EFFECTS OF VITAMIN D_3 ON DUODENAL EPITHELIUM OF CHICKENS: AN ELECTRON MICROSCOPIC STUDY

by

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CHAPTER ONE

INTRODUCTION

Vitamin D has long been known as the antirachitic factor. Mellanby in 1919 demonstrated rickets to be a nutritional disease which could be prevented or cured by the inclusion of cod liver oil in the diet. He also recognized that rapidly growing animals required more antirachitic factor in their diets than did mature animals. However, he attributed the antirachitic powers of cod liver oil to its content of fat-soluble vitamin A (Mellanby, 1919). Shortly thereafter it was discovered that the antirachitic factor was distinct from vitamin A, as it was still active after vitamin A activity in cod liver oil had been destroyed (McCollum et al., 1922a). McCollum and associates suggested that this factor must be classified as a fourth vitamin as it had the properties of regulating bone metabolism and it became known as vitamin D (McCollum et al., 1922b, 1925).

The Influence of Vitamin D on Intestinal Calcium Absorption

It was not until the 1930's that vitamin D was demonstrated to stimulate intestinal calcium absorption (Nicoleysen, 1937a, b). Previously this mode of action had been suggested (Orr et al., 1923)

but it was generally believed that vitamin D acted by decreasing calcium excretion into the colon (Harris, 1934; Nicoleysen and Eeg-Larsen, 1953).

The Intestinal Absorptive Epithelium

The columnar absorptive cells, which are responsible for the absorptive functions of the intestinal epithelium, have been studied previously in detail, in mouse by Zetterqvist (1956) and in rat by Palay and Karlin (1959). These cells are polarized, possessing an apical brush border and a smooth basal surface which rests upon a thin basal lamina. The lateral margins of the cells possess many folds which interdigitate with those of apposing cells. The lateral membranes of adjacent cells are held in close apposition by the juxta-luminal junctional complexes and by desmosomes dispersed along the length of the membranes (Farquhar and Palade, 1963).

The brush border of the absorptive cells is composed of microvilli and has a glycocalyx covering the external surface of the plasma membrane (Ito, 1965). Beneath the brush border there is a band of cytoplasm containing a mesh-work of fine filaments: the terminal web region. The apical cytoplasm, above the nucleus which is located below the mid-point of the cell, contains the Golgi apparatus, granular and agranular endoplasmic reticulum, mitochondria, and lysosomes. The basal cytoplasm

contains mainly mitochondria, granular endoplasmic reticulum, and free polyribosomes.

Calcium transport across the absorptive cell involves the passage of calcium across several barriers: 1) through the glycocalyx and across the plasma membrane of the apical surface; 2) through the terminal web region; 3) through the apical and basal cytoplasm, where cytoplasmic organelles may hinder or facilitate its movement; and 4) across the basal membrane and basal lamina. The term calcium transport will be used in this sense, to imply the passage of calcium into, through, and out of the absorptive cell, while calcium absorption will be used to describe movement of calcium into the absorptive cell only.

In the cases of _in vivo_ and _in vitro_ studies measuring calcium transport, the additional barriers of muscular layers, capillary endothelium, and serosa are included, so that these measurements are not of calcium transport at the level of the absorptive cell only. In studies utilizing everted gut sacs the picture may be further complicated by the creation of an artificial lumen enclosing what was formerly the external environment.

An alternate pathway which calcium may follow, rather than crossing the absorptive cells, may be by penetration of the intercellular spaces of the absorptive epithelium. Evidence for this has been suggested recently by the discovery of permeable junctional complexes in rabbit intestine (Machen et al., 1972).

Site of Intestinal Calcium Absorption

Intestinal absorption of calcium in chicks has been found to be greatest in the duodenum, and to decrease progressing distally in the small intestine *in vivo* (Taylor and Wasserman, 1967; Hurwitz et al., 1967). *In vitro*, using everted gut sacs, the opposite results have been found (Sallis and Holdsworth, 1962; Hurwitz et al., 1967). Hurwitz et al., attributed this discrepency to the thickness of intestinal muscle influencing the transport of calcium in everted gut sac preparations.

Discrimination Among Different Forms of Vitamin D in Chicks

Chicks have long been known to respond poorly to vitamin D_2 in comparison with vitamin D_3 (Steenbock et al., 1932). The ratio of antirachitic efficiency of D_2: D_3 was found to be 1:10 for chicks (Chen and Bosmann, 1964). Similarly, vitamin D_3 has been found to produce an intestinal response of calcium absorption ten times greater than that produced by vitamin D_2 in chicks, while no such discrimination in response is found in rats or humans (Suda et al., 1970). It appears that this discrimination occurs at the metabolic level resulting in rapid excretion of vitamin D_2 or its metabolites into the bile and feces (Imrie et al., 1967; Drescher et al., 1969).

Time Course of Vitamin D- Induced Intestinal Calcium Absorption

The time course of calcium absorption in chick duodenum exhibits a bi- or tri-phasic curve (Wasserman et al., 1971). After the injection of 100 IU of vitamin D_3 into rachitic chicks there is a lag of about eight to ten hours before calcium absorption increases by detectable levels. At about twelve hours calcium absorption has doubled, and it remains at this level until about sixteen to eighteen hours. By twenty-two to twenty-four hours calcium absorption has increased to more than three times the original level. Calcium absorption continues to rise slowly, so that by seventy-two hours it is about four times the original level and remaining fairly constant (Ebel et al., 1969; Wasserman et al., 1971).

The complexity of the curve exhibited by calcium absorption after vitamin D-induction appears to be the result of vitamin D's ability to influence intestinal calcium absorption in more than one manner (Wasserman et al., 1971). It influences at least two processes of calcium absorption: active transport and diffusion. Each of these systems may be quite complex, possibly involving carrier proteins, enzymes, permeability changes and structural reorganization of the luminal cell membrane.

Active Transport

Active transport of calcium across the wall of the small intestine against an uphill gradient was first demonstrated by Schacter and Rosen, using everted gut sacs *in vitro* (Schacter and Rosen, 1959; Schacter et al., 1961). They found this transport mechanism to be saturable, dependent upon oxidative phosphorylation, blocked by metabolic inhibitors, and impaired by vitamin D deficiency. Their results were supported *in vivo* by Wasserman and associates (Wasserman et al., 1961). Sallis and Holdsworth (1962) found that the administration of vitamin D to rachitic chicks stimulated Ca^{45} absorption, and that glycolysis was necessary as an energy supply for this process. They also suggested that enzymes were necessary as at $0^{\circ}C$ calcium transport was reduced to five per cent.

It has been suggested that active calcium transport, affected by vitamin D, occurs at the basal surface of the absorptive cell (Schacter et al., 1966). Martin and DeLuca (1969) found that calcium transport by everted gut sacs was greatly reduced when sodium was absent from the incubation medium. However calcium still entered the absorptive cell freely across the brush border, thus they suggested that sodium was required for active calcium transport across the serosal surface of the cell. On the other hand, Adams and Norman (1970) have suggested that a sodium-calcium diffusional exchange system

exists at the basal membrane and that it operates independently of vitamin D.

Adams and Norman (1970) have confirmed that vitamin D is essential in an active transport system for calcium operating at the luminal surface of the absorptive cell. They found that no active transport of calcium occurred in rachitic chicks, while in vitamin D supplemented chicks calcium was actively transported against an electropotential gradient. As Sallis and Holdsworth (1962) had found previously, anaerobiosis did not depress calcium transport in chicks. In rats the opposite has been found, with anaerobiosis depressing active calcium transport (Schacter and Rosen, 1959; Martin and DeLuca, 1969).

Diffusion

At about the same time as active transport of calcium was discovered, vitamin D was also found to increase the rate of diffusion across everted gut sacs *in vitro* (Harrison and Harrison, 1960). This process of calcium transport was not affected by metabolic inhibitors. Wasserman et al. found that vitamin D could stimulate both the influx and efflux of calcium across rachitic chick intestine (Wasserman and Kallfelz, 1962; Wasserman et al., 1966). Increasing the concentration of calcium in the lumen also stimulated the rates of influx and efflux and the system appeared to be unsaturable. They proposed that

vitamin D would affect mainly diffusion from the lumen by controlling the permeability of the apical membrane, by changing the membrane's structure, and/or inducing a calcium carrier system.

Similarly the intestinal mucosa has been proposed to act as a diffusion barrier. This barrier, which appears to be located at the luminal surface, is lessened by vitamin D (Harrison and Harrison, 1965). It has been suggested that the overall rate limiting step in calcium absorption is its rate of passage across the microvillar membrane (DeLuca, 1969; Adams et al., 1970).

Inhibition of Vitamin D-Induced Calcium Absorption by Actinomycin D

In 1964, Eisenstein and Passovoy found that vitamin D-induced hypercalcemia was not induced in the presence of actinomycin D, an inhibitor of DNA directed RNA synthesis (Eisenstein and Passovoy, 1964). Subsequently several investigators found that actinomycin D, puromycin, and cyclohexamide blocked increases in calcium absorption induced by vitamin D (Norman, 1965, 1966a; Schacter and Karowarski, 1965; Zull et al., 1965, 1966). Norman (1966) found that actinomycin D could be given any time up to five hours after vitamin D administration, yet increased calcium absorption would still be blocked. He also found that vitamin D-replete chicks could be transformed into functionally vitamin D-deficient chicks by giving them actinomycin D for thirty-six to forty-eight hours.

Lag

One of the first indications of the complexity of vitamin D-induced calcium absorption was the time lag observed between vitamin D administration to a rachitic animal and an observable increase in calcium absorption (Carlsson and Hollunger, 1954; Sallis and Holdsworth, 1962; Wasserman, 1962; DeLuca, 1967). In rachitic chicks the lag observed after vitamin D administration was eight to sixteen hours (Wasserman, 1962). This time period was too great to be accounted for as the length of time required for vitamin D to travel to the target tissue.

Part of the lag has been found to be necessary for the conversion of vitamin D into active metabolites. Most of the investigation concerning vitamin D metabolites, their identification, isolation, activity, and conversions has been carried on by two groups: one headed by H.F. DeLuca and the other by A.W. Norman. The biochemistry of vitamin D and its metabolites has been reviewed by DeLuca (1967; 1971a, b), and by Wasserman and Corradino (1971).

Active Metabolites of Vitamin D

Before vitamin D can act upon intestine it must undergo two activating conversions. Vitamin D is first converted into 25-hydroxycholecalciferol (25-HCC) (Blunt et al., 1968) in the liver (Ponchon and DeLuca, 1969; Ponchon et al., 1969; Horsting

and DeLuca, 1969). This conversion is probably carried out by a mitochondrial hydroxylation system, and requires the participation of the cytoplasm (DeLuca, 1971a). 25-HCC is further metabolized to 1,25-dihydroxycholecalciferol (1,25-DHCC) in the kidney (Fraser and Kodicek, 1970; Gray et al., 1971; Holick et al., 1971; Lawson et al., 1971). 1,25-DHCC is the metabolite of vitamin D which is active in intestine, and responsible for promoting calcium absorption (Holick et al., 1971; Frolick and DeLuca, 1971). It is now believed to be the hormonal form of the vitamin, responsible for the maintenance of serum calcium (Tanaka et al., 1972). Other metabolites of vitamin D have also been identified, but are not as active in intestine as 1,25-DHCC (DeLuca, 1971; Suda and DeLuca, 1972).

The conversion of vitamin D to 25-HCC is blocked by actinomycin D and cyclohexamide (DeLuca, 1969). Actinomycin D and cyclohexamide also block the conversion of 25-HCC to 1,25-DHCC, suggesting that 25-HCC induces the systhesis of a factor necessary to carry out the conversion (Tanaka and DeLuca, 1971). Originally it had been proposed that actinomycin D acted directly at the intestinal level, blocking the production of a vitamin D-induced protein (Zull et al., 1966). Now it appears that, at least in rats, the action of 1,25-DHCC is not blocked by actinomycin D at the intestinal level (Tanaka et al., 1971).

Although 25-HCC is more active than the parent vitamin,

and 1,25-DHCC is the most efficient metabolite in inducing intestinal calcium absorption (Suda and DeLuca, 1972), prior conversion of vitamin D into metabolites at sites other than intestine may not always be necessary. Schacter and associates (1964) found that a rapid calcium absorptive response could be produced when vitamin D was placed directly into rat duodenum. The addition of vitamin D to intestinal organ culture has also been found to produce calcium uptake directly by mucosa (Corradino and Wasserman, 1970, 1971). Thus it appears that some conversion of vitamin D to active metabolites could occur locally in intestine or that it may be possible to bypass the conversions to some extent.

Indications of Protein Synthesis in Vitamin D Action

As mentioned previously, the blocking of vitamin D action by actinomycin D implicated protein synthesis in vitamin D activity (Zull et al., 1965, 1966; Norman, 1965, 1966a; Schacter and Karowarski, 1965). This also indicated that the vitamin D molecule did not act directly in calcium transport.

After the administration of tritiated vitamin D, radioactivity was found localized in the nuclear fraction of intestinal mucosa (Haussler and Norman, 1967; Stohs and DeLuca, 1967). Haussler and Norman (1967) and later Lawson et al. (1969) found the radioactivity to be associated mainly with the chromatin,

while Stohs and DeLuca (1967) found the major site of label to be the nuclear membrane. Vitamin D was also found to produce rapid labeling of RNA in intestinal mucosa (Norman, 1966b; Stohs et al., 1967). Thus it became evident that vitamin D induced some type of protein synthesis necessary to carry out the process of calcium absorption.

Vitamin D-Induced Calcium Binding Protein

At about the same time as the actinomycin D and nuclear labeling experiments implicated the synthesis of a transport protein, a vitamin D-induced calcium-binding protein (CaBP) was identified by Wasserman and Taylor (Wasserman and Taylor, 1966; Taylor and Wasserman, 1967) in the supernatant of homogenated chick intestinal mucosa. The presence of this protein could be detected at about the same time as increased levels of calcium absorption were seen in vitamin D-injected rachitic chicks. Subsequently similar vitamin D-induced CaBPs were found to occur in the intestines of rat (Kallfelz et al., 1967; Drescher and DeLuca, 1971a), dog (Taylor et al., 1968), monkey (Wasserman and Taylor, 1971), cow (Fullmer and Wasserman, 1972), pig (Forsyth et al., 1972), and human (Hitchman et al., 1971). CaBP has also been found in the shell gland of laying hens (Corradino et al., 1968) and in chick kidney (Wasserman and Taylor, 1966; Taylor and Wasserman, 1967).

Rachitic chicks were found to have no intestinal CaBP, while rachitic chicks treated with vitamin D_3 had higher intestinal CaBP levels than normal (Wasserman and Taylor, 1966, 1968). Additional vitamin D given to normal chicks on normal diet produced no increase in CaBP synthesis or in calcium uptake (Taylor and Wasserman, 1969). Younger birds possessed higher CaBP levels than mature ones, as did laying compared to non-laying hens (Wasserman and Taylor, 1968).

The time course of appearance and abundance of CaBP were found to correlate well with the appearance and amount of calcium absorption induced by vitamin D_3 treatment of rachitic chicks (Ebel et al., 1969). A similar correlation was found by MacGregor et al. (1970) but not by Harmeyer and DeLuca (1970).

Normally CaBP is absent from chick intestine until the day of hatching (Corradino et al., 1969). In chicks placed on a rachitogenic diet immediately upon hatching, the amount of CaBP doubled for the first four days then decreased to nil at two weeks of age (Taylor and Wasserman, 1972). The synthesis of CaBP has been induced prematurely in organ culture of embryonic chick intestine to which vitamin D_3 was added directly (Corradino and Wasserman, 1970, 1971).

Drescher and DeLuca (1971b) have suggested that CaBP may not be newly synthesized, but the conversion product of a pre-existing protein. They based this on their discovery of a CaBP precursor with a higher molecular weight than CaBP. The

occurance of such a precursor in intestine would allow rapid synthesis of CaBP under the influence of vitamin D stimulation.

Adaption

The phenomenon of adaptation, whereby the efficiency of calcium absorption increases in response to a low calcium diet, was noted by Nicoleysen (Nicoleysen et al., 1953). According to Taylor and Wasserman CaBP was found to increase proportionately to the degree of adaptation shown by chicks (Taylor and Wasserman, 1969; Wasserman et al., 1971). Increased calcium absorption also occurred in chicks on phosphorous deficient but calcium adequate diets, despite high plasma calcium concentrations (Morrisey and Wasserman, 1971). These chicks showed undermineralized bones, indicating that the degree of mineralization of the skeleton may be a controling factor in adaptation.

Intestinal Localization of Vitamin D-Induced Calcium-Binding Protein

Using fluorescent antibody for CaBP, Taylor and Wasserman localized CaBP in chick intestine at the level of light microscopic examination (Wasserman et al., 1969; Taylor and Wasserman, 1970). They found specific fluorescence, indicating the presence of CaBP, over the goblet cells and the brush border region of both absorptive and goblet cells, in both vitamin D_3-treated rachitic and normal

untreated chicks. Non-specific fluorescence only was present over sections from rachitic chicks. The fluorescence over goblet cells decreased in the distal intestine, correlating with decreased levels of CaBP observed in this region (Taylor and Wasserman, 1967). They could not determine whether the brush border fluorescence was associated directly with the microvilli, with the glycocalyx, or with the mucous, but indicated that fluorescence could not be removed by washing. This suggested a firm association of CaBP with some structural component of the brush border. Taylor and Wasserman also suggested that CaBP was formed by the goblet cells, but did not completely rule out the possibilities that CaBP could be formed by both goblet and absorptive cells, or formed in the absorptive and transferred to the goblet cells.

The localization of CaBP at the brush border supported the proposal of Wasserman et al. (1966) that vitamin D may act at the luminal surface of absorptive cells by changing the membrane structure or effecting a calcium carrier system. Wasserman and associates (1969) suggested that CaBP may act in any of three fashions: as a diffusional facilitator, as an intracellular carrier, or as a microvillar concentrator of calcium.

CaBP has been found to complex with lysolecithin and in consequence the affinity of CaBP for calcium is reduced (Wasserman, 1970). Wasserman suggested that CaBP may react with the lecithin-lysolecithin cycle as a means of releasing calcium

in or near the surface membrane. As yet the exact manner in which CaBP functions is unknown.

Vitamin D-Induced Calcium-Dependent Adenosine Triphosphatase of Brush Border

Another protein which may participate in calcium absorption was discovered by DeLuca and his associates: a vitamin D-induced, calcium-dependent adenosine triphosphatase (Ca-ATPase) present in the brush border fraction of intestinal homogenates (Martin et al., 1969; Melancon and DeLuca, 1970). Ca-ATPase was found to be absent, or present only at low levels in the intestinal mucosa of rachitic chicks and rats. Vitamin D treatment was found to produce increases in the amount of Ca-ATPase and in the hydrolysis of ATP. Both increases were proportional to the increase in calcium absorption observed after vitamin D treatment. Magnesium, but not sodium, was required for the Ca-ATPase to function. Melancon and DeLuca (1970) compared the possible role of the Ca-ATPase to that of sodium-stimulated ATPases which supply the energy required for active transport of sodium. They concluded that Ca-ATPase would easily fit into the scheme of active transport of calcium from the lumen into the intestinal absorptive cell.

Alkaline Phosphatase Activity of Brush Border in Relation to Vitamin D

The brush border of intestinal epithelium is the site of alkaline phosphatase activity (Chase, 1963; Ito, 1965; Eicholz and Crane, 1965). Recently vitamin D was found to cause a two to three-fold increase in the levels of alkaline phosphatase in intestinal brush borders of rachitic chicks (Norman et al., 1970). The increase in alkaline phosphatase was found to follow the same time course as the vitamin D-induced increase in calcium absorption, and could be blocked by cyclohexamide. Norman et al. (1970) suggested that these results indicated *de novo* synthesis of alkaline phosphatase in response to vitamin D, and that it was probably involved in calcium absorption. They suggested that it might be necessary to alter another enzyme, such as an ATPase, functioning in a calcium-dependent system which required phosphorylation and hydrolysis.

Filipin Studies

Adams et al. (1970) found that the polyene antibiotic filipin acts upon intestinal mucosa in a manner which mimics that of vitamin D. Filipin, when incubated with rachitic chick epithelium *in vitro*, induced increased calcium transport from mucosa to serosa by an active process (Adams et al., 1970).

They suggested that filipin was able to reorganize the rachitic microvillar membrane structurally, thus activating a previously inactive calcium transport system.

It was also discovered that forty per cent more cholesterol occurred in microvillar membranes from normal than from rachitic chicks (Adams et al., 1970). As filipin required membrane sterol to react, they suggested that proteins occurring in the microvillar region might cover or expose membrane sterol to various extents, and that this might somehow play a role in calcium absorption across the apical membrane.

Wong et al. (1970) found that exposure of intestinal mucosa to filipin for more than eighty minutes produced gross morphological changes in the brush broders of vitamin D-deficient chicks only. This appeared to indicate that a fundamental difference existed between vitamin D-deficient and vitamin D-treated membranes. This was consistant with the hypothesis proposed by Adams et al. (1970) that vitamin D may act by changing the microvillar membrane structure, through the synthesis of proteins which would be active either structurally or catalytically in the microvillar membrane.

Other Factors Influencing Vitamin D-Induction of Intestinal Calcium Absorption

Strontium has long been known to have rachitogenic properties (Shipley et al., 1922). Corradino and Wasserman (1970) found that strontium in the diet of vitamin D_3 replete chicks produced decreased calcium absorption and CaBP production. Subsequently Omdahl and DeLuca (1972) found the inhibitory effect of dietary strontium to be due to a block in the conversion of 25-HCC to 1,25-DHCC in the kidney.

Vitamin D-induced calcium absorption may be inhibited by calcitonin (Olsen et al., 1972). The evidence for this however has been conflicting (Winter et al., 1970), and may be due to the observations of Olsen et al. (1972) that small doses of calcitonin produced inhibition of vitamin D-induced calcium absorption, while large doses produced the opposite effect.

Parathyroid hormone apparently does not affect intestinal calcium absorption induced by vitamin D (Morrisey and Wasserman, 1971; Pavlenko et al., 1972). However some factor elaborated by bone may influence vitamin D-induced intestinal calcium absorption, as indicated by adaptation shown by chicks on low phosphorous diets, even though their serum calcium levels were high (Morrisey and Wasserman, 1971).

Relationship Between Vitamin D Action and Turnover of Intestinal Epithelium

Intestinal epithelial cells have been shown to undergo continuous migration and replacement along the villi, so that the whole population is rapidly and continually renewed (Leblond and Stevens, 1948; Leblond and Messier, 1958). Complete turnover of the cell population on the duodenal villus in chick requires ninety to ninety-eight hours (Imondi and Bird, 1966).

Spielvogel et al. (1972) found that administration of vitamin D for more than 110 hours produced increased villus length in normal and rachitic chicks. However the cells travelled faster so that their turnover times did not change. They concluded that vitamin D can act only on fully differentiated intestinal cells, which are well advanced in their migration up the villus, but does not cause changes in cell size, number or migration rate. They also suggested that the decay or decrease in response to vitamin D administration after long time intervals may be partly due to the turnover of fully differentiated cells. Similarly Reddy (1972) found that germfree rats, which have a longer mucosal life span, showed elevated levels of CaBP, Ca-ATPase, Mg-ATPase, alkaline phosphatase, and of calcium and magnesium absorption.

Many aspects of vitamin D induction of calcium absorption remain as mysteries. It is not known how the systems of diffusion and active transport of calcium interrelate: whether they are initiated by different stimuli, whether they operate under different ranges of conditions, and whether they operate at the same time or as different steps of the same absorptive process. It is also unknown how calcium absorption is altered in adaptation, what feedback stimuli from the rest of the organism affect calcium absorption in the intestine, and what parts of the absorptive process are affected.

How calcium enters the cell, whether it travels complexed to a carrier protein, or is passed from one carrier to another, is also unknown. Neither is it clear how carriers are able to capture and release calcium, or where this occurs. Another question is whether carriers repeat their journeys several times or whether they can only function once and are then destroyed.

The pathways which calcium follows once inside the absorptive cell are unknown. It appears that calcium is stored in the mitochondria when intracellular calcium concentrations rise (Sampson et al., 1970; Borle, 1971). However it is not known how long calcium is stored in the mitochondria, whether the stored calcium is turned over rapidly, or whether this storage is actually a compartment of the calcium transport system.

Another large gap in the knowledge of vitamin D induction of calcium absorption is at the level of the exit of calcium from the absorptive cell. It is not clear whether this process is vitamin D dependent or not, or whether it may be partially vitamin D dependent. Also the mechanisms by which calcium moves across the basal surface are unclear.

The role of the intercellular spaces in the passage of calcium across the intestinal epithelium is another unanswered question. These spaces may represent a system for bypassing the absorptive processes which are centered in the absorptive cell.

Finally, only a few studies have dealt with the subject of vitamin D-induced calcium absorption in relation to the morphology of the intestinal epithelium: Sampson et al. (1970), Taylor and Wasserman (1970), Wong et al. (1970), and Spielvogel et al. (1972). These have covered a few aspects of the effects of vitamin D on intestinal epithelium: the electron microscopic localization of calcium on the brush border and in mitochondria of rat absorptive cells (Sampson et al., 1970); the immunofluorescent localization of CaBP in intestinal epithelium at the light microscopic level (Taylor and Wasserman, 1970); the effects of filipin on the brush border of absorptive cells (Wong et al., 1970); and the relationship between vitamin D-treatment and the turnover of chick intestinal epithelium (Spielvogel et al., 1972). As yet there have been no studies at the electron microscopic level of the effects of vitamin D on the entire absorptive or

goblet cell.

The aim of this study is to add to the present morphological data concerning the action of vitamin D in intestinal epithelium, thereby adding some new understanding to the present knowledge of vitamin D-induced intestinal calcium absorption which has been obtained through biochemical and physiological studies. It is also hoped that this study may provide some basis for new areas of biochemical, physiological, and morphological investigations concerning vitamin D-induced calcium absorption by intestinal epithelium. The present thesis describes in detail the morphology of intestinal epithelium of chick, an animal which has been used frequently in biochemical and physiological studies of vitamin D induction of calcium absorption at the electron microscopic level. The intestinal epithelia of rachitic chicks and chicks adapted to a low calcium diet have also been studied. Changes in the intestinal epithelium of rachitic chicks have been investigated at various time intervals after vitamin D administration when increased calcium absorption is known to occur. It has also been attempted to visually localize calcium in the intestinal epithelial cells of rachitic and vitamin D-injected chicks at the electron microscopic level.

CHAPTER TWO

MATERIALS AND METHODS

a. Vitamin D_3 Repletion Experiments

White Leghorn cockerels were placed on a rachitogenic test diet (Catalogue Number 170660; General Biochemicals, Chagrin Falls, Ohio) two days after hatching. After four weeks on this diet half of the chicks were injected intramuscularly with 500 IU of vitamin D_3 (Sigma Chemical Co., St. Louis, Mo.) dissolved in 0.5 ml. propylene glycol. The remaining chicks were injected with equal amounts of propylene glycol alone. At intervals of 12, 18, 24, 48, and 72 hours following injection chicks from both groups were anesthetized by intraperitoneal administration of ½ ml./100 gm. body weight of 7% chloral hydrate in aqueous solution. They were perfused through the ascending aorta with cold Karnovsky fixative, modified after Sotelo and Palay (1968). This solution contained:

1% paraformaldehyde,

1.25% gluteraldehyde, and

0.05% calcium chloride

buffered to pH 7.4 with 0.067 M sodium cacodylate buffer. The duodenal loop was removed from each animal, cut into small pieces

to facilitate exposure of the villi to the fixative, and further fixed in the same fixative for two hours in the cold.

