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IDENTIFICATION OF VITAMIN D TARGETS BY IMMUNOCYTOCHEMICAL LOCALIZATION OF D-CABP

by

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I. INTRODUCTION AND LITERATURE REVIEW

The antirachitic property of vitamin D has been appreciated for a long time. Active research on vitamin D began at the turn of the century when rickets reached epidemic proportions in children living in industrialized zones. Mellanby in 1919 produced in dogs a disease that closely resembled rickets. He demonstrated that rickets is a nutritional disease which can be prevented and cured by including cod liver oil in the diet. Since McCollum and Davis (1913) demonstrated that butterfat and cod liver oil contained a factor named vitamin A which is essential to the growth and health of animals, Mellanby attributed the antirachitic powers of cod liver oil to vitamin A. McCollum recognized that the properties of the antirachitic substance discovered by Mellanby must be different from those of the fat-soluble vitamin A. The antirachitic activity of cod liver oil could survive aeration and heating at 100 °C for 14 hr, while vitamin A did not withstand this treatment. These experiments demonstrated the existence of a distinct vitamin and was named vitamin D (McCollum, 1922; McCollum, 1925). In Vienna another series of investigations were taking place at the clinical Level. Huldinsky (1919) and Chick et al (1923) independently demonstrated that exposure to sunlight, or artificially produced ultraviolet light was effective in the prevention and cure of rickets. This created some confusion since both cod liver oil and sunlight were recognised as effective antirachitic agents. The reason for the similar effects of seemingly two different treatments was unraveled by Goldblatt and

Soames (1923) who showed that ultraviolet irradiation of rachitic rats resulted in the production of an antirachitic factor in the liver that could be extracted and used to cure other rachitic rats. Steenbock provided a firm basis for the demonstration of ultraviolet activation of previtamin D in food (Steenbock, 1924) in the sterol fraction of skin (Steenbock and Black, 1924) and in plant extracts (Hess et al, 1926). The next step was the isolation and identification of the members of the previtamin D series. The provitamin 7-dehydrocholesterol was isolated from pig skin and chemically characterized by Windaus and Bock (1937). This provitamin is the most common provitamin D occuring in the skin of many vertebrate species (Bills, 1954). Upon irradiation it produces the other major form of vitamin D, vitamin D_3 (Shenck, 1937). Previtamin Ds are also present in the plant kingdom (Bills, 1954; Sebrell and Harris, 1954). Vitamin D_2 is produced by irradiating plant ergosterol (Askew et al, 1931; Windaus et al, 1932). This vitamin is also called ergocalciferol and was for many years the major synthetic form of vitamin D used for the prevention and cure of rickets in man. Thus in the early 1930s, the isolation and identification of the series of vitamin D nutritional compounds had been completed.

During this time, other work was underway to understand the mechanism of action of the antirachitic properties of vitamin D. Howland and Kramer (1921) studied the in vitro calcification of rachitic cartilage, and learned that blood from normal animals produced mineralization of cartilage, while blood from rachitic animals did not. They provided evidence that rickets is caused by a failure of cartilage calcification due to inadequate levels of calcium and phosphorus in the blood. This work helped to focus on the idea that the function of vitamin D was to elevate plasma calcium and phosphorus to levels that would support mineralization.

The large amount of calcium found in the feces of rachitic children led Orr et al (1923) to the conclusion that an important function of vitamin D is to improve gastrointestinal absorption of calcium. The experiments carried out by Nicolaysen (1937a,b) conclusively demonstrated that vitamin D specifically improves intestinal absorption of calcium and that vitamin D does not influence endogenous loss of calcium due to failure of bone mineralization, or excretion of calcium into the colon (Nicolaysen, 1937). He also demonstrated that the intestine had the ability to regulate its efficiency of calcium absorption according to the needs and demands of the organism. The ability to adapt required vitamin D (Nicolaysen et al, 1953). This observation was first confirmed in vitro by Schachter and coworkers (Schachter and Rosen, 1959; Schachter et al, 1961) as well as by a variety of other techniques (Nellans and Kimberg, 1979; Bronner, 1982).

<u>Metabolism of vitamin D</u>

It was assumed that vitamin D acts directly in the healing of rickets and in the promotion of calcium absorption. However Irving in 1944 was the first to recognize that there is a lag period between the administration of the vitamin and the initiation of its physiological response. This lag period is present for every known physiological response of vitamin D (Norman, 1979). Raoul and Gounelle (1958) suggested that the lag period preceding the action of vitamin D may be explained by the requirement for the metabolic conversion of vitamin D into an active form before it can perform its biological function. The principal steps in the metabolism of vitamin D are well known (see the following reviews: Spanos, 1978; Holick and DeLuca, 1978; Wasserman, 1979; DeLuca, 1982; Norman 1979, 1980; Miller, 1983; DeLuca, 1984; Napoli and Horst, 1984). In the stratum germinativum of the skin there are large quantities of 7-dehydrocholesterol which absorb light at 250-310nm and undergo a chemical photolysis to form previtamin D_3 (Holick and Clark, 1978). At body temperature, previtamin D_3 is in thermal equilibrium with vitamin D_3 (Holick and DeLuca, 1978). Vitamin D_3 then binds to a D-binding protein (DBP), which is a globulin (Rikkers and DeLuca, 1967; Rikkers et al, 1969; Imawari et al, 1976; Haddad and Walgate, 1976) and is transported from the epidermis into the blood. Vitamin D_3 accumulates in fat and in the liver where it is enzymatically hydroxylated at carbon 25 in the endoplasmic reticulum of hepatic cells (Bhattacharya and DeLuca, 1974). The resulting 25-hydroxyvitamin D_3 (25-OH-D_3) is the major circulating metabolite of vitamin D in the plasma (Blunt et al, 1968). It acts directly on target tissues only at much higher than physiological concentrations and must be further metabolised. This metabolite is then hydroxylated in position 1 in the mitochondria of the kidney proximal tubule cells to form 1,25 dihydroxyvitamin D_3 (1,25 DHCC, $1,25(OH)_2D_3$ or calcitriol) which is the most potent metabolite of vitamin D (Frazer and Kodicek, 1970; Gray et al, 1971; Holick and Clark, 1978).

Hormonal nature of vitamin D

$1,25(OH)_2D_3$ is produced in a particular cell type to carry out its function in target cells at other locations (Norman, 1980). It fulfills the requirements for inclusion as a true hormone since it modulates the metabolism of its target cells in a specific direction, is active in small amounts and is subject to feedback regulation.

Regulation of vitamin D synthesis

The principal regulators of $1,25(OH)_2D_3$ or calcitriol synthesis are calcitriol itself, the plasma concentration of calcium and phosphorus, and parathyroid hormone (see review Holick and DeLuca, 1978). The most significant enzymatic site for the regulation of vitamin D synthesis is the 25-hydroxycholecalciferol-1-hydroxylase in the kidney (Holick and DeLuca, 1978; Henry, 1980; DeLuca, 1980; Miller, 1983). Boyle et al (1971) were the first to discover that animals on a low calcium diet produced large amounts of $1,25(OH)_2D_3$, whereas animals on high calcium diets produced small amounts of $1,25(OH)_2D_3$. Thus low calcium dietary intake and consequent hypocalcemia markedly stimulated $1,25(OH)_2D_3$ production. Changes in serum calcium concentrations follow an inverse linear relationship with the production of $1,25(OH)_2D_3$ (Boyle at al, 1972). There is a tight relationship and cooperativity between parathyroid hormone and $1,25(OH)_2D_3$ in promoting the mobilization of bone calcium and phosphate (Garabedian et al, 1974) as well as the reabsorption of calcium in the distal convoluted tubules of the kidney (Kleeman et al, 1961).

Other hormonal influences on the synthesis of vitamin D

The main physiological calcium stresses occur during growth spurts, pregnancy and lactation in mammals, egg laying in birds and in all embryos.

In young chicks (Spanos et al, 1976a) and in growing children (Pike et al, 1977) circulating levels of $1,25(OH)_2D_3$ are elevated suggesting that vitamin D may be responsible for the observed changes in calcium absorption . Human growth hormone increases the concentration of intestinal D-CaBP in hypophysectomised rats (Bruns et al, 1983). Growth hormone may regulate plasma levels of $1,25(OH)_2D_3$ during growth (Spanos, 1978).

Plasma levels of prolactin are elevated during late pregnancy and in lactating mammals (Hwang et al, 1971) and egg laying birds (Bolton et al, 1975).The increased calcium absorption observed in these physiological situations may be explained by high levels of circulating $1,25(OH)_2D_3$ (Pike et al, 1977; Spanos et al, 1976b,c).

Prolactin injections stimulate 1-hydroxylase activity (Spanos et al, 1976c) and elevate plasma levels of 1,25$(OH)_2D_3$ in chicks (Spanos et al, 1976c). Inhibition of prolactin secretion in lactating rats causes a marked fall in 1,25$(OH)_2D_3$ plasma levels, while replacement therapy restores the plasma levels to normal (Spanos, 1978). These experiments indicate that prolactin is involved in the regulation of vitamin D under the appropriate physiological conditions.

Studies performed on diabetic rats suggest that vitamin D may be implicated in the production of calcium malabsorption observed in these animals. These animals have decreased calcium absorption (Schneider and Schedl, 1972) and decreased vitamin D-dependent calcium binding protein (D-CaBP) (Schneider et al, 1974). The transport defect can be corrected by the administration of 1,25$(OH)_2D_3$ but not by 25$(OH)D_3$ (Schneider et al, 1975). Indeed serum levels of 1,25$(OH)_2D_3$ are significantly lower in rats with streptozocin-induced diabetes (Schneider et al, 1977). Although the precise point at which insulin affects the renal hydroxylation of 25$(OH)D_3$ is not known, recent studies have demonstrated that insulin is necessary for the maximal stimulation of renal 1,25$(OH)_2D_3$ production by PTH (Wongsurawat and Armbrecth, 1985).

Molecular mechanism of action of 1,25$(OH)_2D_3$

The chemical structure of vitamin D and its metabolites is closely related to the four ring-nucleus of other steroid hormones such as: estrogen, progesterone, testosterone, glucocorticoids and mineralocorticoids. The similarity extends to the need of inserting hydroxyl groups at specific sites in order to confer biological activity on the molecule. Steroid hormones act via a two step mechanism: the hormone binds to a high specificity intracytoplasmic receptor, this complex then binds to nuclear receptors to regulate gene transcription. Very little is known concerning the molecular details of the mechanism of entry of steroid hormones into cells (Norman, 1980). The lipid soluble uncharged molecules are not repelled from the plasma membrane and can easily cross the cell's plasma membrane. Once inside the target cell, the hormone binds to a high specificity cytosolic receptor. This complex travels to the nucleus where it interacts with DNA to stimulate gene transcription (Gorski and Gannon, 1976; Buller and O'Malley, 1976).

Nuclear localization of $1,25(OH)_2D_3$ in target cells

Early work established that after administration of radiolabelled vitamin D and $1,25(OH)_2D_3$ there was a rapid accumulation of the steroid in the intestine (Neville and DeLuca, 1966). This was also demonstrated in the chick (Bilke et al, 1982). Cellular fractionation studies showed that the radiolabeled substance accumulated in the nucleus and that the binding sites in the nuclear fraction were saturable since previous administration of unlabeled $1,25(OH)_2D_3$ prevented the accumulation of 80% of the labelled substance (Stohs and DeLuca, 1967; Haussler et al, 1968; Chen and DeLuca, 1973; Lawson and Wilson, 1974). However there is some question whether biochemical methods can truly establish nuclear localization of 1,25(OH)$_2$D$_3$ since artifactual redistribution can easily occur during fractionation. The synthesis of radiolabeled 1,25(OH)$_2$D$_3$ of high specific radioactivity (Brumbaugh and Haussler, 1974; Napoli et al, 1980) allowed the confirmation of the rapid (within 15-30 min) localization of this hormone in the enterocyte nucleus by radioautography (Jones and Haussler, 1979) and has also demonstrated the existence of many unsuspected vitamin D targets (Stumpf et al, 1979; Stumpf et al, 1980; Narbaitz et al, 1981).

Intracellular 1,25(OH)$_2$D$_3$ receptor protein

Brumbaugh and Haussler (1973) were the first to present evidence demonstrating the existence of a high specificity receptor molecule for 1,25(OH)$_2$D$_3$ in enterocytes. This could not be readily confirmed but with the introduction of careful washing to eliminate proteolytic digestion, and the inclusion of high salt concentrations in the fractionating medium, the existence of a receptor was demonstrated by sucrose density gradient centrifugation and Scatchard analysis of binding characteristics (Norman et al, 1982a). The receptor has been well characterised in both the rat and the chick intestine (Kream et al, 1977; Feldman et al, 1979; Wecksler et al, 1980). It is a macromolecule having a sedimentation rate of 3.7s on sucrose density gradient in chick small intestine and 3.2s in the rat small intestine. The dissociation constant (Kd) of 1,25(OH)$_2$D$_3$ for the chick receptor is 5×10^{-11} M (Mellon and DeLuca, 1979) and

7.4×10^{-11} M for the rat receptor (Wecksler et al, 1979a). The homogeneous receptor has been isolated and it is apparently a single polypeptide of 68,000 daltons (Simpson and DeLuca, 1982). The presence of high affinity binding sites for $1,25(OH)_2D_3$ has been documented in the duodenum of a variety of species eg: frog (Hausler et al, 1982), eel (Marcocci et al, 1982), mouse (Colston et al, 1980) and man (Wecksler et al, 1979b). This observation has been extended to include a wide variety of tissues: kidney, bone, pancreas, (Norman et al, 1982a). Whether the receptor macromolecule is in the cytosol or in the nucleus prior to its interaction with vitamin D hormone is not known since when isolation is carried out in a low salt medium , 90% of the unbound receptors are recovered from the nuclear fraction (Kream et al, 1976). In a high salt medium the receptor appears in the cytosol (Walters et al, 1980). Monoclonal antibodies have been produced against the receptor (Pike, 1983) and application of immunocytochemical methods could resolve this problem.

The receptor has been used by Brumbaugh and Haussler (1974a,b; Haussler et al, 1977, 1980) to develop a competitive binding assay to estimate circulating plasma levels of $1,25(OH)_2D_3$.

The appearance of receptors during development has provided us with an experimental model demonstrating that $1,25(OH)_2D_3$ must interact with its receptor before eliciting a hormonal response in the nucleus. Lactating rat pups are insensitive to exogenous $1,25(OH)_2D_3$ (DeLuca et al, 1982b). Examination of neonate rat pup intestine by sucrose density gradient analysis revealed that receptors can only be detected at weaning (Halloran and DeLuca, 1981) when the intestine begins to respond to $1,25(OH)_2D_3$ (Deluca et al, 1982b). In man a disease called vitamin D-dependent rickets type II is characterized by severe manifestations of rickets, high blood levels of $1,25(OH)_2D_3$ and a lack of target organ responsiveness to the vitamin D hormone (Bell et al, 1978; Rosen et al, 1979). Resistance to vitamin D is thought to be in part due to the lack of receptor molecule for $1,25(OH)_2D_3$ (Eil et al, 1981). Several types of receptor abnormalties have been identified that may explain clinically observed end-organ defects in the action of $1,25(OH)_2D_3$ (Liberman, 1983; Pike 1985). This observation provides us with further evidence demonstrating that $1,25(OH)_2D_3$ must interact with its receptor molecule before it can elicit a target organ response.

Nuclear effects of vitamin D hormone

Administration of vitamin D_3 and $1,25(OH)_2D_3$ to rachitic chicks stimulates general intestinal RNA metabolism (Tsai and Norman, 1973a) and nucleoplasmic DNA-dependent RNA polymerase II but not polymerase I (Zerwekh et al, 1974). Increased chromatin template activity was observed in chick (Haussler, 1968; Zerwekh et al, 1976) and in rat intestine (Hallick and DeLuca, 1969) and kidney (Chen and DeLuca, 1973). It also stimulates RNA synthesis (Norman, 1966; Stohs et al, 1967).

Even intravenous administration of $1,25(OH)_2D_3$ does not totally abolish the time lag before the appearance of the biological response in intestinal calcium transport (Emtage et al, 1974; Spencer et al, 1976). There is a time lapse of at least 4 hours after injection before an increase in calcium absorption can be measured. This lag is probably due to the time-dependent activation of genomic information followed by an increase in mRNA synthesis and specific protein synthesis. Thus certain actions of vitamin D and its active metabolite should be blocked by inhibitors of gene replication, gene transcription and translation processes. Actinomycin D is an antibiotic that binds to the guanine residues of DNA, thereby inhibiting DNA directed protein synthesis (Reich, 1964). In vivo work in several laboratories have provided conflicting results of its effect on vitamin D action. On one hand there are reports showing that actinomycin D does not affect $1,25(OH)_2D_3$ mediated increased intestinal calcium transport in the rat (Tanaka et al, 1971) and chick (Bikle et al, 1978). On the other hand it was found that actinomycin D suppresses the hypercalcemic effects of vitamin D in mice (Eisenstein and Passavoy, 1964) and the rise in calcium transport in rats (Zull et al, 1965) and chicks (Norman, 1965; Lawson and Emtage, 1974). In these studies actinomycin D was used at close to lethal doses and the animals were exposed to high levels of the antibiotic for prolonged periods. These experimental conditions may account for the above discrepancy. Similar difficulties can be circumvented by the use of organ and tissue culture techniques since high concentrations of the compound can be directly administered to the tissue of interest causing rapid and complete inhibition. Corradino (1973a) has demonstrated the adequacy of using organ culture of chick embryonic gut in studying $1,25(OH)_2D_3$ induced calcium transport. He has reported that calcium uptake and calcium transport measured by the everted gut technique is blocked by actinomycin D but also by x amatin , which is an irreversible inhibitor of RNA polymerase I (Scott and Tomkins, 1975) and by cycloheximide (Corradino, 1973a) which blocks peptide bond formation through binding to the large ribosomal subunit (Vasquez, 1974). Recently Franchesi and DeLuca (1981b) have confirmed the adequacy of the in vitro embryonic chick model for the study of the mechanism of action of $1,25(OH)_2D_3$. These authors examined the effects of inhibitors of protein and mRNA synthesis as early as 6 hours after the onset of $1,25(OH)_2D_3$ stimulation of calcium uptake in cultured embyronic enterocytes. They demonstrated that cycloheximide and actinomycin D blocked hormone-dependent calcium uptake, in a completely reversible and partially reversible manner respectively and that protein or RNA synthesis was inhibited 68% and 51% respectively without significant toxic effects. Anisomysin, another inhibitor of polypeptide chain elongation (Scott and Tomkins, 1975) and α amatin also blocked $1,25(OH)_2D_3$ dependent calcium uptake (Franchesi and DeLuca, 1981b). These studies indicate that $1,25(OH)_2D_3$ stimulates mRNA synthesis as the mechanism of activation of intestinal calcium transport.

Vitamin D-dependent calcium binding protein (D-CaBP), a genomic expression of vitamin D hormone action

To date the most studied protein whose de novo synthesis is regulated by $1,25(OH)_2D_3$ is vitamin D-dependent calcium binding protein or D-CaBP.

Avian and mammalian D-CaBPs

D-CaBP was first isolated from the chick intestinal mucosa (Wasserman and Taylor, 1966) and was subsequently purified from tissues of many other animal species (for review see Wasserman et al, 1978; Norman, 1979; Taylor, 1980). Characterization of this protein has demonstrated that two main types of D-CaBPs exist: type 1 (D-CaBP-I) which is of avian duodenal and cerebellar origin, and type 2 (D-CaBP-II) of mammalian duodenal origin. They differ in molecular weight (28,000 and 10,000 daltons respectively) and in the number of high affinity Ca^{+2} binding sites (4 and 2 respectively). Both proteins are highly acidic (pI=4.2-4.7) and have a Ca^{+2} binding constant of 10^{-6}M (see reviews, Wasserman et al, 1978; Wasserman, 1980). D-CaBP-I has been found in the kidney and cerebellum of both avian and mammalian species, whereas D-CaBP-II has been so far detected only in tissues of mammals (Wasserman and Feher, 1977; Wasserman et al, 1978). The amino acid composition of D-CaBP-I of the chick gut (Taylor and Brindak, 1974) and of D-CaBP-II of porcine and bovine gut (Bredderman and Wasserman, 1974; Hofman et al, 1979; Fullmer and Wasserman, 1980, 1981) has been determined. Sequence information on the porcine and bovine intestinal D-CaBP-II has been published (Fullmer and Wasserman, 1981; Szebenyi et al, 1981). The primary structure of D-CaBP-II of the rat duodenum has been deduced from the nucleotide sequence of cDNA clone obtained from rat D-CaBP-II mRNA. Rat duodenal D-CaBP-II differs from the bovine and porcine sequences by 16 and 14 residues respectively, whereas the residues of the calcium binding domain are conserved (Desplan et al, 1983). The 3-D structure of D-CaBP-II from bovine intestine has been recently published (Szebenyi et al, 1981; Szebenyi and Moffat, 1983). Chemical characterization of D-CaBP-I from mammalian kidney has been accomplished in the mouse (Delorme et al, 1983), the rat (Hermsdorf and Bronner, 1975; Pansini and Christakos, 1984),the dog (Sands and Kessler, 1971), and man (Morrisey and Rath, 1974). Recently the presence of D-CaBP-I has been demonstrated in the reptilian kidney (Rhoten et al, 1984). Similarly, characterization of D-CaBP-II of duodenal origin has been achieved in many mammalian species (Fullmer and Wasserman, 1975; Bruns et al., 1977, 1978; Oldham et al, 1980; Gleason and Lankford, 1981; Staun et al, 1984). Both types of D-CaBPs are coded for by a specific mRNA (Thomasset et al, 1983).

Calcium-binding proteins

D-CaBPs belong to a class of low molecular weight intracellular calcium modulated proteins first described by Kretsinger (1976). A high sequence homology found in the calcium binding domain which is called the "EF" hand is characteristic of calcium binding proteins of the Calmodulin superfamily. Other members of this class are not vitamin D-dependent and include: parvalbumin, Calmodulin, troponin C and the brain S-100 protein (see reviews: Kretsinger, 1976; Kretsinger et al, 1982; Goodman et al, 1979; Levine and Williams, 1982; Levine and Dalgarno, 1983).

D-CaBP-II is related to parvalbumin (Szebenyi et al, 1981) which is a low molecular weight (MW=12,000) calcium binding protein found in the sarcoplasm of vertebrate skeletal muscle cells as well as in some neurons where synaptic transmission is triggered by Ca^{+2} influx (Celio and Heizman, 1981). Mammalian D-CaBP and parvalbumin both contain two loop-helix calcium-binding domain, resembling in internal configuration and relationship to one another but differing in amino acid sequence (Szebenyi et al, 1981).

The molecular weight, amino acid sequence, calcium binding constant, and changes in mobility and configuration with calcium binding of D-CaBP-I are similar to Calmodulin. Calmodulin is a ubiquitous protein found in both vertebrates and plants, which regulates a number of intracellular enzyme activities in a calcium dependent manner and binds a class of anti-psychotic drugs called phenothiazines (Cheung, 1980). Antiserum against D-CaBP-I (Thomasset et al, 1982b; Rhoten et al, 1982) and D-CaBP-II (McCann et al, 1983) does not cross-react with calmodulin . Moreover, D-CaBP-I does not stimulate 3'5' cyclic phosphodiesterase and does not bind to phenothiazine (Rhoten et al, 1982). Localization by immunohistochemical techniques have demonstrated that calmodulin and D-CaBP-I differ in their intracellular distribution in the intestinal cell (Thomasset et al, 1981) and in kidney tubular cells (Rhoten et al, 1982). Finally calmodulin concentrations in the intestine do not respond to 1,25(OH)$_2$D$_3$ (Thomasset et al, 1981b). Hence D-CaBP-I and calmodulin are functionally, immunochemically and immunocytochemically distinct proteins (Christakos et al, 1984).

D-CaBPs as molecular markers of 1,25(OH)$_2$D$_3$

The presence of D-CaBP-I in the chick duodenum and of D-CaBP-II in rat duodenum is totally dependent upon vitamin D$_3$ or one of its active metabolites (Wasserman and Taylor, 1966; Corradino and Wasserman, 1971; Drescher and DeLuca, 1971; Bar and Wasserman, 1974; Moriuchi et al, 1975; Corradino et al, 1976; Thomasset et al, 1979a). Vitamin D also induces the formation of D-CaBP-I in the chicken shell gland (Corradino et al, 1968), in the chick kidney (Taylor and Wasserman, 1967; Wasserman et al, 1977; Christakos and Norman, 1980) and in the rat kidney (Hermsdorf and Bronner, 1975; Thomasset et al, 1979a, 1982a, 1983). The most potent inducer of D-CaBP-I in the chick intestine (Corradino, 1973c; Franchesi and DeLuca, 1981a) so far evaluated is 1,25(OH)$_2$D$_3$.

The development of sensitive radioimmunoassays for both type I D-CaBP (MW=28,000) and type II D-CaBP (MW=10,000) has permitted the demonstration of the vitamin D dependence of D-CaBPs in other tissues as well (Christakos et al, 1979; Thomasset et al, 1982b). The vitamin D dependence seems to reflect cell turnover time since D-CaBP can still be detected in tissues which have a slower turnover time than the duodenum after it has disappeared from the enterocytes (Taylor and Wasserman, 1972; Taylor and Brindak, 1974; Spencer et al, 1976; Christakos et al, 1979; Thomasset et al, 1982b).

The concentration of D-CaBP-I in the chick intestine is highly correlated with the amount of radiolabelled 1,25$(OH)_2D_3$ recovered from the intestine (Freidlander et al, 1977). The induction of D-CaBP-I by 1,25$(OH)_2D_3$ is due to an increase in the rate of synthesis of the protein and this in turn is dependent upon the regulated production of the hormone (Friedlander et al, 1977). Indeed the appearance and amount of D-CaBP-I in the chick and D-CaBP-II in the rat intestine has been used as a quantitative index of the vitamin D status of the animal (Bar and Wasserman, 1974; Bronner and Freund, 1975; Marche et al, 1978).

The production of D-CaBP mRNA in the chick (Emtage et al, 1973; Spencer et al, 1976, 1978; Christakos and Norman, 1980; Charles et al, 1981; Siebert et al, 1982) rat (Thomasset et al, 1981a, 1982b) and pig duodenum (Mellersh et al, 1980) is regulated by 1,25$(OH)_2D_3$. This evidence is consistent with a transcriptional regulation of D-CaBP synthesis by 1,25$(OH)_2D_3$. Since D-CaBP is a genomic expression of vitamin D hormone action, this protein can be used as a molecular marker for the presence of 1,25$(OH)_2D_3$ targets.

Vitamin D and the mechanism of intestinal calcium absorption

Intestinal calcium transport is regulated by the need of the organism for calcium. Orr et al, (1923) were the first to demonstrate that vitamin D stimulates calcium absorption. Nicolaysen (1953) noted that an inverse correlation existed between the amount of calcium absorbed by rats and the extent of mineralization of their skeleton. Nicolaysen postulated the existence of an "endogenous factor" that controlled intestinal absorption of calcium according to the prevailing requirements for the proper mineralization of bone. With the discovery of 1,25$(OH)_2D_3$ it became clear that Nicolaysen's endogenous factor is 1,25$(OH)_2D_3$. It is now possible to correlate the conversion of vitamin D to the active steroid hormone with changes in the efficiency of intestinal calcium transport (Norman, 1979).

Of the three major regulators of calcium metabolism, parathyroid hormone, vitamin D_3 and calcitonin, only vitamin D has been demonstrated to have a direct effect on calcium transport in the small intestine (Bronner, 1982). In fact the epithelial cells of the small intestine are the best studied classical vitamin D target cells. The primary effect of the hormone at this site is the optimization of the absorption of calcium and phosphorus. One way vitamin D may stimulate calcium absorption is by increasing the effective surface area. In a morphometric study performed on rats, Sampson and Krawitt (1976) found that vitamin D repletion of rachitic animals increased the height of villi by 27%. This study also showed that microvilli were longer in vitamin D repleted rats. An increase in the height of microvilli as a result of vitamin D replacement has also been demonstrated in the chick duodenum (Jande and Brewer, 1974). The synthesis of actin in the brush border is stimulated by $1,25(OH)_2D_3$ (Wilson and Lawson, 1978). However the molecular events underlying the effects of $1,25(OH)_2D_3$ on calcium translocation are still not completely understood.

The intestinal mucosa is a "leaky" epithelium, i.~~e. the~~ tight junctions which join the cells at the luminal surface are readily permeated by ions in solution (Claude and Goodenough, 1973). Calcium can cross the gut epithelium either by diffusion, moving between the cells (the paracellular shunt pathway) due to the presence of a favorable electrochemical gradient (Walling, 1982) or through the cells by an energy dependent process. Hence at least 2 mechanisms are involved in calcium absorption : a saturable metabolically active process where calcium is translocated through the enterocytes and a non-saturable passive process where calcium travels via the paracellular shunt. Different parts of the intestine differ in their capacity to absorb calcium. In the rat (Pansu et al, 1981) and chick (Wasserman et al, 1968) calcium absorption is greatest in the duodenum, decreased in the jejunum and is the least in the ileum. In a detailed study of calcium absorption in the rat small intestine using the in situ loop procedure, Pansu et al (1981) demonstrated that the diffusional process is present all along the small intestine but that the active process is present only in the proximal portion of the small intestine. The active saturable process varies inversely with calcium intake (Pansu et al, 1981) and age (Zornitzer and Bronner, 1971) being high in young rats and in those on a low calcium regimen. The non-saturable process does not appear to be affected by prior calcium intake (Pansu et al, 1981) and seems similar in young and old animals (Bronner et al, 1981; Zornitzer and Bronner, 1971). According to these investigators these results indicate that the non-saturable component which represents calcium absorption by diffusion is not affected by vitamin D. However other investigators have presented evidence to the contrary. Harrison and Harrison (1960) have shown that vitamin D accelerates the passive transfer of calcium across the everted gut sac in the rat. This was also confirmed in the chick (Norman et al, 1968). In a freeze fracture replication study of chick duodenum, Jande (1976) observed that in the zonula occludens of rachitic chicks fewer ridges are present then in vitamin D replete animals, and many ridges show discontinuities. This morphological observation supports the notion that vitamin D affects absorption by diffusion.

The overall characteristics of the active process of calcium absorption have been well studied (see following reviews: Norman, 1979; Nellans and Kimberg, 1979; Bronner et al, 1982; Walling, 1982). The active process of calcium translocation from the intestinal lumen to the serosal fluid occurs against an electrochemical gradient (Schachter, 1961; Martin and DeLuca, 1969; Walling and Rothman, 1968). This transfer of calcium across the intestinal mucosa is energy dependent since it is inhibited by low temperatures and inhibitors of oxidative phosphorylation.

Schachter and Rosen (1959) were the first to use the everted intestinal sac technique to demonstrate that vitamin D stimulates the transfer of calcium against a concentration gradient in the intestine. It is well established that the active process of Ca^{+2}

absorption is vitamin D dependent (Norman, 1979; Nellans and Kimberg, 1979; Nemere et al, 1984) and that the amount of circulating 1,25$(OH)_2D_3$ varies inversely with calcium intake (Edelstein et al, 1978). In man, net intestinal calcium absorption was found to be positively correlated to plasma 1,25$(OH)_2D_3$ levels (Wilz et al, 1979). The regulation of calcium absorption by the administration of vitamin D is a dose dependent phenomenon (Norman, 1979) and the elapsed time required is a function of the vitamin D status and prior calcium intake (Bronner et al, 1982).

The process of active calcium absorption follows Michaelis-Menten kinetics and in the rat gut it has an apparent Km of about 1mM (Papworth and Patrick, 1970; Walling and Rothman, 1970; Walling and Kimberg, 1974; Nellans and Kimberg, 1978) and may be saturated at a calcium concentration near 2mM. At this and higher concentrations net passive diffusion can occur. Hence the hormonally regulated active process has a major role in determining the calcium flux across the gut when luminal calcium concentrations are too low for net diffusion to occur (Walling, 1982).

The translocation of Ca^{+2} across the intestinal epithelium can be divided into 3 steps. The first step is the entry of Ca^{+2} across the brush border and into the cell. The second step is the movement of Ca^{+2} inside the cell. Once Ca^{+2} reaches the basolateral membrane it is extruded out of the cell and this is the third and final step.

Entry of Ca^{+2} across the brush border.

The intestinal brush border is a complex organelle comprised of the glycocalyx, the microvillar plasma membrane which includes a number of associated digestive enzymes, and elements of the cytoskeleton in the apical cytoplasm. The Ca^{+2} ion most likely interacts with anionic groups associated with the glycocalyx and traverses the microvillar membrane through channels or by interaction with a diffusion facilitating factor. On thermodynamic grounds, both the concentration gradient and electropotential gradient greatly favors the passive entry of calcium into the enterocyte (Nellans and Kimberg, 1979). Surprisingly the initial rate of calcium uptake into the chick and rat enterocytes is a saturable process which must be carrier-mediated and is stimulated by vitamin D (Martin and DeLuca, 1969; Walling and Rothman, 1970; Nellans and Kimberg, 1979; Bronner, 1982). Experiments with isolated brush border membrane vesicles demonstrated that vesicles from vitamin D_3-dosed rats (Miller and Bronner, 1981) or chicks (Rasmussen et al, 1979) accumulated Ca^{+2} at a much faster rate than those from rachitic animals. The molecular nature of vitamin D regulation of calcium entry may involve synthesis of a channel or a carrier site or could also involve a modification of the lipid composition of the plasma membrane. The change in permeability of the brush border membrane brought about by vitamin D hormone could be the result of an increase in plasma membrane fluidity (Rasmussen et al, 1982). Intestinal cells from $1,25(OH)_2D_3$ treated chicks incorporate more phosphatidyl choline (PC) than phosphatidylethanolamine as compared to rachitic chicks (Matsumoto et al, 1981; Wasserman et al, 1982). Both phospholipase A_2 activity and the acylation of lyso-phosphatidyl-choline is enhanced by $1,25(OH)_2D_3$ in rats (O'Doherty, 1979). The increased synthesis and increased incorporation of phospholipids with a higher content of unsaturated groups suggest that the increased brush border membrane fluidity in turn might increase Ca^{+2} permeability (Rasmussen et al, 1982). However spin resonance studies revealed no difference in the fluidity of the brush border membrane which could be ascribed to vitamin D hormone (Putkay et al, 1982). The validity of the fluid state hypothesis has been challenged (Wasserman et al, 1984).

The group of Weiser et al (Feedman et al, 1977; Mac Laughlin et al, 1980) have reported interesting observations on the effect of vitamin D_3 and $1,25(OH)_2D_3$ on Ca^{+2} uptake by a membrane fraction identified to be primarily of Golgi origin. The level of calcium uptake by Golgi membrane vesicles from different regions of the intestine paralelled the level found in the everted sac preparation from these regions, ie duodenum>ileum>jejunum and was vitamin D dependent. Within 15-30 minutes after administration of $1,25(OH)_2D_3$ to rachitic rats they noted a significant uptake of Ca^{+2} by these membranes. This rapid effect of $1,25(OH)_2D_3$ was inhibited by cycloheximide and it was concluded that this effect is dependent on new protein synthesis (Weiser et al, 1981). However the increased calcium uptake was not energy dependent. This $1,25(OH)_2D_3$-stimulated calcium uptake was interpreted as representing the induction of production of Ca^{+2} channels or transport facilitator that would be shuttled to the plasma membrane and could then be responsible for the $1,25(OH)_2D_3$ induced alteration in membrane permeability of the brush border membrane to Ca^{+2}.

Other brush border membrane effects

Intestinal alkaline phosphatase (ALKPase) is found primarily in the brush border membrane (Chase, 1963; Eicholz and Crane, 1965). The relationship between ALKPase activity and vitamin D was first demonstrated by Motzok (1950). Vitamin D_3 induces a two- to three-fold increase in alkaline phosphatase activity in the intestinal brush borders of rachitic chicks (Haussler et al, 1970; Norman et al, 1970; Holdsworth, 1970). Vitamin D also promotes the phosphorylation of alkaline phosphatase (Wasserman and Brindak, 1979; Wilson and Lawson, 1981; DeJonge et al, 1981). The activity of low affinity Ca^{+2},Mg^{+2}-stimulated ATPase active in the mM range of calcium ion concentration was also shown to be increased by vitamin D (Melancon and DeLuca, 1970; Martin et al, 1969; Mircheff et al, 1977; Haussler at al, 1970; Birge et al, 1974). There is a lack of correlation between ALKPase activity and $1,25(OH)_2D_3$ induced calcium transport (Morrisey et al, 1970; Wasserman et al, 1977; Bikle et al, 1979) whereas the low affinity Ca^{+2}-ATPase responds within the same time frame as the calcium absorptive system (Lane and Lawson, 1978). However it seems that these two enzymes may be different expressions of the same molecular complex (Russell et al, 1972). The Ca^{+2}-ATPase portion of the molecule could conceivably pump calcium into the cell, whereas the alkaline phosphatase portion may not be directly involved in 1,25$(OH)_2D_3$ stimulated calcium transport.

There are many other brush border membrane proteins whose synthesis is stimulated by 1,25$(OH)_2D_3$ eg: actin (Wilson and Lawson, 1978), a 80-90 kilodalton protein (Wilson and Lawson, 1977; Rasmussen et al, 1977), a brush border vitamin D-dependent calcium binding protein (IMCal MW=200,000) (Kowarski and Schachter, 1980; Schachter and Kowarski, 1982). This IMCal complex contains 3 distinct activities: high affinity calcium binding (CABC), ALKPase and Ca^{+2}-ATPase. The CABC activity varies in concordance with the intestinal transport mechanism. These investigators have proposed that IMCal is a component of the entry mechanism of Ca^{+2} into the cell.

Vitamin D hormone also brings about alterations in the topography of the chick brush border which are not dependent upon the synthesis of new proteins. Differences in the accessibility of specific proteins to a non-permanent probe (125-I-diazoiodosulfanilic acid) (Norman et al, 1982b) as well as in the release of enzymes by proteolysis and calcium uptake by brush border membrane vesicles have been reported (Nemere et al, 1983) as a result of administration of vitamin D_3 or 1,25$(OH)_2D_3$ to rachitic chicks.

Exactly how all these factors are integrated to produce the observed effect of 1,25$(OH)_2D_3$ on calcium absorption in the intestine still needs to be defined.

<u>Transport of calcium across the intracellular milieu</u>

The ionic calcium concentration of cells in the resting state is in the range of 10^{-7} to 10^{-8}M and possibly lower (Carafoli and Crompton, 1978). Because intracellular Ca^{+2} is important in regulating a wide range of cellular functions (Carafoli and Crompton, 1978) it must be tightly controlled. The low intracellular ionic calcium concentration is maintained by the uptake of Ca^{+2} by endoplasmic reticulum (DeMeis, 1982) and mitochondria (Carafoli, 1982), binding to high affinity calcium binding proteins, extrusion of Ca^{+2} across the plasma membrane by an ATP-dependent pump (Schatzman,1982) and in many cells by a Na /Ca exchange mechanism (Blaustein and Nelson, 1982). Specific hormones can influence the intracellular milieu by causing a transient increase in cytosolic Ca^{+2} concentration either by releasing calcium from intracellular stores and/or by increasing the influx of Ca^{+2} across the plasma membrane. Through interaction with calmodulin and other calcium binding proteins, calcium activation of various enzyme systems occurs. It is through this precisely controlled environment that relatively large amounts of Ca^{+2} must travel during the course of calcium absorption. Bronner (1982) using data from Pansu et al (1981) estimated that the amount of calcium per hour that crosses the intestinal cells is at least $3x10^5$ greater than the free calcium concentration.

There are many proposed mechanisms that could prevent excessively high ionic Ca^{+2} concentration. The possibility that Ca^{+2} is translocated through the cell in vacuoles or in membrane enclosed packets has been proposed (Warner and Coleman, 1975; Davis and Jones, 1981). Jande and Brewer (1974) in an electron microscopic study observed increased endocytotic activity in vitamin D replete chicks. This would provide a means of transporting large amounts of Ca^{+2} through the cell without disturbing the intracellular milieu.

On the basis of results obtained by pyroantimonate precipitation of calcium, Weringer et al (1978) suggested that mitochondria play an important role in sequestering and transporting Ca^{+2} from the apex to the basal region of the cell. Isolated mitochondria accumulate calcium from the surrounding medium using energy-coupled respiration. Calcium uptake can proceed until the extramitochondrial Ca^{+2} concentration is approximately 10^{-6}M, a value in the range of intracytosolic Ca^{+2} concentration (Fiskum and Lehninger, 1980, 1982). From early in vitro studies Borle (1971, 1973) has proposed that the mitochondria serve as an intracellular ion buffer system. Mitochondria have long been thought to be involved in calcium transport across intestinal cells (Hamilton and Holdsworth, 1970; Omdahl and DeLuca, 1973). Vitamin D administered in vivo and in vitro does not affect calcium uptake by mitochondria but seems to favor the ability of the kidney mitochondria to release calcium in vitro (Engstrom and DeLuca, 1962). $1,25(OH)_2D_3$ stimulates the synthesis of a protein in the outer membrane of mitochondria from intestinal cells before maximum calcium transport is recorded (Hobden et al, 1980). However these investigators were unable to find a role for this mitochondrial protein. A small but direct effect of $1,25(OH)_2D_3$ on calcium uptake in vitro has been demonstrated by Bikle et al (1979). These investigators suggested that the increased mitochondrial uptake of calcium due to vitamin D_3 (Engstrom and DeLuca, 1962) was the result of the vitamin D-induced increase in cytosolic calcium. Somlyo et al (1981) assert that the calcium binding constant of mitochondrial membranes is so low in relation to intracellular Ca^{+2} that these organelles cannot regulate intracellular calcium.

