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The Purification And Properties Of An Intestinal Calcium Binding Protein From The Baboon

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to result from read - through of the 'leaky' UGA stop signal. In my opinion this might provide some justification for the existence of the protein doublet as shown here on SDS polyacrylamide gel electrophoresis.

The deduced amino acid sequence for the 9 000 dalton CaBP contains the two 'EF hand' domains described by Meows and Kretsinger, (1975) corresponding to calcium binding sites I and II described by Desplan et al, (1983b). The homology obtained between the site I structure and the sequence located just before and after the first TGA stop codon suggests, after com arison with the structure of other intracellular CaBPs described by Kretsinger (1976) and Meows and Kretsinger, (1975), that the rat 9 000 dalton CaBP mRNA contains the remains of an untranslated structure calcium binding site III. Thus in the rat, the 9 000 dalton CaBP could be the result of early termination of the translation of a vitamin D-induced larger molecule containing four calcium binding sites such as the 28 000 dalton CaBP found in rat kidney and cerebellum. The 9 000 dalton CaBP has been shown to contain only two binding domains.

This evidence could also possibly be used as a guide to determine the existence of the large and small proteins shown here on SDS polyarylamide gel electrophoresis. However no data is available for the baboon to suggest that the small molecular weight proteins result from early termination of translation of a larger CaBP. The data presented by Perret etal, (1985b) might explain the results shown by Davie, (1981) where he showed the formation of a 12-13 000 dalton fraction which he described as being associated with a decline in the 28 000 dalton CaBP fraction. The 12-13 000 dalton fraction also contained two distinct CaBPs.

The suggestion of early termination of translation resulting in a small CaBP instead of a larger one could very well explain the results shown by Alpers et al, (1972) where the authors indentified two CaBPs from human small intestine, one slightly larger than the other with molecular a weight of approximately 20 000 daltons. Delorme et al, (1983) also reported evidence for the presence of two vitamin D-dependent CaBPs in the mouse kidney. One of these had a molecular weight of 25 000 daltons and the other a molecular weight of 10 000 daltons. Both proteins were shown to be vitamin D-dependent and were distinct from each other with respect to their pI values. Alpers et al, (1980) showed the presence of two vitamin D-dependent CaBPs from intestinal mucosa of dogs. It was again demonstrated that there existed a large 57 000 dalton protein as well as a smaller 19 000 dalton protein. It was however stated that these proteins eminated from different parts of the GIT. The new evidence by Perret et al, (1985b) could possibly explain the existence of the multiple proteins in different tissues of different mammalian species. Thus, the recent evidence described in the area of molecular biology could well answer the many unanswered questions as to whether one or two CaBPs exist in specific tissues, in this case the baboon intestine.

In a recent publication by Perret et al, (1985b), their results demonstrated that the same CaBP mRNA species is expressed throughout the digestive tract of the rat. There were however significant differences in the expression of the gene for the 9 000 dalton CaBP and in its regulation by 1,25 dihydroxycholecalciferol from one segment to another. The highest concentration of CaBP mRNA occurred in the duodenum, proximal jejunum and caecum. Lower levels of CaBP mRNA were shown to exist in the ileum. (Taylor et al, 1984). The evidence of the different concentration of CaBP clarifies the evidence by Wasserman et al, (1968)

where the concentration of the CaBP was shown to decrease in the order of duodenum >jejunum >ileum in the chick. It is interesting at this stage to point out that in the chick, no reports of small and large CaBPs have been reported. This could have evolutionary significance as will be discussed later.

Two smaller molecular weight component shown here on the 12,5% SDS gel migrated to a R_f less than the 6 000 daltons. This protein doublet could well have resulted from protease digestion. It has also been shown by Bryant and Andrews, (1983) that when Ca-liganded proteins were stored at - 10°C in solution for long period (up to 6 months) and thawed, the presence of an additional two faint protein staining bands in electrophoresis were seen, whereas initially there had only been one band. When CaBPs were stored at 4°C however, and then thawed, no degradation was shown to be evident. Such changes in storage are also well documented for the bovine CaBP (Fullmer and Wasserman, 1981). These could be the reasons for the small protein doublet with R_f less than 6 000 daltons. As was quoted in the methods and results section, the 12,5% SDS gel contained a low percentage of glycerol in the separating gel which increased the resolving power of the gel. The glycerol possibly decreases the amount of free water space in the separating gel which limits the space in which the protein can migrate thus enabling the protein to be resolved from the dye front.

