and this indicates its similarity to CaBPs from most other species, excluding the rat.

The tissue distribution of the human CaBP has been studied by a number of researchers. A partially purified CaBP was isolated from the human kidney and duodenum with the former showing a molecular weight of 22 000 daltons (Piazolo et al, 1971). The human duodenal and kidney CaBPs migrated equally as rapidly on non-denaturing polyacrylamide gel electrophoresis. This study provided no evidence that the calcium reabsorption in the kidney was under the influence of vitamin D. The calcium binding activity was increased 36-fold over that of the starting material having used heat treatment at 65°C for 15 min , Sephadex G-75/100 gel chromatography and DEAE-cellulose ioxchange chromatography.

A partially purified CaBP was isolated from human intestinal mucosa by Menczel et al, (1971). Electrophoresis showed the presence of at least two bands containing calcium binding activity, with the faster moving band having the major portion of the CaBP activity. The concentration of the intestinal CaBP in the duodenum was found to be five times that in the jejunum. The presence of another two CaBPs was found in the human kidney, one showing a molecular weight of 70 000 daltons and the other of 27 700 (Morrissey and Rath, 1974). The 27 000 molecular weight protein showed as a single band on non-denaturing acrylamide gels and had a specific calcium binding activity 184-fold greater than that of human albumin.

Alpers et al, (1972) showed that there are at least two separate proteins with calcium binding activity in the human duodenum. One of these proteins was shown to have a molecular weight of 20 000 daltons as

measured by non-denaturing polyacrylamide gel electrophoresis. The protein moved rapidly on non-denaturing electrophoresis and displayed CaBP activity. A second slower moving CaBP was also shown on electrophoresis, but the study did not provide any evidence for the vitamin D-dependency of either protein.

Davie, (1981) investigated calcium binding activity in human small intestinal mucosal cytosol. Binding activity was detected in fractions with molecular weights at 28 000 daltons and 900 000 daltons. The appearance of a CaBP at a molecular weight of 12 - 13 000 daltons was associated with a decline in the 28 000 molecular weight fraction due to proteolysis. The 12 - 13 000 dalton fraction contained at least two distinct CaBPs, one of which showed similar properties to the pig protein described by Hitchman and Harrison (1972). Antiserum to this human protein reacted against the 28 000 dalton fraction from cytosol of human small intestinal mucosa and human kidney.

Staun et al, (1984), purified a CaBP from the human kidney. The protein was shown to have a molecular weight of 26 000 daltons on SDS gel electrophoresis and 28 000 daltons by gel filtration. These results are comparable to those obtained for chick intestine, kidney, brain and pancreas, rat kidney and also human renal CaBP. Two purification procedures were used, one in which heat precipitation at 60^{o}C for 15 min followed by gel filtration using Sephadex G-100 and chromatofocusing showed a 200-fold purification over starting material. The second procedure using immunoadsorbant chromatography gave a 500-fold purification over starting material.

A number of studies have been conducted to elucidate some other biochemical properties of mammalian CaBPs. Delorme et al, (1982) compared the net charge of mouse and rat intestinal CaBPs. Although they showed similar molecular weights of 10 000 daltons, their pI values differed significantly. The pI of the rat intestinal CaBP was 8,0 and that of the mouse 4,9. Accordingly the mouse form displayed more anodal migration on gel electrophoresis. The mouse and rat proteins exhibited only partial immunochemical similarity although their amino acid compositions were very similar. In a more recent study by Delorme et al, (1983) on the mouse kidney, two CaBPs were isolated, one with pI of 5,9 and the second with a pI of 4,9. The former protein had a molecular weight of 25 000 daltons and the latter 10 000 daltons which incidently was immunologically identical to the mouse 10 000 dalton CaBP. In another study, Oldham et al, (1980) biochemically characterised the intestinal as well as the parathyroid CaBPs in the dog. The intestinal protein was found to be more acidic in nature having an apparent pI of 4.4 compared with a pI of 5,15 for the parathyroid protein. Finally, a study on the human kidney by Staun et al, (1984), revealed that the protein eluted from gel filtration columns had a pI of 4,5 when subjected to chromatofocusing.

The bovine intestinal CaBP has been studied by the technique of X-ray crystallography at a resolution of 2.3Å. Two calcium binding sites were evident, but one calcium binding domain was larger and had a rearranged loop (Szebenyi et al, 1981). This result has implications for structural predictions of other CaBPs comprising the same homologous family such as calmodulin, troponin C, parvalbumin, S-100 and myosin light chain.

In a study on CaBPs by Bryant and Andrews (1983), the authors characterised CaBPs from pig, sheep, rabbit and cow by their amino acid compositions. The sheep protein was very similar in composition to the bovine protein, and the protein from the pig mucosa had essentially the same composition as that studied by Hofmann et al, (1979). All the proteins contained two tightly bound calcium ions per molecule, a property now considered to be common in mammalian CaBPs. The labile nature of the pig calcium-free protein was demonstrated by the formation of a form which bound only one calcium ion (Bryant and Andrews, 1983).

Studies on normal porcine parathyriod glands by Oldham et al, (1974) indicated that preliminary amino acid analysis of the CaBP showed lysine and aspartic acid to be the predominant amino acids. The calcium binding constant was found to be $9,3 \times 10^{5} M^{-1}$ and there appeared to be two high affinity binding sites for calcium per molecule of protein.

