample Tested	Vol(ml)	Standard BSA (10mg/ml) Vol(ml) added	Measured Protein (mg/ml) I	Measured Protein (mg/ml) II	Final Percentage Ammonium Sulphate in assay
1)					
25% Ammonium Sulphate	0,2	0,6	6,15	6,1	6,25
50% "	"	"	6,20	6,21	12,5
75% "	"	"	6,17	6,1	18,75
100% "	"	"	5,55	5,0	25,0
2)					
25% Ammonium Sulphate	0,4	0,4	4,05	4,22	12,5
50% "	"	"	4,00	4,00	25,0
75% "	"	"	2,00	2,17	37,5
100% "	"	"	2,20	2,20	50,0
3)					
25% Ammonium Sulphate	0,6	0,2	2,25	2,51	18,75
50% "	"	"	2,00	1,90	37,5
75% "	"	"	2,05	2,05	56,25
100% "	"	"	2,10	2,12	75

I and II - Results of protein concentration measured from two separate experiments. Results from 1,2 and 3 are shown in Figure 26-28 respectively.

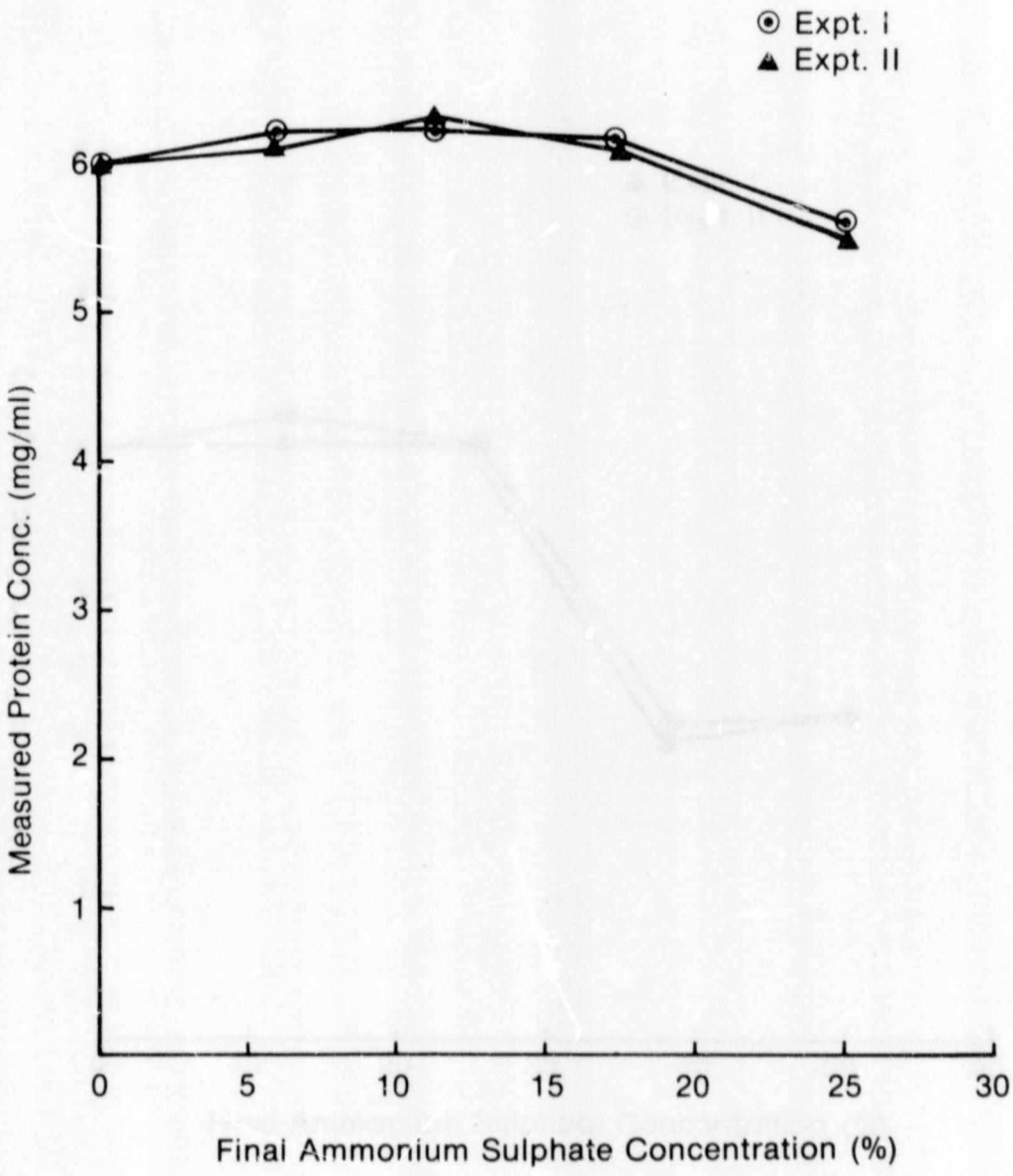


Fig. 26: The effect of increasing concentrations of ammonium sulphate of the Biuret assay using 0,2ml of an ammonium sulphate solution and 0,6ml of standard BSA solution **(10mg/ml)**.