Following fixation the tissues were washed, for at least two hours, with several changes of Millonig's buffer (Millonig, 1961), then post-fixed for one hour in 2% osmium tetroxide in Millonig's buffer. Following post-fixation, the tissues were dehydrated through ten minute changes of: 30, 50, 70, and 90% ethyl alcohol at 4°C; and two changes each of absolute ethyl alcohol and propylene oxide at room temperature. The tissues were then embedded in Araldite 502 (Ladd Research Industries) after Luft (1961) and Coulter (1967).

Thick (1 μ) sections were cut using glass knives on an LKB Ultrotome III, picked up with a wire loop, placed on glass slides, and stained with 0.5% toluidine blue in 1% borax by brief heating on a hot plate. The sections were rinsed with distilled water, dried, then examined by light microscopy for the purpose of orientation.

Thin (about 600 Å) sections were made of suitable areas using glass or diamond knives on an LKB Ultratome III, and picked up on uncoated 200 mesh copper grids. The sections were stained for 40 minutes with a saturated solution of uranyl acetate in 50% ethyl alcohol, then for 5 to 7 minutes using lead citrate, after Reynolds (1963). The grids were then examined at 60 kv in a Phillips EM 300.

b. Normal and Low-Calcium Diet Chicks

This experiment was carried out in cooperation with Dr. D. Harold Copp, of the Department of Physiology, University of British Columbia. The chicks used in this experiment were raised on low-calcium and normal diets in Dr. Copp's laboratory.

White leghorn cockerels were raised for four weeks subsequent to hatching on the following low-calcium diet:

31.5 kg. ground yellow cornmeal

12.9 kg. soybean meal

0.9 kg. distilled dry solubles

0.9 kg. dehydrated grass

0.2 kg. iodized NaCl

0.2 kg. bone meal

0.62 gm. vitamin A palmitate (325,000 IU/gm.)

3.4 gm. riboflavin (24 gm./lb.)

6.0 gm. feed grade $MnSO_4 \cdot 2\ H_2O$

1.0 gm. niacin

45.4 gm. dl-methionine.

The total calcium and phosphorous contents of this diet were 0.236% and 0.447% respectively.

From hatching, the chicks were divided into two groups and their diets supplemented as follows: the normal chicks received supplements of 0.4 IU vitamin D_3/gm. of diet, and 20 gm. $CaCO_3$/gm.

of diet; the low-calcium chicks received only the supplement of 0.4 IU vitamin D_3/gm. of diet.

At four weeks of age the chicks were anesthesized, perfused, and their duodenal loops taken and prepared for electron microscopy as described in the previous section.

c. Mineral Visualization

Small pieces of duodenum from rachitic and twenty-four hour vitamin D_3-replete chicks were fixed by immersion in S-collidine buffered paraformaldehyde, after the technique of Sampson et al. (1970). This fixative contained 4% paraformaldehyde with 5 mM $Cacl_2$ added, and was buffered to pH 7.4 with 0.067 M S-collidine buffer (Bennett and Luft, 1959). The tissues were stored for twenty-four hours in this fixative, rinsed with S-collidine buffer, then post-fixed for one hour in 1.5% osmium tetroxide in 0.067 M S-collidine buffer (pH 7.4) after Sampson et al. (1970). Dehydration and embedding for electron microscopy were carried out as previously described.

Prior to post-fixation, some tissues from each group were washed several times with S-collidine buffer, then placed in 4.13% disodium ethylenediamine tetraacetate (EDTA). The pH of the EDTA was brought to 7.4 with NaOH as suggested by Warshawsky and Moore (1967). This solution was changed several times during the twenty-four hours of treatment. The tissues were then rinsed

many times with S-collidine buffer, and post-fixed for one hour in 1.5% osmium tetroxide in 0.067 M S-collidine buffer (pH 7.4). Dehydration and embedding were carried out in the same manner as for the tissues which were not treated with EDTA.

The material was sectioned as described previously and unstained sections from each group were examined at 60 kv in a Phillips EM 300. Following this some sections were stained with uranyl acetate and lead citrate, as described in the first section, then examined electron microscopically.

d. Acid Phosphatase Localization

Pieces of duodenum from rachitic and twenty-four vitamin D_3-replete chicks were used for the demonstration of acid phosphatase activity using the method of Gomori, as modified by Barka and Anderson (1963). The tissues used in this demonstration were fixed by perfusion with cold Karnovsky fixative, modified by Sotelo and Palay (1968) as described in section (a).

Light Microscopy

Following fixation thick sections (7 μ) were cut using an IEC Microtome-cryostat, picked up on glass slides, coated with Formvar and allowed to dry. The slides were incubated for 30 to 120 minutes in an incubation mixture prepared according to

Barka and Anderson (1963). This incubation mixture contained:

10 ml. 0.1 M tris-maleate buffer, pH 5.0

10 ml. 1.25% sodium β glycerophosphate, pH 5.0

10 ml. distilled water

20 ml. 0.2% lead nitrate (added drop by drop to the solution composed of the first three substances).

An incubation time of thirty minutes produced the best results. Following incubation the slides were washed, treated with 0.5 to 1.0% ammonium sulfide in aqueous solution, washed with water, and then mounted in glycerin jelly prior to examination with the light microscope.

Electron Microscopy

Following fixation, 50 μ sections of the tissue were cut using a Sorvall Tissue Sectioner. These were then incubated in an incubation mixture, of the same composition as that used for acid phosphatase determination at the light microscopic level, for thirty minutes. Following incubation these tissues were post-fixed, dehydrated, embedded, and sectioned for electron microscopic examination as described in Section (a). Unstained sections were examined in a Phillips EM 300, then some sections were stained with uranyl acetate and lead citrate, as previously described, and re-examined.

CHAPTER THREE

RESULTS

The duodenal villi of the chick are covered by a simple columnar epithelium. It is composed of two cell types: absorptive cells and goblet cells.

I Absorptive Cells

A. Normal Chicks

General Morphology

The absorptive cells are columnar in shape with a height of about 40 μ (Figure 1). In cross section they are generally hexagonal in shape with a width of about 7 μ (Fig. 2). Their luminal surface is covered by a brush border. Near the lumen the lateral surfaces of apposing cells are held in close contact by elements of the junctional complexes. Below the complexes the cell membranes form interdigitating folds. The basal surfaces are fairly smooth and rest upon a thin basement lamina.

For descriptive purposes the cytoplasm may be divided into four areas: 1) the brush border; 2) the terminal web; 3) apical cytoplasm; and 4) basal cytoplasm. The last two

regions are separated by the nucleus, which is located below the midpoint of the longitudinal axis of the cell.

1. The Brush Border

The brush border of the absorptive cells is visible with the light microscope and is composed of microvilli (Granger and Baker, 1950). These microvilli have been studied extensively in many animals (Dalton et al., 1951; Zetterqvist, 1956; Palay and Karlin, 1959; Brown, 1962; McNabb and Sandborn, 1964; Ito, 1965a, 1969) including chick (Overton and Shoup, 1964; Lecount and Grey, 1972).

The microvilli are straight fingerlike outgrowths, projecting from the apical surface of the absorptive cell at an angle of about ninety degrees (Fig. 4). In longitudinal sections they appear uniformly spaced and in cross sections they appear equidistant from each other, each microvillus being surrounded by 6 others (Fig. 3). Each microvillus is about 1.8 μ in length and about .09 μ in diameter. The plasma membrane covering the microvilli shows clearly a trilaminar structure and is about 110 Å in width. A mat of very fine filaments, which radiate from the outer surface of the plasma membrane, covers the entire microvillar surface (Figs. 3, 4). This coat has been termed fuzz or glycocalyx by Ito (1965a, 1969), and is considered to be an integral part of the plasma membrane. In chicks, the glycocalyx extends

to a height of 800 to 1100 Å beyond the apical tips of the microvilli.

The core of each microvillus contains many fine filaments which run parallel to its long axis (Zetterqvist, 1956; Palay and Karlin, 1959) (Figs. 3, 4). These filaments extend into the apical cytoplasm as rootlets (Fig. 4), and may be continuous with some of the filaments of the terminal web, as suggested by McNabb and Sandborn (1964).

2. The Terminal Web

The filaments of the terminal web form a network which is arranged in a band across the apical cytoplasm of the absorptive cell (Figs. 4, 5). These filaments are similar to those of the microvillar core in size and in electron opacity. However they differ in orientation so that individual filaments can be followed for only short distances in longitudinal sections of the cell, as described earlier by Cardell et al. (1967). At the level of the terminal web the lateral junctional complexes are seen holding the membranes of adjacent cells in close apposition (Figs. 6, 7). These structures have been found to be similar to those described by Farquhar and Palade (1963).

Between the bases of the microvilli inpocketings of the apical plasma membrane are seen which have been called pinocytotic pits (Palay and Karlin, 1959) or apical pits (Cardell

et al., 1967) (Figs. 4, 6). The terminal web cytoplasm contains rounded vesicles of .06 to .13 μ in diameter, which possess a unit membrane similar to the plasma membrane (Figs. 4, 5). The contents of these vesicles are slightly electron dense. No other organelles except a few scattered polyribosomes and occasionally endoplasmic reticulum of the agranular type are seen in the region of the terminal web.

3. The Apical Cytoplasm

a. Mitochondria

The most obvious organelles of the apical cytoplasm are the mitochondria. They are usually rod-shaped, but may be spherical or curved, or even sometimes show branching (Fig. 8). They have a diameter of approximately .32 μ and may reach more than 4 μ in length. They resemble previously described mitochondria in fine structure (Palade, 1953; Zeterqvist, 1956). Their cristae are oriented transversely, and slightly electron dense granules of up to approximately 600 Å in diameter are seen within their matrix (Fig. 9). In general the mitochondria are most often seen oriented parallel to the long axis of the cell.

b. Lysosomes and Related Bodies

On the basis of their morphology, four distinct types of vesicular bodies are distinguishable: 1) multivesicular bodies; 2) dense bodies; 3) heterophagic or autophagic vacuoles; and 4) peroxisomes.

1. Multivesicular bodies (described as Component II by Zetterqvist, 1956) possess a single unit membrane which encloses several smaller vesicles, each surrounded by its own unit membrane (Sotelo and Porter, 1959) (Fig. 9). Multivesicular bodies are usually about 0.3 to 0.5 μ in diameter, while the smaller vesicles within them are about 0.05 μ in diameter and contain slightly electron dense contents. They are usually seen below the terminal web region.

2. Dense bodies are usually rounded with quite electron dense contents, which appears homogenous in nature (Fig. 11). They are delimited by a single unit membrane and may vary in size, but are usually about 0.5 μ in diameter. They are usually seen between the region of the terminal web and the Golgi apparatus.

3. Autophagic and heterophagic lysosomes are indistinguishable from each other and may be collectively termed secondary lysosomes (DeDuve and Wattiaux, 1966). These vary in size from 0.5 to more than 1.0 μ in diameter and may be rounded or lobular in shape. Their contents include vesicles, ribosomes, portions of mitochondria and endoplasmic reticulum as well as small

electron dense granules and amorphous materials of varying electron density (Figs. 8, 10, 12). One or more unit membranes may surround secondary lysosomes and often they show some degree of myelination of their contents. Secondary lysosomes usually occur between the terminal web and Golgi regions.

4. Peroxisomes or microbodies closely resemble dense bodies in size and in the appearance of their contents especially when observed at low magnifications. The contents of peroxisomes appears slightly less dense and more uniform than that of dense bodies (Fig. 13). Some sections of peroxisomes reveal the presence of a core composed of a bundle of tubule-like structures (Fig. 14a). The membranes of peroxisomes are often seen to be continuous with those of the agranular endoplasmic reticulum (Fig. 14) as previously described by Novikoff and Novikoff (1972). Peroxisomes usually appear scattered in the apical cytoplasm, and several are often seen close to the nucleus (Fig. 15).

c. Endoplasmic Reticulum

i. Agranular Type

Intermingled with the large organelles of the apical cytoplasm are many elements of agranular endoplasmic reticulum. These are most readily visible below the terminal web region (Figs. 4, 5, 13, 14). Some are seen as tubular cisternae, while

many are seen as rounded vesicles of up to 0.1μ in diameter. These are more irregular in outline and have a thinner limiting membrane than the apical vesicles described previously in the terminal web region. The contents of the agranular cisternae appear slightly electron dense. Occasionally they are seen to contain lipid droplets, as described by Cardell et al. (1967) (Fig. 13).

ii. Granular Type

Many cisternae of granular endoplasmic reticulum are dispersed in the apical region, appearing as ribosome studded vesicles or short cisternae (Figs. 8, 13). The material within the cisternae appears slightly electron dense. The granular endoplasmic reticulum cisternae are often seen in close proximity to mitochondria. Occasionally they are seen as small groups of parallel cisternae. Sometimes continuities between agranular and granular endoplasmic reticulum are seen (Fig. 14).

Many free polyribosomes are scattered throughout the apical cytoplasm (Figs. 8, 15).

d. Golgi Apparatus

The Golgi apparatus occupies a supranuclear position (Fig. 1). It resembles previously described Golgi complexes (reviewed by Favard, 1969) and is composed of the usual saccules,

vesicles, and vacuoles (Fig. 15). The vesicles and vacuoles appear to contain slightly electron dense material. The Golgi apparatus of intestinal absorptive cells does not appear as prominent as that of secretory cells.

e. Microtubules

In the apical cytoplasm microtubules are seen oriented approximately parallel to the long axis of the cell (Fig. 17). They also appear among the filaments of the terminal web, as described by Cardell et al. (1967). These microtubules appear similar to those described previously in other cell types by Ledbetter and Porter (1963), and De-Thé (1964).

4. Nuclear Region

The nuclei of absorptive cells are oval when seen in longitudinal section, their long axes parallel to those of the cells (Fig. 1). They measure about 10 μ in length and about 4 - 5 μ in width. Their margins are fairly smooth or slightly irregular and dense clumps of chromatin are seen attached to the inside of the nuclear envelope. The double nuclear envelope shows nuclear pores, similar to those described by Watson (1955, 1959) (Fig. 16). The outer surface of the envelope is ribosome studded. One or more nucleoli are usually visible.

The narrow cytoplasmic regions on either side of the nucleus, when seen in longitudinal sections, contain mitochondria, granular and agranular endoplasmic reticulum, and free polyribosomes.

5. Basal Cytoplasm

Below the nucleus mitochondria similar to those of the apical cytoplasm are seen (Fig. 18). Most of them appear to be cut transversely or obliquely in the longitudinal sections of absorptive cells. Cisternae of granular endoplasmic reticulum are also evident in this region. Usually they are seen in close proximity and lying parallel to the contours of mitochondria. Dense bodies and peroxisomes similar to those of the apical region are also seen occasionally in the basal cytoplasm. Many vesicles of agranular endoplasmic reticulum and many free polyribosomes are visible. The most basal portion of the cytoplasm often appears devoid of organelles other than smooth walled vesicles and free polyribosomes.

6. Intercellular Space

Below the juxta-luminal junctional complex the lateral surfaces of adjacent cells exhibit elaborate interdigitations (Fig. 2). Their membranes are held in close apposition for most

of the length of the cells by numerous desmosomes (Farquhar and Palade, 1963) (Fig. 18). Towards the basal region, large intercellular spaces of up to 0.2 μ in width are seen (Fig. 1). These contain slightly electron dense material. In the cytoplasm some vesicles are visible in contact with the plasma membrane in this region (Fig. 19).

Underlying the basal surfaces of the cells there is a continuous basal lamina of about 300 Å in width (Fig. 20). It appears to be composed of very fine filamentous material of low electron density.

B. Experimental Chicks

The ultrastructure of the duodenal absorptive cells was examined in the following experimental groups: rachitic chicks; vitamin D_3-replete chicks, injected with a single dose of vitamin D_3 at 12, 18, 24, 48, and 72 hours before sacrifice; and chicks raised on a low calcium diet while receiving normal amounts of vitamin D_3. Tissue from each of these groups received the same type of fixation, gluteraldehyde and paraformaldehyde (regular fixation), as did tissue from the normal chicks.

Some aspects of the ultrastructure of the absorptive cells remained similar to the normal situation in all groups, despite experimental treatment. No changes could be discerned in the morphology or size of the nuclei in any group when compared

with the normals. Free polyribosomes were seen throughout the cytoplasm of all groups. Their abundance and appearance did not appear to be affected by experimental treatment. Microtubules were also seen in the cytoplasm of all groups and these did not seem to show any changes in morphology, orientation, or abundance with experimental treatment.

Experimental treatment caused the appearance and/or abundance of some organelles to differ from the situation seen in the normal group. These changes are described in the following sections.

Duodenal tissue from two experimental groups, rachitic chicks and twenty-four hour vitamin D_3-replete chicks, was also fixed using the S-collidine buffered paraformaldehyde technique of Sampson et al. (1970). When material fixed using this technique was examined, electron dense deposits were seen in association with various regions of the absorptive cells. The appearance and location of these deposits will be described under the headings of the various organelles concerned, following the descriptions of their morphologies after experimental treatment and regular fixation.

1. The Brush Border

a. Regular Fixation

In all experimental groups the microvilli appeared to resemble those of the normal animals with respect to morphology, shape, and appearance of their core filaments and rootlets. However the height of the brush border appeared to vary in different experimental groups. The average height of the microvilli appeared to be least in rachitic chicks, about 1.1 μ (Fig. 28) and greatest in eighteen hour vitamin D_3-replete chicks, about 1.9 μ in height (Fig. 30). In the other experimental groups the following average microvillar heights were found: 12 hours, about 1.3 μ ; 24 hours, about 1.5 μ ; 48 hours, about 1.7 μ ; 72 hours, about 1.7 μ ; and low calcium chicks about 1.8 μ (Figs. 27, 29, 31, 32). The rachitic and twelve hour chicks showed the greatest differences in microvillar height compared to the average height seen previously in the normal animals (approximately 1.8 μ) (Fig. 4). The diameter of the microvilli was about the same in both the normal animals and in the experimental groups: approximately .09 μ .

No changes were observed in the structure or distribution of the glycocalyx with experimental treatment.

b. S-Collidine Buffered Paraformaldehyde Fixation

When S-collidine buffered paraformaldehyde fixation technique of Sampson et al. (1970) was used, large electron dense

granules were seen on the microvilli of both rachitic and twenty-four hour vitamin D_3-replete chicks (Figs. 35, 36). These granules varied in size, the largest reaching about 650 Å in diameter. Some large granules appeared to be composed of smaller deposits, and often two or three large granules were seen grouped together. Most of the granular deposits appeared to be located on the outer surfaces of the microvilli rather than inside the microvillar membrane, in both experimental groups.

Most of the electron dense granules seen on the microvilli of vitamin D_3-replete animals were large (Fig. 36), while many more small granules were seen on the rachitic microvilli (Fig. 35). The ratio of granules seen on the rachitic brush border to those seen on the vitamin D_3-replete brush border was approximately 6:1.

The general appearance and electron density of these granules did not seem to be changed when sections were stained with uranyl acetate and lead citrate (Fig. 37). Treatment of the tissue with EDTA for twenty-four hours between fixation and post-fixation caused nearly all of the electron dense granules to be removed from the microvilli of both rachitic and vitamin D_3-replete chicks.

Very fine electron dense deposits were seen over most of the plasma membrane of the brush border in both rachitic and vitamin D_3-replete chicks (Figs. 35, 36). These appeared as lines of increased electron density associated with either leaflet of the

plasma membrane. In the vitamin D_3-replete chicks these deposits seemed to be more evident than in the rachitic chicks.

When sections were stained with uranyl acetate and lead citrate the plasma membrane gained contrast and the fine deposits became less apparent (Fig. 37). EDTA treatment appeared to remove most of the fine deposits from the membranes of both groups (Fig. 38).

2. The Terminal Web

a. Regular Fixation

All experimental groups possessed a reticulum of fine filaments extending across the apical portion of their cytoplasm. In every experimental group this filamentous web appeared morphologically similar to that of the normal chicks.

Apical pits and apical vesicles were seen in the terminal web region of all groups. These were always similar in size and appearance to those seen in the normal group. All groups also possessed free polyribosomes scattered throughout this region, as well as cisternae of agranular endoplasmic reticulum, which were seen chiefly in the lower region of the terminal web. Both the free polyribosomes and the agranular endoplasmic reticulum did not seem to differ from the normal state in either morphology or abundance in any experimental group.

In rachitic chicks and chicks treated with vitamin D_3 for twelve hours, fewer apical pits and vesicles were seen than in normal animals (Fig. 28). By eighteen hours the number of these apical pits and vesicles appeared to have increased (Figs. 30, 39). At twenty-four hours these structures seemed to be seen as often as at eighteen hours (Fig. 31). By forty-eight hours their frequency of appearance seemed to have decreased, and appeared to resemble the situation seen in the normal animals (Figs. 32, 33). Chicks which were fed a low calcium diet seemed to show apical pits and vesicles as frequently as did the eighteen and twenty-four hour vitamin D_3-replete chicks (Figs. 34, 40).

b. S-Collidine Buffered Paraformaldehyde

In duodenum which was fixed with S-collidine buffered paraformaldehyde, large electron dense granules similar to those seen previously on the microvilli of similarly fixed tissue were seen in some of the apical pits (Fig. 35). Their occurrance was infrequent in both rachitic and vitamin D_3-replete chicks. Such granules were never seen within the apical vesicles of either group.

Fine electron dense deposits similar to those seen previously on the plasma membrane of the microvilli were sometimes seen on the limiting membranes of the apical vesicles (Figs. 35, 36). This seemed to be more apparent in the twenty-four hour vitamin D_3-replete than in the rachitic chicks.

After EDTA treatment no large electron dense granules were seen in the apical pits (Fig. 38). Fine electron dense deposits which had been seen on the membranes of the apical pits and vesicles appeared to have been removed, but incompletely, by EDTA. Removal of the large granules and most of the fine membrane deposits by EDTA occurred in both rachitic and vitamin D_3-treated material.

3. Apical Cytoplasm

a. Mitochondria

i. Regular Fixation

Rachitogenic diet and vitamin D_3-repletion appeared to exert no effect on the mitochondria of the apical cytoplasm. When the mitochondria of these animals were compared to those seen previously in the absorptive cells of normal chicks no differences could be discerned in mitochondrial shape, size, or ultrastructure, or in the frequency of appearance and general orientation of the mitochondria (Figs. 21 - 26, 42).

Chicks which were fed a low calcium diet also possessed mitochondria in their apical cytoplasm which resembled those seen in other groups with respect to shape, size, number and orientation. Granules within the matrix of the mitochondria of low calcium diet chicks appeared larger (up to 1200 Å) and more electron dense

than those seen in other groups (Figs. 40, 41). Also granules within the matrix appeared to be more abundant in this group as often several granules could be seen in a cross section of a single mitochondrion.

ii. S-Collidine Buffered Paraformaldehyde Fixation

In rachitic duodenum fixed with S-collidine buffered paraformaldehyde pale granules of up to 600 Å were seen within the mitochondrial matrix (Fig. 43). These granules resembled those seen previously in the matrix of normal, rachitic, and vitamin D_3-replete animals in both size and electron density.

In twenty-four hour vitamin D_3-replete duodenum fixed with S-collidine buffered paraformaldehyde both the electron density and the frequency of appearance of such granules appeared to increase (Fig. 44). They again measured about 600 Å in diameter, smaller than many of the matrix granules seen previously in mitochondria of chicks fed a low calcium diet.

Uranyl acetate and lead citrate staining appeared to increase the electron density of these granules in both rachitic and vitamin D_3-replete tissues fixed with S-collidine buffered paraformaldehyde (Fig. 45). EDTA treatment between fixation and post-fixation of this tissue appeared to alter the granules in twenty-four hour vitamin D_3-replete chicks. After EDTA treatment these granules appeared to be less electron dense and more closely

resembled the pale granules seen within the mitochondrial matrix of rachitic tissue fixed with S-collidine (Fig. 46). EDTA treatment did not appear to affect the appearance of the mitochondrial granules of the rachitic chicks.

b. Lysosomes and Related Bodies

i. Regular Fixation

All experimental groups exhibited the four types of vesicular bodies which were seen in the normal animals: 1) multivesicular bodies, 2) dense bodies, 3) secondary lysosomes, and 4) peroxisomes. The morphologies of all of these bodies were similar to those seen in the normal animals. However, the relative numbers of these bodies were seen to change with experimental treatment.

1) Multivesicular Bodies

Multivesicular bodies were seen in small numbers in all experimental groups. In rachitic animals they did not appear to be present as frequently as in the normal chicks. They continued to appear with less frequency until twenty-four hours after vitamin D_3 repletion when they were seen as often as in the normal animals (Fig. 47). This situation seemed to continue through to seventy-two hours after vitamin D_3 repletion. Chicks which were fed a low calcium diet seemed to possess slightly more multivesicular bodies than did normal animals (Fig. 40).

2) Dense Bodies

Dense bodies seemed to appear more frequently in rachitic than in normal animals (Figs. 21, 48). They remained at about the same level of abundance as in the rachitic animals at twelve (Figs. 22, 49) and eighteen hours (Figs. 23, 50) after vitamin D_3 injection. At twenty-four hours their frequency of appearance seemed to decrease (Fig. 24). A further decrease in their number seemed to have occurred by forty-eight hours. In the forty-eight (Fig. 25) and seventy-two hour (Fig. 26) vitamin D_3-replete chicks and in the low calcium chicks (Fig. 27), dense bodies were seen as frequently as in the normal animals.

3) Secondary Lysosomes

Secondary lysosomes seemed to appear less frequently in rachitic than in normal chicks (Fig. 21). At twelve (Fig. 22) and eighteen hours (Fig. 23) after vitamin D_3 injection secondary lysosomes appeared to become slightly more common than in the rachitic chicks. This was especially evident in the cells which exhibited the most Golgi activity. By twenty-four hours secondary lysosomes appeared to have undergone a further increase and were seen more often than the dense bodies (Figs. 24, 51). The situation remained similar at forty-eight (Figs. 25, 52) and seventy-two hours (Figs. 26, 53) at which times these groups seemed to have received further increases in secondary lysosomes. Low calcium

chicks appeared to resemble these last two groups in the extent of its secondary lysosome population (Figs. 27, 41, 53).