The functional physiology of the mammalian calcium binding protein has been studied in some detail. In <u>in vitro</u> studies of calcium transport in the intestine of the rat and golden hamster by Schachter, (1970), it was indicated that in both species, the level of intestinal CaBP was greater in the duodenum than in the ileum. The levels of CaBP activity were found to be directly related to the degree of calcium transport in the intestine of the golden hamster. However, Kalifelz and Wasserman, (1972) showed the reverse. <u>In vivo</u>, blood vessels penetrate the level of the lamina propira and thus calcium has only to move across mucosal cells and the basal cells before becoming available for removal from the intestine by blood. <u>In vitro</u> calcium moves across mucosa, submucosa, muscularis and serosa before it enters the serosal fluid.

Harrison et al, (1975 a,b) using pigs, investigated the relationship between the concentrations of intestinal CaBP and the presence of rickets. Pigs were made rachitic by diets deficient in both calcium and vitamin D. The experiments demonstrated a poor inverse correlation between the levels of intestinal CaBP and rickets. This paper demonstrated that there was still some question as to the vitamin D-dependency of the pig protein. From the results, it may be theorised that actual residual stocks of vitamin D were not sufficient to prevent rickets under the extra stress of calcium deficiency in the diet. An important point is that the presence of intestinal CaBP cannot be taken as evidence of sufficient quantity of vitamin D (or metabolites) at the gene level to prevent rickets in the pig. It is possible that other factors such as dietary phosphate may stimulate or inhibit CaBP synthesis in addition to vitamin D-metabolites. Similarly, it is possible that factors other than vitamin D-metabolites are active in providing or preventing rickets. It is possible that active metabolites of vitamin D may not act simultaneously on gastrointestinal muscosal tissue and on bone.

A study on the vitamin D-dependent CaBP changes during gestation in pre and post-natal developing rats was carried out by Delorme et al, (1979). During the perinatal period, calcium metabolism is stressed. As intestinal CaBP was considered to be influenced by $1,25(OH)_2D_3$, the measurement of calcium binding a[illegible], with the appearance of CaBP as well, would indicate the role [illegible] D played during this period. The authors reported the variations in CaBP concentrations in the rat during the last 5 days of gestation. These were determined in the maternal duodenum, placenta, foetal membranes and foetal intestine. The CaBP changes were also followed from birth until weaning. The appearance

of the maternal intestinal CaBP which increased on day 19,5 of gestation was consistent with that of calcium intestinal absorption and may be explained by increased $1,25(OH)_2D_3$ production. The placental CaBP rose from day 17,5 until the end of the gestation and may have been related to the flux of calcium from mother to foetus. The placental CaBP was predominantly found in the foetal part of the organ where materno-foetal exchanges occur. The yolk sac was claimed to have synthesised substantial amounts of CaBP. In the foetal membranes, CaBP plateaued from day 17,5 to day 20,5 and decreased on day 21,5. The presence of the CaBP in the foetal placenta and yolk sac suggested that extra stress of calcium deficiency in the diet. An important point is that the presence of intestinal CaBP cannot be taken as evidence of sufficient quantity of vitamin D (or metabolites) at the gene level to prevent rickets in the pig. It is possible that other factors such as dietary phosphate may stimulate or inhibit CaBP synthesis in addition to vitamin D-metabolites. Similarly, it is possible that factors other than vitamin D-metabolites are active in producing or preventing rickets. It is possible that active metabolites of vitamin D may not act simultaneously on gastrointestinal mucosal tissue and on bone, these two tissues might be targets for vitamin D.

In the foetus, intestinal CaBP was detected as early as day 17,5 of gestation and increased markedly during the last day of gestation. From birth, and during the first three weeks of postnatal life, the intestinal CaBP remained unchanged. It did, however, undergo a sharp rise just prior to weaning. The specific distribution of CaBP along the intestine was acquired during intra-uterine life and did not change with either suckling or weaning. The two main changes in intestinal CaBP observed, just before birth and at weaning, may have reflected intestinal maturation and/or variations in vitamin D metabolism.

Thomasset et al, (1979) studied the effect of vitamin D and calcium deficiency on duodenal, jejunal and ileal CaBP and on the plasma calcium and 25-$(OH)D_3$ levels in the growing pig. In vitamin D-deficient pigs, the amount of intestinal CaBP decreased by 33% in proximal duodena and 70% in middle duodenum. It was unmeasurable in the distal ileum. Plasma calcium levels declined significantly and plasma 25-$(OH)D_3$ levels were undetectable. The pigs adapted to the calcium deficient diet by considerably increasing jejunal and ileal CaBP. Thus the study suggested that a low calcum diet promotes CaBP synthesis. This implies that the ileum was more sensitive than the duodenum to the action of $1,25(OH)_2D$. The ileum is not usually considered as an essential target tissue for vitamin D. In this study calcium deficiency was associated with a drop in plasma 25-$(OH)D_3$ levels. This decrease may have been due to a more rapid conversion of 25-$(OH)D_3$ into $1,25(OH)_2D_3$. This phenomenon of increased $1,25(OH)_2D_3$ due to increased 25-$(OH)D_3$-1-hydroxylase activity, has been reported by Boyle et al, (1971) and Hughes et al, (1975) in calcium deficient rats.