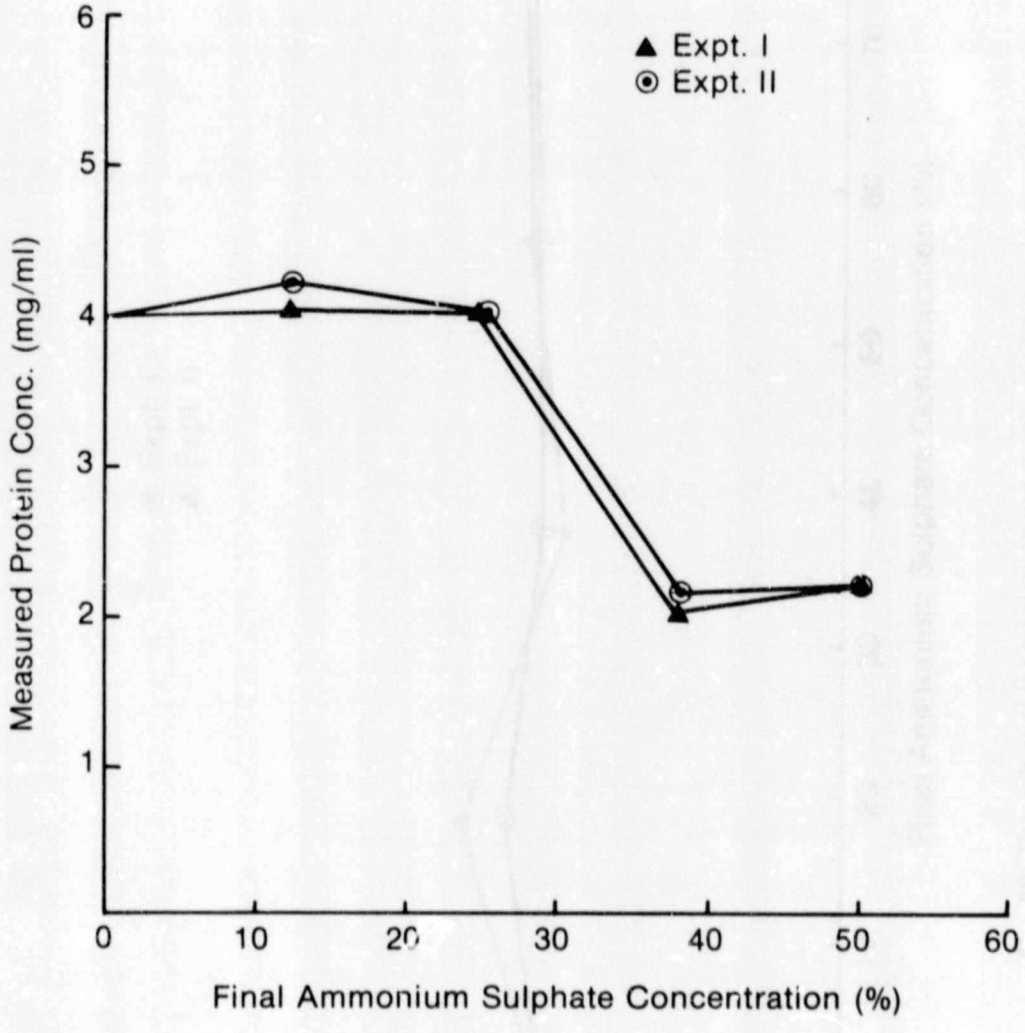


Fig. 27: The effect of increasing concentrations of ammonium sulphate on the Biuret assay using 0,4ml of an ammonium sulphate solution and 0,4ml of standard BSA solution (10mg/ml).