4) Peroxisomes

Peroxisomes seemed to appear more frequently in rachitic than in normal animals, especially in the region of the apical cytoplasm immediately above the nucleus (Figs. 21, 66). At twelve (Figs. 22, 67) and eighteen hours (Figs. 23, 68) after vitamin D_3 repletion these appeared as often or slightly more often than in the rachitic animals. By twenty-four hours their frequency of appearance seemed to have decreased compared to the previous experimental groups (Figs. 24, 69). This trend of decrease seemed to continue and by seventy-two hours their numbers were only slightly greater than in normal chicks (Figs. 25, 26, 70, 71). Animals which were fed a low calcium diet appeared to have as many peroxisomes as did the forty-eight and seventy-two hour groups (Figs. 27, 53).

ii. Acid Phosphatase Localization

Acid phosphatase activity was demonstrated using the Gomori method (as modified by Barka and Anderson, 1963). This produced dense precipitate over the sites of acid phosphatase activity.

At the light microscope level acid phosphatase activity

in the absorptive cells was seen as rounded granules which were concentrated together in a band below the terminal web region. Single granules were also seen scattered below this band, mainly in the upper half of the absorptive cells. Both the band of dark granules and scattered vesicles were seen in rachitic (Fig. 55) and in twenty-four hour vitamin D_3-replete chicks (Fig. 56), the only two groups which were used for acid phosphatase demonstration. No qualitative or quantative differences could be ascertained in the distribution of acid phosphatase granules between rachitic and vitamin D_3-replete chicks.

At the electron microscopic level acid phosphatase activity, indicated by the presence of electron dense precipitate, was localized in vesicular bodies which occupied locations corresponding to those seen with the light microscope. Most of the acid phosphatase positive vesicles were seen below the region of the terminal web in a region corresponding to that occupied by the band of granules seen with the light microscope (Figs. 57, 58, 59).

In both rachitic and twenty-four hour vitamin D_3-replete chicks precipitate indicative of acid phosphatase activity was apparently located over dense bodies and secondary lysosomes. Very little precipitate was seen over the multivesicular bodies and none was seen over the peroxisomes.

In rachitic chicks most of the vesicles showing acid phosphatase activity were dense bodies (Fig. 57). These had

precipitate uniformly distributed over their entire contents (Fig. 59a). Secondary lysosomes showed precipitate distributed over only a portion of their contents.

In twenty-four hour vitamin D_3-replete chicks most of the vesicles showing acid phosphatase activity were secondary lysosomes, covered partially by precipitate (Figs. 58, 59b). Some dense bodies completely covered by precipitate were also seen in this group.

iii. S-Collidine Buffered Paraformaldehyde Fixation

Absorptive cells of duodenum which had been fixed with S-collidine buffered paraformaldehyde showed small electron dense granules of about 80Å aggregated in vesicular bodies. Some rounded vesicles were seen to be almost completely filled with these granules. These vesicles appeared to resemble dense bodies (Fig. 60). Other vesicles, which appeared to be secondary lysosomes, showed such granules in parts of their contents. In these structures the granules were either randomly scattered or organized in rows and even arranged in concentric patterns (Fig. 61). Similar granules were sometimes seen free in the cytoplasm, often close to lysosomes. Multivesicular bodies and peroxisomes were not seen to contain similar granules.

In twenty-four hour vitamin D_3-replete chicks more vesicles which appeared to be lysosomes seemed to contain small

electron dense granules than in the rachitic chicks (Figs. 62, 63). In the twenty-four hour chicks most of these vesicles were seen to be partially filled with these granules and appeared to be secondary lysosomes. More granules also appeared to be free in the cytoplasm of this group.

In sections which had been stained with uranyl acetate and lead citrate it became more difficult to distinguish individual granules from each other within the lysosomes (Figs. 43, 44). EDTA treatment appeared to result in a partial loss of granules from the lysosomes. Fewer lysosomes containing granular deposits were seen after EDTA treatment (Fig. 64). Also the granules within individual lysosomes appeared to be arranged more diffusely than in material not treated with EDTA (Fig. 65). Most of the granular material seen previously in the cytoplasm appeared to be lost after EDTA treatment.

c. Endoplasmic Reticulum

i. Agranular Type

Endoplasmic reticulum of the agranular type was abundant in the apical cytoplasm of all groups. In all experimental groups its morphology appeared to be similar to that of the normal chicks. It appeared either as short tubular cisternae or as rounded vesicles and both forms contained slightly electron dense contents.

The abundance of agranular endoplasmic reticulum did not appear to differ from the normal in rachitic chicks (Fig. 48). Eighteen hours after vitamin D_3 injection agranular rounded vesicles appeared to be present with increased frequency in the apical cytoplasm (Figs. 23, 50). By forty-eight hours the appearance of agranular endoplasmic reticulum appeared to have reverted to the level of occurrance seen in the normal chicks (Fig. 52), and remained as such through seventy-two hours (Fig. 53). The low calcium group of chicks appeared to resemble the eighteen or twenty-four hour chicks in their content of agranular endoplasmic reticulum (Fig. 40).

ii. Granular Type

Endoplasmic reticulum of the granular type in the apical cytoplasm of all experimental groups was morphologically similar to that of the normal animals. Most of it was seen as short cisternae which were in close proximity to the mitochondria and paralleled their contours. Rachitogenic diet, low calcium diet, and vitamin D_3 repletion did not seem to affect the amount of granular endoplasmic reticulum which was seen in association with the mitochondria. Neither was there any noticeable change in the prevalence of small rounded ribosome-studded vesicles with experimental treatment.

Vitamin D_3 repletion however did appear to cause an increase in the occurrance of groups of short cisternae of granular endoplasmic reticulum arranged in parallel rows in twelve hour vitamin

D_3-replete animals (Fig. 67). In vitamin D_3-replete goups taken at later time intervals the amount of granular endoplasmic reticulum arranged in parallel fashion appeared similar to the normal chicks. At seventy-two hours there again appeared to have been an increase in the amount of granular endoplasmic reticulum occurring in parallel cisternae (Fig. 71). In these chicks such groups of cisternae seemed to appear as frequently as in the twelve hour group.

d. Golgi Apparatus

In all experimental groups the Golgi apparatus was seen to occupy a supranuclear position. Its activity, as indicated by the general abundance of saccules, vesicles, and vacuoles, appeared to change in the experimental groups examined. The various elements were seen in all groups and remained morphologically similar to those seen in the normal chicks.

In rachitic chicks Golgi activity appeared greater than in the normal chicks, as more large Golgi vacuoles and prominent stacks of Golgi saccules could be seen (Figs. 21, 66). The larger vacuoles appeared as if they were made up of smaller vacuoles as they had a globular or foamy internal appearance.

Vitamin D_3 treatment caused a further increase in Golgi activity. At twelve hours large vacuoles were the most evident element of the Golgi complex (Figs. 22, 67). Many of these were present so that a large band of Golgi apparatus was seen across

the supranuclear region of most cells. By eighteen hours this band of vacuoles had decreased slightly in prominance but large vacuoles were still the predominant element of the Golgi apparatus (Figs. 23, 68).

At twenty-four hours Golgi activity had decreased further and large vacuoles no longer formed a prominant band. Stacks of Golgi saccules and small vesicles and vacuoles were seen more frequently than in the two preceeding groups (Figs. 24, 69). Levels of activity similar to that of the twenty-four hour chicks seemed to be present in the forty-eight (Figs. 25, 70) and seventy-two hours chicks (Figs. 26, 53). These three groups appeared to exhibit more evidence of Golgi activity than did the normal chicks. Chicks fed a low calcium diet appeared to show as much or slightly more Golgi activity than did the normal chicks (Fig. 54).

4. Basal Cytoplasm

a. Mitochondria

The mitochondria of the basal cytoplasm in all experimental groups appeared similar to those of the normal group in abundance, size, and orientation. They were morphologically similar to normal mitochondria with the same exception seen previously concerning the mitochondria of the apical cytoplasm. This was the presence of granules with increased electron density and size within the mitochondrial matrix of chicks fed a low calcium diet (Fig. 72). These granules were seen

more frequently in this group than were matrix granules in the other groups.

S-collidine buffered paraformaldehyde fixation and EDTA treatment affected the mitochondria of the basal region of rachitic and twenty-four hour vitamin D_3-replete chicks in the same ways as these techniques affected the mitochondria of the apical cytoplasm.

b. Endoplasmic Reticulum

i. Agranular Type

Agranular endoplasmic reticulum present within the basal regions of rachitic and twelve hour vitamin D_3-replete animals resembled that of the normals in both morphology and abundance (Figs. 73, 74). At eighteen hours more rounded, smooth-walled vesicles were seen in the basal region than had been seen previously (Fig. 75). By twenty-four hours the amount of agranular endoplasmic reticulum again resembled that of the normal chicks (Fig. 76). This situation was again seen in forty-eight and seventy-two hour chicks and chicks fed a low calcium diet (Figs. 78, 79).

ii. Granular Type

In all experimental groups cisternae of granular endoplasmic reticulum were seen near the mitochondria and following their contours. The amount of such granular endoplasmic reticulum did not

appear to change with experimental treatment, however many of the cisternae appeared to have become dilated by twelve hours following vitamin D_3 injection. All of the cisternae possessed contents which were slightly electron dense.

Cisternae arranged in parallel rows in the basal region were seen less frequently in rachitic than in normal chicks (Fig. 73). Twelve hours after vitamin D_3 repletion the amount of such cisternae increased and these were seen more frequently than in the normal chicks (Figs. 22, 74). By eighteen hours the abundance of free cisternae of granular endoplasmic reticulum had reverted to resemble the normal situation (Fig. 75). This pattern remained through forty-eight hours after vitamin D_3 injection, and was seen in chicks fed a low calcium diet (Figs. 76, 77, 79).

By seventy-two hours there appeared to have been a large increase in the amount of granular endoplasmic reticulum (Figs. 26, 78). In this group rows of long parallel cisternae often filled the central region of the basal cytoplasm and sometimes extended into the cytoplasm adjacent to the nucleus. Many of these cisternae appeared dilated. This group showed the most prominant basal granular endoplasmic reticulum of any group examined.

c. Vesicular Bodies

Dense bodies and peroxisomes were seen more frequently in the basal cytoplasm of rachitic than normal chicks (Figs. 21, 73). This situation remained through twenty-four hours after vitamin D_3

administration (Figs. 22, 23, 24, 76). At forty-eight hours these bodies appeared to have decreased to levels similar to those seen in the normal animals (Figs. 25, 77). Dense bodies and peroxisomes seemed to appear with similar frequency in the seventy-two hour and low calcium animals as well (Figs. 26, 79). In all groups dense bodies and peroxisomes appeared morphologically similar to those seen in the normal group.

Secondary lysosomes were occasionally seen in the basal cytoplasm of all groups and these bodies appeared normal in structure.

At twelve hours after vitamin D_3 treatment large globular vacuoles with pale electron dense contents appeared in the basal cytoplasm (Figs. 22, 74). These were similar to the large vacuoles seen previously in the Golgi region. They were also seen frequently at eighteen hours (Fig. 23). From twenty-four through seventy-two hours such vacuoles remained visible in the basal cytoplasm but were seen less frequently than in the two previous groups (Figs. 24, 25, 26). In all of the groups from twelve through seventy-two hours similar vacuoles also appeared in the cytoplasm adjacent to the nucleus. They appeared most frequently in this region at twelve and eighteen hours (Figs. 22, 23). Such vacuoles were seen infrequently in the basal regions of normal, rachitic, and low calcium chicks.

5. Intercellular Space

i. Regular Fixation

In all experimental groups juxta-luminal junctional complexes and desmosomes were seen and these did not appear to be structurally different from those of the normal animals. Also extensive interdifitations of the lateral cell membranes were seen in all groups.

In rachitic chicks large intercellular spaces were seen more frequently between the absorptive cells in their basal regions than in the normal chicks (Fig. 73). These appeared to be filled with material of slightly electron dense and globular nature, similar in appearance to the contents of the Golgi vacuoles. Large intercellular spaces with similarly appearing contents seemed to occur as frequently at twelve hours as in the rachitic chicks. At eighteen hours more spaces filled with globular material seemed to appear than previously (Figs. 21, 76). These were seen at the level of the nucleus and below (Fig. 79). The remaining vitamin D_3-injected groups seemed to show as many intercellular spaces filled with globular material as did the eighteen hour chicks (Figs. 24, 25, 26). In these last four groups material of appearance similar to that seen within the intercellular spaces was also seen below the basal membranes of the cells (Figs. 75, 80). The intercellular spaces of low calcium chicks appeared to resemble those of normal chicks in abundance and the nature of their contents.

ii. S-Collidine Buffered Paraformaldehyde Fixation

Both rachitic and twenty-four hour vitamin D_3-replete material showed small electron dense deposits adhering to most of the plasma membrane covering the lateral and basal surfaces after S-collidine buffered paraformaldehyde fixation (Figs. 82 - 85). These deposits appeared as fine granules of dense lines and could be seen on the inner and outer leaflets of the plasma membrane. They appeared to resemble the fine deposits seen previously on the microvillar membranes of similarly fixed material. Sometimes these electron dense deposits were seen to completely fill the intercellular space for short distances. This was especially evident in areas where the lateral membranes formed interdigitations. In both the rachitic and vitamin D_3-replete animals these fine deposits were similar in appearance, location, and abundance.

When sections were stained with uranyl acetate and lead citrate some of the fine deposits seemed to be obscured as the plasma membrane gained more contrast (Fig. 37). EDTA treatment removed most of the fine deposits from the basal and lateral membranes and the intercellular spaces of both rachitic and vitamin D_3-replete chicks (Figs. 86, 87).

II Goblet Cells

Goblet cell fine structure has previously been described in the small intestine of rat, guinea pig, and human. (Palay, 1958; Taylor, 1959; Bierring, 1962; Hollman, 1963; Trier, 1963; Trier and Rubin, 1965). These descriptions are quite similar regardless of the animal or portion of small intestine studied.

Freeman (1962, 1966) has described the secretory cycle of goblet cells in detail. Normally mucous is continually formed and expelled as the cells migrate up the intestinal villus (Jennings and Florey, 1956). Complete expulsion of mucous does not occur without an external stimulus (Florey, 1960; Freeman, 1966). Thus only goblet cells in the mature stage of their cycle are normally seen on the intestinal villus.

A. Regular Fixation

1. General Morphology

The goblet cell of chick duodenum is composed of two major regions: the mucous-filled theca and the basal stalk or foot. The distended theca resting on the long, slender foot give the cell its characteristic 'goblet' shape (Fig. 1). Distension of the theca causes adjacent absorptive cells to be compressed in the apical region. The theca is filled almost entirely with mucous droplets so that only a thin layer of cytoplasm remains surrounding

the mucous mass. The foot contains most of the cell organelles which are tightly packed together, giving this region a greater electron density than the cytoplasm of the surrounding absorptive cells.

The lateral edges of goblet cells form cytoplasmic extensions which interdigitate with those of adjacent absorptive cells (Figs. 1, 3). The juxta-luminal junctional complex is present between goblet and absorptive cells and desmosomes are found holding their lateral membranes in close apposition (Fig. 93). The basal surfaces of goblet cells are usually not as smooth as those of absorptive cells and display several small foot-like projections. The basal lamina is continuous beneath both the goblet and absorptive cells.

2. The Theca

The apical surfaces of goblet cells are extended into microvilli similar to those of absorptive cells (Figs. 88, 89). In goblet cells which are not in the process of expelling mucous a terminal web is visible (Figs. 88, 89). The band of terminal web filaments may appear incomplete or disrupted due to mucous accumulation beneath it. Apical pits and vesicles are not apparent in the terminal web region. Both the brush border and terminal web of goblet cells are more electron dense than those of absorptive cells.

The mucous droplets which pack the theca are of varying electron density and size (Fig. 90). In electron density they range from pale to dark and small areas of increased electron density are visible within many droplets (Fig. 89). The mucous droplets appear granular or fibrillar in composition. This is most apparent in those which are the least electron dense.

Initially each droplet is surrounded by a membrane which it may lose as it becomes larger and coalesces with other droplets (Freeman, 1966). In humans as the droplets become larger they decrease in electron density and become more granular in appearance,while in rats and guinea pigs the droplets retain their electron density (Freeman, 1966). In chicks large mucous droplets of light and dark electron density are seen. Large coalesced droplets may show several distinct areas of varying electron density which have no membranes separating them. Mucous in the process of being expelled is composed of large coalesced droplets of different electron densities (Figs. 90, 91). There appears to be no correlation between droplet size and electron density in chicks.

Surrounding the mucous mass of the theca there is a thin layer of compressed cytoplasm which is usually extremely electron dense (Fig. 1, 2). Here mitochondria, ribosomes, and granular endoplasmic reticulum are readily recognizable. The cytoplasm may project into the mucous mass between the droplets,

and sometimes small portions of cytoplasm become trapped within it. In some cases the mucous appears to have completely enveloped the lateral cytoplasm and to be in direct contact with the plasma membrane (Fig. 90).

Prior to the expulsion of mucous the microvilli become flattened. Then the plasma membrane ruptures allowing mucous and some cytoplasmic components to escape in an apocrine mode of secretion (Palay, 1958; Bierring, 1962; Freeman, 1962, 1966). Trier and Rubin (1965) have suggested that merocrine secretion with the loss of mucous alone is the normal occurrance in the absence of extreme stimulation. In chick duodenum remains of cytoplasmic components are seen in the discharged mucous, although these cells were not specifically stimulated to discharge.

3. The Foot

The foot of the goblet cell is long and slender, comprising about two thirds of the cell's length (Fig. 1). It is about half the width of the basal portions of adjacent absorptive cells. The nucleus is located in the extreme basal portion of the foot and often appears compressed (Fig. 94).

The Golgi apparatus fills most of the foot cytoplasm between the nucleus and the theca (Fig. 92, 93). Dilated stacks of Golgi cisternae and small vesicles are abundant. Many large mucous droplets are seen dispersed in this region. They are of

varying electron density and may lack a limiting membrane or possess an incomplete one. Some droplets appear to be coalesceing in the foot region before they join the mucous mass of the theca.

The remaining foot cytoplasm is extremely electron dense. It contains many cisternae of granular endoplasmic reticulum which contain dense filamentous material. Many free polyribosomes are in evidence in close proximity to the Golgi apparatus. Several mitochondria of the usual structure are seen. Secondary lysosomes, dense bodies, and myelin figures also appear occasionally in the foot region. The entire foot region has the appearance of tight packing of organelles into a small area.

4. The Effects of Vitamin D_3 On Goblet Cell Morphology

No consistant differences could be observed in the appearance of goblet cells when the various rachitic and vitamin D_3-treated groups were compared. The appearance of the mucous mass varied considerably in goblet cells from the same region of the same animal, and in animals belonging to the same experimental group. This variation involved differences in the electron density and the degree of coalescence of droplets within the mucous mass.

The foot regions of all goblet cells examined appeared quite similar with much Golgi apparatus, granular endoplasmic reticulum and free polyribosomes in evidence. They all showed

tight packing and compression of organelles, and strong electron density.

B. S-Collidine Buffered Paraformaldehyde Fixation

1. Rachitic Chicks

Electron dense deposits similar to those seen on the microvilli of the absorptive cells were also seen on the microvilli of the goblet cells (Fig. 95). Many goblet cells seen in section had microvilli which appeared disrupted or distorted due to the presence of mucous secretory material. It was not possible to determine the relative frequency of the deposits seen on goblet cell microvilli as compared to those seen on the absorptive cell microvilli.

2. Vitamin D_3-Injected Chicks

Electron dense deposits were again seen on the microvilli. In goblet cells in the process of expelling, many deposits were seen on the microvilli adjacent to the mucous (Fig. 96). Electron dense deposits were also seen on what appeared to be cytoplasmic remnants, within and surrounding the apical portion of the mucous mass. These deposits were of variable size, the largest being about 250 Å in diameter.

3. Staining

Staining with uranyl acetate and lead citrate did not seem to change the appearance of the deposits on the microvilli of either group or on the mucous mass of the vitamin D_3-treated chicks. As staining enhanced the contrast between pale and dark areas within the mucous, it became more evident that the granules seen in and around the mucous were attached to cytoplasmic remnants (Fig. 97). The small areas of the mucous which possessed the greatest electron density did not show any electron dense deposits. Also very few deposits appeared to be free in the mucous and not associated with cytoplasmic remnants.

4. EDTA

EDTA treatment removed all granules from the microvilli of both rachitic and Vitamin D_3-treated chicks (Fig. 98). Neither could any deposits be seen on the cytoplasmic remnants in and around the apical portion of the mucous mass of the vitamin D_3-treated chicks.

CHAPTER FOUR

DISCUSSION

Site of Electron Microscopic Examination in Relation to Turnover Time of Intestinal Epithelium

Thick sections (approximately 2 μ) of Araldite embedded tissue were examined under the light microscope for selection of suitable areas of tissue, prior to thin sectioning and subsequent electron microscopic examination. In all cases areas in the upper third of the villus, but below its tip, were selected for electron microscopic examination. The tips of the villi show morphological alterations, such as swollen mitochondria and degeneration of the brush border and other cytoplasmic elements, which are undersirable in comparative ultrastructural examinations (Trier and Rubin, 1964).

Intestinal epithelium is known to undergo constant renewal. New cells are formed by mitotic division in the crypts, then migrate up the villi and are extruded at their tips (Leblond and Stevens, 1948). Spielvogel et al. (1972) found that in rachitic chicks vitamin D_3 treatment produced increased villus length and caused the epithelial cells to migrate at faster rates. However, when they considered these two factors together, no change could be found in the overall turnover time of the absorptive epithelium after vitamin D_3 treatment. Their results indicated that the falling

off in response to a single dose of vitamin D_3 was related to renewal of the epithelium, and that vitamin D_3 can act only on fully differentiated cells which have already begun to migrate up the villus.

Spielvogel et al. (1972) found that it took cells twenty hours longer to migrate to the tips of the villi from the bases of the crypts than from the bases of the villi. As calcium absorption doubles by twelve hours after vitamin D_3 repletion in rachitic chicks (Ebel et al., 1969; Wasserman et al., 1971), it would appear that increased calcium absorption due to vitamin D action is not related to the differentiation of a new population of absorptive cells.

In this study the upper part of the villus was chosen for examination in order to compensate for migration of the epithelial cells. Imondi and Bird (1966) found that chick duodenal epithelial cells, formed by mitoses in the crypts, migrated up about half the length of the villus in forty-eight hours. Allowing twenty hours off the total migration time for the cells which would have been in the crypts at the time of injection and not influenced by vitamin D_3 (Spielvogel et al., 1972), by seventy-two hours the last of the cells to be affected by the vitamin should have been nearing the tips of the villi.

Ultrastructural Changes in the Duodenal Absorptive Cells With Vitamin D_3 Deficiency and Repletion

1. The Microvilli

a. Regular Fixation

A general trend towards lengthening of the microvilli after vitamin D_3 repletion was noted. Rachitic chicks had the shortest microvilli (1.1μ) while of the vitamin D_3-replete chicks those killed eighteen hours after vitamin D_3 injection had the longest (1.9μ). The latter group's microvilli appeared to be of comparable length to those of the normal chicks (1.8μ). No change in microvillar diameter was observed in any of these groups.

An inverse relationship between diameter and length has been found to exist during the embryonic development of chick duodenal microvilli (Overton and Shoup, 1964). As no changes in diameter were observed here in association with the changes in length, an actual decrease in microvillar surface area may have occurred in the rachitic chicks.

Microvilli have been found to increase in length and decrease in diameter with the progression of the absorptive cells from the crypts to the tips of the villi (Dalton, 1951; Brown, 1962). As the samples examined here were taken from similar areas on the villi, the differences in microvillar length could not have

been due to the location of the cells.

There has been described a rapid turnover in brush border protein contents (James et al., 1971), and cyclohexamide treatment has been shown to produce a rapid, reversible decrease in microvillar length (LeCount and Grey, 1972). In rachitic chicks, vitamin D_3 repletion has been shown to be followed by a forty per cent increase in the cholesterol content of the brush border plasma membrane (Adams et al., 1970). These findings suggest a relationship between microvillar length and the amounts of protein and cholesterol within their membranes. The protein content of the microvillar membrane may include Ca-ATPase, alkaline phosphatase, and even CaBP, all of which have been implicated in the entry of calcium into the absorptive cell.

Rachitic animals have long been known to have slower growth rates than normal animals (Migicovsky and Emslie, 1947). Wong et al. (1970) found that prolonged exposure to filipin produced morphological changes in the microvilli, with the apparent loss of many of them. This may reflect the inability of rachitic chicks to rapidly renew membrane components which may be exhausted or relocated when calcium absorption is induced by filipin.

Vitamin D-replete membranes have a higher cholesterol content than do rachitic membranes (Adams et al., 1970), yet they appear to be unaffected by filipin treatment. Wong et al. (1970)

therefore suggested that proteins may be present in the vitamin D-treated membranes which mask the cholesterol, preventing it from interacting with filipin. Thus vitamin D treatment may result in the synthesis of proteins which may act as calcium binding sites.

The changes in microvillar length seen in the present experiments may represent: 1. stimulation of the animals' overall protein synthetic activity when the stress of rickets is counteracted by vitamin D_3 repletion, and 2. vitamin D_3-induced synthesis of additional membrane surface areas which would be directly involved in calcium absorption by the cell. That is, the synthesis of binding sites, of enzymes associated with the plasma membrane, and of components necessary for structural alteration and/or rapid renewal of the microvillar plasma membrane.

b. S-Collidine Buffered Paraformaldehyde

In chick duodenum which was fixed using the S-collidine buffered paraformaldehyde technique for mineral visualization of Sampson et al. (1970), large electron dense granules were seen adhering to the absorptive cell microvilli, more frequently in rachitic than in vitamin D_3-replete animals. Most of these granules appeared to be adhering to the outer surface of the microvillar membrane. Very small electron dense deposits were also seen adhering to either the inner or outer surface of the

plasma membrane, and these appeared to be more common in vitamin D_3-replete than in rachitic animals. Both types of deposits were removed by EDTA treatment, indicating that they may have been calcium.

In rat intestinal absorptive cells, Sampson et al. (1970) also observed large electron dense granules adhering to the microvilli and these occurred more frequently in rachitic than in vitamin D-replete rats. However, most of the large granules which were seen by Sampson et al. appeared to adhere to the inner surface of the microvillar membrane. They suggested that calcium was able to cross the microvillar membrane in the absence of vitamin D, but remained in this region to form granular deposits unless mobilized by the action of vitamin D.

Oschman and Wall (1972) found small electron dense deposits, similar to the small deposits seen in chick, adhering to the inner leaflet of the microvillar membrane in cockroach midgut caeca when calcium-containing fixatives were used, and suggested that these deposits represented binding sites for calcium. In the present experiment, the presence of more such small deposits on vitamin D_3-replete than on rachitic microvilli suggests that the former possess more sites for calcium binding on the microvillar surface. These binding sites could participate in calcium absorption either by diffusion or by active transport. The presence of fewer large granules on the vitamin D_3-replete

microvilli may indicate that more calcium becomes bound to the membrane and absorbed, leaving less calcium to precipitate into large, randomly distributed granules. The difference observed in the site of formation of large granules in chicks and in rats (Sampson et al., 1970) may indicate that the two species differ in their methods of calcium absorption. Alternately, it may indicate that species differences lead to the formation of different artifacts using this type of mineral visualization technique.

