A SEARCH FOR VITAMIN D DEPENDENT CALCIUM BINDING PROTEINS IN THE RAT NERVOUS SYSTEM

by

© Harout Hasséssian

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

Department of Pharmacology and Therapeutics
McGill University
Montréal, Québec
Canada

ISBN 0-315-38193-0

TABLE OF CONTENTS

RESULTS

ABSTRACT

Rats vitamin D deficient from utero were compared to vitamin D replete controls to test for vitamin D dependent calcium binding proteins in the soluble (100 000 X g supernatant) fraction from cerebellum, spinal cord and sciatic nerve. The proteins were subjected to polyacrylamide gel electrophoresis (PAGE) and stained with a carbocyanine dye. Calcium binding proteins form J complexes with the dye and appear as blue bands on polyacrylamide gels (Campbell, MacLennan and Jorgensen, 1983).

Samples prepared from three anteroposterior divisions, "cervical", "thoracic" and "lumbar" of the spinal cord, showed six blue staining bands of molecular weight: 107, 101, 80, 75, 60 and ≃ 14 kilodaltons. The rat younger than 49 days postpartum did not show this 80 kilodaltons band on carbocyanine dye stained gels. All the blue bands present in the spinal cord PAGE profile except the 80 kilodaltons band were also found in the sciatic nerve. The cerebellum has four blue staining bands of the following molecular weights: 116, 51, 28 and ≃ 14 kilodaltons. In this study blue staining bands were found to represent acidic proteins which can be precipitated with barium sulfate as can calcium binding proteins of plasma and bone (Malhotra, 1979). After removal of sodium dodecyl sulfate (SDS) from gels, calcium ligating bands were located by a radioautographic method (Anthony and Babitch, 1984). The cerebellum contained a 28 and a 14 kilodaltons band detectable in this way. This 28 kilodaltons calcium binding band may represent the 28 kilodaltons calcium binding protein ($CaBP_{28K}$) of rat kidney and brain (Baimbridge, Miller and Parkes, 1982; Schreiner, Jande, Parkes, Lawson and

Thomasset, 1983) for which vitamin D dependence has been shown in the kidney (Thomasset, Parkes and Cuisinier-Gleizes, 1982). Only one calcium binding band of 14 kilodaltons molecular weight was found in the spinal cord or the sciatic nerve. Neither the 28 nor the 14 kilodaltons bands could be shown to be vitamin D dependent.

The results of this study show that blue staining by the carbocyanine dye can not be used as a reliable indicator of calcium binding proteins and suggest that $CaBP_{28K}$ does not exist in the spinal cord or sciatic nerve of the rat.

RESUME

La présence de protéines liant le calcium a été comparée dans la fraction soluble (100 000 X g) d'extraits de cervelet, de moelle épinière et de nerf sciatique entre des rats carencés en vitamine D dès le début de la vie intra-utérine, et des rats témoins non carencés. Les protéines de ces fractions ont été séparées sur gel polyacrylamide à électrophorèse puis colorées au carbocyanine. Les protéines liant le calcium forment des complexes en forme de J avec ce colorant et apparaissent sous forme de bandes bleues sur des gels de polyacrylamide (Campbell, MacLennan and Jorgensen, 1983).

Les échantillons préparés à partir des régions "cervicale", "thoracique" et "lombaire" de la moelle épinière, montrent six bandes bleues dont les poids moléculaires sont: 107, 101, 80, 75, 60 et ≃ 14 kilodaltons. Les échantillons prélevés de rats âgés de moins de 49 jours après la naissance ne montrent pas la bande de 80 kilodaltons sur les gels colorés à la carbocyanine. A l'exception de cette bande de 80 kilodaltons, toutes les autres bandes bleues présentes dans le patron électrophorétique de la moelle épinière sont aussi retrouvées dans celui du nerf sciatique. Le cervelet a quatre bandes bleues correspondant aux poids moléculaires suivants: 116, 51, 28 et ≃ 14 kilodaltons. Dans cette étude, il est apparu que les bandes bleues représentent des protéines acides, lesquelles peuvent être précipitées par le sulphate de barium tout comme les protéines liant le calcium provenant du sang et des tissus osseux (Malhotra, 1979). Après l'élimination du sulphate de sodium dodécylé des gels, les bandes

liant le calcium ont été révélées par radioautographie (Anthony and Babitch, 1984). Par cette méthode, des bandes de 28 et 14 kilodaltons ont été mises en évidence dans le cervelet. Il est possible que la bande de 28 kilodaltons liant le calcium soit la même protéine que celle ($CaBP_{28K}$) retrouvée dans le rein et le cerveau du rat (Baimbridge, Miller and Parkes, 1982; Schreiner, Jande, Parkes, Lawson and Thomasset, 1983) et pour laquelle une dépendance à la vitamine D a été démontrée dans le rein (Thomasset, Parkes and Cuisinier-Gleizes, 1982). Une seule bande de 14 kilodaltons liant le calcium a été retrouvée dans la moelle épinière ainsi que dans le nerf sciatique. Aucune des deux bandes de 28 ou de 14 kilodaltons n'a montré de dépendance à la vitamine D.

Les résultats de cette étude montrent que, la coloration bleue obtenue avec le colorant carbocyanine ne peut être utilisée comme indicateur fiable pour l'identification des protéines liant le calcium, et suggèrent que la protéine $CaBP_{28K}$ n'existe pas tant au niveau de la moelle épinière que dans le nerf sciatique du rat.

ACKNOWLEDGEMENTS

I wish to express gratitude to Dr. A.C. Cuello for giving me the opportunity to complete a thesis in the Department of Pharmacology and Therapeutics.

I would like to thank Dr. B. Collier for his valuable advice during my candidature. I thank Dr. A. Tenenhouse for taking me into his laboratory and Drs. E. Ishac and R. Mosqueda, for their supportive friendship. I appreciate the comradeship of Mr. M. Ghahremani and Mrs. B. Glijer and acknowledge the proficient secretarial work of Ms. F. Larue. Not least of all, I would like to thank my parents for making the pursuit of my ambition possible.

STATEMENT OF THE PROBLEM

Only two soluble calcium binding proteins are known to be vitamin D dependent. They are the 28 kilodaltons protein ($CaBP_{28K}$) first discovered in chick intestine (Wasserman and Taylor, 1963; Wasserman, 1980) and the mammalian intestinal 9-10 kilodaltons calcium binding protein ($CaBP_{9K}$) (Warembourg, Perret and Thomasset, 1985). Immunoreactivity to both these proteins exist in the rat kidney, and the antigen is vitamin D dependent (Thomasset, Desplan and Parkes, 1983). In the brain there is immunoreactivity to the 28 kilodaltons protein but it is not vitamin D dependent (Taylor, 1974; Thomasset, Parkes and Cuisinier-Gleizes, 1982).

Inability to demonstrate a vitamin D dependence for $CaBP_{28K}$ in the brain, has been attributed to the fact that central nervous system neurons do not divide after embryonic development (Taylor, 1974). Cells of the intestinal mucosa and the kidney continue to divide during the life of the rat (Messier and LeBlond, 1960). The implication of Taylor's hypothesis is that; whether a cell will have vitamin D dependent calcium binding protein in the G_o state or not is dependent on whether the gene coding for this protein is induced in the preceding G_1 period of the cell cycle.

Unlike vitamin D deficient animal models used in the past. The rats in this study have not been exposed to vitamin D during development in the womb or throughout their postpartum life. The experiments described in this thesis were designed to determine if any soluble (100 000 X g supernatant) calcium binding proteins present in the nervous system of vitamin D replete rats are absent from these vitamin D deficient animals.

INTRODUCTION

1.1.1 Source, Fate and Action of 1,25-Dihydroxycholecalciferol in the Rat.

Cholecalciferol (vitamin D_3) regulates calcium and phosphorus homeostasis in the rat and other species (DeLuca and Schnoes, 1983; DeLuca, 1985; Karsenty, Lacour, Ulmann, Pierandrei and Drueke, 1985). In vitamin D_3 deficiency, the intestinal absorption of calcium and phosphorus is reduced, producing a drop in the product of calcium and phosphorus in the extracellular fluid (Weinstein, Underwood, Hutson and DeLuca, 1984). Symptoms of neuromuscular irritability, tetany and sometimes seizures are produced; bone mineralization is impaired and growth is retarded (DeLuca, 1984).

Vitamin D_3 is thought to be derived from 7-dehydrocholecalciferol by a photochemical reaction in the malpighian layer of skin (Lawson and Davie, 1979; Bonjour, Trechsel, Muller and Scholer, 1985). Ultraviolet irradiation of 290-320 nm wavelength splits the bond between carbon atoms 9 and 10 in 7-dehydrocholecalciferol (Uskokovic, Partridge, Narwid and Baggiolini, 1980). The product is a previtamin D, that slowly equilibrates with cholecalciferol (Holick, Frommer, McNeill and Potts, 1977; DeLuca, 1985). Conversion to cholecalciferol requires the rotation of ring A by 180° around the single bond between carbon atoms 6 and 7, and the formation of a double bond between carbon atoms at positions 10 and 19 (Uskokovic, Partridge, Narwid and Baggiolini, 1980). Presumably cholecalciferol is absorbed into the subepidermal microcirculation and transported to the liver via the plasma (Norman, 1979; Stanbury, Mawer, Taylor and de Silva, 1980). To date,

regulation of the photochemical reaction has not been demonstrated (DeLuca, 1985).

Cholecalciferol is inactive and must be metabolized to its active form, 1,25-dihydroxycholecalciferol, via an intermediate metabolite 25-hydroxycholecalciferol (DeLuca and Schnoes, 1983; DeLuca, 1985). Two systems are capable of hydroxylating cholecalciferol at carbon 25; one a 50 kilodaltons, microsomal, mixed function monoxygenase dependent on flavoprotein and a cytochrome P-450 (Yoon and DeLuca, 1980; Hayashi, Noshiro and Okuda, 1986), the other a mitochondrial system which hydroxylates cholecalciferol and other sterols at carbon 25 (Bjorkhem, Holmberg, Oftebro and Pederson, 1980). Which of the two is physiologically most important has been a controversal issue (Fraser, 1980; Henry, 1980) and a lot of work has been done to support one system or the other. The picture that emerges from these reports is that the mitochondrial hydroxylase is of "high capacity, low affinity" and the microsomal hydroxylase "low capacity, high affinity" (Fukushima, Nishii, Suzuki and Suda, 1978; Bjorkhem and Holmberg, 1979). This being true, it would be logical to conclude that the microsomal system is more important when the concentration of cholecalciferol is low and the mitochondrial system is important when the concentration is high (Bjorkhem, Holmberg, Oftebro and Pedersen, 1980; DeLuca, 1985).

The rate limiting reaction in the synthesis of 1,25-dihydroxy-cholecalciferol is the 1-hydroxylation, the site of which in the non-pregnant rat is the kidney (Henry, 1980; DeLuca, 1985). The 1-hydroxylase is a mitochondrial enzyme, and the data compiled thus far support either a mixed function oxidase or a peroxidase mechanism for cytochrome P-450 in catalazing this hydroxylation (Warner, 1983). The 1-hydroxylase activity is regulated in response to the organism's

requirement for calcium and phosphorus (DeLuca, 1984). At least eleven different factors have been implicated in controlling the 1-hydroxylase activity (Henry, 1980; Fraser, 1980; Kumar, 1984). These include plasma levels of: calcium, phosphorus, parathyroid hormone, 1,25-dihydroxycholecalciferol and other hormones.

When synthesis of 1,25-dihydroxycholecalciferol is suppressed, the 24-hydroxylation of 25-hydroxycholecalciferol is activated and 24-hydroxylation is inhibited when 1,25-dihydroxycholecalciferol synthesis is stimulated (DeLuca, 1985). No biologicaly significant role has been found for the 24-hydroxylated metabolite (Jarnagin, Brommage, DeLuca, Yamada and Takayama, 1983: Brommage, Jarnagin, DeLuca, Yamada and Takayama, 1983; DeLuca, 1985).

1,25-dihydroxycholecalciferol enters the plasma where it is transported by vitamin D binding protein (DBP) (Cooke, 1986). 1,25-dihydroxycholecalciferol is taken up by intestine, bone, kidney and possibly other tissues in which it may also have a function (Stumpf, Sar, Reid, Tanaka and DeLuca, 1979; Stumpf, Sar, Clark, Lieth and DeLuca, 1980; Jande, Maler and Lawson, 1981; Peterfy and Tenenhouse, 1982; DeLuca and Shnoes, 1983; Tsoukas, Provedini and Manolagas, 1984; Costa, Blau and Feldman, 1986; Warembourg, Perret and Thomasset, 1986). The action of 1,25-dihydroxycholecalciferol has been best studied in the intestine, where the hormone binds to a high affinity (K_d = $5x10^{-11}$ M) and low capacity, cytosolic receptor that is likely to be required for the translocation of the hormone into the nucleus (Kream and DeLuca, 1977; Walters, Hunziker and Norman, 1980). The importance of this receptor has been demonstrated in man. Individuals defective in the cytosolic receptor (vitamin D_3-dependent rickets type II) are hypocalcemic and have secondary

hyperparathyroidism although plasma levels of 1,25-dihydroxycholecalciferol may even be higher than normal in some cases (Pike, Allegretto, Kelly, Donaldson, Marion, Mangelsdorf and Haussler, 1984).

Termination of 1,25-dihydroxycholecalciferol action is by conversion, mainly in the liver, kidney and intestine, to inactive metabolites (Kumar, 1984). Within a 24 hour period, 25-30% of a 1,25-dihydroxycholecalciferol dose will be oxidized to calcitroic acid (9,10-secocholesta-5,7,10(19)triene-24,25,26,27-tetranor-1α,3β-23-oate) and excreted in the bile (Onisko, Esvelt, Schnoes and DeLuca, 1980). Of the remainder, about 10% will be hydroxylated to 1,24,25-trihydroxycholecalciferol by renal mitochondria (Kleiner-Bossaller and DeLuca, 1974; Kumar, 1984) and 20-25% will be excreted in the bile as glucuronide and sulfate metabolites (Kumar, Nagubandi, Mattox and Londowski, 1980). 15-20% of the glucuronide derivative is probably reused by the rat after hydrolysis to the free vitamin (Kumar, 1984).

In mucosal cells of the intestine, 1,25-dihydroxycholecalciferol induces the synthesis of a specific calcium binding protein ($CaBP_{9K}$) (Wasserman, Corradino and Taylor, 1968; Thomasset, Parkes and Cuisinier-Gleizes, 1982).

1.2.1 The $CaBP_{9K}$ and $CaBP_{28K}$ of Rat.

Constituting 2% of the soluble proteins in the absorptive layer of the duodenal mucosa in the rat (Marche, Le Guern and Cassier, 1979; Taylor, Gleason and Lankford, 1984; Warembourg, Perret and Thomasset, 1985), is a 7 500 - 14 500 daltons molecule ($CaBP_{9K}$) with two high affinity ($K_d = 1 \times 10^{-8} - 1 \times 10^{-5}$ M) calcium binding sites (Thomasset, Desplan,

Moukhtar and Mathieu, 1981; Desplan, Heidmann, Lillie, Auffray and Thomasset, 1983). $CaBP_{9K}$ is also found in the jejunum, ileum and cecum but in less than one fifth the amounts of the duodenum (Thomasset, Parkes and Cuisinier-Gleizes, 1982). The function of $CaBP_{9K}$ remains unknown. It was demonstrated that 1,25-dihydroxycholecalciferol mediated calcium transport is independent of de novo protein synthesis (Bikle, Zolock, Morrissey and Herman, 1978) indicating that $CaBP_{9K}$ cannot be directly responsible for the 1,25-dihydroxycholecalciferol stimulated transport.

Antibodies raised against $CaBP_{9K}$ will immunoreact with antigens in the segments distal to the loop of Henle and proximal to the mid collecting duct in the rat renal cortex (Schreiner, Jande, Parkes, Lawson and Thomasset, 1983). The distribution of this immunoreactivity correlates well with the location of renal cells that have been shown to take up 1,25-dihydroxycholecalciferol into the nucleus (Stumpf, Sar, Narbaitz, Reid, DeLuca and Tanaka, 1980; Manillier, Farman, Bonjour and Bonvalet, 1985). Another investigation found no correlation between cells that take up 1,25-dihydroxycholecalciferol and the distribution of the immunoreactivity (Kawashima and Kurokawa, 1982), probably because they could not distinguish between specific nuclear uptake and non-specific cytosolic uptake in their homogenates (Schreiner, Jande, Parkes, Lawson and Thomasset, 1983). A study using cytosol from whole kidney has demonstrated a 3.3 S high affinity binding component for 1,25-dihydroxycholecalciferol which is believed to be the cytosolic receptor for the hormone (Chandler, Pike and Haussler, 1979).

In the rat kidney and cerebellum there is a cytosolic 28 kilodaltons calcium binding protein ($CaBP_{28K}$) with four high affinity calcium binding sites ($K_d = 1 \times 10^{-8} - 1 \times 10^{-5}$ M) (Baimbridge, Miller and Parkes, 1982; Thomasset, Desplan and Parkes, 1983). $CaBP_{28K}$

binds alkaline earth metals, in order of decreasing affinity, as follows: calcium > strontium > barium > magnesium (Taylor, 1980). It also exists in duodenal enterocytes but in smaller amounts (Moriuchi, Yamanouchi and Hosoya, 1975; Thomasset, Parkes and Cuisinier-Gleizes, 1982; Schreiner, Jande, Parkes, Lawson and Thomasset, 1983). This 28 kilodaltons protein is localized in the renal cortex and is found almost exclusively in the distal convoluted tubule with some amounts detectable in the collecting duct (Schreiner, Jande, Parkes, Lawson and Thomasset, 1983). The kidney and cerebellar $CaBP_{28K}$ have been compared: they are identical immunologicaly and behave similarly on two dimensional isoelectric focusing/ electrophoresis, they are acidic (pI = 4.3) and have very similar amino acid compositions with a high content of aspartic and glutamic acid residues (Van Eldik, Zendegui, Marshak and Watterson, 1982; Intrator, Baudier, Elion, Thomasset and Brehier, 1985). The peptide maps obtained after tryptic and chymotryptic digestion, in the absence of calcium, are identical and the amino terminal sequence of both proteins is identical.

The major difference is their vitamin D dependence; the kidney protein is dependent on the vitamin D status of the animal and the cerebellar protein is not (Thomasset, Parkes and Cuisinier-Gleizes, 1982). Thirty day old vitamin D deficient rats have in their cerebellum $CaBP_{28K}$ equal in quantity to controls and the amount does not change after the rats are injected intraperitoneally with 100 ng/100 g body weight of 1,25-dihydroxycholecalciferol (Thomasset, Parkes and Cuisinier-Gleizes, 1982). The serum calcium concentration in the treated animals rose from about 7 to 12 mg/dl and the $CaBP_{28K}$ in the gut and the kidney rose to normal levels. When the distribution of radiolabelled 1,25-dihydroxycholecalciferol, in the brain, is compared to that of

immunoreactive $CaBP_{28K}$ there is a poor relationship (Jande, Maler and Lawson, 1981). Notably no detectable 1,25-dihydroxycholecalciferol or its receptor has been reported in the cerebellum where $CaBP_{28K}$ is found in large amounts (Stumpf, Sar, Clark, Lieth and DeLuca, 1980; Thomasset, Parkes and Cuisinier-Gleizes, 1982).