Purified baboon CaBP fractions from Sephadex G-100 gel chromatography were also assessed by a novel method of counter-ion electrophoresis. This method of electrophoresis was developed by Ueng and Bronner, (1979) and was used to detect the presence of the rat intestinal CaBP in eluted fractions. This technique which has only been reported once in the literature by these researchers proved to be successful in identifying

the presence of the baboon CaBP as well as highlighting the vitamin D-dependency of this CaBP.

The molecular weight of the intestinal CaBP was measured by three different methods namely Sephadex gel chromatography, SDS polyacrylamide gel electrophoresis and HPLC analysis. None of the methods yielded an accurate value because of the limitations of the separation techniques used. The molecular weight of protein determined by Sephadex G-100 gel chromatography was found to be approximately 11 900 daltons. This value is an approximation because the CaBP protein eluted was shown to be smaller than the smallest molecular weight marker used on the calibration curve, namely, cytochrome c. The molecular weight of the intestinal CaBP protein was also determined by Sephadex G-75 gel chromatography. A protein of molecular weight of approximately 11 220 daltons was determined. Clearly, for accurate measurement of the molecular weight of the CaBP, lower molecular weight standards should have been used such as aprotinin or insulin. The molecular weight of the baboon CaBP determined by Sephadex gel chromatography in the studies here are in approximate agreement with results shown by Fullmer and Wasserman, (1975) for five mammalian intestinal CaBPs, as well as results reported for the rat by Gleason and Lankford, (1981).

An anomalous chromatographic protein profile shown in Figure 13 was compared to numerous other experiments using Sephadex G-100. In Figure 13 a CaBP peak is eluted very close to a large protein peak of approximately 20 000 daltons (Ve/Vo = 1,80). Although the CaBP peak was eluted in the same place in all subsequent experiments, the major protein component eluted much earlier from these columns. This anomalous result was not observed in any of the subsequent chromatographic separations. The reason for this anomalous result could have been due to proteolytic

degradation and/or disaggregation of the protein, causing it to be eluted later with a smaller molecular weight as shown.

The molecular weight of the CaBP was also determined by SDS gel electrophoresis. The estimated molecular weights of the two bands shown in Figure 11 were between 11 - 12 500 daltons. The graph of R_f versus molecular weight showed that the region between cytochrome c (12 500 daltons) and aprotinin (6 000 daltons) was non-linear thus this again is an estimation only. The approximate molecular weight of 11-12 500 CaBP determined by SDS electrophoresis is similar to other mammalian intestinal CaBPs described and discussed earlier in this dissertation. HPLC was also used as a technique to determine the molecular weight of the baboon intestinal CaBP. The method of the HPLC has to date been used to identify the amino acid composition of the CaBP (Fullmer and Wasserman, 1979) and very recently was used after tryptic digestion of rat skin extract to determine the composition of the rat skin CaBP. HPLC has yet however to be used in the determination of the molecular weight of the intact active molecules (MacManus et al, 1985). The protein columns used in this study had limitations in that the low molecular marker proteins could not be resolved, thus detracting from an accurate assessement of the molecular weight. A purified protein was eluted which had no biological activity, thus the merits of this procedure are in doubt.

Experiments involving isolation and partial purification of the intestial CaBP from baboons on different diets demonstrated the vitamin D-dependancy of the CaBP. The animals on the vitamin D-deficient diets (groups I and II) for fourteen months, showed biochemical, radiological and histological signs of rickets (Pettifor et al, 1981).

Mucosal extracts from groups I, II, III and V were treated as described in Section 2.1, 2.1.1 and 2.3.2. The CaBP activity was evident in the intestinal mucosa of three of the four groups of animals. The most important result obtained from these dietary experiments was the absence of CaBP activity in the Group II animals when compared to the Group V or control animals. This result thus provides evidence for the vitamin D-dependency of the the baboon intestinal protein. Although the results indicate increased CaBP activity in Group III mucosal extracts after Sephadex G-100 gel chromatography, a comparison of the ratios of the CaBP activity to absorbance at 280mm for Group V and Group III animals showed no real difference for the two groups as shown in Table 8. The increased CaBP activity in the extracts from Group III animals could have significant, as a diet low in calcium is supposed to stimulate production of $1,25(OH)_2D_3$ which leads to translation of CaBP. The results reported here unfortunately did not highlight this. CaBP activity shown for the Group I animals on vitamin D-deficient diets plus phosphate is an interesting result. Phosphate has been reported to stimulate production of $1,25(OH)_2D_3$ via renal Vitamin D hydroxylase and thus the small amount of CaBP is translated. (Norman, 1979; Ross and Henry, 1979; Fraser, 1980; Norman et al, 1982).