2. Apical Pits and Vesicles

Endocytosis

Pinocytosis was first described by Lewis (1931) who saw macrophages trapping fluid droplets within folds of their undulating plasma membranes. With the advent of electron microscopy this term has been used widely to describe processes which may be similar in their end results, but not in their mechanics, e.g. the elevation of folds which surround droplets and incorporate them into the cytoplasm, shallow inpocketing of the plasma membrane to form vesicles, and deep inpocketing of the plasma membrane as channels which bud off vesicles at their tips (Fawcett, 1965). As a result there has been a profusion of descriptive terminology in order to describe these processes more accurately and in attempts to distinguish them from one another (reviewed by Stockem

and Wohlforth-Botterman, 1969). The term 'endocytosis' to indicate engulfment of material by invagination of the plasma membrane has recently gained the widest use in electron microscopic descriptions, and will be used here.

Endocytosis has been described as functioning in the uptake of proteins and colloidal materials by the ileal and jejunal absorptive cells of newborn rat and mouse (Clark, 1959), in antibody absorption by jejunal absorptive cells of newborn mammals (Kraehenbuhl and Campiche, 1969; Rodewald, 1970), and in antibody absorption from swallowed amniotic fluid by the intestinal absorptive cells of fetal rat (Orlic and Lev, 1973). The endocytotic activity of these systems is apparently very great, and results in the formation of large vacuoles in the apical cytoplasm of the absorptive cells. Clark (1959) suggested that the disappearance of these vacuoles as the animals mature may structurally indicate the increased selectivity which adult animals exercise in their endocytotic activity. Recently it has been demonstrated that macromolecular absorption by endocytosis continues to occur in the intestinal absorptive cells of adult rat (Walker, et al., 1972).

In the present experiments the apical surfaces of the absorptive cells in all groups showed apical pits and vesicles. However, these appeared more frequently in vitamin D_3-replete than in rachitic chicks. By eighteen hours after vitamin D_3

injection this increase was quite apparent. As CaBP has been implicated in calcium absorption (Wasserman and Taylor, 1966; Taylor and Wasserman, 1967) and has been localized at the brush border of the absorptive cells (Taylor and Wasserman, 1970), increased evidence of endocytosis after vitamin D_3 repletion might indicate that a CaBP-calcium complex is absorbed via this mechanism.

Chicks which had been adapted to a low calcium diet showed as many apical pits and vesicles as did the vitamin D_3-replete chicks of eighteen and twenty-four hours. Furthermore the absorptive cells of chicks fed a low calcium diet showed more multivesicular bodies in their apical cytoplasm than did the other groups. In intestinal absorptive cells these bodies may be formed as a result of endocytotic activity (Cardell et al., 1967). These two observations suggest that endocytosis may function in the increased efficiency of calcium absorption seen in adaption (Wasserman and Taylor, 1968).

Endocytosis requires the adsorption of the material to be ingested onto the mucopolysaccharide layer (glycocalyx) covering the cell membrane (reviewed by Stockem and Wohlforth-Botterman, 1969). This condition could be satisfied by the binding of the CaBP-calcium complex to some component of the glycocalyx, or by the binding of calcium to CaBP if this protein is already structurally part of the glycocalyx.

The quantity of the inducer adsorbed also appears to be an important factor in the induction of endocytosis (Stockem and Wolfarth-Botterman, 1969). If CaBP may act as an inducer, then increased CaBP synthesis during adaptation (Wasserman and Taylor, 1968) could fulfil the requirement of a large amount of inducer, resulting in the improved efficiency of calcium absorption through the endocytosis of calcium complexed to CaBP. A similar situation might also occur when rachitic chicks are treated with vitamin D_3, as this is followed by the synthesis of greater amounts of CaBP than are found in normal animals. When normal chicks are treated with additional vitamin D_3, their levels of CaBP and calcium absorption do not increase (Wasserman and Taylor, 1968; Taylor and Wasserman, 1969). This might indicate that these animals do not require an additional increase in their calcium absorption, consequently do not synthesize any additional inducer.

An energy source is required for endocytosis (Miller, et al., 1965). This might be the function of the brush border Ca-ATPase (Martin et al., 1969; Melancon and DeLuca, 1970) in calcium absorption. Endocytosis can be blocked by inhibitors of glycolysis and by low temperatures (reviewed by Jacques, 1969). Sallis and Holdsworth (1962) found that similar conditions would block calcium absorption. Again, inhibitors of oxidative phosphorylation block both endocytosis (Jacques, 1969) and active transport of calcium (Schacter and Rosen, 1959). It has also been

suggested by Norman et al. (1970) that the rise in brush border alkaline phosphatase activity, paralleling increased calcium absorption, might represent part of the Ca-ATPase system (a two step phosphorylation and calcium-dependent hydrolysis process).

It has been suggested that endocytosis may function in the intestinal absorption of the intrinsic factor-vitamin B_{12} complex (Mackenzie and Donaldson, 1969). Calcium is required for vitamin B_{12} absorption to occur (Mackenzie and Donaldson, 1969), and will also induce endocytosis in amoebae (Brandt and Freeman, 1967). These findings suggest that endocytosis and calcium absorption are not incompatible.

Competition is also involved in endocytosis (Jacques, 1969). Calcium, strontium, and magnesium have been shown to compete with each other in a common absorptive pathway (Hendrix et al., 1963).

An important part of any endocytotic system is the replacement of membrane which is 'lost' from the surface in the formation of endocytotic vesicles (Bennett, 1956). Wong et al., (1970) found that prolonged exposure to filipin produced morphological changes in the microvilli of rachitic chicks. These were seen as shortening and the apparent loss of microvilli. As these changes were seen long after filipin induced increased calcium absorption they appeared to be a result, not a cause, of the increased calcium absorptive activity. Filipin-stimulated

calcium absorption was also found to be active, temperature sensitive, and affected by metabolic inhibitors (Adams et al., 1970). These conditions suggest that filipin could have induced endocytotic absorption of calcium, which finally resulted in depletion of the apical membrane. That large amounts of membrane are rapidly translocated by endocytosis has been found in other systems, e.g. amoebae are able to swallow one half of their surface area during twenty minutes of endocytotic activity (Chapman-Andresen, 1965).

Palay and Karlin (1959) suggested that endocytosis in intestinal absorptive cells may occur so rapidly that only a few apical pits and vesicles are caught in section. They also calculated that a few vesicles seen in one section would represent a very large volume when taken over the entire cell surface for a unit of time, such as one hour. Thus the apical pits and vesicles seen in these experiments may represent a large surface area available for the absorption of calcium.

Diffusion

Vitamin B_{12}, when present in large amounts in the intestinal lumen, is absorbed by a diffusional process in an amount proportionate to the concentration of the vitamin available (reviewed by Mackenzie and Donaldson, 1969). This process results in the absorption of only a fraction of the vitamin B_{12}

from the lumen. However when the amount of vitamin B_{12} available is small, absorption occurs much more efficiently and utilizes the gastric intrinsic factor (Mackenzie and Donaldson, 1969). As calcium appears to be absorbed in the intestine by more than one process (see introduction), its absorption could operate in a manner similar to that of vitamin B_{12}.

When the calcium concentration in the intestinal lumen is high, absorption might take place by a diffusional process (Schacter, 1963; Harrison and Harrison, 1966; Wasserman et al., 1966; Wasserman and Taylor, 1969) in which vitamin D effects structural changes in the apical membrane or activates a carrier system, the overall effect of which would be to reduce the diffusional barrier to calcium. It is known that when the calcium supply is low, or the physiological demands for calcium are great, adaptation occurs resulting in more efficient calcium absorption (Wasserman and Taylor, 1968; Wasserman et al., 1970). This could indicate that an active transport process (Schacter and Rosen, 1959; Wasserman et al., 1961; Sallis and Holdsworth, 1962) capable of greater specificity towards calcium is activated when the luminal calcium concentration is low or a great uptake of calcium is needed. This process could be endocytosis.

CaBP may be able to act in more than one manner. It may act as a diffusional facilitator, as suggested by Wasserman et al. (1969), at high luminal calcium concentrations and as part of a

carrier system at low calcium concentrations, or when a large increase in calcium absorption is necessary as when rickets is relieved. In the first case a system for release of calcium from CaBP may be provided, utilizing phospholipase A and the lecithin-lysolecthin cycle (Wasserman, 1970). In the second case another mechanism would be required to release calcium from its carrier within the cell. If this method of calcium absorption is endocytosis then this would involve releasing calcium from the endocytotic vesicles.

Therefore the increase in apical pits and vesicles observed with vitamin D_3 repletion and in chicks adapted to a low calcium diet is suggestive of the involvement of endocytosis in the process of calcium absorption in intestinal absorptive cells.

S-Collidine Buffered Paraformaldehyde Fixation

Large electron dense granules, similar to those seen adhering to the microvilli, were sometimes seen within the apical pits of absorptive cells fixed with S-collidine buffered paraformaldehyde (Sampson et al., 1970), in both rachitic and vitamin D_3-replete animals. Similar granules were never seen within the apical vesicles of either group. Small electron dense deposits, similar to those seen adhering to the microvillar plasma membrane.

were also seen on the membranes of the apical pits and vesicles. These were more apparent in vitamin D_3-replete than in rachitic animals, as were the small deposits on the microvillar membranes. Both types of electron dense deposits were removed by EDTA treatment, as were the microvillar deposits, indicating that they may have been calcium.

The observation of large granules within the apical pits indicates that calcium may accumulate within them. However, as these granules were seen in both groups, yet were never seen within the apical pits, their presence probably indicated random accumulation of calcium as in the formation of large microvillar granules, and does not indicate that such large particles of calcium are actually engulfed by the cells. It seems more significant that small electron dense deposits were seen adhering to the membranes of the apical pits and vesicles, and apparently more frequently in the vitamin D_3-replete animals. This indicates that calcium may become bound to some carrier on the glycocalyx or plasma membrane and subsequently become endocytosed.

The observation of small electron dense deposits over most of the microvillar membrane in vitamin D_3-replete chicks is not inconsistant with endocytosis of calcium if, as previously mentioned, the entire microvillar surface is mobile. Calcium might be bound anywhere on the microvillar surface and subsequently diffuse across the plasma membrane, or the bound calcium might be carried into apical pits as the membrane is relocated.

3. Lysosomes

a. The Vacuolar Apparatus

It was noted that the lysosomal population of the absorptive cells of rachitic chicks changed in response to vitamin D_3 repletion. With time secondary lysosomes became more abundant, while dense bodies, which had formed the predominant portion of the lysosomal population in rachitic animals, appeared less frequently. Chicks which were fed low calcium diet showed more dense bodies in their absorptive cell cytoplasm than did normal animals. Multivesicular bodies, which were seen infrequently in rachitic animals, appeared to increase after vitamin D_3 repletion and were seen most frequently in low calcium chicks. As previously mentioned, the presence of increased numbers of multivesicular bodies may indicate that these cells have undergone increases in their endocytotic activity.

The lysosomal system has been reviewed extensively, e.g. by: DeDuve and Wattiaux (1966), Wattiaux (1969), Dingle and Fell (1969), and Dingle (1972). Presently the terminology of DeDuve and Wattiaux (1966) has been used.

The lysosmal system has been described by the above mentioned authors as composing the 'vacuolar apparatus', which functions in heterophagy (digestion of material originating from outside of the cell) and autophagy (digestion of cellular components).

Primary lysosomes appear to originate from the Golgi apparatus or from agranular endoplasmic reticulum. The granular endoplasmic reticulum may also participate by synthesizing acid hydrolases which are somehow transferred to the Golgi complex (Cohn et al., 1966). The primary lysosome may act as a storage granule, until it meets a pre-lysosome or phagosome and fuses with it, forming a secondary lysosome where digestion of the phagosomal material occurs. A heterophagic phagosome may be an endocytotic vesicle or a multivesicular body, which may be formed form endocytotic vesicles (Cardell et al., 1967). An autophagic phagosome is formed from some portion of the cytoplasm which has become sequestered. A secondary lysosome can continue to fuse with phagosomes until its digestive abilities have been exhausted. Finally a residual body or post-lysosome composed of undigested material remains. Some cells are able to excrete the residual material or lysosomal digestive activities into their external environment e.g. leukocytes (Zucker-Franklin and Hirsch, 1964).

As the activities of the vacuolar apparatus are continuous, it is not always easy to classify the components, when seen in electron microscopic examination, into such clearly defined groups. It seems that in the material examined in these experiments the uniform dense bodies represented primary lysosomes, while the non-uniform secondary lysosomes were digestive vacuoles.

Some vesicles of the latter group may have been residual bodies as these are not easily distinguished from digestive vacuoles by visual examination. Neither was it possible to distinguish secondary lysosomes which had originated by heterophagy from those which had originated by autophagy. However, the changes seen in the lysosomal population did not appear to be due to increased autophagy as cytoplasmic sequestration and degeneration, and extensive or extremely large secondary lysosomes were encountered infrequently.

At the level of electron microscopic examination multivesicular bodies, dense bodies, and secondary lysosomes of intestinal absorptive cells have previously been demonstrated to possess acid phosphatase activity (Behnke, 1963; Moe et al., 1965). Moe et al. (1965) also demonstrated acid phosphatase activity to be present in the extremities of the Golgi saccules. In the present experiments precipitate indicative of acid phosphatase activity appeared over dense bodies and secondary lysosomes. The patterns of electron dense precipitate which were seen over these bodies confirmed that in rachitic animals dense bodies made up the largest portion of the lysosomal population, while in vitamin D-replete animals secondary lysosomes were predominent.

This increase in secondary lysosomes seen after vitamin D_3 repletion seems to be consistant with the possibility that calcium may be absorbed by endocytosis. Increased endocytotic

activity would result in more digestive bodies or secondary lysosomes being formed by the fusion of endocytotic vesicles or phagosomes with inactive primary lysosomes or dense bodies, which predominated the lysosomal population in rachitic chicks. If the calcium-CaBP complex enters the cells in endocytotic vesicles, then digestion of these vesicles by secondary lysosomes would allow calcium to be liberated from its binding protein and enter the cytoplasm.

b. S-Collidine Buffered Paraformaldehyde Fixation

In material which was fixed according to the method of Sampson et al. (1970), small electron dense deposits were seen in the lysosomes: secondary lysosomes and dense bodies. Similar dense deposits have been mentioned in many descriptions of lysosomes and are sometimes described as being ferritin-like (e.g. DeDuve and Wattiaux, 1966). Ferritin has been found to enter acid phosphatase positive vacuoles or lysosomes (Kryzowska-Gruca and Schiebler, 1967).

Lysosomes containing small deposits appeared more frequently in vitamin D_3-replete than in rachitic chicks, and appeared to become less frequent in the former after EDTA treatment. This indicates that some of the deposits may be EDTA labile and possibly calcium. This supports the suggestion that calcium may be liberated from a binding protein of the endocytotic vesicles

by lysosomal enzymes.

Further investigation is required to determine the exact nature of these mineral-like granules within the lysosomes.

4. Peroxisomes

When the Gormori technique for demonstration of acid phosphatase activity (as modified by Barka and Anderson, 1963) was used, peroxisomes failed to demonstrate any acid phosphatase activity, thus could be readily distinguished from dense bodies and other lysosomes. Peroxisomes were seen to be more abundant in the supranuclear cytoplasm of rachitic than normal chicks, and following vitamin D_3 repletion they became abundant in the basal cytoplasm as well.

Peroxisomes have been described recently in guinea pig and rat duodenal absorptive cells by Novikoff and Novikoff (1972). The peroxisomes seen in these experiments resembled those seen by Novikoff and Novikoff in their general appearance and in their association with elements of the agranular endoplasmic reticulum. However, the peroxisomes of chick duodenum were seen to contain a central core or nucleoid composed of a bundle of tubule-like structures while the peroxisomes of guinea pig and rat duodenum lacked such a structure (Novikoff and Novikoff, 1972). Peroxisomes of other tissues have been shown to contain cores of tubule-like structures, and urate oxidase activity has been demonstrated to be

localized on these cores (reviewed by Baudhuin, 1969).

The function of peroxisomes in intestinal absorptive cells as well as other cell types is unclear (Baudhuin, 1969; Nivokoff and Novikoff, 1972). Peroxisomes have been found in cells which convert non-carbohydrate precursors to carbohydrate, and glycoxylate bypass enzymes have been isolated from them in lower organisms. It has thus been suggested that they may function in gluconeogenesis (DeDuve and Baudhuin, 1965; Baudhuin, 1969). In intestine they may function in lipid metabolism, as suggested by Novikoff and Novikoff (1972). Possibly the peroxisome population of intestinal absorptive cells may respond to the stress of rickets and to the stimulatory stress produced by vitamin D_3 repletion by producing extra fuel for mitochondrial respiration or by producing some of the carbohydrates which become part of the glycocalyx.

5. The Golgi Apparatus

The Golgi apparatus was quite conspicuous in all groups studied. Its activity appeared to be greatest in the absorptive cells at twelve and eighteen hours after vitamin D_3 repletion. After this time large vacuoles decreased in number, but many saccules and small vesicles were still present.

In cell types other than intestinal absorptive cells, e.g. goblet cells, the Golgi complex is active in producing large amounts of secretory products (Peterson and Leblond, 1964; Neutra

and Leblond, 1966). In intestinal absorptive cells the Golgi complex has been shown to participate in lysosome production (Moe and Behnke, 1965; Bennett and Leblond, 1971) and in glycocalyx and cell coat formation (Bennett, 1970; Bennett and Leblond, 1970).

The increased Golgi activity observed here after vitamin D_3 repletion may result in increased synthesis of lysosomal enzymes necessary for increased digestive activity in the vacuolar apparatus, possibly to release calcium which may enter the cells in endocytotic vesicles. It may also participate in increased secretion of components of the glycocalyx. This activity could be the renewal of elements of the glycocalyx which are rapidly depleted or relocated during increased calcium absorption, as well as the vitamin D-induced synthesis of various elements not present in the glycocalyx of rachitic chicks.

6. Endoplasmic Reticulum

a. Agranular Type

This organelle has been implicated in steroid synthesis (Christiensen and Fawcett, 1961), in drug detoxification in liver (Fouts, 1961), in lysosome formation (DeDuve and Wattiaux, 1966), and, as the sarcoplasmic reticulum of muscle, in calcium transport

(Constantin et al., 1965). In intestinal absorptive cells it has been shown to function in lipid absorption (Cardell et al., 1967), and in peroxisome formation (Novikoff and Novikoff, 1972).

Rounded vesicles of agranular endoplasmic reticulum were found to be very abundant after vitamin D_3 administration to rachitic chicks. This was especially evident at eighteen hours, when the entire cytoplasm appeared to be full of small vesicles. Some of these may have originated through endocytosis or from the Golgi apparatus, as within the general cytoplasm these are not readily distinguishable from rounded vesicles of agranular endoplasmic reticulum.

The exact signifigance of this proliferation of agranular endoplasmic reticulum is unclear. It may be involved in increased synthesis of lysosomal components, and may also participate in increased synthesis of peroxisomes. As well, the agranular endoplasmic reticulum may function in the synthesis of increased membrane steroid, reflected in the increased brush border cholesterol levels found by Adams et al. (1970) after vitamin D_3 repletion. Another possibility is that it may play some role in increased intracellular transport following vitamin D_3 repletion, and this may include intracellular transport of calcium.

b. Granular Type

The granular endoplasmic reticulum appeared to be less prominant in rachitic than in normal chicks. After vitamin D_3

repletion it became more extensive and showed many dilated cisternae. This was most evident at twelve and seventy-two hours.

Granular endoplasmic reticulum is known to participate in protein synthesis (Palade et al., 1962). It is also known that rachitic animals have slower growth rates than normal (Migicovsky and Emslie, 1947). Vitamin D_3 has previously been demonstrated to stimulate overall protein synthesis, as well as CaBP synthesis, in rachitic chicks (MacGregor et al., 1970). Similarly, increased levels of alkaline phosphatase (Norman et al., 1970) and of Ca-ATPase (Martin et al., 1969; Melancon and DeLuca, 1970) are found after vitamin D repletion.

Therefore the proliferation of granular endoplasmic reticulum and the increase in dilated cisternae may represent stimulation in the overall protein synthetic activity of intestinal absorptive cells which occurs when vitamin D_3 repletion relieves the stress of rickets. As well, these changes may represent synthesis of proteins specifically involved in the mechanism of calcium absorption i.e. Ca-ATPase, alkaline phosphatase, and possibly CaBP as well as other participating carrier proteins or enzymes which are as yet unidentified.

In the time course for calcium absorption following vitamin D_3 repletion in rachitic chicks (Ebel et al., 1969; Wasserman et al., 1971) calcium absorption doubles during the

initial twelve hours and reaches a maximum level by seventy-two hours. Therefore the proliferations of granular endoplasmic reticulum seen at these times may indicate that the vitamin D-induced synthesis of proteins involved in calcium absorption is greatest at these times. An initial increase in protein synthesis would be required to begin the absorptive process. By seventy-two hours an additional boost in protein synthesis might be required to maintain calcium absorption at maximum levels, as at this time the participating proteins would be required in the greatest quantities and would be most rapidly depleted.

7. Mitochondria

a. Regular Fixation

Ions of many types have been found to produce electron dense granules in the mitochondrial matrix: calcium, strontium, and barium (Peachy, 1964); potassium and sodium (Weiss, 1955); and phosphate (Greenawalt et al., 1964). Iron has been shown to produce granules not only in the mitochondrial matrix, but also within the cristae and outer mitochondrial chamber (Oki et al., 1965).

The absorptive cells of small intestine of rachitic rats show only a few such granules within their mitochondrial matrix and their number and electron density increases following vitamin D_2 repletion (Sampson et al., 1970). These granules remain following

microinceration, but are reduced in size, which is suggestive that absorptive cell mitochondria may have a vitamin D-dependent organic component with the ability to bind calcium. In cells transporting large amounts of calcium the ability of the mitochondria to accumulate calcium is apparently utilized as an intracellular buffer system (Borle, 1971).

Slightly electron dense granules, similar to those described by Palade (1953), were seen in the mitochondrial matrix of all chicks examined. Large electron dense granules resembling those seen by Sampson et al. (1970) were seen only in chicks which were fed a low calcium diet. This suggests that these animals, which adapt to the low availability of calcium by increasing the efficiency of vitamin D-induced intestinal calcium absorption (Taylor and Wasserman, 1969; Wasserman et al., 1971), may also respond by increasing the efficiency of their mitochondrial buffering system, or by accumulating more calcium within this compartment. This might involve increased production of the vitamin D-dependant organic component of mitochondria (Sampson et al., 1970) which binds calcium within the matrix or an increase in its affinity for calcium.

The presence of large granules within the mitochondria of low calcium diet chicks may have been influenced by the long time period over which they were fed this diet. These chicks would have become fully adapted to this situation, thus would have responded

by efficiently absorbing the available calcium. Thus the demand for calcium exit from these cells may have been less than the immediate physiological demand placed upon the vitamin D_3-replete cells after removal of the stress of rickets. Rapid exit of calcium from the latter cells may have not allowed large mitochondrial granules to accumulate, or the entry and exit of calcium in these cells may have nearly balanced so as not to place large demands on the mitochondrial buffering system.

DeLuca et al. (1960) observed mitochondrial swelling and disruption in kidney mitochondria isolated from rachitic rats but not in those isolated from vitamin D-treated rats. Such changes, when observed in the mitochondria of chick absorptive cells, showed no correlation to the vitamin D state of the animals, but appeared rather to be artifacts produced in fixation.

b. S-Collidine Buffered Paraformaldehyde Fixation

Rachitic absorptive cells, fixed using S-collidine buffered paraformaldehyde to allow better visualization of mineral (Sampson et al., 1970), showed few mitochondrial granules and these were only slightly electron dense. The mitochondrial granules appearing vitamin D_3-replete cells, fixed by the same method, appeared more frequently and possessed greater electron density. These granules were more electron dense than those in the groups prepared by regular fixation, except for the chicks fed a low calcium

diet. In the latter group the mitochondrial granules were larger and appeared more frequently than in the vitamin D-replete material fixed with S-collidine buffered paraformaldehyde.

The observation here of increased occurrence of electron dense mitochondrial granules following vitamin D repletion is consistant with the observations of Sampson et al. (1970), using the same fixation technique on rats. Treatment of vitamin D_3-replete duodenum of chick with EDTA caused the mitochondrial granules to lose their electron density so that they resembled those seen in rachitic chicks. This observation is consistant with the suggestion of Sampson et al. (1970) that these granules are composed of calcium bound to some organic component. However, the granules seen in vitamin D_3-replete chicks were not as large, nor did they occur as frequently as those seen in vitamin D-replete rats (Sampson et al., 1970). This may be due to species differences in the rates of calcium absorption and/or of calcium transport out of the absorptive cells, following vitamin D repletion, or in the physiological demands for calcium following the removal of rickets. This might result in differences in the demand placed upon their mitochondrial buffering systems and their accumulation of mitochondrial granules.

8. Lateral and Basal Plasma Membranes and the Intercellular Space

a. S-Collidine Buffered Paraformaldehyde Fixation

The intercellular space of absorptive epithelium has been found to serve as a transport channel for various substances including: lipid (Palay and Karlin, 1959; Cardell et al., 1967), protein (Rodewald, 1971), and vitamin B_{12} (Weisberg et al., 1968). In frog skin epithelium the size of the intercellular spaces has been shown to change in response to the rate of ion (sodium) transport across the epithelium (Voûte and Ussing, 1968).

In the present experiments, the absorptive cells of duodenum fixed using S-collidine buffered paraformaldehyde (Sampson et al., 1970) possessed small electron dense deposits along their basal and lateral membranes and within the intercellular space in both rachitic and vitamin D_3-replete chicks. Similar deposits were seen previously on the absorptive cell membranes of cockroach midgut caeca when these were prepared using fixatives which contained calcium (Oschman and Wall, 1972).

Material entering the intercellular spaces is usually pictured as having to pass through the absorptive cells, as the juxta-luminal junctional complexes prevent free entry of materials into these regions directly from the lumen (Farquhar and Palade, 1963). Lanthanum has been used as a tracer to investigate the permeability of junctional complexes. Overton (1968) found that

the juxta-luminal junctional complexes of mouse duodenum were impermeable to lanthanum. However, recently it has been found that lanthanum can penetrate the junctional complexes of rabbit ileum and gallbladder (Machen et al., 1972). Thus the tissues of at least some species possess 'leaky' junctional complexes, through which ions can pass. Whether chick duodenal junctional complexes possess this property has not been demonstrated.

It has been suggested that lanthanum may stain by displacing calcium (Doggenweiler and Frenk, 1965). Thus junctional complexes which are permeable to lanthanum may also be permeable to calcium.

In the present experiments it was found that EDTA treatment removed the small electron dense deposits from the lateral and basal plasma membranes and from within the intercellular spaces, indicating that these deposits may have been calcium. Similar deposits were seen on the microvilli, and within the intercellular spaces they appeared to occur both apcial to and at the level of the juxta-luminal junctional complexes, as well basal to them. This suggests that material forming deposits may have entered the intercellular spaces directly from the lumen and that chick duodenum may possess leaky junctional complexes.