In the brain $CaBP_{28K}$ is specific to neurons, with the highest quantities in the Purkinje cells of the cerebellum (15 µg/mg total soluble cerebellum protein) and the dentate gyrus of the hippocampus (1.71 µg/mg total soluble protein) (Baimbridge, Miller and Parkes, 1982). The $CaBP_{28K}$ concentration increases linearly from embryonic day 17 to a maximum at post gestational day 30, remains constant until day 70 and has dropped markedly by day 210 (Thomasset, Parkes and Cuisinier-Gleizes, 1982). There is no correlation between the period over which the concentration of this cerebellar protein rises and the period in which Purkinje cells proliferate. The Purkinje cell population originates on embryonic day 14 (Rodier, 1977).

A role that has been suggested for $CaBP_{28K}$ in the central nervous system is one of intracellular buffer for calcium (Jande, Maler and Lawson, 1981; Baimbridge and Miller, 1984). In attempting to correlate $CaBP_{28K}$ levels with hyperexcitability of neurons, Baimbridge and Miller conducted the following series of experiments. When kindling stimuli via electrodes chronically implanted in the midline commissural pathway were given to rats, $CaBP_{28K}$ content decreased progressively and was significantly lower (16.6%, $P < 0.01$) than controls after only 10 kindling trials (Baimbridge and Miller, 1984). In the hippocampus $CaBP_{28K}$ is found exclusively in the dentate granule and CA1/CA2 pyramidal cells, together with scattered interneurons (Baimbridge and Miller, 1984).

Commissural kindled rats loose $CaBP_{28K}$ selectively from dentate granule cells (Miller and Baimbridge, 1983). It is significant that the major projection of the dentate gyrus is to the CA3/CA4 pyramidal region which is a focus for epileptiform activity (Wong, Traub and Miles, 1984). It needs to be determined however, whether the drop in $CaBP_{28K}$ following kindling is a causative factor or a consequence to the seizure activity.

1.3.1 Soluble Non Vitamin D Related Calcium Binding Proteins of the Nervous System.

Four low molecular weight, soluble calcium binding proteins: parvalbumin, calmodulin, $CaBP_{28K}$, and the heterogenous group of proteins designated S-100 (the acronym signifies their partial solubility in saturated ammonium sulfate at neutral pH, Van Eldik, Zendegui, Marshak and Watterson, 1982), are known to exist in the nervous system of the rat. Other than $CaBP_{28K}$, for which vitamin D-dependence has been shown in the kidney, there is *no* evidence that they are vitamin D dependent (Van Eldik, Zendegui, Marshak and Watterson, 1982). Nucleotide sequence homologies within the genes coding for these four calcium binding proteins suggests that they have evolved from a common progenitor (Desplan, Heidmann, Lillie, Auffray and Thomasset, 1983; Epstein, Means and Berchtold, 1986).

They share a calcium binding structure designated an EF hand that was originally described in parvalbumin (Kretsinger, 1981). This domain is formed by two helical regions, which in parvalbumin are the E-helix, the F-helix, and a twelve amino acid loop connecting them. $CaBP_{28K}$ contains an additional calcium binding structure that differs from the EF hand by the nature of the calcium ligating atoms (Seamon and Kretsinger, 1983). In the EF hand it is mainly oxygens from amino acid

side chains that ligate the calcium and in the second type of calcium binding site in $CaBP_{28K}$ the calcium chelating atoms of oxygen are derived from carbonyl groups in the peptide backbone (Van Eldik, Zendegui, Marshak and Watterson, 1982).

1.3.2 Parvalbumin.

Parvalbumin is a 12 kilodaltons, acidic, calcium binding protein which in mammals is found in high concentrations in fast twitch muscle (Blum, Lehky, Kohler, Stein and Fischer, 1977; Heizmann, 1984).

An immunologically identical protein has been found in the central nervous system (Gosselin-Rey, Pironf and Gerday, 1978; Celio and Heizmann, 1981). Very small immunoreactive neurons and positive punctate structures can be seen in Rexed lamina IIb of the spinal cord dorsal horns. Motoneuron terminals in the ventral horns and myelinated axons in the dorsal funiculi were also labelled. In spinal ganglia, immunolabelling was restricted to the large cells and in the peripheral nerves to large diameter axons (Celio and Heizmann, 1981). During development, brain parvalbumin begins to increase at postnatal day 5, reaches a maximum at postnatal day 20 and remains constant thereafter (Berchtold and Means, 1985). The increase during development is only slight and the maximum parvalbumin level is much lower than in fast twitch muscle.

Type three parvalbumin is the best studied, the properties of the others are assumed to be indistinguishable from the carp muscle protein. Carp parvalbumin binds two calciums with a dissociation constant of 1×10^{-7}M. The dissociation constant for magnesium is 1×10^{-4} and that for sodium is 1×10^{-2}M (Van Eldik, Zendegui, Marshak and Watterson, 1982). The parvalbumin molecule consists of six alpha helical regions

denoted by letters A-F. Calcium ions are bound by the peptide regions between helices C and D and between helices E and F (Epstein, Means and Berchtold, 1986).

The CD and EF hands each form octahedral calcium binding structures that are related to each other by an approximate 2 fold axis (Seamon and Kretsinger, 1983). The six ligands in the octahedral structure are mainly side chain oxygen atoms of the protein (aspartate-90, aspartate-92, aspartate-94 and glutamate-101). A peptide carbonyl oxygen (lysine-96) is the ligand at one vertex, and water bonded to glycine-98 is the oxygen ligand at the other vertex of the loop (Van Eldik, Zendegui, Marshak and Watterson, 1982). Although a lot is known about the three dimensional structure of parvalbumin, its function in the central nervous system is still obscure (Berchtold and Means, 1985).

1.3.3 Calmodulin.

Calmodulin, a monomeric protein, has been purified and characterized from several mammalian sources including brain (Klee, Crouch and Richman, 1980; Means, Tash and Chafouleas, 1982). It is a 148 residue, 16 790 daltons, acidic protein (pI = 3.9-4.3) containing 27 glutamic acid and 22 aspartic acid residues (Watterson, Sharief and Vanaman, 1980; Cormier, 1983). Partition of calmodulin between the soluble and particulate fractions in brain, depends on the presence of calcium or ethylene glycol-bis (aminoethylether)N,N'-tetraacetic acid (EGTA) in the homogenizing medium. In rat brain 58% of the total calmodulin is particulate in the presence of 0.1 mM calcium chloride, versus 78% in the presence of 2 mM EGTA (Kakiuchi et al, 1978).

Calmodulin is resistant to elevated temperatures. A solution containing calmodulin can be heated for 5 minutes at 90°C with no appreciable loss of biological activity, as assessed by the ability to stimulate phosphodiesterase or myosin light chain kinase (Means, Tash and Chafouleas, 1982). Although calmodulin is heat resistant, it is not truly heat stable. The half life of the protein at 100°C is 7 minutes (Beale, Dedman and Means, 1977). In the presence of calcium, calmodulin will resist denaturation by 8 molar urea or 1% sodium dodecyl sulfate (Dedman, Potter, Jackson, Johnson and Means, 1977).

It is established that calmodulin contains four metal ion binding sites of the EF hand type (Van Eldik, Zendegui, Marshak and Watterson, 1982). These calcium binding sites are in domains I (beginning at the NH_2-terminal portion) through IV (the COOH-terminal portion of the protein) (Watterson, Sharief and Vanaman, 1980). About 70% of the amino acids in domains I and III are identical and a similar homology exists between domains II and IV (Means, Tash and Chafouleas, 1982). Reports on the affinity and specificity of the binding sites for calcium vary considerably (Klee, Crouch and Richman 1980; Prozialeck and Weiss, 1985). The latest model predicts that: the four sites are equal, bind both calcium and magnesium with positive cooperativity and have dissociation constants in the micromolar and millimolar range for calcium and magnesium respectively (Iida and Potter, 1986).

In the absence of calcium, 40% of the protein exists in an α-helical configuration (Dedman, Potter, Jackson, Johnson and Means, 1977). The binding of calcium to calmodulin increases the helicity to greater than 50% and exposes a highly lipophilic region of the molecule (Tanaka and Hidaka, 1980). This change in conformation is mandatory for the

regulation of all calmodulin modulated molecules described thus far (Means, Tash and Chafouleas, 1982). Calmodulin is viewed as an intracellular receptor for calcium, regulating the activity of various molecules in a calcium dependent manner (Kretsinger, 1981).

1.3.4 S-100

S-100 describes a heterogenous population of acidic (pI = 4.1), low molecular weight, calcium binding proteins, composed of two structurally related subunits α and β (Van Eldik, Zendegui, Marshak and Watterson, 1982).

S-100b constitutes almost 90% of the S-100 proteins and is a dimer composed of two 10 500 daltons β-subunits (Baudier, Labourdette and Gerard, 1985). Each β-subunit contains one high affinity ($K_d = 2x10^{-5}$ M) and two low affinity ($K_d = 1x10^{-4}$ M) calcium binding sites of which the high affinity site is of the EF hand type (Isobe, Ishioka and Okuyama, 1981; Baudier, Labourdette and Gerard, 1985). S-100a protein is a dimer of α and β-subunits (Masure, Head and Tice, 1984). The 10 400 daltons α-subunit is characterized by: the presence of a tryptophan which is absent from the β-subunit, and the presence of a single cysteine unlike the β-subunit which has two (Isobe, Ishioka and Okuyama, 1981). Three percent of the S-100 proteins is formed by $S\text{-}100_o$ which is an α-subunit dimer (Masure, Head and Tice, 1984).

Originally isolated from brain and thought to be specific to glial cells, S-100 protein has now been reported to be present in a number of tissues (Suzuki, Nakajima and Kato, 1982). In the rat, the highest levels of S-100 protein are found in the central nervous system, adipose tissue and the trachea while in the liver it is absent (Hidaka et al., 1983; Michetti, Dell'Anna, Tiberio and Cocchia, 1983). The folliculostellate cells of the

pituitary (Nakajima, Yamaguchi and Takahashi, 1980), satellite cells of the adrenal medulla (Cocchia and Michetti, 1981), and the skin (Suzuki, Nakajima and Kato, 1982) contain lower amounts but significantly higher than several other tissues that were tested from the rat (Hidaka et al., 1983). The larger portion of S-100 in the central nervous system is in glia, although a small amount exists in neurons (Packman, Blomstrand and Hamberger, 1971; Tabuchi and Kirsch, 1975). In the peripheral nervous system, it is primarily a neuronal protein (Miani et al, 1972).

The majority of these studies have used antibodies raised against S-100 protein and can not make a distinction between the distribution of S-100a from that of S-100b. The function of S-100 in these tissues is not known (Suzuki, Nakajima and Kato, 1982; Van Eldik, Zendegui, Marshak and Watterson, 1982; Baudier, Labourdette and Gerard, 1985).

METHODS

2.1.1 Experimental Animals.

Twenty-one day old female Sprague Dawley rats were maintained on a vitamin D free diet (ICN Nutritional Biochemicals; Clevaland, Ohio, diet AIN 76A to which vitamin D has not been supplemented) for 8 weeks (Table 2.1). These rats, termed the parental generation, were then mated with male Sprague Dawleys receiving Purina Rat Chow (#5012, Ralston Purina Co., U.S.A.) and all subsequent generations from these matings was kept on the vitamin D free diet. The first filial generation (F_1) females were mated with normal males to produce the vitamin D deficient rats (F_2) used in this study. Rats were housed in a colony room, isolated from external light and lit by an incandescent source filtered for UV wavelengths. The vitamin D deficient rats used for experimentation were between 35 and 90 days old. The Sprague Dawley controls were purchased from Charles River (Charles River Canada Inc., St-Constant, Que.) and maintained on Purina Rat Chow. Both experimental and control animals were on a 12 hour dark and 12 hour light schedule in rooms maintained at 22°C.

2.2 Preparation of Samples.

2.2.1 Dissection.

During the dissection speed and prevention of contamination with other tissues was a preoccupation. Rats were exsanguinated by

AMERICAN INSTITUTE OF NUTRITION
AIN-76 SEMIPURIFID DIET, Rat or Mouse

This diet may be modified but will be listed as a modified AIN-76 Semipurified Diet *Trademark pending

Composition:	
Casein Purified High Nitrogen	20.0%
DL-Methionine	0.3%
Corn Starch	15.0%
Sucrose	50.0%
Fiber (Alphacel non-nutritive bulk)	5.0%
Corn Oil	5.0%
AIN Mineral Mix	3.5%
AIN Vitamin Mix	1.0%
Choline Bitartrate	0.2%

The exact formulation for this diet recommended by the American Institute of Nutrition is intended for growth and maintenance during the first year of life. Investigators should be aware that diets high in sucrose can be cariogenic, and that some strains of rats fed such diets may develop kidney lesions after extended periods.

The diet has been found to be satisfactory for reproduction and lactation in both rats and mice.

If used for deficiency studies, modifications will be necessary. If used in ultra-clean environment, several trace elements should be added (*Fed. Proc. 33, 1748, 1758, 1767, 1773, 1974*).

Note: See Second Report of the ad hoc Committee on Standards for Nutritional Studies.

AIN MINERAL MIXTURE 76

Composition:	gm/Kg of mixture
Calcium Phosphate, Dibasic ($CaHPO_4$)	500.00
Sodium Chloride (NaCl)	74.00
Potassium Citrate, Monohydrate ($HOC(COOK)(CH_4COOK)_4 \cdot H_2O$)	220.00
Potassium Sulfate (K_2SO_4)	52.00
Magnesium Oxide (MgO)	24.00
Manganous Carbonate (43-48% Mn)	3.50
Ferric Citrate (16-17% Fe)	6.00
Zinc Carbonate (70% ZnO)	1.60
Cupric Carbonate (53-55% Cu)	0.30
Potassium Iodate (KIO_3)	0.01
Sodium Selenite ($Na_2SeO_3 \cdot 5H_2O$)	0.01
Chromium Potassium Sulfate ($CrK(SO_4)_2 \cdot 12H_2O$)	0.55
Sucrose, finely powdered	118.00

AIN VITAMIN MIXTURE 76

Composition:	per Kg of mixture
Thiamine HCl	600.0 mg
Riboflavin	600.0 mg
Pyridoxine HCl	700.0 mg
Nicotinic Acid	3.0 gm
D-Calcium Pantothenate	1.6 gm
Folic Acid	200.0 mg
D-Biotin	20.0 mg
Cyanocobalamin (Vit. B-12)	1.0 mg
Retinyl Palmitate (Vit. A), Pre-mix (250,000 IU/gm)	1.6 gm
dl-α-Tocopheryl Acetate	

Table 2.1 Composition of the vitamin D deficient diet.

decapitation after light ether anaesthesia. Using scissors, the vertebral column was separated from the animal and the spinal cord removed. The cords were put into Sorval centrifuge tubes containing the appropriate medium and kept on ice. Approximately 400 mg of spinal cord was obtained from a 250 gm rat and one spinal cord homogenized in 700 µl of medium produced a soluble fraction with a concentration of about 4.5 mg/ml protein.

2.2.2 Homogenizing Medium.

250 mM sucrose was used as the homogenizing medium. It was prepared in 250 ml volumes and kept refrigerated. In the experiments where the homogenizing medium was changed, the additives were pipetted from stock solutions adjusted to pH 7.2. The protease inhibitor PMSF (phenylmethanesulfonylfluoride) was purchased from Sigma Chemical Co., St.Louis, U.S.A..

2.2.3 Solubilization.

Using a Brinkman polytron set at maximum speed, the tissue was homogenized on ice with 4-5 strokes of about three seconds each. The polytron tip was cleaned after each tube to prevent cross contamination. The homogenate was centrifuged; first at 800 X g for 15 minutes then twice at 100 000 X g for 60 minutes each. The supernatant was collected after each centrifugation step and transferred to a new tube. The final

collected sample was about 250 µl in volume. This was pipetted into a glass tube, labelled and kept in the freezer (-20°C) if it was not used immediately.

2.2.4 Concentration of Samples.

Only when necessary, samples were concentrated using vacuum dialysis on ice with 800 nm pore size (12 000 MW cutoff) collodion bags. Although proteins smaller than the cutoff such as $CaBP_{9K}$ would be lost during dialysis, there was no apparent difference between the PAGE profile of concentrated samples and those that were not dialysed.

2.3 Polyacrylamide Gel Electrophoresis.

2.3.1 Preparation and Running of Polyacrylamide Gels.

A discontinuous buffer system was used for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) by the method of Laemmli (1970) as described by Hames and Ricwood (1981). 250 µl samples were prepared in the following way: an aliquot of the cytosolic fraction was diluted in a solution of 2% SDS, 1% DTT (dithiothreitol), 0.08% tracking dye (bromophenol blue), 10% glycerol and completed to 250 µl using reservoir buffer. The samples were immersed in boiling water for 5 minutes and allowed to cool to room temperature prior to loading into wells.

Polyacrylamide gels (1.5 mm thick) of 5% stacking and 10% separating gel were prepared from 30% acrylamide stock solution, 0.38M Tris-HCl (pH 8.8, separating gel buffer), 0.125M Tris-HCl (pH 6.8, stacking gel buffer)

and SDS (0.1% in separating gel, 0.05% stacking gel). Polymerization was catalyzed using TEMED (N,N,N^1,N^1,-tetramethylethylenediamine) and ammonium persulfate.

The reservoir buffer contained 0.0075M SDS plus 0.41M glycine and was adjusted to pH 8.3 with Tris-Base. Ten well slab gels with 100 µl sample/well were run at 12.5 mA/gel through the stacking gel and 35 mA/gel through the separating gel.

The following molecular weight standards (Biorad Chemical Division, Richmond, Cal. 94804) were used: phosphorylase B (92 500), bovine serum albumin (66 200), ovalbumin (45 000), carbonic anhydrase (31 000), soybean trypsin inhibitor (21 500), and lysozyme (14 400).

2.3.2 Staining of Gels.

The gels were fixed in solutions of 10% acetic acid and 25% isopropyl alcohol for 24 hours, then with 10% acetic acid and 10% isopropyl alcohol for 24 hours, and finally with 10% isopropyl alcohol for 12 hours. Duplicate gels were stained individually in solutions of Coomassie Brilliant Blue (Reisner, Nemes and Bucholtz, 1975) or the light sensitive carbocyanine dye (Stains-all) (Campbell, MacLennan and Jorgensen, 1983).

The carbocyanine dye (Sigma Chemical Co., St.Louis); was prepared as described by Campbell, MacLennan and Jorgensen (1983). This procedure has been optimized for blue staining of calcium binding proteins. The gels were stained by shaking overnight in a plastic box (19x14x16cm) containing 250 ml of solution and wrapped in aluminium foil.