D-CaBPs and calcium absorption

The biochemical change that correlates most closely with the effects of vitamin D and its active metabolite on the adaptive changes of calcium absorption by the duodenum, is the concentration of D-CaBP in the small intestine. The amount of circulating $1,25(OH)_2D_3$ varies inversely with calcium intake as does the duodenal content of D-CaBPs (Morrissey and Wasserman, 1971; Freund and Bronner, 1975; Edelstein et al, 1978; Ueng et al, 1979; Buckley and Bronner, 1980; Pansu et al, 1981). Freidlander (1977) demonstrated that there is a relationship between D-CaBP-I levels in the chick duodenum and renal 1 alpha-hydroxylase activity in conditions where changes in dietary calcium and phosphorus intake have occured.

There is parallelism between the D-CaBP-I content and active calcium absorption in the different segments of the chick intestine

(Taylor and Wasserman, 1967). There is a correlation between the affinity of chick duodenal D-CaBP-I for different divalent cations and the efficiency of intestinal absorption of the same cations (Taylor and Wasserman, 1967). In the neonatal rat, there is no saturable component of calcium absorption before weaning (Bronner et al, 1982). The intestine of young rat pups does not respond to vitamin D (Halloran and DeLuca, 1981) and there is no detectable D-CaBP-II until after the pups are weaned (Ueng et al, 1979). Furthermore there is a linear correlation between the maximum saturable flux (Jmax) of the saturable component of calcium transport and D-CaBP-II content in the rat duodenum (Armbecht et al, 1979; Pansu et al, 1981).

The above observations suggest that D-CaBPs may be directly involved in active calcium transport. It was thought that D-CaBP-I played an important role in the initiation of calcium transport in the intestine, since D-CaBP-I was thought to be localized on the surface of the brush border membrane (Taylor and Wasserman, 1970). However subsequent reports have demonstrated that D-CaBP-I is in fact an intracytoplasmic protein (Morrissey et al, 1978b,c; Jande et al, 1981a; Taylor, 1981; Thorens et al, 1982; Roth et al, 1982). This is consistent with the observation that D-CaBP-I is synthesized on free ribosomes (Spencer et al, 1978) as are other proteins that are not destined to be exported out of the cell.

Studies on the relationship of the chronological appearance of D-CaBP-I and the initiation of intestinal calcium transport induced by vitamin D and its active metabolites have yielded contradictory results. Early studies reported that vitamin D-stimulated calcium absorption occurs before the appearance of D-CaBP-I. These studies used the Chelex ion exchange assay to detect the appearance of calcium binding protein in the supernatant of duodenal mucosa preparation (Harmeyer and DeLuca, 1969). However this assay is characterized by poor sensitivity. By analysing duodenal cytosolic extracts with the more sensitive radial immunoassay, D-CaBP-I synthesis was found to start 4-12h after pharmacological levels of 1,25 $(OH)_2D_3$ were administered to vitamin D-deficient chicks (Ebel etal, 1969; Zerwek et al, 1976; Morrissey et al, 1978a; Kreutter et al, 1983) and embryonic organ cultures (Corradino, 1973a, 1973b). Small amounts of D-CaBP-I were also measured by radioimmunoassay 12h after cholecalciferol (Spencer Emtage et al, 1974) or 5-6h after $1,25(OH)_2D_3$ administration (Morrissey et al, 1978a; Siebert et al, 1982; Shinki et al, 1982) and 5-8h after $1,25(OH)_2D_3$ administration by rocket immunoelectrophoresis (Spencer et al, 1976a; Spencer et al, 1978b). Administration of vitamin D_3 and $25(OH)D_3$ increases the rate of biosynthesis of D-CaBP-I as measured by [3-H] leucine incorporation several hours before an increase in calcium transport was noted (MacGregor et al, 1970; 1971). To detect D-CaBP-I with a greater sensitivity, Spencer and coworkers (Spencer et al, 1976a,b; 1978b) administered $1,25(OH)_2D_3$ to rachitic chicks and examined the ability of duodenal polysomes to synthesize D-CaBP-I in a cell-free system. D-CaBP-I was consistently found among the translation products after 2h by immunoprecipitation and subsequent purification by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Using similar procedures Siebert et al (1982) detected the protein at 3h which was the earliest time studied. Increased D-CaBP-I levels have been detected by radial immunoassay 2h after 1,25$(OH)_2D_3$ administration and its increased concentration paralleled increased calcium absorption in the chick gut (Wasserman et al, 1982). Recent investigations performed in cultured embryonic chick duodenum using computer analysis of two-dimensional gel electrophoresis demonstrated that D-CaBP-I is induced within the first hour of exposure to DHCC in amounts that are too small to be detected by commonly used immunoassays (Bishop et al, 1983; 1984). Small amounts of D-CaBP could perform a catalytic function by modulating the activity of enzymes affecting calcium transport through the cell. D-CaBP-II added in vitro was shown to stimulate the activity of two intestinal plasma membrane enzymes in the rat: brush border ALKpase and basolateral Ca^{+2}-ATPase (Freund and Borzemsky, 1979; Freund, 1982). These investigators also presented evidence that D-CaBP-II binds to brush border membranes and basolateral membranes. Using a photoaffinity probe for D-CaBP-I, Norman and Leathers (1982) demonstrated that there is a specific interaction between chick intestinal D-CaBP-I and bovine intestinal ALKpase. This interaction occurs only in the presence of Ca^{+2} suggesting that this ion may mediate conformational changes in the D-CaBP molecule thus facilitating the interaction of D-CaBP with other cellular constituents. As discussed earlier the participation of ALKpase in calcium absorption is doubtful. The function of the association of D-CaBP-I with ALKpase in the molecular process of calcium absorption remains obscure.

Since D-CaBP is an intracellular protein it could serve as an intracellular calcium buffer. The concentration of D-CaBP-I correlates with the efficiency of Ca^{+2} absorption but not with the total amount of absorbed Ca^{+2}. Bar and Hurwitz (1979) demonstrated that D-CaBP-I concentrations varied directly with the percent absorption but inversely with the total amount of calcium absorbed by chicks fed diets containing different amounts of calcium. If D-CaBP-I acted as a buffer, the results should have been reversed.

Kretsinger et al (1982) developed a theoretical model suggesting that D-CaBP-I acts by increasing the diffusibility of Ca^{+2} and thus the availability of calcium at the baso-lateral membrane where Ca^{+2} extrusion takes place. The concentration of calcium would then be of great importance when the available Ca^{+2} in the duodenum is low. Feher (1983) devised an in vitro system to test this idea. A diffusional cell was divided into three compartments with dialysing membranes. The presence of chick duodenal D-CaBP-I in the middle compartment enhanced the translocation of Ca^{+2} from compartment 1 to compartment 3. No such enhancement was observed when bovine albumin was placed in the compartment. This evidence supports the hypothesis that D-CaBP could accelerate the transit of Ca^{+2} through the cell interior.

The discovery of the presence of D-CaBP in many other cell types that do not translocate calcium challenges the hypothesis that D-CaBP is exclusively involved in calcium translocation.

Extrusion of calcium across the baso-lateral membrane

The calcium storing capacity of intracellular systems is limited. Even though intracellular organelles and high affinity calcium binding proteins may transiently modulate intracellular calcium, calcium pumps in the plasma membrane are the primary regulators of intracellular Ca^{+2} concentration over the long term. The transfer of calcium from the cytosol to the extracellular fluid across the baso-lateral membrane is an energy dependent process. Two types of systems have been identified: a high affinity Ca^{+2}-dependent ATPase and a Na^{+} /Ca^{+2} countertransport system (Murer and Kinne, 1980; Blaustein and Nelson, 1982).

Evidence for a Ca^{+2}-dependent ATPase activity was provided by Martin and Deluca (1969), Mircheff and Wright (1976) and more recently by Ghijsen and van Os (1979; Ghijsen et al, 1980; van Os et al, 1981). The latter investigators have purified this enzyme and determined its molecular weight (MW=115,000). This Ca^{+2}-ATPase seems to be exclusively associated with the baso-lateral membrane (Mircheff and Wright, 1976; van Os et al, 1981). Ghijsen et al (1982) demonstrated that the activity of the basolateral Ca^{+2}-ATPase correlates well with the ATP-dependent uptake of calcium by everted basolateral membrane vesicles. The activity of this calcium pump is increased by vitamin D and its active metabolite in the rat (Martin and DeLuca, 1969; Ghijsen et al, 1982) and chick intestine (Meyer and Wasserman, 1983). In red blood cells, calmodulin has been shown to stimulate calcium extrusion across the plasma membrane (Larsen and Vicenzi, 1979). In the intestinal cell Calmodulin may stimulate the Ca^{+2}-ATPase of the baso-lateral membrane (Nellans and Popovitch, 1981; Ghijsen et al, 1982) However Nemere (1984) did not observe any effect of calmodulin on Ca^{+2}-ATPase activity. Calmodulin does not appear to be vitamin D dependent (Halloran et al, 1980; Thomasset et al, 1981b; Rhoten et al, 1982).

Earlier experiments indicated that Na^+ is not required for the uptake of calcium at the mucosal surface but it is required for the appearance of calcium on the serosal side (Martin and DeLuca, 1969; Adams and Norman, 1970; Birge et al, 1972). Ca^{+2} extrusion appears to be dependent on the presence of external Na^+ in many cell types (for review see Blaustein and Nelson, 1982) indicating the widespread occurence of a Na^+/Ca^{+2} exchange system in many animal tissues. A Na^+/Ca^{+2} exchange system which is dependent on a Na^+ gradient not subject to regulation by $1,25(OH)_2D_3$ (Ghijsen et al, 1983) has been identified in the basolateral membrane of rat intestinal cells.

In summary, the effects of vitamin D on the intestinal epithelial cells are complex and diverse. $1,25(OH)_2D_3$ alters the overall morphology of the intestinal villi as well as the protein, lipid and phospholipid composition of the different components of the enterocyte. How the pleiotropic effects of vitamin D_3 are integrated into the molecular mechanism of action of vitamin D on calcium absorption is still not completely resolved. Possible models interrelating the diverse effects have been suggested by many investigators (Bilke et al, 1979; Norman et al, 1982b; Wasserman et al, 1984).

Identification of vitamin D targets

There are various ways available to identify steroid hormone target organs and cells.

Radiolabelled 1,25$(OH)_2D_3$ was first used to determine the nuclear localization of 1,25$(OH)_2D_3$ thus identifying the duodenum as a prime vitamin D target (Stohs and DeLuca, 1967). Radiolabelled 1,25$(OH)_2D_3$ of a higher specific activity (Brumbaugh and Haussler, 1974; Napoli et al, 1980) made it possible to identify by radioautography a wide variety of vitamin D target cells in the rat (Stumpf et al, 1979; 1980; Narbaitz et al, 1981).

The biochemical isolation and characterization of a high affinity receptor macromolecule for 1,25$(OH)_2D_3$ has been carried out in a wide range of species and tissues (see review Norman et al, 1982a) thus identifying "new" vitamin D targets.

As discussed earlier one of the best known genomic expressions of 1,25$(OH)_2D_3$ is the regulation of D-CaBPs synthesis. The production of antibodies against D-CaBPs offers two main avenues of investigation. Quantitative estimation of D-CaBP-I in chick tissues (Christakos, 1979) and D-CaBP-I and II in rat tissues (Thomasset et al, 1982b) has been carried out by radioimmunoassay. With this technique the presence of D-CaBPs has been detected only in tissues where intracytosolic 1,25$(OH)_2D_3$ receptors have been demonstrated. The exact cellular localization of D-CaBPs can be visualized by immunocytochemistry and offers another possibility to identify vitamin D target cells within an organ. Here again a wide variety of cell types containing D-CaBP have been identified as vitamin D targets. (Jande et al, 1981a; Jande et al, 1981b; Jande et al, 1982; Schreiner et al, 1983; Lawson et al, 1984; Schreiner et al, 1985). Results obtained by radioautography in rat tissues (Stumpf et al, 1979; 1980) are in good agreement with those obtained by immunocytochemical localization of D-CaBP.

Evaluation of fixatives

Good fixation is a prerequisite to obtain consistent localization (Mukai and Rosai, 1980). In the case of D-CaBP-I poor fixation results in loss or diminution of antigen localization due to diffusion and/or adsorption of the antigen into the surrounding tissues. This problem is even more crucial for the localization of protein antigens at the EM level.

Using routine non-coagulating fixatives such as paraformaldehyde or fixation by freeze-thawing which are commonly used methods in immunofluorescence studies, D-CaBP-I was first thought to be localized in the goblet cells and in the brush border of chick enterocytes (Taylor and Wasserman, 1970). By using freeze substitution where the translocation of proteins is substantially prevented, it was demonstrated that D-CaBP-I is an intracytoplasmic protein of the enterocytes (Jande et al, 1981a; Taylor, 1981). Evaluation of different fixatives (Jande et al, 1981a) led to the conclusion that coagulating fixatives which precipitate proteins very rapidly such as Carnoy's and Bouin's give the most satisfactory results for immunocytochemical studies at the light microscopic level. In addition they were able to show that paraformaldehyde with or without glutaraldehyde is an inadequate fixative for immunocytochemical studies of D-CaBP-I_a at the electron microscope (EM) level.

These different methods have contributed to confirm one of the most unexpected new developments in vitamin D endocrinology: vitamn D targets are also present in cells that are not involved in transepithelial calcium transport. The wide distribution of vitamin D targets indicates that the vitamin D endocrine system extends beyond the classical target organs (duodenum, kidney, laying hen shell gland, bone). The most striking examples of non-classical targets are : neurons, pancreas B cells, reticular cells in the marginal zones of the spleen and reticular cells in the thymic cortex. Thus vitamin D targets can be divided into 2 main classes: the classical targets and the non-classical targets which unlike the classical targets are not directly involved in calcium translocation.

Vitamin D and the immune system

Vitamin D-deficient rickets has been frequently associated with recurrent infections (Stroder, 1975). Early clinical studies implicated defective neutrophils as the cause of increased susceptibility to infections (Stroder, 1975). Peripheral lymphocytes have shown decreased mobility and an impaired capacity to phagocytoze (Stroder and Kasal, 1970). Vitamin D-deficient mice also had depressed inflammatory responses: both macrophages and bone marrow polymorphonuclear leukocytes were observed to have impaired spontaneous migration and phagocytic capacity. These functions were corrected in vitro by incubating macrophages from vitamin D-deficient animals with $1,25(OH)_2D_3$ (Bar-Shavit, 1981).

There is an increasing body of evidence suggesting that $1,25(OH)_2D_3$ plays a regulatory role in the differentiation and maturation of leukocytes. $1,25(OH)_2D_3$ can induce in vitro the differentiation of murine and human myeloid leukemia cells into macrophages or granulocytes (Abe et al, 1981; Miyaura et al, 1981; Tanaka et al, 1982). $1,25(OH)_2D_3$ also induces the maturation of U937 cells which is a human monocyte line (Amento et al, 1984). Administration of $1,25(OH)_2D_3$ to mice which were inoculated with leukemia cells prolonged their survival (Homma et al, 1983). This suggests that $1,25(OH)_2D_3$ may be useful in the chemotherapy of certain leukemias.

Calcium is important in the proliferation of activated lymphocytes (Perris et al, 1968) and in the production of "hormones" of the lymphoid system such as the interleukins and interferons. Lymphocytes have been known to respond to the calcemic peptide hormones (parathyroid hormone and calcitonin) (Tsoukas et al, 1984). The presence of intracellular receptors specific for $1,25(OH)_2D_3$ in monocytes and in T lymphocytes indicate that these cells are indeed vitamin D targets. However, unlike monocytes, T lymphocytes express the intracytosolic receptor for 1,25(OH)$_2$D$_3$ only when they are activated by mitogenic lectins or Epstein Barr virus (Provvedini et al, 1983; Badha et al, 1983).

Miyakoski et al (1981) and Tsoukas et al, (1984) demonstrated that 1,25(OH)$_2$D$_3$ suppresses mitogen induced lymphoproliferation of human peripheral blood lymphocytes. 1,25(OH)$_2$D$_3$ inhibits the production of the growth promoting lymphokine, interleukin-2, by human T lymphocytes activated in vitro by phytohemagglutinin (Tsoukas et al, 1984). These investigators also found that 1,25(OH)$_2$D$_3$ inhibits the generation of cytotoxic T cells and antibody production by B cells. The production of interleukin-1, a lymphokine which modulates the function of T and B cells, by stimulated U937 cells is increased by 1,25(OH)$_2$D$_3$ (Amento et al, 1984). These observations suggest that the interaction between a classical hormone and the hormone-like products of cells of the immune system may play a role not only in leukocyte differentiation but also in other immune reactions. 1,25(OH)$_2$D$_3$ may be considered as a potential immune regulatory hormone (Stravos et al, 1984; Tsoukas et al, 1984). This adds a new link to the interaction between the endocrine and immune system as exemplified by the immunosuppressive effect of glucocorticoids (Cupps and Fauci 1982).

Objectives

D-CaBP is the only known genomic expression of 1,25(OH)$_2$D$_3$ which is the most potent metabolite of vitamin D. One of the fundamental questions concerning the mechanism of action of vitamin D is the function of D-CaBP in vitamin D targets.

As we have seen there are two types of vitamin D targets: classical targets which are directly involved in calcium homeostasis and non-classical targets which are not involved in transepithelial calcium transport.

The first part of this investigation is concerned with the possible function of D-CaBP in classical vitamin D target cells. Calcium pumps of the plasma membrane are thought to be the primary regulators of intracellular calcium concentration. These pumps are especially important in tissues such as the duodenum where calcium must first cross the epithelium to be absorbed into the bloodstream. In this context the aim of our investigations was to determine the effects, if any, of vitamin D-deficiency on the enzymatic activity of the putative calcium pumps (alkaline phosphatase, Na^{+}, Ca^{+2}-ATPase and Ca^{+2}, Mg^{+2}-ATPase) and to establish whether D-CaBP modulates the catalytic activity of these plasma membrane enzymes.

The primary objective of our present work concerned the localization of D-CaBP in both classical and non-classical target cells, using immunocytochemical methods at the light and electronmicroscopic level. The rationale for the work is the fundamental importance of information on the identity and intracellular distribution of D-CaBP's to the understanding of the mechanism of action of vitamin D.

II.MATERIALS AND METHODS

1) Animals

Fertilised white Leghorn eggs were obtained from a local poultry farm. They were incubated in a forced air incubator. One day old hatchlings of both sexes were provided by the Animal Disease Research Institute (ADRI, 801 Fallowfield Rd., P.O. box 11300, Station H, Nepean, Ont. Canada, K2H 8P9). Tissues were obtained from the following animals; C.D. rats (Charles River, 188 Lasalle, St. Constant, Que. Canada, J0L 1X0) ranging from 7 day old pups to 4 week old animals of both sexes; C57BL/6J mice (Jackson Laboratories, Animal resource division, Bar Harbour, Maine, USA, 04609) Paris R3 white mice of both sexes 3 to 4 week old (Jackson Laboratories); 2 month old New Zealand white rabbits (Ferme La Pro, C.P. 114, Stuckley-Sud, Que. Canada, J0E 2J0); frogs (Rana pipiens, AniLab, 16-3291 Ste Foy, Ste Foy, Que. Canada, G1X 3V2); gold fish (Carassius auratus, Tropical Fish Supplies, 999 Helena St., Fort Erie, Ont. Canada L2A 4K2). Pig tissues were obtained from a local slaughter house. Monkey (Macacca fascicularis) was perfused through the aorta with 0.5% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for about 10 minutes then 2- to 3mm kidney slices were fixed by immersion in Carnoy's fixative. Human kidney material was fixed in AFA (ethyl alcohol 70%, formaldehyde 20% and acetic acid 5%).

2) Chick diets

Normal chicks were fed Purina accu-start (Na=0.15%, Ca=0.9%, P=0.7%, vitamin A= 5,500 IU/kg, vitamin D= 1,000 IU/kg and vit E= 10 IU/kg). Rachitic chicks were kept from the time of hatching in an ultraviolet-free environment and were fed a synthetic vitamin D-deficient diet prepared according to Barnes et al (1973). Total Ca and P content in the diet was 1.06% and 0.9% respectively. Vitamin D-deficient diet containing high calcium was prepared as above but 50g of $CaCO_3$ per kg of feed was added producing a 2% calcium supplement.

3.1) Fractionation of chick duodenal mucosa

Chicks were killed by decapitation. The proximal duodenum was dissected out and the pancreas was removed. The duodenum was slit open and thoroughly washed with ice cold isotonic saline. The duodenum was gently blotted with bibulous paper and the mucosa was scraped with a teflon coated spatula. All subsequent steps were done on ice or in a 5°C cold room. The duodenal scrapings of 2 chicks were combined and were homogenized in 40ml of 5mM Na_2EDTA, pH 7.5 using a Dounce homogenizer. The homogenate was centrifuged at 45,000g for 20 min at 0-4°C. The supernatant thus obtained contained mostly soluble proteins and the pellet contained cellular organelles. The activity of Na^+,K^+-ATPase (Fujita et al, 1972), Na^+,Ca^{+2}-ATPase (Birge et al, 1972), Ca^{+2},Mg^{+2}-ATPase and alkaline phosphatase (Fujita et al, 1972) was measured in the whole homogenate, the supernatant, and the pellet.

3.2) Cell fractionation

Cell fractionation was carried out to isolate brush border from basolateral membrane in order to study the effects of vitamin D on localized membrane enzymes that may be involved in calcium transport.

Chicks were killed by decapitation. Duodenal scrapings were obtained as described above. One chick provided sufficient material for each experiment. All subsequent steps were done on ice or in a cold room.

The fractionation schedule of Mircheff and Wright (1976) which permits isolation of brush border and basolateral membranes from the same tissue sample was followed.

The duodenal scrapings were suspended in 15ml of 5mM Na^{+2}EDTA, homogenized in a Dounce homogenizer with 10 strokes of the tight pestle and centrifuged at 45,000g for 20 min. Final homogenization was performed with a loose pestle. The supernatant (S_0) was discarded. The pellet (P_0) was resuspended in 30ml of fractionation buffer. The fractionation buffer contained: 250mM sorbitol, 12.5mM NaCL, 0.5mM Na_2EDTA, and 5mM imidazole-histidine buffer at pH 7.5. The resuspended pellet was homogenized with 37 strokes and then diluted to 75ml and centrifuged at 450g for 10 min. The supernatant (S_1) was kept and the pellet (P_1) was resuspended in 30 ml of isolation buffer, homogenized with 30 strokes and diluted to 50ml and centrifuged at 450g for 10 min. The supernatant (S_2) was kept and the pellet (P_2) was resuspended in 30ml of isolation buffer, homogenized with 15 strokes and centrifuged at 450g for 10 min. S_1, S_2 and S_3 were pooled and centrifuged at 55,000 for 1hr to obtain P_3. P_3 was resuspended in 30ml of fractionation buffer, homogenized with 15 strokes and centrifuged at 450g for 10 min. This was repeated 3 times (P_4-P_6). The supernatants (S_4-S_6) were discarded. P_6 was resuspended in 30ml of fractionation buffer, homogenized with 15 strokes and centrifuged at 32g for 10 min. This was repeated and P_8 was discarded. S_7 and S_8 were centrifuged at 55,000g for 1hr to obtain the final pellet P_9. All centrifugation were done at 0-4°C.

4) Analytical assays

Na^+,K^+-ATPase was measured in the medium of Fujita et al (1972). The final incubation volume of 1ml contained 3mM ATP (Sigma, Chemcal Co., P.O. box 14508, St.Louis, Mo. 63178, USA), 5mM $MgCl_2$, 10mM KCL, 100mM NaCL in 0.1M Tris-HCL buffer at pH 7.40. The incubation period was 30 minutes at 37°C. The reaction was stopped with 2ml of ice cold 5% trichloroacetic acid. After standing at room temperature for 15 minutes the reaction mixture was centrifuged for 20 min at the highest speed in a clinical centrifuge. Inorganic phosphate released was measured with the method of Fiske and Subbarow using a commercially available kit (Sigma #670).

Ca^{+2},Mg^{+2}-ATPase was measured in a total volume of 1ml containing 5mM ATP (Sigma), 2mM $CaCL_2$, 0.5mM $MgCL_2$ in 50mM Tris-HCL buffer at pH7.4. The effect of calcium concentration was measured in the presence of 0.5mM Mg^{+2} and varying the Ca^{+2} concentration from 0.5mM to 10mM. The effect of pH was measured in the presence of 2mM Ca^{+2} and 0.5mM Mg^{+2} at various pH.

Na^+, Ca^{+2}-ATPase (Birge et al, 1972) was measured in the presence of 3mM ATP (Sigma), 100mM NaCL and 5mM $CaCL_2$. The mixture was incubated at 37°C for 1hr before it was stopped with 2ml of 10% trichloroacetic acid, centrifuged and the inorganic phosphate released was measured by the Fiske and Subbarow method (Sigma kit #670).

Alkaline phosphatase was measured with the method of Fujita et al (1972). The final incubation volume of 1 ml contained 5mM p-nitrophenyl phosphate (Sigma), 0.25mM $ZnCL_2$, 0.25mM $CaCL_2$ and/or 5mM $MgCL_2$ in a 50mM Tris-maleate buffer at pH 9.3. The reaction was allowed to proceed for 10 min at 37°C. The reaction was quenched by the addition of 3ml 0.1N NaOH. The amount of p-nitrophenol released was read with a Pye Unicam spectrophotometer at 400nm.

Enzyme activities were determined in duodenal homogenate aliquots adjusted to 100μg/ml protein concentration with the appropriate buffer in each case. Total protein was measured according to Lowry et al (1951). The final protein concentration in the incubation medium was 10μg/ml . All enzyme activities were determined in duplicate and are expressed as the mean of each set of measurements.

5) EM study of P_4 and P_9

The pellets of two different fractionations were fixed in the centrifuge tube in freshly made 1/2 Karnovsky fixative (Karnovsky, 1965; Sotelo and Palay, 1968) on ice overnight. All subsequent steps were carried out at 0-4°C unless stated otherwise. The pellet was thoroughly washed with 0.1M, pH 7.4 phosphate buffer. The pellet was then fixed for 1 hr with 1% OsO4 in 0.1M phosphate buffer. The fixed pellet was again washed in phosphate buffer and then it was gently removed from the centrifuge tube. The middle part containing the whole thickness of the pellet was cut into small rectangles and was dehydrated in a graded series of alcohol and then in two changes of propylene oxide at room temperature. The blocks were infiltrated with araldite. Thin sections were cut with an LKB ultramicrotome. The sections were stained with uranyl acetate and lead nitrate. The sections were viewed with a Philips 300 electron microscope.

6.1) Total blood calcium measurements

To determine the effect of the rachitogenic, vitamin D-deficient diet on the blood calcium level of chicks, blood samples were collected by intracardial puncture. The blood was allowed to clot at room temperature for 1 hr, rimmed and left overnight in the refrigerator. The serum was removed and centrifuged. Total blood calcium was colorimetrically measured using the Fisher diagnostic kit DK 1100-20 (Fisher Scientific, 112 Colonnade Rd. Nepean, Ont.

Canada). Total blood calcium was routinely measured in all experiments involving normal and rachitic chicks.

6.2) Total calcium measurements in chick duodena

To correlate the amount of calcium present in duodena of rachitic and normal chicks with the activity of membrane enzymes possibly involved in the extrusion of calcium from the cell, total calcium was measured in duodena of fasted (hence non-transporting enterocytes) and of fed (hence actively transporting enterocytes) chicks, both from rachitic and normal animals.

All glassware used was thoroughly cleaned in 1N HCL, rinsed several times with distilled water and oven dried.

Four week old rachitic and normal chicks were used in this study. Chicks were killed by decapitation, the duodenum was quickly dissected out and the pancreas was removed. The duodenum was thouroughly washed with cold isotonic saline and then gently blotted to removed excess water. The mucosa was scraped with a teflon coated spatula and placed in a weighing vial. The vial was weighed on a 5 decimal point Mettler balance and then placed at 70°C for 24 hrs. The vial containing the tissue was left to cool down to room temperature before it was weighed again. The tissue was digested overnight in 0.8ml of concentrated HNO_3 in an LKB sonicator at setting number 2. The completely digested tissue was neutralised with 8 drops of NH_4OH and then diluted to a final volume of 5ml. The total calcium content was estimated by atomic absorption on a Varian

AA5 atomic absorption spectrophotometer.

7) Immunocytochemistry

7.1) Antisera.

Antisera against chick duodenal D-CaBP-I were prepared according to Spencer et al (1976) and were kindly provided by Dr. D.E.M. Lawson (AFRC Institute of Animal Physiology, Babvaham, Cambridge, England. Antiserum against human cerebellar D-CaBP-I was kindly provided by Dr C. O. Parkes (Department of Physiology, University of British Columbia, Vancouver, B.C.) and was prepared according to Baimbridge et al.(1980). The antiserum against the smaller molecular weight ~~rat~~ duodenal D-CaBP-II was kindly provided by Dr. M. Thomasset (INSERM, U. 120, Le Vesinet, France) and was prepared according to the method of Marche et al (1977).

7.2) Tissue preparation for light microscopic localization of D-CaBPs

Tissues from at least four animals from the species described in section 1 (except for human and monkey, where only one sample was available) were dissected out and 2-3 mm thick slices were fixed by immersion in Carnoy's fixative for 3h at room temperature. This fixative has been demonstrated to reflect the true localization of D-CaBP (Jande et al, 1981; Taylor, 1981). The fixed tissues were.

then thoroughly washed in 90% ethyl alcohol and processed for paraffin sectioning. Four um thick serial sections were used for immunohistochemical staining.

7.3) Short indirect method (SIM) of immunocytochemical localization of D-CaBPs at the light microscopic level.

Paraffin slides of tissues were brought down to 30% alcohol. After rinsing in 0.05M Tris buffered saline at pH 7.6 (TBS) the sections were pre-adsorbed with undiluted normal goat serum, (NGS, Gibco, 2260 A Industrial St., Burlington, Ont. Canada, L7P 1A1.) and incubated for 30 min at room temperature. All incubations were done in a moist chamber under gentle continuous agitation on a rotator-tectator. The NGS was drained off and the antiserum diluted with TBS was directly applied. Chick anti-duodenal D-CaBP-I and rat anti-duodenal D-CaBP-II were diluted 1:100 and human anti-cerebellar D-CaBP-I was diluted 1:300. The proper working dilution was found in preliminary experiments by staining chick duodenum and cerebellum and rat duodenum, kidney and cerebellum with 1:50-1:10000 serial dilutions of the antiserum. The dilutions that gave the darkest staining with least background staining were used. The sections covered with antisera were incubated for 2h at room temperature. The slides were jet sprayed with TBS and immersed in TBS for 3 changes of 5 min each. The sections were again incubated for 30min in undiluted NGS. The NGS was drained off and rabbit anti-IgG conjugated with peroxidase (Bionetics, Intermedico, 150 Consmer Rd.

suite 306, Willowdale, Ont. Canada, M2J 1P9) diluted 1:50 was applied to the slides for 30 min. The slides were again jet sprayed and washed as above. The localization of D-CaBP was visualized by incubating the slides in 3,3'diaminobenzidine-HCL (DAB-HCL, Sigma) 0.05%, H_2O_2 0.001% in 0.05M Tris-HCL buffer at pH 7.6 for 10-15 min at room temperature.

Endogenous peroxidase was inhibited according to the method of Heyderman and Neville (1977). Three types of control sections were used: some sections were incubated with the supernatant of the antibody antigen mixture at the point of equivalence, some sections were incubated with non-immune rabbit serum, finally the antiserum step was omitted on some sections.

All antisera were decomplemented for 30 min at 56°C.

7.4) PAP peroxidase-antiperoxidase method for the immunocytochemical localization of D-CaBP (Sternberger, 1979)

The PAP method affords a greater sensitivity for the localization of antigens than SIM. Paraffin sections of tissues were brought down to 50% alcohol, The slides where rinsed in TBS before being incubated with 3% NGS for 30 min. The serum was decanted and diluted D-CaBP antiserum containing 1% NGS was applied for 46 hrs in a sealed container at 10°C. Antiserum against chick D-CaBP-I was diluted 1:5,000, antiserum against human D-CaBP-I was diluted 1:10,000 and antiserum against rat D-CaBP-II was diluted 1:2,000. The appropriate antiserum dilution was determined in preliminary experiments as described previously. The slides were returned to room temperature during the final hour of incubation in antiserum. The sections were jet washed with buffer and washed in three 5 min changes of buffer. The link antiserum (goat anti-rabbit immunoglobulin, Bionetics) diluted 1:20 was applied to the sections for 30 min. The sections were again jet sprayed and washed in buffer. The PAP conjugate (goat peroxidase anti-peroxidase, Bionetics) was diluted 1:100 with Tris buffer containing 1% NGS and the sections were incubated for 30 min. The sections were jet sprayed and washed in buffer as above. To visualize the localization of D-CaBP, the sections were immersed for 10-15 min in a freshly prepared solution of 0.05% DAB-HCL (Sigma), 0.01% H_2O_2 in 0.05M Tris-HCL pH 7.6.

After incubation in DAB the slides were washed with double distilled water. Some slides were counterstained with hematoxylin-eosin (H and E) or periodic acid-Schiff reagent and hematoxylin (PAS) for easier identification of the various structures. The slides were then dehydrated and mounted in permount.

Control sections were stained with non-immune rabbit serum at the same dilution as the antiserum. In other control sections the antiserum step was omitted. All antisera were decomplemented at 56°C for 30 min before use. Endogenous peroxidase was inhibited by the method of Heyderman and Neville (1977).

Because of the possible hazardous nature of DAB all steps involving this chemical were performed in a fume hood. After DAB was used it was neutralized with an equal volume of Javex.

7.5) Evaluation of fixatives for the electron microscopic localization of D-CaBP-I

Four week old chicks were anesthetised with chloral hydrate (35%, 0.1ml/100gr of body weight). Each chick was perfused intracardially with 20ml of isotonic saline at room temperature followed by 20-30ml of one of the fixatives listed below. Each fixative was tested on at least two different animals whereas acrolein was used on at least 4 different animals. The duodenum and cerebellum was removed and about 2mm thick sagittal sections were fixed by immersion at 0-4°C unless stated otherwise. All fixatives were prepared to contain 0.1M sodium cacodylate at pH 7.4.

a) 4% paraformaldehyde followed by overnight fixation in 4% paraformaldehyde.

b) 0.1% glutaraldehyde with 4 % paraformaldehyde for 10 min. and immersion fixed in 4% buffered paraformaldehyde overnight.

c) 0.1% glutaraldehyde with 4 % paraformaldehyde then immersion fixed in the same fixative overnight.

d) 0.5% glutaraldehyde then immersion fixed at room temperature for 2hrs. Free aldehyde groups were blocked by rinsing the tissue in 0.5M NH_4CL for 2hrs at room temperature (Thorens et al, 1982).

e) 10% acrolein (Kodak, Fisher Scientific) with 0.03% $CaCL_2$ then immersion fixed for 10, 20 and 30 min.

f) 20% acrolein (Kodak) with 0.03% $CaCL_2$ then immersion fixed for 20 min.

g) 2% acrolein (Polyscience, distilled, Analychem Corp. Ltd. 7300 Victora Park Ave, Unit G, Markham, Ont. Canada, L3H 1J1) then immersion fixed for 1hr and 2 1/2hrs.

h) 2% acrolein (Polyscience, distilled) containing 0.25% glutaraldehyde then immersion fixed for 2 1/2 hrs.

i) 3% acrolein (Polyscience, distilled) then immersion fixed for 20, 30, 45 and 60 min.

j) 5% acrolein (Polyscience, distilled) then immersion fixed for 5, 10, 15 and 20 min.

After fixation all tissues were thoroughly washed in cacodylate buffer in order to prevent nonspecific adsorption of antiserum and secondary antibodies.

Because of the hazardous nature of acrolein, all steps involving this chemical were performed under a fume hood. Acrolein waste was neutralized with an equal volume of 10% sodium bisulfite solution.

7.6) Localization of chick cerebellar and duodenal D-CaBP-I prior to embedding (Sternberger, 1979).

The duodenum and cerebellum of two different four week old chicks were fixed in 10% acrolein (kodack) for 20 minutes and then thoroughly washed in cacodylate buffer. Forty µm thick coronal and sagittal sections of the cerebellum and longitudinal sections of the duodenum were cut with a Smith-Farquar tissue chopper. Free floating sections were equilibrated in 0.1M Tris-HCL pH 7.4 buffer for one hour with a minimum of 3 changes. All incubations were done under continuous slow rotation at room temperature. The sections were then soaked for another hour in 1% NGS (Gibco) diluted in Tris buffer. The sections were drained and were incubated for 2 h in antiserum diluted 1:100. The sections were washed in buffer for 2 h with a minimum of 3 changes and were soaked again for 1 h in 1% NGS. The sections were drained once more and incubated in antirabbit goat peroxidase conjugate (Miles Laboratories, 77 Belfield Rd., Rexdale Ont. Canada, M9W 1G6) diluted 1:50 with Tris buffer for 2 h. The sections were then washed overnight in buffer with several changes. The sections were incubated with 3,3' diaminobenzidine-HCL (Sigma) 0.5%, H_2O_2 0.001% in Tris-HCL pH 7.6 for 10-15 min on ice. Control sections were incubated with non-immune rabbit serum instead of the antiserum. All sera were decomplemented at 56°C for 30 min before use. After washing the sections for half an hour in buffer some sections were collected and mounted on glass slides in glycerin jelly for light microscopy.

Sections destined for electron microscopy were osmicated in 1% OsO_4 in 0.1M cacodylate buffer pH 7.4 for 1 h on ice. After dehydration the sections were embedded in araldite. The sections were cured in plastic microscope slide "sandwiches" thus ensuring that the sections remained flat. The area of interest was found under a light microscope and the "sandwich" was then glued to an araldite support. Thin sections were cut with an LKB ultramicrotome and the sections were studied with a Phillips 300 electron microscope with and without uranyl acetate staining.

7.7) Post-embedding electron microscopic localization of chick duodenal D-CaBP-I

The duodenum of three different chicks was fixed in 5% acrolein (Polyscience) for 15 min at 0-4°C and then thoroughly washed in cacodylate buffer. In one experiment part of the duodena were embedded in araldite while in subsequent experiments the duodena were embedded in Lowycril K4M at -20°C (JBEM, P.O. box 693, Pointe Claire, Dorval, Quebec, Canada, H9R 4S8). Thin sections were placed on 300 mesh nickel grids. In one experiment we attempted to localize D-CaBP-I on araldite sections with the PAP method (Sternberger, 1979).

The sections were etched by flotation for 3 min at room temperature on a drop of 5% H_2O_2 on a glass slide. The sections were jet sprayed with isotonic saline. Subsequent reagents were applied by floating the sections in shallow depressions made in a sheet of parafilm in covered petri dishes at room temperature unless stated otherwise. All dilutions were made in pH 7.6 Tris-HCL buffer. All buffer solutions were filtered through a 20 µm Millipore filter before use and antisera were decomplemented at 56°C for 30 min .

The sections were floated on a drop of normal goat serum (NGS) diluted 1:30 for 5 min followed by blotting. The sections were then floated on a drop of diluted anti-D-CaBP and control sections were floated on a drop of non-immune rabbit serum (NRS) for 48h at 2-5°C. For the last 1-2 h of incubation, the sections were brought up to room temperature. Serial dilutions (1:100-1:10,000) of the antiserum and NRS in buffer containing 1% NGS were tested. The jet washed and blotted sections were incubated in NGS (1:30 dilution) for 5 min followed by blotting. The sections were incubated in link antiserum (goat anti-rabbit antiserum, Bionetics) diluted 1:10 for 15 min followed by a jet wash and blotting. Another incubation in NGS was followed by incubation in PAP conjugate (goat peroxidase anti-peroxidase, Bionetics) diluted 1:50 in buffer containing 1% NGS for 15 min. The grids were held in a forceps and immersed for 10 min with continuous agitation in freshly prepared 0.0125% 3,3' diaminobenzidine-HCL (Sigma), 0.025% H_2O_2 in 0.05M Tris-HCL pH 7.6. The grids were then rapidly transferred to another beaker containing double distilled water and kept under continuous agitation. The sections were jet washed with double distilled water, blotted and placed on a filter paper. Finally the sections were floated on a drop of 4% OsO_4 in a porcelain dish for 25 min. The sections were jet washed with double distilled water, dried and studied with a Phillips 300 electron microscope.

7.8) Localization of chick duodenal D-CaBP-I with colloidal gold coated with goat anti-rabbit IgG.

The recent development of Low temperature embedding with the resin Lowicryl K4M (Carlemalm et al, 1980; Kellenberger et al, 1980) offers better preservation of antigenicity, fine structure as well as a lower degree of non-specific interaction between the immunological reagents and the embedded tissue than araldite or epon resins. Localization with colloidal gold to visualize provides us with a highly specific labelling technique while preserving fine detail.

Chick duodenum fixed with acrolein was embedded in 3 different media: Epon (Coulter, 1967), Araldite (Coulter, 1967) and Lowicryl K4M (JBEM). Thin sections were cut with an LKB and Sorval ultramicrotome and placed on nickel grids.

All dilutions of the reagents were made with 20mM Tris-HCL buffer pH 8.2 which was filtered through a 20u Millipore filter. Incubations were performed by floating the grids on a drop of 1% ovalbumin solution (Sigma) for 5 min and then transferring them directly to a drop of anti-D-CaBP-I diluted in 1% ovalbumin for 2 h in a moist chamber. Serial dilutions of antiserum (1:100-1:10,000) were tested to find the dilution giving the best results. The antiserum step was omitted from the control sections. The grids were then jet washed with Tris buffer, dipped in the same buffer 3 times and then blotted. The sections were then floated on a drop of colloidal gold suspension absorbed with goat anti-rabbit immunoglobulin (GAR-G10, Janssen Life Science product, SPI, P.O. box 44, Station S, Toronto, Ont. Canada, M5M 4L6) and diluted 1:20 with Tris buffer containing 1% ovalbumin. The sections were again jet washed and dipped 2 times before being blotted and fixed on a drop of 1% glutaraldehyde. After fixation the grids were washed twice in double distilled water and left to dry. The sections were stained for 4 min in the dark with uranyl acetate and then for 30 seconds in lead citrate. The grids were studied with a Phillips 300 electron microscope.