As these electron dense deposits showed similar patterns of distribution in both rachitic and vitamin D_3-replete chicks it

appears that the intercellular spaces may form a pathway for calcium transport across chick absorptive epithelium which may function independently of the action of vitamin D_3. Such transport would probably occur by diffusion, and the movement of calcium by this pathway would be measured along with calcium transported via the absorptive cells in studies utilizing everted gut sac preparations.

It was suggested by Oschman and Wall (1972) that electron dense deposits found adhering to the membranes of cells of cockroach midgut caeca, when fixed using calcium-containing fixatives, represented binding sites for calcium. Thus it may be that the deposits seen adhering to the lateral and basal membranes of absorptive cells of chick duodenum may represent areas across which calcium is transported out of the absorptive cells.

b. Regular Fixation

The intercellular spaces of chick duodenal epithelium were often dilated, or showed vacuole-like structures, towards the bases of the cells. These vacuolar structures became quite prominent at eighteen hours after vitamin D_3 repletion, and remained so in the remaining experimental groups through seventy-two hours. Their contents was slightly electron dense and globular in appearance, similar to the contents of the larger Golgi vacuoles.

Similar material was also seen accumulated below the basal membranes of absorptive cells in the same experimental groups.

As the globular material within the intercellular spaces resembled the contents of Golgi vacuoles and increased in abundance after Golgi activity had increased, following vitamin D_3 repletion, it may have originated in the Golgi apparatus. The nature and function of this material is unknown. Neither is it apparent whether this material is directly related to vitamin D's action on the absorptive epithelium or whether it is involved in vitamin D-induced calcium absorption.

Site of Vitamin D-Induced Calcium Binding Protein Synthesis

Taylor and Wasserman (1970), using a fluorescent antibody localization technique, observed specific fluorescence over the goblet cells and the brush border of all duodenal intestinal epithelium of vitamin D_3-replete chicks. They observed that absorptive epithelium from rachitic chicks showed non-specific fluorescence only. They interpreted these results as indicating that the goblet cells release CaBP, which subsequently becomes associated with the brush border where it participates in calcium absorption. They could not determine the exact location of CaBP in the brush border, nor the site of CaBP synthesis. They suggested that it could be synthesized in the goblet cells, in the absorptive cells then transferred to the goblet cells, or in both

cell types, but that the first case would be the most likely.

In the present experiments goblet cells of normal, rachitic, vitamin D_3-replete, and low-calcium diet chicks were examined electron microscopically to find ultrastructural changes which could be correlated with the synthesis and secretion of CaBP by these cells. No such consistant differences could be found. In all groups individual goblet cells were found to vary in the appearance of their mucous mass (size, electron density, and degree of coalescence of the mucous droplets). The same variations were found in all groups with no apparent differences in the frequency of appearance of these variations. It seems that these differences were due to normal variations in the secretory activity of the individual cells. The foot regions of the goblet cells of all groups were very similar in appearance.

When goblet cells in material fixed with S-collidine buffered paraformaldehyde (Sampson et al., 1970) were examined, it was found that those from vitamin D_3-replete animals possessed many electron dense deposits on the cytoplasmic remnants in and around the apical portion of the mucous mass as it was expelled. In this group many more electron dense deposits were seen on the microvilli adjacent to the mucous mass than in the rachitic animals. EDTA treatment removed these electron dense deposits suggesting that they may be calcium.

These observations indicate that CaBP, after its synthesis is induced by vitamin D, may be released from the goblet cells in association with the cytoplasmic remnants in the mucous secretion. In the goblet cells of the groups fixed by regular fixation no changes could be seen in the qualitative or quantitative nature of these remnants. Thus it remains questionable whether the remnant compartment is large enough to represent all the CaBP necessary for calcium absorption. Also it cannot be determined from the present observations whether the CaBP would be physically or chemically associated with these remnants, or how it would be released from them and distributed over the entire brush border.

These observations are similar to those of Taylor and Wasserman (1970) in that they indicate where CaBP may be located at some stage in its lifespan, but do not indicate conclusively where CaBP is actually synthesized. A major reason to question synthesis of CaBP by the goblet cells is that CaBP also appears in chick kidney (Wasserman and Taylor, 1966; Taylor and Wasserman, 1967) which is devoid of goblet cells, and in hen shell gland (Corradino et al., 1968) which contains some goblet cells (Schraer and Schraer, 1971). In neither of these tissues has the site of CaBP synthesis been determined.

Taylor and Wasserman (1970) found that the specific fluorescence indicative of CaBP could not be removed from the microvillar surface by washing. This observation favoured the

possibility of CaBP synthesis by the absorptive cells, as it indicated a form attachment of CaBP to the glycocalyx or plasma membrane. The glycocalyx cannot be removed by washing, is synthesized within the absorptive cells, and is renewed rapidly (Ito, 1965a, b; 1969). Therefore if CaBP is associated with the glycocalyx it may have a similar origin. Alternately, CaBP may rapidly adhere to the glycocalyx after being released from the goblet cells and resist removal by mechanical means.

Leblond and associates have found that the absorptive cell coat rapidly incorporates label originally appearing in the Golgi apparatus (Neutra and Leblond, 1966; Bennett, 1970; Bennett and Leblond, 1971). In the present experiments, Golgi activity was found to increase after vitamin D_3 repletion. This may result in a rapid turnover of the glycocalyx, allowing carriers or binders involved in vitamin D-induced calcium absorption to take positions on the glycocalyx. Increased amounts of granular endoplasmic reticulum and dilation of the cisternae was also found in the absorptive cells after vitamin D_3 repletion, and although this does not indicate what proteins are being synthesized it is not unfavourable to CaBP synthesis by the absorptive cells.

The three possibilities as to the site of CaBP synthesis proposed by Taylor and Wasserman (1970) all have some merit. As CaBP appears to be released by the goblet cells it would be

"economical" for them to synthesize this protein. However, the protein synthetic activities of the absorptive cells, and the origin of the glycocalyx within these cells indicates that CaBP could be synthesized here as well, and secreted in association with the glycocalyx. Alternately these cells may synthesize CaBP then relay it to the goblet cells by some form of exocytotic and endocytotic activity. Vesicles with these apparent functions are seen at the lateral plasma membranes of both goblet and absorptive cells. The final possibility is that CaBP may have a dual origin and be produced by both cell types. The exact site of CaBP synthesis, as well as its exact location in the brush border, will require autoradiographic and antibody localization techniques to be performed at the electron microscopic level.

Conclusions

In summary, the following sequence, based on the present experiments and the results of previous authors, is proposed as a possible model for vitamin D_3-induced calcium absorption in chick duodenum.

Vitamin D_3, in the form of 1,25-DHCC, acts on the absorptive cells inducing the formation of transport or binding proteins, enzymes, and components necessary for structural alteration of the plasma membrane, all of which facilitate calcium

absorption by the absorptive cells. It may also act on the goblet cells, inducing the release of CaBP, and possibly the synthesis of this protein by these cells. The method by which calcium absorption occurs may depend on the luminal calcium concentration or on the physiological demands exerted through the animal's metabolism. When the luminal calcium concentration is low, or the physiological demands for this ion are great, absorption may occur mainly via an active transport process which exerts great specificity. This process may be endocytosis. When the physiological demands for calcium are not very great, or it is available in excess, then its absorption may occur mainly by diffusion. These two processes may act at the same time, as may other processes of calcium absorption which are as yet undiscovered.

In endocytosis, calcium adsorbed to the glycocalyx, or complexed to CaBP, is adsorbed by the formation of endocytotic vesicles. Energy for this process may be derived from a process involving Ca-ATPase and alkaline phosphatase. As the apical membrane is mobile, calcium adsorbed anywhere on its surface might eventually enter an apical pit and be endocytosed. The endocytotic vesicles would traverse the terminal web region, and in the apical cytoplasm would fuse with a primary lysosome, either directly, or subsequent to joining a multivesicular body. The primary lysosome so activated would become a secondary lysosome or digestive vacuole, and would continue to fuse with endocytotic

vesicles. Within the digestive vacuole calcium would be released from CaBP then liberated into the cytoplasm.

Calcium absorption by diffusion may occur anywhere on the microvillar surface. This may involve structural changes in the membrane, and the participation of binding and/or carrier proteins. The overall effect of these would be to reduce the diffusional barrier to calcium exerted by the plasma membrane. The lecithin-lysolecithin cycle may be involved in this process by releasing calcium from CaBP in the plasma membrane.

The endoplasmic reticulum and Golgi apparatus may participate in both systems through the synthesis of necessary proteins, steroids, and components of the glycocalyx. The Golgi complex and endoplasmic reticulum may also participate through the synthesis of lysosomal enzymes.

Once calcium has entered the absorptive cells, regardless of how it has entered, it may be taken up by the mitochondria, in order to maintain physiological calcium concentrations within the cytoplasm. Finally it is released across the basal and lateral plasma membranes by an active transport or a diffusionsl exchange system involving sodium. An alternate pathway for calcium transport across the absorptive epithelium may occur independent of the action of vitamin D by diffusion across 'leaky' junctional complexes and through the intercellular spaces.

It will require further experimentation, utilizing autoradiography, and antibody labelling and cytochemical techniques at the electron microscope level to determine the sites of synthesis and the functional locations of the carriers and enzymes involved in vitamin D-induced intestinal calcium absorption. Additional study will be required to determine the roles of endocytosis and the vacuolar apparatus, and of the intercellular spaces in the absorption and transport of calcium. Further studies, at the biochemical ultrastructural levels, will be necessary to fully understand the role of the Golgi apparatus, endoplasmic reticulum, mitochondria, and peroxisomes, or of their products, in vitamin D-induced intestinal calcium absorption.

CHAPTER FIVE

SUMMARY

1. The ultrastructure of the columnar absorptive cells and goblet cells of duodenum was studied by electron microscopic examination in normal, rachitic, vitamin D_3-replete chicks and chicks fed a low calcium diet.

2. The absorptive cell microvilli decreased in length during rickets, and increased following vitamin D_3 repletion. No changes in microvillar diameter accompanied the changes in length.

3. Apical pits and vesicles appeared to decrease in number in rachitic chicks, and to increase following vitamin D_3 repletion. Many apical pits and vesicles were seen in the absorptive cells of chicks adapted to a low calcium diet.

4. In rachitic chicks the lysosomal population of the absorptive cells was composed mainly of dense bodies. After vitamin D_3 repletion this population changed, and became composed mainly of secondary lysosomes. This was confirmed by the demonstration of acid phosphatase activity.

5. Multivesicular bodies appeared least frequently in rachitic and most frequently in low calcium chicks.

6. Peroxisomes increased in number, as compared with the normals, in rachitic chicks. During the first eighteen hours following

vitamin D_3 repletion, peroxisomes remained numerous, then subsequently decreased in number.

7. It was found that peroxisomes of chick duodenal absorptive cells contain a central core, composed of tubule-like structures.

8. The Golgi complex of absorptive cells showed the most activity during the first eighteen hours following vitamin D_3 repletion.

9. Agranular endoplasmic reticulum was most abundant in the absorptive cells at eighteen and twenty-four hours following vitamin D_3 repletion.

10. Granular endoplasmic reticulum was less extensive in rachitic than in normal chicks, and following vitamin D_3 repletion it became extensive and showed many dilated cisternae. The granular endoplasmic reticulum was most well developed at twelve and seventy-two hours following vitamin D_3 repletion.

11. Electron dense granules within the mitochondrial matrix occurred most frequently, and were the largest and most electron dense in chicks adapted to a low calcium diet.

12. The intercellular spaces of the absorptive epithelium showed many dilations filled with globular material following vitamin D_3 repletion. Material of similar appearance was also seen accumulated below the basal plasma membranes of the absorptive cells. This material was similar in appearance to the contents of the Golgi vacuoles.

13. No changes were seen in the ultrastructure of the goblet

cells which could be related to the vitamin D_3 states of the chicks.

14. Duodenum from rachitic and twenty-four hour vitamin D_3-replete chicks was prepared according to the mineral visualization technique of Sampson et al. (1970) and electron dense deposits were found in the following regions of the absorptive cells:

a. Large electron dense granules were seen adhering to the microvillar surface, more frequently in rachitic than in vitamin D_3-replete chicks.

b. Small electron dense deposits were seen adhering to the microvillar plasma membrane, more frequently in vitamin D_3-replete than in rachitic chicks.

c. Large electron dense granules, similar to those seen on the microvilli, were sometimes seen in the apical pits, but never within the apical vesicles of either group. Small electron dense granules, similar to those seen on the microvillar plasma membrane, were seen more frequently on the limiting membranes of apical pits and vesicles in vitamin D_3-replete than in rachitic chicks.

d. Similar small electron dense granules were seen adhering to the lateral and basal plasma membranes, and within the intercellular spaces, with similar frequency in both groups.

e. Granules within the mitochondrial matrix appeared more frequently and were of greater electron density in vitamin D_3-replete

than in rachitic chicks.

f. Small electron dense granules were seen more frequently in the lysosomes of vitamin D_3-replete than in rachitic chicks.

g. EDTA treatment removed almost completely the electron dense granules and deposits cited in a, b, c, and d. EDTA treatment caused the electron dense granules within the mitochondrial matrix of vitamin D_3-replete chicks to resemble the pale granules of rachitic chicks. This treatment caused partial removal of the granules within the lysosomes.

15. Small electron dense granules were seen adhering to the cytoplasmic remnants within the apical part of the goblet cell mucous mass in vitamin D_3-replete, but not in rachitic chicks, fixed according to the mineral visualization technique. Electron dense deposits occurred more frequently on the microvilli adjacent to the mucous mass of vitamin D_3-replete than of rachitic chicks. These deposits were removed by EDTA treatment.

16. A hypothetical model of vitamin D-induced intestinal calcium absorption in chick is proposed to correlate these ultrastructural observations with the work of previous authors. This includes the proposals that calcium absorption by active transport may involve endocytosis and the vacuolar apparatus, that the ultrastructural changes observed in the Golgi apparatus and endoplasmic reticulum may be related to the synthesis of components involved in calcium absorption by active transport and diffusion, and that the goblet

cells may participate through the release of CaBP. It is also suggested that the intercellular spaces may represent diffusional pathways for calcium, independent of the action of vitamin D.

FIGURES

List of Abbreviations Used in Figures

AP	Apical pit
AV	Apical vesicle
BB	Brush border
BL	Basal lamina
CH	Chromatin
CY	Cytoplasm
D	Desmosome
DB	Dense body
DG	Electron dense granules
F	Filaments
FT	Foot
G	Golgi complex
GC	Goblet cell
GS	Golgi saccule
GV	Golgi vesicle
I	Interdigitation
ICS	Intercellular space
L	Lysosome
M	Mitochondrion
MA	Macula adherens
MC	Mitochondrial crista
MG	Mitochondrial granule
MVB	Multivesicular body
MU	Mucous droplet
N	Nucleus
P	Peroxisome
PR	Polyribosome
R	Rootlet
RER	Granular endoplasmic reticulum
SER	Agranular endoplasmic reticulum
SL	Secondary lysosome
TH	Theca
TJ	Juxta-luminal junctional complex
TW	Terminal web
VAC	Vacuole
ZA	Zonula adherens
ZO	Zonula occludens

All figures, unless otherwise indicated, material illustrated was fixed by regular fixation and stained with uranyl acetate and lead citrate.

Fig. 1. Longitudinal section through the duodenal epithelium of normal chick. Several columnar absorptive cells and a goblet cell are seen. The lateral surfaces of the absorptive cells are held in close apposition apically by the juxta-luminal junctional complex (TJ). Below the junctional complex they form numerous interdigitations (I), and below the level of the nucleus (N) dilations of the intercellular space (ICS) appear. The major divisions of the absorptive cell cytoplasm can be seen: the brush border (BB); the terminal web (TW); and the apical and basal cytoplasm, separated by the nucleus. These last two ctyoplasmic regions are seen to contain mitochondria (M), lysosomes (L), and granular endoplasmic reticulum (RER). The Golgi apparatus (G) is located in the supranuclear region. The two major regions of the goblet cell are visible: the mucous filled theca (TH) and the slender foot (FT) containing the basally located nucleus. A thin basement lamina (BL) underlies the epithelium. x 7,980.

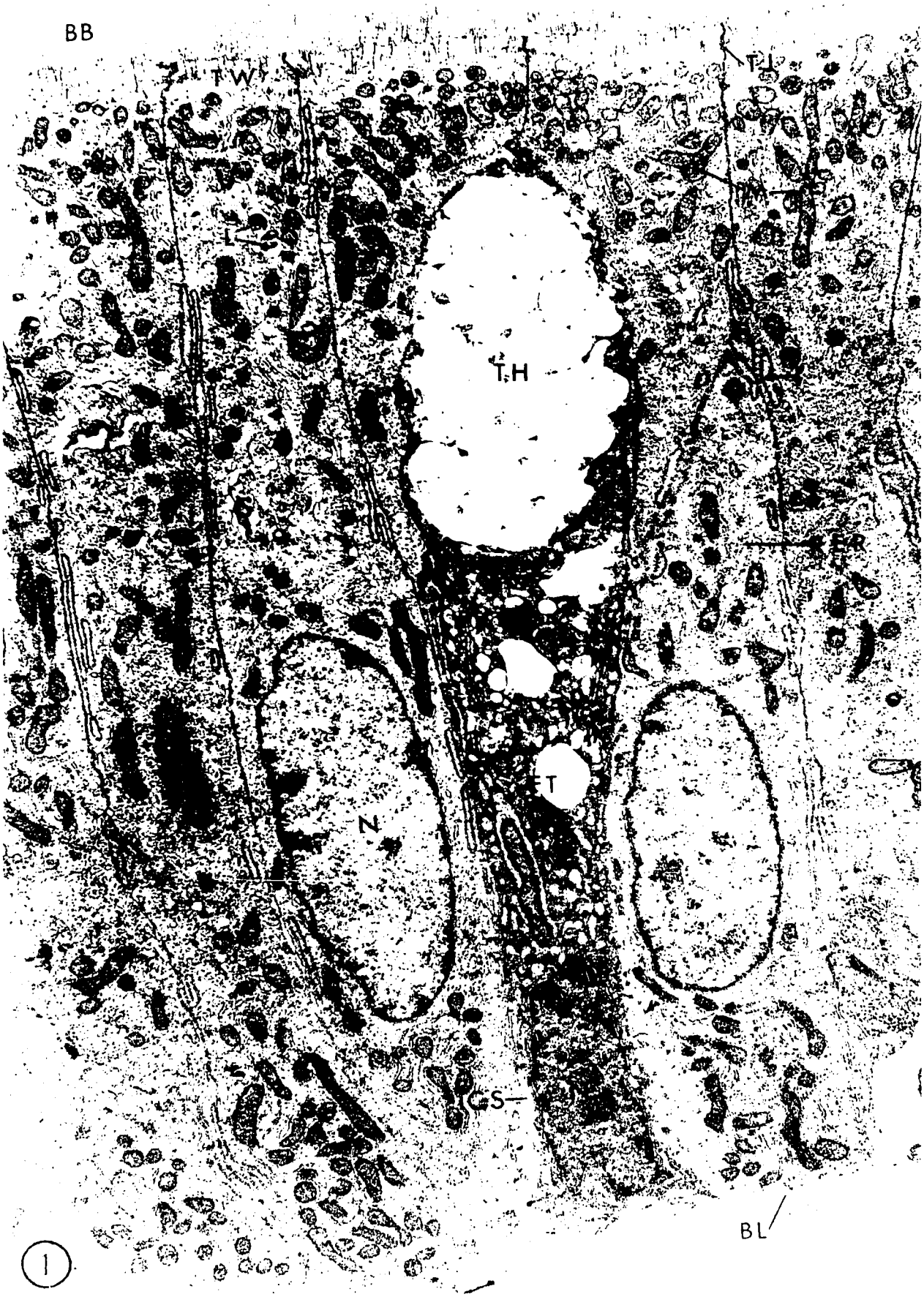
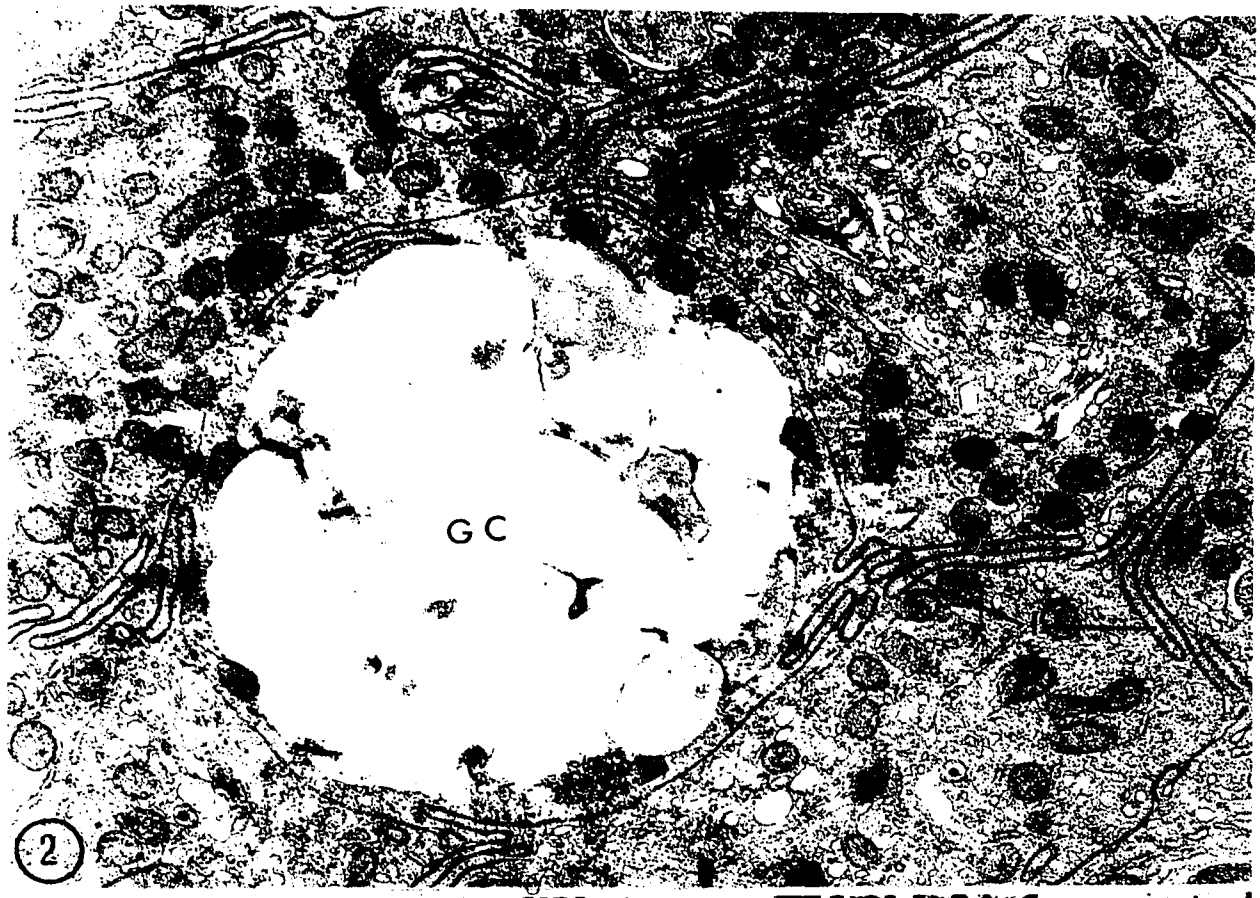

BB
TW
T
T.H
ER
T
N
GS
BL
1

Fig. 2. Cross section of several absorptive cells and a goblet cell (GC) through the supranuclear region of a normal chick. The absorptive cell at upper right shows the roughly hexagonal form characteristic of these cells in cross section. The lateral membranes of both the goblet and absorptive cells form extensive interdigitations. X 9,690.

Fig. 3. Oblique section through the absorptive cell brush border of normal chick. Filaments forming the glycocalyx are visible at right above the tips (*) and between the microvilli radiating from their membranes (⟶). The trilaminar structure of the plasma membrane of the microvilli and the filaments (F) forming the core of the microvilli are clearly visible. Some of the microvilli are arranged in hexagonal formation. X 75,240.