The method of counter-ion electrophoresis was used to confirm the presence or absence of the CaBP. The results confirmed the previous finding using Sephadex G-100 chromatography. The presence of CaBP activity for groups I, III and V and the absence of binding for group II was clearly shown. This result thus confirms the vitamin D-dependency of the protein.

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The baboon intestinal CaBP has also been isolated by Gleason and Lankford (1982). Their study showed that the baboon intestinal protein had similar electrophoretic behaviour, when compared to CaBPs isolated from other mammals excepting for the rat. The study however failed to establish both the molecular weight and the vitamin D-dependency of the protein.

The data thus far reported in this dissertation indicate that the baboon intestinal CaBP, like those described for many other animals, contains at at least one or maybe two low molecular weight CaBPs which is/are vitamin D-dependent. The baboon CaBP was also found by Ross et al, (unpublished) to have a pI of 4,2 which would seem to compare quantitatively with the data described for the mouse 10 000 dalton intestinal CaBP. These results constitute the first definitive report on both molecular weight and vitamin D-dependency of an intestinal CaBP from a higher primate.

Throughout the course of this research work certain problems were experienced with the methodology. Initially, data obtained by the Chelex-assay and Biuret assays were inconsistent and the possible presence of ammonium sulphate contamination was considered. It was thus decided to investigate the anomalies in these two important assays. The effects of ammonium sulphate on these assays were subsequently studied.

In the first experiment in which increasing concentrations of ammonium sulphate caused decreases in the percentage of the total counts measured in the absence of CaBP, the inhibition was caused by the ammonium sulphate in the system. The ammonium sulphate of higher concentrations might have caused quenching to occur, hence the decreased

counts. In the second experiment increasing concentrations of ammonium sulphate could have displaced $^{45}CaCl_2$ from the CaBP thus producing a decrease in the counts observed. This led to an underestimation of CaBP activity in the presence of a final ammonium sulphate concentration of ammonium sulphate. The decrease in counts which was maximal at an ammonium sulphate concentration of about 80% could also have occurred due to the precipitation of some of the CaBP.

The large increase in counts of $^{45}CaCl_2$ observed in the supernatant of the background tubes could be explained in that the increasing concentration of ammonium sulphate inhibits the binding of $^{45}CaCl_2$ with the Chelex-resin. The reason for the subsequent decrease in the counts after an initial large increase cannot be explained. The experiments thus showed the need for all fractions containing ammonium sulphate to be thoroughly dialysed before assessing CaBP activity.

A similar set of experiments was also carried out to determine the effect of increasing concentrations of ammonium sulphate on the Biuret assay. It was shown that in the Biuret assay increasing concentrations of ammonium sulphate in the presence of varying amounts of standard protein BSA (10mg/ml) caused changes in absorbance readings and consequently decreases in the measured protein concentrations. This was however not true for all protein concentrations and was most significant in the case where equal quantities of protein and ammonium sulphate were used. The decrease in protein content from 4mg/ml to 2mg/ml represents a 50% decrease. This occurred at a final ammonium sulphate concentration of between 75 and 99%. The reason for this phenomenon as well as the other results cannot be explained. All protein samples were subsequently thoroughly dialysed before being analysed by the Biuret Assay.

Finally, an experiment to examine the effects of using buffers containing EDTA and $CaCl_2$ on the Chelex assay were carried out. The data seemed to indicate that EDTA has a higher binding affinity for Ca^{++} than Chelex-resin, as the results indicated that nearly 50% of counts remained in supernatant in the presence of EDTA. In contrast, the buffer containing $CaCl_2$ merely caused changes in the specific activity of the isotope and showed no significant effect on the counts remaining in the supernatant. The presence of the $CaCl_2$ in the buffer thus slightly decreases the capacity of the Chelex-resin for binding $^{45}CaCL_2$ thus giving a slightly higher result than that shown for the background counts.