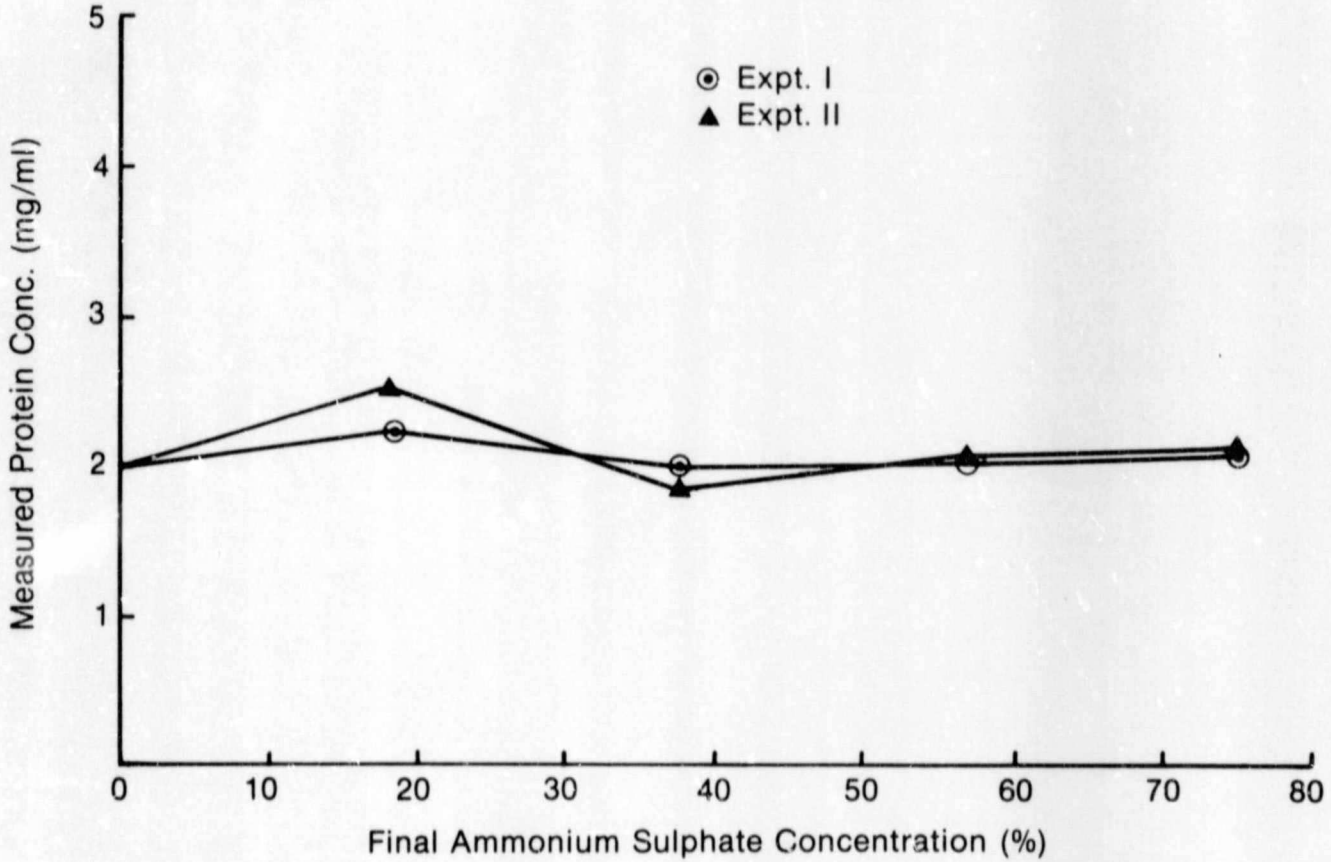


Fig. 28: The effect of increasing concentrations of ammonium sulphate on the Biuret assay using 0,6ml of an ammonium sulphate solution and 0,2ml of standard BSA solution (10mg/ml).