8) Measurement of antibody production in normal and rachitic chicks

One day old hatchlings were received from ADRI and were divided into 3 groups. One group of six chicks was fed normal chick feed, the second group of eight chicks was fed a rachitogenic diet containing high calcium and the third group of twelve chicks was fed a rachitogenic diet without calcium. When the chicks were 3 weeks old they were injected intracardially with 0.1 ml of a 0.5% suspension of sheep red blood cells (SRBC) in normal saline. Controls received the same amount of normal saline. Five days later the chicks were anesthetized with 35% chloral hydrate (0.1ml/100gr of body weight). Blood was collected by intracardiac puncture and divided into 2 aliqots, one for total serum calcium determination and the other for the estimation of antibody titers. Antibody titers were determined by the microtest method (Gross and Seigel, 1979). Twenty five ul of saline was placed in each well of a 96-well plate with conical shape depressions. Serial dilutions of 25µl plasma aliquots were made and 25µl of a 0.75% suspension of erythocytes was added to each well. The plates were sealed and incubated overnight at 36°C. The highest serum dilution resulting in agglutination was determined and the antibody titer was expressed as Log 2 of its reciprocal value.

9) Statistical methods

Means, standard deviation, standard error of the mean (S.E.M.) and Student's t test were calculated according to standard procedures (Snedecor and Cochcran, 1968).

III.RESULTS

Section 1: The movement of Calcium in chick duodenal mucosal cells.

1.1) The effect of vitamin D-deficiency on alkaline phosphatase, Ca^{+2}, Mg^{+2}-ATPase, Na^{+}, Ca^{+2}-ATPase and Na^{+}, K^{+}-ATPase

Calcium pumps in the plasma membrane are thought to be the primary regulators of intracellular calcium concentration. However the factors which regulate the activity of enzymes involved in the transmembrane flux of calcium are not well known. Since the concentration of duodenal D-CaBP is responsive to the administration of calcitriol in rachitic chicks, it seems possible that D-CaBP plays a catalytic role in the regulation of the activity of plasma membrane enzymes such as Ca^{+2}, Mg^{+2}-ATPase and Na^{+}, Ca^{+2}-ATPase which are believed to be involved in the process of calcium extrusion. A survey was therefore carried out of the effects of vitamin D-deficiency on the activities of alkaline phosphatase, Na^{+},K^{+}-ATPase, Na^{+}, Ca^{+2}-ATPase and Ca^{+2},Mg^{+2}-ATPase (Materials and Methods section 4) to determine whether the activities of membrane-bound duodenal enzymes are affected by the vitamin D-status of the chick.

Chicks kept from the time of hatching on a semi-synthetic vitamin D-free diet were typically rachitic when they were 4 weeks old. They had a lower body weight and lower total serum calcium concentration (Table 1) than the control animals; they experienced difficulty in standing up and upon dissection a "rosary" rib cage became apparent.

There was a 3 fold decrease in the alkaline phosphatase (ALK-Pase) activity in the duodenal homogenates of rachitic chicks compared to the activity in normal animals (Table 2).

In the absence of reliable and consistent data in the literature, it was deemed necessary to conduct a preliminary series of experiments to determine the optimum calcium concentration for the measurement of Ca^{+2},Mg^{+2} -ATPase acivity in the presence and absence of 0.5mM Mg^{+2} (Graph 1). In the presence of 0.5mM Mg^{+2} ATPase activity displayed Michaelis-Menten kinetics. Maximal activity was reached in the presence of 0.5mM Mg^{+2} when the concentration of calcium in the incubation medium was 2mM. Since 2mM Ca^{+2} and 0.5mM Mg^{+2} provided the most suitable conditions to measure Ca^{+2},Mg^{+2}-ATPase activity, these concentrations were subsequently used in the determinations. In the presence of 2mM calcium and 0.5mM magnesium, the activity of Ca^{+2},Mg^{+2} -ATPase was significantly decreased ($p<.05$) in rachitic chicks (Table 2).

A statistically significant difference was also found in the ATP-ase activity of duodenal homogenates obtained from rachitic and normal chicks and measured in the presence of 100mM sodium and 2mM calcium ($p<.05$) (Table 2). There was no significant difference in Na^{+},K^{+}-ATPase activity between normal and rachitic chick duodenal homogenates.

Centrifugation of the homogenate at 45,000g for 20min resulted in a supernatant and pelleted fraction (Materials and Methods section 3.1). The supernatant contained soluble proteins, including

D-CaBP and calmodulin, while the pellet contained brush border and basolateral plasma membranes along with cellular organelles. Table 3 summarizes the activities of Ca^{+2},Mg^{+2}-ATPase and Na^{+},Ca^{+2}-ATPase in the supernatant, pellet and whole homogenate obtained from normal and rachitic chicks. The activities of both of these enzymes were significantly reduced in the total homogenates and in the supernatants obtained from rachitic chicks, while the activities remained essentially unchanged from normal values in the pelleted fractions. These results suggest the existence of a factor, possibly D-CaBP, which is present in the supernatant of duodenal homogenates obtained from normal chicks but which is absent in preparations obtained from rachitic chicks. Such a factor may be involved in the full activation of both enzymes.

In order to study in more detail the effects of vitamin D and D-CaBP on the activity of these enzymes as well as to identify whether Ca^{+2},Mg^{+2}-ATPase and Na^{+},Ca^{+2}-ATPase are brush border or basolateral membrane enzymes, I attempted to purify the brush border and basolateral membranes simultaneously from the duodenum of the same chick, using the method of Mircheff and Wright (1976) (Materials and Methods section 3B). Table 4 shows the distribution of the main plasma membrane markers in duodenal homogenate fractions obtained from a normal 3 week old chick. According to the procedure used, pellet 4 (P_4) should be enriched in basolateral membranes and pellet 9 (P_9) in brush border components. Pellet 4 was indeed enriched in Na^{+},K^{+}-ATPase which is a marker enzyme for basolateral membranes. Since the properties of the Ca^{+2},Mg^{+2}-ATPase present in the brush border are different from that in the basolateral membrane (Mircheff and Wright, 1976; Van Os et al, 1981; Ghijsen et al, 1982). I studied the effect of various Ca^{+2} concentrations and pH on the enzyme activity in P_4 and P_9. The Ca^{+2},Mg^{+2}-ATPase present in the pellets behaved differently, P_4 having a much higher activity at all calcium concentrations tested (Graph 2) and a sharply biphasic activity at various pH levels (Graph 3). This indicated that some separation of brush border from basolateral membranes was achieved during the fractionation procedure.

Four trials were attempted to separate the basolateral membranes from the brush border fraction. The sucrase activity which is a marker for brush border membranes was always higher in P_4 than in P_9 indicating either that the brush border membrane preparations were contaminated by basolateral membranes or that substantial damage to the brush border membrane constituents occured.

In addition to the biochemical determinations of enzyme activity, an electron microscopic study of pellets 4 and 9 was also carried out in order to assess the purity of the fractions. In P_9 intact nuclei were present and the brush border elements were deformed. In P_9 numerous membrane vesicles were present in addition to mitochondria. The success of Mircheff and Wright's (1976) method is heavily dependent on the fact that the brush borders remain intact. It seems that chick intestine may be more fragile than rat intestine used by Mircheff and Wright. Even when homogenization was reduced to a minimum, sucrase activity remained highest in P_4 as shown in Table 4. This indicated that the brush border and basolateral membranes were never adequately separated. To this date all successful simultaneous separation of brush border from basolateral membranes have been achieved only in the rat (Douglas et al, 1972; Murer et al, 1976; Fujita et al, 1976). Hence it is possible that the presently available techniques are not suitable to use with chick duodenum as the experimental tissue.

1.2) Relationship between total calcium content and vitamin D status in duodenal mucosa from fed and fasted chicks

Originally it was hoped that by correlating the localization and activity of duodenal membrane marker enzymes with concentration changes of calcium and D-CaBP it could be determined whether D-CaBP played a direct role in vitamin D-mediated calcium absorption.

A quantitative study of the total calcium present in the duodenal mucosa of 4 week old normal and rachitic chicks was carried out (Materials and Methods section 6.2). The chicks were further subdivided into 2 subgroups: the animals in one subgroup were fasted for 18h (non-transporting duodenum) while those in the second subgroup were fed normally (transporting duodenum). In the duodenal mucosa of normal and rachitic chicks which were fasted overnight there was no significant difference in the total calcium content as measured by atomic absorption spectrophotometry (Table 5) between the two groups. In the duodenal mucosa obtained from rachitic feeding chicks the calcium concentration increased almost twofold. The amount of calcium present in feeding and fasted normal chick duodenal mucosa was not significantly different from that measured in rachitic fasted chicks. The above results as well as data in the literature, indicate that the most likely explanation for the increased intracellular total calcium concentration is decreased extrusion.

Section 2: Evaluation of fixatives.

2.1) Evaluation of fixatives electron microscopic localization of D-CaBP-I in vitamin D target cells.

In an extended series of experiments fixatives which are used in EM studies were evaluated at the light microscopic (LM) level. The duodenum of 3-4 week old Leghorn chicks were fixed as described previously (Materials and Methods section 7.4) embedded in paraffin and stained for D-CaBP-I according to the short indirect method (SIM). The staining was compared with the results obtained with freeze substituted material (Jande et al, 1981a). The duodenum was considered to be the best test material since diffusion and adsorption of the protein antigen to mucus contained in the goblet cells can easily be detected at the LM level.

Fixation in paraformaldehyde and/or glutaraldehyde resulted in inconsistent and uneven deposition of the reaction product in the enterocytes along the length of the villi (Fig. 1). Along the sides of the villi some goblet cells and brush borders were positive. These fixatives penetrate relatively slowly and their fixation power is weak. Considerable immunoreactivity can be lost by the diffusion of the protein out of the cell.

Acrolein is an aldehyde with a very rapid penetrating power (Sabattini et al, 1964) and can thus destroy enzymatic activities by over-fixation. However by using various concentrations and fixation times and [illegible]uate balance between fixation and preservation of immunogenicity was found (Table 6). The best fixation was achieved with a 10% buffered solution of acrolein (Kodak) for 20 min (Fig. 2). Control sections were always devoid of reaction product (Fig. 3). It should be noted that different batches of acrolein gave variable results depending on the different amounts of inhibitor which had to be applied. Distilled acrolein from Polysciences is more purified and gave more consistent results from one batch to another. A 5% buffered solution of acrolein from Polysciences for 10-13 min at 0-4°C gave similar results to the acrolein from Kodak (Table 7). In an effort to improve the quality of fixation, acrolein in combination with glutaraldehyde or paraformaldehyde was also tried out as shown in Table 8. None of these combinations gave better results than fixation with acrolein alone.

Thus on the basis of light microscopic studies, a 20 min. fixation with 10% acrolein (Kodak) was considered to be a suitable fixative.

For the study of pre-embedding staining of D-CaBP-I at the EM level, chick cerebellum was used as the test tissue. The reason for this choice was the proven poor penetration of the antiserum and of the peroxidase conjugate into chick duodenal enterocytes. Since Purkinje cells are large, they are sectioned at the time of preparation with the tissue chopper allowing for a better penetration of the antiserum and other immunoreagents.

Paraffin sections of cerebellum fixed with acrolein as described above were stained with chick anti-D-CaBP-I using the SIM. Very dense reaction product was observed throughout the Purkinje cell bodies (Fig. 4). The entire dendritic tree was distincly outlined. The positive axonal processes of the Purkinje cells could be traced to the deep cerebellar nuclei. Neurons in this region were devoid of reaction product and were outlined by the synaptic endings of the Purkinje cell axons. Control sections stained with non-immune rabbit serum or with the supernatant from antigen and antiserum mixture at the equivalence point showed no staining (Fig. 5). When 40 um thick cerebellar sections were observed with the light microscope prior to osmication, Purkinje cell somata and their dendrites showed the presence of dark brown reaction product. No staining was observed in other neuronal or neuroglial cells in either the granular or in the molecular layer. These observations are similar to those published earlier by Jande et al (1981a).

2.2) Ultrastructural localization of D-CaBP-I in the chick cerebellar Purkinje cells

Cerebellar tissue preservation with acrolein was adequate as cellular morphology at the EM level was good. The electron dense reaction product was seen throughout the cytosol of Purkinje cells (figs 6-9) extending into the dendrites as well as into the axonal collaterals and axons reaching into the deep cerebellar nuclei (figs 10-14). The dense reaction product indicating immunoreactivity against D-CaBP-I was seen in between the various cellular organelles and actually obscured the various small structures such as polyribosomes, microfilaments and microtubules (figs 6-9). The mitochondria were completely surrounded by the granular reaction product. The mitochondrial matrix as well as the cristae appeared similar to those of negative cells (Fig. 13). The rough endoplasmic reticulum was covered with reaction product to the extent that the granular ribosomes were obscured. The reaction product was never seen inside the cisternae (Figs. 7-9). Elements of the Golgi apparatus failed to show electron dense reaction product (Fig. 9). The nucleus in some cells was completely devoid of reaction product while in others it was just as electron dense as the cytoplasm (Fig. 6).

In the basal portion of the dendritic trees, the localization was similar to that in the perikaryon except that blank spaces were seen in between the dendritic organelles. The organelles such as mitochondria and the various elements of the smooth endoplasmic reticulum were all covered with reaction product (Fig. 10). In the dendritic spines, reaction product was distributed along the inner surface of the plasma membrane, on the outer mitochondrial membrane as well as on the occasional cisterna of the smooth endoplasmic reticulum. The postsynaptic densities also showed the presence of electron dense reaction product (Fig. 13).

In the Purkinje cell axonal recurrents and axons from the olivary nucleus neurons (Jande et al, 1981b), electron dense material was seen adsorbed onto the cytoplasmic surfaces of all structural elements: the plasma membrane, microtubules, mitochondriae and synaptic vesicles (Figs. 12,14). The myelin sheath of axons going to the deep cerebellar nucleus was devoid of reaction product (Fig. 14)

Material fixed in 0.1% glutaraldehyde containing 4% paraformaldehyde was also examined. The localization was similar to that seen with acrolein; however, immunoreactivity in the perikaryon was greatly decreased. One conspicuous difference was a much larger number of positive dendritic spines in the material fixed in glutaraldehyde (Fig. 15). Some of the postsynaptic densities appeared to show a higher concentration of reaction product as well. Tissue fixed in 4% paraformaldehyde showed poor cytology and greatly decreased immunoreactivity compared to material fixed with acrolein. Postsynaptic densities in the dendritic spines appeared to be more electron dense (Fig. 16).

2.3) Ultrastructural localization of D-CaBP-I in chick duodenal enterocytes

One of the major drawbacks of the preembedding method is the poor penetration of the antiserum and immunoreagents which are molecules of relatively high molecular weight. This problem is circumvented by using the postembedding method where immunoreagents are uniformly exposed over the entire area of the section. A further improvement in the exact localization of the antigen in question can be achieved by using colloidal gold as an immuno-marker in embedded material since it does not obscure cytological details. However, application of the colloidal gold technique (as well as the PAP method) to chick duodenum that was embedded in araldite or Epon and then stained for D-CaBP-I did not show any consistent staining.

On the other hand, duodenum that was embedded in Lowicryl K4M and stained with the colloidal gold method retained its immunoreactivity towards D-CaBP-I. Colloidal gold indicating the presence of immunoreactivity towards D-CaBP-I was present throughout the cytoplasm and nucleus of the enterocytes (Figs. 17,19-21). The marker was usually associated with denser areas where cytoplasmic proteins have precipitated. The mitochondrial membranes and matrix were negative and no colloidal gold was present in the cisternae of the rough endoplasmic reticulum (Fig. 20) and Golgi apparatus. In the nucleus the euchromatin was positive (Fig. 20). No marker was present in the intercellular spaces (Figs. 17,18,21) and only background staining was present in the mucus of goblet cells (Fig. 19) and in the brush border (Figs. 17,18).

These experiments demonstrated that D-CaBP-I is indeed an intracytoplasmic protein. The evaluation of the preparative methods (fixation and embedding) is an important step when localization of a low molecular weight protein such as D-CaBP-I is attempted. Acrolein, unlike glutaraldehyde has given satisfactory results as a fixative for studies of immunocytochemical localization at the EM level .

Section 3: Classical vitamin D targets in soft tissues.

3.1) Light microscopic localization of D-CaBP-I in the chick kidney and of D-CaBPs in the vertebrate kidney.

The kidney is a classical vitamin D target involved in the regulation of the biotransformation of $25(OH)D_3$ into $1,25(OH)_2D_3$. In 1971 Sands and Kessler found that the cortex of the dog kidney had a higher calcium binding capacity than the medulla. Since then the presence of D-CaBP-I has been well documented in the chick (Taylor and Wasserman, 1972), reptilian (Rhoten et al, 1984) and in mammalian kidney: human (Morrissey and Rath, 1974; Staun et al, 1984), pig (Arnold et al, 1975), rat (Hermsdorf and Bronner, 1975; Pansini and Christakos, 1984) and mouse (Delorme et al, 1983).

Using antisera against chick duodenal D-CaBP-I, human cerebellar D-CaBP-I and rat duodenal D-CaBP-II, I have localized D-CaBP-I in the embryonic and adult kidney of the chick, as well as in mouse, rat, frog, pig, monkey and human kidney and D-CaBP-II in the rat kidney.

3.1.1) Evaluation of specificity of antisera

In order to evaluate the specificity and cross reactivity of the antisera, sections of cerebellum and duodenum of both chick and rat were stained with the antisera. It was found (table 9) that the antisera against chick and human D-CaBP-I stained the Purkinje cells in the cerebellum of both animals (Fig. 24, 28) and also the duodenal enterocytes of the chick (Fig. 22). The antiserum against rat duodenal D-CaBP-II stained the enterocytes of the rat only (Fig. 27), as described earlier by Marche et al.(1979b). This antiserum did not stain any structures in the cerebellum of either the chick or the rat (Figs. 25, 29). Both the PAP and the SIM techniques yielded similar results. However, the former gave superior localisation without any background staining at all.

3.1.2) Localization of D-CaBP-I in the vertebrate kidney.

Staining with antiserum against D-CaBP-I of human cerebellar origin gave identical results to those obtained with antiserum against chick duodenal D-CaBP-I. However staining with anti-human D-CaBP-I did not give as much reaction product deposition as staining with anti-chick D-CaBP-I. The following description therefore applies to both antisera unless stated otherwise.

The dark brown reaction product was always seen intracellularly in certain cells of the nephron and was never seen in any cells of the interstitium.

a) Embryonic chick kidney

In 2 day old chick embryo pronephros, no structures were stained (Fig. 30). In day 5 chick embryo mesonephros, cells of the mesonephric duct were positive and as the connecting and midsegment tubules were forming more and more cells became positive (Fig. 31).

The glomeruli, main segment and the portion of the mesonephric duct in contact with the tubal ridge was negative (Fig. 31). In 14 day old mesonephros (Fig. 32) the main segment tubules could be identified by their brush borders and these tubules were negative. D-CaBP-I was present in the cytoplasm of cells in the mid- and connecting segment. The portion of the mesonephric duct in contact with the tubal ridge always remained negative (Fig. 33). In the metanephros, the distal tubules surrounding the central veins stained between day 10 and 11 of incubation. On day 10 just a few scattered tubules were positive (Fig. 34) but from day 12 onwards the number of stained tubules rapidly increased (Fig. 35). All other structures remained negative.

b) Chick kidney

In the 4 week old chick kidney ~~the~~ reaction product was seen in the straight as well as in the convoluted segment of the distal tubules which surround the central vein (Fig. 36). All other structures including the glomeruli, proximal convoluted tubules and medullary ducts were negative (Figs. 36,37). Control sections incubated with non-immune rabbit serum did not show any reaction product at all. The localization of D-CaBP-I in the chick embryonic and adult kidney is summarized in Fig. 38.

c) Rat, mouse, frog, pig, monkey and human kidney

Control sections of all the tissues investigated were devoid of reaction product (Fig. 39). The following description applies to all the species studied except for the goldfish kidney which did not show any staining. The most dense accumulation of reaction product was seen in the cells of the distal convoluted tubules (Fig. 40,41). The reaction product was distributed throughout the cytoplasm and was only infrequently found in the nuclei of these cells. The distribution of the reaction product was not homogeneous in the cytoplasm since the basal regions of the positive cells stained lighter than the apical portions (Fig. 42). Usually only isolated cells were positive near the beginning of the distal convoluted segments (Fig. 41) while the number of positive cells gradually increased along the length of the tubule. Thus there was no precise demarcation of the limit between the ascending limb of Henle's loop and the distal convoluted tubule. The initial segment of the collecting tubule also gave a marked positive reaction. Towards the distal portion of the collecting tubule, again there was no sharp demarcation between stained and unstained cells. The number of positive cells gradually decreased over a short distance, and finally ceased altogether. The cells of the macula densa were always negative. The renal corpuscle as well as the juxtaglomerular cells of the afferent arteriole were always negative.

In the suckling rat pup, in addition to the localization described above, interspersed positive cells were present all along the collecting duct (Fig. 43). In the monkey kidney, in addition to the localization described above; the reaction product was also seen in cells along the entire length of the collecting tubules, i.e.,in the arched and staight portions as well as in the papillary ducts

(Fig. 44,45). The intracellular distribution of reaction product was more homogenous than in the cells of the distal convoluted tubules (Fig. 45).

3.1.3) Localization of D-CaBP-II in the rat kidney.

Of all the kidney sections studied, positive reaction with anti- rat duodenal D-CaBP-II was observed only in the kidney of the rat. Only cells of specific segments of the nephron were stained and all the other cells as well as the components of the interstitium were negative.

The strongest staining was seen in the same cells as described above for immunoreactivity with anti- D-CaBP-I, i.e., the distal convoluted tubules and the initial segment of the collecting duct (Fig. 46). The cells of the thick ascending limb of Henle's loop and throughout the length of the collecting tubules, i.e., the arched, straight and papillary ducts, also stained positively (Fig. 47,48). The staining was always intracellular and the reaction product was concentrated in the apical cytoplasm of positive cells (Fig. 49). The cells of the macula densa showed a faint staining and the reaction product was seen mostly in the apical region of the cell (Fig. 50). Staining of D-CaBP-II in the suckling rat pup was identical to that of weaned rats (Fig. 46-50).

The overall staining intensity with anti-D-CaBP-II was less than that obtained with anti-D-CaBP-I. No reaction product was ever seen in the lumen of the tubules or in any cells of the renal corpuscle or the juxtaglomerular cells of the afferent arterioles. A summary of the staining reactions of the various regions of the nephrons in the specimens studied is presented in Fig. 51.

3.2) Light microscopic localization of D-CaBPs in the salivary glands of the suckling and adult rat

Vitamin D-dependent calcium binding activity in the rat salivary glands was first reported by Freund, Volpe and Witkowski (1977). The methodology used by these investigators did not permit the exact localization of D-CaBP nor its characterization.

The present experiments were therefore conducted to localize D-CaBP-I and II in the sublingual, submandibular and parotid glands of suckling rats.

The dense reaction product indicating the presence of D-CaBP-II was present in the cytoplasm of the cells of the entire ductal system i.e. from the intercalated ducts to the excretory ducts in all 3 types of salivary glands (Figs. 52,53,54). In the parotid the staining of the ductal cells was denser than that observed in the other two salivary glands. In rats older than 1 month, the staining was drastically reduced and appeared extremely faint. Positive results were obtained only with antiserum against D-CaBP-II. No staining was obtained when sections were incubated with anti-D-CaBP-I (Fig. 55). Control sections incubated with non-immune rabbit serum did not show any staining.

3.3) Light microscopic localization of D-CaBPs in the developing incisor and molar teeth of the rat.

Most studies involving teeth have focused on the effects of vitamin D-deficiency on the mineralization of dentin (Becks and Ryder, 1931; Schour and Ham, 1934; Yoshiki et al, 1974; Engstrom et al, 1977). Studies were conducted therefore to establish whether vitamin D target cells are present in the rat tooth.

The incisor and molar teeth of 7 to 9 day old rat pups were stained with antiserum against D-CaBP-I and D-CaBP-II. The ameloblasts of the continuously erupting rat incisor and developing molar tooth were the only structures that stained with anti- serum against D-CaBP-I (Fig. 56). No structures were stained with non-immune serum (Fig. 57) or anti-serum against D-CaBP-II (Fig. 58).

The terminology of Warshawsky (Leblond and Warshawsky, 1979) is used to describe the developmental stages of the ameloblasts. The developing ameloblast facing the pulp in the zone of presecretion did not contain any D-CaBP-I (Figs. 59a,60a). The first sign of staining for D-CaBP-I was observed in the cytoplasm of ameloblasts in the zone of presecretion facing the dentin (Figs. 59b,60b). In the initial portion of the zone of secretion, the smooth- surfaced ameloblasts were homogeneously stained for D-CaBP-I (Figs. 59c,60c). In the major portion of the zone of secretion where the ameloblasts mature, the reaction for D-CaBP-I became more intense and extended, into the developing Tome's processes (Figs. 56, 59d,60d). The presence of D-CaBP-I was also observed in the region of shortened and reduced ameloblasts in the zone of maturation (Figs. 59e,f, 60e,f).

Section 4: Non-classical vitamin D target cells

4.1) Neurons

Since the presence of D-CaBP-I has been demonstrated in central nervous system neurons (Jande et al, 1981a, 1981b; Roth et al, 1981; Baimbridge and Miller, 1982; Rabie et al, 1983; Feldman and Christakos, 1983; Garcia-Segura et al, 1984), the involvement of D-CaBP in calcium translocation in classical vitamin D target organs has been debated. The cerebellum contains the highest amount of D-CaBP in the CNS (Taylor, 1974; Baimbridge et al, 1981, 1982), localized in the Purkinje cells (Jande et al, 1981a). Because of the lack of turnover of neurons, the vitamin D-dependency of D-CaBP-I in the cerebellum could be demonstrated only after chronic administration of high doses of calcitriol (Taylor, 1977). By following the development of D-CaBP-I staining in the chick embryonic cerebellum and retina it was established that D-CaBP-I appeared early in ovo during the ontogeny of the Purkinje cells and in certain retinal cells thus explaining the lack of responsiveness of cerebellar D-CaBP-I to the vitamin D status of older chicks (Taylor, 1974).

Using the PAP technique and antiserum against chick duodenal D-CaBP-I and human cerebellar D-CaBP-I, the cellular localization of D-CaBP-I was investigated in the cerebellum and retina of embryonic chick, 4 week old normal and rachitic chicks, and in the cerebellum of the goldfish, frog, mouse, rat, and rabbit. Although both antisera gave identical results, staining with antiserum against human D-CaBP-I was always fainter than with antiserum against chick D-CaBP-I.

4.1.1) Cerebellum

In chick embryonic cerebellum, small fibers near the pia mater were faintly labelled on day 9 of incubation (Fig. 61). On day 10 the external granular layer could be identified at the surface of the cortex. Some positive fibers were thicker than those labelled on day 9 and in the deep nucleus some fibers were positive (Fig. 62). On day 12, positive cell bodies could be recognised as migrating Purkinje cells forming the boundary between the molecular and granular layers (Fig. 63). The morphological maturation of Purkinje cells in the following days could then be easily followed (Figs. 64,65). In the 16 day old cerebellum, staining for D-CaBP-I was seen throughout the cell body, axon and the dendritic tree of the Purkinje cells, expanding into the granular layer (Fig. 66). In the hatching chick (Fig. 67), staining was found to be very similar to that described for the 4 week old chick by Jande et al (1981).

In the goldfish (Fig. 68), frog (Fig. 69), rat (Fig. 70,71), mouse (Fig. 72) and rabbit (Fig. 73) staining for D-CaBP-I in the cerebellum was always present throughout the Purkinje cell body, including the dendritic tree and axonal process. Antiserum against human cerebellar D-CaBP-I also stained the Purkinje cells, but the staining intensity was not as dark as that obtained with the antiserum against chick duodenal D-CaBP-I (Fig. 71). This demonstrates the widespread occurence of D-CaBP-I in neurons as well as the interspecies cross reactivity between the antisera and antigen from different species and tissues.

4.1.2) Retina

Since only specific neuronal cell populations of the central nervous system have been shown to contain D-CaBP-I and since the retina is an extension of the diencephalon, an investigation of the possible presence of D-CaBP-I in the retina was carried out.

2.1.2.a) The 4 week old normal chick retina

The histology of the chick retina is well known (Hodges, 1974) and the positive retinal neurons have been identified according to their location. Inner to the pigment epithelium is the receptor cell layer which contains various morphologically distinct types of photoreceptor cells (Meyer and Cooper, 1966; Morris and Shorey, 1967). All receptor cells (RC) have been found to contain the dark reaction product indicative of the presence of D-CaBP-I. Various structural regions of the receptors, namely the inner segment, the cytoplasm surrounding the nucleus, some of the nuclei and all synaptic pedicles were positive for D-CaBP-I (Figs. 74,75). There was a sharp demarcation at the outer limiting membrane, because the outer nuclear region showed considerably lesser amounts of D-CaBP-I staining (Fig. 75). Only occasional receptor cell nuclei showed positive staining. The synaptic pedicles were always darkly stained and appeared as a distinct layer. Muller cells, which take part in the formation of the external limiting membrane through their junctional complexes with the receptor cells did not show any staining (Fig. 75).

The outer plexiform layer (OPL) where the pedicles of the receptor cells form synaptic connections with the horizontal (HC) and bipolar cells (BC) was positive (Fig. 75). Quite often in the outer plexiform layer, two stratifications were distinguishable: an outer one which represents the synaptic pedicles of the photoreceptors and an inner one which is composed of the cell processes of the horizontal and bipolar cells. Further inwards is the inner nuclear layer (INL) which contains four types of cells; horizontal, bipolar, Muller and amacrine cells (AC). The main cell body of the horizontal cell, including the nucleus and the cellular processes taking part in the formation of the inner plexiform layer were positive (Figs. 74,75). It appears that most of reaction product was deposited on the outer side of the nucleus (Fig. 75). The nuclei of the bipolar cells are situated in the outer half of the INL. Only a few scattered nuclei in this region were positive (Fig. 75). Muller cells which begin at the outer limiting membrane and end at the inner limiting membrane were completely negative. In the INL, closest to the inner plexiform layer (IPL), are the amacrine cell bodies. Although it was not possible to identify the different types of these cells as described by Cajal (1972), it is apparent that some of the positive cells are indeed amacrine cells (Fig. 75). Their location in the INL and their branching pattern in the IPL support such a conclusion. In a number of positive amacrine cells their branching cellular processes could be seen extending into the IPL where they take part in synaptic connections (Fig. 75).

In the avian retina the IPL is made up of many horizontal layers as a consequence of the stratification of the synapses between the bipolar, amacrine and ganglion cells. In our preparations five layers could be distinguished due to the deposition of the reaction product; two of these layers were very prominent (Fig. 74,75).

Control sections incubated with normal rabbit serum did not show any reaction product in the entire retina (Fig. 76). The pecten which is a projection of the ventral surface of the avian eye into the vitreous body is a thin, darkly pigmented lamina folded fanwise upon itself (Hodges, 1974). This structure did not contain any reaction product for D-CaBP-I (Fig. 77).

4.1.2.b) The 4 week old rachitic chick retina

The overall staining for D-CaBP-I in the retina of chicks fed a rachitogenic diet for 4 weeks was fainter than in the retina of chicks fed a normal diet. The receptor cells showed less reaction product but each cell's synaptic pedicle was positive and thus the outer region of the outer plexiform layer was well defined by the dark reaction product (Figs. 78,79). The horizontal cells, their main cell body, the nucleus and their cellular processes in the outer plexiform layer were completely negative (Fig. 78). This was very clear in the tangential section of the retina (Fig. 79). Some of the bipolar and amacrine cells were positive (Figs. 78,79). The various layers in the IPL were less distinct (Fig. 79). Some ganglion cells were also positive. Apart from the absence of reaction product in the horizontal cells, no other major difference from the normal retina has been found.

c) The retina of the chick embryo and of the growing chick.

In the embryonic retina the first faint staining for D-CaBP-I appeared on day 9 in scattered neurons in the lower third of the inner nuclear layer; these neurons are probably amacrine cells (Fig. 80). By day 12 staining in amacrine cells increased and scattered ganglion cells were also positive (Fig. 81). On day 16, the inner segment of receptor cells were positive (Fig. 82). On day 17, staining in the receptor cells increased and bipolar cells were positive. Some layers of the inner plexiform layer where amacrine cells synapse with ganglion cells were positive (Fig. 83). On day 18 (Fig. 84) the staining pattern was already similar to that of hatchlings (Figs. 85,86). The receptor cells, especially the segments situated in the outer nuclear layer were positive for D-CaBP-I. The horizontal cell bodies along with their nucleus and extensions to the outer plexiform layer were but faintly positive

(Figs. 85,86). Some amacrine cells and probably some bipolar cells were also positive (Figs. 85,86). Interior to the inner nuclear layer, no difference was observed from the normal 4 week old retina. Stratification of the OPL became evident in the retina of the 2 week old normal chicks. The outer stratum, formed by the synaptic pedicles of the receptor cells and the inner stratum formed by processes of the horizontal cells, were visible due to the positive staining of the horizontal cells (Fig. 87). No major difference was observed in the inner layers of the hatchling retina when compared to the retina of the 4 week old normal chick.

In the retina of chicks fed a rachitogenic diet for 2 weeks, the cell bodies and synaptic pedicles of the receptor cells were positive (Fig. 88). The soma and the processes of the horizontal cells that take part in the formation of the outer plexiform layer were completely negative (Fig. 88). In the more interior layers no major differences between the rachitic and normal retina were noted.

d) Mouse and rat retina

In the retina of the white and black mouse and also in the rat, similar neuronal types were positive. Thus the following description applies equally to the staining characteristics of the retina in these rodents. Only barely detectable amounts of D-CaBP-I were present in the receptor cells (figs. 89,90). The horizontal cell soma and their processes stained very darkly, rendering the OPL very prominent (figs. 89,90). In the INL, the cell bodies and nuclei of many amacrine cells were positive (figs. 89,90). In some of these cells the single main process was seen to reach out into the IPL (figs. 89,90). Three horizontal lines in the IPL were very prominent because of their dense staining for D-CaBP-I. Many of the ganglion cells as well as their axons forming the optic tract were positive (figs. 89,90).

In the retina of the rat, antiserum against D-CaBP-II did not stain any of the structures.

e) The rabbit retina

In the rabbit retina (figs. 91,92) as in the case of the mouse and the rat, only very faint staining was present in the inner segment and no staining was observed in the receptor cell pedicle. Here again the OPL was the most prominent layer because of the dark positive staining in the horizontal cells. The INL is very thin and bipolar cells along with Muller cells were negative. However some amacrine cells were stained. Horizontal stratification was not evident in the IPL similarly to the situation in the chick retina.

f) The frog retina

The inner segment of the frog receptor cells as well as their pedicles were faintly positive (Fig. 93). Some distinct horizontal cells stained darkly. In the INL the cytoplasm as well as the nuclei of many bipolar cells were also positive (Fig. 93). Some amacrine cells at the border of the INL and IPL were positive (Fig. 93). In the IPL many sublayers stood out because of the positive staining of amacrine and ganglion cell fibers which synapse in this layer. Many ganglion cell bodies along with their axons forming the optic tract were also positive.

4.1.3) Other neurons

In the 10 day old chick embryo some neurons in the ventral and dorsal horns of the spinal cord were positive for D-CaBP-I (figs. 94,95). There was no reaction product in the neuroglial cells (figs. 94,95). The neurons of the spinal ganglia were negative (Fig. 96). In the chick ganglion nodosum, some neurons were densely stained (Fig. 97).

In the rat small intestine the parasympathetic ganglion cells of Auerbach's plexus were positive for D-CaBP-I. No reaction product was present in any other structure of the villi or surrounding tissue (Fig. 98).

4.2) The stomach

4.2.1) Enterochromaffin cells of the rat and the chick.

Inokuchi et al (1982) have shown that in the rat stomach 2 morphologically distinct types of cells are present which contain 5-hydroxy-tryptamine : pyramidal and spindle shaped cells. Both types of cells were recognizable with D-CaBP-I staining, however the spindle shaped cells appeared to be more numerous (Fig. 99). Enterochromaffin cells in the chick stomach were also positive for D-CaBP-I.

4.2.2) Parietal cells of the rat gastric glands.

In the rat stomach parietal cells were lightly positive when sections were stained with rat anti-D-CaBP-II (Fig. 100).

4.3) Endocrine glands

4.3.1) The chick and rat adrenal gland

The adrenal gland is composed of two main types of cells: the cortical cells of mesodermal origin and the medullary cells which are derived from the neural crest. In the avian adrenal there is no definite boundary between cortical and medullary cells (Hodges, 1974). Immunocytochemical localization of D-CaBP has shown that D-CaBP-I is present only in the cells of the cortex (Fig. 101).

In the rat adrenal only antiserum against rat D-CaBP-II gave positive results. Here again the cortical cells are faintly positive and there was no staining in the medulla (Fig. 102).

4.3.2) Other endocrine glands

There was no staining for any D-CaBPs in the rat and chick thyroid and parathyroid.

4.4) The chick and rat skin

The skin is a unique organ involved in the synthesis, storage and release of $1,25(OH)_2D_3$ precursors. Esvelt et al (1980) demonstrated that $1,25(OH)_2D_3$ can cause a fourfold increase in skin dehydrocholecalciferol content and could thus exert a positive feedback on the production of $1,25(OH)_2D_3$.

In the rat, antiserum against D-CaBP-I applied to sections of skin gave negative results (Fig. 103). However, reaction product indicating the presence of D-CaBP-II was observed in the upper layers of the stratum granulosum and in keratinized cells of the stratum corneum of the skin (Fig. 104) and in the upper layers of the stratum granulosum of the esophagus (Fig. 105). Similar results were obtained when antiserum to D-CaBP-I was applied to chick skin preparations (Fig. 106).

4.5) The chick and rat ovary

In 1982 Dokoh et al demonstrated the presence of $1,25(OH)_2D_3$ receptors in cultured hamster ovary and in hen ovary. The presence of D-CaBPs was investigated in the ovaries of the developing chick embryo, the chick and the rat.

4.5.1) The embryonic and 6 week old chick ovary.

The first sign of sexual differentiation in the chick embryonic ovary is the proliferation and thickening of the germinal epithelium which begins at day 7 of incubation (Romanoff, 1960). The first signs of staining for D-CaBP-I appeared in scattered cells of the germinal epithelium in the 8 day ovary (Fig. 107) and increased in intensity during the next two days. Large primordial cells which are evenly distributed in the cortex did not contain any reaction product. As the primordial cells are dividing and forming oogonia, they cluster into small groups which form lobulations of the surface epithelium. By day 10, these lobulations or cortical cords are well differentiated. The dividing oogonia are surrounded by prefollicular cells which stain darkly for D-CaBP-I (Fig. 108). There was some D-CaBP-I staining present in cells of the medulla in the right ovary (Fig. 109). In the hatching chick the prefollicular cells were darkly stained (Fig. 110). As prefollicular cells became follicular cells they lost their D-CaBP-I reactivity so that in the 6 week old chick ovary only the germinal epithelial cells were positive (Fig. 111). The follicular cells surrounding the oocytes were negative. In the chick embryonic testis no staining was observed.

4.5.2) The rat ovary

In the 1 month old rat ovary only antiserum against D-CaBP-II gave positive results. Reaction product was observed in the cytoplasm of the follicular cells surrounding small oocytes. Theca and granulosa cells surrounding mature oocytes were lightly positive. All other structures in the ovary remained negative (Fig. 112,113).

4.6) Chick lymphoid organs

The demonstration of 1,25(OH)$_2$D$_3$ receptors in bovine thymus by Reinhardt et al (1982) and evidence demonstrating the immunosuppressive power of calcitriol (Manogalas and Deftos, 1984; Tsoukas et al, 1984) has raised the possibility that vitamin D is involved in functions of the immune system.

4.6.1) The localization of D-CaBP-I in the lymphoid organs of growing chicks.

Localization of D-CaBP-I was attempted in the thymus, spleen and bursa of Fabricius of the growing chick using monospecific antiserum against chick duodenal D-CaBP-I. The chicks used varied from 20 day old chick embryos to chicks 6 weeks of age.

4.6.1.a) Thymus and spleen.

In the thymus of 20 day old chick embryos, the cortical epithelial cells (ERC) were homogeneously labelled with reaction product throughout the cell body and cellular processes. The nuclei were also labelled (Fig. 114). In the spleen, some reticular cells in the marginal zones (MZ) between the red and white pulp were labelled with reaction product in the nucleus and throughout the cytoplasm (Fig. 115). The localization of positive D-CaBP-I cell types (cortical ERC in the thymus and reticular cells in the MZ of the spleen) remained the same throughout the growth period, although the intensity of staining and the number of positive cells varied with age. The staining of thymic cortical ERC's increased with age reaching maximal intensity in the 2 week old normal chicks (Fig. 116). As chicks matured Hassal's corpuscles in the thymic medulla also became positive (Fig. 116). In the two week old normal spleen, MZ were well demarcated by intensely labelled reticular cells (Fig. 117). In the four week old normal chick staining in the thymic cortex was reduced compared to the 2 week old chicks, but scattered ERC cell bodies and Hassal's corpuscles were still positive (Fig. 118). In the spleen overall staining was also reduced, but in some areas the density of positive reticular cells allowed the delineation of the MZ (Fig. 119). In the 6 week old normal chick, staining in the thymic cortex was further reduced becoming barely detectable. Scattered cortical ERC cell bodies were still positive and Hassal's corpuscles remained stained. In the spleen the overall distribution of D-CaBP-I was similar to that observed in the 4 week old normal chick.