Four methods of protein determinations were used. The reason for using the three major assays was to find the method with the least variation and the one with the highest degree of accuracy. The Bio-Rad assay is much quicker than the Lowry assay (5 min as apposed to 30-40 min) and only requires one reagent compared to three. The Bio-Rad assay is also free from most of the chemical interferences which limit the Lowry assay and the Biuret assay. This last point is especially true for interference of ammonium sulphate and EDTA. The Biuret, Lowry and Bio-Rad assays were however used for protein determination in this work, with the last two showing fewer inconsistencies. The absorbance method was used for quick determination of protein content during chromatographic experiments.

In summary, although the baboon intestinal CaBP has been only partially purified, the new data established will substantiate the evidence already reported for the low molecular weight CaBPs in mammals. The presence of a doublet of low molecular weight clearly needs further investigation. The molecular weight of the intestinal CaBP reported here

agrees well with those reported for the guinea pig, cow, pig, horse, mouse and rat. The vitamin D-dependency of the CaBP has been shown in a number of animals including the calf, mouse, rat and dog but has not been shown for the baboon intestinal CaBP.

The dissertation has shown that there are still many areas that need clarification in regard to the physiological, biochemical characterisation and vitamin D-dependency of this family of proteins under study. Although not discussed or researched here, the functional role that the intestinal CaBP plays in the area of molecular biology and its relationship to role CaBP plays in calcium transport needs more investigation. From the information discussed in this dissertation, some interesting patterns seem to have emerged, although more data would be required to verify these. It appears that the avian species and those lower on the evolutionary scale the amphibians, have intestinal CaBPs of molecular weights of about 28 000 daltons. The prominent mammalian intestinal CaBPs thus far studied have molecular weights of about 11 000 daltons, i.e. less than one half of their avian counterparts. The molecular size of the kidney CaBP does not seem to vary with species. Molecular weights similar to the size reported for the chick CaPB are the rat and human CaBPs.

Thus, the indication is that the intestinal CaBP seems to have been modified during evolution, yielding a protein of less than one half the size of the chick protein. In the kidney, either the 28 000 dalton type persisted from avian to mammalian species or the 11 000 dalton intestinal

type evolved. Recent evidence by Perret et al, (1985a) revealed the absence of cross-hybridisation between intestinal CaBPs and kidney and cerebellar forms. This evidence clearly indicates the lack of homology between CaBPs from different tissues. Thus, the idea of early termination of translation of a larger protein resulting in the appearance of a smaller protein might possibly only occur if the CaBPs originate from the same tissue type. The reason for the apparent difference in the nature of the kidney protein during evolution has until now not been addressed. It also seems that antisera produced against the different mammalian intestinal CaBPs are not species specific as demonstrated by Rhoten et al, (1984) for the green anole and the rat kidney.

If this pattern of antigenic reactivity and molecular size holds as more information becomes available, it would suggest that the larger CaBP represents a more primitive type of intestinal protein whereas the more highly evolved smaller variety is capable of functioning at one half to one third of its molecular size. Research by Perret et al, (1985a) showed that the lack of homology is not consistent with the previously reported model for the evolution of intracellular CaBPs by Desplan et al, (1983b). The 9 000 dalton CaBP could have arisen as the result of transcription of a larger molecule containing 4 calcium binding sites, with subsequent gene duplication of this sequence. But to be consistent with the observed lack of cross-hybridisation this event must have occurred early in their evolutionary development, since early time they have diverged independently. The addition of the two bases at positions 216 and 236, required to maximize the homology between site-I and site-III- like structure described by Desplan et al, (1983b), supports such a divergence. A fortiori mRNAs of other members of the intracellular CaBP family, such as calmodulin and troponin C (Means and

Kretsinger, 1975 ; Kretsinger, 1976), could not hybridise since their evolutionary divergence probably began even earlier than that of the 28 000 dalton and 9 000 dalton CaBPs.

The existence of a large primitive and a small highly evolved CaBP, however, would be difficult to explain when one considers that the human CaBPs so far isolated and characterised exist in both the small as well as the large molecular weight forms in the same tissues. Man in comparison to amphibian, avian and lower mammalian species is highly evolved and therefore retention of the high and low molecular weight proteins could be due to adaptation through the evolutionary cycle.