GC
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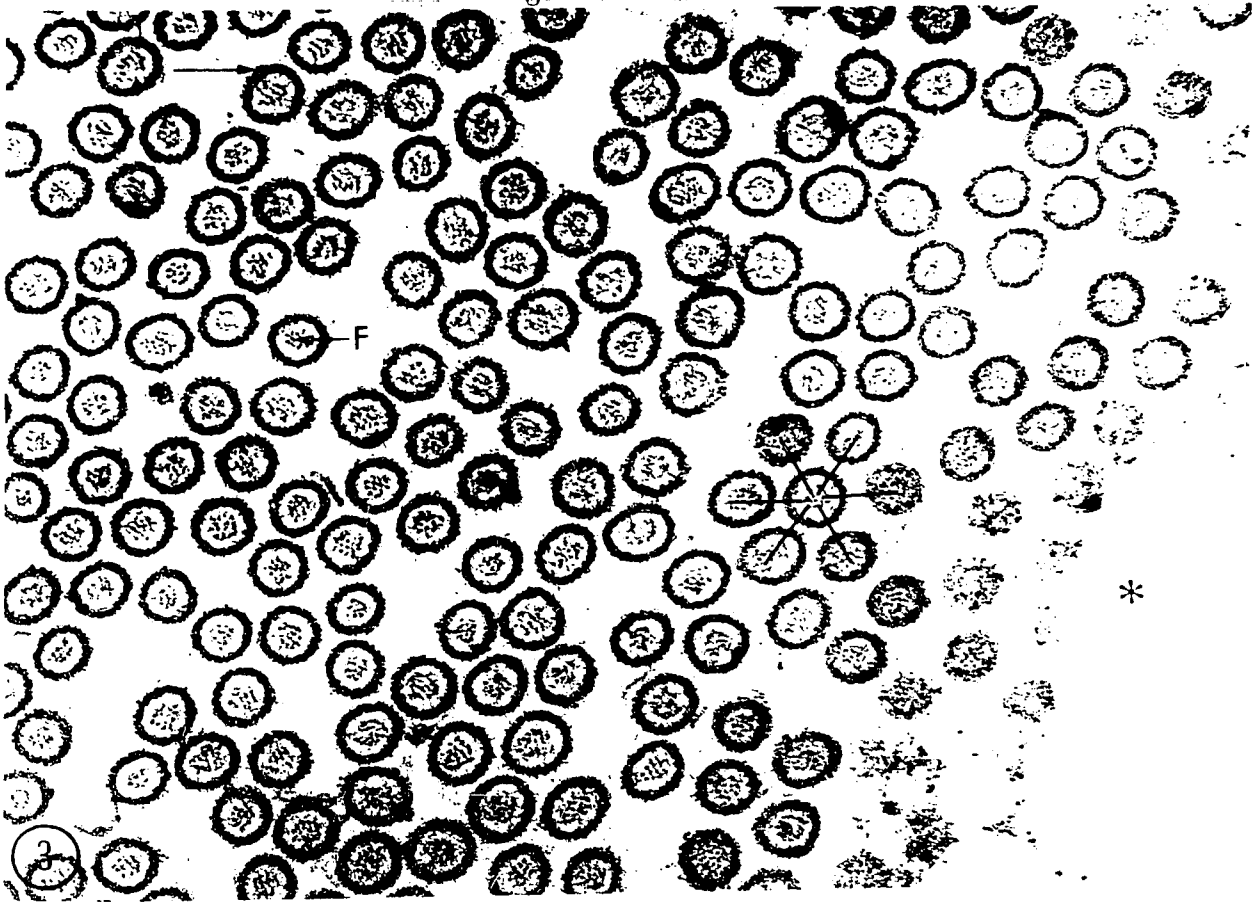
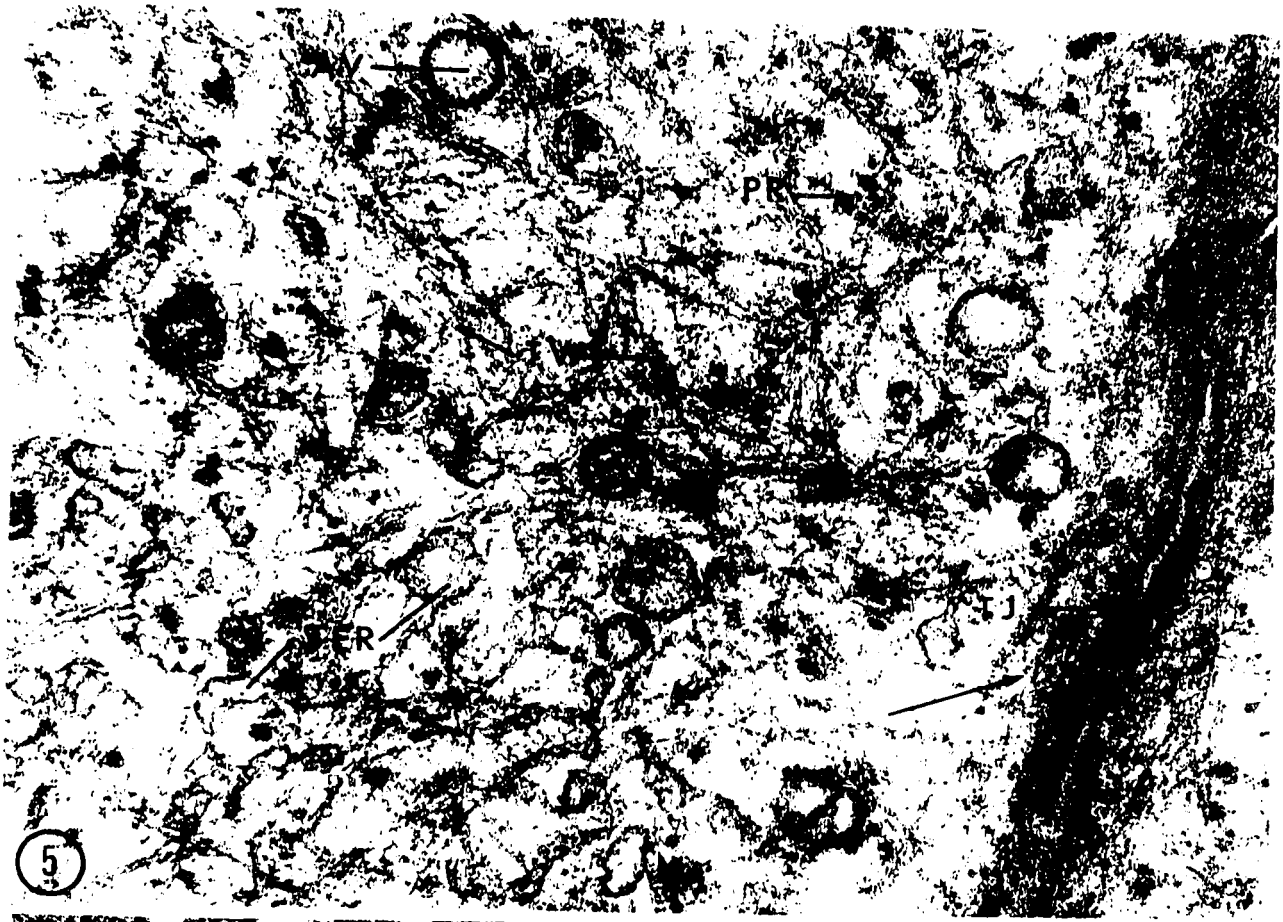

F
*
3

Fig. 4. Longitudinal section of microvilli of normal chick. The glycocalyx (*) is visible above and between the microvilli. The core filaments can be seen to extend into the region of the terminal web as rootlets (R). The trilaminar structure of their plasma membrane is again clearly visible. The microvilli are spaced equidistantly along the apical surface of the cell. Between their bases apical pits (AP) are seen as depressions of the plasma membrane. The filaments of the terminal web (TW) form a band across the apical cytoplasm. Within the terminal web region are seen apical vesicles (AV), vesicles of agranular endoplasmic reticulum (SER), and occasionally free polyribosomes (PR). Mitochondria and granular endoplasmic reticulum appear below the terminal web, and these organelles are seen in the terminal web region. X 62,700.

4

Fig. 5. Oblique section through the filamentous network of the terminal web of normal chick. At right a portion of the juxta-luminal junctional complex appears. The filaments which parallel the plasma membrane at the junctional complex are seen to be continuous with those of the terminal web (⟶). Several apical vesicles with slightly electron dense contents are visible. At the lower left agranular endoplasmic reticulum is abundant. X 62,700.

Fig. 6. and 7. Longitudinal sections through the juxta-luminal junctional complex of normal chick. The three areas of the junctional complex, as described by Farquhar and Palade (1963), are visible in both figures: the zonula occludens (ZO), the zonula adherens (ZA), and the macula adherens (MA). In fig. 6 an apical pit (A P) is seen to the left of the junctional complex. In fig. 7 the filaments of the macula adherens and the terminal web are seen to be continuous (⟶). Fig. 6 X 62,700. Fig. 7 X 75,240.



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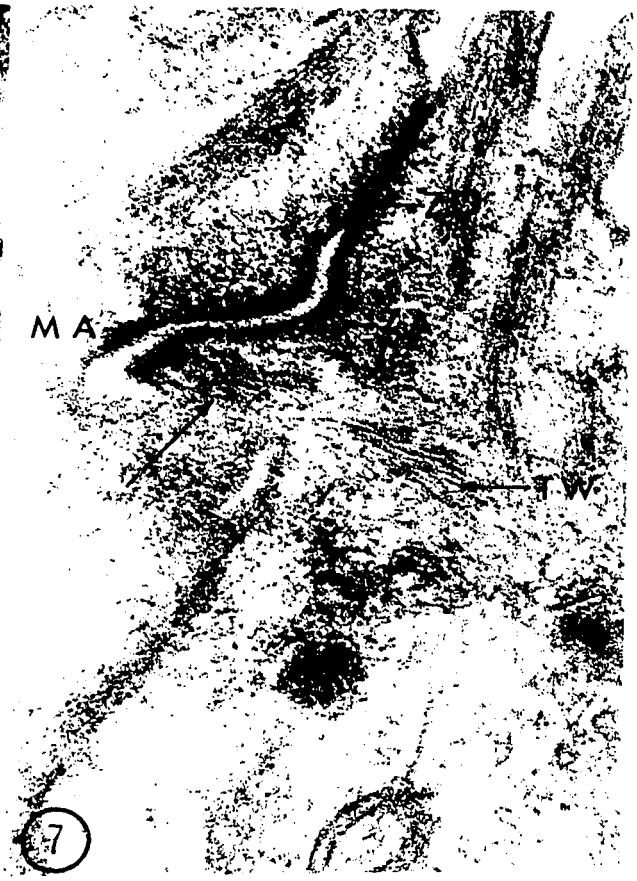
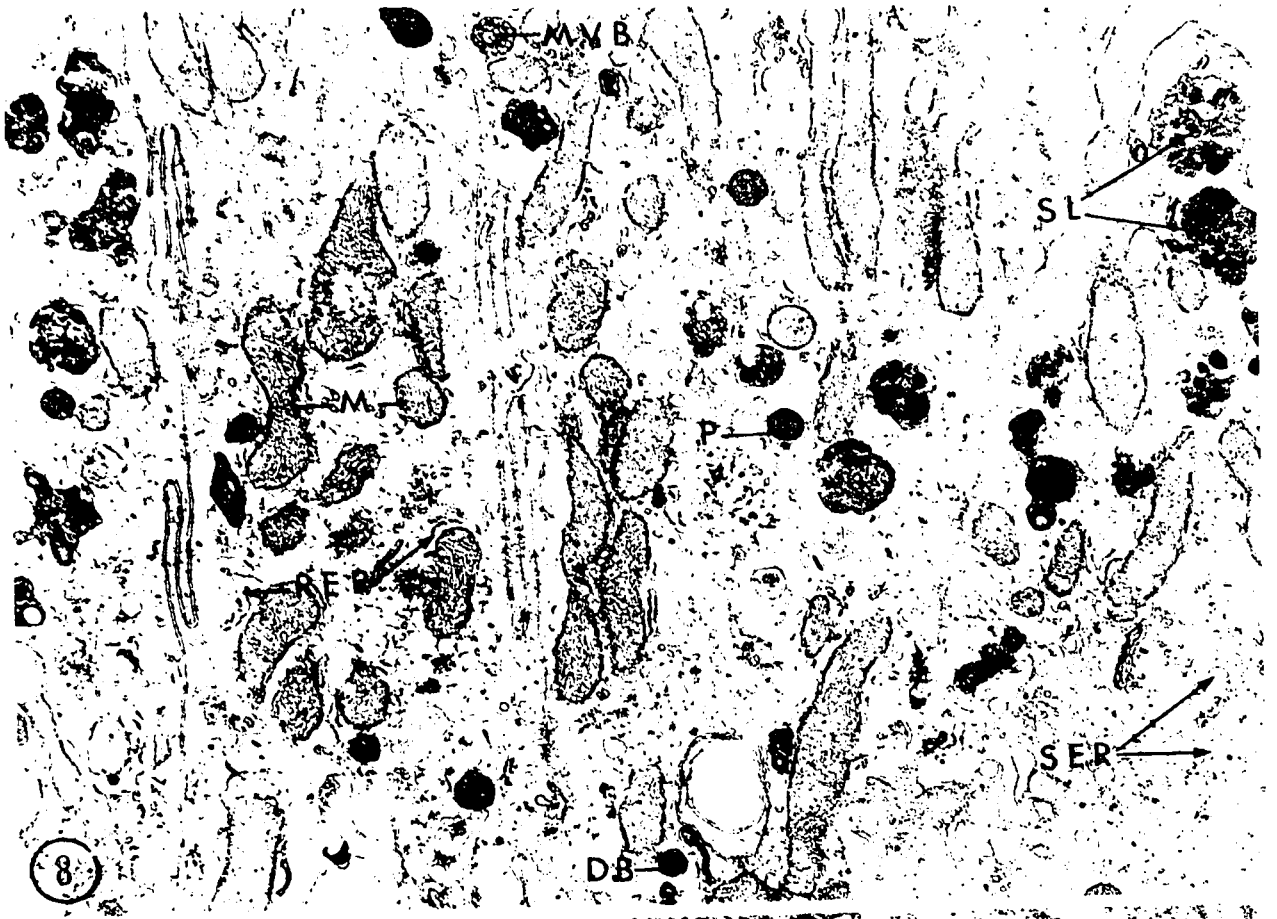


7

Fig. 8. Longitudinal section of the upper half of the apical cytoplasm of the absorptive cells of normal chick. Many mitochondria are visible, most of them oriented parallel to the long axis of the cell. Several secondary lysosomes (SL) and peroxisomes (P) are visible. In the lower portion of the figure a dense body (DB) is visible. Dense bodies are not easily distinguishable from peroxisomes at low magnifications. Cisternae of granular endoplasmic reticulum are seen in close proximity to mitochondria. Rounded and tubular cisternae of agranular endoplasmic reticulum, and free polyribosomes are abundant throughout this region. X 13,970.

Fig. 9. Section through the apical cytoplasm below the terminal web of normal chick. Several multivesicular bodies (MVB) are seen. Note their single limiting membrane, enclosing many smaller vesicles, each with its own limiting membrane ($\longrightarrow$). Several mitochondria are visible with transversely oriented cristae (MC) and slightly electron dense granules (MG) within their matrix. X 47,880.



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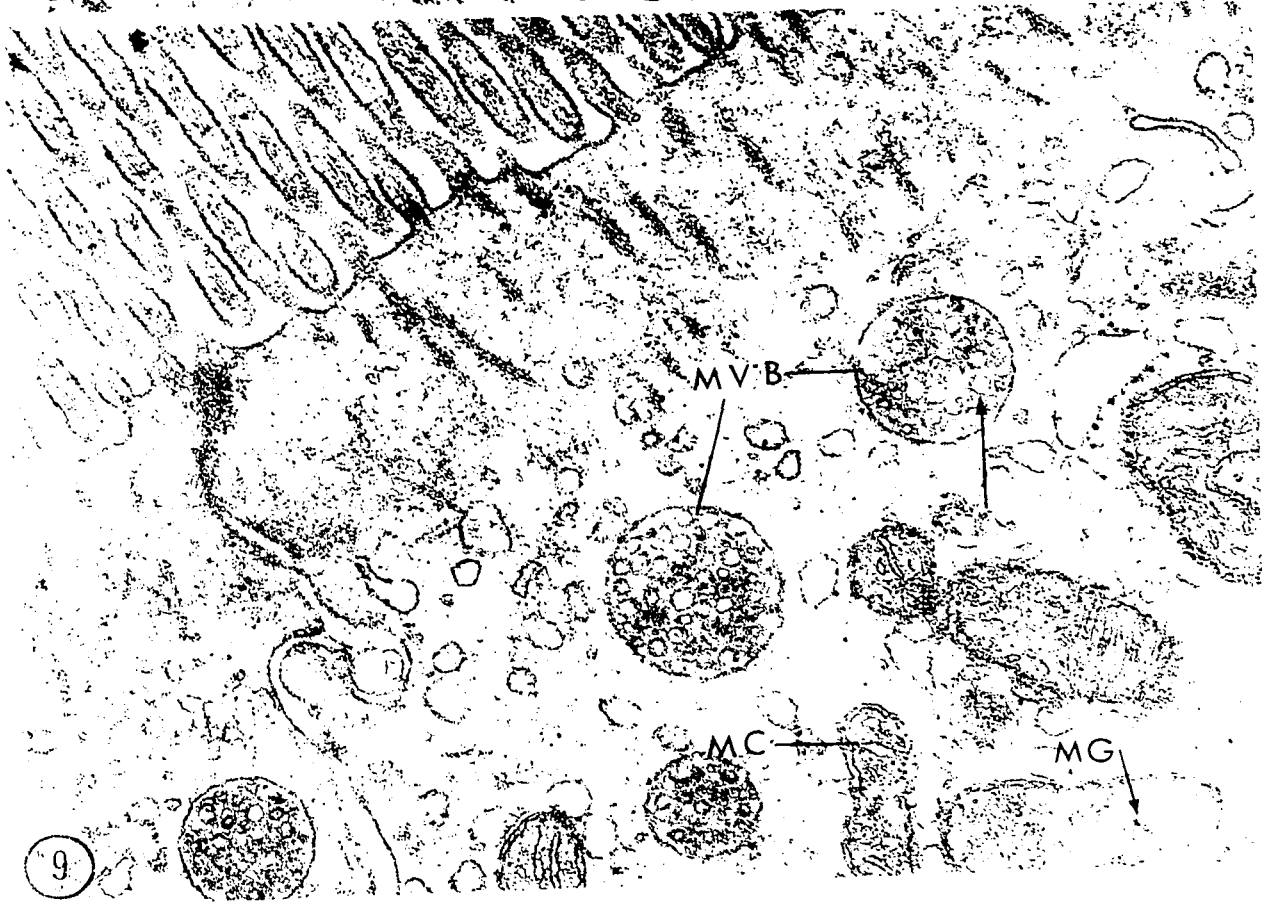

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Fig. 10. Apical cytoplasm at higher magnification. The secondary lysosomes are seen to contain heterogenous material of varying electron density. They vary in shape from rounded to lobular. X 55,860.

Fig. 11. A dense body. Its contents appear highly electron dense and fairly homogenous. Note the single limiting membrane, best visible on its lower left side (⇨). X 47,880.

Fig. 12. Several extremely large secondary lysosomes of chick fed a low calcium diet. They contain a vast array of cytoplasmic organelles. Extensive myelination is seen within the largest of the three (⇨). X 39,900.

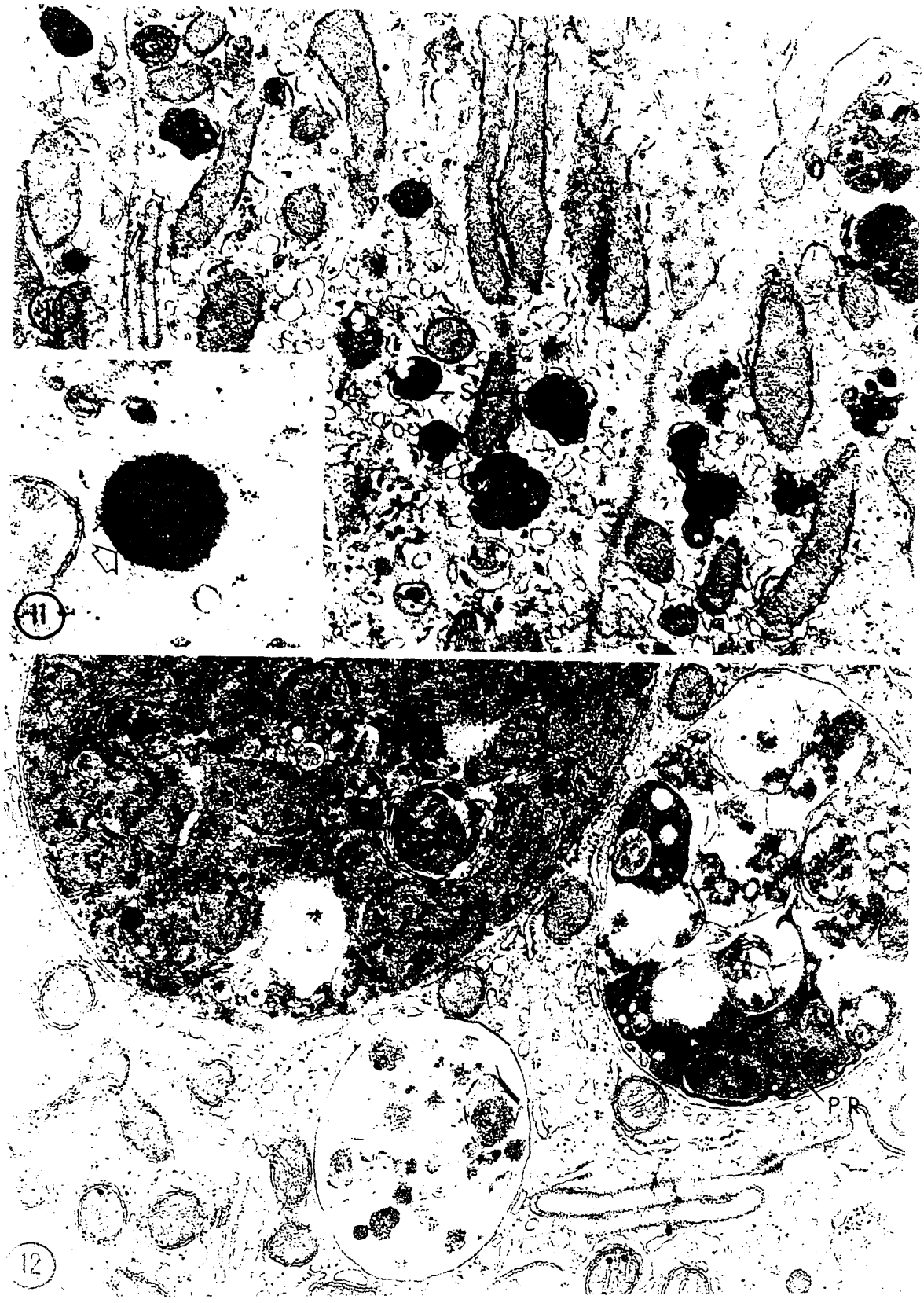
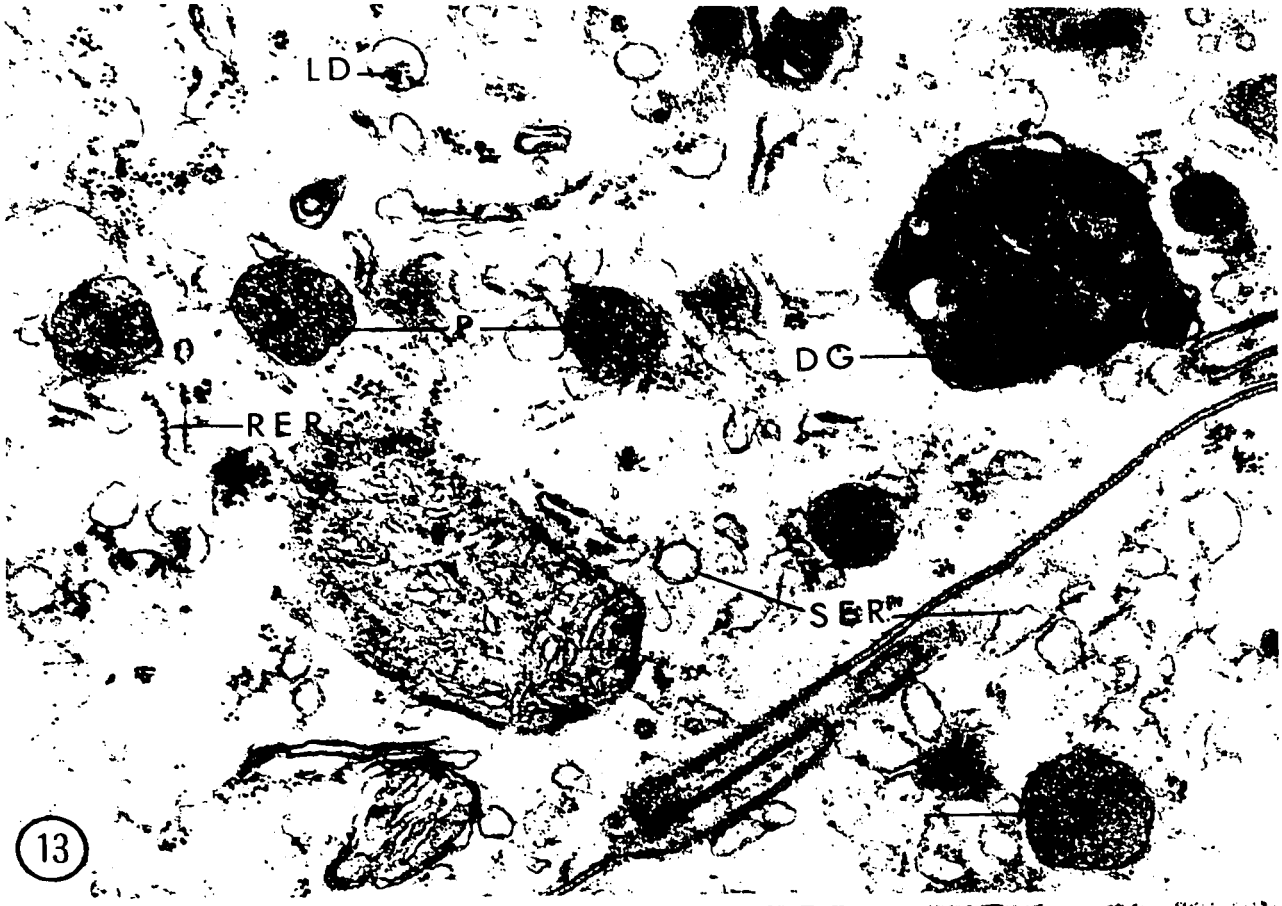

11
12
PR

Fig. 13. Secondary lysosome and several peroxisomes of normal chick. Many small electron dense granules (DG) are seen within the secondary lysosome. The contents of the peroxisomes appear less dense than that of dense bodies at higher magnifications. Some sections of peroxisomes show a core which appears more electron dense than the surrounding material. Sections of vesicular and tubular cisternae of agranular endoplasmic reticulum are also visible. In the upper portion of the figure one of these is seen to contain electron dense lipid droplets. X 39,900.

Fig. 14. Peroxisomes and endoplasmic reticulum of normal chick. Continuity can be seen between the peroxisomal limiting membrane and a membrane of agranular endoplasmic reticulum (⇨). Continuity between granular and agranular endoplasmic reticulum is seen at many sites (⟶). X 61,650.

14a. Inset shows a peroxisome with a clearly visible central core. The core appears to be composed of tubule-like structures. X 55,860.

14b. Inset shows two peroxisomes with their central cores cut in oblique section, appearing as a band across each peroxisome. X 55,860.