The duplicate gel was stained for 30 minutes with 200 ml 0.5% coomassie brilliant blue (Biorad R-250) in a pyrex dish (22x12x5cm) and by

shaking. The coomassie stained gels were destained using 250 ml of 20% acetic acid 10% methanol solution, changed frequently over a 12 hour period.

2.3.3 Drying of Gels.

Gels were dried at 80°C using the Biorad Slab Gel Drier. The drier was connected to a vacuum pump through a 1000 ml flask which was immersed in methanol cooled with dry ice. Biorad Filter Paper Backing was wetted and spread on the drier. The gel was placed onto the backing directly from the solution in which it was sitting and covered with Biorad Dialysis Membrane Backing.

2.3.4 Densitometry.

Densitometry of the carbocyanine dye stained gels were performed in the dark. The absorbance pattern at 605 nm, of gel tracks were produced using an EC910$_1$ densitometer and a Pharmacia pen recorder (Pharmacia, Uppsala, Sweden). Different proteins will bind the carbocyanine dye to different extents, hence quantitative determination of the amount of a particular protein after it is stained requires a standard curve for that protein. Because none of the proteins were purified, it was not possible to produce a standard curve thus the relative quantitation of protein bands by this method is not precise.

2.3.5 Photography of Gels.

Photographs were taken in the department using gels directly from destaining (Coomassie Blue stained gels) or the carbocyanine dye solutions. Gels were illuminated from beneath and during photography of the carbocyanine dye stained gels all ambient light was removed. Kodacolor VR100 or Ektachrome 200 film was used with a yellow filter. Processing of the film was done at a commercial photography laboratory.

2.4.1 Calmodulin, Parvalbumin and Radioactive Calcium.

The radioactive calcium was purchased from ICN Biomedicals (ICN Biomedicals Inc., Irvine California 92715). The radionuclidic purity is 99+% and the specific activity of the lots that were used range from 6.18-9.42 mCi/mg calcium. Calmodulin (phosphodiesterase 3':5-cyclic nucleotide activator from bovine brain) was bought from Sigma as was rabbit muscle parvalbumin.

2.5 Assays and Molecular Weight Determination.

2.5.1 Determination of Protein Concentration.

Protein concentrations were determined with a modified Lowry assay (Lowry, Rosebrough, Farr and Randall, 1951). Two percent sodium carbonate in H_2O (solution A), 1% copper sulfate (solution B_1), 2% sodium citrate (solution B_2), 1N sodium hydrozide, and phenol reagent diluted 1:2 with H_2O were used. 200 µl of the protein sample dilutions

were made alkaline with 200 µl of 1N sodium hydroxide. After 10 minutes, 2 ml of solution C (98% solution A, 1% solution B_1, 1% solution B_2) was added. 10 minutes later 200 µl of the folin reagent was pipetted and the absorbance was read at 750 nm 20 minutes afterwards.

2.5.2 Serum Assays.

Calcium and phosphorus in the serum were measured using specific diagnostic kits from the Lancer division of Sherwood Medical, St.Louis, MO. Serum parathyroid hormone (PTH) was measured with the midmolecule radioimmunoassay kit (Immuno Nuclear Corporation, Stillwater, MN). No serum assays for vitamin D were conducted, as this animal model has been repeatedly tested in this laboratory for serum vitamin D and has been shown to be vitamin D deficient (Glijer, Peterfy and Tenenhouse, 1985).

2.5.3 Determination of Protein Band Molecular Weights.

1:20 diluted Biorad low molecular weight protein standards were run adjacent to samples on each gel. With the relative mobility (Rf=distance migrated by the band/distance migrated by the dye front) of these standards and the log of their known molecular weights, a linear regression program of the Hewlett Packard model 33E calculator was used to form a standard curve. Using individual curves for each gel and the calculator program, the molecular weight of identifiable bands was determined by their relative mobility. The mean of separate molecular weight determinations was used as the molecular weight of that band.

2.6 Detection of Calcium Binding Proteins.

2.6.1 Batch Chromatography.

Batch chromatography was performed on ice. In a Sorval tube 1.5 ml of DEAE Cellulose (Whatman) was equilibrated to pH 8.2 by washing 10 times with 5 ml of phosphate buffer. 900 µg of protein sample (400 µl) was added. The tube was sealed with parafilm and inverted repeatedly over 10 minutes to allow mixture of the sample with the gel. The mixture was centrifuged (≈ 1 min, 800 X g) and the supernatant collected for analysis. This process was repeated with 500 µl of 0.025, 0.05, 0.1, 0.2 and 0.5M sodium chloride solutions, the supernatant being collected each time for analysis.

2.6.2 Barium Sulfate Precipitation of Proteins.

Barium sulfate precipitation of proteins was performed on ice by a modification of the method of Li and Olson (1967). 35 mg/ml of Barium sulfate was added to the 100 000 X g supernate in a conical tube, and covered with parafilm. The mixture was inverted intermittently over a period of 2 hours. The solution was transferred to a centrifuge tube and spun for 20 minutes at 1 500 X g and 4°C. The pellet was washed twice with 0.25 times supernate volume of 0.1M potassium oxalate in 0.15M sodium chloride solution. Using a fine tip spatula the pellet was stirred in the potassium oxalate during each washing and then centrifuged for 2 minutes at 800 X g. The resultant pellet was washed twice, using the same technique as above in 0.25 times supernate volumes of 0.15M sodium chloride

solution. Protein was eluted from the Barium sulfate by a wash with 0.25 times supernate volume of 0.2M sodium citrate solution. During this wash the pellet was disrupted using a fine tip spatula and inverted intermittently over a 1 hour period. The mixture was centrifuged at 800 X g for 2 minutes and the supernatant collected.

2.6.3 Detection of Calcium Binding Proteins by ^{45}Calcium Radioautography.

All incubations were done at room temperature with solutions prepared that day. Following electrophoresis ^{45}calcium binding was performed as described by Anthony and Babitch (1984) which is a modification of two previously reported methods (Schibeci and Martonosi, 1980; Maruyama, Mikawa and Ebashi, 1984). A non-stained gel was put into a plastic box (19x14x6cm) and washed for 24 hours by shaking with 250 ml of 25% isopropyl alcohol-10% acetic acid, then with 250 ml of 10% isopropyl alcohol-10% acetic acid for a second 24 hours. A third wash was performed in 250 ml of H_2O for 30 minutes. For 60 minutes the gels were incubated with 0.4mCi/L of ^{45}calcium chloride with constant shaking, in non SDS reservoir buffer (pH 8.4) containing 1mM magnesium chloride and 0.1M potassium chloride. The gel was then washed with non SDS buffer, containing 1mM magnesium chloride and 0.1M potassium chloride, by shaking for 80 minutes. A final wash was performed in 250 ml of H_2O by shaking for 10 minutes. The gels were dried and exposed to Kodak X-AR film for varying lengths of time (2-7 days).

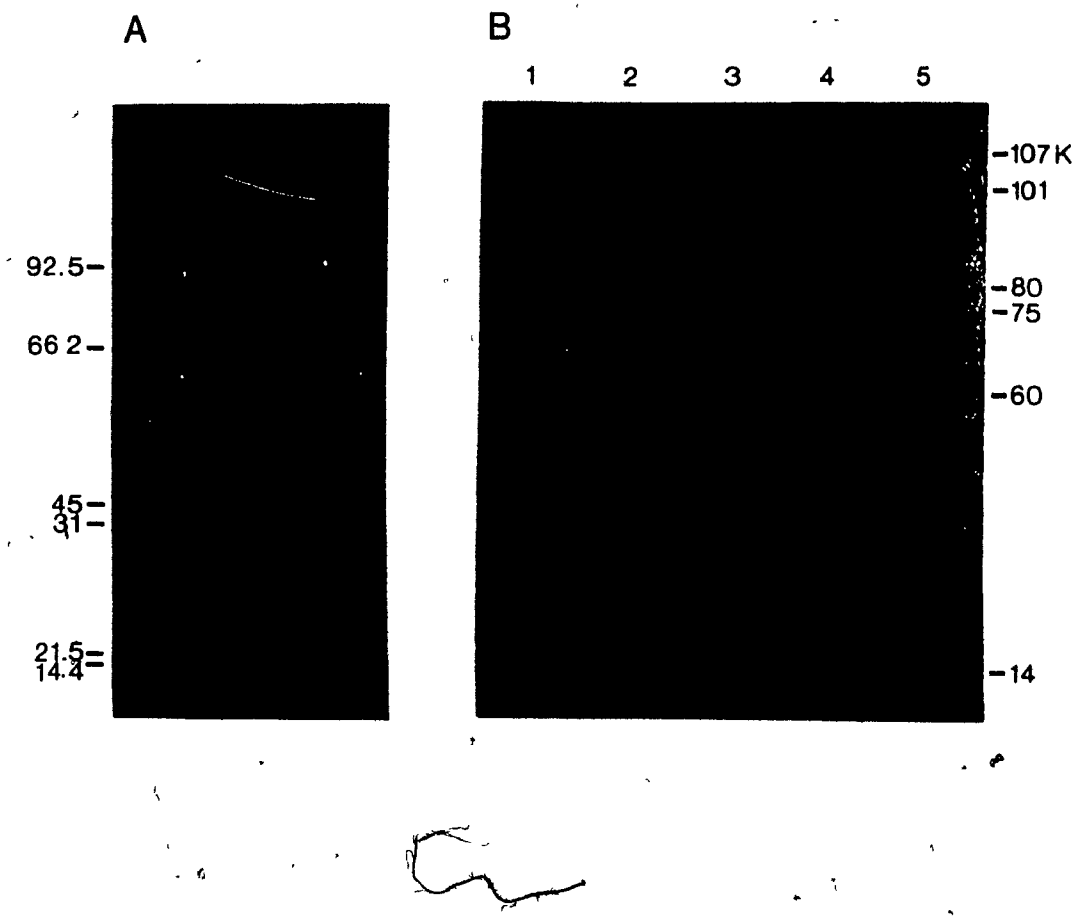
RESULTS

3.1.1 Polyacrylamide Gel Electrophoretic Profile of the Rat Spinal Cord.

Soluble protein from spinal cord was subjected to PAGE and stained with the carbocyanine dye to determine if any bands will stain blue. Female and male spinal cords from animals of ages between 21 and 90 days old were analyzed. Gels stained with the carbocyanine dye exhibited both pink and blue bands although there were fewer bands apparent than on coomassie blue stained gels. The bands that stain blue are believed to represent interaction of the carbocyanine dye with macromolecules in a one-to-one ratio of dye to anion site and have absorbance maxima between 600-615 nm (Bean, Shepard, Kay and Walwick, 1965; Campbell, MacLennan and Jorgensen, 1983). Calmodulin was run on a gel and stained with the carbocyanine dye. It appears as a blue band at the dye front (Figure 3.1 A).

Tissue obtained from male and female rats were indistinguishable from one another on gels stained with either coomassie blue or the carbocyanine dye (Figures 3.1 and 3.2). Protein samples from spinal cords of rats younger than 49 days show five blue staining bands on gels stained with the carbocyanine dye (Figures 3.1 B and 3.2 A). These bands are of the following estimated molecular weights: 107, 101, 75, 60 and ≃ 14 kilodaltons. The molecular weights are the means calculated from a statistical sample of 222-260 rats. Samples from rats older than 49 days exhibit an additional band of 80 kilodaltons, but no 28 kilodaltons blue staining band was detected in either age group (Figure 3.3 A,B). Duplicate gels stained with coomassie blue show no difference between spinal cords from young or mature animals (Figures 3.1 C and 3.2 B).

Figure 3.1. A,B,C. PAGE of the soluble fraction of spinal cord proteins from male rat, and of calmodulin. These are 10% acrylamide gels (29:0.8 ratio of acrylamide: bis-acrylamide) and migration is toward the anode. (A) Calmodulin run adjacent to protein standards and stained with the carbocyanine dye. (B) Gel stained with the carbocyanine dye. In lanes 1, 2, 3 are samples from 21, 35 and 42 day old rats. In lanes 4 and 5 are run samples from 49 and 56 day old rats. An 80 kilodaltons blue band appears in the 49 and 56 day animal samples. (C) Coomassie blue stained duplicate to the gel shown in Figure 3.1 A.


A
B
1
2
3
4
5
92.5
66 2
45
31
21.5
14.4
107K
101
80
75
60
14

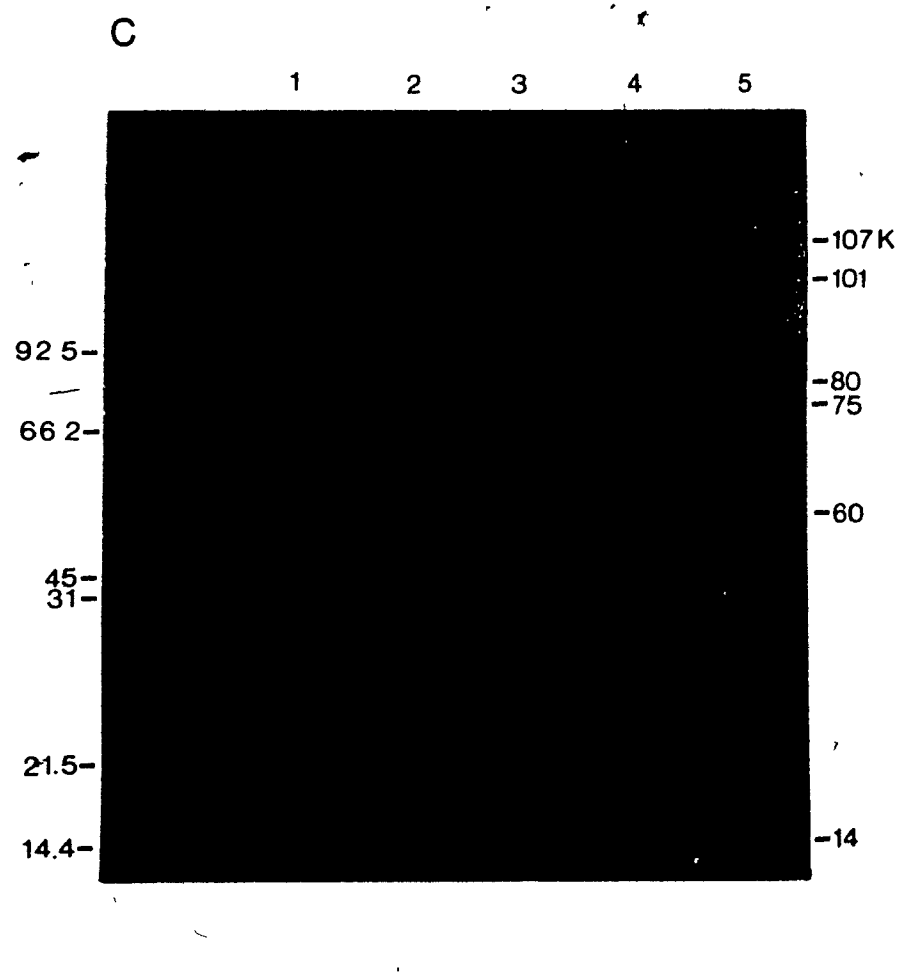
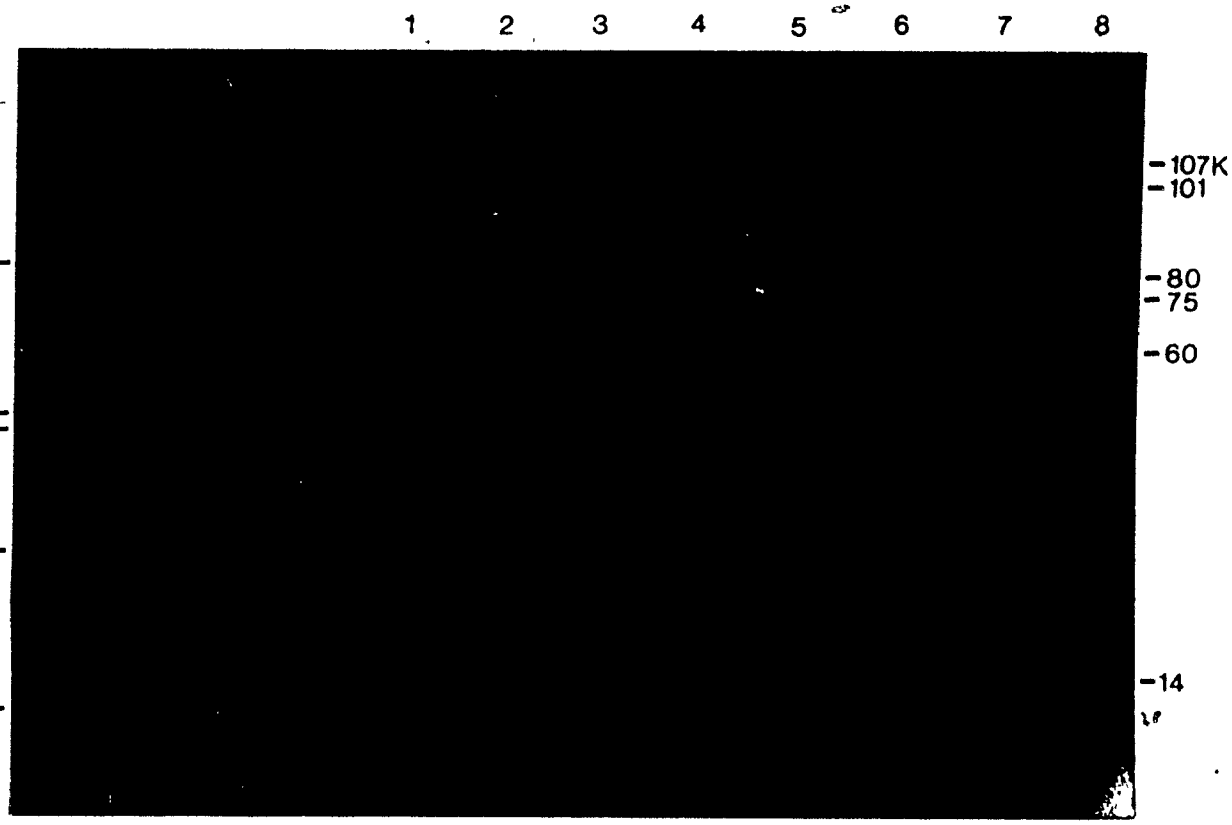

C
1
2
3
4
5
92 5
66 2
45
31
21.5
14.4
107K
101
80
75
60
14

Figure 3.2 A,B. Separation of female rat spinal cord proteins as described in Figure 3.1. Lanes 1, 2, 3, 4, 5, 6, 7 and 8 represent samples from 21, 28, 35, 42, 49, 56, 63 and 70 day old rats respectively. (A) Gel stained with the carbocyanine dye. An 80 kilodaltons blue band is present in samples from 49, 56 and 63 day old rats. (B) Coomassie blue stained duplicate to the gel shown in Figure 3.2 A.