3.4.3 THE EFFECT OF EDTA AND $CaCl_2$ ON THE CHELEX-100 UPTAKE OF $^{45}CaCl_2$

Table 11 shows the effects Tris pH 8,0 of buffers containing 1mM EDTA and 5mM $CaCl_2$ on the Chelex-100 resin uptake of $^{45}CaCl_2$.

Table 11 EFFECTS OF EDTA AND $CaCl_2$ ON THE CHELEX-100 UPTAKE of $^{45}CaCl_2$

Test Sample	Mean Counts (cpm)
Total counts	55 569
Background counts	519
Buffer containing 15mM Tris, 1mM EDTA	40 000
Buffer containing 15mM Tris, 5mM $CaCl_2$	549

The results show that in the presence of EDTA nearly 80% of the counts remain in the supernatant. In contrast, the presence of 5mM $CaCl_2$, which changes the specific activity of the isotope, has no significant effect on the counts remaining in the supernatant, that is the resin effectively removes all the calcium in solution.

CHAPTER 4

DISCUSSION AND CONCLUSION

The aim of this dissertation was to purify and characterise an intestinal CaBP from the baboon (Papio ursinus). The results obtained show both the effectiveness as well as the shortfalls of the purification procedures used. The dissertation also highlights areas of new information with regard to the vitamin D-dependency and molecular size of the intestinal CaBP from the higher primate.

The purification procedures used were based on methods already described for the isolation of the vitamin D-dependent CaBP from the chick, rat and baboon. The work was also based in part on the purification methods described for the isolation of the bovine CaBP.

Results indicated that of the three initial procedures used, that is ammonium sulphate fractionation, heat and pH precipitation, ammonium sulphate fractionation achieved the greatest degree of purification. Results obtained from ammonium sulphate fractionation compare favourably to those shown by Wasserman et al, (1968) and Fullmer and Wasserman, (1975). The purification achieved by these researchers, however, was lower than that shown here. They obtained purifications of between 3 - 6-fold over crude starting materials whereas we achieved approximately a 20-fold purification. The reason for this could be that in the purification of the chick CaBP there was more inactive protein present therefore resulting in a lower purification. Another reason for the different results obtained could have been due to the species difference.

The results shown in Table 4 also indicated that ammonium sulphate fractionation to 99% saturation resulted in the precipitation of some inactive protein thus failing to effect further purification. These results, are in direct contrast to those reported by Friedlander and Norman, (1980) where the authors demonstrated further purification at this step for CaBP from the chick intestine. This method has only been employed in the purification of the chick CaBP and has not been employed for any small molecular weight mammalian CaBPs.

A second isolation and purification procedure was carried out using pH precipitation as this method was specifically developed for the purification of a mammalian CaBP, namely bovine CaBP. This method of purification was tried, as the ammonium sulphate method described by Wasserman was only used for the purification of the chick CaBP. Results comparing the ammonium sulphate fractionation and the pH precipitation procedures indicated that the specific activity of the 0-75% ammonium sulphate fraction was greater than any of the fractions purified by the pH precipitation procedure. It was thus decided that results from the pH precipitation procedure would not be useful in effecting purification. A further experiment was however carried out using a pH 6,5 supernatant fraction just to qualify the latter statement.