In summary, although the baboon intestinal CaBP has only been partially purified, the new data established with regard to the vitamin D dependency as well as ideas on the evolutionary development would add to the vast knowledge already in existence for these CaBPs. The new information generated by this research will hopefully serve to clarify some of the unsubstantiated data existing on the molecular size, number of proteins in a particular tissue as well as the vitamin D-dependency in higher mammals such as primates, with special reference to man.

CHAPTER 5

APPENDIX I

The protein curves shown below are representative of typical assays performed during experimentation

TABLE 12: STANDARD BIURET ASSAY

TUBE NO	STANDARD SOLUTION(ml)	Av.Abs.	Conc. (mg/ml.)
1	0,8	0,560	8
2	0,6	0,465	6
3	0,5	0,375	5
4	0,4	0,320	4
5	0,2	0,160	2
6	0	0	0

FIGURE 29 shows typical standard Biuret curve. Standard protein concentration of BSA (10mg/ml) was used.

TABLE 13: STANDARD LOWRY ASSAY

TUBE NO	STANDARD SOLUTION (ml)	Av. Abs.	Conc. (µg/0,5ml)
1	0,0	0,0	0
2	0,005	0,075	10
3	0,01	0,130	20
4	0,015	0,195	30
5	0,02	0,250	40

FIGURE 30 Shows typical standard Lowry curve. Standard protein concentration of BSA (1,0 mg/ml) was used.