Bruns et al, (1982) described the regulation of CaBP in the intestine and placenta of mice. The researchers altered the animals' mineral metabolism by feeding pregnant mice high quantities of calcium and strontium. The high calcium diets decreased CaBP content in both the maternal placenta and intestine by 58% and 45% respectively. In the strontium fed mice, CaBP was reduced by 67% and 60% in the placenta and intestine respectively. Foetal mineral accumulation was also markedly reduced by some 70%. Administration of $1,25(OH)_2D_3$ to strontium fed mice allowed for an increase in CaBP. However, with similar treatment placental CaBP was unaffected. A similar pattern was observed with calcium-fed mice. The administration of $1,25(OH)_2D_3$ to mothers in

amounts supplied to give an intestina response was not sufficient to increase placental production of CaBP.

A number of studies on the cellular and subcellular localisation of the mammalian CaBP have been carried out. Lippiello, (1974) observed CaBP to be predominantly associated with the distal tubular cells in both bovine and chick kidney using species specific antisera. The pancreatic islets of rat, cat, dog, mouse and chick, yielded positive antibody reaction, indicating the presence of CaBP (Morrissey et al, 1975). The distribution of the peroxidase end product suggested that CaBP was located intracellularly in the B-cells.

Studies on the localisation of the human intestinal CaBP were carried out using immunohistochemical techniques (Morrissey, 1975). The study utilised antisera prepared against CaBP isolated from the human kidney. A non-specific fluorescence was noted in goblet cells. Morrissey et al, (1975) using peroxidase conjugated antisera, reported the presence of CaBP in the intracellular spaces of the human jejunum. The authors noted that 25(OH)vitamin D administered to rachitic children restored the normal pattern of fluorescence as seen in healthy persons. The protein appeared to be associated with the lateral and basal membranes with none or little at the absorptive surface.

The most recent studies have extended the number of species examined and provided the first ultrastructural localisation of CaBP employing anti pig intestinal CaBP antiserum. Lippiello, (1974) observed the CaBP to be predominantly associated with the distal tubular cells in bovine and chick kidney using special specific antisera. CaBP was located in pig intestine in the cytoplasm of absorptive cells and inconsistently at

absorptive surface, but not in the goblet cells (Arnold et al, 1976). Using the same technique as Morrissey et al, (1975) a study on the kidney tissues isolated from the monkey, dog, cat, mouse, man and the chick using antisera prepared against CaBP isolated from human kidney, was carried out. CaBP was found to be associated with intracellular membranes, or cytosol of cells in both proximal and distal convoluted tubules, straight segments and in the thin loops in the papilla but not in the cells of the glomerulus.

Marche et al, (1978) studied the cellular localisation of the vitamin D-dependent CaBP in the duodenum of the rat. The use of indirect immunofluorescence and immunoperoxidase methods, indicated the presence of a CaBP which was exclusively detected in the villus part of the duodenal mucosa. Moreover, the CaBP was detected mainly in the supranuclear region of the cytoplasm of the absorptive cells and also at the level of the basal laminae. The CaBP was not demonstrated either in the nuclei or associated with the brush border membrane of absorptive cells. The CaBP was detected neither in the goblet cells nor in subepithelial layers. The results agreed well with the view that the CaBP may act as an intracellular buffer by protecting the cell against too high calcium concentrations.

The same authors (Marche et al, 1980) studied the rat intestinal CaBP in relation to the age of the cell in isolated epithelial cell populations from rat duodenum. They attempted to correlate CaBP with thymidine kinase activity. CaBP distribution along the length of the villus appeared as a gradient, increasing from the crypt to the tips of the villus. The CaBP concentrations in cells were shown to be negatively correlated with thymidine kinase activity. This indicated that the CaBP

was absent in the crypt cells and appeared in differentiated cells with the development of the brush border. Thus it was suggested that CaBP, like alkaline phosphatase, could be considered as an indicator of enterocyte maturation.

Studies, although limited at this stage, have been carried out on the molecular biology of the mammalian CaBP. Mellersh and Tomlinson, (1980), showed evidence for two molecular weight forms of pig intestinal CaBP. They extracted and translated pig duodenal mRNA in wheat germ cell-free systems. Total nucleic acids were extracted from duodenal mucosa and fractionated by affinity chromatography on oligo (dT) - cellulose. The poly adenylated mRNA obtained in this way, was less than 1% of the total nucleic acids. In the wheat germ cell free translation system it stimulated incorporation of [^{3}H] - leucine into protein 8 - 10-fold over endogenous protein synthesis. The translated [^{3}H] - leucine containing proteins were immunoprecipitated with a double antibody technique and found to contain 5% of total protein as specifically-precipitated authentic CaBP. On SDS, immunoprecipitated protein comigrated with pure CaBP (9 000 daltons). Like CaBP, this translated protein was heat stable and had the same pI as the pure protein. Like mature CaBP, itself did not contain methionine. (Fullmer and Wasserman, 1975). However, when only [^{35}S]- methionine was used in translation, a protein of 11 000 daltons was found to be immunoprecipitated with antisera raised against pure pig CaBP. The immunoprecipitated protein was found to be 5% of total protein synthesised and on SDS gel electrophoresis comigrated with a faint higher molecular weight band. Methionine is used to initiate protein synthesis in all prokaryotes. The absence of this amino acid in pig CaBP reported earlier has interesting implications.