Staining variations between individuals is to be expected but I have encountered a striking variation in the density of positive reticular cells in the thymic cortex and MZ of the spleen of 3-4 week old normal chicks from different broods. In most birds the staining pattern in the thymus and the spleen was essentially the same as that described above for the 4 week old normal chicks (Fig. 118,119). However, I occasionally encountered a brood where the thymic cortex of 4 week old normal chicks was very densely labelled by positive cortical ERC (Fig. 120), whereas in the spleen there were only a few scattered positive reticular cells, making it difficult to distinguish the marginal zones (Fig. 121).

4.6.1.b) Bursa of Fabricius.

In the bursa of 20 day old embryos no staining was observed. In the 4 week old normal bursa of Fabricius some staining was present in scattered cells of the medulla (Fig. 124). Since staining in the bursa was always rather faint, it was difficult to assess any changes in staining intensity which might have occured with age.

4.6.2) Lymphoid organs in rachitic chicks.

In chicks fed a rachitogenic diet for 4 weeks, staining in the cortical ERC and Hassal's corpuscles (Fig. 122) and reticular cells in the MZ of the spleen (Fig. 123) was generally reduced in intensity and occasionally was entirely absent.

All control sections incubated with non-immune rabbit serum instead of anti-D-CaBP-I were entirely negative (Fig. 125).

4.6.3) The effect of amprolium on D-CaBP-I in the chick thymus and spleen

During the winter months chicks are prone to coccydiosis and the antibiotic amprolium is added to their diet as a safegard against infection.

It seemed that the specimens that showed very dense staining in thymic cortical ERC came from broods that were raised during the summer months. To test the hypothesis that amprolium may have an effect on D-CaBP-I concentration in the chick thymus and spleen, a brood of winter hatchlings was divided into two groups, one group receiving normal diet while the other group received the same diet supplemented with 0.0125% amprolium.

The results have shown that staining in thymic and spleen sections obtained from chicks fed the diet supplemented with amprolium was less intense than in comparable sections from chicks fed the normal diet.This difference was noticable in one week old chicks only; with advancing age this difference disappeared.

4.6.4) The effect of vitamin D-deficiency on antibody production against sheep red blood cells in the chick.

Patients with vitamin D-deficient rickets are prone to infections (Stroder and Schneider, 1975). Early clinical studies implicated defective neutrophils as the cause of increased susceptibility to infections (Stroder and Schneider, 1975). Recently calcitriol has been shown to be important not only in the regulation of macrophage function (Bar-Shavit et al, 1981) but also in the differentiation and maturation of leukocytes (Abe et al, 1981;

Tanaka et al, 1982; Amento et al, 1982; Suda et al, 1984). The presence of calcitriol receptors in activated lymphocytes (Tanaka et al, 1982; Provvedini et al, 1983; Bhalla et al, 1983) is indicative of a direct action of calcitriol on T and B cells which are responsible for the humoral response. The presence of D-CaBP-I in the reticular cells of the thymus suggest that vitamin D could also act indirectly on the cellular and humoral response by regulating the microenvironment of T cell maturation. An experiment was carried out in order to determine whether the humoral response of the immunological system is influenced by the vitamin D status of chicks.

One day old hatchlings were divided into 3 groups. One group received normal chick feed, the second group received the rachitogenic diet containing a calcium supplement and the third group received the rachitogenic diet without supplementation. The rachitic group containing the calcium supplement was included in order to keep blood calcium level close to normal so that the possible effect on antibody production would be due to a deficiency of vitamin D and not to low blood calcium.

The antibody titers in the rachitic chicks maintained for 4 weeks on diets with and without calcium supplementation were reduced by more than 50% compared to the titers found in the normal chicks ($p<.05$; Table 10).

There was no statistically significant difference in antibody titers between the two groups of rachitic chicks, i.e. the groups with and without calcium supplement. Hence the decreased antibody production in rachitic chicks was related to the vitamin D deficiency and was not due to lowered blood calcium levels.

Section 5: Control tissues.

Vitamin D receptors have been demonstrated in a wide variety of tissues. The liver and muscle tissue are not considered vitamin D targets since these tissues do not posses intracytoplasmic receptors for calcitriol (Norman et al, 1982). Indeed in the rat and chick liver (Fig. 126), skeletal (Fig. 127), cardiac (Fig. 128) and smooth muscle (Fig. 2) there was no sign of positive staining for either D-CaBP-I or D-CaBP-II antisera.

IV.DISCUSSION AND CONCLUSIONS

The function of D-CaBP in intestinal calcium absorption

The effect of vitamin D on intestinal calcium absorption has been studied in great detail (see reviews; Avioli, 1972; Zerwekh, 1978; Bilke et al, 1979; Nellans and Kimberg, 1979; Norman, 1979; Kenny, 1981; Walling, 1982). A main center of attention has been the role of D-CaBP in transepithelial calcium transport. The following evidence has been used to support the hypothesis that D-CaBP has a primary role in vitamin D-stimulated calcium translocation as a carrier protein: the protein has been found in tissues where extensive movements of calcium takes place: in the duodenum (Taylor and Wasserman, 1974), kidney (Schreiner et al, 1983) and in the laying hen shell gland (Corradino et al, 1968; Jande at al, 1981). The production of intestinal D-CaBP is under the control of vitamin D which is a key factor in the regulation of calcium absorption by the intestine. Intestinal D-CaBP content and calcium absorption decreases in parallel with age (Wasserman and Taylor, 1968, 1969; Bar and Hurwitz, 1972; Bruns et al 1977). There is correlation between the binding affinity of D-CaBP for the alkaline earth series (Ca>Sr>Ba>Mg) and the relative absorption of these ions (Taylor and Wasserman, 1969). Finally there is parallelism between the D-CaBP content and absorption of Ca^{+2} in different segments of the intestine (Taylor and Wasserman, 1967).

Recently evidence has been accumulating against this hypothesis. Since the decay of D-CaBP-response to 1,25$(OH)_2D_3$ is much slower than that of the calcium transport system (Spencer et al, 1978), these investigators suggested that D-CaBP may be necessary for sustaining but not for initiating the enhanced rate of calcium absorption. D-CaBP could act as a buffer for intracellular calcium in its transport across the mucosal cells. An argument against the intracellular buffer proposal is that the concentration of D-CaBP correlates with the efficiency of Ca^{+2} absorption and not with the total amount of absorbed Ca^{+2} (Bar and Hurwitz, 1979). If D-CaBP acted as an ionic calcium buffer this relationship should be reversed.

Recent experiments performed with cultured embryonic chick duodenum using computer analysis of two dimensional gel electrophoresis demonstrated that D-CaBP-I is induced before vitamin D-stimulated calcium absorption occurs, and appears in amounts that are too small to be detected by commonly used immunoassays (Bishop et al, 1983;1984). Small amounts of D-CaBP could perform a catalytic role in regulating cell membrane calcium pumps. Indeed our results suggest that there may be a factor, most likely D-CaBP, which is lacking in the duodenum of rachitic chicks, which could modulate the activity of Ca^{+2},Mg^{+2} -ATPase and Na^{+}, Ca^{+2}-ATPase. There is evidence indicating that the translocation of calcium from the intestinal lumen to the plasma involves the uptake and concentration of calcium in mucosal tissue (Schachter et al, 1960, 1961; Avioli, 1969). In view of previously reported data, our observations of higher mucosal calcium content in feeding rachitic chicks can best be interpreted as a reflection of decreased calcium extrusion. However, direct proof of the involment of D-CaBP in the transport of Ca^{+2} is lacking. There are no reports of experiments in which antibodies directed specifically against the Ca^{+2} binding site of D-CaBP block the absorption of Ca^{+2}. The production of antibodies directed to one specific site of a molecule is now possible with the refined technique of monoclonal antibody production.

One of the most recent areas in vitamin D research is the discovery that vitamin D targets are not solely confined to calcium transporting cells but involve a wide variety of cells many of which have been demonstrated to contain D-CaBP (Jande et al, 1982; Lawson et al, 1984). This finding does not support the contention that D-CaBP is involved in transcellular calcium transport. It is possible that D-CaBPs have a single molecular function which has been incorporated in a variety of physiological processes (Jande et al, 1981b).

Kretsinger et al (1982) developed a theoretical model suggesting that D-CaBP acts by increasing the availability of ionic calcium to the basolateral cell membrane where Ca^{+2} extrusion occurs. Feher (1983) has devised an in vitro system to test this idea using a 3 compartmental diffusional cell. He demonstrated that D-CaBP accelerates the transit of ionic calcium through the diffusional cell. Hence D-CaBP could accelerate the transit of Ca^{+2} through the cell interior, increasing its availability to the calcium pumps of the plasma membrane. To date, this hypothesis is the most compatible with the results presented in this thesis and in the published literature.

Immunohistochemical localization of D-CaBP

In any immunohistochemical study, tissue preparation is a crucial point. It is imperative that the fixative used should preserve the structural integrity of the tissue and preserve the "in situ" locus without destroying the immunogenicity of the protein antigen under investigation (Kuhlmann, 1976; Miller, 1972; Steinberger, 1974). Protein antigens may be divided into 3 main categories. The first category includes structural proteins such as collagen, keratin, muscle proteins, and the proteins of microtubules and microfilaments. The second category of immobilized proteins comprises those proteins which are enclosed within a membranous vesicle (e.g. endocrine secretions) and proteins which are an integral part of a membrane (e.g. plasma membrane receptors of lymphocytes, hydrolytic enzymes of the brush border membrane). The third and final category includes soluble proteins which upon homogenization of the tissue and fractionation will remain in the supernatant (e.g. D-CaBPs, calmodulin, among others). These proteins present a special problem for the immunohistochemist because diffusion and adsorption to foreign sites may be more rapid than fixation, resulting in an artifactual localization. A case in point is D-CaBP-I and II. These D-CaBPs are low molecular weight acidic proteins that are considered water soluble since upon tissue homogenization and fractionation, over 80% of the total is recovered in the supernatant.

Using routine non-coagulating fixatives (paraformaldehyde) and freeze thawing techniques, D-CaBP-I was first thought to be localized in goblet cells and in the brush border of the enterocytes (Taylor and Wasserman, 1969). By using freeze substituted material where translocation of protein antigens is prevented, it was demonstrated that D-CaBP-I is in reality an intracytoplasmic protein of the absorptive cells (Jande et al,1981; Taylor, 1981). When a soluble intracytoplasmic antigen such as D-CaBP-I and II is to be localized by immunocytochemistry , it cannot be contained by coagulation of the immediately surrounding membrane. Rather the protein itself must be irreversibly precipitated as quickly as possible since in an aqueous milieu D-CaBP can migrate from its true site in the absorptive cell cytoplasm to bind to goblet cell mucus which is rich in calcium. Different fixatives have been evaluated by Jande et al (1981) and these investigators demonstrated that coagulating fixatives such as Carnoy's and Bouin's that precipitate proteins very rapidly gave the most satisfactory results and could be used routinely to localize D-CaBP at the light microscope level in various tissues (Jande et al, 1982).

This problem is even more crucial in electron microscopic studies since these same investigators have shown that paraformaldehyde with or without glutaraldehyde is inadequate for immunocytochemical localization of D-CaBP-I. Among aldehydes commonly used in EM studies, acrolein is the fastest penetrating

(Sabattini et al, 1964) and an evaluation of this fixative to localize D-CaBP-I has been presented. Results obtained with this fixative were similar to those obtained with freeze substituted material. Because of the very rapid penetration and fixation by acrolein, well fixed tissue can be obtained in a much shorter time than by freeze substitution. It has been demonstrated that D-CaBP-I in the duodenum and cerebellum is indeed a cytosolic protein and is not associated with any membranous structure.

While these experiments were undertaken the use of acrolein as a fixative for immunocytochemical localization of pituitary hormones at the electron microscopic level has been reported (Smith and Keefer, 1982) showing the potentially wide range of application of this fixative in immunohistochemical studies.

Specificity of antisera

The absence of D-CaBP staining in tissues that are not vitamin D targets and have no demonstrated $1,25(OH)_2D_3$ receptors (see review by Norman et al, 1982b; Pike; 1985) i.e. the liver and muscle tissue demonstrates the specificity of the technique used to localize D-CaBP type I and II.

The interspecies reactivity of D-CaBP was established using antisera directed against chick duodenal and human cerebellar D-CaBP- I to identify CaBP-positive cells in the distal convoluted tubules of the kidney and in Purkinje cells of the cerebellum in different species. The specificity of the staining reaction was proven by the negative results which were obtained when non-immune rabbit serum was substituted for the specific antisera.

Rat enterocytes gave a positive staining only when antiserum against D-CaBP-II from rat duodenum was used. Similar results were reported by Marche et al (1979 a,b). Contrary to the interspecies reactivity obtained with D-CaBP-I, tissue and species specificity can usually be demonstrated in the case of D-CaBP-II (Wasserman et al., 1977; Thomasset et al, 1983). In accordance with this observation, sections of chick and rat cerebellum, chick duodenum and kidney from several mammalian species consistently showed lack of deposition of reaction product when stained with antiserum against rat duodenal D-CaBP-II. This suggests the lack of cross reactivity of anti-D-CaBP-II with that of anti-D-CaBP-I.

These observations along with the available information on amino acid composition (see review by Wasserman et al, 1978) suggest that D-CaBP-I is highly conserved throughout the vertebrate phylum; the point is also demonstrated by the immunoreactivity of antiserum against chick intestinal D-CaBP-I with D-CaBP-I from a wide range of species (Wasserman and Fullmer, 1982). Mammalian D-CaBP-II is also very similar among many species (Wasserman et al, 1977; Thomasset et al, 1983). Surprisingly antiserum against mammalian intestinal D-CaBP-II is species specific. This may be explained by the fact that the calcium binding sites are highly conserved and as a consequence less or not immunogenic, while only minor sites where amino acid changes have occured may act as immunogenic determinants.

To study the role of the different immunogenic determinants, monoclonal antibodies specifically directed against the calcium-binding sites of the CaBP molecule could be useful. Furthermore, such antibodies would be of help in understanding the function of D-CaBP eg. by blocking the calcium binding sites we could investigate the effects on calcium transport in the enterocytes, calcium spikes in the cerebellum, cellular division of oogonia, etc.

The kidney

The principal sites of regulation of calcium homeostasis are: the intestine, the skeleton and the kidney. Current evidence suggest that vitamin D has a direct effect in the kidney in enhancing tubular reabsorption of calcium relative to the tubular reabsorption of sodium. The administration of $1,25(OH)_2D_3$ to vitamin D-depleted thyroparathyroidectomized rats fed a low phosphate diet results in increased tubular reabsorption of calcium (Steele et al, 1975; Puschett et al, 1975). The calcium clearance ratio at comparable sodium clearance ratios is significantly lower in vitamin D-repleted than vitamin D-depleted rats (Costanzo et al, 1974).

From a biochemical point of view, a direct action of $1,25(OH)_2D_3$ on the renal tubules is most probable since a specific receptor protein for $1,25(OH)_2D_3$ (Christakos and Norman, 1981) as well as for D-CaBP is present in the kidney. Taylor and Wasserman (1967) first reported the presence of D-CaBP-I in the chick kidney. Christakos and Norman (1981) demonstrated that the vitamin

D-dependent increase of D-CaBP-I in chick kidney is accompanied by a parallel increase in the amount of mRNA coding for D-CaBP-I. Furthermore, chick intestinal and kidney D-CaBP-I have a similar half life and a similar time course of induction with a maximum at 8-10 hours after 1,25(OH)$_2$D$_3$ stimulation. However the maximal response in terms of D-CaBP-I concentration in the kidney is much smaller than in the intestine.

The present observations on the distribution of D-CaBP-I staining in the cells of the distal convoluted tubules and in the initial segment of the collecting tubules of the rat, pig, monkey and human kidney have been published (Schreiner et al, 1983) and are similar to other reports in the current literature (Morrissey et al, 1978a, b; Rhoten and Christakos, 1981; Christakos et al, 1981; Rhoten and Christakos, 1981; Roth et al, 1981, 1982; Taylor et al, 1982). Our results confirm the absence of D-CaBP-I in the cells of the macula densa and renal corpuscles.

The staining of D-CaBP-II described in the rat kidney establishes for the first time the presence of immunoreactive sites for D-CaBP-II in all the cells that also contain D-CaBP-I, as well as in some additional cells, i.e. in cells of the thick ascending limb of Henle's loop, the arched straight and papillary portions of the collecting ducts and of the macula densa. The immunocytochemical localization of D-CaBP-II in the kidney of mammals has not been reported previously. Marche et al (1977) reported the binding of anti-serum against rat duodenal D-CaBP-II to rat renal cortical proteins. However, at that time the kidney D-CaBP found in the rat

(Hermsdorf and Bronner, 1975), dog (Sands and Kessler, 1971), and human (Morrissey et al, 1974; Piazolo et al, 1971) was reported to be in the 28,000 molecular weight range. Thus Marche et al (1977) remarked only that their "results required further investigation". Murray et al (1974, 1975) who isolated pig duodenal D-CaBP-II and raised antisera against it, reported immunoreactivity of the pig renal cortical extract to the above antisera. There is now biochemical evidence demonstrating the presence of both D-CaBP-I and II in the mouse kidney (Delorme et al, 1983). To date, only D-CaBP-I has been isolated from rat kidney; its molecular weight has also been determined (Pansini and Christakos, 1984). On the basis of my results and from the evidence available in the literature, it is reasonable to assume the presence of D-CaBP-II in the rat kidney as well as in the kidney of other mammalian species. The presence and localization of D-CaBP-II has been confirmed in the mouse and rat kidney (Rhoten et al, 1985).

Using an autoradiographic technique to determine the nuclear uptake of 1,25$(OH)_2D_3$ Stumpf et al (1979) have identified the cells of the distal convoluted tubules as vitamin D target cells .These are precisely the cells that are positive for D-CaBP-I and II. In addition, weakly positive cells for 1,25$(OH)_2D_3$ have also been described in the ascending limb of Henle's loop, cells of the macula densa and those of the collecting ducts (Stumpf et al, 1979, 1980). These cells contain immunoreactive sites to D-CaBP-II antiserum. Thus in the target cells of the rat kidney there is a clear correlation between the demonstration of hormone uptake and the immunocytochemical localization of the product of hormone action.

Micropuncture studies in the rat have shown that the bulk of calcium filtered at the glomerulus is absorbed in the proximal tubules (Lassiter et al, 1963; Frick et al, 1965; Sutton et al, 1975; Costanzo et al, 1978, 1980; Greger et al, 1978; Bengele et al,1980; Suki and Rouse, 1981), which did not show any staining for either D-CaBPs. Evidence supporting passive and /or active calcium transport across the proximal convoluted tubules has been reported (Sutton and Dirks, 1975; Ullrich et al, 1976). The cells of the proximal tubules are morphologically similar to the duodenal enterocytes and are also the site of calcium absorption. As in the duodenum, the question whether both passive and active mechanisms of calcium absorption are operative and their relative importance remains controversial. Calcium absorption in the proximal convoluted tubules takes place in cells that do not contain any D-CaBP. Hence the presence of this protein is not an absolute requirement for calcium translocation in the kidney.

Calcium is also absorbed in the loop of Henle (Sutton and Dirks, 1975) and this is assumed to take place in the thick ascending limb where D-CaBP-II has been localized. There is no information regarding the mechanism of calcium transport in the loop of Henle of the rat. Parathyroid hormone-sensitive adenylate cyclase is present in the cortical thick ascending limb (Morel, 1981) but its role in regulating tubular calcium absorption is unknown.

The cells of the distal convoluted tubules which contain both

D-CaBP-I and D-CaBP-II represent the site of calcium absorption were the steepest calcium gradient is present (Lassiter et al, 1963; Agus et al, 1973). This is where the fine regulation of calcium absorption occurs (Goldberg et al, 1976). Costanzo and Windhager (1980) demonstrated active calcium absorption in the distal convoluted tubules of the rat which is increased by parathyroid hormone (PTH). They speculated that the variability of the effect of PTH on calcium transport among individual tubules may be the result of the presence of heterogeneous cell types that are not affected to the same extent by PTH. This functional heterogeneity in the distal convoluted tubules may also be related to the presence of D-CaBP-I and II. Indeed I have demonstrated that some cells contain both proteins while others contain one or the other or none at all. This different localization may be an indication of the differences in the manifestations of function by D-CaBP-I and II.

In the rat the transition of connecting tubule to cortical collecting duct is gradual and there may be intermingled cell types present (Kriz et al, 1978). In the weaned rat the localization of D-CaBP-I in cells of the initial portion of the cortical collecting duct correlates well with the localization of PTH-sensitive adenylate cyclase (Morel, 1981) and with PTH sensitive ^{45}Ca efflux (Greger et al, 1978). D-CaBP-I in weaned rats was absent from segments of the collecting ducts where PTH-insensitive calcium efflux occurs. The magnitude of calcium transport in these tubules is low and occurs by an unknown mechanism (Bengele et al, 1980). In the suckling rat interspersed cells throughout the entire length of the collecting duct system were positive for D-CaBP-I; similar situation prevailed in the monkey where all the cells of the entire collecting duct system were positive for D-CaBP-I suggesting that these sites may be included in the regulation of calcium absorption and may represent different handling of the renal calcium load in these animals. However physiological studies are not available to support this hypothesis.

In the present study of the kidney, duodenal enterocytes and Purkinje cells D-CaBP has always been found in the cytosol and not in association with any cell membrane, confirming the observations of Roth et al (1981, 1982) Thus a direct involvement of D-CaBP in the initial stage of calcium absorption across an epithelial membrane is highly improbable.

Nonetheless, vitamin D has a direct effect in enhancing calcium reabsortion. The detailed mechanism of interaction between $1,25(OH)_2D_3$ and the kidney in the maintenance of calcium homeostasis remains to be clearly defined.

The chick embryonic kidney

The chick duodenum is one of the tissues containing the highest concentration of D-CaBP-I (Christakos et al, 1979). It is absent from the chick embryonic duodenum and appears only on the day of hatching when calcium absorption begins. At this time there is a rapid synthesis of the protein (Corradino et al, 1969). The reason for the inability of the intestine in normal embryos, to synthesize detectable quantities of D-CaBP-I is not understood, since high affinity cytosolic receptors for $1,25(OH)_2D_3$ are present in the gut of 15 day old embryonic enterocytes (Oku et al, 1976; Seino et al, 1982) and radioactive $1,25(OH)_2D_3$ accumulates in their nucleus (Narbaitz et al, 1980). Also the egg contains cholecalciferol and its metabolites (Moriuchi and DeLuca, 1974; Frazer and Emtage, 1976). The renal enzyme 25(OH) cholecalciferol 1-hydroxylase has been detected in day 9 mesonephros (Bishop and Norman, 1975).

The results presented in this thesis suggest that as in the duodenum, the appearance of D-CaBP-I in the embryonic kidney is related to the initiation of function in the vitamin D target cell. During embryonic development 3 sets of kidneys are produced. The pronephros which is non-functional (Romanoff, 1960) did not show any D-CaBP-I staining. The onset of mesonephric function appears during the fifth day of incubation (Romanoff, 1960), the time when D-CaBP-I positive tubules appear. The metanephros begins to function around the tenth day of incubation (Romanoff, 1960) and staining in the distal convoluted tubules appeared at that time. This also corresponds to the time when $1,25(OH)_2D_3$ is produced in detectable amounts by the mesonephros and then by the metanephros (Kubota et al, 1980).

Taylor and Wasserman (1972) have measured D-CaBP-I by radioimmunoassay in the chick mesonephros and metanephros on the tenth day of incubation. Because of the small size of mesonephric tissue of younger embryos they were not able to demonstrate the presence of D-CaBP-I in younger mesonephros. Radioautographic studies carried out in 16 day old metanephros by Narbaitz et al (1982) demonstrated that the cells of the distal convoluted tubules accumulated tritiated 1,25$(OH)_2D_3$. The cells of the distal convoluted tubules are the cells that contain D-CaBP-I in the embryonic chick metanephros as well.

The rat salivary glands

In man, the salivary glands do not normally play an important role in regulating water and electrolyte balance because most of the secreted fluid is later reabsorbed in the alimentary tract. In animals without widely distributed sweat glands, saliva provides the fluid for evaporative cooling during heat stress and may therefore play an important role in the regulation of water and electrolyte balance (Schneyer et al, 1972).

The first indication that salivary glands could be vitamin D targets came from Tenenhouse and Goodwill (1978) who showed that protein secretion from parotids of vitamin D-deficient rats was greatly altered under isoproterenol stimulation. The rat parotid (Goodwin et al, 1978) and submandibular gland (Stumpf et al, 1980) accumulate radioactive vitamin D metabolites. Vitamin D-dependent calcium binding activity measured by the chelex resin method was found in the rat salivary glands (Freund and Withowski, 1977). This methodology does not permit the identification of the nature of the vitamin D target cells nor the type of D-CaBP present.

By using an immunocytochemical method, I have demonstrated that

D-CaBP-II but not D-CaBP-I is present in the cytoplasm of the cells of the entire duct system in the suckling rat parotid, submandibular and sublingual salivary glands. The parotids always gave darker staining indicating that this gland may contain a higher concentration of D-CaBP-II. This correlates well with the results of Freund, Volpe and Witkowski (1977) who demonstrated that calcium binding activity was higher in the parotid than in the submaxillary and sublingual glands. The presence of D-CaBP-II in the salivary glands has been confirmed by radioimmunoassay (Thomasset et al, 1982). The observed decrease of D-CaBP-II staining in the salivary glands in older rats is similar to the situation in the duodenum where D-CaBP-II concentrations decrease with age (Zornitzer et al, 1971).

Under the influence of parathyroid hormone the handling of calcium by the salivary gland is similar to that of the kidney (Schneider et al, 1977). Indeed, Glijer et al (1984) proposed that vitamin D is necessary for the synthesis of a protein(s), possibly D-CaBP-II, which is essential for the utilization of extracellular calcium in the secretion process of the parotid. I have demonstrated that D-CaBP-II is present in the cells of the distal convoluted tubules and collecting ducts which is the site of fine regulation of calcium excretion. It is tempting to speculate that D-CaBP-II may have a similar function in the salivary glands and in the kidney.

The developing rat incisor and molar teeth

Most studies in this context have focused on the effects of vitamin D on the formation of dentin (Becks and Ryder, 1931; Yoshiki et al, 1974; Engstrom et al, 1977). The effect of vitamin D on dentin formation is not a direct effect on odontoblasts but is due to decreased serum calcium levels (Yoshiki et al, 1974). The absence of D-CaBPs from the odontoblasts further substantiates this finding. However, the presence of D-CaBP-I in the ameloblasts suggests that these are vitamin D target cells.The presence of D-CaBP-I in the ameloblasts and the possible function of vitamin D in tooth formation has been confirmed by other investigators (Celio et al,1984; Taylor, 1984). Antibodies to D-CaBP-II did not stain any structure of the tooth in ~~my~~ experience, although Taylor et al (1984) have reported the presence of immunoreactive sites in the ameloblasts to antiserum against rat duodenal D-CaBP-II.

This is one of the instances where results obtained by radioautography are in disagreement with immunocytochemical localization of D-CaBPs. Radioautographic studies have indicated that the pulpal cells are vitamin D targets, while the odontoblasts and the ameloblasts did not accumlate radioactive cholecalciferol (Kim et al, 1983).

Our understanding of the function of D-CaBP-I in the ameloblasts is hindered by the lack of understanding of the function of D-CaBP generally. There is evidence indicating that a fundamental difference exists in the processing of calcium by secretory ameloblasts and odontoblasts. The odontoblasts may have a mechanism to concentrate calcium (Boyde and Reith, 1977; Reith and Boyde, 1978), possibly an ATP-dependent calcium pump (Granstrom and Linde, 1981). On the other hand ameloblast do not concentrate calcium, and calcium transit through these cells is very rapid (Boyde and Reith, 1977; Reith and Boyde, 1978). These cells may function as a gate regulating the passage of calcium into enamel (Reith and Boyde, 1979). It is then possible that D-CaBP as suggested earlier may increase the diffusibility of calcium thus increasing the calcium load the cell can translocate. It is interesting to note that the odontoblasts and the cells of the proximal convoluted tubules of the kidney are similar in that they are actively engaged in calcium translocation but do not contain any D-CaBPs.

Neurons

D-CaBP-I was originally isolated from chick duodenum (Wasserman and Taylor, 1966). The presence of D-CaBP-I in the central nervous system (CNS) has been demonstrated by biochemical means in the chick cerebellum (Taylor, 1974) and in the rat (Feldman and Christakos, 1983). Since D-CaBP-I immunoreactivity was found in the cerebellum of the gold fish, frog, chick, mouse, rat and rabbit, it is reasonable to assume that this protein is present in the CNS of all vertebrates.

Jande et al (1981b) were the first to report the complete mapping of D-CaBP-I in the chick and rat brain . These findings have been confirmed by many investigators (Roth et al, 1981; Baimbridge and Parkes, 1981; Baimbridge and Miller, 1982; Baimbridge et al, 1982; Feldman and Christakos, 1983; Garcia-Segura et al, 1984). The cochlear hair cells and vestibular hair cells of the rat inner ear are also D-CaBP-I positive (Rabie et al, 1983). In the chick some sympathetic neurons of the ganglion nodosum (Hodges, 1974) while in the rat small intestine parasympathetic ganglion cells of the plexus of Auerbach are positive for D-CaBP-I. I have extended the list of D-CaBP positive cells to include certain neurons in the dorsal and ventral horn of the chick spinal cord.

The function of D-CaBP-I in neurons is still not clear. Since D-CaBP-I is present only in specific neurons, it cannot have an obligatory role in neurotransmission. On the basis of available electrophysiological data, Jande et al (1981b) suggested that there may be a correlation between the presence of D-CaBP-I and the production of voltage dependent calcium spikes. These sites also contain benzodiazepine receptors which potentiate the ability of GABA to open cation channels (Squires and Saederup, 1982). D-CaBP-I could prevent a calcium surge in the cell by rapidly redistributing calcium throughout the cell and making it available to the intracellular calcium regulating mechanisms. There is some evidence that vitamin D can alter neurotransmitter metabolism and neuronal function. Chronic vitamin D-deficiency in the rat increases the dopamine concentration in the cortex and in the median eminence of the hypothalamus (Baski and Hughes, 1982).

Nonetheless the vitamin D-dependency of D-CaBP-I in the brain has been difficult to prove (Taylor, 1974). D-CaBP-I synthesis in the chick brain is stimulated only after chronic administration of high doses of 1,25$(OH)_2D_3$ (Taylor, 1976). I have shown that in the developing chick embryonic cerebellum D-CaBP-I can be detected as early as day 9 of incubation. Since D-CaBP-I is present early in ontogeny and since neurons do not turn over, it is possible that the vitamin D status of the growing chick has little effect on D-CaBP-I concentrations in the CNS.

On the other hand, the comparative developmental study of the retina from normal and rachitic chicks demonstrates the vitamin D-dependency of the synthesis of D-CaBP-I in the horizontal cells. These neurons are the only known neurons that acquire D-CaBP-I during the first week post-hatching and are thus more sensitive to the vitamin D status of the growing animal. The vitamin D-dependency of D-CaBP-I in the horizontal cells of the chick retina (Schreiner et al, 1985) has been demonstrated for the first time.

The study of the retina demonstrated a wide variation in the concentration of D-CaBP-I in the receptor cells. Staining was most pronounced in the chick receptor cells and weakest in the albino rat. There is evidence that calcium acts as a transmitter in the light induction of receptors (Hagina, 1972; Yoshikami et al, 1980; Hendriks et al, 1974). The different D-CaBP-I reactivities observed in the chick and rat retina may represent different calcium loads imposed on the receptors. In all the vertebrate retinas studied, some amacrine cells were always positive. There are several types of amacrine cells in the vertebrate retina which contain different peptides such as: acetylcholine (Nichols and Roelle, 1968), glycine, GABA (Marshall and Voaden, 1974a,b), dopamine (Araki et al, 1983), indolamine (Hauschild and Laties, 1973; Floren, 1979), enkephalin (Brecha et al, 1979; Tornqvist et al, 1981), somatostatin (Kirsch and Leonhardt, 1979; Buckerfield et al, 1981; Eskay et al, 1980; Tornqvist et al, 1981; Ellis et al, 1983), TRH (Eskay et al, 1980; Tornqvist et al, 1981), substance P (Fukuda et al, 1981; Karten and Brecha, 1980; Eskay et al, 1980), glucagon and neurotensin (Tornqvist et al, 1981). Evidence concerning the role of these neuropeptides has suggested that these may function as neurotransmitters (Tornqvist et al, 1981). In the retina the release of TRH, somatostatin and substance P is calcium dependent under depolarizing conditions (Eskay, 1980) and could be related to the presence of D-CaBP-I.

In all the species studied with the exception of the rabbit, positive horizontal layers in the IPL were found. These layers represent synaptic contacts between the D-CaBP-I positive amacrine cells and bipolar and ganglion cells.

In the rat, mouse and frog retina, most of the ganglion cells along with their axonal fibers were positive for D-CaBP-I. In contrast, in the chick only a few ganglion cells were positive while the axonal fibers were negative. This correlates well with the observations of Feldman and Christakos (1983) who noted that the rat optic tract fibers are positive for D-CaBP-I, while Roth et al (1981) demonstrated that the chick optic tract fibers are negative. The functional significance for this disparity in D-CaBP-I content between two homologous structures remains to be established.

Because of the localization of 1,25$(OH)_2D_3$ in certain

D-CaBP-I positive neurons (Stumpf et al, 1980), the CNS can be considered a vitamin D target organ (Jande et al, 1981b).

Gastric endocrine cells

Stumpf et al (1979) demonstrated that dispersed individual cells in the basal portion of the gastric glands where gastric endocrine cells are localized, accumulate $1,25(OH)_2D_3$. In the rat stomach cells ecorresponding to the above localization were strongly positive for D-CaBP-I. These cells are morphologically very similar to enterochromaffin cells containing 5-hydroxytryptamine (Inokuchi et al, 1982). Simultaneous immunohistochemical demonstration of D-CaBP-I and of either gastrin or 5-hydroxytryptamine could establish the identity of the D-CaBP-I cells. Parietal cells which were slightly labelled for D-CaBP-II could be considered vitamin D target cells.

The presence of D-CaBP-I in gastrin producing cells and D-CaBP-II in the parietal cells would be of interest. Calcium must be ionized in order to be absorbed (Schachter et al, 1960) and gastric acidity could be an important factor in calcium absorption (Ali and Evans, 1967; Mahoney and Hendricks, 1972). Gastrin is a physiological activator of acid secretion by the parietal cells of the stomach. Evidence that both parietal and gastrin-producing cells are vitamin D target cells would open the possibility that vitamin D can simultaneously control calcium absorption by the solubilization of calcium salts via gastrin-hydrochloric acid stimulation and the rate of calcium absorption by duodenal enterocytes. Furthermore, interactions between calcium, calcemic hormones (mainly calcitonin and parathyroid hormone) and gastrointestinal hormones might enable the organism to respond to changes in feeding patterns by modulation of the absorption of dietary calcium in an integrated, beneficial fashion (Cooper et al, 1978). However, Selking et al (1982) have demonstrated that vitamin D-induced hypercalcemia in parathyroidectomized rats does not influence the concentration of gastrin in the serum, or the number of antral gastrin producing cells.

Endocrine glands

Since intracellular calcium is important in the process of cellular secretion and D-CaBP is present in the cortical cells of the adrenal it seemed possible that D-CaBP plays a role in the modulation of endocrine secretion. However, D-CaBP is not present in the thyroidal follicular cells nor in the cells of the parathyroid, consequently D-CaBP does not appear to have an obligatory role in endocrine secretion.

The skin

The skin is a unique organ for the synthesis, storage and release of vitamin D. The presence of D-CaBP-II in the rat skin and D-CaBP-I in the chick skin indicate that the skin is also a vitamin

D target. This is further substantiated by the presence of high specificity 1,25$(OH)_2D_3$ receptors in mouse (Horiuchi et al, 1985) and rat skin (Simpson and DeLuca, 1980). It has been shown that the rat skin accumulates 1,25$(OH)_2D_3$ (Stumpf et al, 1979). There is evidence demonstrating that 1,25$(OH)_2D_3$ is involved in the differentiation and development of the skin (Kuroki et al, 1985). Calcium is an important factor in the regulation of cellular division (Perris et al, 1968) and may be related in some way to the presence of D-CaBPs in the skin.

The ovary

Dokoh et al (1982) demonstrated the presence of high affinity receptors for 1,25$(OH)_2D_3$ in cultured hamster and hen ovaries. Physiological doses of 1,25$(OH)_2D_3$ allowed the production of specific, dose-dependent inhibition of cell growth in cultured hamster ovary.

~~In~~ the developmental study of the chick gonad, I have detected D-CaBP-I in the germinal epithelial cells as early as day 8 of incubation. At this time a thickened germinal epithelium differentiates a female gonad from a male gonad. The most active mitotic activity of oogonia occurs between day 12-17 (Hughes, 1963). During this time prefollicular cells are rapidly becoming D-CaBP-I positive and are surrounding the oogonia. It is possible that the prefollicular cells are necessary to create and regulate the proper microenvironment for the regulation of the division of the oogonia.

Calcium is important in the regulation of cellular division and the presence of D-CaBP-I in the prefollicular cells may indicate a special requirement of the oogonia for calcium. However in the rat ovary D-CaBP-II was found only in the follicular cells.

The chick lymphoid organs

In the present study the presence of D-CaBP-I in the chick thymus, spleen and bursa of Fabricius was established. Consequently, these organs can be considered vitamin D targets. This conclusion is further supported by the decreased D-CaBP-I staining in the rachitic chick thymus and spleen. Furthermore, cytosolic receptors for $1,25(OH)_2D_3$ have been isolated from the bovine thymus (Reinhardt et al, 1982) and the rat thymic epithelial cells (ERC) have been identified as $1,25(OH)_2D_3$ targets by radioautography (Stumpf et al, 1981).

Immune competence in the chick develops only some time after hatching, but precursors of immunocompetent cells are present in the thymus and bursa at least 6-7 days before hatching (Szenberg, 1976; Metcalf and Moore, 1971). At hatching the thymus and bursa are fully lymphoid whereas the spleen is immature and may be the cause of the reported immunological incompetence (Isacson, 1959; Papermaster et al, 1962). In the growing chick immune competence develops rapidly during the first month of life (Motika, 1975; Seto, 1976). The population of T lymphocytes in the thymus reaches a peak in 2 week old chicks and then steadily declines to reach a stable level at 9 weeks (Potworowski, 1971). It is during this period that the most rapid progression in D-CaBP-I staining has been observed in the chick thymic cortical ERC and in the reticular cells of the marginal zones of the spleen. Full adult responsiveness to soluble antigens is achieved at 6 weeks of age (Wolfe and Dilks, 1948), at a time when I observed only a faint staining in the thymic cortical ERC and decreased numbers of positive reticular cells in the marginal zones of the spleen. In the thymus and the bursa, reticular cells are thought to create a microenvironment important for the maturation of T and B lymphocytes, respectively (Bryant, 1974; Jordan et al, 1979; Rompanen and Sorvari, 1980). It is possible that D-CaBP-I in the reticular cells of the thymus are implicated in the maintenance and regulation of the microenvironment. Avian thymus extracts have hormonal properties but no particular cell type has been associated with the hormone production (Kendall, 1980). In mice, reticular cells have been identified as thymic hormone containing cells (Savino et al, 1982) and these stain in a pattern somewhat similar to that of D-CaBP-I positive thymic ERC of the chick.

In the spleen the white pulp develops during the first week after hatching and T lymphocytes rapidly populate the periarteriolar sheaths during the second week after hatching (Metcalf and Moore, 1971). This correlates well with the observation that the most impressive staining of spleen reticular cells occured in the marginal zones of 2 weeks old chicks, a time when T lymphocyte migration into the white pulp is very rapid. The marginal zones represent an important pathway for T and B lymphocyte migration

(Brelinska and Pilgrim, 1983) and it is there that blood borne antigens are first localized (Nossal et al, 1966; Mitchell and Abbt, 1971; Brown et al, 1973). The D-CaBP-I positive reticular cells of the chick spleen resemble the metallophils described in the marginal zones of the rat spleen (Satodate et al, 1971). The reticular cells of the different splenic compartments do not represent an homogeneous population as demonstrated by cytochemical (Muller-Hermelink et al, 1974) and morphological criteria . An ultrastuctural study would be required to determine the particular reticular cell population D-CaBP-I positive cells belong to.

I have shown that staining for D-CaBP-I in the reticular cells of the chick thymus and spleen increased with age to reach a maximum in 2 week old chicks. Staining decreased at a varied rate in the thymus and spleen of chicks from different broods as indicated by the striking variation in staining for D-CaBP-I in 4 week old chicks. This may indicate that the expression of D-CaBP-I in the thymic and splenic ERC is not only age- and vitamin D-dependent but may also be under the control of some as yet unknown factor(s).

The presence of $1,25(OH)_2D_3$ receptors in activated T and B lymphocytes suggest a direct action of vitamin D on lymphocytes. The demonstration of vitamin D targets in reticular cells of the thymus suggest that vitamin D may act indirectly on the immune system by regulating the microenvironment of maturing T cells.

The reduced capacity of rachitic chicks to produce antibodies against sheep red blood cells may be due either to decreased numbers of B and perhaps T helper cells or to the impaired function of B and perhaps T helper cells or to the impaired function of B and perhaps T helper cells. The production of antibodies against the cell membrane of lymphocytes permits tagging of T or B lymphocytes with a fluorescent label and estimate the number of either type of lymphocytes in the blood and lymphoid organs (Tseng and Glick, 1982). The effect of vitamin D-deficiency on the population of T and B lympocytes in the blood and in the lymphoid organs could thus be studied and could be correlated to the rate of mitosis of maturing lymphocytes in the lymphoid organs. The injection of colchicine arrests cellular mitosis and the rate of mitosis of lymphocytes in the thymus and bursa can be estimated by counting the numbers of metaphase plates present in the thymus and bursa (Perris and Whitfield, 1967). The effect of vitamin D on the integrity of the function of T and B cells could be assessed by a study of surface markers of the lymphocytes in normal and rachitic chicks. The surface markers of T cells can be studied by the rosetting technique (Peacock and Towar, 1980) and by the phytoagglutinin response (Peacock and Towar, 1980). B lymphocyte function can be assessed by the EAC rosette assay (Peacock and Towar, 1980).