A


1
2
3
4
5
6
7
8
92.5
45
31
21.5
14.4
107K
101
80
75
60
14

B

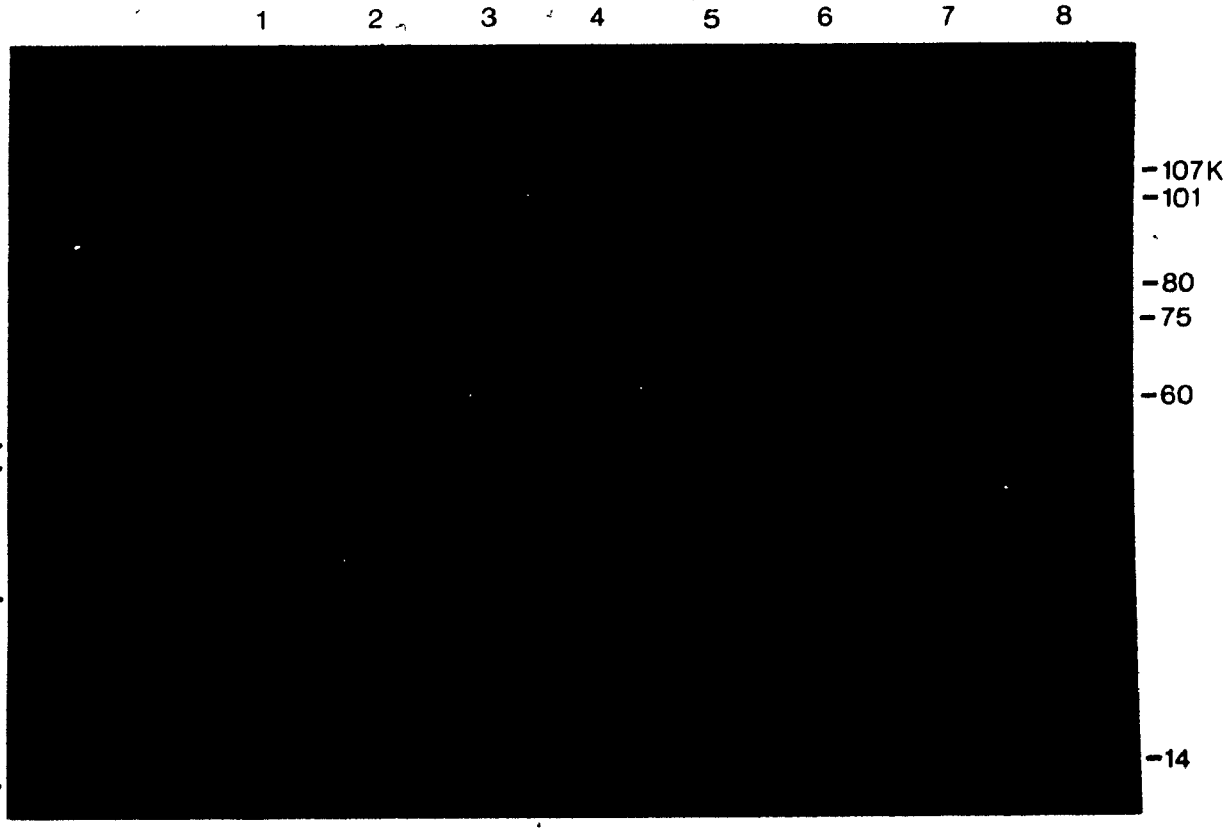
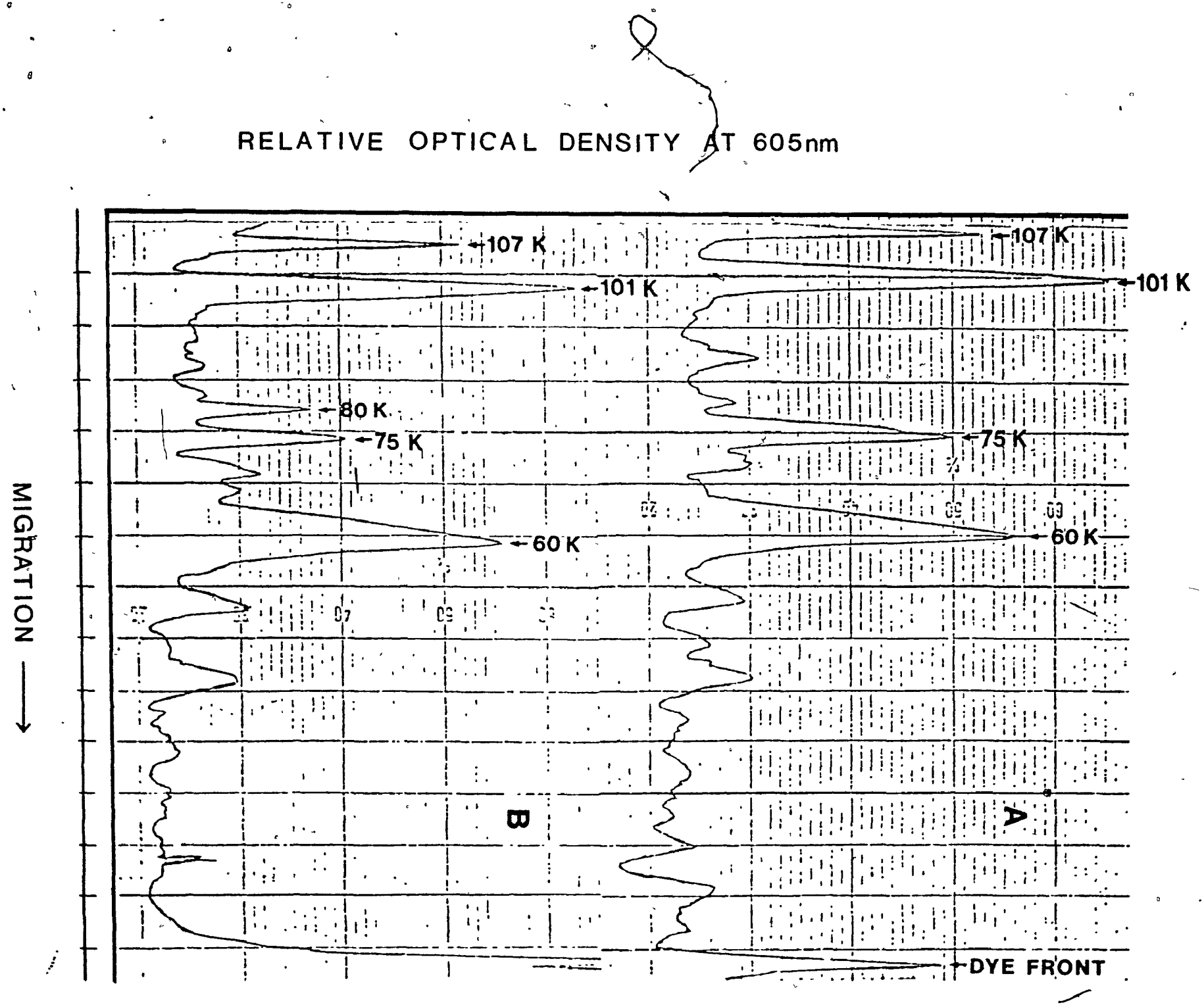

1
2
3
4
5
6
7
8
92.5
66.2
45
31
21.5
14.4
107K
101
80
75
60
14

Figure 3.3 A,B. Densitometric analysis of gels stained with the carbocyanine dye. (A) Sample from a 31 day old male rat. (B) A sample taken from a 52 day old male rat, showing both 80 and 75 kilodalton bands.


RELATIVE OPTICAL DENSITY AT 605nm
MIGRATION
107 K
101 K
80 K
75 K
60 K
107 K
101 K
75 K
60 K
B
A
DYE FRONT

3.1.2 Effect Produced on the PAGE Profile by Different Homogenizing Media.

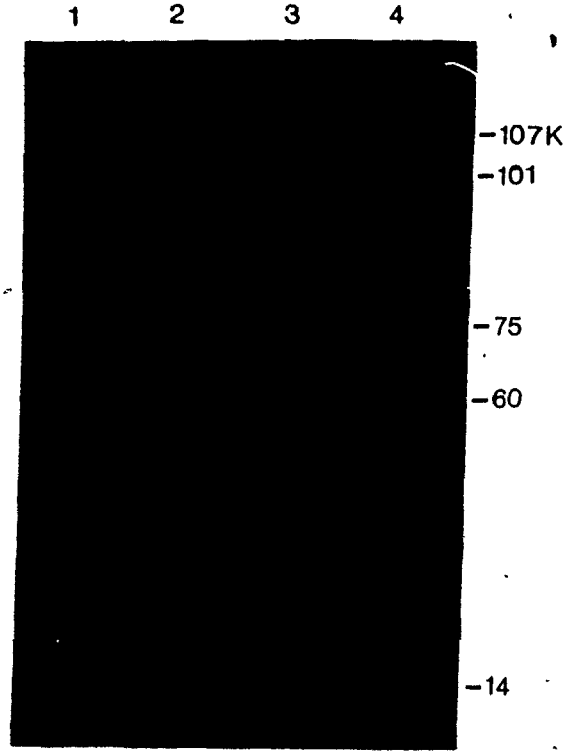
The original medium used was 0.25M sucrose a non-ionic solution isotonic with plasma. Divalent cations are known to affect the activity of hydrolytic enzymes (Fullmer, Wasserman, Hamilton, Huang and Cohn, 1975; Van Eldik, Zendegui, Marshak and Watterson, 1982) that alter the tertiary structure of the calcium binding proteins and divalent cations may interfere with the staining of macromolecules by the carbocyanine dye (Caday and Steiner, 1985). Once the PAGE profile of the spinal cord in the absence of added cations was established, experiments were conducted to determine what effect added cations or chelation of metal ions have on this profile.

Four divalent cations were tested separately: 1 mM calcium chloride, 5 mM magnesium chloride, 5 mM manganese chloride, 1 mM barium chloride and the chelator of metal ions EDTA (ethylenediamine tetraacetate) was tested at 2 mM concentration.

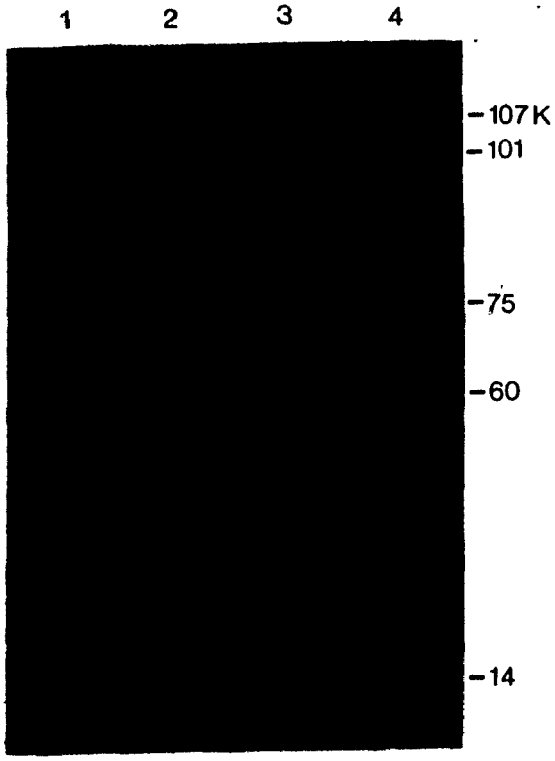
This experiment demonstrated that, the PAGE pattern of soluble extract from spinal cord tissue homogenized in medium without added divalent cations, is different from tissue homogenized with added cations (Figure 3.4 A,B). Samples of the 0.25M sucrose and sucrose with 2 mM EDTA media stain identically. The 107 and 101 kilodalton bands are not visible on gels stained with either the carbocyanine dye or coomassie blue, when divalent cations are added to the homogenizing medium (lanes 2 and 4 in Figure 3.4 A,B). When the tissue is homogenized with added divalent cations, there is no apparent blue band at 75 kilodaltons when the gel is stained with the carbocyanine dye, and at 60 kilodaltons the blue staining is weaker on both coomassie and carbocyanine dye stained gels (lanes 2 and 4 in Figure 3.4 A and B). Of the media that were tested 0.25 M sucrose

Figure 3.4 A,B. The effect produced on the PAGE profile of the spinal cord soluble fraction by different homogenizing media. Samples run in lanes 2, 3 and 4 had in the homogenizing medium, in addition to 0.25 M sucrose, 1 mM calcium chloride, 2 mM EDTA and 5 mM magnesium chloride respectively. (A) Gel stained with the carbocyanine dye. The 107, 101 and 75 kilodalton blue bands are not visible and blue staining at the 60 kilodaltons site is weaker in the samples to which divalent cations were added to the homogenizing medium. (B) Coomassie blue stained duplicate to the gel shown in A. The staining in the 60 kilodaltons area is reduced and the 107 and 101 kilodalton bands have not stained in lanes 2 and 4.

A



B



and 0.25 M sucrose with 2 mM EDTA produced the greatest number of blue bands in carbocyanine dye stained gels. There was no apparent advantage to adding EDTA thus 0.25 M sucrose by itself was used as the homogenizing medium. In this system too many bands are present to determine if there is a migration shift of any macromolecule as described by Burgess and coworkers (1980) for calmodulin after it has bound calcium.

3.1.3 Use of Protease Inhibitor.

Addition of the protease inhibitor PMSF (phenylmethane-sulfonylfluoride) to produce a final concentration in the homogenization medium of 0.1 mM, did not alter the spinal cord soluble fraction pattern as seen on PAGE. The samples shown in Figure 3.4 A and Figure 3.4 B contain PMSF. Note that the sucrose lane profile is not different from previous figures in which PMSF was not present, hence no PMSF was used in subsequent experiments.

3.1.4 Spinal Cord PAGE Profile of "Cervical", "Thoracic" and "Lumbar" Segments.

To test for any regional differences in the blue bands produced by the carbocyanine dye, spinal cords were separated into three segments by cutting at the level of the first and the last rib dividing the cord into: "cervical" (most proximal), "thoracic" (middle), and "lumbar" (distal) segments. Soluble protein extracts from each segment were run separately. There was no difference among these three levels or between any of these divisions and the entire cord (Figure 3.5).

3.2.1 Polyacrylamide Gel Electrophoretic Profile of Rat Cerebellum.

Based on the fact that calcium binding proteins on SDS-gels are stained blue by the carbocyanine dye (Campbell, MacLennan and Jorgensen, 1983), it should be possible to demonstrate a blue staining band corresponding to $CaBP_{28K}$ (Thomasset, Parkes and Cuisinier-Gleizes, 1982) in the PAGE profile of the soluble fraction from rat cerebellum.

The cerebellum from rats of the following ages: 28, 35, 42, 49, 56, 63 and 70 days were prepared as described in the methods for the spinal cord. 300 µg of total soluble protein was run in each well on the rational that this quantity permitted good separation of proteins in the spinal cord experiments. The concentrations of $CaBP_{28K}$ in the cerebellum is reported to be 15 µg/mg total soluble protein (Baimbridge, Miller and Parkes, 1982) hence it is estimated that 4.5 µg of $CaBP_{28K}$ is present per lane. 4 µg of calsequestrin stains very strongly with this dye (Campbell, MacLennan and Jorgensen, 1983).

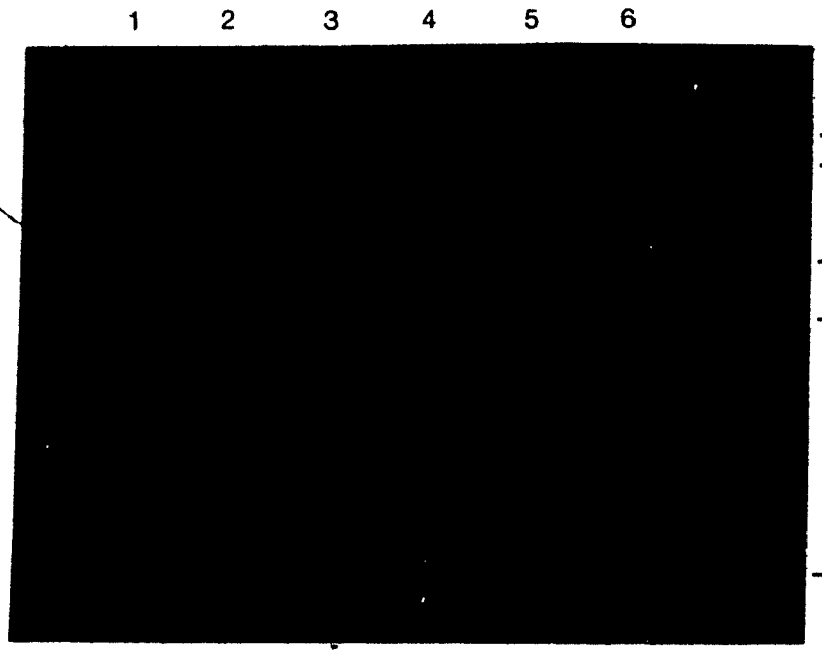
The PAGE profile showed four blue staining bands of the following estimated molecular weights: 116, 51, 28 and ≈ 14 kilodaltons (Figure 3.6). These four bands were present in samples from rats of all the ages between 28 and 70 days that were tested.

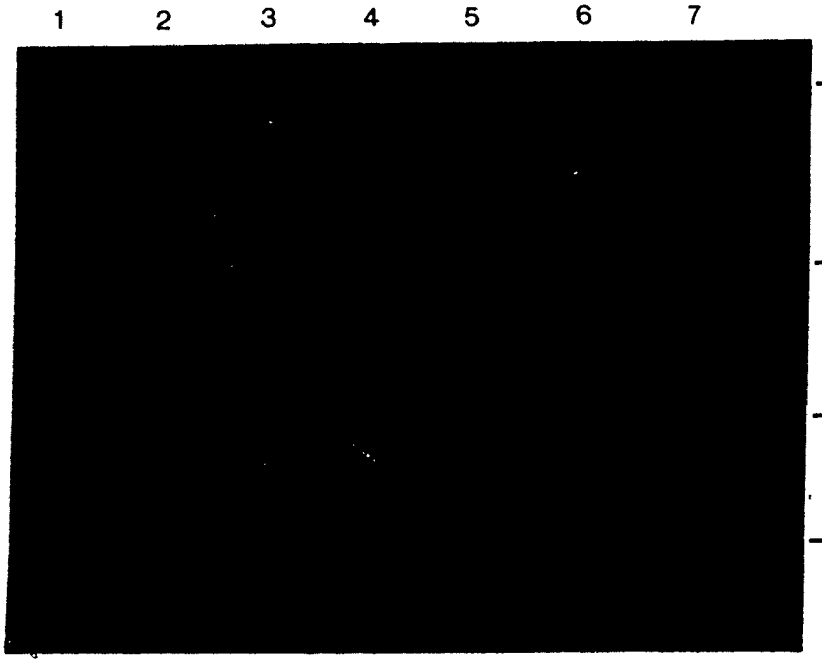
The 28 kilodaltons band can represent $CaBP_{28K}$. This band does not correspond to the molecular weight of any other soluble calcium binding protein of the rat central nervous system that is described in the literature (Van Eldik, Zendegui, Marshak and Watterson, 1982).