In another study, Thomasset et al, (1983) extracted mRNA from rat duodenum, kidney and cerebellum and translated it in a cell free reticulocyte system in the presence of L[S^{35}]- methionine. The vitamin D-dependent CaBPs were identified by immunoprecipitation using antibodies specific to duodenal (7 500 daltons) and cerebellar CaBPs (28 000 daltons). When the duodenal mRNA was translated, the immunoprecipitated polypeptide obtained using antibodies specific to duodenal CaBPs, comigrated with the small CaBP. Only the addition of unlabelled small duodenal CaBP prevented the immunoprecipitation of the major protein. Likewise, when mRNA extracted from the kidney and cerebellum was translated, the product immunoprecipitated by antibodies specific to large mammalian CaBP was electrophoretically similar to pure (28 000 daltons) protein, being displaced only by addition of unlabelled large CaBP. This suggested that two distinct mRNAs coding for small and for large vitamin D dependent CaBPs are expressed in specific tissues of the rat. Among these tissues investigated namely duodenum, kidney and cerebellum, the small CaBP gene was expressed only in the duodenum, while the large gene expression was confined to the kidney and cerebellum. Thus, the availability of the specific mRNAs are tools for investigating at the gene level the control of CaBP synthesis by vitamin D in intestine, kidney, brain and other tissues.

Recent studies on the rat 9 000 dalton CaBP mRNA using a complementary DNA have been carried out by Perret et al, (1985a). The rat possesses two cholecalciferol - induced CaBPs. The 9 000 dalton CaBP is mainly concentrated in the duo denum while the 28 000 dalton protein is located in the kidney and cerebellum. The mRNA encoding the 9 000 dalton CaBP has been characterised using the cloned cDNA, pC109, synthesised from rat duodenal 9 000 dalton CaBP mRNA (Desplan et al, 1983a). Nucleotide sequence analysis of this cDNA shows the presence of

two stop codons, TGA and TAG, at positions 207 and 271, respectively, of the 3 untranslated regions. The cDNA hybridised mRNA, isolated from rat duodenum, directs the cell free synthesis of two proteins precipitable by antisera to 9 000 dalton intestinal CaBP. A major protein comigrates with the 9 000 dalton CaBP whereas a minor product corresponds to a protein which is larger by 2 000 daltons. The minor protein appears to result from read-through of the 'leaky' UGA stop signal. No protein band which was immunoprecipitable with 28 000 dalton CaBP antiserum was detected when cDNA - hybridised mRNA from rat kidney and cerebellum was translated in a cell-free system. The data from this study, indicates that there are distinct genes coding for each rat CaBP.

Having discussed the two major categories of vitamin D-dependent CaBPs it is important to devote a brief section to methods of purification and identification of these proteins that have been described.

1.5 Overview of Methods of Purification and identification of vitamin D-dependent CaBPs

Purification procedures originally developed for the chick intestinal CaBP by Wasserman et al, (1968) have, with many modifications, been successfully employed in the isolation of CaBPs from many other species.

The isolation procedure generally involved the preparation of an aqueous supernatant solution from a homogenate of mucosal tissue as a starting material. This was subjected to ammonium sulphate fractionation and gel filtration chromatography. One research group used preparative discontinuous acrylamide gel electrophoresis. On occasions, due to the heat stability of the CaBPs, heat treatment at 60 - 70^{o}C replaced the

ammonium sulphate step in order to precipitate approximately 50% of the inactive protein.

Friedlander and Norman (1980) devised a procedure using a two step ion-exchange chromotography procedure in which an active CaBP was eluted with buffers containing EDTA and $CaCl_2$ respectively. This method was a modification of procedure by Wasserman et al, (1968). The technique and the final stages of the purification takes advantage of the change in intrinsic charge upon binding calcium. The principle behind the use of EDTA and $CaCl_2$ in these buffers was based on the acidic nature of the CaBP. Initially, the CaBP binds strongly to the ion-exchange column, equilibrated with the pH8,0 buffer. The EDTA in the buffer maintains a low free concentration of calcium ions, which might bind with the CaBP, causing it to pass through the column. Once no more protein was measured on eluted fractions from the column, Tris $CaCl_2$ pH8,0 buffer was pumped onto the column and the CaBP eluted as a single protein peak.

Yet another novel purification procedure has been used, namely the method of chromatofocusing to purify and simultaneously determine the iso-electric point of the particular CaBP (Staun et al, 1984). This method employs a linear pH gradient which is generated in the column by exploiting the buffering action of the exchanger and using amphoteric buffers which have even buffering capacity over a wide range of pHs. Proteins elute from such gradients in order of their iso-electric points. Focusing effects occur which result in sample concentration, band sharpening and very high resolution.

The Chelex method described by Wasserman et al, (1968) has been extensively used in detecting the calcium binding activity of the protein. Recently radioimmunoassays, immunoprecipitation and

immunocytochemical methods have been used successfully to identify, localise and characterise the vitamin D-dependent intestinal CaBPs as well as CaBPs from many other organs from various species.