A third purification procedure involving heat precipitation was carried out. This method was employed because the CaBP has been shown to be heat stable (Bredderman and Wasserman, 1974). These authors have also shown that increases in the amount of inactive protein content decreases the specific activity of the active protein which could have affected subsequent purifications. However, although many of the authors have commented on heating crude supernatant fractions for various times

ranging from 5 - 15 min, they have not considered or systematically investigated the effect of time of heating the crude supernatant fractions. It has been shown here that there is a rapid decrease in protein concentration with time. This is followed by a slow and then rapid increase in CaBP activity as measured by the Chelex-assay. This method showed that the specific activity of the protein was optimal after 20 min of heating but thereafter declined. The decline in the specific activity could have resulted from prolonged heating thus possibily denaturing the active protein. The other phenomenon observed pertaining to the heating experiment was the increase in the CaBP activity which became optimal at 20 min. This result could be explained by the fact that heating could have possibly inactivated an inhibitor present in the crude supernatant thus resulting in this increase in CaBP activity. The decline in the CaBP activity could have been due to prolonged heating i.e. denaturing of CaBP.

Comparing the specific activities from ammonium sulphate fractionation and heat precipitation, the latter shows a value slightly less than the former, namely, 0,13 (Figure 3) as opposed to 0,15 (Table 5). The overall purification achieved by the ammonium sulphate fractionation procedure was approximately 10-fold whereas that achieved by heat treatment was about 8,7-fold over the starting material. The ammonium sulphate fractionation procedure also achieved purifications of 16 and 20-fold over starting material. The variances could have been due to the different amounts of protein processed each time. The physical condition of the intestinal mucosa could also have produced these variances, as on occasions, intestinal mucosa was seen to be traumatised after surgery. Ulcers were often evident in the mucosa.

Further purification of the baboon intestinal CaBP was effected using Sephadex G-100 and Sephadex G-75 gel filtration chromatography. This step produced a CaBP fraction of specific activity of 0,55 (Ca*Pr/Ca*R/mg protein) for the Sephadex G-100 experiment. This represented a 3,7-fold increase in purification over the ammonium sulphate fractionation and an overall 37-fold purification over starting material.

In the case of Sephadex G-75, the specific activity of the pooled and concentrated CaBP peak was found to be 0,89 (Ca*Pr/Ca*R/mg protein) compared to 0,13 for the starting material, which had also been subjected to heat treatment. This represented a 6,8-fold increase in the purification of the protein. Overall, heating and Sephadex G-75 gel chromatography achieved a 59-fold purification over the starting material. Thus comparing the overall purification achieved, heat treatment and Sephadex G-75 gel chromatography in this set of experiment was the better. The specific activity of the Sephadex G-100 fraction was lower than that shown by Wasserman et al, (1968) in his purification of chick protein, that is 0,55 compared to 1,01, however, a 4,4-fold purification was over the ammonium sulphate fraction was achieved which is not dissimilar to that reported here. The specific activity of the Sephadex G-75 fraction purified from heat treatment could not be compared to results shown by Gleason and Lankford, (1982) in their purification of the baboon intestinal CaBP as neither specific activities nor purifications were quoted by these authors.

The results thus far obtained showed that either heat or ammonium sulphate precipitation as well as either Sephadex G-100 or G-75 could effect further purification of the protein. The protein and CaBP activity profiles of Sephadex G-100 reported here compared favourably qualitatively with those shown and described by Fullmer and Wasserman,

(1975) in their purification and characterisation of intestinal CaBPs from cow, pig, horse and guinea pig. The specific activities obtained here compared more favourably with the results shown for the pig in their study. The specific activities achieved after Sephadex G-100 gel chromatography for CaBPs for the other animals, however, were almost 4 times higher, although the overall purification achieved were similar. The guinea pig showed the lowest degree of purification, namely 2,2-fold as opposed to the pig which showed a 3,3-fold purification over the ammonium sulphate fractions. The overall purifications achieved ranged from 7-fold (chick) to 22-fold (pig) over the original starting material. The overall purifications achieved in results reported from this dissertation ranged from 18 - 36-fold for the CaBP after Sephadex G-100 gel chromatography. These results are comparable and at the same time are slightly higher than the results reported by Fullmer and Wasserman, (1975) for different mammalian species. The pig result seemed to be the only one comparable with results obtained here.