It seems that the crucial time period for the maturation of the chick lymphoid system is the 2nd week post hatching. At this time the chicks are not yet completely vitamin D-deficient and the experiments described above would therefore indicate the effects of low levels of vitamin D on maturing lymphocytes. In order to evaluate the effects of vitamin D deficiency on the lymphoid system of the chick, experiments would have to be carried out on cultured cells and tissues kept in a completely synthetic medium. Lymphoid organs of chick embryos produced by rachitic hens would provide the ideal working material. However since vitamin D and its metabolites are necessary for the development of the chick embryo, the production of viable embryos in the absence of vitamin D is extremely difficult.

The demonstration of vitamin D targets in such a wide variety of cell types indicates that the scope of action of vitamin D is not limited to calcium homeostasis in the classical definition but is implicated in a wide range of physiological processes.

V.SUMMARY

1) The activity of enzymes that may modulate calcium extrusion from duodenal enterocytes, Ca^{+2}, Mg^{+2}-ATPase and Na^{+}, Ca^{+2}-ATPase is significantly lower in duodenal homogenates from rachitic chicks than in similar homogenates obtained from normal chicks.

2) Differences were found in the subcellular distribution of the activity of the above mentioned enzymes in homogenate fractions prepared from the duodenum of normal and rachitic chicks. It appears that a factor may be present in the soluble fraction of the duodenal mucosa of normal chicks, which is absent in a corresponding preparation from rachitic chicks. This factor may contribute to the regulation of the activity of Ca^{+2}, Mg^{+2}-ATPase and Na^{+}, Ca^{+2}-ATPase. The possibility exists that this factor is D-CaBP.

3) Calcium accumulates in the duodenal mucosa of fed rachitic chicks compared to normal controls. The level of calcium in fasted normal chicks does not change with feeding and remains similar to the level of calcium in the duodenal mucosa of fasted rachitic chicks. The accumulation of calcium in the duodenal mucosa of fed rachitic chicks could be the result of reduced calcium extrusion due to decreased activity of the enzymatic pumps.

4) The presence of D-CaBP is not absolutely required for calcium

translocation since it is absent from certain cells (eg. the proximal convoluted tubules cells of the kidney, the odontoblasts of the tooth) that are known to be sites of calcium transport.

5) The presence of D-CaBP-I seems to be related to the development of function in certain vitamin D target cells. Thus it was demonstrated that correlation exists between the development of function and the appearance of D-CaBP-I in the embryonic duodenum, mesonephros and metanephros. The pronephros which is non-functional does not contain any D-CaBP-I positive cells. Furthermore, the development of the immunological competence of the growing chick parallels the time of appearance of D-CaBP-I in the lymphoid organs.

6) The wide variety of cell types containing D-CaBP-I (table 11) which are not involved in calcium translocation confirms the hypothesis that D-CaBP may have a molecular function which has been integrated into a variety of physiological processes. Since D-CaBPs are considered to be molecular markers of vitamin D target cells, the identification of a wide range of cells containing D-CaBPs considerably widens the scope of vitamin D research.

APPENDIX I

ABBREVIATIONS

A: Ameloblasts.

ADRI: Animal desease research institute.

AL: ascending thick limb of Henle's loop.

BC: Bipolar cells.

CD: collecting duct.

CS: connecting segment.

D: dentin.

DAB-HCL: 3, 3' diaminobenzidine-HCL.

DCT: distal convoluted tubule

DHCC: 1,25-dihydroxycholecalciferol or calcitriol or 1,25$(OH)_2D_3$.

E: enamel.

ERC: epithelial reticular cell.

FL: fiber layer.

G: glomerulus.

GC: ganglion cell

HC: horizontal cells.

H and E: Hematoxylin and eosin.

INL: inner nuclear layer.

IPL: inner plexiform layer.

MCD: medullary collecting duct.

MD: macula densa.

MD: main duct.

MiS: mid-segment.

MT: mesonephric tubule.

MS: main segment.

MZ: marginal zone.

NGS: normal goat serum.

O: odontoblast.

ONL: outer nuclear layer.

OLM: outer limiting membrane.

OPL: outer plexiform layer.

PAP: peroxidase anti-peroxidase method of antigen localization.

PAS: periodic acid Schiff.

PCT: proximal convoluted tubules.

PCV: posterior cardinal vein.

PF: paraformaldehyde.

Pi: inorganic phosphate.

PND: primary nephric duct.

PnP: p-nitro-phenol.

2

RC: receptor cell.

SIM: short indirect method of antigen localization.

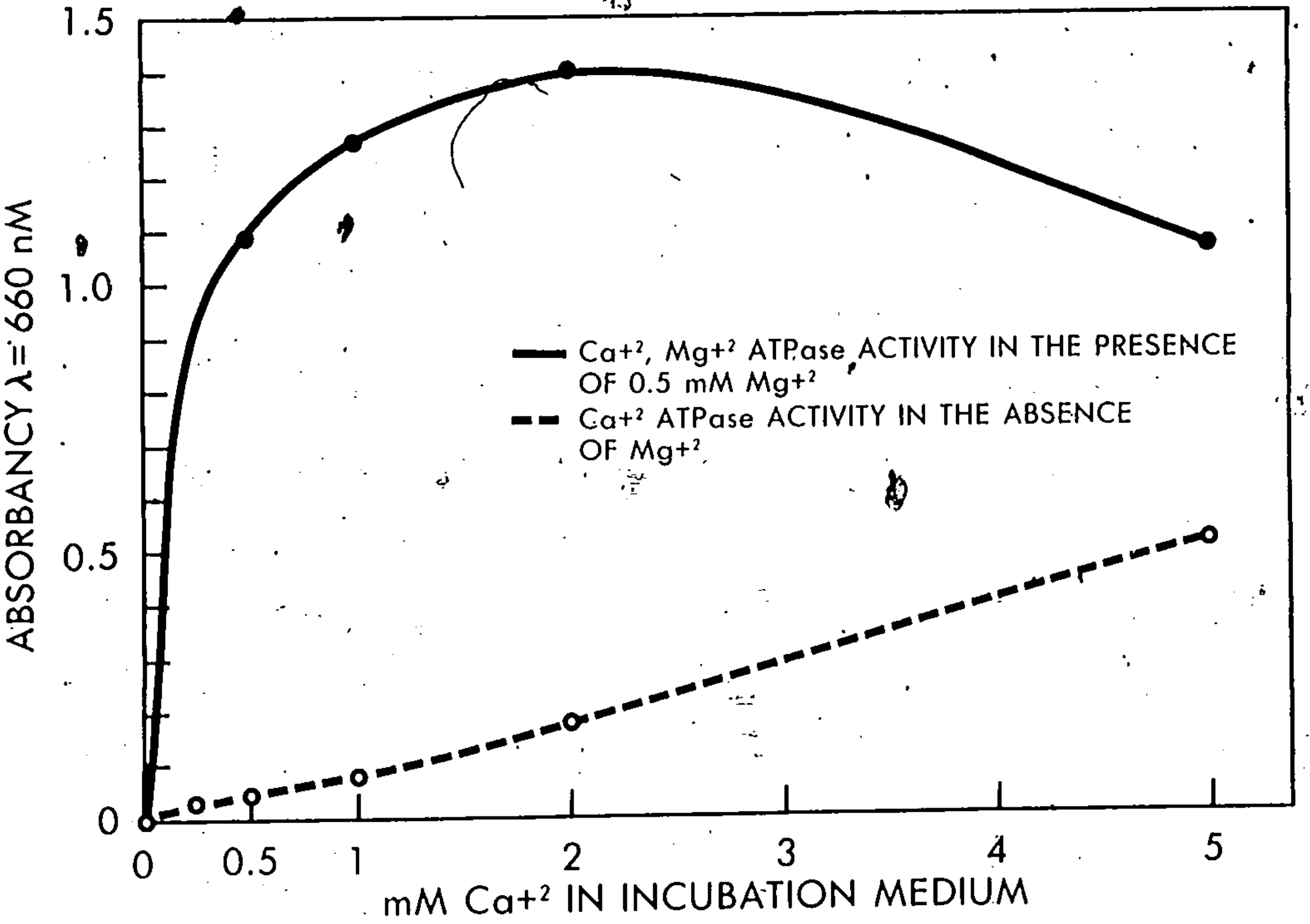
TAL: thick ascending limb of Henle's loop.

TBS: Tris buffer saline.

TR: tubal ridge.

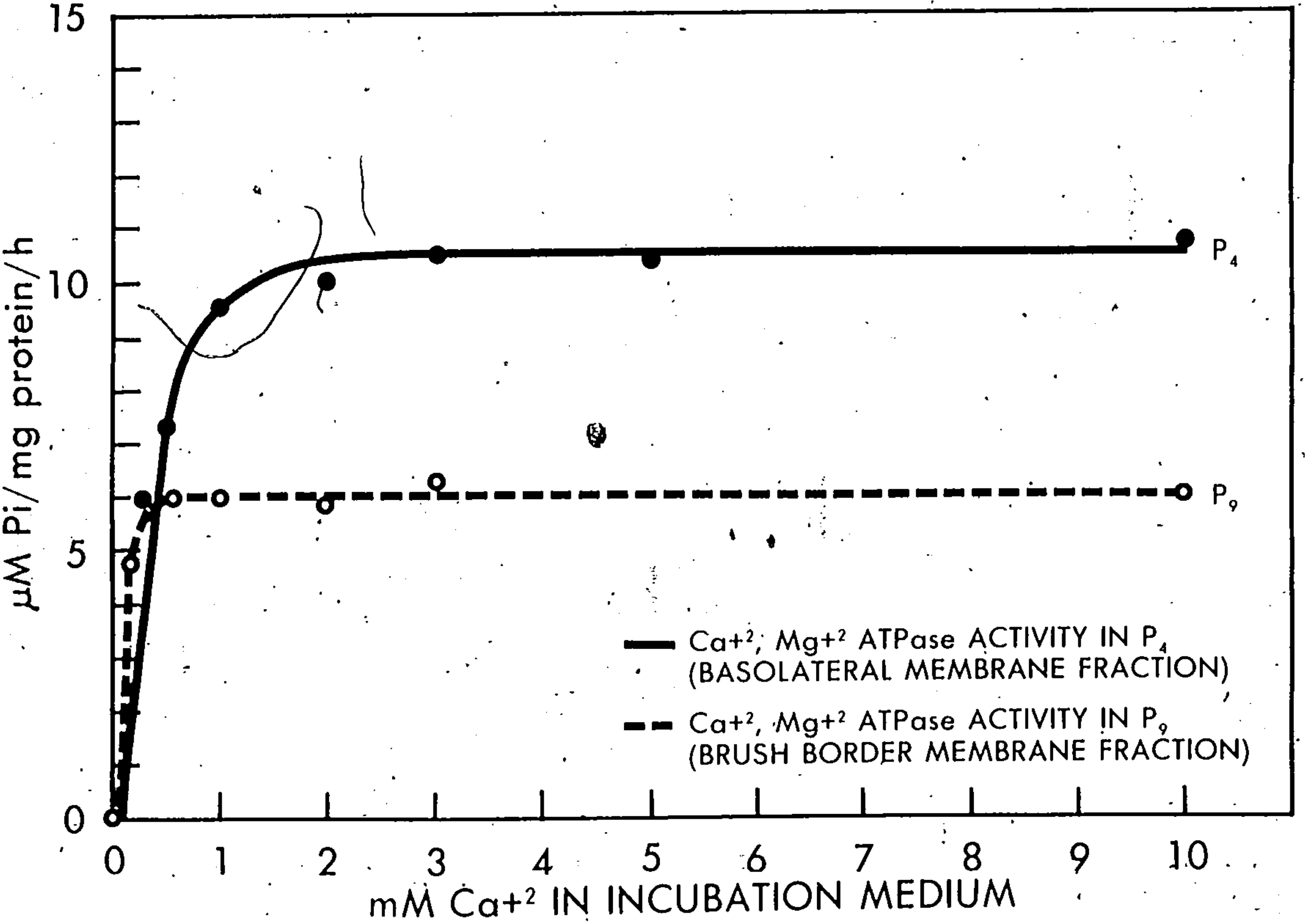
APPENDIX II

THE EFFECT OF VARYING [Ca^{+2}] AND OF [Mg^{+2}] ON THE Ca^{+2}, Mg^{+2} ATPase ACTIVITY MEASURED IN THE WHOLE HOMOGENATE OF A NORMAL CHICK DUODENUM



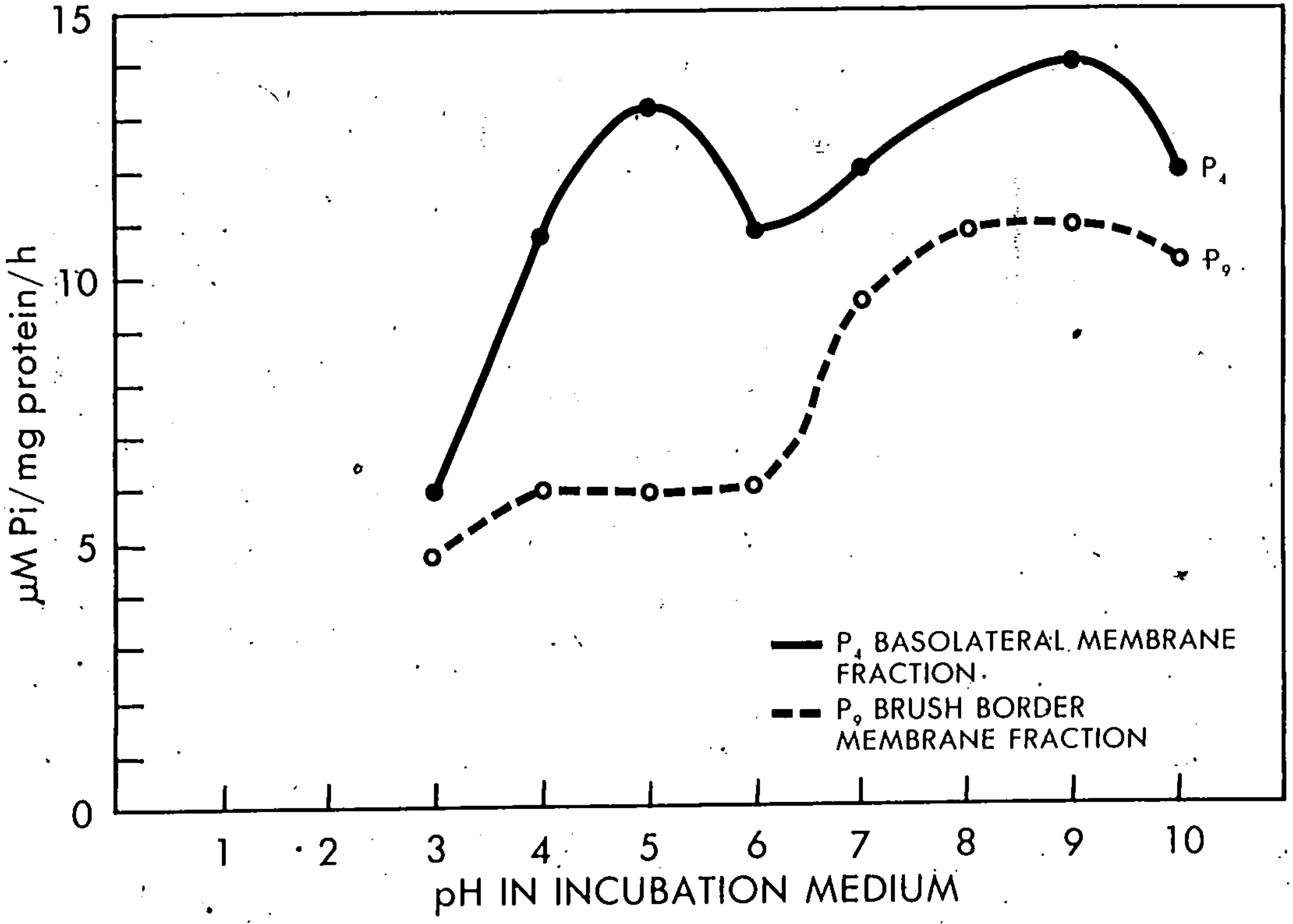
Graph 1- The effect of varying Ca^{+2} and Mg^{+2} concentrations on the Ca^{+2}, Mg^{+2}ATPase activity measured in the whole homogenate of a normal chick duodenum.

THE EFFECT OF VARYING [Ca^{+2}] ON Ca^{+2}, Mg^{+2} ATPase IN THE PRESENCE OF 0.5mM Mg^{+2}



Graph 2- The effect of varying Ca^{+2} concentrations on the activity of the Ca^{+2}, Mg^{+2}ATPases in the basolateral and the brush border membrane fraction from a normal chick duodenum.

THE EFFECT OF pH ON Ca^{+2}, Mg^{+2} ATPase ACTIVITY IN THE BASOLATERAL AND BRUSH BORDER MEMBRANE FRACTIONS



Graph 3- The effect of pH on the activity of Ca^{+2}, Mg^{+2}ATPases in the basolateral and brush border membrane fractions from a normal chick duodenum.

APPENDIX III

TABLE 1

Body weight and concentration of serum calcium in 4 weeks old normal and rachitic chicks.

	Normal n=6	Rachitic n=12
Body weight gm+S.E.M.	240±5	148±6
Serum calcium mg%+S.E.M.	9.1±0.3	7.2±0.4
		$p<0.005$

Each animal was weighed twice and calcium serum measurements were performed in duplicates. The average of these measurements were used in the calculations.

Values are expressed as the mean+standard error of the mean (S.E.M.). Significance of difference of the means of serum calcium concentrations was calculated according to Student's t-test.

TABLE 2

The effect of vitamin D-deficiency on ALK-Pase, Ca^{+2}-ATPase, Mg^{+2}-ATPase, Ca^{+2}, Mg^{+2}-ATPase, Na^{+}, Ca^{+2}-ATPase and Na^{+}, K^{+}-ATPase in whole homogenates of chick duodenum.

Enzyme	Ion concentrations	Enzyme activity in Normal	Rachitic chicks	
ALK-Pase	0.25mM Zn^{+2} 0.25mM Ca^{+2} 5mM Mg	7.58±1.51 1205.6±241.1	2.22±0.25 (1) 418.2±83.6 (2)	$p<.01$
ATPases				
	none	0.55±0.9 89.7±41.5	0.14±0.1 * 11.6±4.0 **	
	2mM Ca^{+2}	4.77±0.80 739.8±98.1	3.02±0.33 431.7±39.0	
	0.5mM Mg^{+2}	2.83±0.47 441.3±69.5	1.93±0.52 276.7±33.8	
	2mMCa^{+2} 0.5mM Mg^{+2}	7.89±1.30 1160.9±110.7	5.6±0.4 851.4±60.5	$p<0.05$
	100mM Na^{+} 2mM Ca^{+2}	5.46±0.91 841.8±81.9	3.52±0.29 542.0±64.2	$p<0.05$
	100mM Na^{+} 10mM K^{+} 5mM Mg^{+2}	5.84±1.09 1083.2±184.4	6.60±1.02 1050.2±187.7	$p\geq.05$

Enzymatic activities were measured in duplicate and the average was used in the calculations. In all cases n=6 for normal and n=12 for rachitic chicks except for ALK-Pase and Na^{+}, K^{+}-ATPase activities where n=5 for both normal and rachitic chicks.

The mean values ± standard error of the mean (S.E.M.) are given. Significance of differences between the means of normal and rachitic chicks were calculated according to Student's t test.

ALK-Pase activities are expressed as (1) nM of p-nitrophenol released per mg protein per 15 min (nM PNP/mg protein/15 min) and (2) nM of p-nitrophenol released per gr of wet tissue per 15 min (nM PNP/gr tissue/15 min).

ATP-ase activities are expressed as * uM of inorganic phosphate released per mg protein per h (uM P /mg protein/h) and as ** uM of inorganic phosphate released per gr of wet tissue per h (uM P /gr tissue/h).

TABLE 3

Ca^{+2}, Mg^{+2}-ATPase and Na^{+}, Ca^{+2}-ATPase activities in whole homogenate, supernatant and pellet obtained from normal and rachitic chick duodenum.

Enzyme	Homogenate	Supernatant	Pellet
Normal chicks n=6			
Ca^{+2}, Mg^{+2}-ATPase	7.89±1.3* 1160.9±110.7**	1.65±0.27 101.0±26.6	4.61±0.51 748.0±124.8
$p<0.05$			
Na^{+}, Ca^{+2}-ATPase	5.46±0.91 841.8±81.9	1.33±0.22 84.7±22.8	3.03±0.51 504.9±85.2
$p<0.05$			
Rachitic chicks n=12			
Ca^{+2}, Mg^{+2}-ATPase	5.6±0.40 851.4±60.5	0.34±0.02 26.6±6.4	4.93±0.33 706.2±61.7
$p\geq0.05$			
Na^{+}, Ca^{+2}-ATPase	3.52±0.29 542.4±64.2	0.4±.03 23.0±6.9	3.01±0.20 430.3±52.1
$p\geq0.05$			

ATPase activities are expressed in * uM of inorganic phosphate released per mg protein per hr (µM P_i/mg protein/h) and ** uM of inorganic phosphate released per gr wet tissue per h (µM P_i/gm tissue/h).

Mean values ± standard error of the mean (S.E.M.) are given. Significance of differences of the means between the activities in the homogenate and the pellet was calculated according to Student's t-test.

TABLE 4

Distribution of principal enzyme markers among the main fractions obtained by differential centrifugation of duodenal homogenates from 3-4 week old chicks. For details see Materials and Methods section 3.2.

Fraction	S_0	P_0	P_4	P_9
Total protein mg	29.2 30%	68.6 70%	17.1 18%	3.6 4%
ALK-Pase mM PNP/mg protein/h	1399 9.3%	13775 90.7%	6852 45.1%	12003 79.1%
Sucrase mM glucose/mg protein/h	4.2 19.6%	17.2 80.4%	7.4 34.4%	0.6 3%
Na^+, K^+ -ATPase mM Pi/mg protein/h	-	603.7 100%	205.2 34%	28.8 4.8%

S_0=initiial supernatant
P_0=initial pellet
P_4=basolateral membrane fraction
P_9=brush border membrane fraction.

Since the homogenate was very viscous and difficult to sample, the total initial quantities were taken as the sums of the amounts present in S_0 and P_0. All enzyme determinations were performed twice and the mean of the determinations are presented.

TABLE 5

Atomic absorption measurements of total calcium in the duodenal mucosa of fasted and non-fasted normal and rachitic chicks.

Feeding chicks	mMCa/l cell water *
normal n=6	2.99±0.29
rachitc n=8	7.19±1.07 p<.01
Fasted chicks	
normal n=6	2.78±0.34
rachitic n=8	2.51±0.26 p≥.05

Each determination was repeated twice and the average was used to calculate the mean of each group.

* The total calcium concentration in the duodenal mucosa is expressed as mM of calium per liter of cell water (mMCa/l cell water)

The mean values±standard error of the mean are given (S.E.M.). Significance of difference between the means of each group was calculated according to Student's t-test.

TABLE 6

Evaluation of paraformaldehyde and glutaraldehyde as fixatives for the localization of D-CaBP-I.

Fixative	D-CaBP-I localization at L.M. level in the chick duodenum
a)4% paraformaldehyde (PF) overnight	Inconsistent staining, in absorptive cells cytoplasm and brush border, goblet cells, and in lamina propria.
b)0.1% glutaraldehyde and PF 10min then PF overnight	Patchy and inconsistant staining as above.
c)0.1% glutaraldehyde with PF overnight	Same as above
d)0.5% glutaraldehyde 2h	Same as above

For details of fixatives see Materials and Methods section 7.5.

TABLE 7

Evaluation of acrolein as a fixative for the localization of D-CaBP-I.

Fixative	D-CaBP-I localization in the chick duodenum
e) 10% acrolein	
10min	Consistent staining of the nucleus and cytoplasm of absorptive cells. Brush borders and goblet cells are negative. Some leaching.
20min	Very consistent, comparable to freeze substituted material.
30min	Consistent as above but decreased immunoreactivity
f) 20% acrolein	Consistent as above but decreased immunoreactivity.
g) 2% acrolein (distilled)	
1h	Consistent staining as above but only at the tip of villi. Reduced immunoreactivity along the sides of villi.
2h	As above but even more reduced immunoreactivity.
h) 3% acrolein (distilled)	
20min	Consistent staining but only at very tip of villi.
30-45-60min	Very little immunoreactivity left.

Table 7

i) 5% acrolein (distilled)

5min	Consistent and homogeneous staining of absorptive cells but only at tip of villi.
10min	Consistent and homogeneous staining of absorptive cells throughout villi. Comparable to freeze substituted material.
15min	Consistent as above but decreased immunoreactivity.
20min	Even more decreased immunoreactivity.

TABLE 8

Acrolein in combination with other aldehydes.

Fixative	D-CaBP-I localization in chick duodenum.
j) 10% acrolein 20min then 4% glutaraldehyde	Inconsistent staining, leaching and decreased immunoreactivity.
k) 10% acrolein with 4% glutaraldehyde for 20 min	Inconsistent leaching and decreased immunoreactivity
l) 10% acrolein with 4%PF for 4h	Consistent only at very tip of villi
m) 10% acrolein then 4%PF for 4h	Consistent only at very tip of villi
n) 10% acrolein in 4%PF for 20min followed by 4%PF	Consistent at tips of villi. Best results amongst combination trials but inferior to acrolein alone
o) 2% acrolein (distilled) with 0.25% glutaraldehyde for 2 1/2 h	Improved cytology but decreased immunoreactivity as in g

For details of fixatives see Materials and Methods section 7.5.

TABLE 9

Staining of rat and chick cerebellum and duodenum with antisera against chick duodenal and human cerebellar D-CaBP-I and rat duodenal D-CaBP-II.

Antiserum against D-CaBP from:	tissues: cerebellum chick	cerebellum rat	duodenum chick	duodenum rat
Chick duodenum	+	+	+	-
Human cerebellum	+	+	+	-
rat duodenum	-	-	-	+

- indicates no staining

+ in the cerebellum the cytoplasm of Purkinje cells is positive and in the duodenum the cytoplasm of enterocytes is positive.

TABLE 10

The effect of vitamin D-deficiency on antibody production against sheep red blood cells in the chick.

Diet	Serum Calcium mg%	Antibody titers
normal n=6	10.61±0.94	21.73±5.26
Rachitic with calcium supplement n=7	10.40±1.49	7.71±1.70
Rachitic n=11	7.35±1.29	9.81±1.73

Antibody titers are expressed as the $\log_2$ of the reciprocal value of the highest serum dilution resulting in agglutination. For details see Materials and Methods section 8.

Values are expressed as the mean ± S.E.M. Significance of differences between the means were calculated using Student's t-test.

TABLE 11

Summary of the immunocytochemical localization of D-CaBP-I in chick and rat tissues and of D-CaBP-II in rat tissues.

Tissues	D-CaBP-I		D-CaBP-II
	chick	rat	rat
Duodenum			
enterocytes	+	-	+
parasympathetic neurons		+	-
neurons			
Kidney			
pronephros	-		
mesonephros			
mesonephric duct	+		
midsegment	+		
metanephros			
glomerulus	-	-	-
PCT	-	-	-
TDL	-	-	-
TAL	-	-	+
PCT	-	-	-
DCT.	+	+	+
macula densa	-	-	+
collecting tubule	-	+	+
collecting duct	-	-	+
Tooth			
odontoblast		-	-
ameloblast		+	-
dental pulp		-	-
Salivary glands			
acini		-	-
ducts		-	+
Neurons			
Purkinje cells	+	+	-
Spinal cord neurons	+		
Spinal ganglion	-		
Ganglia nodosum	+		
Certain retinal neurons	+	+	-
Stomach enterochromaffin cells	+	+	-

Adrenal gland			
cortical cells	+	-	+
medullary cells	-	-	-
Thyroid gland	-	-	-
Parathyroid gland	-	-	-
Ovary			
prefollicular cells	+		
germinal epithelium	+		
granulosa and theca cells	-	-	+
Skin			
keratin layer	+	-	+
granulosa layer	+	-	+
Lympoid organs			
thymic cortical ERC	+		
Hassal's corpuscles	+		
splenic reticular cells	+		
bursal reticular cells	+		
Muscle			
Skeletal	-	-	-
cardiac	-	-	-
smooth	-	-	-
Liver	-	-	-

+ positive intracellular staining
- no staining detected

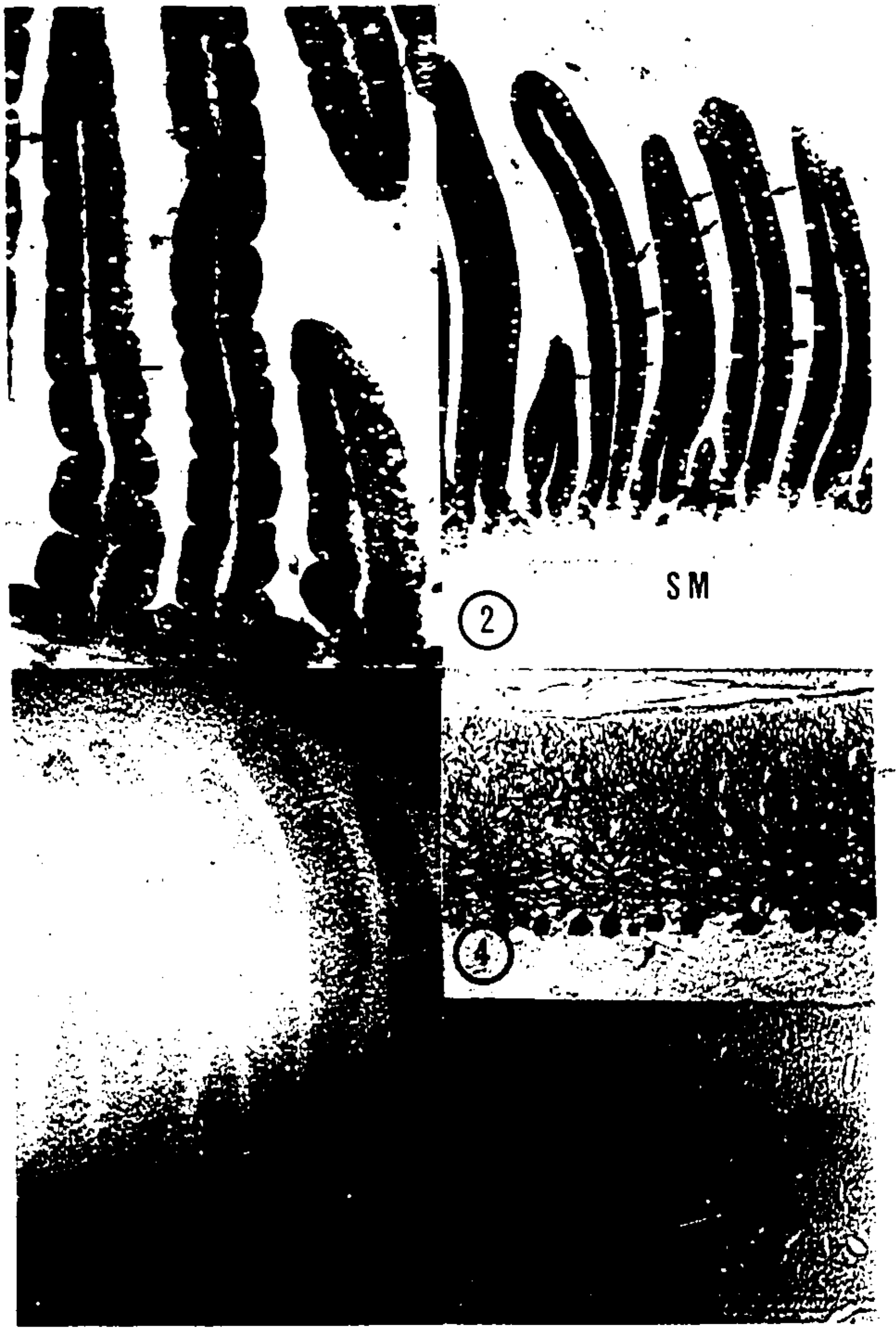

SM
②
④

Fig. 1 -A section of chick-duodenum fixed in 0.5% glutaraldehyde according to Thorens et al (1982). Note the uneven distribution of reaction product in enterocytes along the villi and also the occasional positive goblet cells (arrows). X100.

Fig. 2 - A section of chick duodenum fixed for 20 minutes in 10% acrolein (Kodak). Note the overall good fixation of the tissue. The dark brown reaction product is present in all the absorptive cells of the villi. The goblet cells (thin arrow), the lamina propria (thich arrow) and the smooth muscle (SM) of the duodenal wall are completely negative. X85.

Fig. 3 - A control section of the same duodenum as above stained with non-immune rabbit serum. X85.

Fig. 4 - A sagittal section through the chick cerebellum fixed for 20 minutes in 10% acrolein (Kodak). Note that the entire body of the Purkinje cells, the dendrites and the axons (arrow) show intense staining with the reaction product. X170.

Fig. 5 - A control section of chick cerebellum fixed as in Fig. 4 but stained with non-immune rabbit serum. No reaction product is seen in the Purkinje cell bodies (arrows), axons and dendrites. X170.

S

Fig. 6 - A section of chick cerebellar cortex showing parts of two Purkinje cells (PC). The section is from the surface of the block and thus empty spaces (S) are present. Note the large amount of reaction product in the PC bodies and their dendrites (D). The nucleus is also darkly stained. Another neuron in the molecular layer (*) is completely negative. X7,880.


7

Fig. 7 - Part of a Purkinje cell showing dark reaction product (*) in between the various organelles such as mitochondria and endoplasmic reticulum (hollow arrows). Note the adsorption of reaction product on the outer membrane of the mitochondria (thin arrows). X62,700.

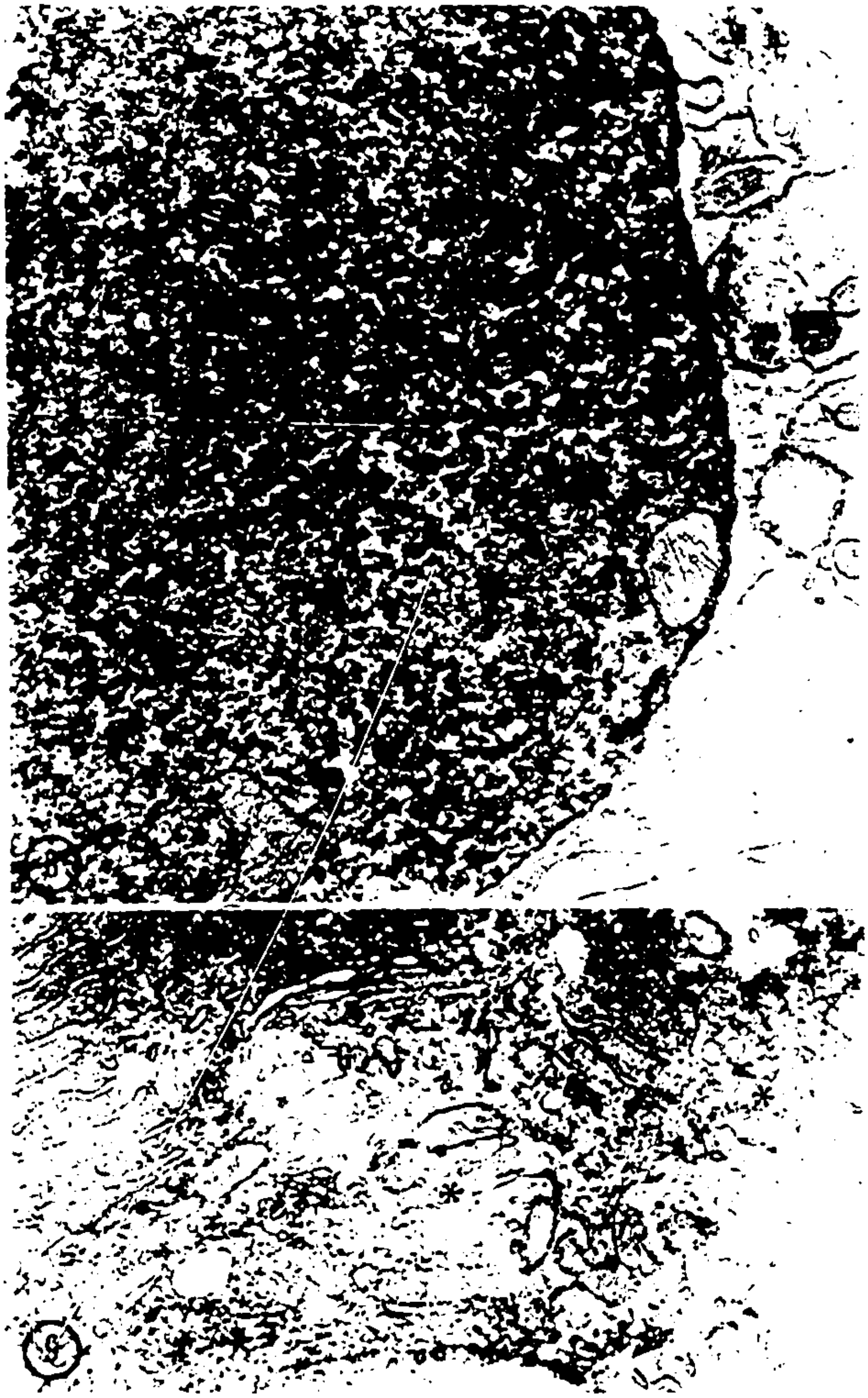
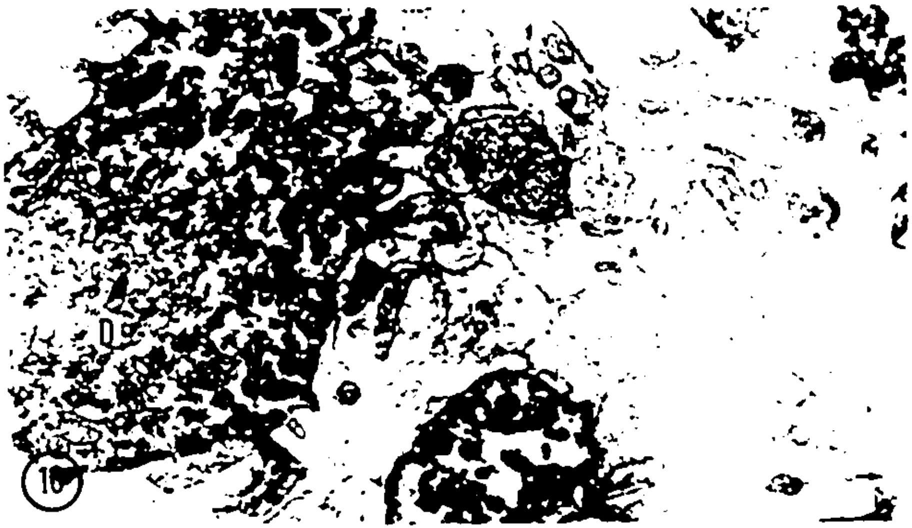


Fig. 8 - Medium power electron micrograph of a Purkinje cell and some of the adjacent tissue. Notice the overall dark color of the Purkinje cell because of the cytosolic distribution of the reaction product. There was no reaction product in the lumen of the rough surfaced endoplasmic reticulum (thin arrow). X25,650.

Fig. 9 - Part of a Purkinje cell body showing a Golgi region (G). The reaction product is present (*) in the cytoplasm surrounding the Golgi region but not inside this organelle. X25,650.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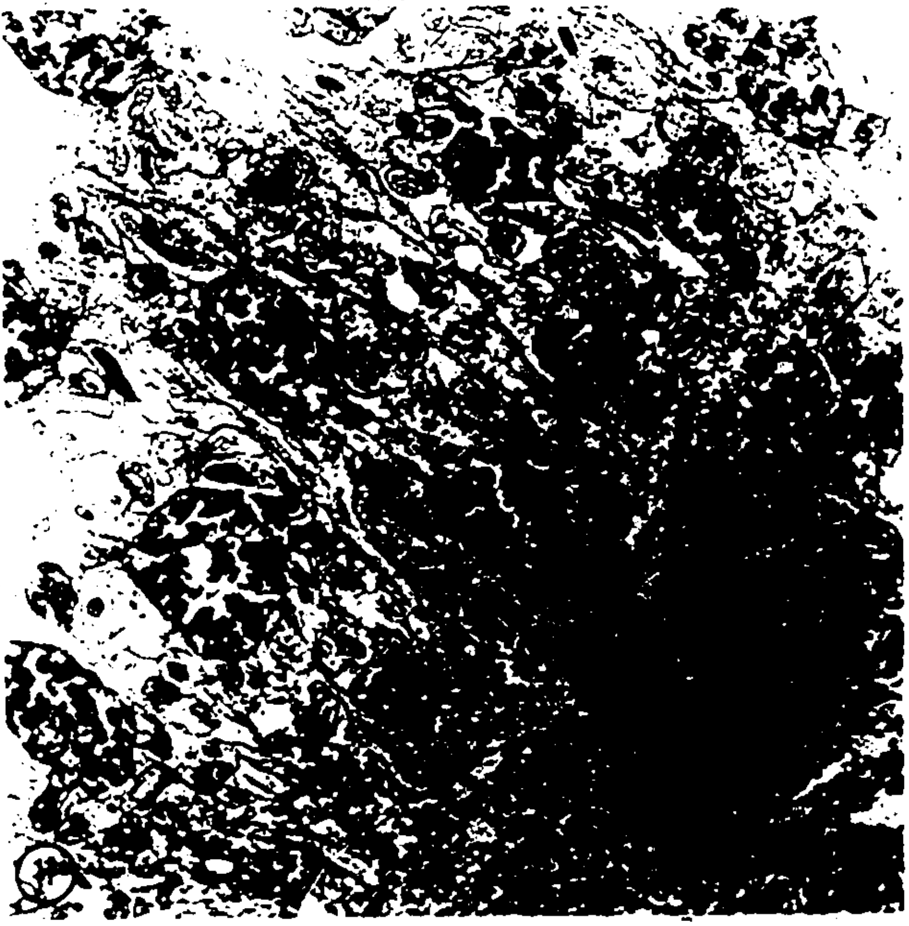

Fig. 10 - A section cutting the Purkinje cell dendrites (D) in cross section. The reaction product is seen in the entire dendrite section. The distribution of the reaction product is similar to that in the soma. Some of the dendritic spines (thin arrow) and Purkinje cell axons (A) are labelled. X16,200.