Counter-ion electrophoresis described by Ueng and Bronner, (1979) was specially developed to identify the CaBP. The method is an extension of non-denaturing analytical gel electrophoresis. Radioactive $^{45}CaCl_2$ was added to the lower buffer chamber or anode. The principle used in the method is that $^{45}CaCl_2$ moves towards the cathode, the protein migrates toward the anode and subsequently the radioactive labelling and final identification of the CaBP in the slices occur.

Other methods of purification of the various avian and mammalian CaBPs include, Sephadex G-75/100 gel chromatography, DEAE-Sephacel/Cellulose ion-exchange chromatography, SDS polyacrylamide gel electrophoresis, and HPLC analysis. It must be stated that heat treatment followed by some form of gel permeation chromatography and ion-exchange chromatography has been the most widely used purification protocol. SDS polyacrylamide gel electrophoresis has nearly always been used to determine the molecular weight of the protein or to determine its anodic mobility. The use of HPLC, counter-ion electrophoresis and the method of the two-step ion-exchange chromatography has been used very sparsely by researchers. The usefulness of the widely used procedures are highlighted by the effectiveness of the purification of the various proteins achieved. The widely used procedure will be highlighted in the results reported here and in the discussion of this dissertation.

The aim of this study was to isolate, purify and characterise the intestinal CaBP from the baboon. The advantages and disadvantages of the various purification techniques were also investigated.

CHAPTER 2 : MATERIALS AND METHODS

2.1 PREPARATION OF INTESTINAL MUCOSA

Animals were obtained from two sources:

Mature baboons (Papio ursinus) were obtained from the Department of Surgery, University Witwatersrand Medical School. These baboons were donor animals forming part of a renal transplantation study. Immature baboons were obtained from a study on calcium deficiency rickets carried out at the Council for Scientific and Industrial Research - National Food Research Institute in Pretoria. Both groups of animals were fed on a semi-synthetic diet (Pettifor et al, 1981) (See Appendix II).

The whole duodenum and 30cm of jejunum were obtained from the animals approximately 15 - 20 min after death. All subsequent isolation and purification steps were carried out at between 0 - 4° C.

The intestinal material was immediately placed in a beaker of cold saline, cooled, rinsed with saline and slit open. The slit intestinal tissue was then mopped on dry tissue paper. The mucosal layer was scraped from the underlying muscle tissue with a thin glass slide. The mucosal scrapings were then snap-frozen using dry ice and stored at -20°C until used. For experimental purposes, the mucosal scrapings were thawed and homogenised in 3 - 4 volumes of a buffer mixture (TKN Buffer) whose composition was 137mM Tris pH 7,4, 120mM NaCl, 4,74mM KCl, and 0,01% ß - mercaptoethanol.

The homogenisation was carried out using either a Potter Elvejhem homogeniser with a teflon pestle or an Ultraturrax homogeniser. The

crude homogenate was centrifuged at 30 000xgmax for 30 min. The resulting crude supernatant was treated in one of three different ways.

2.2 FRACTIONATION OF BABOON INTESTINAL MUCOSA EXTRACT

2.2.1 AMMONIUM SULPHATE FRACTIONATION. (Wasserman et al, 1968 and Friedlander and Norman, 1980).

The supernatant of the crude homogenate was sequentially brought to 75 and 99 % saturation over one hour by the slow addition of solid crushed ammonium sulphate to a final concentration of 476g/l and 690g/l respectively to a solution being stirred with a magnetic mixer.

Each solution was allowed to stir for approximately two hours at $0^{o}C$ and then centrifuged at 30 000xgmax for 30 min. Samples from each supernatant fraction were dialysed extensively against TKN buffer.

The pellets resulting from each fraction were redissolved in and dialysed against TKN buffer. The supernatants and pellet solutions were subsequently concentrated in an Amicon stirred cell using a UM2 membrane. These fractions were frozen and stored at $-20^{o}C$ for further investigations.

2.2.2 HEAT PRECIPITATION OF BABOON INTESTINAL MUCOSA EXTRACT (Gleason and Lankford, 1981; Bryant and Andrews, 1983)

A study to establish the optimum heating time was carried out on a supernatant fraction of an intestinal mucosal extract. The supernatant

of the crude homogenate was stirred in a stainless steel beaker preheated to between 60 - 65°C in a temperature controlled water bath at 60 - 65°C. The supernatant was heated for approximately 5 min to allow it to reach 65°C. Fractions were then taken from the beaker every 5 min for 35 min and immediately cooled to 0°C in ice. The cooled samples were then centrifuged at 30 000xgmax for 20min. The resulting supernatants from these fractions were frozen, stored and later analysed for protein content and calcium binding activity.

All subsequent heat purification procedures were carried out for the optimal heating time of 20 min.

2.2.3 MODIFIED PROCEDURE USING pH PRECIPITATION METHOD. (Wasserman, July 1979 privileged communication).

The scraped intestinal mucosa was homogenised in 2 - 4 volumes of distilled water and centrifuged at 23 000xgmax for 1h at 4°C. The resulting intestinal mucosal supernatant was filtered through cheesecloth, and 6N HCl was added dropwise to the stirred supernatant until the pH reached 4,0. The solution was centrifuged at 23 000xgmax for 10 min. The resulting supernatant was again filtered through cheesecloth and to this 1N NaOH was added dropwise to bring the pH to 6,5. The solution was again centrifuged and concentrated by ultrafiltration or by lyophilisation. The resultant pH 6,5 supernatant fraction was stored at -20°C for further biochemical investigation.