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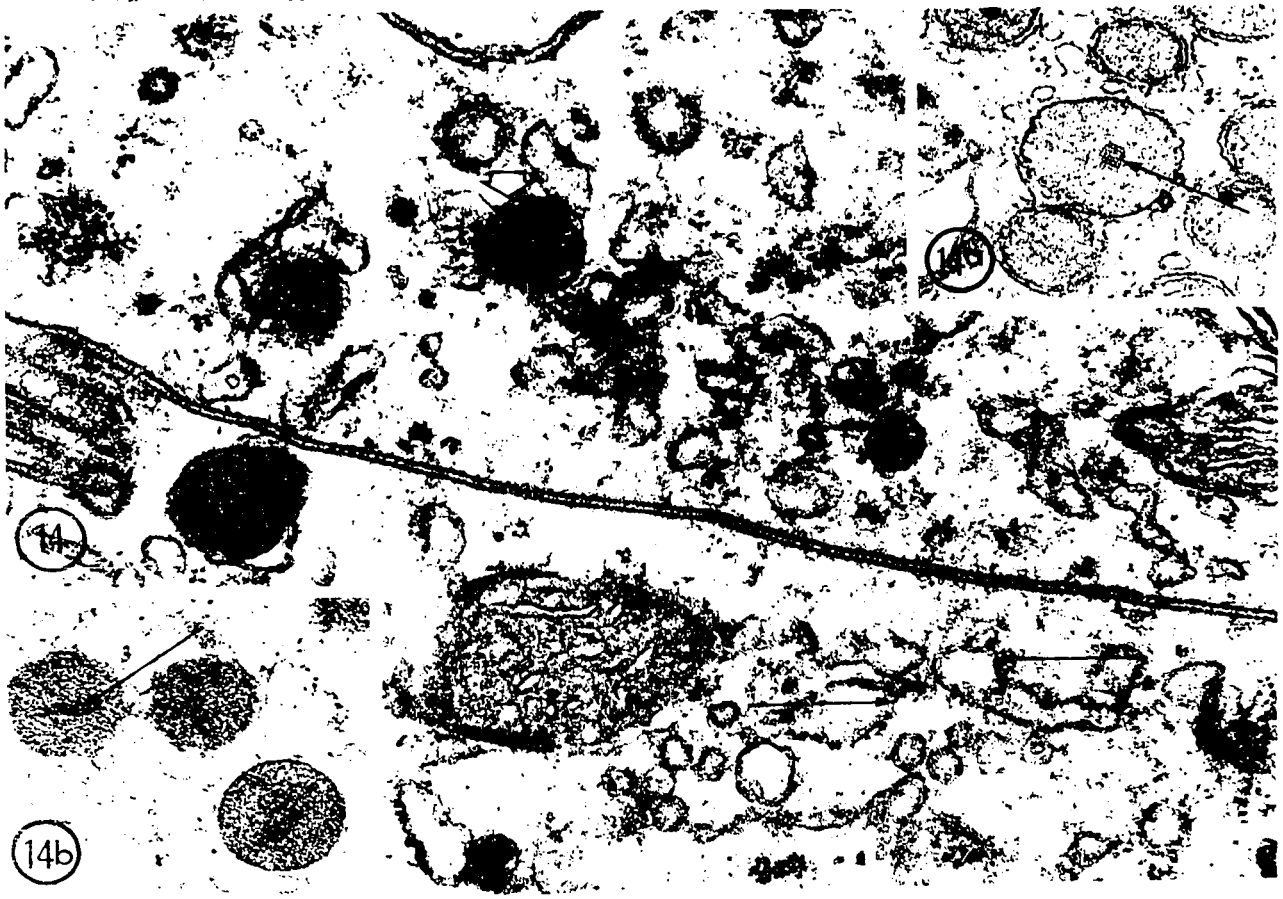


14

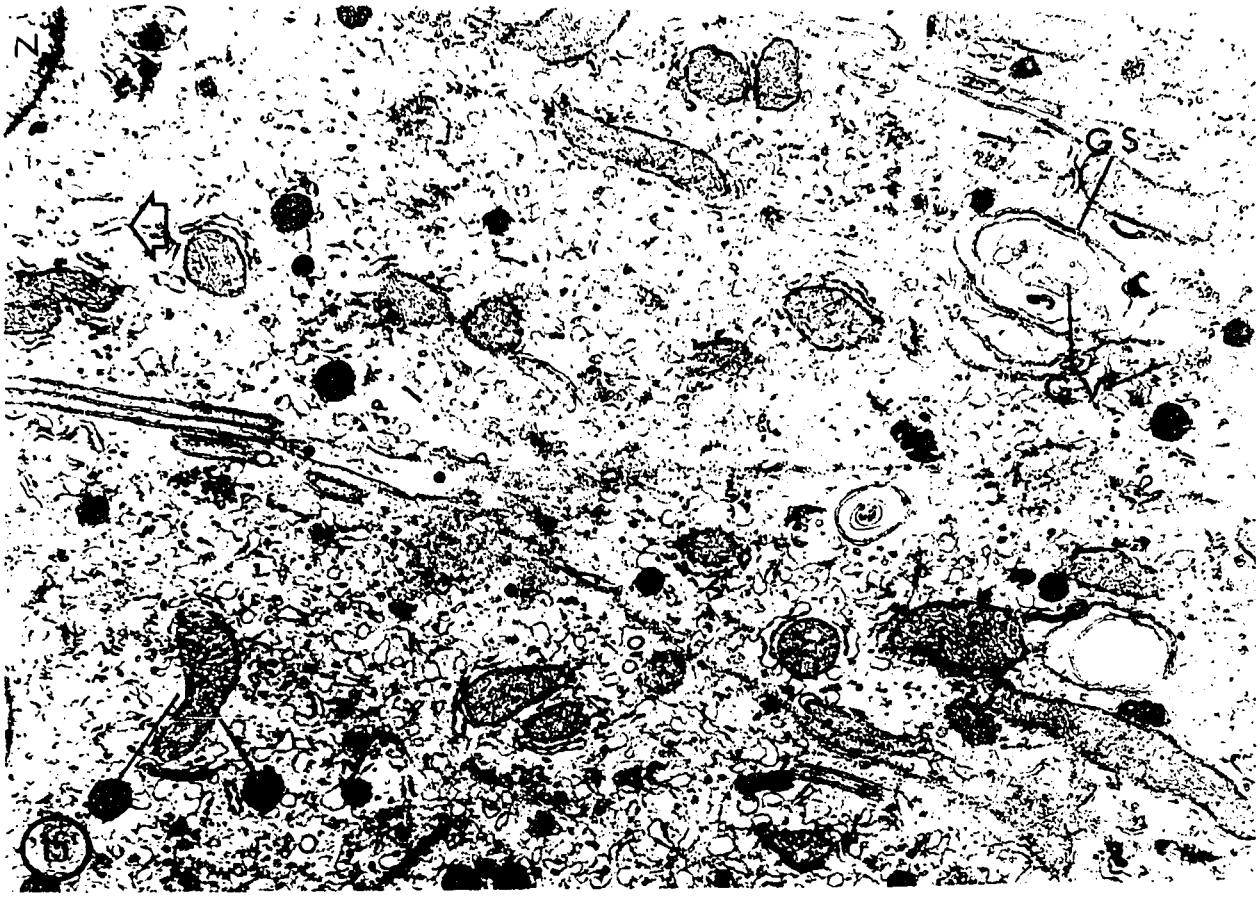
14b

Fig. 15. The suprenuclear region of the apical cytoplasm. The Golgi complex is seen in this region. It shows evidence of some activity. It is composed of stacks of flattened saccules (GS), the innermost of which is dilated with slightly electron dense contents as are the extremities of the other saccules. Vesicles (GV) appear close to the saccules. Several short cisternae of granular endoplasmic reticulum are seen close to the nucleus (⇩). Many peroxisomes are seen above the nucleus. X 13,965.

Fig. 16. A portion of the nucleus is seen in this figure. The double nature of the nuclear membrane is visible. It is ribosome studded facing the cytoplasm (⟶). Note the pore in the nuclear membrane (⇩). Clumps of chromatin (CH) are seen along the margins of the nucleus inside the inner membrane. A portion of the nucleolus is visible.

16a. Inset shows a higher magnification of the pore.

Fig. 17. A microtubule, longitudinally sectioned for part of its length is visible (⇩). Eighteen hour vitamin D_3-replete chick. This microtubule appears to parallel the contour of the adjacent mitochondrion. X 62,700.



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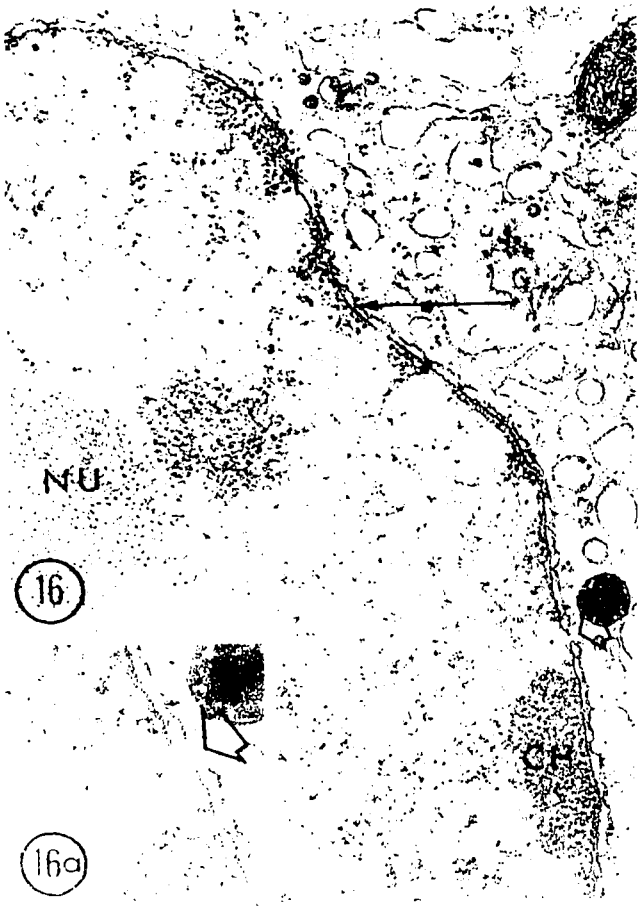

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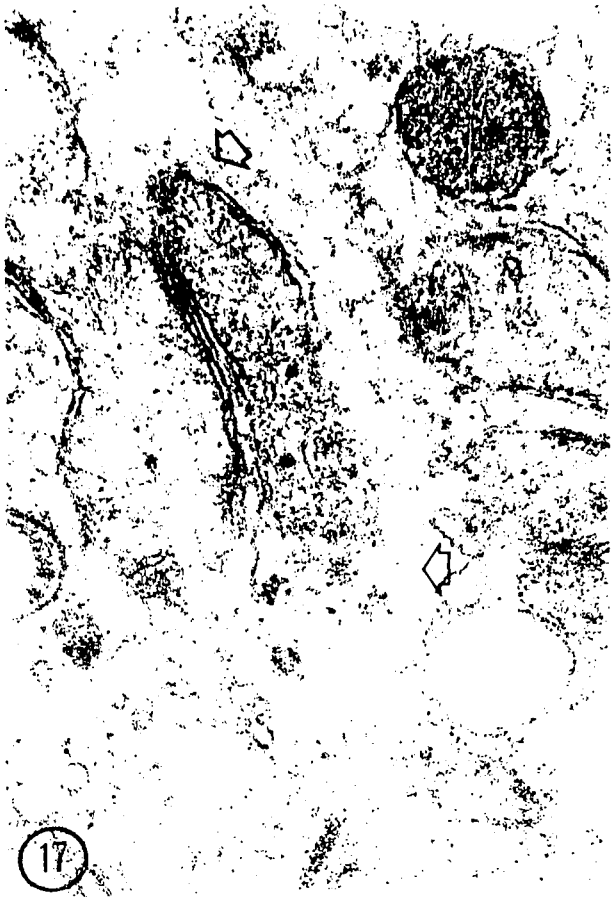
16a



17

Fig. 18. Oblique section through the basal absorptive cell cytoplasm of normal chick. Mitochondria similar in appearance to those of the apical region are numerous. Granular endoplasmic reticulum is abundant, much of it in close proximity to the mitochondria. The most basal portion of this region (seen at left) contains mainly free polyribosomes. A desmosome (D) can be seen holding adjacent cell membranes in close apposition. X 20,235.

Fig. 19. A round vesicle is seen in close proximity to the lateral plasma membrane (V) of an absorptive cell. Two similar vesicles can be seen with their limiting membranes in direct continuity with the plasma membrane. X 31,350.

Fig. 20. The basal portion of three absorptive cells. A dilation is seen in the intercellular space between the lateral plasma membranes of two cells. Below the basal plasma membrane is seen the basal lamina. X 16,100.


RER
D
18
V
19
IC
BL
20

Fig. 21. Longitudinal section of the absorptive epithelium of rachitic chick. Note the abundance of dense bodies and peroxisomes in the apical cytoplasm. The Golgi apparatus appears fairly active. Most of the granular endoplasmic reticulum appears to be in close proximity to mitochondria. X 6,380.

Fig. 22. Longitudinal section through the absorptive epithelium of twelve hour vitamin D_3-replete chick. Note the increase in Golgi activity, seen in the band of Golgi vacuoles across the apical cytoplasm. Several vacuoles similar to the Golgi vacuoles are seen in the basal cytoplasm. Also note the proliferation in granular endoplasmic reticulum in the basal cytoplasm, and in the supranuclear region. The lysosomal population is again composed mainly of dense bodies. Many peroxisomes again appear. X 6,380.


TW
DB
IC
21


S
A
A

Fig. 23. Longitudinal section through absorptive epithelium of eighteen hour vitamin D_3-replete chick. The lysosomal population is still composed mainly of dense bodies. The Golgi apparatus is still active, although the band of vacuoles does not appear quite as prominant as in Fig. 22. Many dilated cisternae of granular endoplasmic reticulum are seen in close proximity to mitochondria. Note the large number of rounded vesicles of granular and agranular endoplasmic reticulum throughout the cytoplasm. Dilations are seen in the intercellular space which contain material similar in appearance to the contents of the Golgi vacuoles. X 6,380.


SL
DB
G
P

Fig. 24. Longitudinal section through the absorptive epithelium of twenty-four hour vitamin D_3-replete chick. Relatively more secondary lysosomes are seen than in the three previous figures. The Golgi complex shows less activity than in Figs. 22 and 23. Several large dilations appear in the intercellular space. X 6,380.

ICS
24

Fig. 25. Longitudinal section through the absorptive epithelium of forty-eight hour vitamin D_3-replete chick. Note the further increase in the relative number of secondary lysosomes and decrease in dense bodies. Peroxisomes appear less frequently than in the previous four figures. The Golgi apparatus shows activity similar to that seen at twenty-four hours. X 6,380.

Fig. 26. Longitudinal section through the absorptive epithelium of seventy-two hour vitamin D_3-replete chick. Note the increased amount of granular endoplasmic reticulum; it almost fills the entire basal cytoplasm. Peroxisomes appear to have decreased in number compared to forty-eight hours. X 6,380.

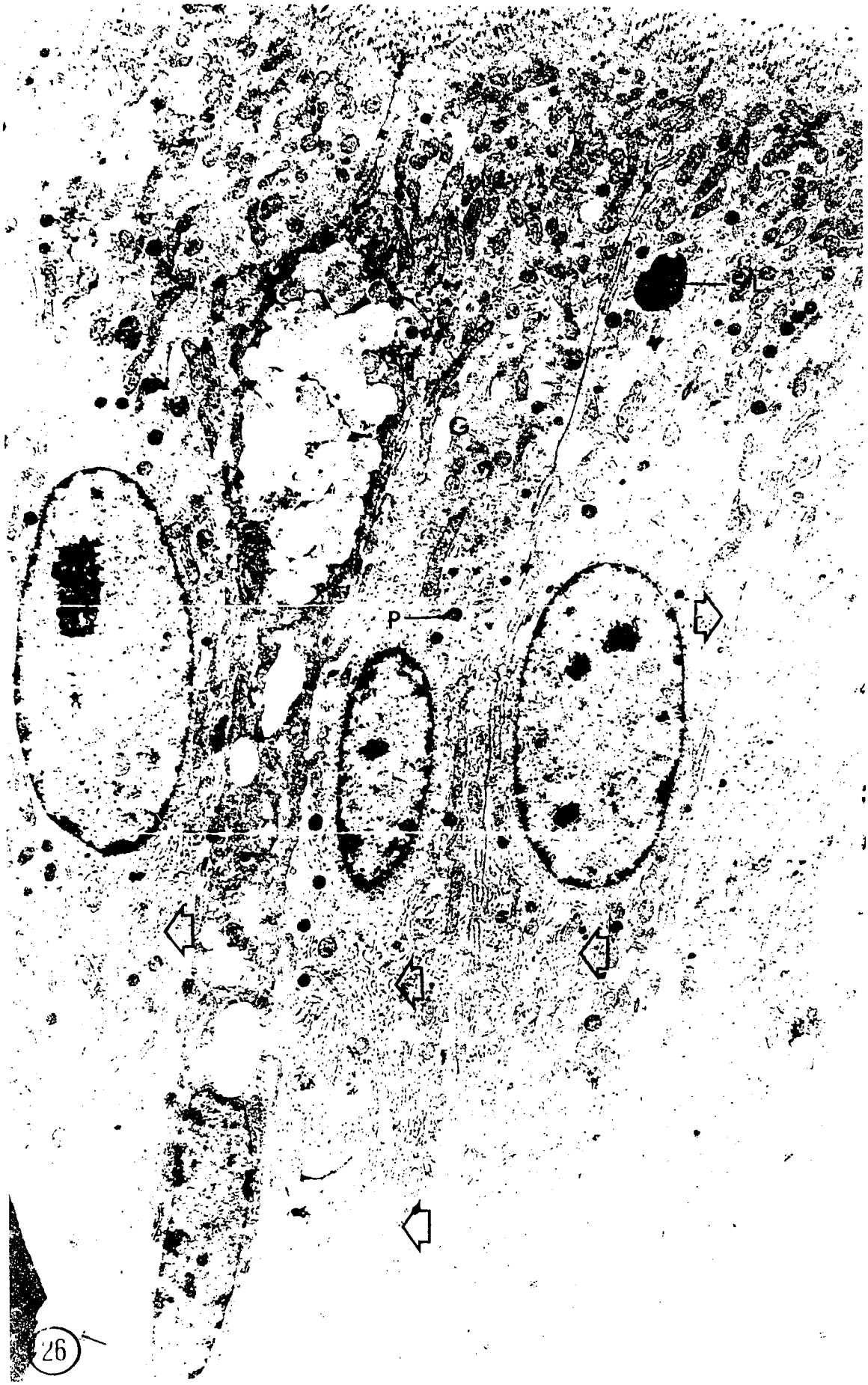


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Fig. 27. Longitudinal (slightly oblique) section through the absorptive epithelium of chick fed a low calcium diet. The lysosomal population shows approximately equal numbers of dense bodies and peroxisomes. The Golgi apparatus does not show much activity. Granular endoplasmic reticulum is associated mainly with mitochondria. Agranular endoplasmic reticulum is abundant in the supranuclear region. Peroxisomes appear in the cytoplasm in numbers similar to those seen at forty-eight hours. X 7,980.

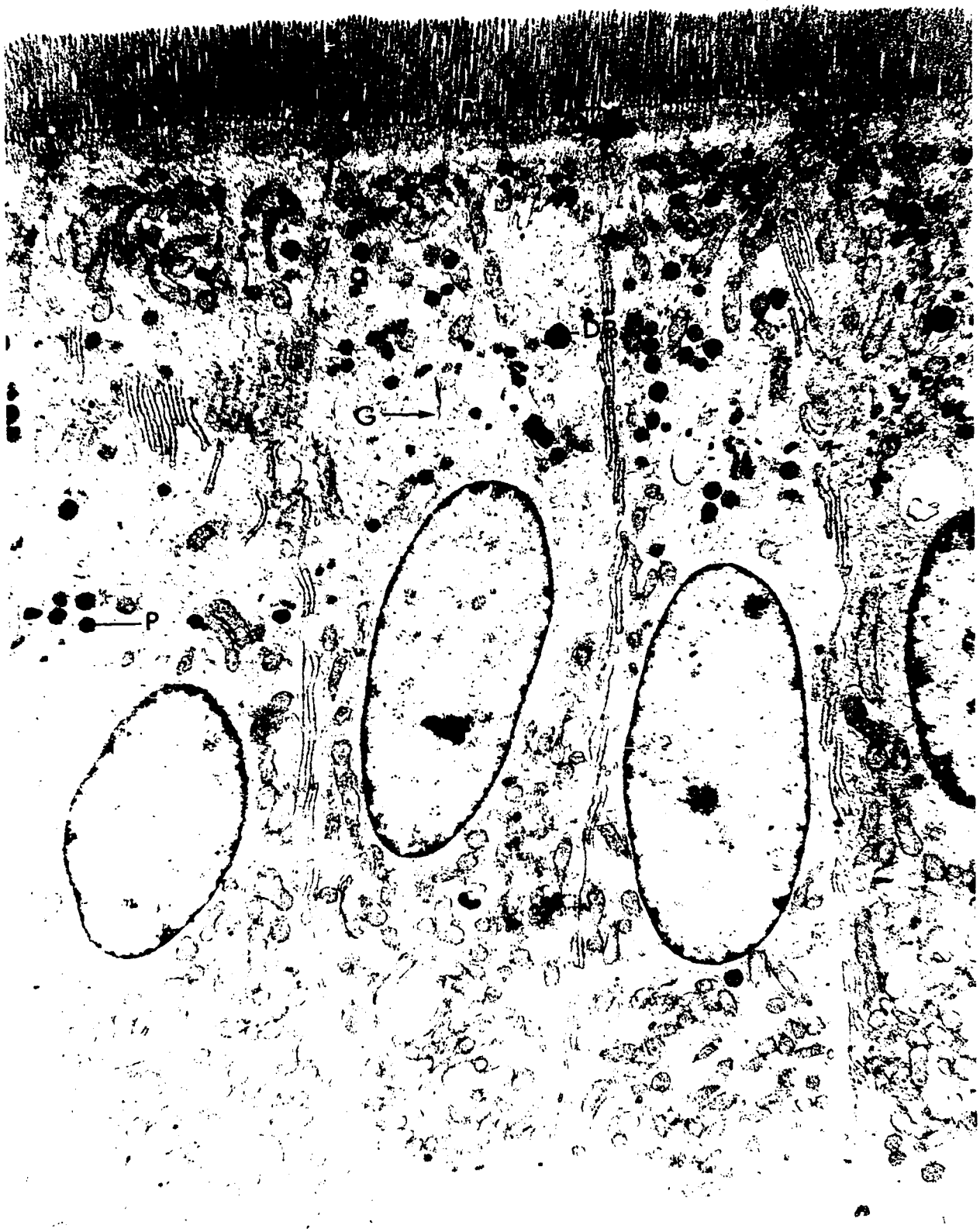

DB
G
P

Fig. 28. Brush border and terminal web regions of rachitic chick. The microvilli appear similar to those seen in normal chick (Fig. 4) in shape, and in the morphologies of the plasma membrane, core filaments, and rootlets. The glycocalyx is not readily visible in this figure, but can be seen to be of normal appearance in Fig. 48 (also of rachitic chick). The microvilli appear shorter than normal. Apical pits and vesicles are seen less frequently than normal; a single apical pit and few apical vesicles can be seen. The remainder of the terminal web region resembles that of the normal chick. X 62,700.

Fig. 29. Brush border and terminal web regions of twelve hour vitamin D_3-replete chick. The microvilli are slightly longer than in Fig. 28. Apical pits and vesicles seem to appear as frequently as in the rachitic chick. Two obvious apical vesicles are seen among the filaments of the terminal web. X 62,700.

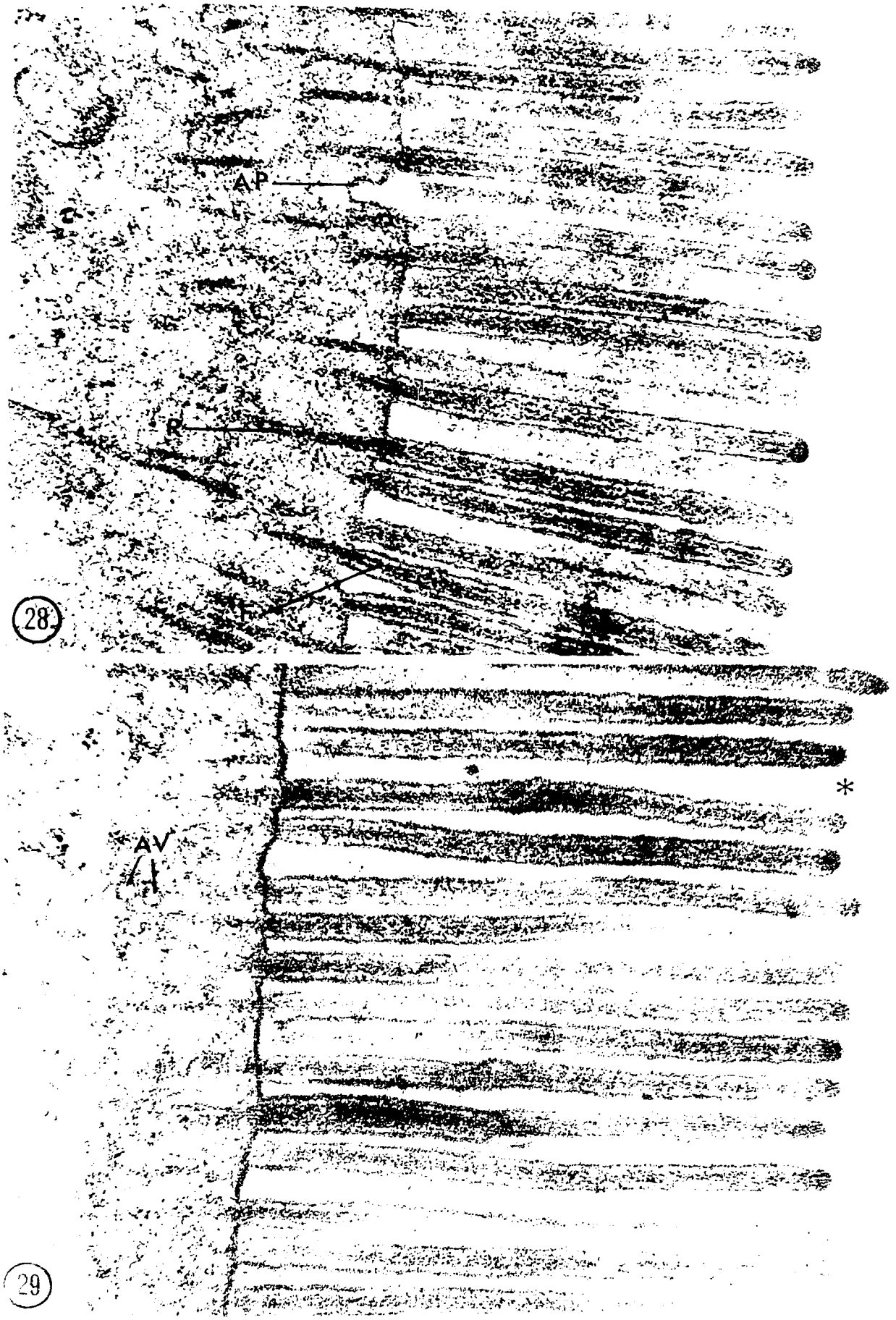
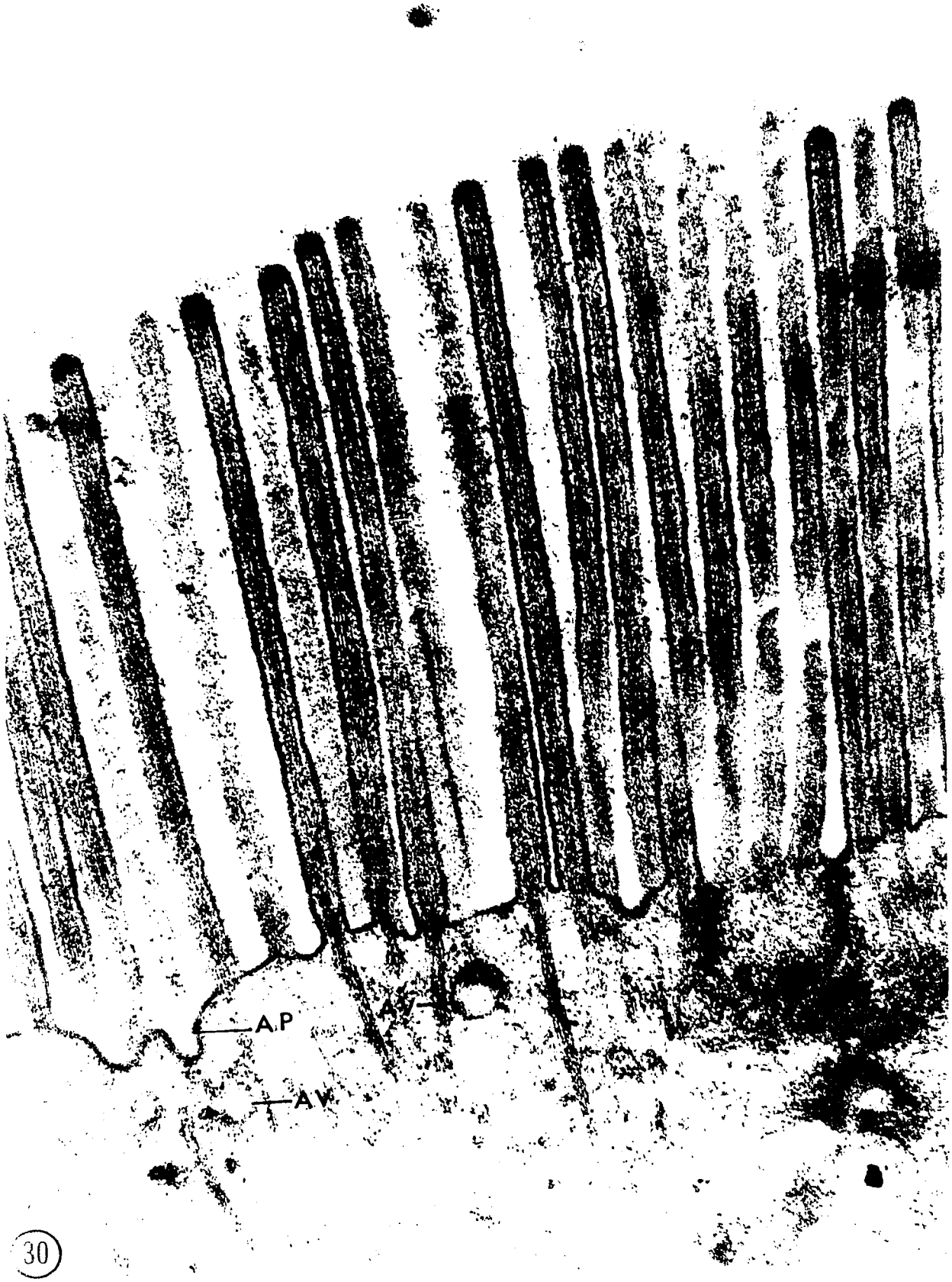