A further purification technique using DEAE-Sephacel ion-exchange chromatography following heat precipitation and Sephadex G-100 gel chromatography was carried out. A combined CaBP fraction which comprised the two peak fractions eluted off the DEAE-Sephacel ion-exchange column had a specific activity higher than that measured for the fraction eluted off Sephadex G-100 column. This indicated a 1,7-fold purification of the protein. The overall purification of the CaBP was approximately 31-fold from the starting material. In the experiment using DEAE-Sephacel ion-exchange chromatography, the method required a stepwise elution of the protein. The active proteins were eluted with the low ionic strength buffer. It was shown in a small experiment (results not quoted here) that CaBP activity could only be measured at a concentration of NaCl

lower than 150mM. The results showed that the $1/Kd^{o}$ values in Chelex-assay increased hyperbolicaly after a NaCl concentration of 150mM. Two CaBP peaks were eluted in starting buffer off the column. The implication of this would indicate that the pI of the protein is basic i.e. comparable to that described by Bruns et al, (1980) for the rat CaBP which has a pI of 8,0. However, Ross et al, (unpublished) have determined the pI of the baboon CaBP to be 4,2.

One would therefore surmise that experimental errors might have caused this result to occur. However, the choice of the pH of the buffer used which was pH7,5 should have caused the protein to bind to the ion-exchange resin and be eluted later, thus leading one to think that the result reported by Ross et al, (unpublished) could be correct. The pH of buffer used for the chromatography experiment for a protein with a pI of 4,2 would possibly have had to have been more basic, i.e. possibly pH 8 - 9 instead of pH 7,5 used here.

An experiment was carried out using a pH 6,5 supernatant protein fraction on DEAE-A25 Sephadex ion-exchange chromatography. This procedure which was carried out as described Wasserman, (1979). The procedure was later published by Friedlander and Norman, (1980). In the results obtained from this experiment, a protein fraction was shown to be eluted as a single peak, confirming Friedlander and Norman's results, but the presence of EDTA in the buffer made it impossible to measure the presence of CaBP activity. Unfortunately no activity was evident even after dialysis. This anomaly could be explained by the presence of residual EDTA in the buffer which could have a higher Kd for calcium than

does CaBP for the $^{45}CaCl_2$ isotope in the assay. The reason for dialysis failing could have been that the membrane initially used in dialysis was not adequate thus allowing the CaBP to dialyse out because of too large a pore size for this low molecular weight protein. However, this experiment did not work, but the fraction in question was electrophoresed on non-denaturing as well as SDS polyacrylamide gel electrophoresis. The resulting protein fraction could have been passed through a Sephadex G-10 column in preference to dialysis to remove residual EDTA, but this was not done.

Non-denaturing electrophoresis on a 10% slab gel showed the presence of a single band of protein from the fraction eluted off a DEAE-A25 Sephadex column which did not correspond with any of the other bands from the other fractions electrophoresed. This phenomenon could have been due to the marked increase in the specific activity of this protein which was not visible in the crude fractions. This pH 6,5 supernatant protein fraction was then subjected to SDS gel electrophoresis which showed the existence of a number of protein staining bands. The 12,5% SDS result (not shown) indicated that this protein raction from DEAE-25 Sephadex gel chromatography was not pure. This SDS electrophoresis procedure, however, did not resolve the major protein component of low molecular weight from the dye front. Subsequent experiments using a modified SDS procedure on the protein fractions obtained from ion-exchange chromatography were successful, see Figure 11.

On the basis of the various purification procedures attempted, a protocol was chosen as described in Section 2.7 and the results therefrom reported in Section 3.1.8 in Tables 6 and 7. Having expressed the results obtained in two different ways, a CaBP protein was shown to be purified approximately 30-fold from the starting crude supernatant. The combination of heat precipitation, Sephadex G-100 gel chromatography and DEAE-Sephacel ion-exchange chromatography showed a significant purification of the protein. These results cannot be compared with those obtained by Gleason and Lankford, (1982) in their isolation of an intestinal CaBP from the baboon (Papio cyanocephalus), as no quantitative data was shown by these authors. It must be pointed out that the use of ammonium sulphate, Sephadex G-100 and DEAE-Sephacel ion-exchange chromatography in earlier experiments achieved an overall purification of 118-fold, whereas the chosen protocol achieved only a 30-fold purification. The 118-fold purification is close to that determined by Staun et al, (1984) in their purification of the CaBP from human kidney. Their analytical procedure, however, differed slightly at the end where chromatofocusing was used instead of ion-exchange chromatography. They achieved a 200-fold purification over starting material using this protocol. Further purification was achieved and the overall purification increased to 500-fold when they employed the method of immunoadsorbent chromatography.