Fig. 11 - A section cutting the Purkinje cell dendrites (D) in cross section. Notice the reactivity in the dendrites and its spines (thin arrow). A positive axon (A) is also present. X9,120.

Fig. 12 - A section of an axon and a dendrite. In the axon, the reaction product is adsorbed to the plasma membrane (thin arrow), microtubules (hollow arrows) and synaptic vesicles. In the dendrite the distribution is similar to that previously described and the synaptic densities are also positive. X41,000.

Fig. 13 - A dendritic spine at higher magnification. The reaction product is seen adsorbed to the cytoplasmic surfaces of various organelles. Note the reaction product associated with the synaptic density. X50,000.

Fig. 14 - An axon in the deep cerebellar nucleus. The myelin sheath (M) is negative and the distribution of reaction product is similar to that described in other axons. X7,260.

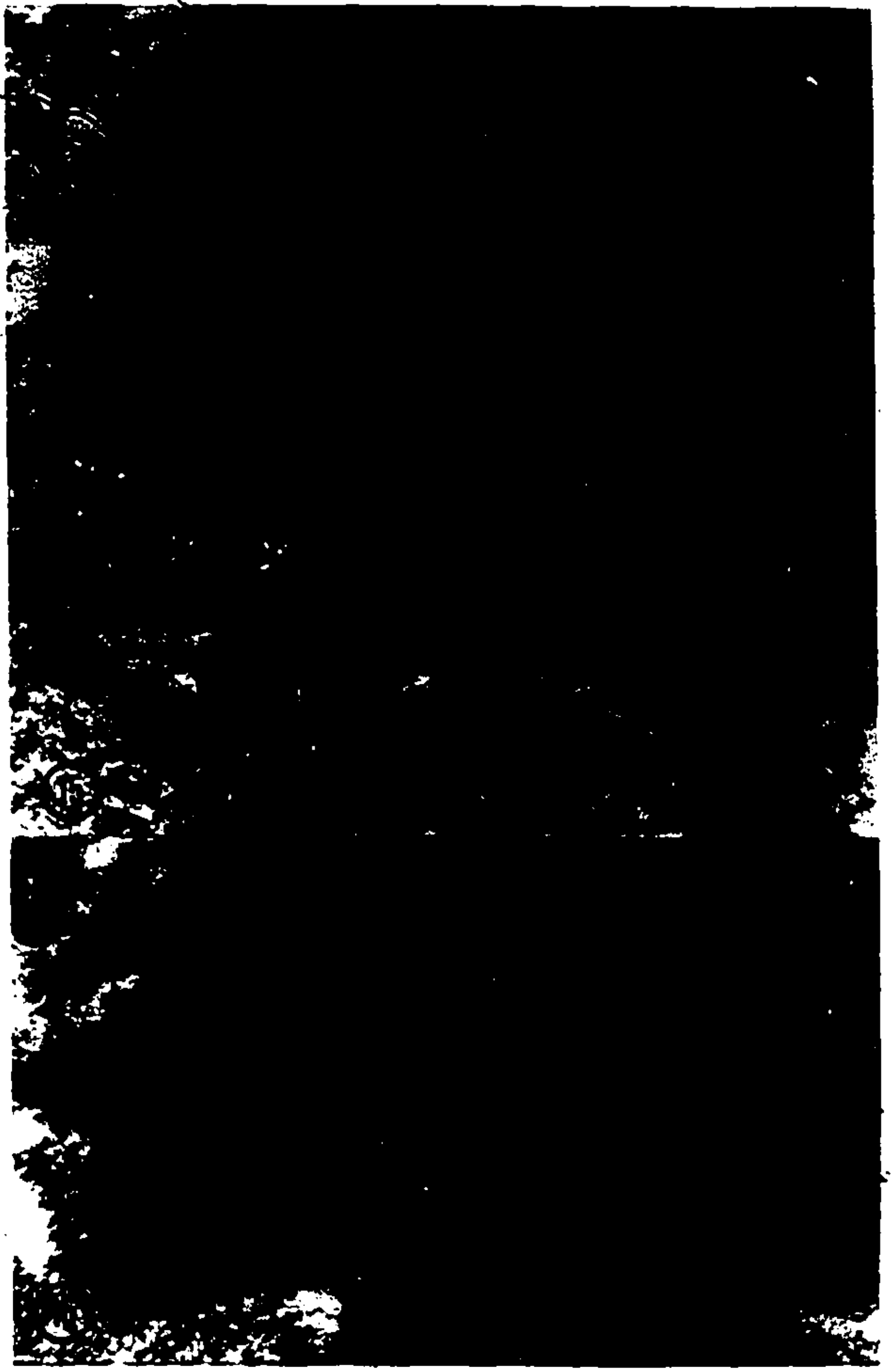

Fig. 15 - A dendrite surrounded by dendritic spines from material fixed in 0.1% glutaraldehyde with 4% paraformaldehyde. Note that dendritic spines contained more reaction product than those in Fig. 10 and 11 which are from material fixed with acrolein. X5,590.

Fig. 16 - A portion of a section of a Purkinje cell dendrite from material fixed with 4% paraformaldehyde. The cytology is poor and immunoreactivity in the Purkinje cell soma is much decreased. However reaction product is still associated with the synaptic densities (arrows) of the dendritic spines. X14,520.

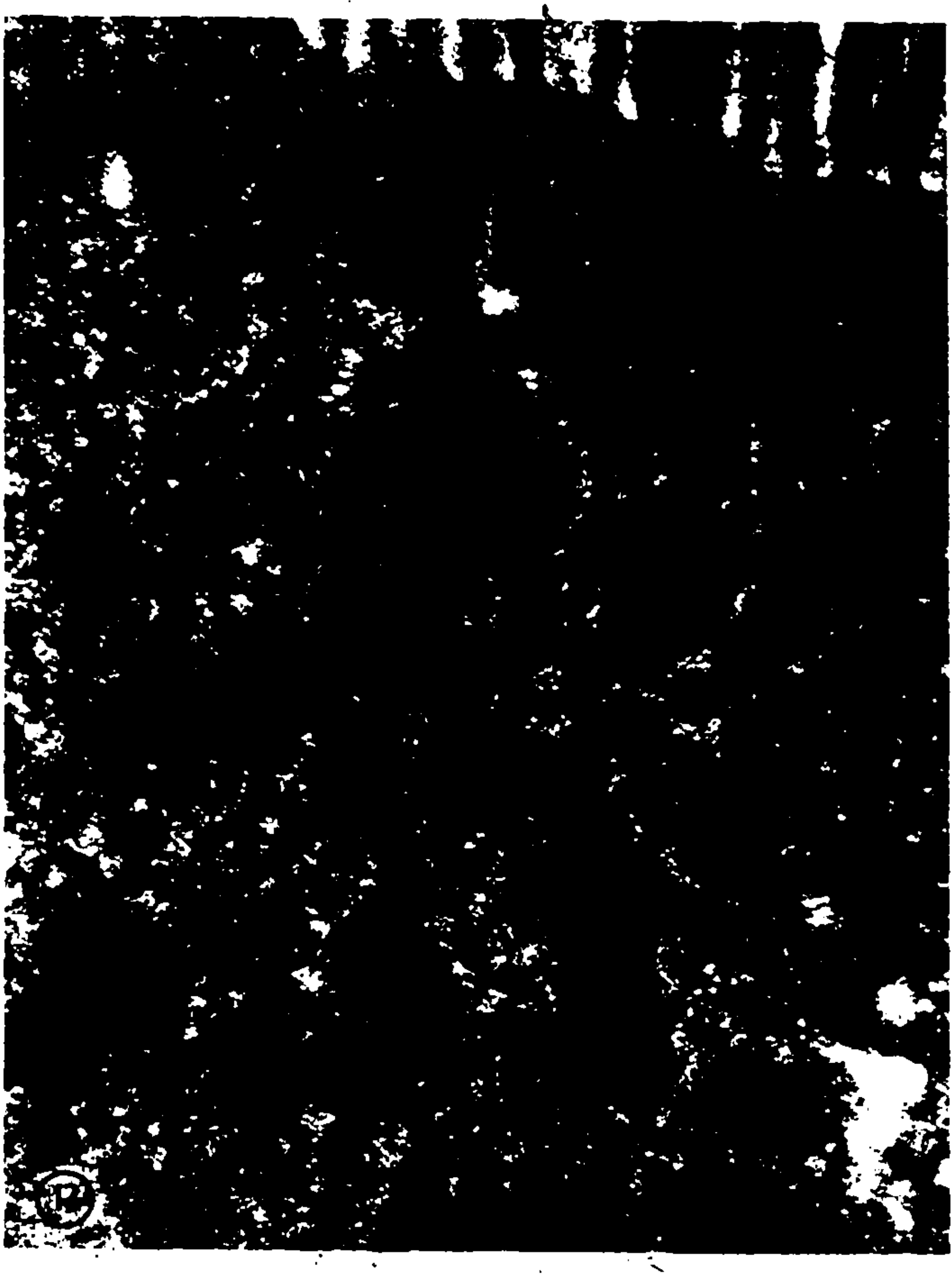

Fig. 17 - A portion of a section of chick duodenum fixed with 5% acrolein (distilled, Polysciences) then embedded in Lowycril K4M. D-CaBP-I was localized with the colloidal gold method. Colloidal gold indicating the presence of immunoreactivity against D-CaBP-I is present throughout the absorptive cell cytoplasm. Colloidal gold particles are associated with denser staining areas of the cytoplasm and only background levels are present in the mithochondrial matrix (M) and brush border region (BB). The junctional complex constituted of : the zonula occludens (ZO), the zonula adherens (ZA) and a series of desmosomes (D) is negative. X14,520.

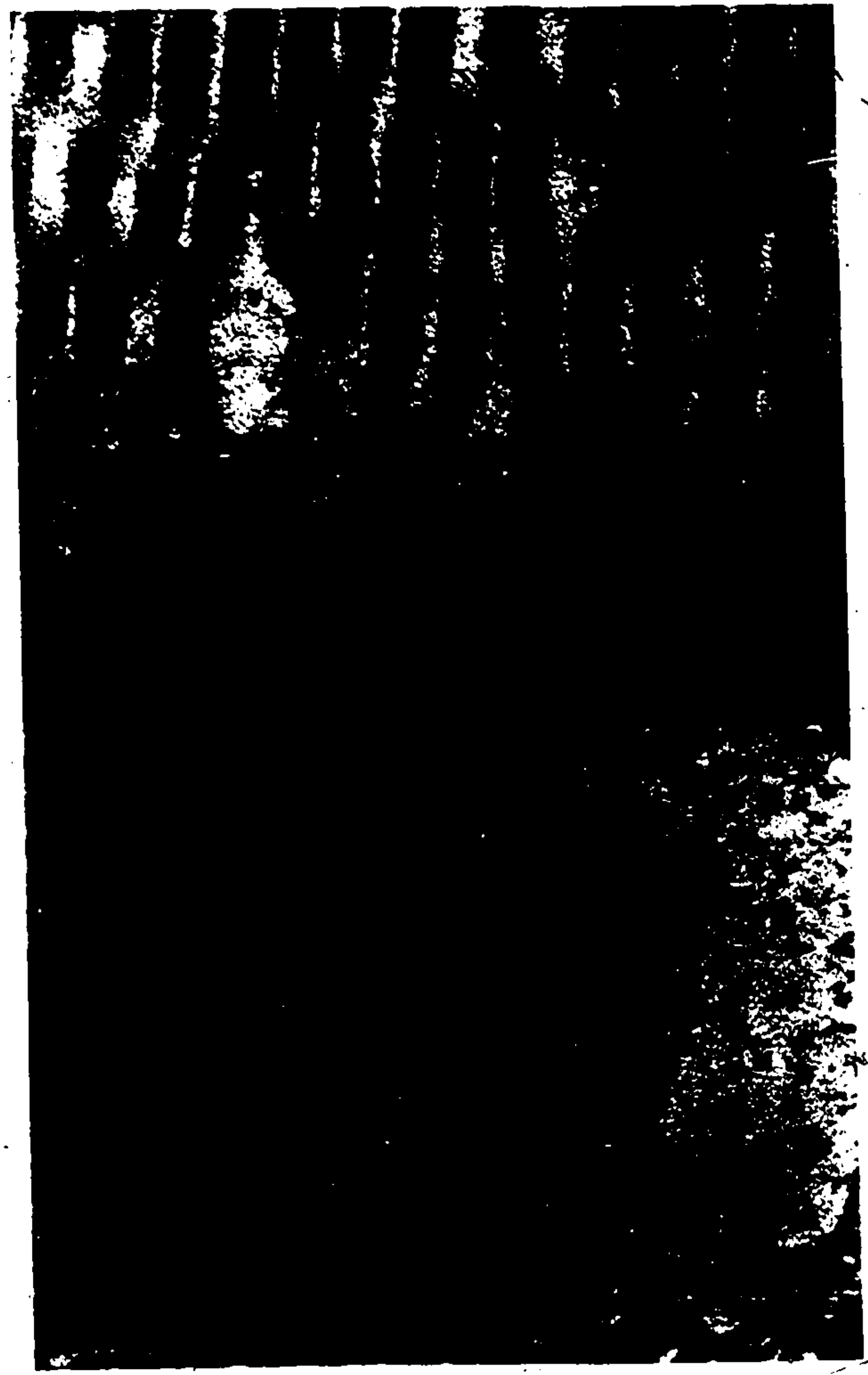

Fig. 18 - High magnification of the brush border region of a chick duodenal enterocyte stained as described in Fig. 17. Only background staining is present in the microvilli (MV). The microfilaments (arrows) in the terminal web were also negative. X23,100.

Fig. 19 - Portion of a positive enterocyte next to a goblet cell stained as described in Fig. 17. Note that only background staining is present in the goblet cell mucous (GM). X9,075.

Fig. 20 - Portion of an enterocyte stained as in Fig. 17. Colloidal gold is present in the cytoplasm. There is no colloidal gold in the rough endoplasmic reticulum cisternae (arrows) and in the mitochondrial matrix (M). In the nucleus the euchromatin is positive. The nucleolus (NU), heterochromatin and nuclear envelope (NE) are negative. X18,480.

Fig. 21 - The basal portion of an enterocyte. The heavily labelled cytoplasm of the enterocyte is highly contrasted with the lamina propria (LP) where only background level of staining is present. X18,050.

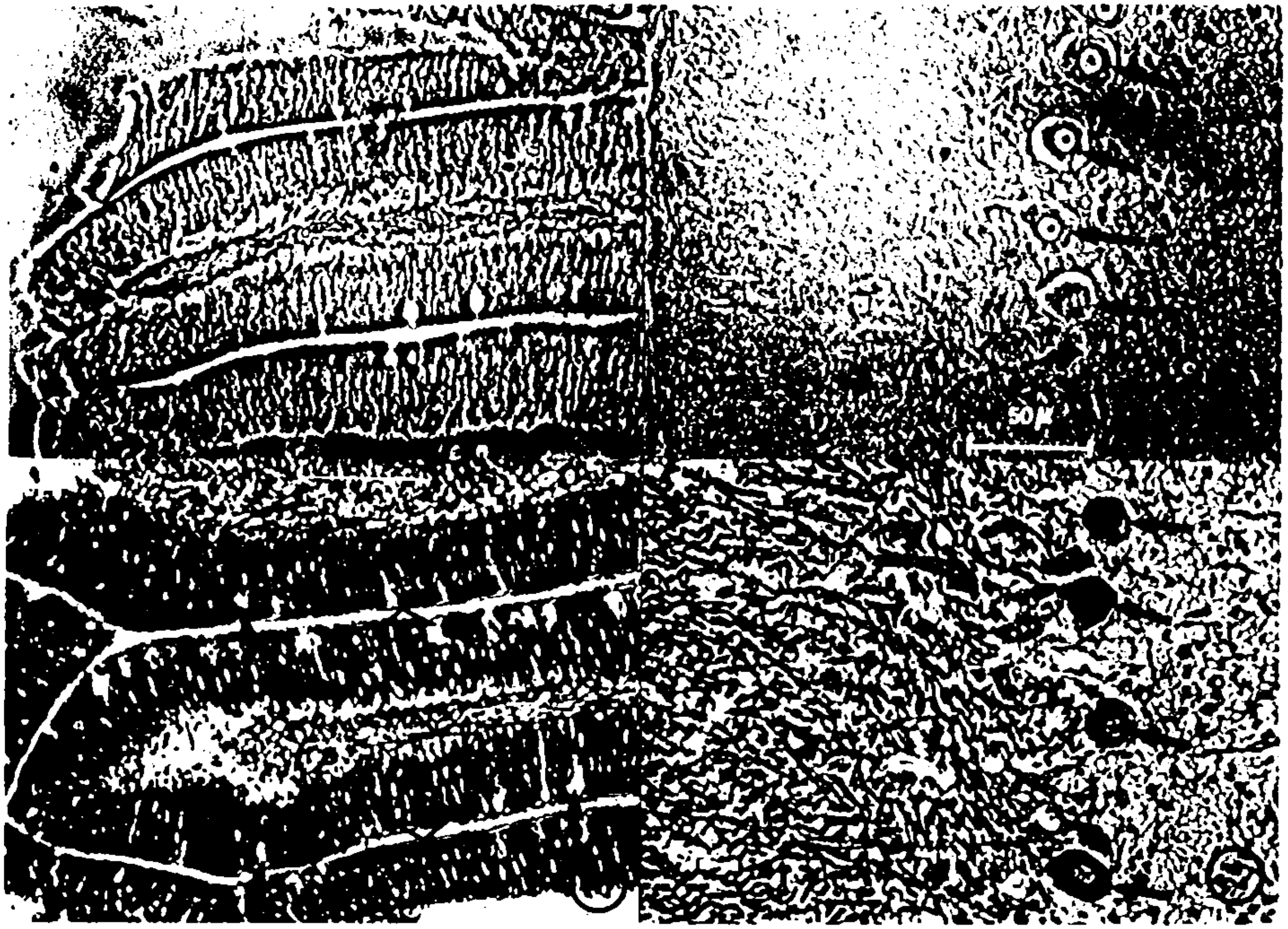
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Fig. 22 - A phase contrast picture of chick duodenum stained with anti-chick D-CaBP-I by the PAP method. The cytoplasm and nucleus of the enterocytes are positive. The brush border (hollow arrows) and goblet cells (full arrows) are negative. X250.

Fig. 23 - A phase contrast picture of the same duodenum as above but stained with anti-rat D-CaBP-II. All stuctures described above are negative. X250.

Fig. 24 - A phase contrast picture of chick cerebellum stained with anti-chick D-CaBP-I by the PAP method. Reaction product is present in the Purkinje cells (arrows) soma, dendritic tree and axon. X250.

Fig. 25 - A phase contrast picture of the same cerebellum as above but stained with anti-rat D-CaBP-II. The Purkinje cells (arrows) are all negative. X250.

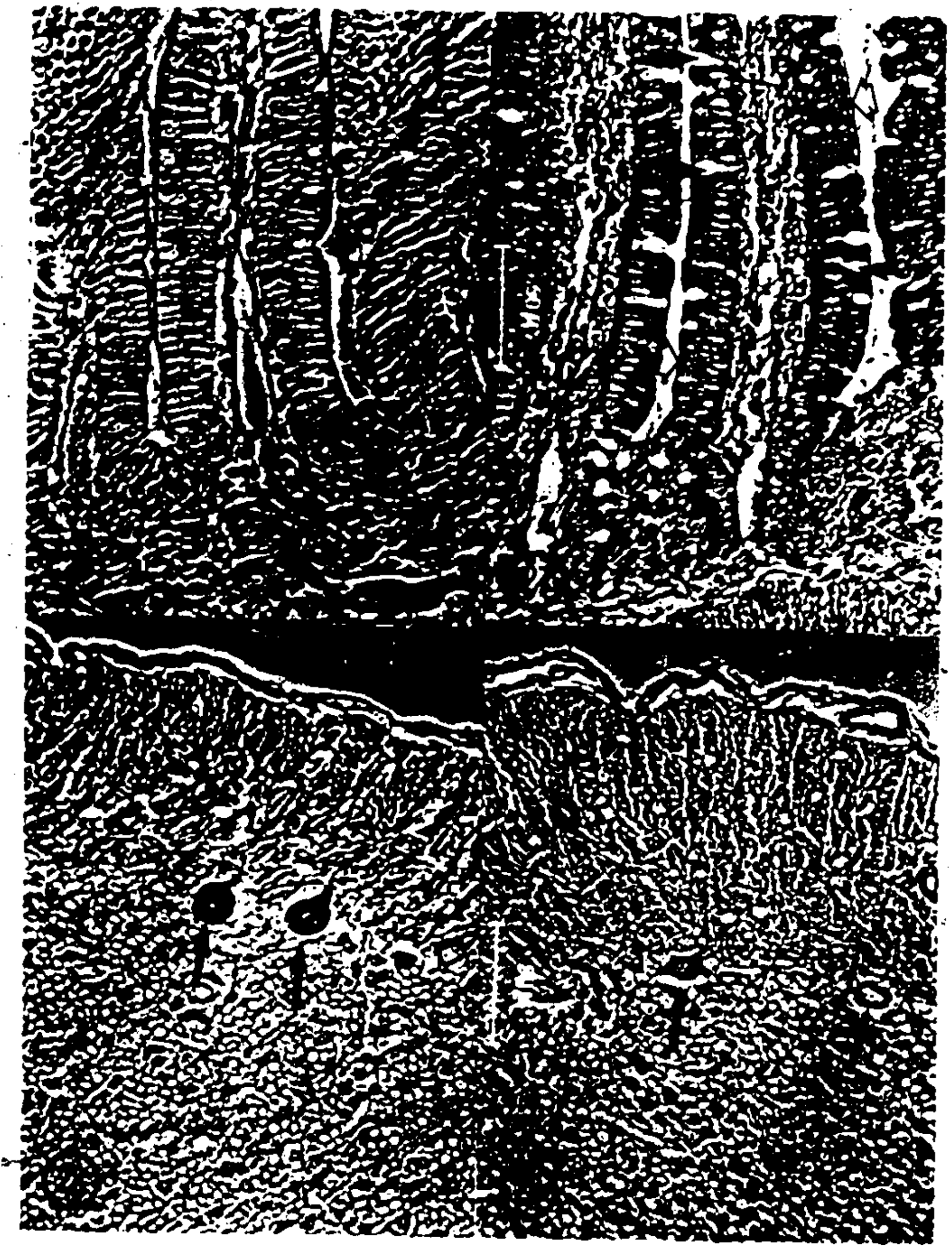

Fig. 26 - A phase contrast picture of a portion of rat duodenum stained with anti-chick D-CaBP-I by the PAP method. The enterocytes and goblet cells are all negative. X250.

Fig. 27 - A phase contrast picture of the same rat duodenum as above but stained with anti-rat D-CaBP-II. The enterocytes are positive. The brush border (hollow arrows) and goblet cells (full arrows) are all negative. X250.

Fig. 28 - A phase contrast picture of a portion of rat cerebellum stained with anti-chick D-CaBP-I by the PAP method. The Purkinje cells (arrows) are positive. X250.

Fig. 29 - A phase contrast picture of the same rat cerebellum as above but stained with anti-rat D-CaBP-II. The Purkinje cells (arrows) are negative. X250.

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Fig. 30 - A portion of a cross section of a 2 day old chick embryo stained with anti-chick D-CaBP-I by the PAP method and counterstained with H and E. The primary nephric duct (PND) is completely negative as well as the developing mesonephric tubule (MT). Also shown is the dorsal aorta (DA) and posterior cardinal vein (PCV) . X720.

Fig. 31 - A portion of a cross section of a 5 day old chick embryo stained as above. The cells of the mesonephric duct (MD) and connecting segments (CS) are heavily labelled. In the mid segment (MiS) scattered cells are positive. The section of the mesonephric duct in contact with the tubal ridge (TR) is negative. The developing glomeruli (G) and main segment (MS) are also negative. X720.

Fig. 32 - A portion of a 14 day old chick mesonephros stained with anti-D-CaBP-I and counterstained with PAS. The brush border of the cells of the main segment (MS) is well developed. These cells along with the glomerulus (G) are negative. The darkly positive cells belong to the midsegment. X720.

Fig. 33 - Another portion of the same section described above showing that the section of the mesonephric duct (MD) which is in contact with the tubal ridge (TR) remained negative throughout the development of the mesonephros. X720.

36

37

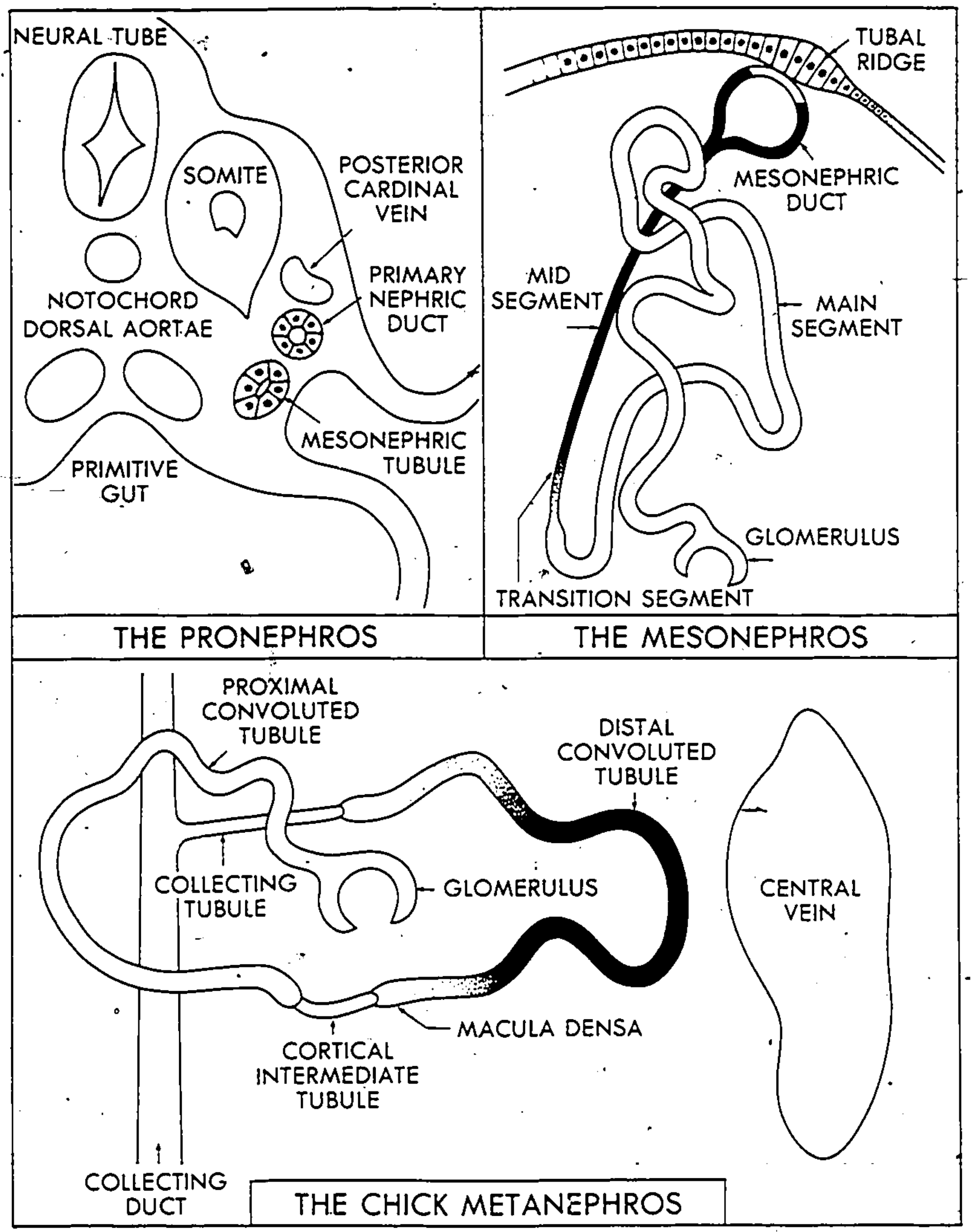
Fig. 34 - Portion of a section of a 10 day old embryonic metanephros stained with anti-chick D-CaBP-I with the PAP method and counterstained with H and E. The distal convoluted tubules (DCT) which are developing nearby a central vein (CV) are heavily labelled. X720.

Fig. 35 - Portion of a section of a 12 day old embryonic metanephros stained as above. The proximal convoluted tubules (PCT) are negative. The number of positive distal convoluted tubules (DCT) surrounding central veins (CV) is rapidly increasing. X720.

Fig. 36 - Portion of a section of the kidney cortex of a 4 week old chick stained with anti-chick D-CaBP-I by the PAP method and counterstained with PAS. The proximal convoluted tubules (PCT), macula densa (MD) and glomeruli (G) are negative. The reaction product is seen inside the cells of the distal convoluted tubules (DCT) which surround the central vein. X250.

Fig. 37 - Portion of the same kidney section as in Fig. 36 showing the medulla. The medullary collecting ducts (MCD) and the remaining thick and thin segments of the medullary loops are all negative. X250.

LOCALIZATION OF D-CaBP-I IN THE CHICK PRONEPHROS, MESONEPHROS AND METANEPHROS

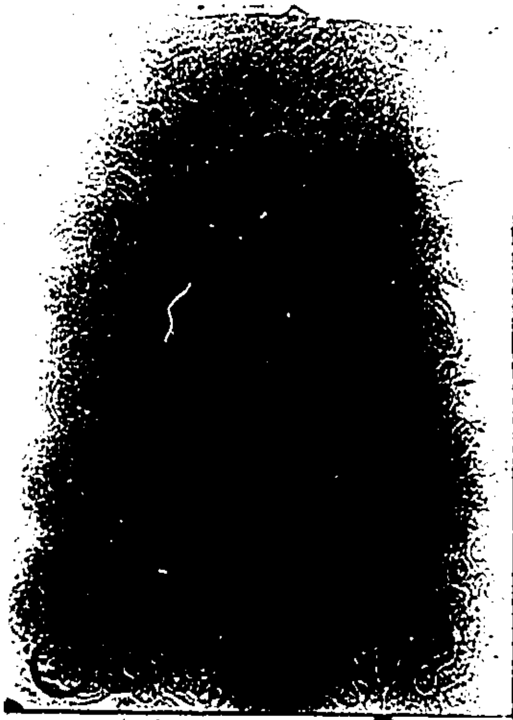


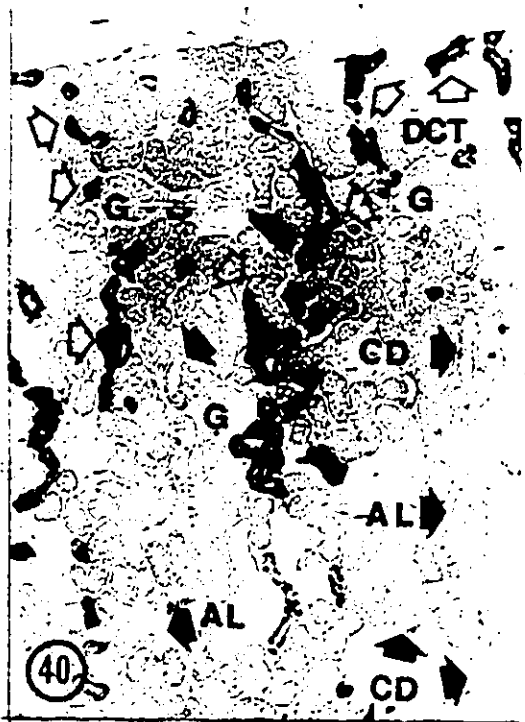
THE PRONEPHROS

THE MESONEPHROS

THE CHICK METANEPHROS

Fig. 38 = Schematic representation of D-CaBP-I staining in the chick embryonic and adult kidney. Isolated positive cells are represented by stipled areas whereas regions where all the cells along the tubules are positive are indicated in black.





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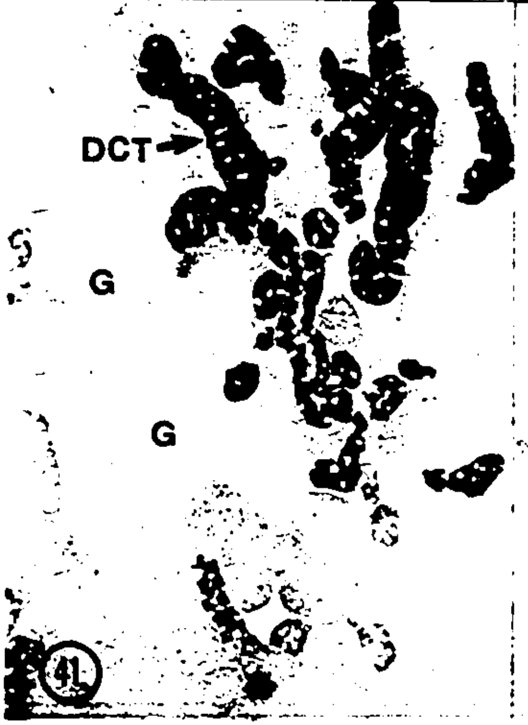

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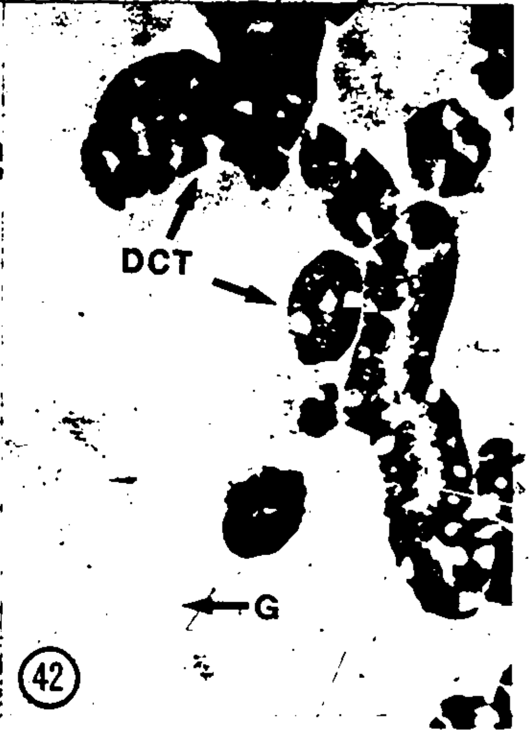

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Fig. 39 - Control section of rat kidney reacted with non-immune rabbit serum instead of anti-D-CaBP-I using the short indirect method (SIM). All the components of the kidney including the glomeruli (G) and collecting ducts (CD) are negative. X68.

Fig. 40 - Section of rat kidney adjacent to that of Fig. 39 stained with anti-chick D-CaBP-I by SIM. Positive cells of the distal convoluted tubules (DCT) are indicated by hollow arrows. The glomeruli (G) the ascending limbs of Henle's loop (AL) and the collecting ducts (CD) are negative and are indicated by solid arrows. X68.

Fig. 41 - Portion of rat renal cortex stained with anti-chick D-CaBP-I by the PAP method. The glomeruli (G) and surrounding proximal convoluted tubules are negative. The dark brown DAB deposits are seen inside the cells of the distal convoluted tubules (DCT). X140.

Fig. 42 - Higher magnification of a part of Fig. 41, which shows the intracellular distribution of the reaction product in the cells of the distal convoluted tubules (DCT). The basal regions (arrows) stained more lightly than the apical region of the cells. X360.

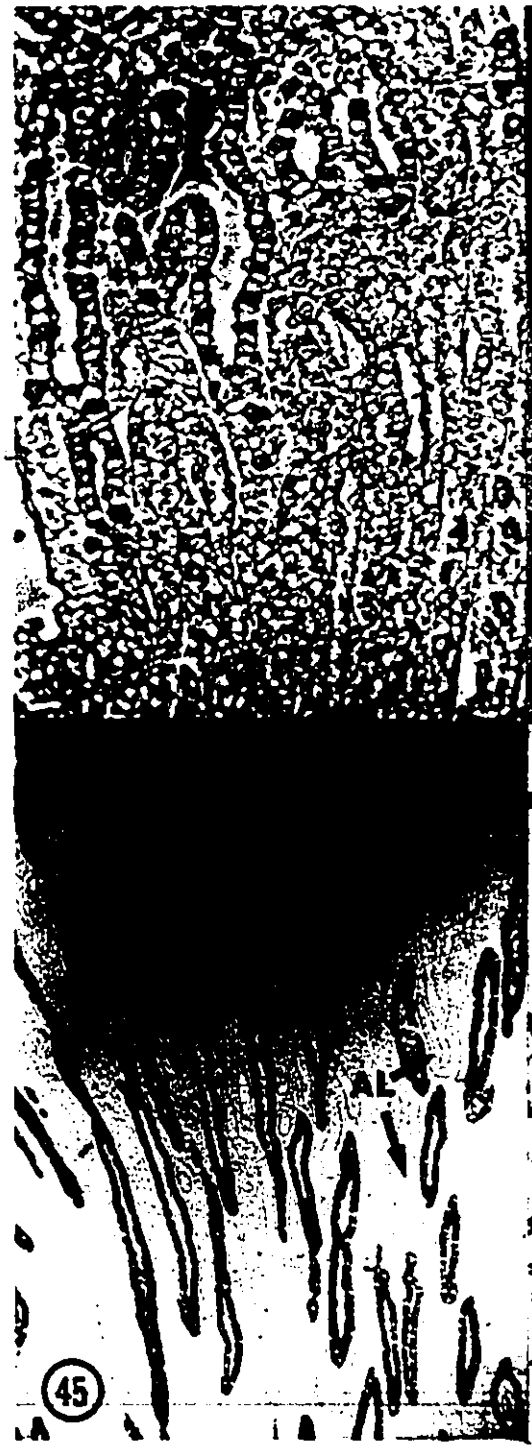

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Fig. 43 - Phase contrast picture of a portion of a 1 week old rat pup kidney stained with anti-chick D-CaBP-I by the PAP method. Interspersed cells along the collecting ducts (CD) are positive. X250.

Fig. 44 - Section of monkey renal medulla stained with anti-human D-CaBP-I. Note that the reaction product is distributed in all the cells of the collecting duct (CD) to the very tip of the papillary ducts (PD). X11.

Fig. 45 - Higher magnification of a portion of the monkey renal medulla seen in Fig. 44. The dense reaction product is found inside the cells of the collecting ducts (CD). The ascending thick limb of the loops of Henle (AL) are negative. X68.

Fig. 46 - Section of rat renal cortex stained with anti-rat D-CaBP-II using SIM. Compare with Fig. 10, which shows an adjacent section stained with D-CaBP-I. Hollow arrows indicate sites along the distal convoluted tubules (DCT) positive for D-CaBP-II. In Fig. 40 these same tubules are positive for D-CaBP-I. Solid arrows indicate tubular segments in the collecting ducts (CD) and the ascending thick limb of the loop of Henle (AL), all the cells are positive for D-CaBP-II. The glomeruli remain negative. X68.

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Fig. 47 - Rat renal subcortex and outer medulla stained with anti-rat D-CaBP-II using the PAP method. Reaction product is seen in the cells of the thick ascending limb of the loops of Henle (AL) and the cells of the collecting ducts (CD). X68.

Fig. 48 - Rat renal subcortex and outer medulla stained with anti-rat D-CaBP-II using SIM. D-CaBP-II , unlike D-CaBP-I is found in all the cells of the collecting ducts (CD), and the distal tubule is positive even before it becomes convoluted. X68.

Fig. 49 - High magnification of a glomerulus with its surrounding tubules in rat renal cortex stained with anti-rat D-CaBP-II by the PAP method. The dense reaction product is concentrated in the apical cytoplasm of the cells of the distal convoluted tubules (DCT). Compare with Fig. 42, which shows the intracellular distribution of D-CaBP-I. X360.

Fig. 50 - Portion of rat kidney cortex stained with anti-rat D-CaBP-II with the PAP method and counterstained by PAS-hematoxylin. The brush border of the proximal convoluted tubules (PCT) is evident. Reaction product is present in the cells of the distal convoluted tubules (DCT). Cells of the macula densa (MD) show a faint staining in their apical cytoplasm. X275.