3.3 Partial Purification of Bands that Stain Blue with the Carbocyanine Dye.

Figure 3.5. Separation of the soluble fraction from spinal cord segments of two 40 day female rats. The first three lanes from left to right are "cervical", "thoracic" and "lumbar" segments of one rat. The next three lanes represent samples from another rat, run in the same order.

Figure 3.6. The soluble fraction of male rat cerebellum separated by PAGE as described in Figure 3.1. Each lane represents the sample from a rat 7 days older than the one run in the lane to the immediate left. The sample from a 28 day rat is run in lane 1.


1
2
3
4
5
6
-107K
-101
-75
-60
-14


1
2
3
4
5
6
7
-116K
-51
-28
-14

3.3.1 Anion Exchange Batch Chromatography.

Batch chromatography, using DEAE-cellulose (diethylamino-ethyl-cellulose), was performed on the soluble fraction of spinal cord homogenates from 60 day old male rats in an attempt to partially purify proteins that stain blue with the carbocyanine dye. This experiment demonstrates that the blue staining bands are only eluted off the anion exchange resin with 0.5 M sodium chloride, suggesting that they are highly acidic proteins (Figure 3.7 A). Acidity of the protein however does not correlate with blue staining, a finding that has been reported previously (Campbell, MacLennan and Jorgensen, 1983).

3.3.2 Barium Sulfate Precipitation of Proteins.

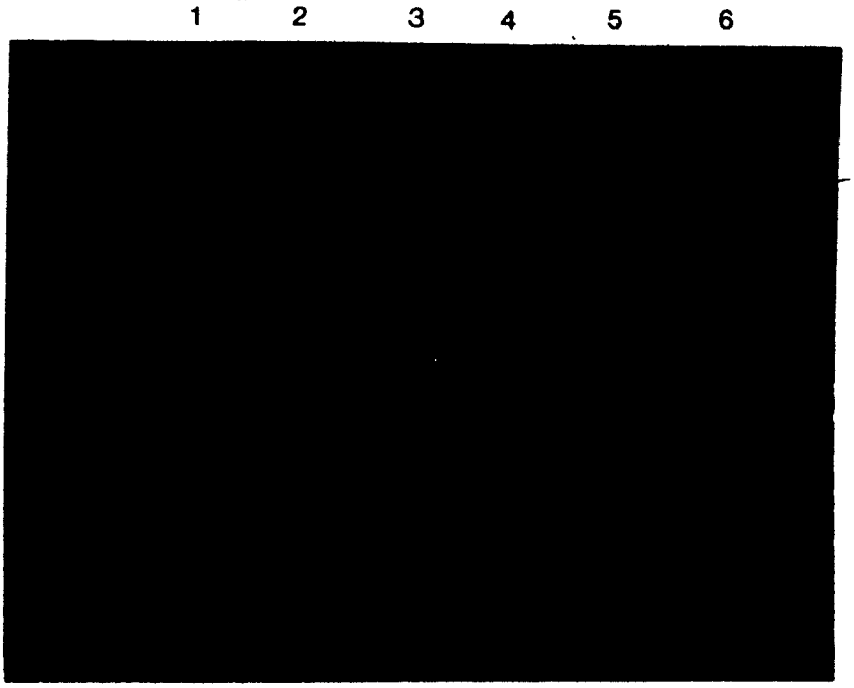
Barium sulfate is used to precipitate proteins that contain a high proportion of gamma-carboxyglutamic acid residues. It is a method most frequently used for the purification of clotting factors from plasma (Malhotra, 1979; Lian, Reit, Roufosse, Glimcher and Gallop, 1979). With this technique it was possible to demonstrate that only macromolecules which form the J complex with the carbocyanine dye are barium sulfate precipitable (Figure 3.8 A).

3.4.1 Binding of Radioactive Calcium to Bands on Polyacrylamide Gels.

A radioautographic method which has been shown to detect calcium

Figure 3.7 A,B. Elution of J complex forming bands off DEAE cellulose. Standards are run on the far left. Non-adsorbed macromolecules are in lane 1, subsequent lanes show proteins eluted using 0.025, 0.05, 0.1, 0.2 and 0.5 M sodium chloride. The 107 and 101 kilodalton bands are not apparent in any of the collected fractions, indicating that they have either not eluted off the DEAE cellulose or that they have deteriorated. (A) Gel stained with the carbocyanine dye and (B) its coomassie blue stained duplicate.

A



B

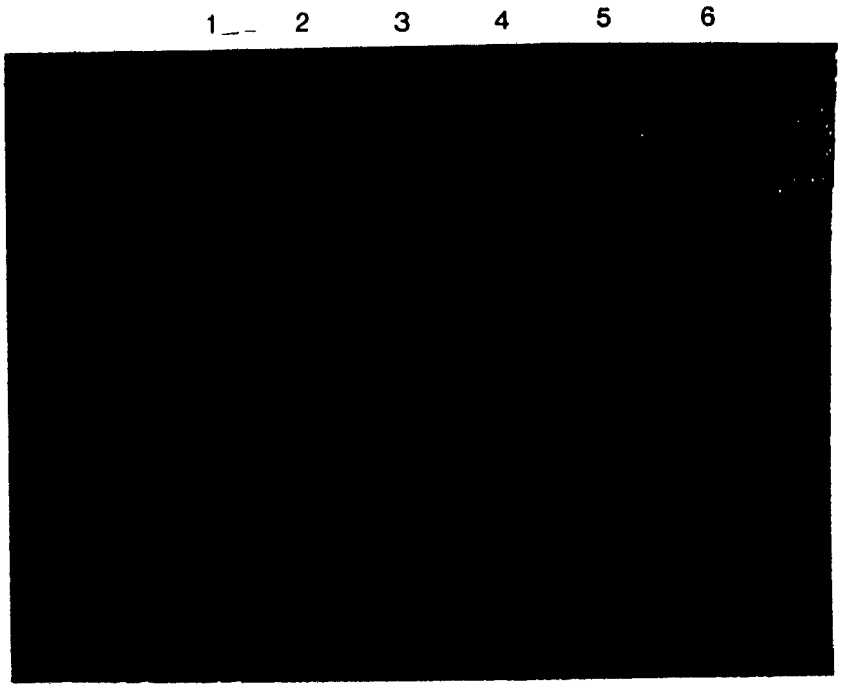
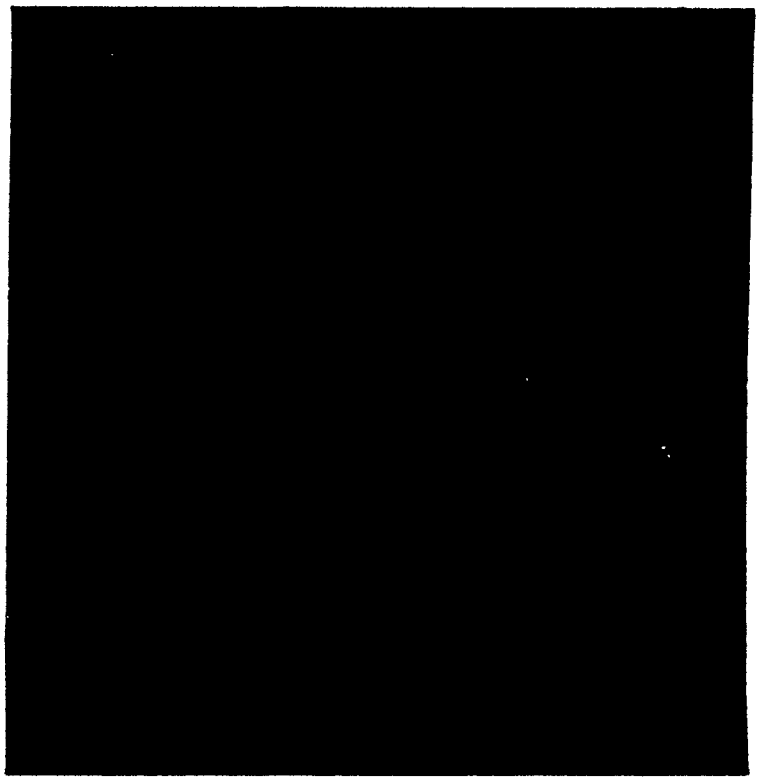
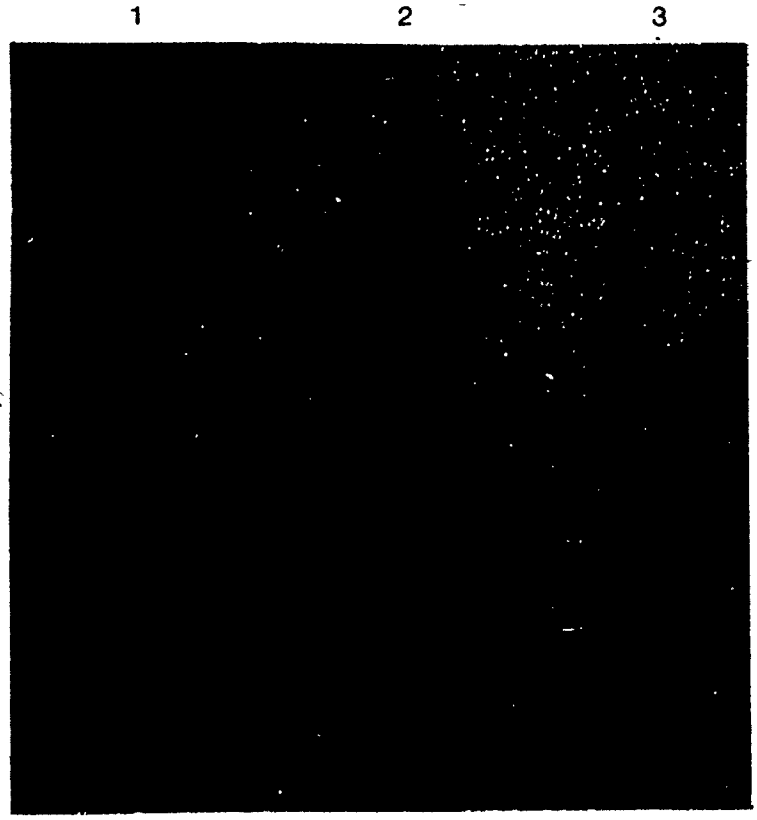


Figure 3.8 A,B. Barium sulfate precipitated proteins. (A) Carbocyanine stained gel with standards run in the first lane. The fraction of proteins that precipitated from solution are run in lane 3 and the rest in lane 2. (B) Coomassie blue stained duplicate to the gel described in A. The high molecular weight bands are missing, indicating that they are either strongly adsorbed to barium sulfate and can not be eluted even with the 0.2 M sodium citrate that was used, or that they have deteriorated.

A



B



binding proteins in polyacrylamide gels (Schibeci and Martonosi, 1980; Anthony and Babitch, 1984) was used to determine whether the carbocyanine dye stained blue bands bind ^{45}calcium. 50, 100, 250, and 500 µg of total protein from soluble extracts of spinal cord homogenates were run in separate lanes on a 10% acrylamide gel. No labelling above background was detectable in lanes containing 50 and 100 µg of total protein. The lane in which 500 µg of protein was run had about twice the quantity of radioactive calcium bound as the lane containing 250 µg of protein (Figure 3.9). The labelling is markedly higher than the background, resembles a PAGE band, and is absent from the lane in which standards were run (Figure 3.10, lane 1). It is specific to lanes in which protein samples were run and directly dependent on the quantity of protein run in the lane (Figure 3.9). These factors argue against any artefactual precipitation of the radioactive calcium or binding to the tracking dye.

When a radioautographic experiment was performed on a 10% acrylamide gel in which barium sulfate precipitable proteins were run separate from non barium sulfate adsorbed proteins, again only one band was labelled (Figure 3.10). The band was in the lane with the barium sulfate precipitable proteins and it had a relative mobility of 1. Although this calcium binding band found in the spinal cord of the rat stains blue with the carbocyanine dye, every blue staining band does not bind calcium:

Figure 3.9. Radioautograph of a gel with different amounts of total spinal cord soluble fraction protein in each lane. Lanes 1, 2, 3 and 4 have 500, 250, 100 and 50 µg of sample respectively. Labelling can be detected only in the lanes with 250 and 500 µg of total protein.

Figure 3.10. Radioautograph of a similar gel to that shown in Figure 3.8 A. The labelled band is in the lane with barium sulfate precipitable proteins. Standards are run in lane 1 and show no labelled bands.

1 2 3 4

1 2 3

3.5 Calcium Binding Proteins of the Vitamin D Deficient Rat.

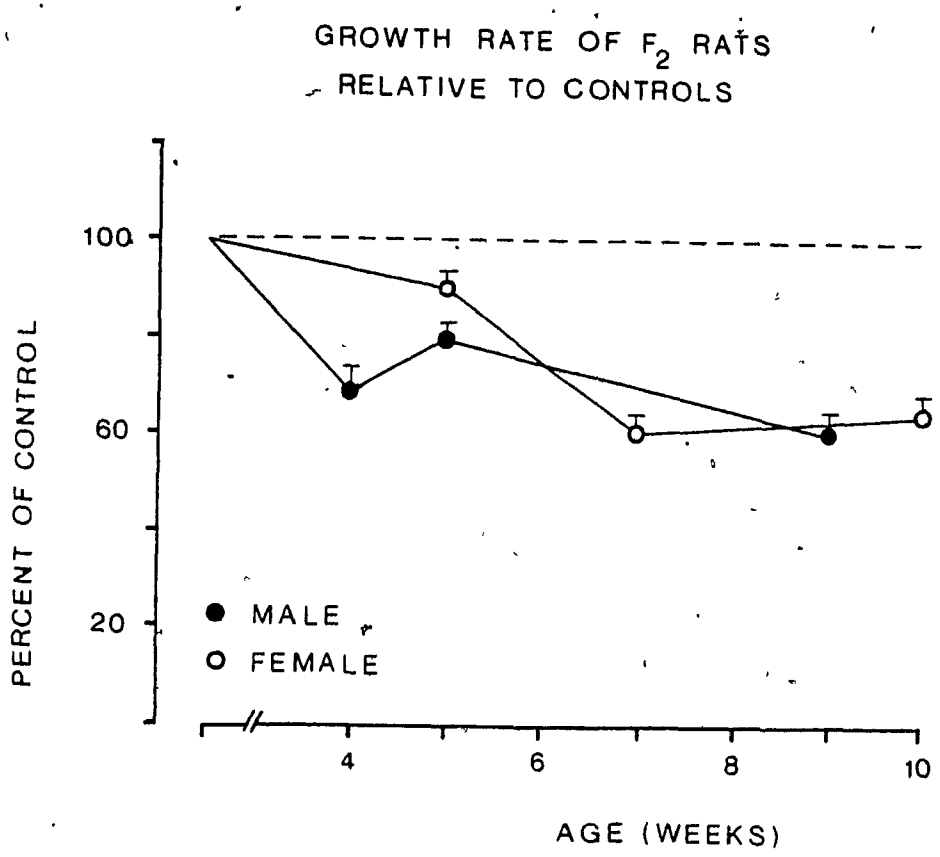
3.5.1. The Vitamin D Deficient Rat Model.

The parental and the F_1 generation rats show retarded growth such that after 8 weeks on the vitamin D free diet they are less than 70% the weight of rats on purina rat chow (Glijer, Peterfy and Tenenhouse, 1985). At 8 weeks both generations have calcium in their serum that is about one half the normal concentration (Glijer, Peterfy and Tenenhouse, 1985). Their serum phosphorus concentration is normal and they have secondary hyperparathyroidism as evidenced by the high level of parathyroid hormone (PTH) in their serum (Glijer, Peterfy and Tenenhouse, 1985).

Disappearance of 1,25-dihydroxycholecalciferol in the serum of rats from the parental generation is slow. After 8 weeks on the vitamin D free diet the serum concentration of 1,25-dihydroxycholecalciferol is 13.3 pg/ml, approximately 25% of that found in the serum of control rats (Glijer, Peterfy and Tenenhouse, 1985). Twelve weeks after they are started on the vitamin D deficient diet, 1,25-dihydroxycholecalciferol is still detectable in the serum of parental generation females although in amounts close to the detection limit of the assay (10 pg of 1,25-dihydroxycholecalciferol in a 10 ml plasma sample). The F_1 generation has no detectable 1,25-dihydroxycholecalciferol in their serum (Glijer, Peterfy and Tenenhouse, 1985).

Symptoms of severe vitamin D deficiency are apparent in the F_2 rats. Animals of this colony exhibit retarded growth (Figure 3.11), have an awkward gait and are the only generation on the vitamin D free diet that develop full motor seizures. F_2 rats are severely hypocalcemic, with plasma calcium one half of controls (Table 3.1), they have PTH levels in the plasma elevated 3 fold and their phosphate is normal (Table 3.1).

Figure 3.11. Body weight of F_2 rats as a percent of controls. Each point represents a mean $\pm$ the standard error of the mean (N = 4-25).



VITAMIN D STATUS	SERUM CALCIUM	SERUM PHOSPHORUS	SERUM PARATHYROID HORMONE
	mg/dl	mg/dl	pmole/L
D+	10.40 ± 0.35 (3)	9.17 ± 1.01 (3)	62.4 ± 14.30 (3)
D-	4.52 ± 0.32 (11)	7.93 ± 0.45 (4)	180.0 ± 25.50 (4)

Table 3.1 Serum concentration of calcium, phosphorus and parathyroid hormone in control and vitamin D deficient rats used in this study. The N values are given in brackets.

3.5.2 Binding of Radioactive Calcium to Parvalbumin and the Soluble Fraction of Cerebellum from Vitamin D Replete and Deficient Rats.

A known calcium binding protein was used to test this radioautographic technique. Seven micrograms of parvalbumin, on a 10% acrylamide gel, was clearly labelled with the radioactive calcium (Figure 3.12). The soluble fraction of cerebellums from F_2 male rats and vitamin D replete controls were then compared. They are identical as demonstrated by the calcium binding bands on the radioautograph (Figure 3.12). Four hundred micrograms of soluble protein from vitamin D deficient and vitamin D replete rat cerebellum subjected to PAGE, showed label at Rf=1.0 and at a site of Rf=0.70. These sites correspond to the 28 kilodaltons and dye front bands that stain blue with the carbocyanine dye (Figure 3.6).