It thus seems contradictory to have chosen the latter purification protocol in this dissertation. However, due to time constraints for repeat experiments as well as lengthly dialysis of ammonium sulphate fractions and reduced fractionation time using heat precipitation for 20 min instead of approximately 3 hours, led one to choose the latter protocol. The 3 step protocol employed here also used a single buffer system throughout which enabled one to complete the overall purification

in a very much shorter time. From the results shown in Table 4 and Tables 6 and 7, it is evident that ammonium sulphate purifies a fraction with high specific activity, and with low protein concentration.

Further evaluation of this purification protocol was carried out by subjecting the purified fractions to a modified SDS polyacrylamide gel electrophoresis procedure. The results showed the purification of two low molecular weight protein staining bands, one with R_f corresponding to cytochrome c and the smaller protein with an R_f less than cytochrome c with approximate molecular weight of 11 000 daltons. The purification effected from crude material through Sephadex G-100 gel chromatography was evident. The purification at this stage showed 2 protein bands and it was not evident in the sample wells containing the Sephadex G-100 sample as well as the samples from DEAE-Sephacel ion-exchange chromatography which was the CaBP. This result compares with results observed by Mellersh and Tomlinson, (1980) in which the authors observed 2 CaBPs, one migrating on SDS gel electrophoresis with a molecular weight of 11 000 daltons and a second migrating with a molecular weight of 9 000 daltons.

The 11 000 molecular weight protein in their work was only seen to be translated in presence of [^{35}S]-methionine. However, Fullmer and Wasserman (1975) showed in amino acid analysis, that the cow, pig, horse and guinea pig CaBPs contain no methionine residues. Although the wheat germ system in which Mellersh and Tomlinson, (1980) conducted their experiments was thought not to have any processing activity, it seems possible that the high-molecular weight form of CaBP (11 000 daltons) was synthesised and partially processed to a protein of lower molecular weight in this system. The partial processing although not quite clear

could be one of the reasons for the existence of the 12 500 dalton and 11 000 dalton proteins shown here on SDS polyacrylamide gel electrophoresis.

Desplan et al, (1983a) reported the existence of only one mRNA species coding for rat intestinal CaBP with a molecular weight of 7 500 daltons. This protein co-migrated with authentic CaBP as judged by immunoprecipitation with antiserum raised against rat intestinal CaBP and subsequent indentification by polyacrylamide gel electrophoresis. (Thomasset et al, 1981; Thomasset et al, 1982).

Studies by Thomasset et al, (1983) showed that mRNA extracted from rat duodenum, kidney and cerebellum was translated in a cell free reticulo lysate system in the presence of L-[^{35}S]-methionine. Vitamin D-dependent CaBPs were identified by immunoprecipitation using antibodies specific to duodenal CaBP (7500 daltons) and cerebellar CaBP (28 000 daltons) as well kidney CaBP (28 000 daltons). This data indicated that two distinct mRNAs coding for small and large vitamin D-dependent CaBPs were expressed in specific tissues in the rat.

All this evidence however, still fails to clarify the existence of the two CaBPs of law molecular weight reported here. However recent work carried out by Perret et al, (1985a) which followed on from research carried out by Desplan et al, (1983a) possibly provides an insight into clarifying the results reported here. Perret et al, (1985a) showed that cDNA - hybridised mRNA, isolated from rat duodenum, directed the cell-free synthesis of two proteins precipitable by antisera to the 9 000 dalton CaBP. The authors showed that a major protein comigrated with the 9 000 dalton CaBP whereas a minor product corresponded to a protein which was larger by 2 000 daltons (11 000 daltons). The minor protein appeared