LOCALIZATION OF D-CaBPs IN THE VERTEBRATE KIDNEY

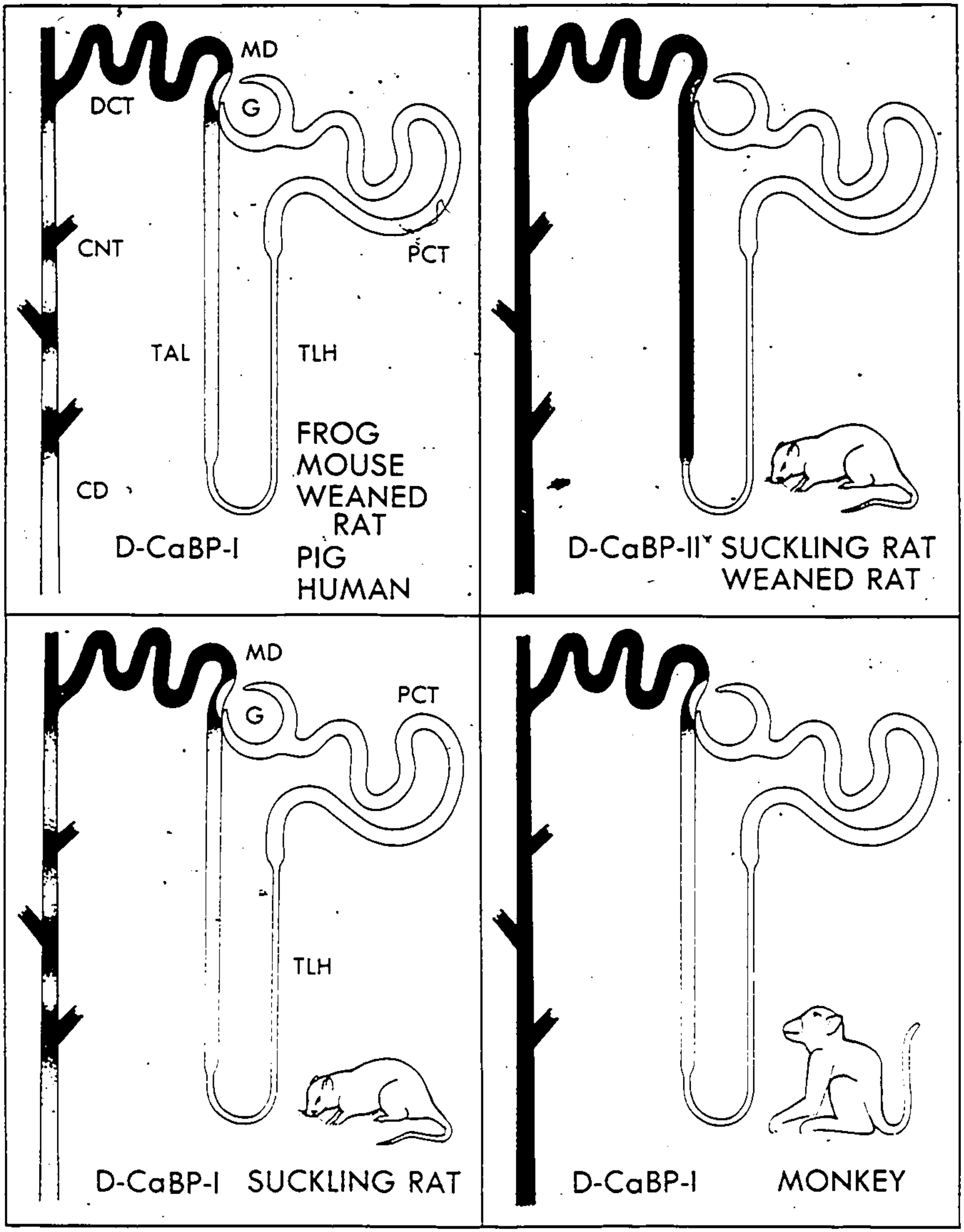


Fig. 51 - Schematic representation of the localization of D-CaBP-I in the nephrons of the rat, mouse, frog, pig, monkey and human and of D-CaBP-II in the rat nephron. Interspersed positive cells are indicated by stippled areas whereas regions where all the cells along the tubules are positive are indicated by solid areas. Abbrevations are: (G) glomerulus, (MD) macula densa, (PCT) proximal convolted tubules, (TLH) thin limb of the loop of Henle, (TAL) thick ascending limb, (DCT) distal convoluted tubule, (CNT) connecting tubule, (CD) collecting duct.

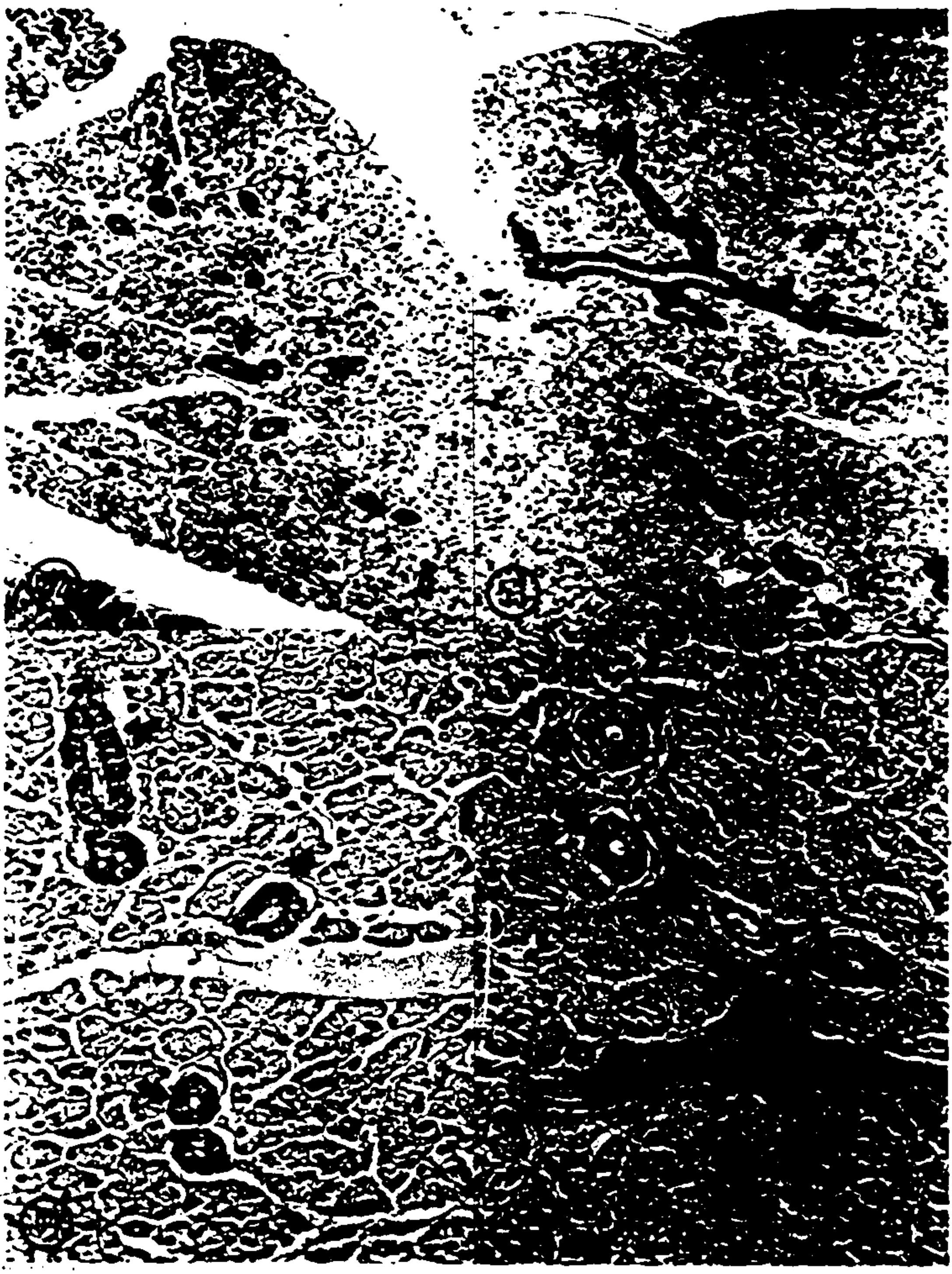
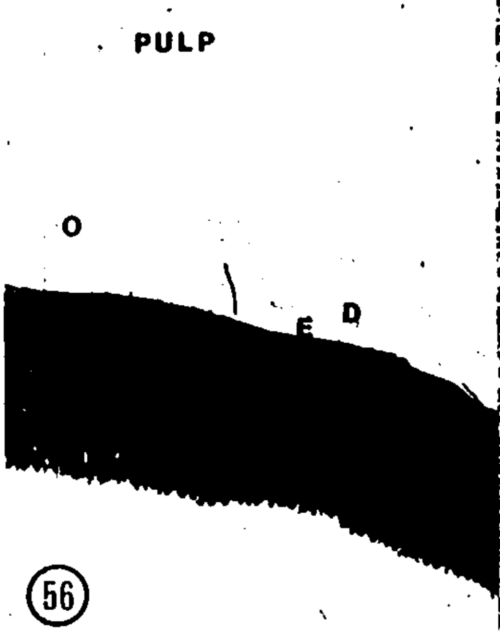


Fig. 52 - A portion of a section of the parotid of a 1 week old suckling rat stained with anti-rat D-CaBP-II by the PAP method and counterstained with H and E. The ductal cells (arrows) contain reaction product in the cytoplasm and nucleus. The acini are negative. X100.

Fig. 53 - A portion of a section of the submaxillary of a 1 week old suckling rat stained with anti-rat D-CaBP-II by the PAP method and counterstained with H and E. The ductal cells are positive (arrows). X100.

Fig. 54 - Phase contrast picture of a higher magnification of a rat parotid gland stained with anti-rat D-CaBP-II by the PAP method. The intralobular ductal cells are positive (arrows). X250.

Fig. 55 - Phase contrast picture of a section adjacent to that of Fig. 54 stained with anti-chick D-CaBP-I by the PAP method. The ductal cells (arrows) and acini are all negative. X250.



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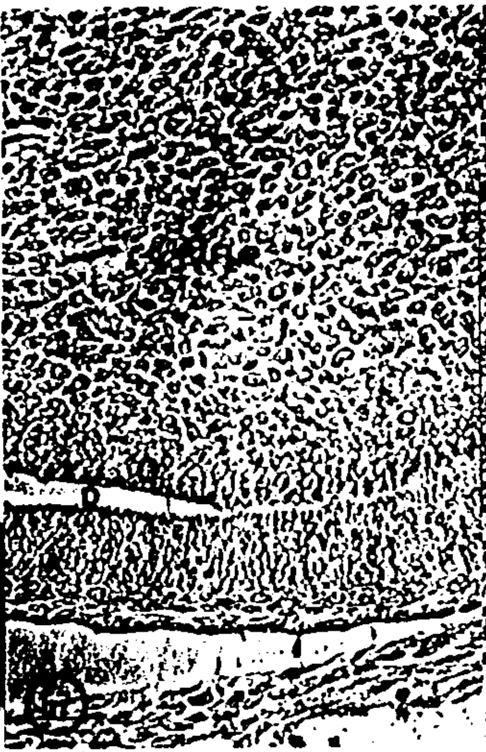

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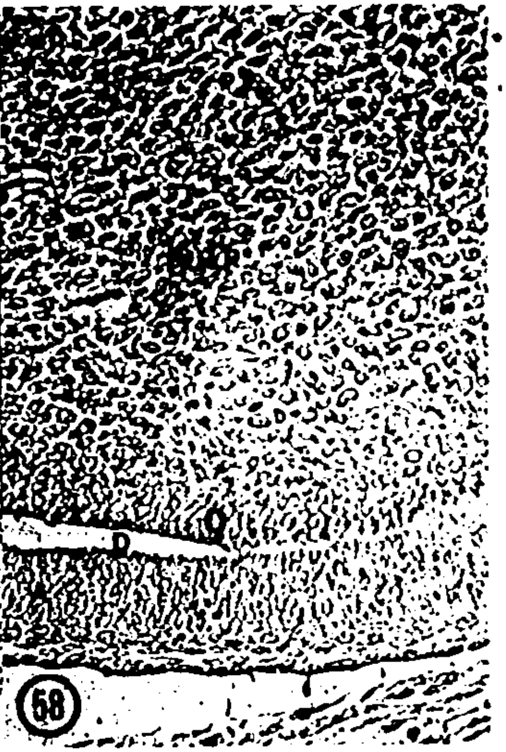
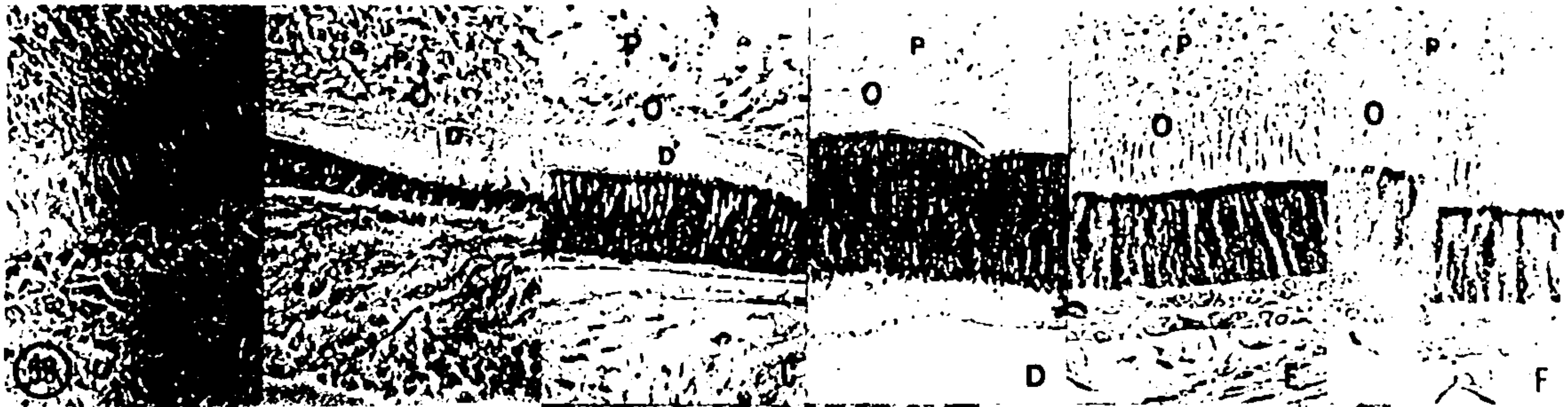


(58)

Fig. 56 - A portion of a rat incisor tooth stained with anti-chick D-CaBP-I. The dark reaction product is present throughout the cytoplasm of the ameloblasts and Tome's processes. The nuclei of the ameloblasts are darkly labelled. All other structures including the enamel (E), the dentin (D) the odontoblasts (O) and the pulp cavity (PULP) were completely negative. X250.

Fig. 57 - A phase contrast picture of a section adjacent to that of Fig. 56 that was stained with non-immune serum. All structures are negative including the ameloblasts (A). Structures are labelled as in Fig. 56. X250.

Fig. 58 - A phase contrast picture of a section adjacent to that of Fig. 57 stained with antiserum against rat D-CaBP-II. All structures are negative and are labelled as in Fig. 56. X250.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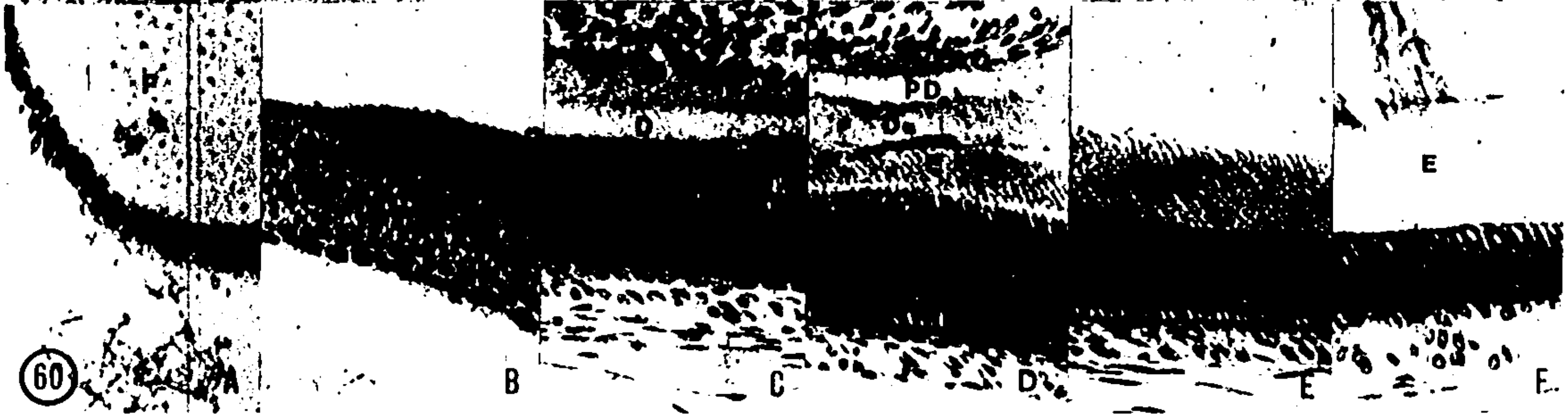
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Fig 59a-f - Phase contrast microscopy of the different stages of maturation of rat ameloblasts stained with anti-chick D-CaBP-I.

Fig 60a-f - Ameloblasts stained as above and then counterstained with H and E.

The zones in figs 59 and 60 are labelled as follows:

a) The zone of presecretion where there is no D-CaBP-I staining including the ameloblasts (A) facing the pulp cavity (P).

b) The zone of presecretion facing dentin (D). The odontoblasts (O) are negative and ameloblasts (A) are beginning to show positive staining.

c) The initial portion of the zone of secretion where the smooth surfaced ameloblasts are heavily labelled for D-CaBP-I.

d) The major portion of the zone of secretion. The reaction product extends throughout the cytoplasm and the Tome's processes of the ameloblasts. The enamel (E), dentin (D), predentin (PD) and odontoblasts are all negative.

e) The zone of maturation where the positive ameloblasts begin to shorten in length.

f) The zone of maturation where the shortened ameloblasts are still positive.

All pictures X100.

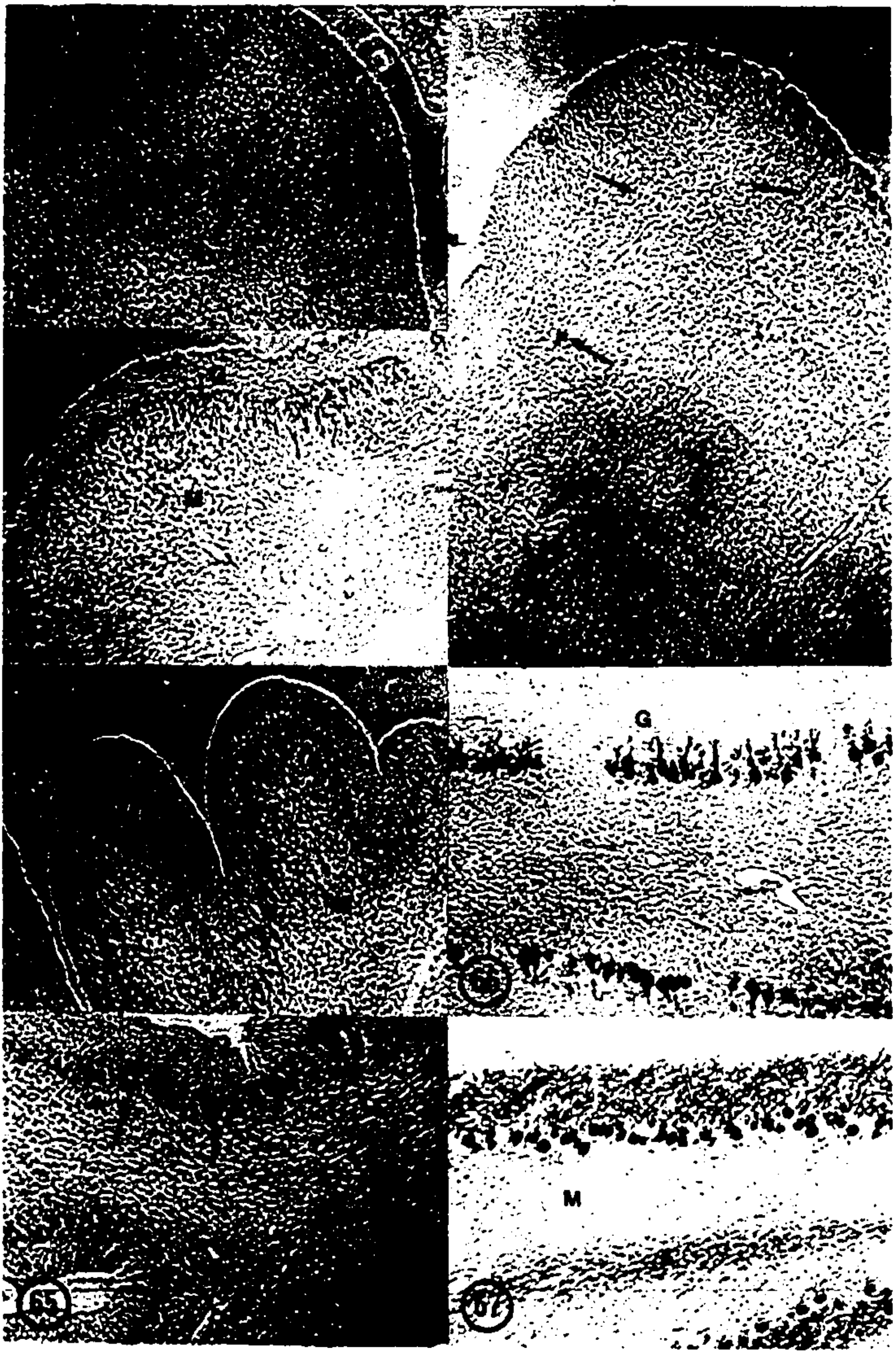
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Fig. 61 - Phase contrast picture of a portion of a 9 day old chick embryonic cerebellum stained with anti-chick D-CaBP-I using the PAP technique. Small scattered fibers (arrows) are positive. X100.

Fig. 62 - Phase contrast picture of a portion of a 10 day old chick embryonic cerebellum stained as above. Positive fibers are thicker and more numerous (arrows) than on day 9 especially in the region of the deep nucleus (DN). The granular cell layer is labelled G. X100.

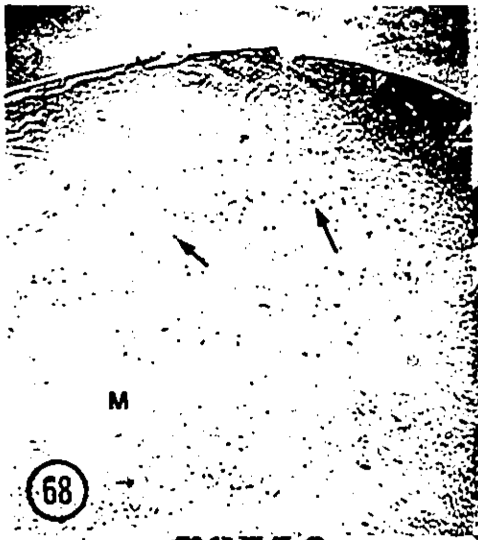
Fig. 63 - Phase contrast picture of a portion of a 12 day old chick embryonic cerebellum stained as in Fig. 61. Positive cells forming the boundary between the granular (G) and molecular layer (M) can be recognised as Purkinje cells. X100.

Fig. 64 - Phase contrast picture of a portion of a 13 day old chick embryonic cerebellum stained as in fig 61. Positive Purkinje cells are forming a continuous layer between the granular (G) and molecular (M) layer. X100.

Fig. 65 - Phase contrast picture of a portion of a 14 day old chick embryonic cerebellum stained as in Fig. 61. The positive Purkinje cells are beginning to mature. On some cells (arrows) the beginning of the main dendrite can be distinguished. X100.

Fig. 66 - Phase contrast picture of a portion of a 16 day old chick embryonic cerebellum stained as in Fig. 61. The main dendrite of the positive Purkinje cells is growing into the granular layer (G). X100.

Fig. 67 - Phase contrast picture of a portion of a hatching chick cerebellum stained as in Fig. 61. The dendritic tree of the Purkinje cell is almost complete. The positive axons (A) of the Purkinje cells can be seen in the molecular layer (M). X100.



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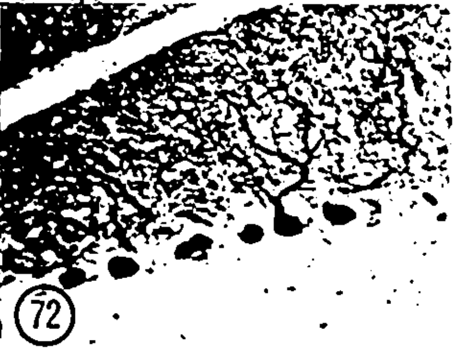
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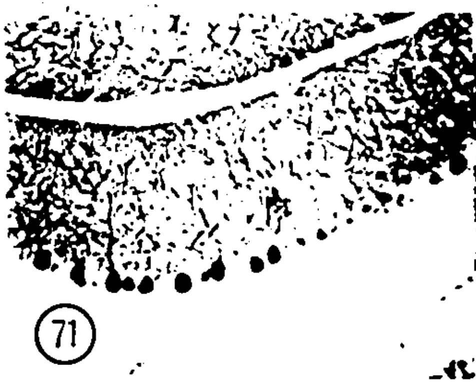
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Fig. 68 - Phase contrast picture of a portion of a section of the cerebellum of a gold fish stained with anti-chick D-CaBP-I. The Purkinje cells (arrows) which are scattered in the molecular layer (M) are positive. X100.

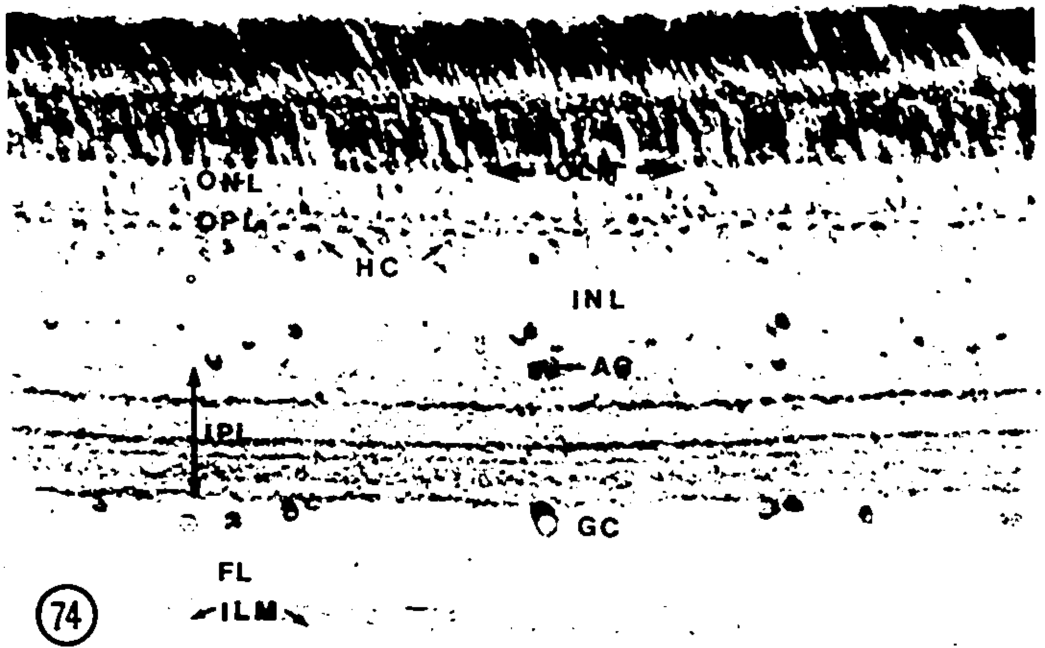
Fig. 69 - A portion of a section of the cerebellum of a frog stained with anti-chick D-CaBP-I. The Purkinje cells (arrows) are positive. X100.

Fig. 70 - A portion of rat cerebellum stained with anti-chick D-CaBP-I. Reaction product is present throughout the cytoplasm of the Purkinje cells. X100.

Fig. 71 - A portion of rat cerebellum stained with anti-human D-CaBP-I. The Purkinje cells are also positive but staining is not as intense as in Fig. 71. X100.

Fig. 72 - A portion of mouse cerebellum stained with anti-chick D-CaBP-I. The Purkinje cells are positive. X100.

Fig. 73 - A portion of rabbit cerebellum stained with anti-chick D-CaBP-I. The Purkinje cells are darkly stained. X100.



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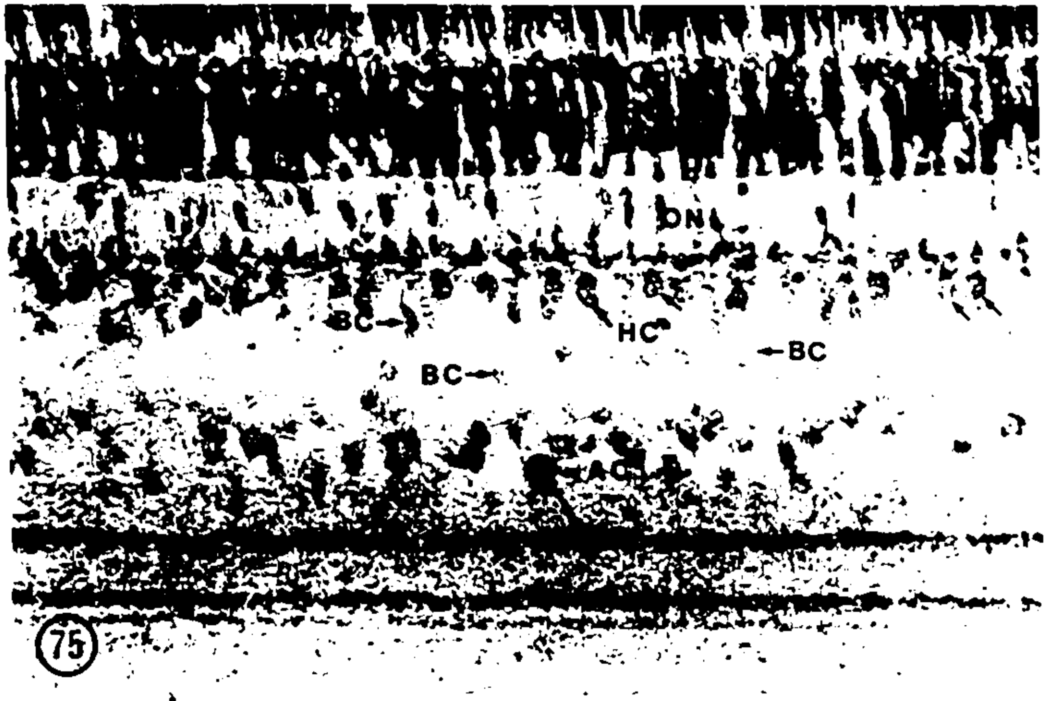

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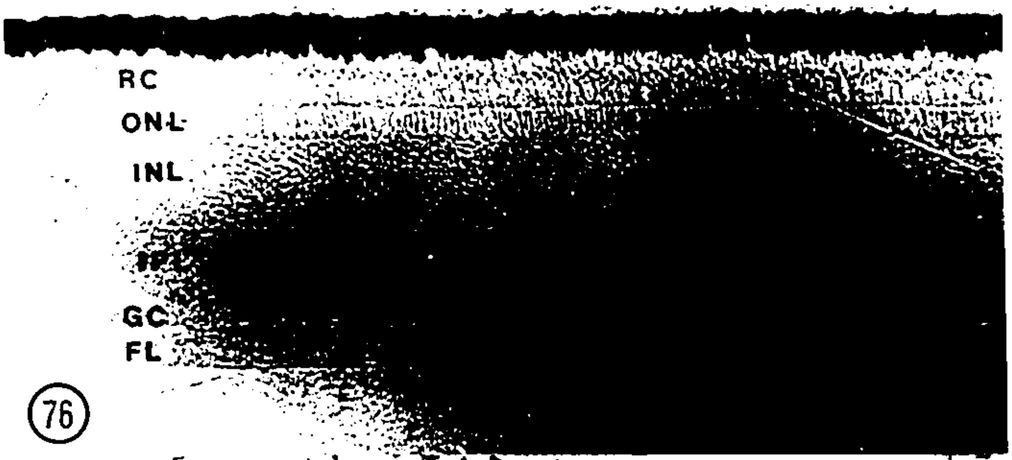
Fig. 74 - A vertical section of a normal chick retina stained with anti-chick D-CaBP-I using the PAP method. The inner segment of the receptor cells (RC) are positive for D-CaBP-I. At the outer limiting membrane (OLM) there is a sharp boundary because of the lighter staining of the outer nuclear layer (ONL). In the outer plexiform layer (OPL) the positive pedicles of the RC and the positive processes and cellular bodies of the horizontal cells (HC) form two distinct layers. In the inner half of the inner nuclear layer (INL) positive amacrine cells (AC) are seen. In the inner plexiform layer (IPL) at least five horizontal bands can be distinguished. A few scattered ganglion cells (GC) are positive. The fiber layer (FL) and the inner limiting membrane (INL) are negative. X400.

Fig. 75 - Higher magnification of normal chick retina stained with anti-chick D-CaBP-I. Here again the inner segments of the receptor cells (RC) are positive. The outer nuclear layer (ONL) stains lightly. The pedicles of the RC are positive. The inner nuclear layer (INL), horizontal cells (HC) and probably some bipolar cells (BC) are also positive. Amacrine cells (AC) containing D-CaBP-I are present close to the INL. One AC in particular is labelled and its sole process is seen reaching into a D-CaBP-I positive, inner plexiform layer. X562.5.



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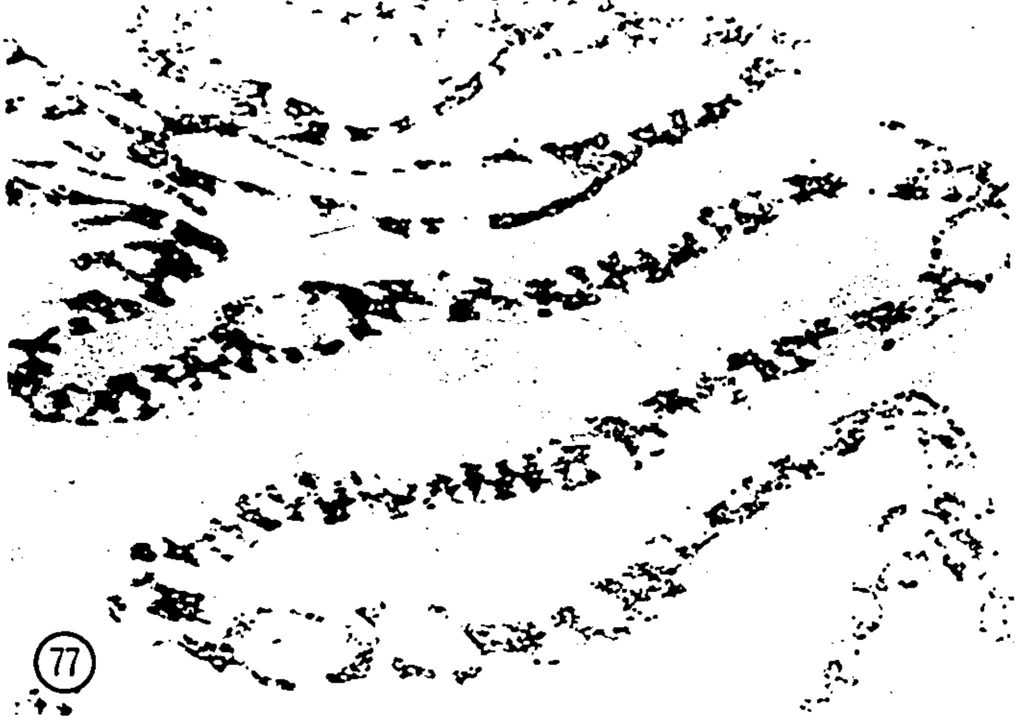

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Fig. 76 - Vertical section of a normal chick retina incubated with non-immune serum (control). All the components of the retina including receptor cells (RC), the outer nuclear layer (ONL), the outer plexiform layer (OPL), the inner nuclear layer (INL), the inner plexiform layer (IPL), the ganglion cell layer (GC), and the fiber layer (FL) were negative. X225.

Fig. 77 - Portion of a normal chick pecten incubated with anti-chick D-CaBP-I by the PAP method. The polymorphic intervascular cells are heavily laden with pigment granules but there is no reaction product. X375.


INL
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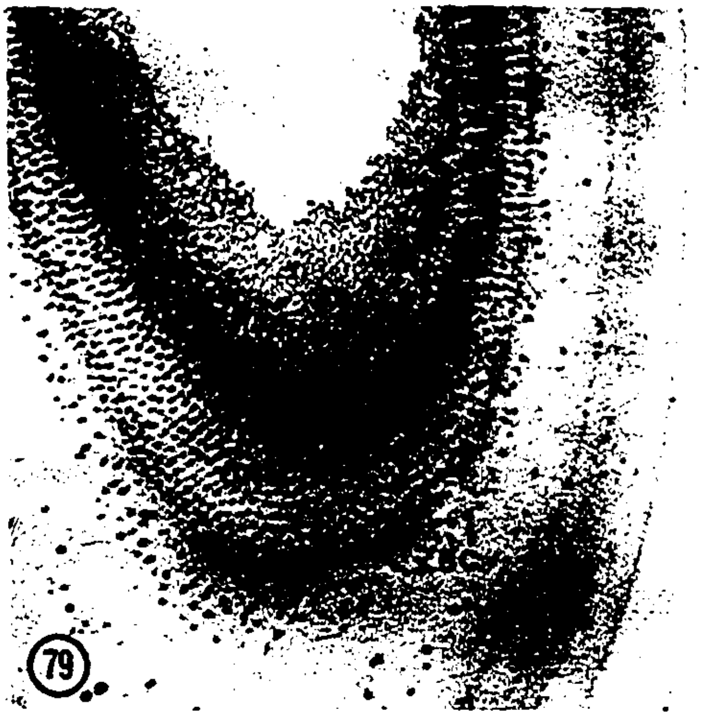
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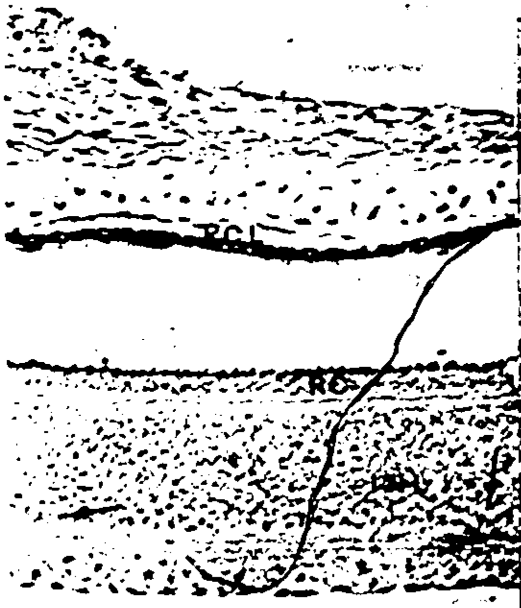
Fig. 78 - Vertical section of a retina from a 4-week old rachitic chick, stained with anti-chick D-CaBP-I by the PAP method. In the outer plexiform layer (OPL) only the pedicles of the receptor cells are clearly positive and the horizontal cells are negative. In the inner nuclear layer (INL), scattered positive cells are present, some are probably amacrine cells, while othe~~rs~~ may be bipolar cells. Note scattered positive ganglion cells. X225.

Fig. 79 - Tangential section of a retina from a 4-week old rachitic chick stained with anti-chick D-CaBP-I. The inner segment of the receptor cells (RC) as well as the cytoplasm in the outer nuclear layer (ONL) are positive. Horizontal cells in the outer plexiform layer (OPL) are not clearly seen. Some cells of the group labelled BC are probably positive bipolar cells. X225.



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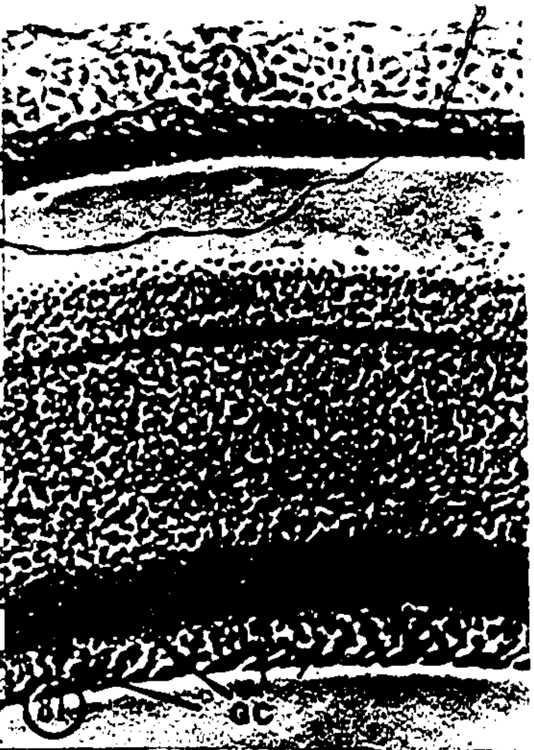

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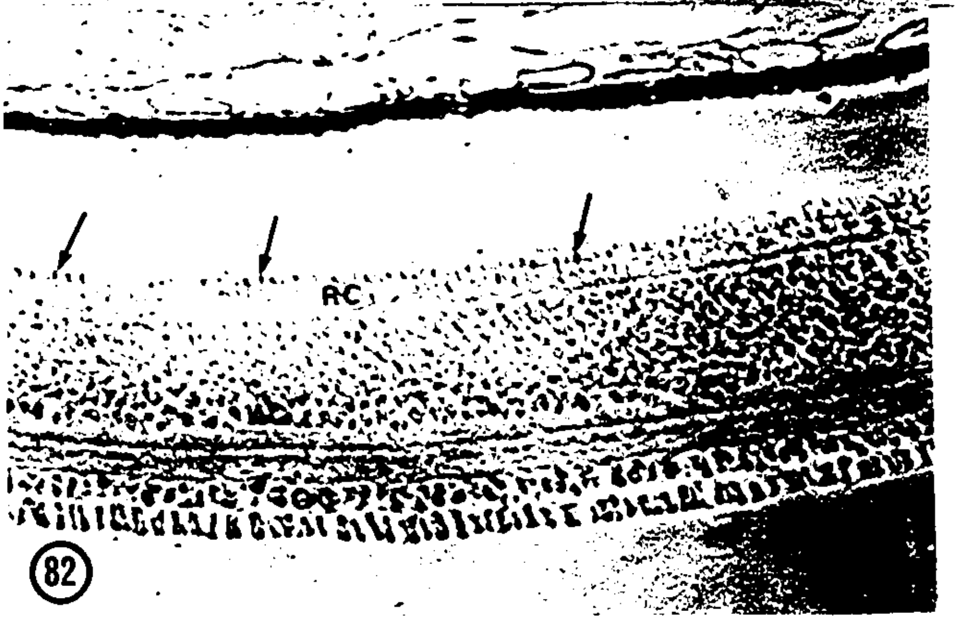

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Fig. 80 - Phase contrast picture of a vertical section of a retina from a 9 day old chick embryo stained with anti-chick D-CaBP-I. Scattered amacrine cells (arrows) in the lower third of the inner nuclear layer (INL) displayed a faint positive reaction. The pigment cell layer (PCL) and receptor cell layer (RC) are also labelled. X250.