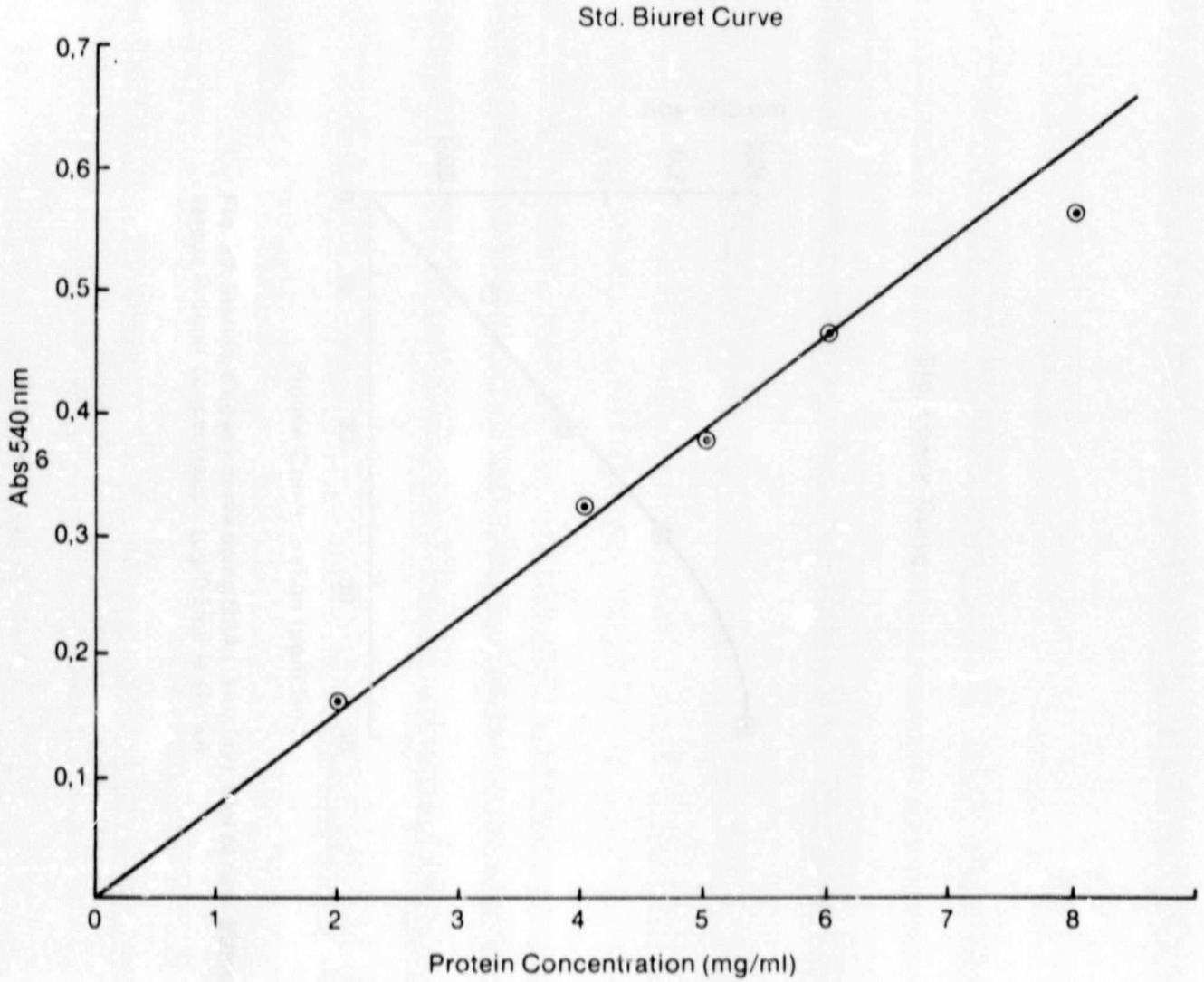


Fig. 29: Standard Biuret curve using BSA (10mg/ml). Plot of Abs. 540nm versus Protein concentration (mg/ml) is shown.

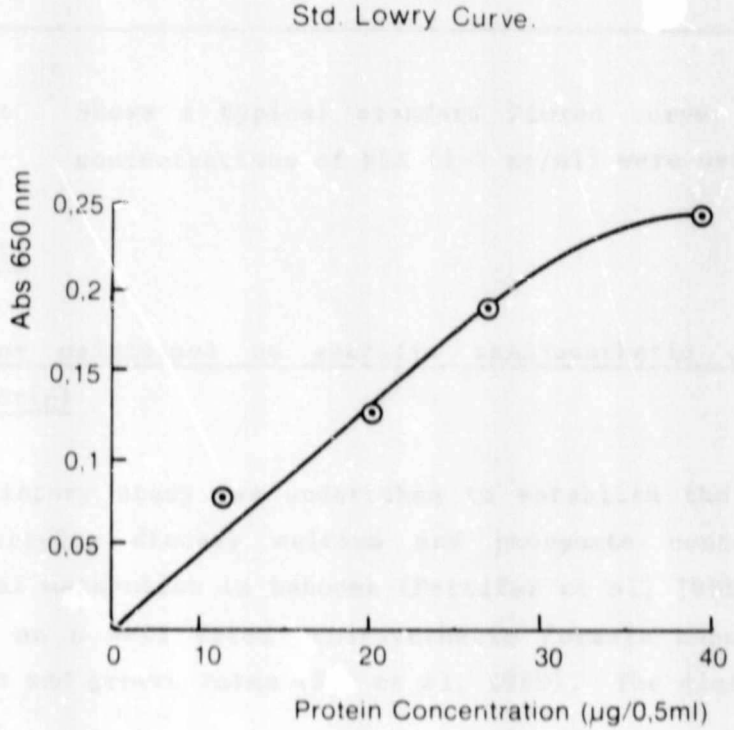


Fig. 30: Standard Lowry curve using BSA (1mg/ml). Plot of Abs. 650nm versus Protein concentration (µg/0,5ml) is shown.

TABLE 14: STANDARD BIORAD ASSAY

TUBE NO	STANDARD SOLUTION (ml)	Av. Abs.	Conc.(µg)
1	0,0	0,0	
2	0,1	0,117	100
3	0,1	0,196	200
4	0,1	0,288	300
5		0,396	400
6		0,479	500

FIGURE 31: Shows a typical standard Biorad curve. Standard protein concentrations of BSA (1-5 mg/ml) were used.

APPENDIX II

a) Baboons maintained on specific semi-synthetic diets at C.S.I.R. (Pretoria)

The dietary study was undertaken to establish the effects of cereal and varying dietary calcium and phosphate contents on bone and mineral metabolism in baboons (Pettifor et al, 1981). The diets were based on a well tried semi-synthetic formula known to sustain good health and growth rates (Sly et al, 1980). The diet consisted of;

15% casein
10% sugar
57,3% dextrin
10% sunflower-seed oil
4% vitamin mixture
3,7% mineral mixture

In the dietary experiments six groups of baboons were used. Each group comprised of seven animals aged between 6 and 12 months. All the baboons were males, with the exception of two animals in group 3 which were reported to be female. The average mass of the animals ranged from 4 - 8 Kg.