3.5.3 The Soluble Fraction of Spinal Cord and of Sciatic Nerve from Vitamin D Deficient Female Rat.

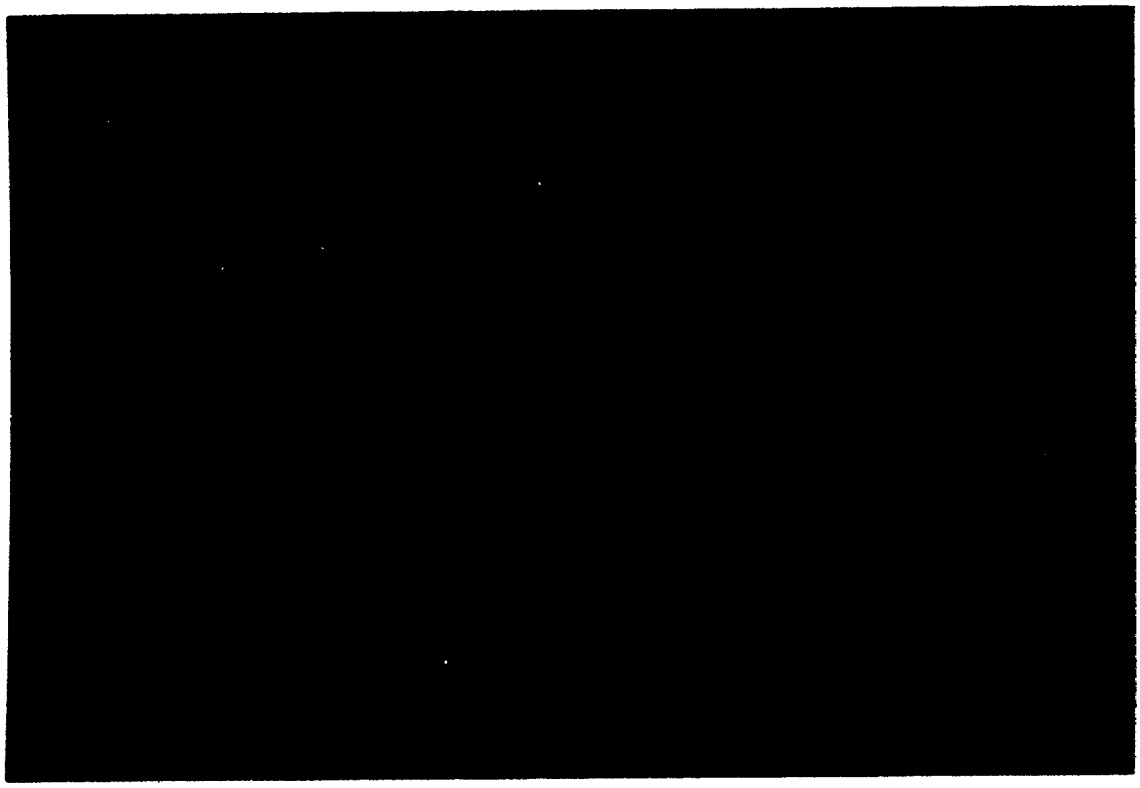
The soluble fraction from spinal cord and sciatic nerve of vitamin D replete and F_2 vitamin D deficient female rats were compared. Among 35, 60 and 90 day old rats, there was no difference between the pattern of samples run on polyacrylamide gels (Figure 3.13 shows only 90 day old rats), or the number and molecular weight of the calcium ligating bands. All the J complex forming bands present in the spinal cord pattern, except one, were found in the PAGE profile of the sciatic nerve. The 80 kilodaltons blue band was absent from the sciatic nerve. The sciatic nerve samples like the spinal cord had only one calcium binding protein (Figure 3.14).

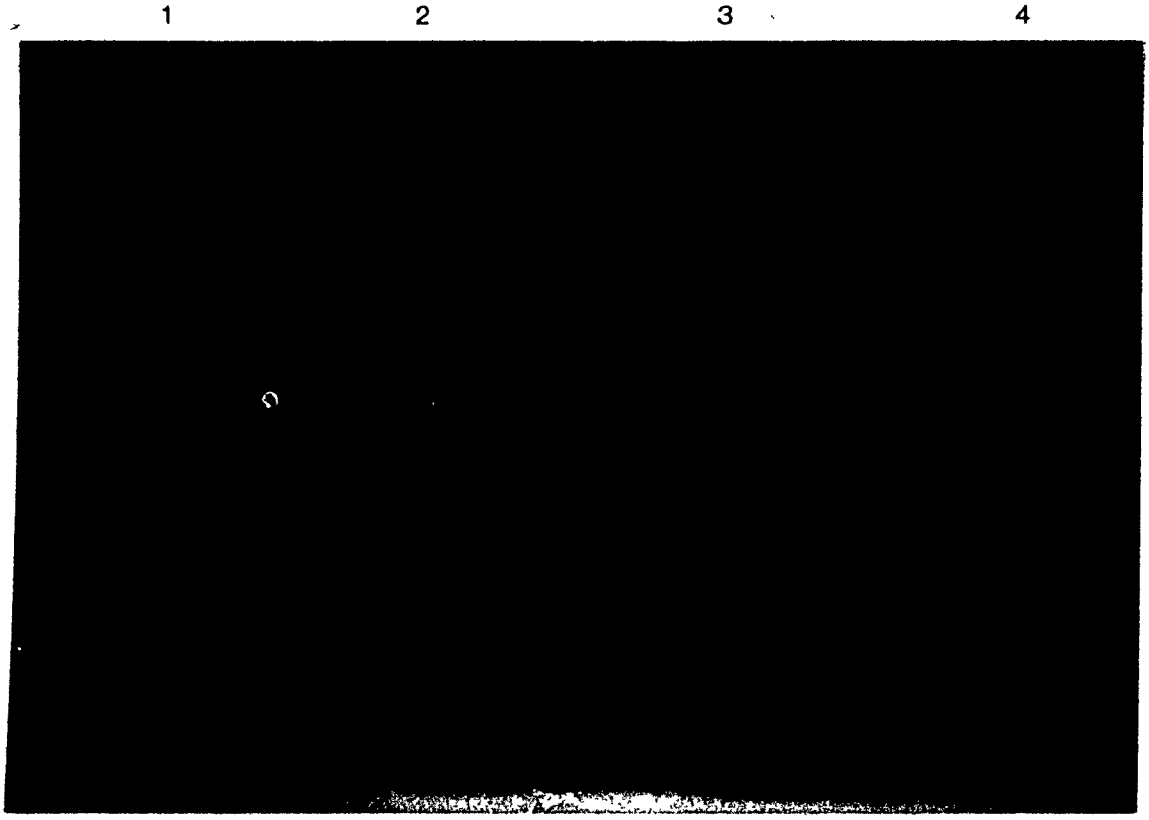
Figure 3.12. Radioautograph of parvalbumin and the soluble fraction of cerebellums from vitamin D replete and vitamin D deficient male rats. Lane 1 has 7 μg of parvalbumin while lanes 2 and 3 contain 400 μg of total protein from the cerebellar soluble fraction of vitamin D replete and vitamin D deficient rats respectively.


1
2
3

Figure 3.13. Separation of the soluble fraction of sciatic nerve and spinal cord proteins, from vitamin D deficient and control rats. Run in the first two lanes are spinal cord samples from 90 day female, vitamin D deficient rats. Run in the third lane is the pooled soluble fraction of sciatic nerve proteins from these two animals. The last lanes are spinal cord and sciatic nerve samples of 90 day control rats.

Figure 3.14. Radioautograph of a gel on which spinal cord and sciatic nerve samples from 90 day old female, vitamin D deficient and control rats are run. Four lanes are present: the first two lanes are samples from vitamin D deficient rat spinal cord and sciatic nerve respectively, the second two are spinal cord and sciatic nerve samples from control rats. All the lanes contain only one band that binds radioactive calcium. In each lane the band has a relative mobility of 1.


1
2
3
4
5
6


1
2
3
4

DISCUSSION

The objective of this project was to rapidly screen several segments of the rat nervous system for calcium binding proteins and to determine whether these proteins can be shown to be vitamin D dependent using this model. The methods that were used, though somewhat crude, are rapid and sufficiently sensitive for our purposes. As all the J complex forming bands did not bind calcium in the radioautographic experiments, blue staining by the carbocyanine dye can not be used as a reliable indicator of calcium binding proteins. It appears that any acidic protein with several anionic sites will stain blue.

The J-state arises through reaction of individual dye molecules with particular sites and is a function of dye configuration and concentration (Bean, Shepard, Kay and Walwick, 1965; Campbell, MacLennan and Jorgensen, 1983). As the average spacing between anionic groups in macromolecules is increased, there is a shift from the alpha band to the J band (Green, Pastewka and Peacock, 1973). The carbocyanine dye interacts with anionic sites on chromotropes that include: carboxyl, phosphate, sulphate and cysteic acid groups. Carboxyl groups are most frequently associated with the J-state (Bean, Shepard, Kay and Walwick, 1965).

Known calcium binding proteins: calsequestrin, calmodulin, troponin C and S-100, on polyacrylamide gels, interact with the carbocyanine dye and appear as blue bands with absorbance maxima at 600-615 nm (Campbell, MacLennan and Jorgensen, 1983). The carbocyanine dye forms J complexes with six SDS gel bands of the soluble fraction from spinal cord homogenates of rats 49 days of age or older.

All the known soluble calcium binding proteins of the EF hand type are acidic (Kretsinger, 1981; Wasserman, 1980) but all acidic proteins need not be "calcium binding proteins" (Williams, 1980). Having several glutamic acid residues will make the protein capable of interacting with the carbocyanine dye to produce the J state and precipitable by barium sulfate (Figure 3.8 A) (Malhotra, 1979).

The 80 kilodaltons band that appears on carbocyanine dye stained gels, in rats older than 49 days, does not correlate with any known vitamin D related phenomenon and does not correspond in molecular weight to any of the central nervous system soluble EF hand type, calcium binding proteins (Van Eldik, Zendegui, Marshak and Watterson, 1982). It is not likely to be a high affinity calcium binding protein since it did not bind calcium in the radioautographic experiments.

The bands that were shown to bind calcium are composed of macromolecules that bind calcium selectively. The other bands that stain blue with the carbocyanine dye are not labelled with the radioactive calcium, possibly because the magnesium in the incubation medium is able to compete effectively with calcium for the non-specific anionic sites. Reports have appeared in the literature that demonstrate renaturation of proteins after removal of SDS from the gel by washing. Olden and Yamada (1979) showed that several proteins retain their antigenic properties, while Lacks and Springhorn (1980) have reported that several monomeric enzymes regain their activity. The original radioautographic method, to the one that was used in this thesis, was shown to detect as little as 2 µg of the high affinity calcium binding protein of sarcoplasmic reticulum (Schibeci and Martonosi, 1980). This sensitivity was increased 40 fold by the modification of Anthony and Babitch (1984). With the radioautographic

method that was used in this thesis they were able to detect as little as 0.5 µg of any EF hand type calcium binding protein.

Like calmodulin, parvalbumin and S-100, the Rf=1.0 bands (Figures 3.9 and 3.12) migrate in the 14 kilodaltons range and bind calcium. The above three proteins have different properties (Berchtold, Heizmann and Wilson, 1983) that can be used to determine which of them if any, constitute this Rf = 1.0 band. Because this band was also present in rachitic rat samples it was not analyzed further.

In this laboratory, the definition of vitamin D deficiency in experimental rats, requires that no 1,25-dihydroxycholecalciferol be detectable in a sample of serum. The assay which is used has a detection limit of 10 pg 1,25-dihydroxycholecalciferol in a 10 ml plasma sample. Although no serum 1,25-dihydroxycholecalciferol assay was done for the animals that were used as vitamin D deficient, the F_1 rats have been repeatedly examined by members of the laboratory (Glijer, Peterfy, Tenenhouse, 1985) and were found to have no detectable 1,25-dihydroxycholecalciferol in their serum. Furthermore the serum levels of calcium that were measured in the F_2 rats (Table 3.1), and the retarded growth they exhibit (Figure 3.11), strongly argue that these rats are deficient in vitamin D. The F_2 rats are the only generation that show full motor seizures, indicating that the effects of vitamin D deficiency are even more severe in this generation than those preceding it.

It is demonstrated that the rat cerebellum has a 28 kilodaltons calcium binding protein which is absent from the spinal cord (Figures 3.9 and 3.12). The labelling in the 28 kilodaltons range appears as a doublet. The significance of this is not known, however it has been observed for $CaBP_{28K}$ in tissue homogenates subjected to PAGE (Celio, Scharer, Morrison, Norman and Bloom 1986). The only cerebellar calcium

binding protein of 28 kilodaltons molecular weight that is described in the literature is $CaBP_{28K}$. The calcium binding band that was detected in this study, migrates on gel electrophoresis as 28 kilodaltons and the band is split as is the $CaBP_{28K}$ band when the homogenate fraction containing $CaBP_{28K}$ is subjected to PAGE. No 28 kilodaltons calcium binding protein other than $CaBP_{28K}$ has been reported for the rat cerebellum. These observations suggest that the 28 kilodaltons calcium binding protein which was found in the cerebellum (Figure 3.12) is $CaBP_{28K}$, however there is no immunological study in this thesis to confirm it. The 28 kilodaltons protein is present in both the vitamin D deficient rat cerebellar samples and the vitamin D replete ones. As previous studies which did not use this animal model, no vitamin D dependent calcium binding protein was found in the cerebellum, the spinal cord or sciatic nerve of the rat. From the samples which were dialyzed, any protein which is smaller than the 12 kilodaltons cutoff will be lost. No attempts were made to look for proteins smaller than 12 kilodaltons in this study.

The different $CaBP_{28K}$ response of tissues, to changes in vitamin D status, has been attributed to different rates of cell turnover (Taylor, 1974; Thomasset, Parkes and Cuisinier-Gleizes, 1982). $CaBP_{28K}$ is also found in the chick intestine, kidney and brain (Taylor, 1980). Wasserman and Taylor (1972) found that the rate of disappearance of $CaBP_{28K}$ from the intestine and kidney of rachitic chicks correlates with the rate of elimination of ^{3}H-thymidine from prelabelled DNA in cells containing $CaBP_{28K}$. Thomasset and her colleagues (1982) reported that although the level of $CaBP_{28K}$ in the kidney and intestine of the rat is reduced by vitamin D deprivation, the $CaBP_{28K}$ in the cerebellum is not. Cells of the gut turnover at a rate of about once every 48-96 hours (Birge and Alpers, 1973; Lawson, 1978) while those of the central nervous system do not turn over (Messier and Leblond, 1960).

These authors make a correlation between vitamin D dependence of $CaBP_{28K}$ in the tissue and cell turnover but give no suggestions why the two should be related. Clues may be obtained from work that has been done in other hormone systems. As described by Palmiter (1983), exposure of the avian oviduct at day 14 to estrogen for two days, leaves the oviduct permanently committed to respond to progesterone. Similar irreversible steroid effects are the basis of phenotypic sex determination by androgens (Palmiter, 1983). In both these systems the changes persist in the absence of the primary hormone stimulus. It has been suggested that these hormones produce changes in chromatin proteins (e.g. methylation of cytosine residues) that results in the continual transcription of the effected gene (Palmiter, 1983). If induction of genes by vitamin D is permanent, then brain cells exposed to vitamin D at any part of their cell cycle will continue producing $CaBP_{28K}$. In renal and intestinal tissue there are always new cells that are born which have not been exposed to vitamin D and who will respond to the hormone. The vitamin D deficient samples that were tested in this thesis are from tissue whose cells have not been exposed to vitamin D at any part of the cell cycle.

If the 28 kilodaltons calcium binding protein that was found in the cerebellum is $CaBP_{28K}$, why then could it not be shown to be vitamin D dependent. The answer to this is not known, however it is reasonable to speculate the following. Although the serum 1,25-dihydroxycholecalciferol level may be an indication of the liver and kidney hydroxylase activities, other enzymes may be synthesizing 1,25-dihydroxycholecalciferol in specific parts of the brain. How well the serum 1,25-dihydroxycholecalciferol reflects the level at target sites in the central nervous system is not known. The brain may be "self-sufficient" in vitamin D.

A point to consider is the fact that the pH optima for calcium binding by $CaBP_{28K}$ is pH 6.3 and pH 9.3 (Ingersoll and Wasserman, 1971). The gel and radioautographic experiments were conducted at pH 8.4. To determine the pH optima for calcium binding Ingersoll and Wasserman (1971) used the Chelex 100 assay. The interpretation of this assay is difficult because it does not measure specifically $CaBP_{28K}$ binding of calcium. Binding by other molecules will be measured as well. However, they produce data showing that the maximal binding activity of $CaBP_{28K}$ is at pH 6.3 and at pH 8.3 only 84% of this maximum is obtained. This difference in pH is not likely to be responsible for the absence of any detectable $CaBP_{28K}$ in the spinal cord. The activity at pH 8.4 is still high and if the protein did exist it would appear in the carbocyanine dye stained gels. The rational for using pH 8.3 is that it is a pH well above most protein isoelectric points. All calcium binding proteins are likely to be capable of chelating calcium at this pH although it may not be optimal for any particular one.

Absence of the blood-brain barrier and a different neuroglia type from that of the central nervous system were reasons why the sciatic nerve of the two animal types was compared. The sciatic nerve is composed of two individual nerves, the Tibial and Common Peroneal bound in a sheath of connective tissue. The origin of these nerves is the spinal cord. They are formed by the fusion of spinal nerves from the level of L4 to S3. The neuroglia in the periphery, the Schwann cells, are physiologically different from the central oligodendroglia (Raine, 1981). The sciatic nerve samples show a Rf = 1.0 calcium binding band and although this tissue is free of the blood brain barrier, no vitamin D dependent calcium binding protein could be detected. It can not be said if the Rf = 1.0

bands in the central and peripheral nervous systems represent the same protein nor if the proteins are located in neuron or glia.

Although after intravenous injection 1,25-dihydroxycholecalciferol can be traced in central neurons (Stumpf, Sar, Clark, Lieth and DeLuca, 1980), the ability of 1,25-dihydroxycholecalciferol and 25-hydroxycholecalciferol to cross the blood brain barrier is small (Taylor, 1974). Only 2.2% of [^{3}H]1,25-dihydroxycholecalciferol injected into the carotid artery was taken up after a single passage through the brain (Gascon-Barré and Huet, 1983). The radiolabelled hormone that does cross the blood brain barrier is localized at sites that correlate poorly with the distribution of $CaBP_{28K}$ immunoreactivity (Jande, Maler and Lawson, 1981). Furthermore no receptor for 1,25-dihydroxycholecalciferol has been found in the cerebellum (Thomasset, Parkes and Cuisinier-Gleizes, 1982). Thus a reassessment of 1,25-dihydroxycholecalciferol target cells and of current concepts for its mode of action is required.

Several proteins of comparable molecular weight can share determinants but need not be identical. It is important therefore, to establish which of these brain antigens, recognized by $CaBP_{28K}$ antibody, is indeed calcium binding. A radioautographic method such as the one used in this study is useful for such an analysis. Using calcium binding function together with an assay for the 1,25-dihydroxycholecalciferol receptor, it may be possible to make a better correlation between vitamin D target cells and $CaBP_{28K}$ in the central nervous system.