2.3 GEL FILTRATION CHROMATOGRAPHY

2.3.1 GEL FILTRATION ON SEPHADEX G - 100 AND SEPHADEX G - 75 COLUMNS

Gel filtration chromatography was performed by downward elution on a 2,6cm x 90cm column containing Sephadex G - 100/G - 75 (Superfine) (Pharmacia Uppsala, Sweden). The Sephadex G - 100/G - 75 was first swollen in and washed thoroughly with TKN buffer and then allowed to pack in the column overnight by downward elution at approximately 0,73 ml/min with TKN buffer. Three column volumes were collected before these columns were calibrated. The columns were calibrated by the addition of solutions each containing 10 mg of blue dextran, and one of 20 mg of bovine serum albumin (68 000 daltons), 15 mg of ovalbumin (43 000 daltons), 15 mg of chymotrypsinogen (23 000 daltons), 10 mg of myoglobin (17 200 daltons) or 10 mg of cytochrome c (12 500 daltons). The column was eluted with TKN buffer and one ml fractions were collected on a Gilson automatic fraction collector. The eluted fractions containing either blue dextran or proteins were monitored by measuring their absorbance at 280nm. The void volume, Vo, was determined from the elution volume of the blue dextran solution. The respective elutions volumes, Ve, for each of the standards was also measured. The Ve/Vo ratios for the proteins were plotted against the log of their molecular weights.

A sample of partially purified CaBP was placed on the calibrated columns and eluted in the same way as the standard proteins. The eluted fractions were assayed for calcium binding activity by the Chelex assay and protein content by spectrophotometric analysis at 280nm. The active fractions were pooled, concentrated by ultra-filtration on an Amicon using a UM2 membrane and frozen until further needed. The molecular

weight of the CaBP was determined by reference to a plot of the Ve/Vo vs log molecular weight of standard molecular weight proteins. In some later gel filtration chromatography experiment a buffer consisting of 20mM Tris pH 7,5, 30mM NaCl was used which was the same as the initial elution buffer for DEAE - Sephacel ion-exchange chromatography. This was done so as not to necessitate lengthy dialysis before further purification could be effected. G-75/G-100 curves were extrapolated so as to determine the molecular weight of the CaBP eluted from each of the columns. The extrapolation had to be made as the eluted CaBP fell outside the range of standard molecular weight proteins used for the calibration curve.

2.3.2 GEL FILTRATION CHROMATOGRAPHY OF AMMONIUM SULPHATE FRACTIONATED SAMPLES FROM BABOONS FED ON SEMI-SYNTHETIC DIETS

Intestinal mucosal extracts from the four groups of baboons which had previously been subjected to ammonium sulphate fractionation, were chromatographed on Sephadex G-100 columns. Elution of the proteins was as described in Section 2.3.1. In each of the four experiments, 80 fractions of 3,3 ml each was collected. The amount of protein applied to the Sephadex G-100 columns was different for each of the experiments. The Vo's for each of the four experiments ranged from 154 - 158 ml. The protein content of each of the four fractions applied to Sephadex G-100 is described in Table 8. The protein content of the eluted fractions was measured by reading their absorbances at 280 mm on a spectrophotometer. The CaBP activity of the eluted fractions was also measured. The peak fractions resulting from each experiment were pooled and concentrated.

2.4 CALCIUM BINDING ASSAY (Briggs and Fleishman, 1965))

The Chelex - 100 resin (Bio Rad mesh size 100 - 200 Bio Rad Laboratories Richmond California) was washed at least 10 times in TKN buffer and allowed to settle in a measuring cylinder. The resin was finally diluted in TKN buffer so that 0,2 ml resin suspension contained 0,05 - 0,1 ml of packed resin.

A test sample of protein (10 - 60 µg) was pipetted into a small test tube followed by the addition of Chelex resin in suspension and then by an appropriate dilution of $^{45}CaCl_2$ (N.E.N. Boston, U.S.A.). The tube was vortexed for fifteen seconds and centrifuged at approximately 3 000 rpm for ten min. An aliquot of the supernatant was pipetted into a plastic vial containing 4 ml of Aquagel (Chemlab) and counted in a Packard scintillation counter model 3255. For volumes of sample, Chelex resin and radioactivity added in the assay, see Table 2

TABLE 2: Volumes of reagents used in the CaBP Assay

Components	Tube No I*		Tube No II°		Tube No III+	
	Macro	Micro	Macro	Micro	Macro	Micro
$^{45}CaCl_2$	0,1ml	0,02ml	0,1ml	0,02ml	0,1ml	0,02ml
TKN Buffer	1,2ml	0,125ml	1,0ml	0,10ml	-	-
Chelex-100	-	-	0,2ml	0,025ml	0,2ml	0,025ml
Protein Sample	-	-	-	-	1,0ml	0,1ml

Tube No I* = Total Blank - total cpm in assay

Tube No II° = Background counts after binding of Chelex - 100 resin.

Tube No III+ Tubes containing protein fractions to be assayed.

The $^{45}CaCl_2$ was diluted to give total counts of approximately 6,8 x 10^4 cpm and 2,8 x 10^4 cpm in the macro and micro methods respectively.