Fig. 81 - Phase contrast picture of a vertical section of a retina from a 12 day old chick embryo stained with anti-chick D-CaBP-I. Staining in scattered amacrine cells (AC) is denser than in day 9 retina. Some ganglion cells (GC) are also positive. X250.

Fig. 82 - Phase contrast picture of a vertical section of a retina from a 16 day old chick embryo stained with anti-chick D-CaBP-I. There is a faint positive staining in the inner segment (arrows) of the receptor cells (RC). Scattered amacrine (AC) and ganglion cells (GC) are also present. X250.

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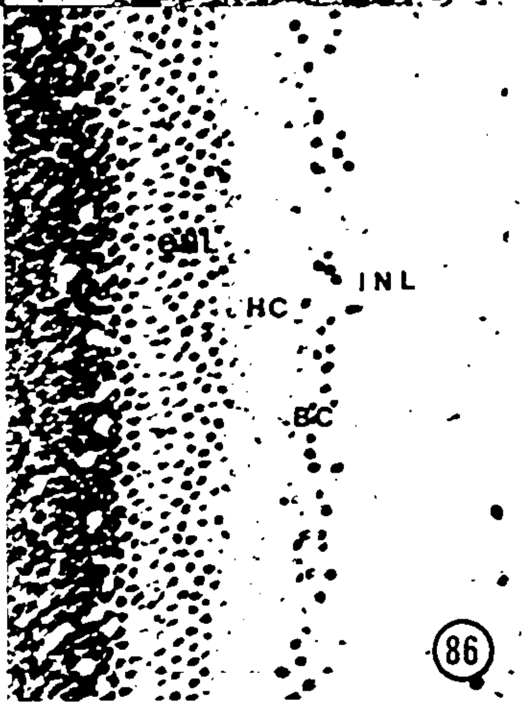

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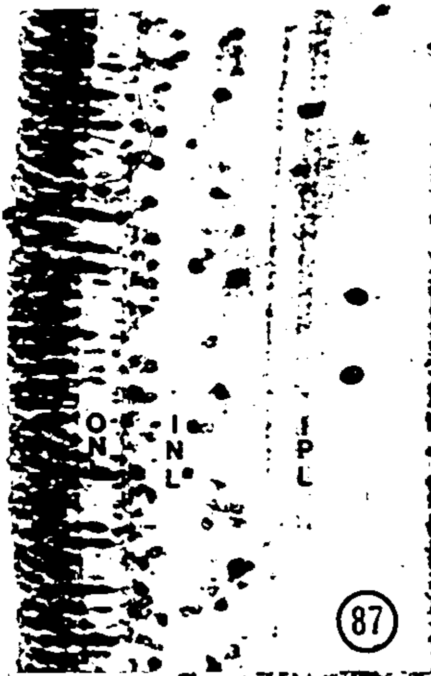
Fig. 83 - Phase contrast picture of a vertical section from a 17 day old chick embryo stained with anti-chick D-CaBP-I. In addition to receptor cells (RC), amacrine cells (AC) and ganglion cells (GC), some bipolar cells (BC) are positive. Some layers of the inner plexiform layer (IPL) are positive X250.

Fig. 84 - Phase contrast picture of a vertical section from a 18 day old chick embryo stained with anti-chick D-CaBP-I. The staining pattern is similar to that described for Fig. 25. X250.

Fig. 85 - Vertical secton of a retina from a 1-day old chick stained with anti-chick D-CaBP-I. The receptor cells, probably some bipolar cells (BC), some amacrine cells (AC).and ganglion cells are positive. The horizontal cells (HC) are only faintly positive. X365.

Fig. 86 - Tangential section of a retina from a 1-day old chick stained with anti-chick D-CaBP-I. In the outer nuclear layer (ONL) the cytoplasm and the nuclei of the receptor cells are positive. In the inner nuclear layer (INL), horizontal cells (HC) are faintly positive while probably some bipolar cells (BC) and some amacrine cells at the bottom of the INL are positive. X365.



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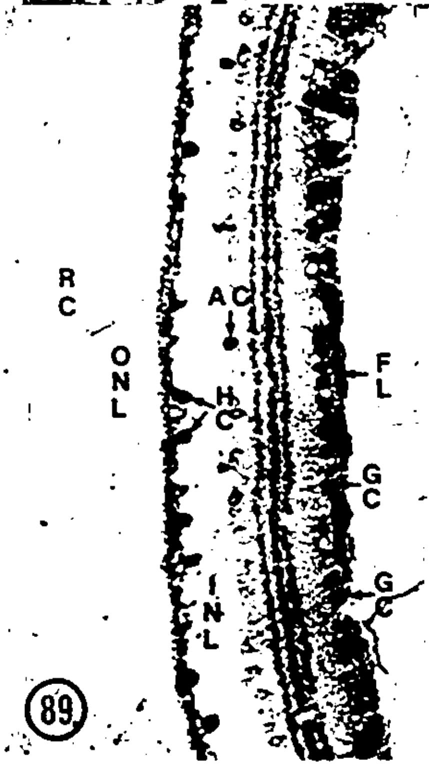

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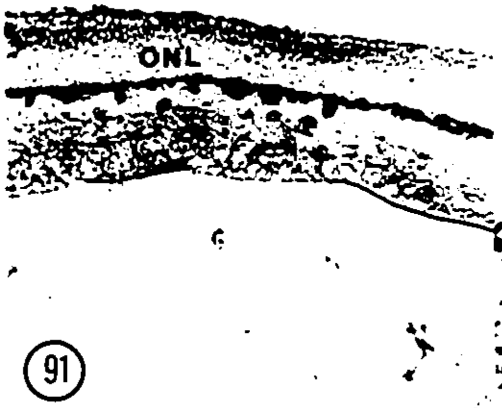
(90)

Fig. 87 - Vertical section of a 2-week-old normal chick retina stained with anti-chick D-CaBP-I. In the outer nuclear layer (ONL) some of the cell bodies of the receptors cells are positive. In the inner nuclear layer (INL) horizontal cells, probably some bipolar cells and some amacrine cells are positive. In the inner plexiform layer (IPL) three positive bands are visible. X365.

Fig. 88 - Vertical section of 2-week-old rachiti~~c~~ chick retina stained with anti-chick D-CaBP-I. Receptor cell (RC) inner segment and cell bodies in the outer nuclear layer (ONL) are positive. RC pedicles also stained for D-CaBP-I but horizontal cells (HC) are negative. Probably some bipolar cells (BC), some amacrine cells and some ganglion cells are positive. X365.

Fig. 89 - Vertical section of a mouse retina stained with anti-chick D-CaBP-I. The receptor cells (RC) have but a trace amount of D-CaBP-I. In the inner nuclear layer (INL) horizontal cell bodies and their processes are densely stained. Some amacrine cells (AC) are also positive. In the inner plexiform layer three horizontal lines are distinctly stained. Most ganglion cells (GC) and their fibers (FL) are positive. X225.

Fig. 90 - Section of a rat retina stained with anti-chick D-CaBP-I. Here again receptor cells (RC) are but very lightly stained. Horizontal cells (HC) and some amacrine cells (AC) are positive. Most ganglion cells (GC) and their fibers (FL) are positive. X225.



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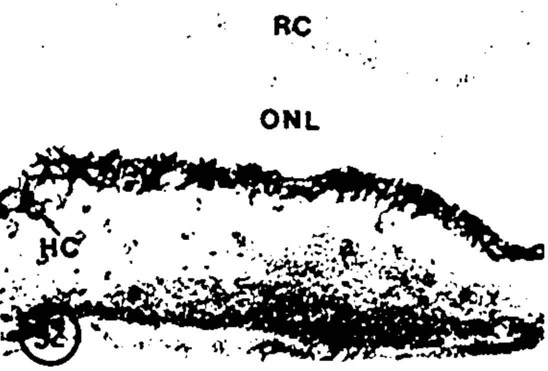

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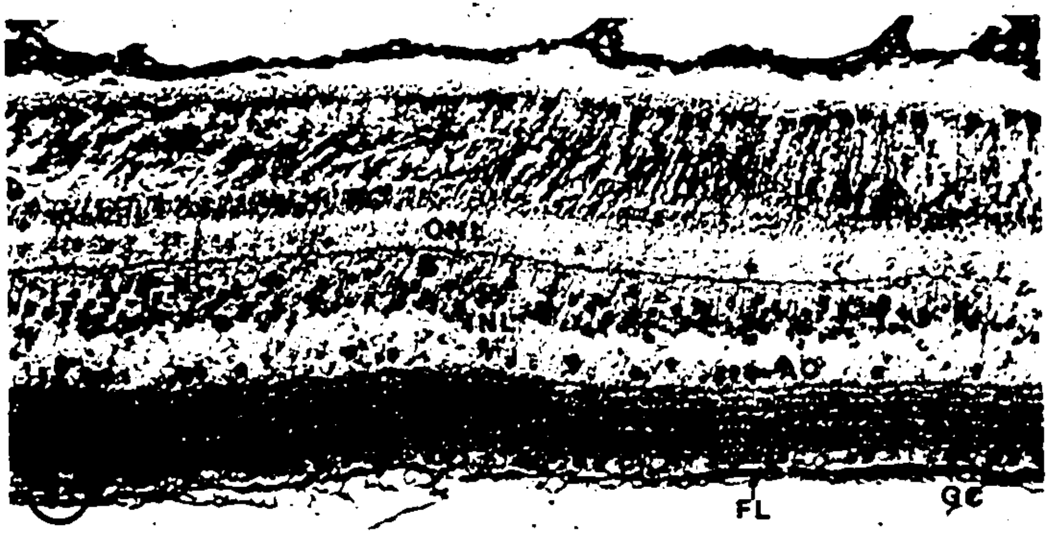

Fig. 91 - Section of a rabbit retina stained with anti-chick D-CaBP-I. Receptor cells (RC) are very faintly positive. Horizontal cells are the most densely stained cells. Some amacrine cells are also positive. X225.

Fig. 92 - Tangential section of a rabbit retina showing the extensive branching of D-CaBP-I positive horizontal cell processes. X225.

Fig. 93 - Section of a frog retina stained with anti-chick D-CaBP-I. The inner segments of receptor cells (RC), as well as the cell bodies in the outer nuclear layer (ONL) and their pedicles are faintly positive. Horizontal cells (HC), bipolar cells and some amacrine cells (AC) in the inner nuclear layer (INL) are positive. Four horizontal lines in the inner plexiform layer are positive. Many ganglion cells (GC) and their fibers (FL) are positive. X225.

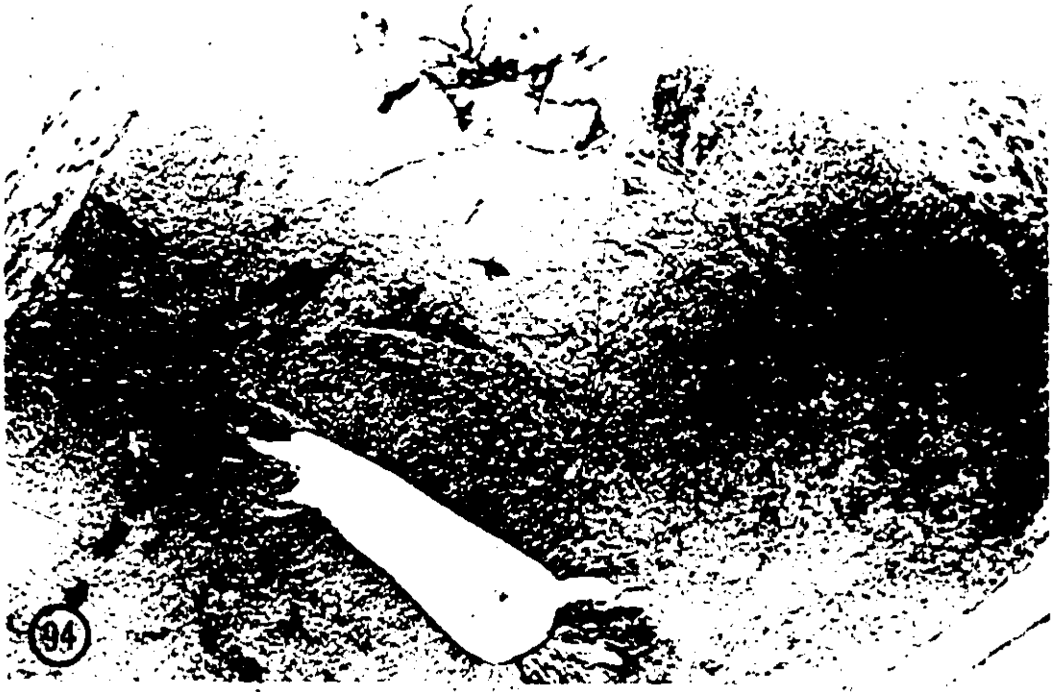

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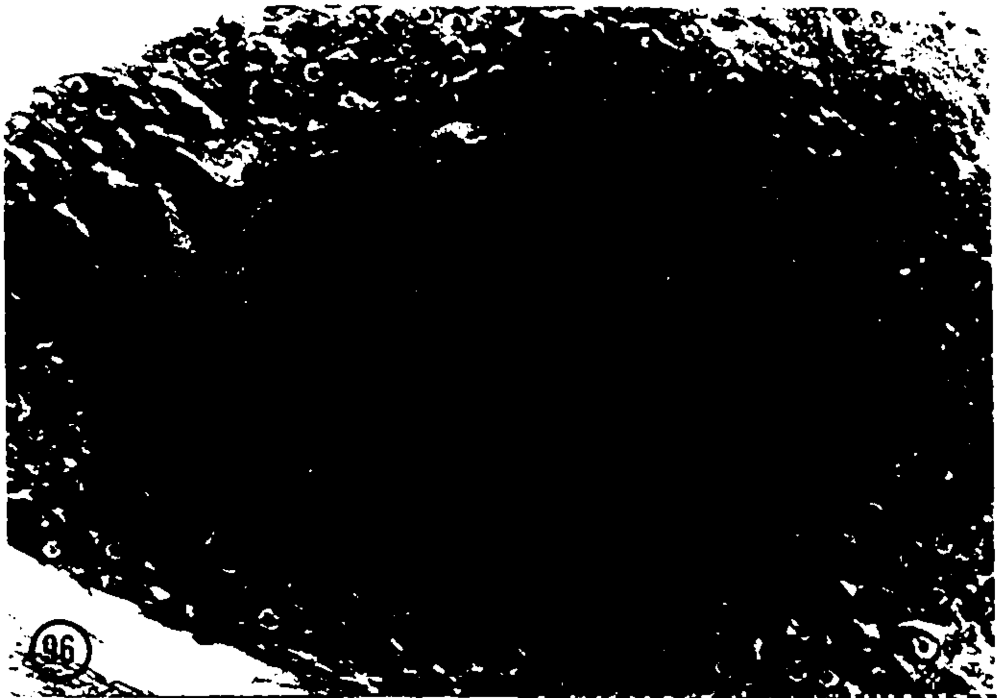
Fig. 94 - A portion of the spinal cord of a 10 day old chick embryo stained with anti-chick D-CaBP-I by the PAP method and counterstained with H and E. Reaction product is present throughout the cytoplasm of certain interneurons in the dorsal and ventral horns. Motor neurons (M) ependymal (E) and glial cells are negative. X275.

Fig. 95 - Higher magnification of D-CaBP-I positive neurons in the ventral horn. The smaller nuclei (arrows) belong to glial cells which are negative. X320.



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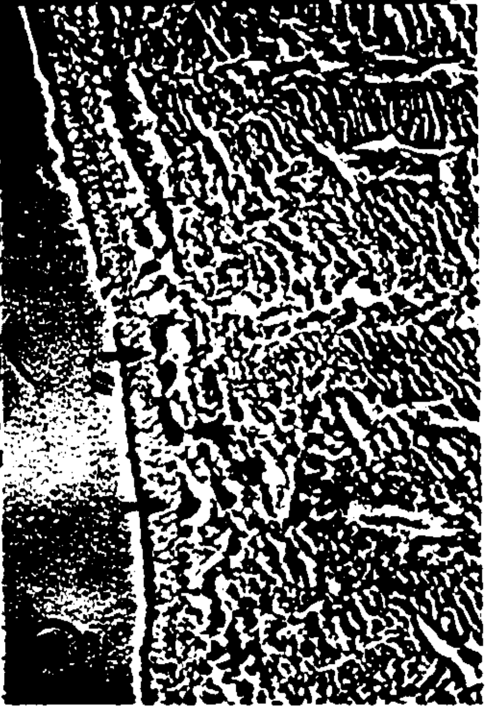

Fig. 96 - A spinal ganglion of a 10 day old chick embryo stained with anti-chick D-CaBP-I by the PAP method and counterstained with H and E. All the neurons are devoid of reaction product. X375.

Fig. 97 - A portion of the ganglion nodosum of a 4 week old chick stained with anti-chick D-CaBP-I with SIM. The nucleus and cytoplasm of certain neurons is darkly labelled. Positive axons (A) are also present. X420.

Fig. 98 - Phase contrast picture of a portion of a rat duodenum stained with anti-chick D-CaBP-I by the PAP method. Parasympathetic neurons (arrows) located in between the smooth muscle layers are darkly stained. All structures of the villi including the enterocytes (E) are negative. X100.

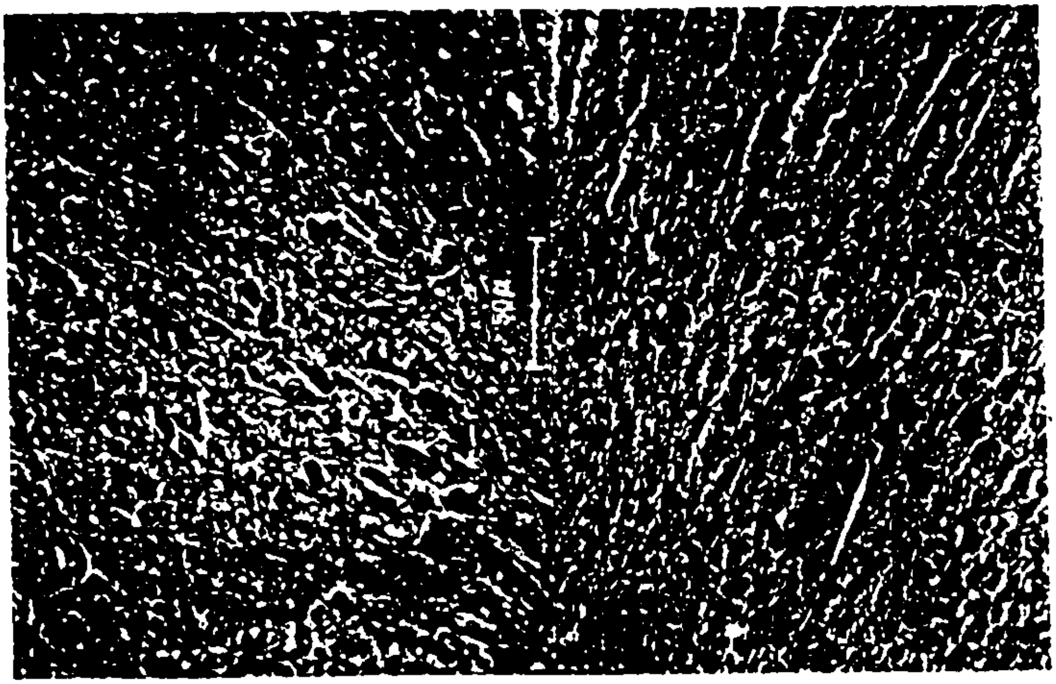
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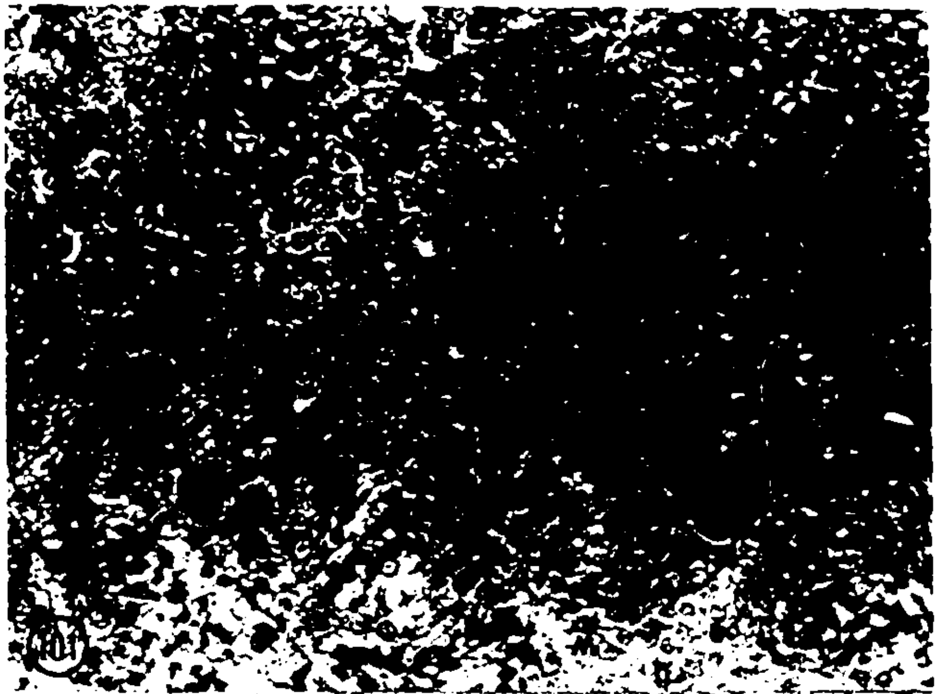
Fig. 99 - Phase contrast picture of a portion of a section of the fundic stomach of a rat stained with anti-chick D-CaBP-I. Interspersed pyramidal (thick arrows) and spindle shaped cells (thin arrows) in the basal portion of the gastric glands are positive. X250.

Fig. 100 - Phase contrast picture of a section adjacent to that of Fig. 99 stained with anti-rat D-CaBP-II. Parietal cells are slightly positive (arrows). X250.



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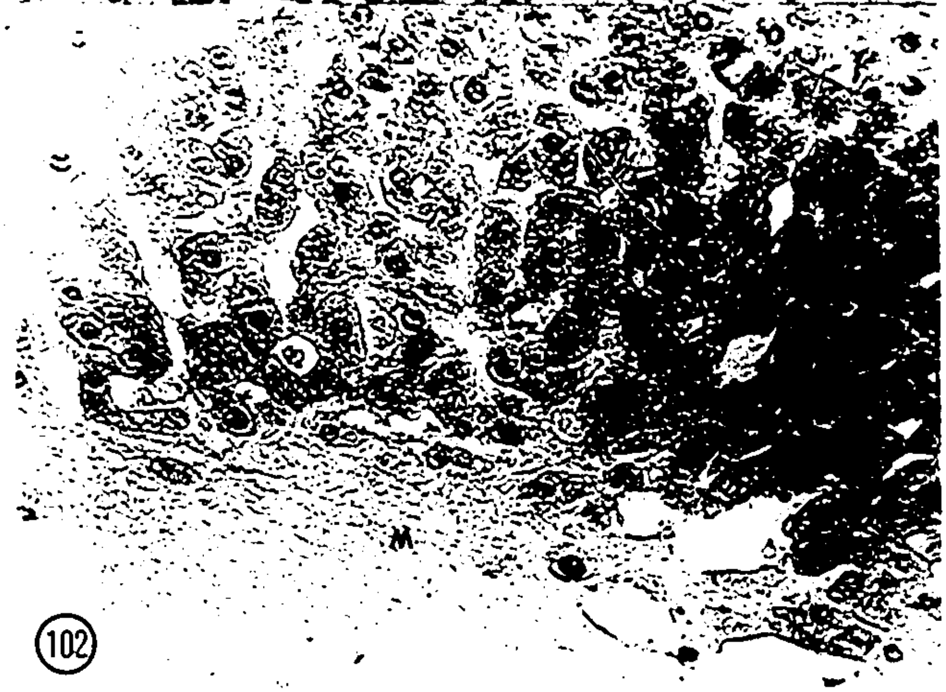

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Fig. 101 - A portion of a chick adrenal gland stained with anti-chick D-CaBP-I and counterstained with H and E. The cortical cell (arrows) are positive. The medullary cells (M) do not contain any reaction product. X275.

Fig. 102 - A portion of a rat adrenal gland stained with anti-rat D-CaBP-II. The cortical cells are positive. All structures in the medulla (M) are negative. X250.

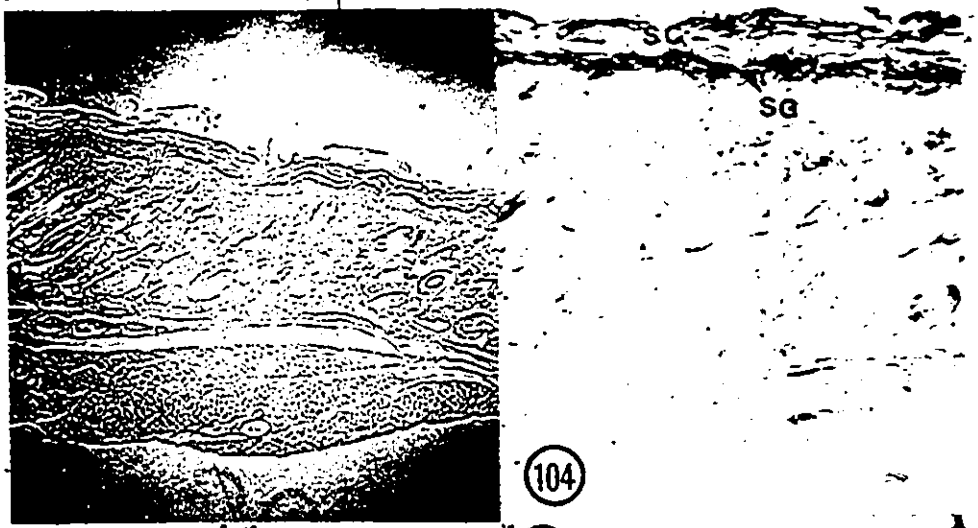

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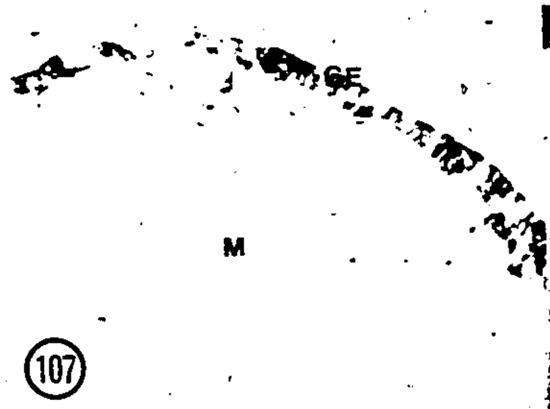
Fig. 103 - Portion of a section of rat skin stained with anti-chick D-CaBP-I. All structures including the stratum corneum (SC), granulosum (SG), spinosum (SS) and basale (SB) are negative. X50.

Fig. 104 - Portion of a section of rat skin stained with anti-rat D-CaBP-II adjacent to that of fig 103. The stratum corneum (SC) and cells of the stratum granulosum (SG) are positive. X100.

Fig. 105 - Portion of a section of rat esophagus stained with anti-rat D-CaBP-II. The stratum corneum (SC) and the cells of the stratum granulosum (SG) are positive. Note that some cells in the stratum spinosum are heavily labelled. X100.

Fig. 106 - Portion of a section of chick skin stained with anti-chick D-CaBP-I. The stratum corneum (SC) and the cells of the stratum granulosum (SG) are positive. X100.



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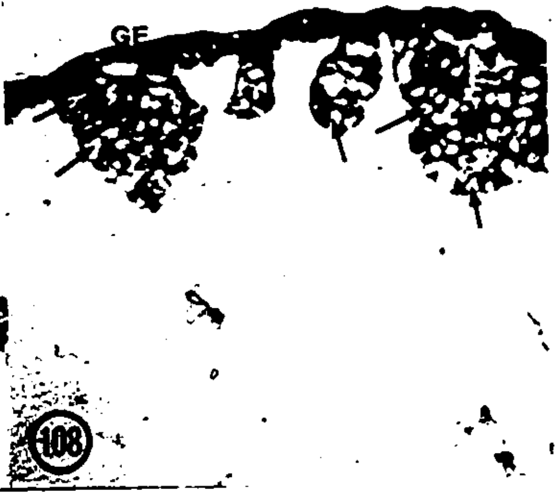

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Fig. 107 - A portion of a section of a 8 day old chick embryonic ovary stained with anti-chick D-CaBP-I. Some positive staining is present in the cells of the germinal epithelium (GE). Structures of the medulla are not stained (M). X256.

Fig. 108 - A portion of a section of a 14 day old chick embryonic ovary stained with anti-chick D-CaBP-I. The germinal epithelial cells (GE) are positive for D-CaBP-I. The prefollicular cells are densely labelled for D-CaBP-I and completely surround the dividing oogonia. The oogonia (arrows) in the cortical cords are completely negative. X256.

Fig. 109 - Another portion of Fig. 108 showing the right (RO) and a portion of the left ovary (LO). Notice that the development of the right ovary does not keep pace with that of the left ovary. In the right ovary there is no germinal epithelium and no cortical cords. There are a few unorganised D-CaBP-I positive cells in the medulla. X256.

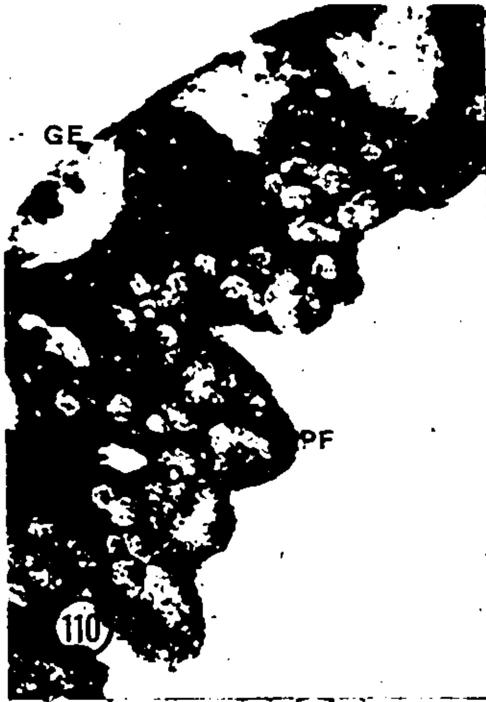

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Fig. 110 - A portion of a section of the ovary of a hatching chick stained with anti-chick D-CaBP-I and counterstained with H and E. Here again the germinal epithelial (GE) cells and prefollicular cells (PF) surrounding the oogonia (O) are densely labelled for D-CaBP-I. X256.

Fig. 111 - A portion of a section of a 6 week old chick ovary stained with anti-chick D-CaBP-I. At this age only the germinal epithelial (GE) cells are positive. The follicular cells surrounding the maturing oocytes are negative. X100.

Fig. 112 - The ovary of a 1 week old rat pup stained with anti-rat D-CaBP-II and then counterstained with H and E. The follicular cells are positive. The follicular cells surrounding the primary follicles (PF) are more densely labelled than those surrounding growing and maturing follicules. X128.

Fig. 113 - Higher magnification of a portion of the rat ovary of Fig. 112. The follicular cells surrounding the primary follicles are positive (arrows). X250.


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Fig. 114 - Section of the thymus of a 20 day old chick embryo stained with anti-chick D-CaBP-I by the PAP method. In the cortex (C) fine cellular processes (arrows) and cell bodies of cortical epithelial cells (ERC) are densely labelled with reaction product. In the medulla (M) no structures are stained. X250.

Fig. 115 - Section of the spleen of the same 20 day old chick stained with anti-chick D-CaBP-I by the PAP method. Positive reticular cells in the marginal zones (MZ) delimit the developing white pulp areas (*). At this stage the bulk of the spleen is made up of red pulp (RP). X250.

Fig. 116 - A two week old normal chick thymus stained with anti-chick D-CaBP-I by the PAP method. In the cortex (C) ERC are densely stained throughout their cytoplasm and in the medulla (M) Hassal's corpuscles (HC) are also labelled. X100.

Fig. 117 - The spleen of the same two week old normal chick stained with anti-chick D-CaBP-I by the PAP method. Numerous, heavily labelled reticular cells in the marginal zones (MZ) demarcate the boundary between the red (RP) and white pulp (*). X100.


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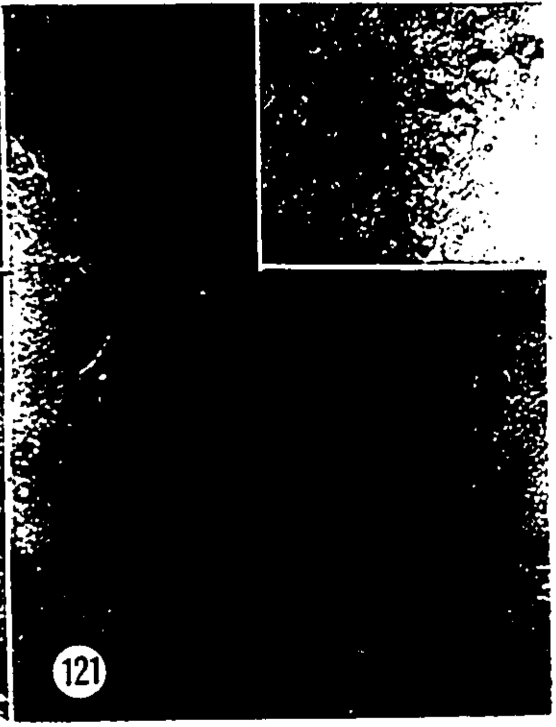

121

Fig. 118 - Section from a 4 week old normal chick thymus stained with anti-chick D-CaBP-I by the PAP method. Staining in the cortex (C) is very much decreased. Scattered ERC cell bodies are still positive and Hassal's corpuscles (HC) in the medulla (M) are also positive. X100.

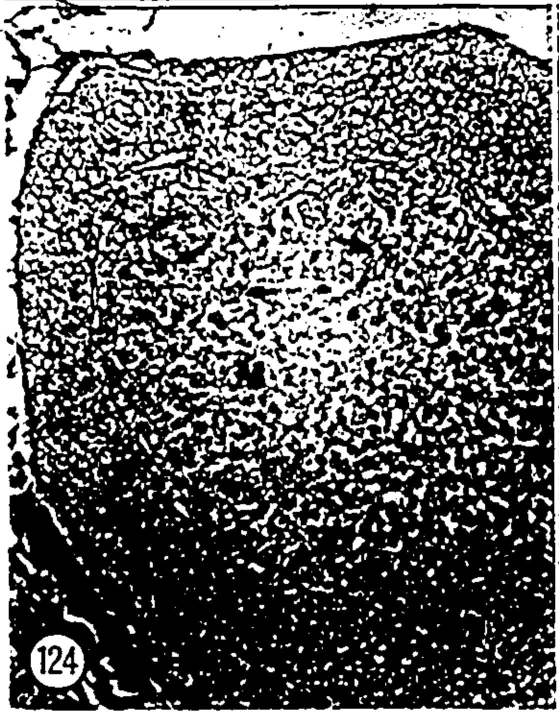
Fig. 119 - Section of the spleen of the same 4 week old normal chick stained as in Fig. 118. Most of the marginal zones (MZ) are still well marked off by D-CaBP-I positive reticular cells. X100.

Fig. 120 - Section of a 4 week old thymus stained with anti-chick D-CaBP-I by the SIM. The cortex (C) is heavily labelled by positive ERC. In the medulla (M) Hassal's corpuscles (HC) are positive. When compared with Fig. 118 it shows the striking variation in the staining of cortical ERC encountered in 4 week old normal chicks from different broods. X100.

Fig. 121 - Section of the spleen of the same 4 week old normal chick of Fig. 120 reacted with anti-chick D-CaBP-I by the SIM. Positive reticular cells in the marginal zones (MZ) are so few that it is difficult to distinguish the red and white pulp. X32. The inset shows a positive reticular cell at a higher magnification. X320. When compared with Fig. 119 it shows the striking variation in staining of reticular cells in the marginal zones in the spleen of 4 week old normal chicks from different broods.


122


123


124


125

Fig. 122 - Section of the thymus of a 4 week old vitamin D-deficient chick stained with anti-chick D-CaBP-I by the PAP method. Only a faint staining of ERC in the cortex (C) can be observed. In the medulla (M), Hassal's corpuscles (HC) were faintly postive. X100.

Fig. 123 - Section of the spleen of a 4 week old vitamin D-deficient chick stained with anti-chick D-CaBP-I by the PAP method. Only a few scattered reticular cells in the marginal zones (MZ) are positive. X100.

Fig. 124 - Phase contrast picture of a follicle of the Bursa of Fabricius from a 4 week old normal chick stained with anti-chick D-CaBP-I with the PAP method. Scattered positive cells are present (arrows) in the medulla (M). No reaction product is seen in the cortex (C). The well developed capillary network (CAP) dividing the cortex from the medulla can be identified. X320.

Fig. 125 - Phase contrast picture of a control section of a follicle of the Bursa of Fabricius adjacent to that of Fig. 124, stained with non-immune rabbit serum instead of anti-chick D-CaBP-I by the PAP method. All the components of the cortex (C) and medulla (M) are negative. X320.

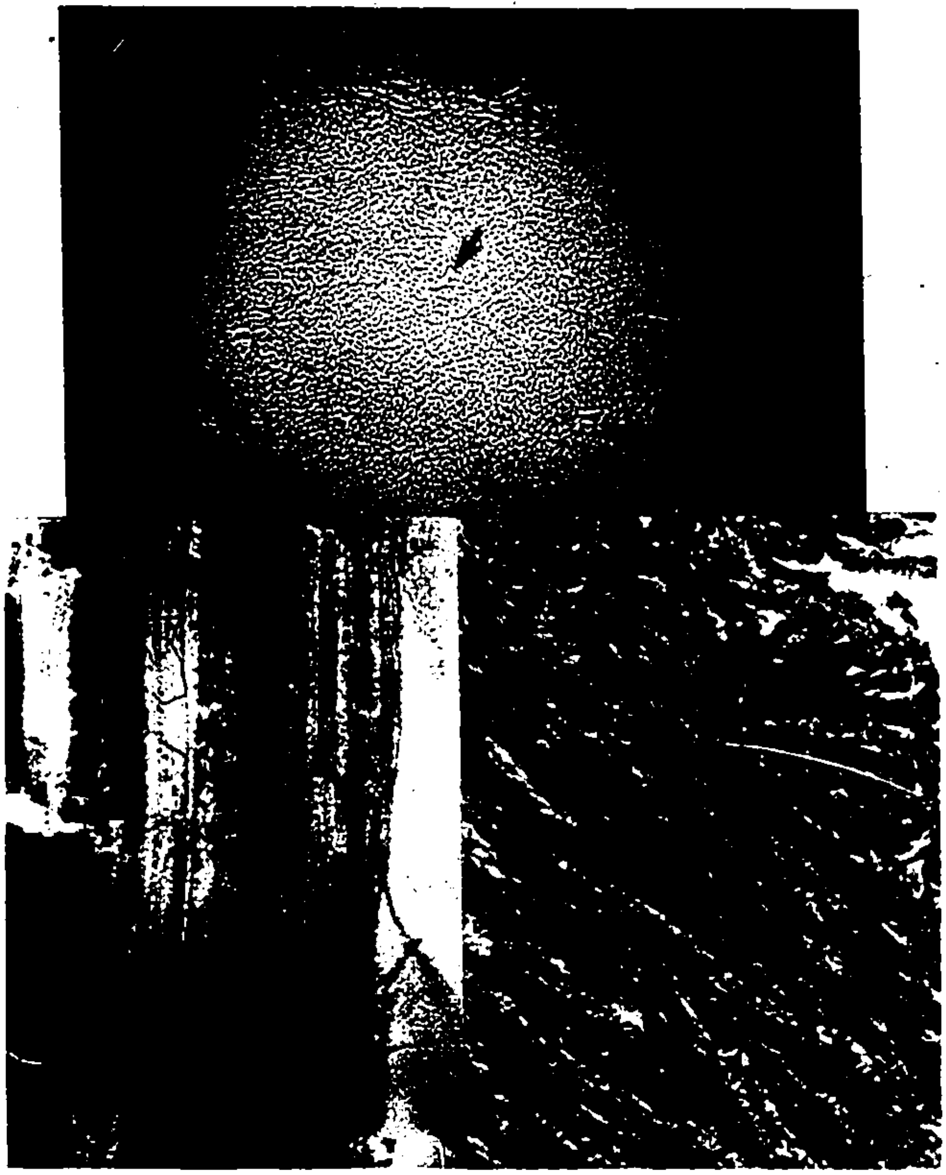


Fig. 126 - Phase contrast picture of a portion of chick liver stained with anti-chick D-CaBP-I by SIM. All structures including the hepatic cells are negative. The central vein of the lobule is indicated by an arrow. X100.

Fig. 127 - Portion of a section of chick skeletal muscle stained with anti-chick D-CaBP-I by SIM. All structures are negative. X250.

Fig. 128 - Portion of a section of chick cardiac muscle fibers stained with anti-chick D-CaBP-I by SIM. All structures of the cardiac fibers (CF) and Purkinje fibers (PF) are negative. X250.

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