Some investigators have suggested that a role of $CaBP_{28K}$ in the mammalian central nervous system is one of intracellular calcium buffer, possibly preventing the spread of intracellular calcium (Jande, Maler and Lawson , 1981) and that reduction of intracellular $CaBP_{28K}$ produces

hyperexcitability of the neuron (Baimbridge and Miller, 1984). Significantly, Purkinje cells show tetrodotoxin (TTX) insensitive spontaneous bursting (Llinas and Sugimori, 1980a). Calcium spiking and calcium dependent potassium changes are the main events underlying this behavior (Llinas and Sugimori, 1980b). Entry of calcium through voltage dependent channels triggers a potassium conductance that moves the membrane potential away from threshold. This calcium and potassium activity is viewed as the most significant self-regulatory mechanism in long term excitability of Purkinje cells (Llinas and Sugimori, 1980a). When the calcium current is surpressed, however, Purkinje cells behave in an erratic fashion, producing prolonged and unregulated periods of high frequency firing. These observations do not support the buffer hypothesis for $CaBP_{28K}$. The calcium activated potassium channel is found in nearly every excitable cell (Hille, 1984) making control of membrane excitability universally possible through this mechanism. This leaves the protein without an attractive hypothesis for its function.

Although the study reported here did not show any vitamin D dependent calcium binding protein in the spinal cord, cerebellum, or sciatic nerve, the idea of vitamin D having an action on neurons is highly intriguing. Vitamin D induced alterations of the lipid composition of membranes has been demonstrated as early as 1972 in the chick intestine (Goodman, Haussler and Rasmussen). Subsequently Rasmussen and his collaborators (1982) have reported that administration of 1,25-dihydroxycholecalciferol to isolated brush border membrane vesicles produces the following primary effects: an increase in the rate of phosphatidylcholine synthesis and an increase in the content of

polyunsaturated fatty acids in the phosphatidylcholine fraction. Both these events can be demonstrated in the presence of cycloheximide.

More recently Drueke and his colleagues (1985) showed in the rat, simultaneous increases of the influx velocity of calcium and the ratio of phosphatidylcholine to phosphatidylethanolamine after enterocyte vesicles taken from vitamin D replete rats were incubated in a medium containing 100 pM of 1,25-dihydroxycholecalciferol. Other investigators (Hay, Mawer and Hassam, 1982) have reported that vitamin D deficient animals fed a diet in which essential fatty acids were replaced by saturated fatty acids, do not show an increase in intestinal calcium absorption when treated with 1,25-dihydroxycolecalciferol. Further, O'Doherty (1979) has shown that 1,25-dihydroxycholecalciferol stimulates the activity of phosphatidylcholine deacylation and reacylation enzymes in intestinal mucosal cells of the vitamin D deprived rat.

The membrane effects of 1,25-dihydroxycholecalciferol have been integrated into a working hypothesis (Rasmussen, Matsumoto, Fontaine and Goodman, 1982). 1,25-dihydroxycholecalciferol stimulates the synthesis of phosphatidylcholine in the brush border membrane, and this phospholipid fraction undergoes metabolism involving deacylation-reacylation. The reacylation leads to an increase in the content of polyunsaturated fatty acids in the phosphatidylcholine fraction. These two changes in membrane phospholipid composition result in an increase in membrane fluidity, which in turn brings about a change in the rate of calcium transport across the brush border membrane. Rasmussen and coworkers (1982) speculate that the composition of annular lipids surrounding calcium carriers is effected by vitamin D.

These studies prompted Bikle and his collaborators (1984) to test whether membrane fluidity plays a role in regulating calcium flux across the chick intestinal brush border membrane. Many observations they made agree with previous reports, however there was a discrepancy in time between the 1,25-dihydroxycholecalciferol stimulated increase in calcium transport and the increase in membrane fluidity, unlike Matsumoto's study (1981) which found that membrane fluidity preceded or increased simultaneously with the increase in calcium transport rate. Bikle and coworkers (1984) concluded that modulation of membrane fluidity is an important means of regulating calcium transport across the intestinal brush border membrane, but the means by which 1,25-dihydroxycholecalciferol regulates calcium transport across this membrane does not involve a general increase in membrane fluidity. Together these reports demonstrate that 1,25-dihydroxycholecalciferol affects the lipid composition of intestinal membranes, however the relationship of these changes to calcium transport is still controversial.

An action on the membrane of neurons producing a change in the membrane resistance to ions can have drastic effects on the membrane potential and conduction of the action potential (Hille, 1984). Thus the classical thought of vitamin D action (ie. induction of genes) as well as the hypothesis in which 1,25-dihydroxycholecalciferol produces effects via an action directly on membranes, raise many fascinating questions that need to be addressed in relation to the nervous system. Only further investigation will determine the importance of vitamin D in nervous system physiology.

REFERENCES

Anthony, F.A. and Babitch, J.A. (1984).
Improved Detection of Calcium-Binding Proteins in Polyacrylamide Gels.
J. Neuroscience Meth. 12:79-89.

Baimbridge, K.G., Miller, J.J. and Parkes, C.O. (1982).
Calcium Binding Protein Distribution in the Rat Brain.
Brain Res. 239:519-525.

Baimbridge, K.G., Mody, I. and Miller, J.J. (1983).
Cortical Distribution and Localization of Calcium Binding Protein in Rats and Epileptic (EL) Mice.
Society for Neuroscience 13th Annual Meeting Abs. 144.1.

Baimbridge, K.G. and Miller, J.J. (1984).
Hippocampal Calcium Binding Protein During Commissural Kindling-Induced Epileptogenesis: Progressive Decline and Effects of Anticonvulsants.
Brain Res. 324:85-90.

Baudier, J., Labourdette, G. and Gerard, D. (1985).
Rat Brain S100b Protein: Purification, Characterization, and Ion Binding Properties. A comparison with Bovine S100b Protein.
J. Neurochem. 44:76-84.

Beale, E.G., Dedman, J.R. and Means, A.R. (1977).
Isolation and Regulation of the Protein Kinase Inhibitor and the Calcium-Dependent Cyclic Nucleotide Phosphodiesterase Regulator in the Sertoli Cell-Enriched Testis.
Endocrinology 101:1621-1634.

Bean, R.C., Shepard, W.C., Kay, R.E. and Walwick, E.R. (1965).
Spectral Changes in a Cationic Dye Due to Interaction with Macromolecules. III. Stoichiometry and Mechanism of the Complexing Reaction.
J, Phys. Chem. 69:4368-4379.

Berchtold, M.W., Heizmann, C.W. and Wilson, K.J. (1983).
Ca^{2+}-Binding Proteins: A Comparative Study of their Behavior During High-Performance Liquid Chromatography Using Gradient Elution on Reverse-Phase Supports.
Analyst. Biochem. 129:120-131.

Berchtold, M.W. and Means, A.R. (1985).
The Ca^{2+}-Binding Protein Parvalbumin: Molecular Cloning and Developmental Regulation of mRNA Abundance.
Proc. Natl. Acad. Sci. 82:1414-1418.

Bikle, D.D. (1985).
Osteomalacia and Rickets. In Cecil Textbook of Medicine, 17th edition, Wyngaarden, J.B. and Smith, L.H. editors. W.B. Saunders Co., Philadelphia.

Bikle, D.D., Whitney, J. and Munson, S. (1984).
The Relationship of Membrane Fluidity to Calcium Flux in Chick Intestinal Brush Border Membranes.
Endocrinology 114:260-267.

Bikle, D.D., Zolock, D.T., Morrissey, R.L. and Herman, R.H. (1978).
Independence of 1,25-$(OH)_2$-D_3 mediated calcium transport from de novo RNA and protein synthesis.
J. Biol. Chem. 253:484-488.

Birge, S.J. and Alpers, D.H. (1973).
Stimulation of Intestinal Mucosal Proliferation by Vitamin D.
Gasteroenterology 64:977-982.

Bjorkhem, I. and Holmberg, I. (1979).
On the 25-Hydroxylation of Vitamin D_3 in vitro Studied with a Mass Fragmentographic Technique.
J. Biol. Chem. 254:9518-9524.

Bjorkhem, I., Holmberg, I., Oftebro, H., and Pedersen, J.I. (1980).
Properties of a Reconstituted Vitamin D_3 25-Hydroxylase from Rat Liver Mitochondria.
J. Biol. Chem. 255:5244-5249.

Blum, H.E., Lehky, P., Kohler, L., Stein, E.A. and Fischer, E.H. (1977).
Comparative Properties of Vertebrate Parvalbumins.
J. Biol. Chem. 252:2834-2838.

Bonjour, J.Ph., Trechsel, U., Muller, K. and Scholer, D. (1985).
Increase in Skin 7-Dehydrocholesterol Induced by an Hypocholesterolemic Agent Associated with Elevated 25-Hydroxyvitamin D_3 Plasma Level. In, Vitamin D, Chemical, Biochemical and Clinical Update. Norman, A.W. et al. Editors, de Gruyter, Berlin.

Brommage, R., Jarnagin, K., DeLuca, H.F., Yamada, S. and Takayama, H. (1983).
1-but not 24-hydroxylation of Vitamin D is Required for Skeletal Mineralization in Rats.
Am. J. Physiol. 244:E298-E304.

Burgess, W.H., Jemiolo, D.K. and Kretsinger, R.H. (1980).
Interaction of calcium and calmodulin in the presence of sodium dodecyl sulfate.
Biochim. Biophys. Acta 623:257-270.

Caday, C.G. and Steiner, R.F. (1985).
The Interaction of Calmodulin with the Carbocyanine Dye (Stains-All).
J. Biol. Chem. 260:5985-5990.

Campbell, K.P., MacLennan, D.H. and Jorgensen, A.D. (1983).
Staining of the Calcium Binding Proteins: Calsequestrin, Calmodulin, Tropinin C, and S-100, with the Cationic Carbocyanine Dye "Stains-All".
J. Biol. Chem. 258:11267-11273.

Carpenter, M.B. (1978).
Core Text of Neuroanatomy 2nd Edition, Williams and Wilkins, Baltimore, p. 178.

Celio, M.R. and Heizmann, C.W. (1981).
Calcium Binding Protein Parvalbumin as a Neuronal Marker.
Nature, 293:300-302.

Celio, M.R., Scharer, L., Morrison, J.H., Norman, A.W. and Bloom, F.E. (1986).
Calbindin immunoreactivity alternates with cytochrome C-oxidase-rich zones in some layers of the primate visual cortex.
Nature 323:715-717.

Chandler, J.S., Pike, J.W. and Haussler M.R. (1979).
1,25-Dihydroxyvitamin D_3 Receptors in Rat Kidney Cytosol.
Biochem. Biophys. Res. Commun. 90:1057-1063.

Cocchia, D. and Michetti, F. (1981).
S-100 Antigen in Satellite Cells of the Adrenal Medulla and the Superior Cervical Ganglion of the Rat. An Immunochemical and Immunocytochemical study.
Cell Tiss. Res. 215:103-112.

Cooke, N.E. (1986).
Rat Vitamin D Binding Protein-Determination of the Full-Length Primary Structure from Cloned cDNA.
J. Biol. Chem. 261:3441-3450.

Costa, E.M., Blau, H.M. and Feldman, D. (1986).
1,25-Dihydroxyvitamin D_3 Receptors and Hormonal Responses in Cloned Human Skeletal Muscle Cells.
Endocrinology 119: 2214-2220.

Cormier, M.J. (1983).
Calmodulin: The Regulation of Cellular Function.
In Calcium in Biology, Spiro, T.G. editor.
John Wiley and Sons, New York.

Dedman, J.R., Potter, J.D., Jackson, R.L., Johnson, J.D. and Means, A.R. (1977).
Physiochemical Properties of Rat Testis Ca^{2+} Dependent Regulator Protein of Cyclic Nucleotide Phosphodiesterase: Relationship of Ca^{2+}-Binding, Conformational Changes and Phosphodiesterase activity.
J. Biol. Chem. 252-8415-8422.

DeLuca, H.F. and Schnoes, H.K. (1983).
Vitamin D: recent advances
Annu. Rev. Biochem. 52:411-439.

DeLuca, H.F. (1984).
The Metabolism and Functions of Vitamin D in Advances in Experimental Medicine and Biology, vol. 196 Chrousos, G.P., Loniaux, D.L. and Lipsett, M.B.
Plenum Press, New York and London.

DeLuca, H.F. (1985).
The Vitamin D-Calcium Axis-1983 in Calcium in Biological Systems Rubin, R.P., Weiss, G.B. and Putney, J.W. Editors.
Plenum Press, New York and London.

Desplan, C., Heidmann, O., Lillie, J.W., Auffray, C. and Thomasset, M. (1983).
Sequence of Rat Intestinal Vitamin D-Dependent Calcium-Binding Protein Derived from a cDNA Clone.
J. Biol. Chem. 258:13502-13505.

Drueke, T., Lacour, B., Lucas, P.A., Karsenty, G. and Mauriat, F. (1985).
Rapid Changes in Enterocyte Membrane Phospholipid Content and Ion Transport Induced by 1,25-Dihydroxyvitamin D_3 (Calcitriol), in Vitamin D. A Chemical, Biochemical and Clinical Update.
Walter de Gruyter & Co., Berlin.

Epstein, P., Means, A.R. and Berchtold, M.W. (1986).
Isolation of a Rat Parvalbumin Gene and Full Length cDNA.
J. Biol. Chem. 261:5886-5891.

Fraser, D.R. (1980).
Regulation of the Metabolism of Vitamin D.
Physiol. Rev. 60:551-613.

Fukushima, M., Nishii, U., Suzuki, M. and Suda, T. (1978).
Comparative Studies on the 25-Hydroxylations of Cholecalciferol and 1α-Hydroxycholecalciferol in Perfused Rat Liver.
Biochem. J. 170:495-502.

Fullmer, C.S., Wasserman, R.H., Hamilton, J.W., Huang, W.Y. and Cohn, D.V. (1975).
The Effect of calcium on the Tryptic Digestion of Bovine Intestinal Calcium Binding Protein.
Biochem. Biophys. Acta 412:256-261.

Gascon-Barre, M. and Huet, P.M. (1983).
Apparent ^3H 1,25-dihydroxyvitamin D_3 Uptake by Canine and Rat Brain.
Am. J. Physiol. 244:E266-271.

Glijer, B., Peterfy, C. and Tenenhouse, A. (1985).
The Effect of Vitamin D Deficiency on Secretion of Saliva by Rat Parotid Gland in vivo.
J. Physiol. 363:323-334.

Goodman, D.B.P., Haussler, M.R., and Rasmussen, H. (1972).
Vitamin D_3 Induced Alteration of Microvillar Membrane Lipid Composition.
Biochem. Biophys. Res. Commun. 46:80-86.

Gosselin-Rey, C., Piront, A. and Gerday, C. (1978).
Polymorphism of Parvalbumins and Tissue Distribution.
Biochimica et Biophysica Acta 532:294-304.

Green, M.R., Pastewka, J.V. and Peacock, A.C. (1973).
Differential Staining of Phosphoproteins on Polyacrylamide Gels with a Cationic Carbocyanine Dye.
Anal. Biochem. 56:43-51.

Hames, B.D. and Ricwood, D. (1981).
Gel Electrophoresis of Proteins: A Practical Approach. IRL Press Limited, London and Washington D.C.

Hay, A.W.M., Hassam, A.G., Crawford, M.A., Stevens, P.A., Mawer, E.B. and Case, C. (1979).
The Ability of 1,25-Dihydroxycholecalciferol to Alter the Fatty Acid Composition of Phosphoglycerides in Rat Intestinal Mucosa and Smooth Muscle. In: Vitamin D Basic Research and Its Application.
De Gruyter, Berlin.

Hay, A.W.M., Mawer, E.B. and Hassam, A.G. (1982).
The Role of Essential Fatty Acids in Vitamin D-Dependent Calcium Absorption in the Intestine, in Vitamin D, Chemical, Biochemical and Clinical Endocrinology of Calcium Metabolism.
Walter de Gruyter & Co., Berlin.

Hayashi, S., Noshiro, M. and Okuda, K. (1986).
Isolation of a Cytochrome P-450 that Catalyzes the 25-Hydroxylation of Vitamin D_3 from Rat Liver Microsomes.
J. Biochem. 99:1753-1763.

Henry, H.L. (1980).
"Regulation of Vitamin D Metabolism" in Vitamin D, Molecular Biology and Clinical Nutrition, Marcel Dekker.

Heizmann, C.W. (1984).
Parvalbumin, an Intracellular Calcium-Binding Protein: Distribution, Properties and Possible Roles in Mammalian Cells.
Experientia 40:910-921.

Hidaka, H., Endo, T., Kawamoto, S., Yamada, E., Umekawa, H., Tanabe, K. and Hara, K. (1983).
Purification and Characterization of Adipose Tissue S-100b Protein.
J. Biol. Chem. 258:2705-2709.

Hille, B. (1984).
Ionic Channels of Excitable Membranes.
Sinauer Associates Inc.

Holick, M.F., Frommer, J., McNeill, S. and Potts, J.T. (1977).
In "Vitamin D: Biochemical, Chemical and Clinical Aspects Related to Calcium Metabolism" A.W. Norman et al. editors,
de Gruyter, Berlin.

Iida, S. and Potter, J.D. (1986).
Calcium Binding to Calmodulin. Cooperativity of the Calcium-Binding Sites.
J. Biochem. 99:1765-1772.

Ingersoll, R.J. and Wasserman, R.H. (1971).
Vitamin D_3-Induced Calcium-Binding Protein.
J. Biol. Chem. 246:2808-2814.

Intrator, S., Baudier, J., Elion, J., Thomasset, M. and Brehier, A. (1985).
Identity Between Rat Renal and Cerebellar Cholecalcins (28 000 MW Cholecalciferol-Induced CaBP). Comparison with Bovine Brain Caligulin. In, Vitamin D. A Chemical, Biochemical and Clinical Update. Walter de Gruyter & Co., Berlin.

Isobe, T., Ishioka, N. and Okuyama, T. (1981).
Structural Relation of Two S-100 proteins in Bovine Brains; Subunit Composition of S-100a Protein.
Eur. J. Biochem. 115:469-474.

Jande, S.S., Maler, K. and Lawson, D.E.M. (1981).
Immunohistochemical Mapping of Vitamin D Dependent Calcium Binding Protein in Brain.
Nature 294:765-767.

Jarnagin, K., Brommage, R., DeLuca, H.F., Yamada, S. and Takayama, H. (1983).
1-but not 24-Hydroxylation of Vitamin D is Required for Growth and Reproduction in Rats.
Am. J. Physiol. 244:E290-E297.

Kakiuchi, S., Yamazaki, R., Feshima, Y., Uenishi, K., Yasuda, S., Kashiba, A., Sobue, K., Ohshima, M. and Nakajima, T. (1978).
Membrane-Bound Protein Modulator and Phosphodiesterase.
Adv. Cyclic Nucleotide Res. 9:253-263.

Karsenty, G., Lacour, B., Ulmann, A., Pierandrei, E. and Drueke, T. (1985)
Early Effects of Vitamin D Metabolites on Phosphate Fluxes in Isolated Rat Enterocytes.
Am. J. Physiol. 248:G40-G45.