2.5 EXPRESSION OF BINDING DATA FOLLOWING CaBP ASSAY

The binding activity was given in terms of the Ca*Pr/Ca*R ratio per ml of supernatant fluid in assay as described by Wasserman et al, (1968), or as units/mg protein where a unit is defined as cpm/ml of supernatant fluid in assay.

2.6 ION-EXCHANGE CHROMATOGRAPHY

2.6.1 DEAE - A25 SEPHADEX ION-EXCHANGE CHROMATOGRAPHY (Wasserman Privileged communication, 1979)

DEAE-A25 Sephadex (Pharmacia) was extensively washed and equilibrated in 15mM Tris pH 8,0 1mM EDTA, 60mM NaCl. The packed Sephadex column (2,6cm X 35cm) was then equilibrated by passing 3 to 4 void volumes of the above buffer through the column. After this procedure, a partially purified protein sample, the pH 6,5 supernatant fraction, was dialysed against the starting buffer and then loaded onto the column. The flow rate of the column was approximately 0,9ml/min and 5ml or 10ml fractions were collected. The protein content of the fractions was measured by spectrophotometric analysis at 280nm. When the absorbance readings at 280nm had returned to zero, a second buffer containing 15mM Tris pH 8,0, 5mM $CaCl_2$ and 50mM NaCl was pumped onto the column and a number of fractions were collected. The protein content of each fraction was monitored.

2.6.2 DEAE-SEPHACEL ION-EXCHANGE CHROMATOGRAPHY (Bryant and Andrews 1983).

DEAE-Sephacel (Pharmacia) was extensively washed and equilibrated with 3 to 4 volumes of a buffer containing 20mM TrisHCl pH 7,5, 30mM NaCl. A 1cm X 10 cm column was packed and equilibrated at a flow rate of 0,44 ml/min. A protein fraction having previously been chromatographed on a Sephadex G-100 column and eluted in 20 mM Tris pH 7,5, 30mM NaCl was pumped onto the column following which 2ml fractions were collected.

The protein content of each fraction was monitored on a spectrophotometer at a wavelength of 280nm. As soon as the protein content measured zero, stepwise addition of starting buffer solution containing firstly 150mM NaCl and then 2M NaCl was pumped onto the column. Fractions (2ml) were collected and measured for CaBP activity and protein content with the exception of the 2M NaCl fractions in which only protein content was measured. All active fractions eluted were pooled and subjected to SDS polyacrylamide gel electrophoresis to determine the purity and molecular weight. All water used in this method was pretreated with cation exchange resin Chelex - 100 Resin (100 - 200 mesh) to decrease the calcium content of the buffers. All the active fractions were pooled, concentrated and redialysed against H_2O to reduce salt content and then electrophoresed on SDS polyacrylamide gel electrophoresis for identification purposes.

2.7 METHOD DESCRIBING A PARTICULAR PURIFICATION PROTOCOL FOR THE PURIFICATION OF BABOON INTESTINAL CALCIUM BINDING PROTEIN

The various purification procedures were scrutinised and having obtained results from each one, a protocol involving three procedures was chosen to purify the intestinal CaBP.

The supernatant fraction from baboon intestinal mucosa (260ml) was subjected to ammonium sulphate fractionation (5ml) and heat precipitation at 65°C for 20 min (255ml) in two separate experiments. Material from the latter procedure was further purified by Sephadex G-100 gel chromatography and then by DEAE-Sephacel ion-exchange chromatography.

A supernatant obtained after heating an intestinal mucosal extract for 20 min at 65°C was concentrated to 80 ml. A 16ml aliquot of this fraction was loaded onto a Sephadex G-100 column. The peak CaBP fraction obtained after Sephadex G-100 gel chromatography was pooled and concentrated down to a volume of 16ml. A 3ml aliquot of this fraction was loaded onto a DEAE-Sephacel ion-exchange column. The purified fractions obtained from each of the procedures were then subjected to Chelex-100 assay. The results are shown in two different ways, Table 6 shows the changes in specific activity and the purification, whereas Table 7 shows the yield as well as the purification-fold of the protein. The ammonium sulphate procedure is shown for comparitive purposes only.

2.8 ANALYTICAL POLYACRYLAMIDE DISCONTINUOUS ROD AND SLAB GEL ELECTROPHORESIS (Davis et al, 1964)

The running buffer containing 25 mM Tris and 190 mM Glycine pH 8,3 was prepared and left to cool at 4°C. The rods were assembled in a gel former. For slab gels, 14cm X 16cm glass plates were assembled. The plates were assembled with 0,75mm spacers and sealed with clamps. Rod and slab gels (7,5% and 10%) were prepared by mixing appropriate volumes of the following solutions. Acrylamide (5ml) of 29,2% Acrylamide (Biorad), 0,8% Bisacrylamide (Biorad); Separating gel buffer (2,5ml); containing 300mM Tris HCl pH8,9 and (0,23ml) Temed, made to 100ml with distilled H_2O; Freshly prepared ammonium persulphate solution 0,30ml (0,1%(w/v)) and H_2O, (12ml).