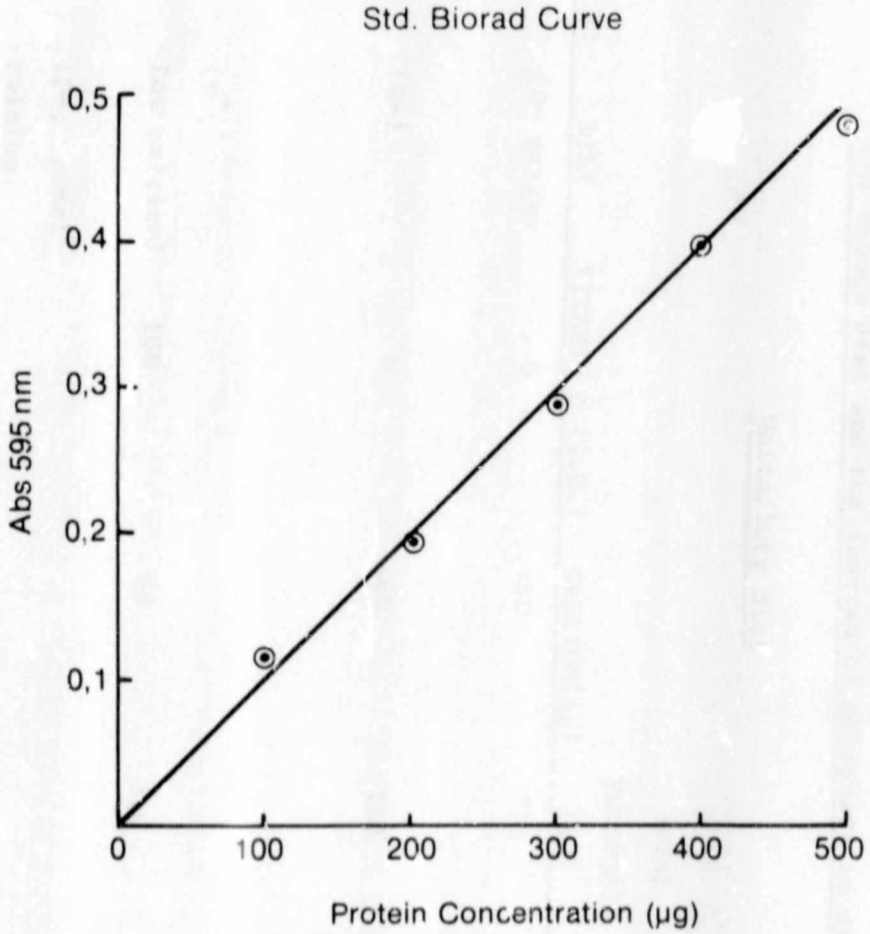


Fig. 31: Standard Biorad curve using BSA (1-5mg/ml). Plot of Abs. 595nm versus Protein concentration (µg) is shown.

For the information required in this dissertation, only animals representative of groups I, II, III and V were used. The groups IV and VI dietary regimens were not of importance and therefore results and diets not quoted here.

All the baboons were housed indoors in specifically designed animal houses. The only source of light was incandescent electric light. The mineral content of the four groups of baboons' diets is represented in Table 15 below.

TABLE 15: The calculated calcium, phosphate and vitamin D content of the baboon diet and the sources of phosphate in diet.

Units/100g diet

GROUP NO	DIET	VITAMIN D (I.U.)	CALCIUM(mg)	TOTAL PHOSPHATE (mg)	PHOSPHATE SOURCE
I	(D- maize)	0	400	310	Casein + maize
II	(D-)	0	400	310	Casein + inorganic salt
III	(D^+, Low calcium)	100	40	310	"
V	(D+, normal calcium control)	100	400	310	"

All animals were sacrificed at the end of the 18 month experiment.

b) Baboons maintained on specific diets at Department of Surgery, University of the Witwatersrand Medical School

All the baboons were maintained on a commercially prepared diet. The baboons used were made available by the Department of Surgery for both transplantation and CaBP studies.

CHAPTER 6

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