Kawashima, H. and Kurokawa, K. (1982).
Localization of Receptors for 1,25-Dihydroxyvitamin D_3 along the Rat Nephron.
J. Biol. Chem. 257:13428-13422.

Kay, R.E., Walwick, E.R. and Gifford, C.K. (1964a).
Spectral Changes in a Cationic Dye Due to Interaction with Macromolecules. I. Behavior of Dye Alone in Solution and the Effect of Added Macromolecules.
J. Phys. Chem. 68:1896-1906.

Kay, R.E., Walwick, E.R. and Gifford, C.K. (1964b).
Spectral Changes in a Cationic Dye Due to Interaction with Macromolecules. II. Effects of Environment and Macromolecule Structure.
J. Phys. Chem. 68:1907-1916.

Klee, C.B., Crouch, T.H. and Richman, P.G. (1980).
Calmodulin.
Ann. Rev. Biochm. 49:489-515.

Kleiner-Bossaller, A. and DeLuca, H.F. (1974).
Formation of 1,24,25-Trihydroxyvitamin D_3 from 1,25-dihydroxy-vitamin D_3.
Biochim. Biophys. Acta 388:489-495.

Kream, B.E. and DeLuca, H.F. (1977).
A Specific Binding Protein for 1,25-Dihydroxyvitamin D_3 in Rat Intestinal Cytosol.
Biochem. Biophys. Res. Commun. 76:735-738.

Kretsinger, R.H. (1981).
Calcium-Modulated Proteins, in Mechanisms of Selective Signalling by Calcium.
Neurosciences Res. Prog. Bull., Vol 19, No 3.

Kumar, R., Nagubandi, S., Mattox, V.R., and Londowski, J.M. (1980).
Enterohepatic Physiology of 1,25-Dihydroxyvitamin D_3.
J. Clin. Invest. 65:277-284.

Kumar, R. (1984).
Metabolism of 1,25-Dihydroxyvitamin D_3.
Physiol. Rev. 64:478-504.

LaPorte, D.C., Wierman, B.M. and Storm, D.R. (1980).
Calcium-Induced Exposure of a Hydrophobic Surface on Calmodulin.
Biochemistry 19:3814-3819.

Laemmli, U.K. (1970).
Cleavage of Structural Proteins During the Assembly of the Head of Bacteriophage T_4.
Nature (London) 227:680-685.

Lacks, S. and Springhorn, S.S. (1980).
Renaturation of Enzymes after Polyacrylamide Gel Electrophorosis in the Presence of Sodium Dodecyl Sulfate.
J. Biol. Chem. 255: 7467-7473.

Lawson, D.E.M. (1978).
Biochemical Responses of the Intestine to Vitamin D, in "Vitamin D", Academic Press.

Lawson, D.E.M. and Davie, M. (1979).
Aspects of the Metabolism and Function of Vitamin D, in "Vitamins and Hormones". Munson, P.L., Diczfalusy, E., Glover, J. and Olson, R.E. editors,
Academic Press.

Li, L.F., and Olson, R.E. (1967).
Purification and Properties of Rat Prothrombin.
J. Biol. Chem. 242:5611-5616.

Lian, J.B., Reit, B., Roufosse, A.H., Glimcher, M.J. and Gallop, P.M. (1979).
Osteocalcin Content in Normal, Rachitic and Vitamin K, Antagonized Bone, in "Vitamin K Metabolism and Vitamin K Dependent Proteins". Suttie, J.W. editor,
University Park Press.

Llinas, R. and Sugimoni, M.J. (1980a).
Electrophysiological Properties of <u>in vitro</u> Purkinje Cell Dendrites in Mammalian Cerebral Slices.
J. Physiol. (London) 305:197-213.

Llinas, R. and Sugimoni, M.J. (1980b).
Electrophysiological Properties of <u>in vitro</u> Purkinje Cell Dendrites in Mammalian Cerebral Slices.
J. Physiol. (London) 305:171-195.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall (1951).
Protein Measurement with the Folin Reagent.
J. Biol. Chem. 193:265-275.

Malhotra, O.P. (1979).
Effect of Gamma-Carboxyglytamic Acids on Properties of Prothrombins and their Fragments, in "Vitamin K Metabolism and Vitamin K Dependent Proteins".
Suttie, J.W., Editor, University Park Press.

Manillier, C., Farman, N., Bonjour, J.-P. and Bonvalet, J.-P. (1985).
$1,25(OH)_2D_3$ Binding Along the Rat Nephron: Autoradiographic Study in Isolated Tubular Segments.
Am. J. Physiol. 248:F296-F307.

Marché, P., Le Guern, C. and Cassier, P. (1979).
Immunocytochemical Localization of a Calcium-Binding Protein in the Rat Duodenum.
Cell Tissue Res. 197:69-77.

Maruyama, K., Mikawa, T. and Ebashi, S. (1984).
Detection of Calcium Binding Proteins by Radioactive Calcium Autoradiography on Nitrocellulose Membrane after Sodium Dodecyl Sulfate Gel Electrophoresis.
J. Biol. Chem. 95:511-519.

Masure, H.R., Head, J.F. and Tice, H.M. (1984).
Studies on the α-subunit of Bovine Brain S-100 Protein.
Biochem. J. 218:691-696.

Matsumoto, T., Fontaine, O. and Rasmussen, H. (1981).
Effect of 1,25-Dihydroxyvitamin D_3 on Phospholipid Metabolism in Chick Duodenal Mucosal Cell. Relationship to its Mechanism of Action.
J. Biol. Chem. 256:3354-3360.

Means, A.R., Tash, J.S. and Chafouleas, J.G. (1982).
Physiological Implications of the Presence, Distribution and Regulation of Calmodulin in Eukaryotic Cells.
Physiol. Rev. 62:1-39.

Messier, B. and LeBlond, C.P. (1960).
Cell Proliferation and Migration as Revealed by Radioautography after Injection of Thymidine-H^3 into Male Rats and Mice.
American Journal of Anatomy 106:247-265.

Miani, N., De Renzis, G., Michetti, F., Correr, S., Olivieri, C., Sangiacomo and Caniglia, A. (1972).
Axonal Transport of S-100 Protein in Mammalian Nerve Fibers.
J. Neurochem. 19:1387-1394.

Michetti, F., Dell'Anna, E., Tiberio, G. and Cocchia, D. (1983).
Immunochemical and Immunocytochemical Study of S-100 Protein in Rat Adipocytes.
Brain Res. 262:352-356.

Miller, J.J. and Baimbridge, K.G. (1983).
Biochemical and Immunohistochemical Correlates of Kindling Induced Epilepsy: Role of Calcium Binding Proteins.
Brain Res. 278:322-326.

Moriuchi, S., Yamanouchi, T. and Hosoya, N. (1975).
Demonstration of Two Different Vitamin D-Dependent Calcium-Binding Proteins in Rat Intestinal Mucosa.
J. Nutr. Sci. Vitaminol. 21:251-259.

Nakajima, T., Yamaguchi, H., and Takahashi, K. (1980).
S-100 Protein in Folliculostellate Cells of the Rat Pituitary Anterior Lobe.
Brain Res. 191:523-531.

Norman, A.W. (1979).
Vitamin D: The Calcium Homeostatic Steroid Hormone.
Academic Press.

O'Doherty, P.J.A. (1979).
1,25-Dihydroxyvitamin D_3 increases the activity of the intestinal phosphatidylcholine deacylation-reacylation cycle.
Lipids 14:75-83.

Olden, K. and Yamada, K.M. (1979).
Direct Detection of Antigens in Sodium Dodecyl Sulfate-Polyacrylamide Gels.
Anal. Biochem. 78:483-490.

Onisko, B.L., Esvelt, R.P., Schnoes, H.K. and DeLuca, H.F. (1980).
Metabolites of 1α, 25-dihydroxy-vitamin D_3 in Rat Bile.
Biochemistry 19:4124-4130.

Packman, P.M., Blomstrand, C. and Hamberger, A. (1971).
Disc Electrophoretic Separation of Proteins in Neuronal, Glial and Subcellular Fractions from Cerebral Cortex.
J. Neurochem. 18:479-487.

Palmiter, R. (1983).
Hormone Action. In: Biochemistry, Zubay, G.L., Editor.
Addison-Wesley Publishing Company. Reading, Massachusetts.

Peterfy, C. and Tenenhouse (1982).
Vitamin D Receptors in Isolated Rat Parotid Gland Acinar Cells.
Biochem. Biophys. Acta. 721:158-163.

Pike, J.W., Allegretto, E.A., Kelly, M.A., Donaldson, C.A., Marion, S.L., Mangelsdorf, D.J. and Haussler, M.R. (1984).
1,25-Dihydroxyvitamin D_3 Receptors: Altered Functional Domains are Associated with Cellular Resistance to Vitamin D_3. In, Advances in Experimental Medicine and Biology, vol. 196, Chrousos, G.P., Loniaux, D.L. and Lipsett, M.B. editors.
Plenum Press, New York.

Plum, F. (1985).
Ataxia and Related Gait Disorders. In, Cecil Textbook of Medicine, 17th edition, Wyngaarden, J.B. and Smith, L.H. editors. W.B. Saunders Co., Philadelphia.

Price, P.A. (1985).
The Vitamin K-Dependent Bone Protein and the Action of 1,25-Dihydroxytamin D_3 on Bone. In, Calcium in Biological Systems.
Rubin, R.P., Weiss, G.B. and Putney, J.W. editors.
Plenum Press, New York.

Prozialeck, W.C. and Weiss, B. (1985).
Mechanism of Pharmacologically Altering Calmodulin Activity. In, Calcium in Biological Systems.
R.P. Rubin, Weiss, G.B., and Putney, J.W. Editors.
Plenum Press, New York and London.

Raine, C.S. (1981).
Neurocellular Anatomy in "Basic Neurochemistry", Seigel, G.J., Albers, R.W., Agranoff, B.W., Katzman, R., editors. Little Brown and Company, Boston.

Rasmussen, H., Matsumoto, T., Fontaine, O., and Goodman, D.B.P. (1982).
Role of Changes in Membrane Lipid Structure in the Action of 1,25-Dihydroxyvitamin D_3.
Federation Proc. 41:72-77.

Reisner, A.H., Nemes, P. and Bucholtz, C. (1975).
The Use of Coomassie Brilliant Blue G250 Perchloric Acid Solution for Staining in Electrophoresis and Isoelectric Focusing on Polyacrylamide.
Anal. Biochem. 64:509-516.

Rodier, P.M. (1977).
Correlations Between Prenatally-Induced Alterations in CNS Cell Populations and Postnatal Function.
Teratology 16:235-246.

Seamon,K.B. and Kretsinger, R.H. (1983).
Calcium-Modulated Proteins. In: Calcium in Biology. Spiro, T.G., Editor.
John Wiley and Sons, New York.

Schibeci, A. and Martonosi, A. (1980).
Detection of Ca^{2+}-Binding Proteins on Polyacrylamide Gels by ^{45}Ca Autoradiography.
Anal. Biochem. 78:483-490.

Schreiner, D.S., Jande, S.S., Parkes, C.O., Lawson, D.E.M. and Thomasset, M. (1983).
Immunocytochemical Demonstration of Two Vitamin D Dependent Calcium Binding Proteins in Mammalian Kidney.
Acta Anat. 117:1-14.

Spencer, R., Charman, M., Wilson, P. and Lawson, D.E.M. (1978).
The Relationship Between Vitamin D-Stimulated Calcium Transport and Intestinal Calcium-Binding Protein in the Chicken.
Biochem. J. 170:93-101.

Stanbury, S.W., Mawer, E.B., Taylor, C.M. and De Silva, P. (1980).
The Skin, Vitamin D and the Control of its 25-hydroxylation: an Attempted Integration. Mineral and Electrolyte Metabolism, 3:51-60.

Stumpf, W.E., Sar, M., Reid, F.A., Tanaka, Y. and DeLuca, H.F. (1979).
Target Cells for 1,25-$(OH)_2$-Vitamin D_3 in Duodenum, Jejunum, Ileum, Colon, Stomach, Kidney, Skin, Pituitary and Parathyroid.
Science 206:1188-1190.

Stumpf, W.E., Sar, M., Clark, S.A., Lieth, E. and DeLuca, H.F. (1980).
Target Neurons for 1,25(OH) Vitamin D_3 in Brain and Spinal Cord.
Neuroendocrinol. Lett. 2:297-301.

Stumpf, W.E., Sar, M., Narbaitz, R., Reid, F.A., DeLuca, H.F. and Tanaka, Y. (1980).
Cellular and Subcellular Localization of 1,25-$(OH)_2$-Vitamin D_3 in Rat Kidney.
Proc. Natl. Acad. Sci. USA 77:1149-1153.

Suzuki, F., Nakajima, T. and Kato, K. (1982).
Peripheral Distribution of Nervous System-Specific S-100 Protein in Rat.
J. Biochem. 92:835-838.

Tabuchi, K. and Kirsch, W.H. (1975).
Immunocytochemical Localization of S-100 Protein in Neurons and Glia of Hamster Cerebellum.
Brain Res. 92:175-180.

Tanaka, T. and Hidaka, H. (1980).
Hydrophobic Regions Function in Calmodulin Enzyme(s) Interactions.
J. Biol. Chem. 255:11078-11080.

Taylor, A.N. (1974).
Chick Brain Calcium Binding Protein: Comparison with Intestinal Vitamin D Induced Calcium Binding Protein.
Arch. Biochem. Biophys. 161:100-108.

Taylor, A.N. (1980).
Vitamin D Dependent Calcium-Binding Proteins. In: Vitamin D, Molecular Biology and Clinical Nutrition. A. W. Norman, Editor.
Marcel Dekker Inc. New York and Basel.

Taylor, A.N., Gleason, W.A. and Lankford, G. (1984).
Immunocytochemical Localization of rat Intestinal Vitamin D-dependent calcium-binding protein.
J. Histochem. Cytochem. 32: 153.

Thomasset, M., Desplan, C., Moukhtar, M. and Mathieu, H. (1981).
Primary Translocation Product of mRNA Coding for Rat Duodenal Vitamin D-Dependent Calcium-Binding Protein.
FEBS Lett. 134:178-182.

Thomasset, Desplan and Parkes, (1983).
Rat Vitamin-D-Dependent Calcium-Binding Proteins. Specificity of mRNAs for the 7500-Mr Protein from Duodenum and the 28 000-Mr Protein from Kidney and Cerebellum.
Eur. J. Biochem. 129:519-524.

Thomasset, M., Parkes, C.O. and Cuisinier-Gleizes, P. (1982).
Rat Calcium Binding Proteins: Distribution Development and Vitamin D Dependence.
Am. J. Physiol. 243:E483-E488.

Tsoukas, C.D., Provedini, D.M. and Manolagas, S.C. (1984).
1,25-Dihydroxyvitamin D_3: A Novel Immunoregulatory Hormone.
Science 224:1438-1439.

Uskokovic, M.R., Partridge, J.J., Narwid, T.A. and Baggiolini, E.G. (1980).
Synthesis of Vitamin D Metabolites and Analogs, in Vitamin D Molecular Biology and Clinical Nutrition, Norman, A.W. editor, Marcel Dekker Inc.

Van Eldik, L.J., Zendegui, J.G., Marshak, D.R. and Watterson, D.M. (1982).
Calcium Binding Proteins and the Molecular Basis of Calcium Action.
Int. Rev. Cytol. 77:1-61.

Walters, M., Hunziker, W. and Norman, A. (1980).
Unoccupied 1,25-dihydroxyvitamin D_3 receptors. Nuclear/Cytosolic Ratio Depends on Ionic Strength.
J. Biol. Chem. 255:6799-6805.

Warembourg, M., Perret, C. and Thomasset, M. (1985).
Localization of Cholecalcin (9 000 MW Cholecalficerol- Induced CaBP) Messenger RNA in Rat Duodenum and Placenta by in situ Hybridization, in Vitamin D, Chemical, Biochemical and Clinical Update. de Gruyter, Berlin.

Warembourg, M., Perret, C. and Thomasset, M. (1986).
Distribution of Vitamin D-Dependent Calcium-Binding Protein Messenger Ribonucleic Acid in Rat Placenta and Duodenum.
Endocrinology 119:176-184.

Warner, M. (1983).
25-Hydroxyvitamin D Hydroxylation.
J. Biol. Chem. 258:11590-11593.

Warner, M. and Tenenhouse, A. (1985).
Age Related Changes in Calcium Homeostasis in Rats with Chronic Vitamin D Deficiency, in "Vitamin D". A Chemical, Biochemical and Clinical Update. de Gruyter, Berlin.

Wasserman, R.H. (1980).
Vitamin D Dependent Calcium Binding Proteins, in "Calcium Binding Proteins: Structure and Function", Siegel, F.L. Carafoli, E., Kretsinger, R.H., MacLennan, D.H. and Wasserman, R.H. editors.
Elsevier North Holland Inc.

Wasserman, R.H. and Taylor, A.N. (1963).
Vitamin D_3 Inhibition of Radiocalcium Binding by Chick Intestinal Homogenates.
Nature (London) 198:30-32.

Wasserman, R.H. and Taylor, A.N. (1972).
Metabolic Roles of Fat Soluble Vitamins D, E and K.
Annu. Rev. Biochem. 41:926-928.

Wasserman, R.H., Corradino, R.A. and Taylor, A.N. (1968).
Vitamin D-Dependent Calcium Binding Protein. Purification and Some Properties.
J. Biol. Chem. 243:3978-3986.

Watterson, D.M., Sharief, F. and Vanaman, T.C. (1980).
The Complete Amino Acid Sequence of the Ca^{2+}-Dependent Modulator Protein (Calmodulin) of Bovine Brain.
J. Biol. Chem. 255:962-975.

Weber, K. and Osborn, M. (1969).
The Reliability of Molecular Weight Determinations by Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis.
J. Biol. Chem. 244:4406-4412.

Weinstein, R.S., Underwood, J.L., Hutson, M.S., and DeLuca, H.F. (1984).
Bone Histomorphometry in Vitamin D-Deficient Rats Infused with Calcium and Phosphorus.
Am. J. Physiol. 246:E499-E505.

Williams, R.J.P. (1980).
A General Introduction to the Special Properties of the Calcium Ion and their Deployment in Biology. In: Calcium-Binding Proteins: Structure and Function. Siegel, F.L., Carafoli, E., Kretsinger, R.H., MacLennan, D.H. and Wasserman, R.H. Editors.
Elsevier North Holland Inc.

Wong, R.F., Traub, R.D. and Miles, R. (1984).
Epileptogenic Mechanism as Revealed by Studies of the Hippocampal Slice. In: Electrophysiology of Epilepsy. Schwartzkroin, P.A. and Wheal, H.V. Editors.
Academic Press. London.

Yoon, P.S. and DeLuca, H.F. (1980).
Resolution and Reconstitution of Soluble Components of Rat Liver Microsomal Vitamin D_3 25-Hydroxylase.
Arch. Biochem. Biophys. 203:529-541.