Before addition of ammonium persulphate the solutions were slowly mixed and degassed for 10 - 15 min. The final solution of the separating gel was then poured into slab or tube of rod gel to a height of 13 - 14cm for the former and 7-8cm for the latter. The gels in each case were then overlayed with 0,5ml - 1,0ml running buffer.

The stacking gel solution was prepared by mixing and addition of appropriate volumes of Acrylamide 2,5ml of 10% acrylamide, (Biorad) and 2,5% Bis-acrylamide (Biorad); Stacking gel buffer (1,25ml); 50mM Tris pH 6,8 with 48ml 1N HCl and (0,460ml) Temed, made to 100ml with distilled water;

Riboflavin Solution (2,5ml) 0,4 mg/10ml riboflavin, and H_2O, (5ml). This solution was mixed slowly and added to the top of the separating gel. In the case of the slab gel, the solution was added after the well former was in place. The gels were then left for approximately 1h to set. In both instances, due to use of riboflavin as polymerising agent in the stacking gels, the gels were left for approximately 1h under U.V. light for gels to polymerise. Once the gels had polymerised, they were either used immediately or stored in the fridge. The volumes shown above are for a 7,5% separating gel.

Samples were prepared by taking known amounts of protein (5 - 80 µg) and overlaying them directly to the top of the rod gel or into the sample well of slab gel. All samples to be electrophoresed contained tracking dye composed of 0,25% bromophenol-blue. Samples were dissolved in 25% Tris HCl pH 6,8; 10% glycerol and 10 µl of bromophenol-blue.

Electrophoresis was carried out at 4mA/tube for rod gels and 25mA for slab gels. In both cases, electrophoresis was carried out until the marker dye was approximately 1cm from the bottom of the gel. For rod gels, proteins were allowed to stack for 15 min at 2mA/tube. For slab gels, proteins were allowed to stack for 30 - 45 min at 12 mA.

The slab and rod gels were fixed and stained as described in Section 2,10. Rod gels were scanned using a densitometer (Quick Scan from Helena Laboratories), to show the presence of protein bands.

Protein concentrations from 5 - 80 µg were placed on rod gels and electrophoresed. Scans of gels were made showing in each case both the top and the bottom of gel. The sensitivity of the scanner was not the same for each of the gels scanned. The time scale was also not the same for each scan.

2.9 COUNTER-ION ELECTROPHORESIS (Ueng and Bronner, 1979)

In this procedure, a slight modification of conventional disc gel electrophoresis as described by Davis, (1964) was performed at 4°C by including 0,4mM $CaCl_2$ (13,400 cpm ^{45}Ca/nmol) in 700ml of buffer in the lower buffer chamber. The running buffer was 25mM Tris, 192mM glycine pH 8,3. Disc gels measuring 0,3cm x 10cm were prepared to contain a 10% separating gel and a 4,5% stacking gel. The solution containing the sample (30 µl containing about 60 µg protein) was layered on top of the stacking gel and electrophoresed at 4°C for approximately 4h at 4mA/tube to minimise heat generation.

The gel was removed from the glass rod using conventional removal techniques and then snap frozen on a glass plate using dry ice and immediately sliced into 2-3mm slices using a ruler as measure. The slices were then placed into premarked glass vials containing 1ml of a 30% H_2O_2 solution and left overnight in an oven at 55 - 60°C to solubilize. The vials were then cooled to room temperature (22°C) and Aquagel (Chemlab) was added to each vial and the radioactive solutions counted on a Packard liquid scintillation counter.

A single disc gel was kept separate, fixed and stained in Coomassie Brilliant Blue in 7% acetic acid overnight and destained in 7% acetic acid.

This disc served to compare the protein staining profile with the calcium binding component. In a particular experiment in which protein fractions from 4 groups of baboons on semi-synthetic diets had been chromatographed on Sephadex G-100 gel chromatography, 120 µg of each of the pooled and eluted protein fractions, was loaded onto each rod gel. This was done so that results could be accurately compared.

2.10 SDS POLYACRYLAMIDE GEL ELECTROPHORESIS FOR THE DETERMINATION OF PURIFICATION AND ESTABLISHMENT OF THE MOLECULAR WEIGHT OF THE BABOON CaBP (Laemmli, 1970 and King and Laemmli, 1971)

The Biorad system for electrophoresis was used. Firstly, the running buffer which contained 25mM Tris, 192mM Glycine pH 8,3 and 0,1% SDS was prepared and left to cool with constant stirring in the electrophoresis tank at $4^{o}C$. The plates (14 cm X 16 cm) were then cleaned thoroughly and finally rinsed with distilled water and dried in air. The plates were assembled into the system using a 0,75mm spacer and sealed with clamps. The separating gel was poured by addition of a solution containing Acrylamide 5,5 ml of 40% acrylamide solution (38,9% Acrylamide (Biorad), 1,1% Bisacrylamide (Biorad); Glycerol (1,5ml), (75% (v/v); Separating gel buffer (8,65ml); 1,5 M Tris pH 8,8 and 0,4% SDS; H_2O(1,82ml); Temed (0,033ml); Freshly prepared ammonium persulphate solution (0,02ml), (10% w/v).

Before addition of Temed and ammonium persulphate, the solution was slowly mixed and degassed for 10 - 15 min. The final solution of separating gel was then poured into the plate to a height of 13 - 14 cm. The gel was then overlayed with 0,5-1,0ml running buffer.