AP
R
28
AV
*
29

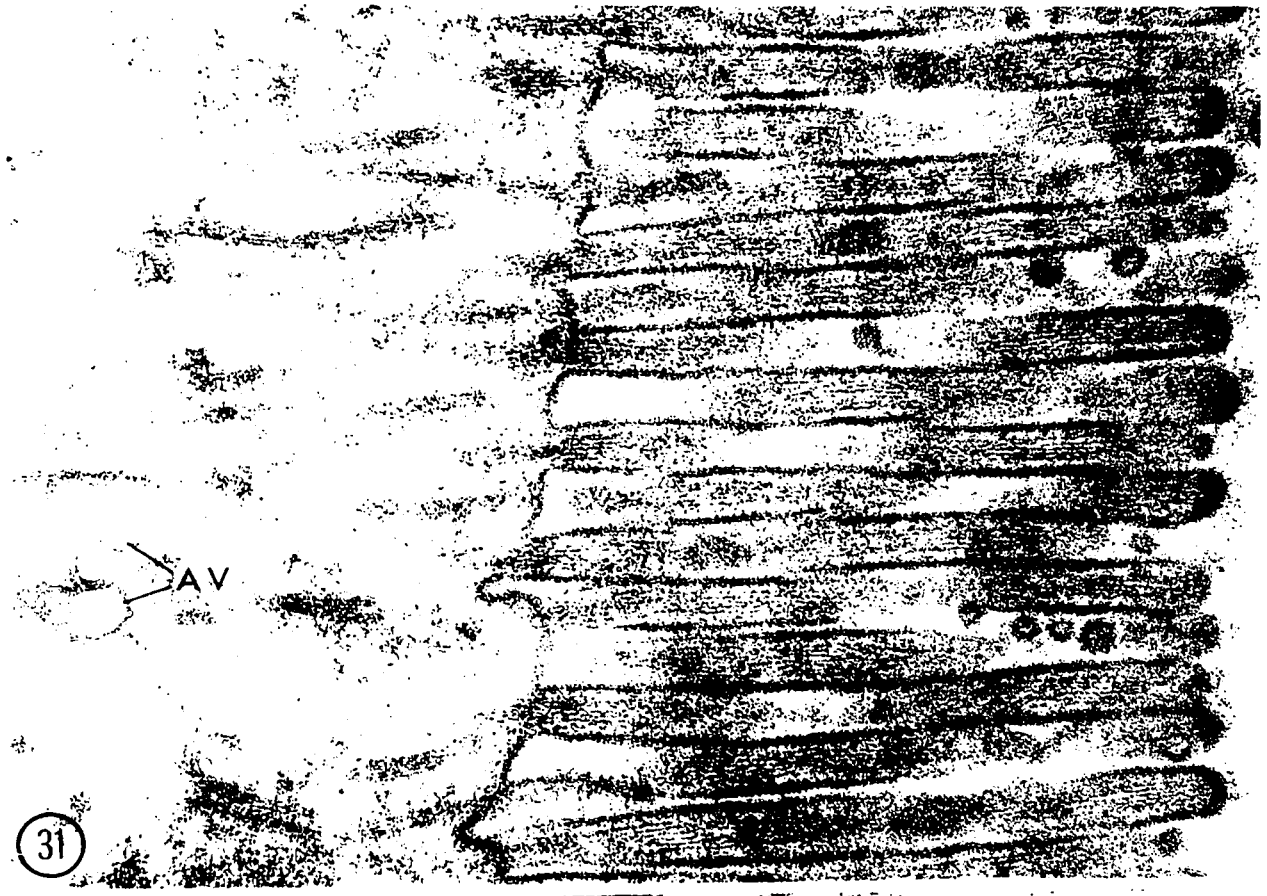
Fig. 30. Brush border and terminal web regions of eighteen hour vitamin D_3-replete chicks. The microvilli are of normal appearance and are slightly longer than in normal chicks. Apical pits and vesicles are seen more frequently than in rachitic, normal and twelve hour vitamin D_3-replete animals. X 62,700.



30

Fig. 31. Brush border and terminal web region of twenty-four hour vitamin D_3-replete chick. The microvilli appear obliquely sectioned in this figure. Apical pits and vesicles appear to be as abundant as at eighteen hours. X 62,700.

Fig. 32. Brush border and terminal web regions of forty-eight hour vitamin D_3-replete chick. The microvilli resemble those of normal chicks in length and morphology. Fewer apical pits and vesicles are seen than at eighteen and twenty-four hours (Figs. 30, 31). These appear about as frequently as in normal chicks (Fig. 4). X 62,700.


AV
31

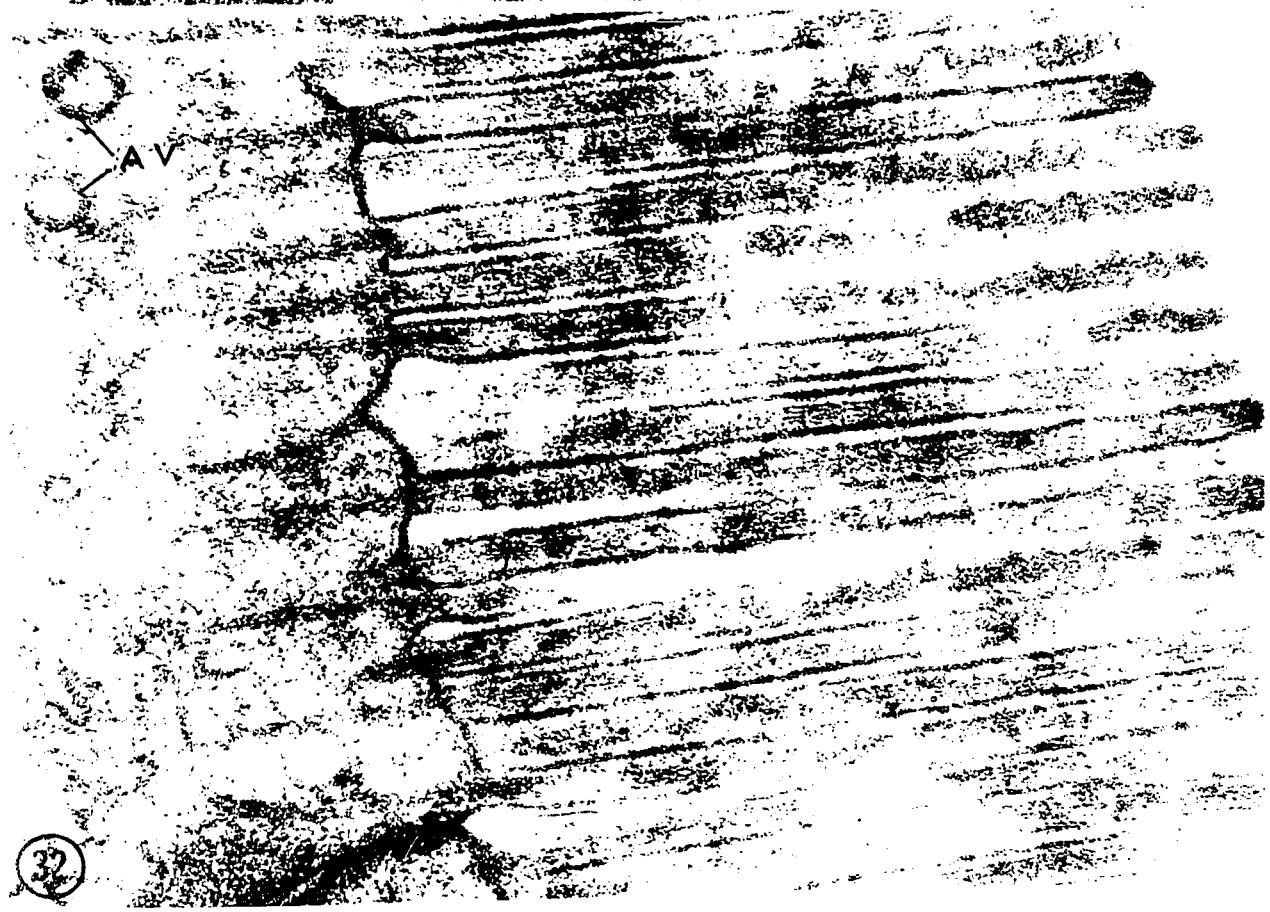
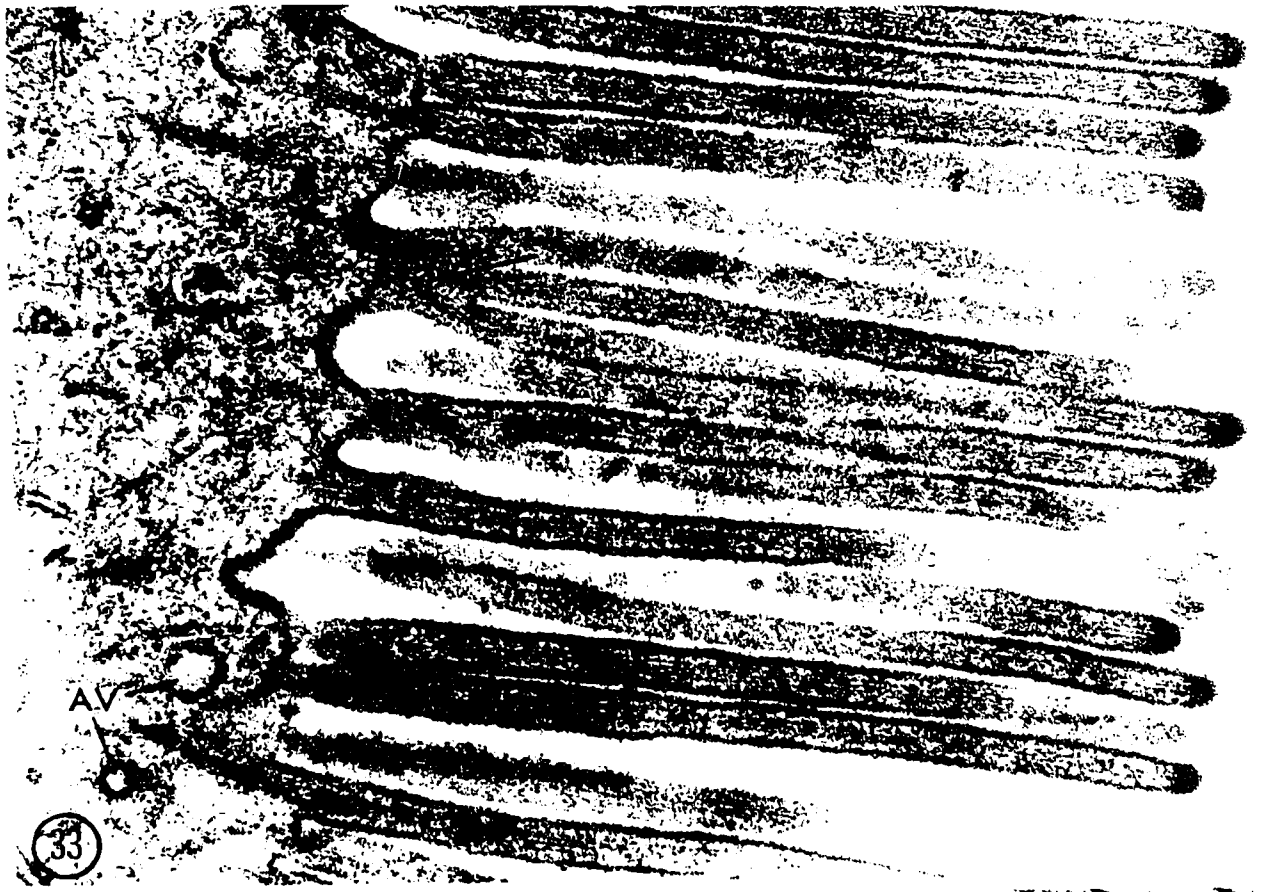

AV
32

Fig. 33. Brush border and terminal web regions of seventy-two hour vitamin D_3-replete chic. Branching of microvilli, a situation which is seldom seen, is visible here. The central core filaments of the two branches appear to meet (⟶). Apical pits and vesicles appear as frequently as at forty-eight hours (Fig. 32); several are visible in this figure. X 62,700.

Fig. 34. Brush border and terminal web regions of chick fed a low calcium diet. The microvilli are normal in length and appearance. Branching of microvilli is again visible. The terminal web region appears morphologically normal. An apical pit cut in oblique section and a few apical vesicles are in evidence. X 62,700.



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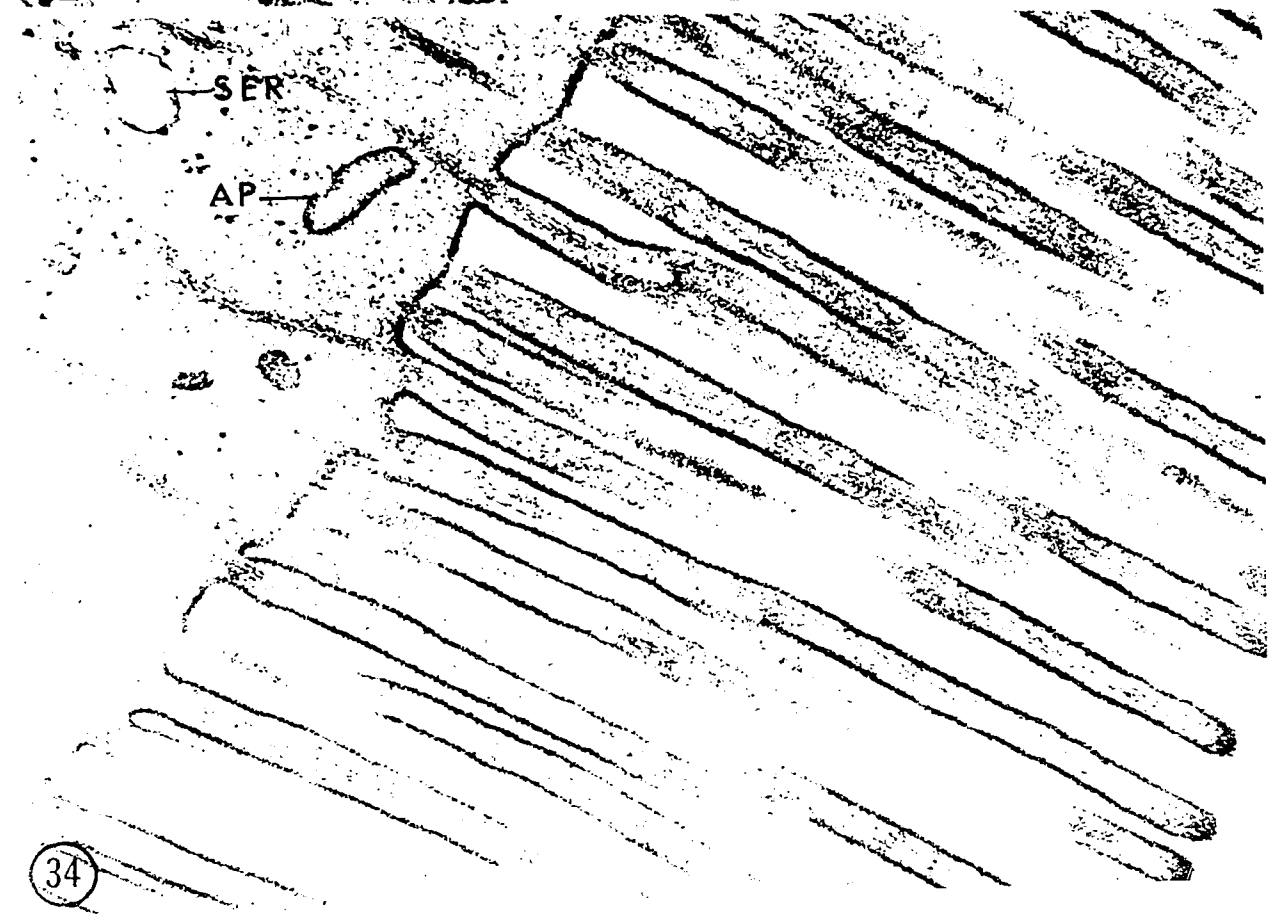


34

Figs. 35 to 38 illustrate material fixed with S-collidine buffered paraformaldehyde (Sampson et al., 1970).

Fig. 35. Brush border of rachitic chick absorptive cells. Many electron dense granules of varying size are seen adhering to the outer surfaces of the microvilli. Some of these granules appear to form small groups. Similar granules are seen at the bases of the microvilli and one appears to be in an apical pit which is out of the plane of sectioning (⇨). No large granules are seen in the apical vesicles. Fine electron dense deposits can be seen adhering to the plasma membrane in several areas (→). Unstained preparation. X 50,160.

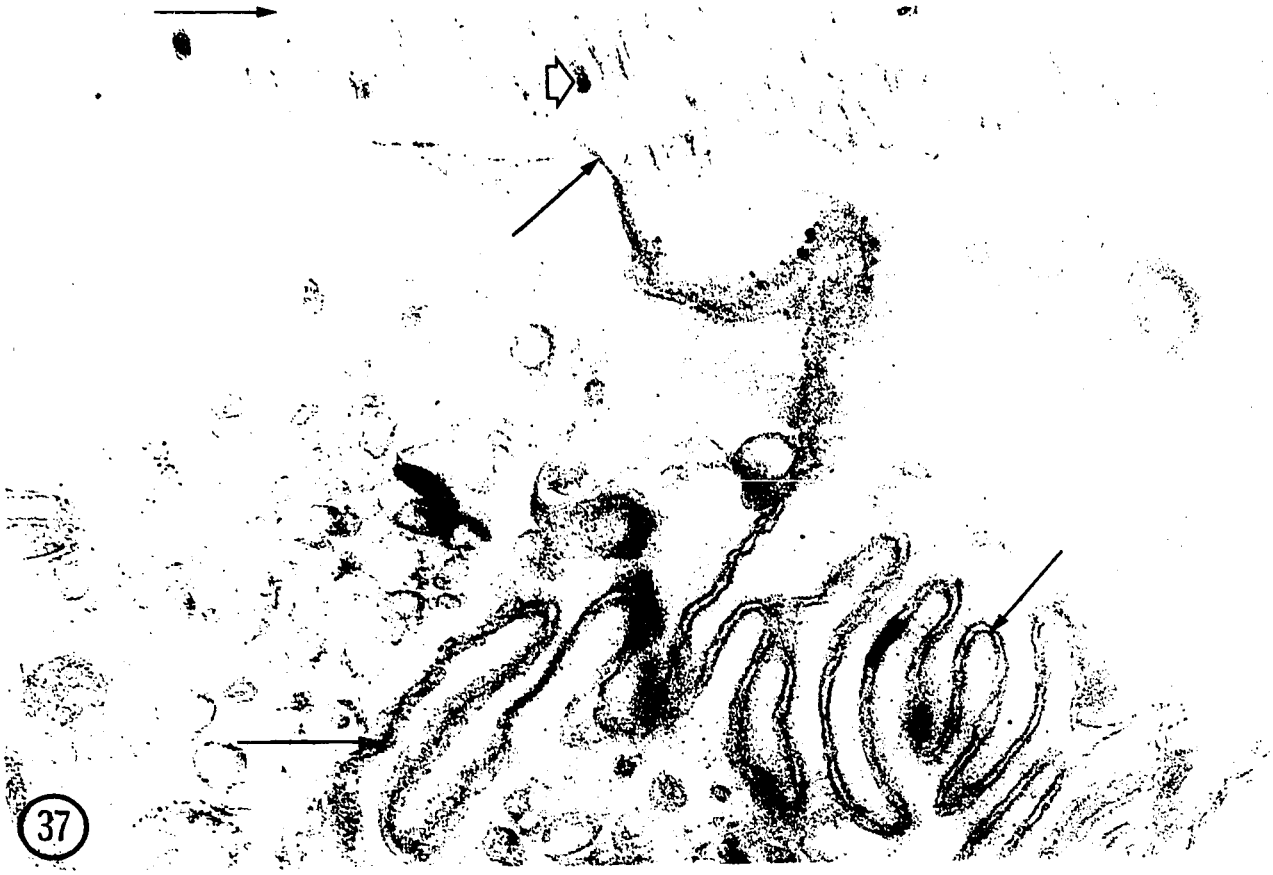
35a. Inset, from same material as in Fig. 35, shows to apical pits containing large electron dense granules similar to those in Fig. 35. Small electron dense deposits are again visible on the plasma membrane (⇨). Unstained preparaton. X 62,700.

Fig. 36. Brush border of twenty-four hour vitamin D_3-replete chick. Large electron dense granules similar to those in Fig. 35. These are less numerous. Small electron dense deposits (→) appear more obvious than in rachitic chicks and are seen over most of the apical surface. They appear to adhere to either the inner or outer side of the plasma membrane in various places. Similar fine electron dense deposits can be seen on the limiting membranes of the apical vesicles but none of these contains large electron dense granules. Unstained preparation. X 50,160.


35a
35
36

Fig. 37. Brush border and terminal web region of twenty-four hour vitamin D_3-replete chick. Stained with uranyl acetate and lead citrate. Staining does not appear to alter the appearance of the large electron dense granules (⇨). The plasma membranes appear more uniformly electron dense after staining, but small electron dense deposits can still be seen adhering to them (⟶). Small electron dense deposits are in evidence along the lateral plasma membranes and within the intercellular space. X 47,900.

Fig. 38. Brush border of twenty-four hour vitamin D_3-injected chick, treated with EDTA. The large electron dense deposits are no longer in evidence on the microvilli, or in the apical pits. Some areas of the membrane show greater electron density than others (⟶) but small electron dense granular deposits are not seen on the membrane. Unstained preparation. X 50,160.



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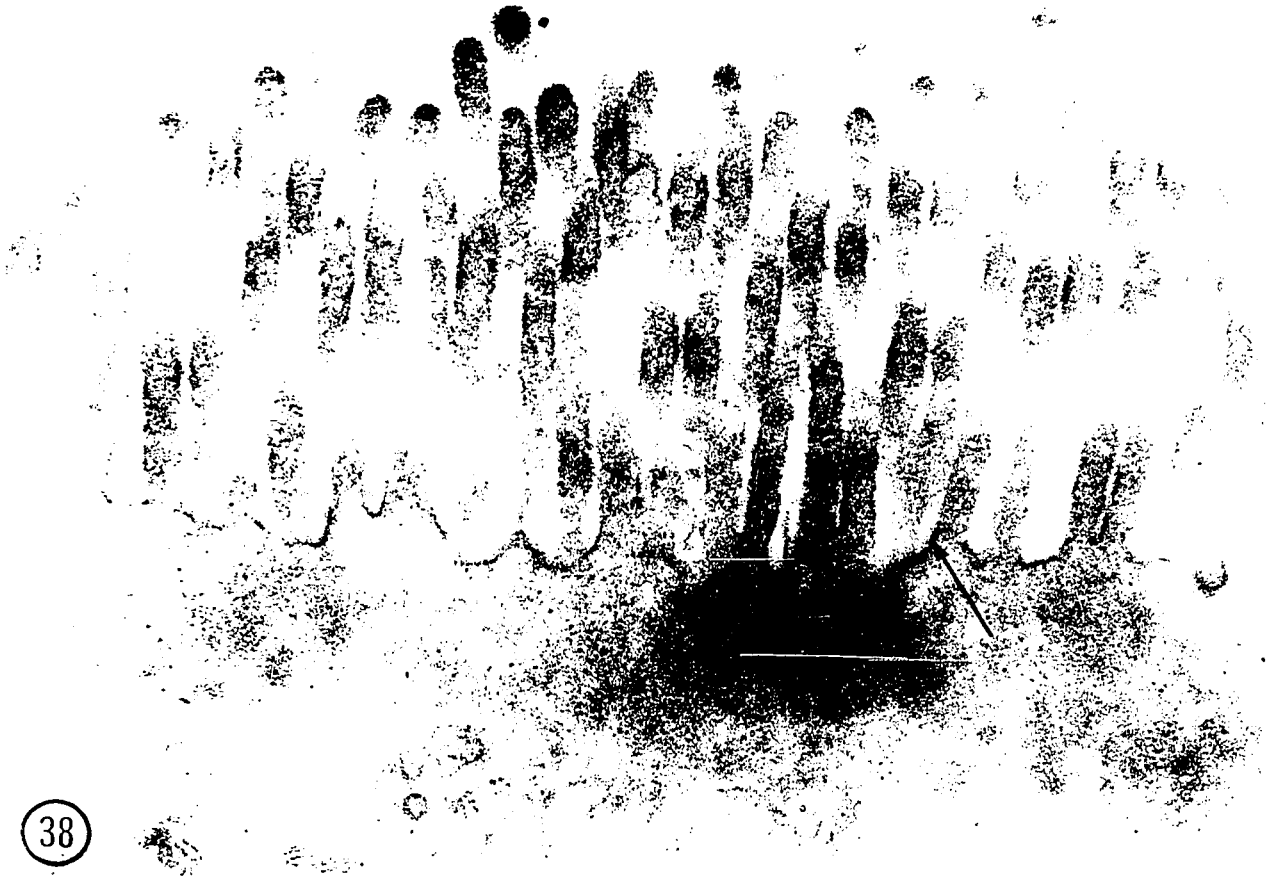
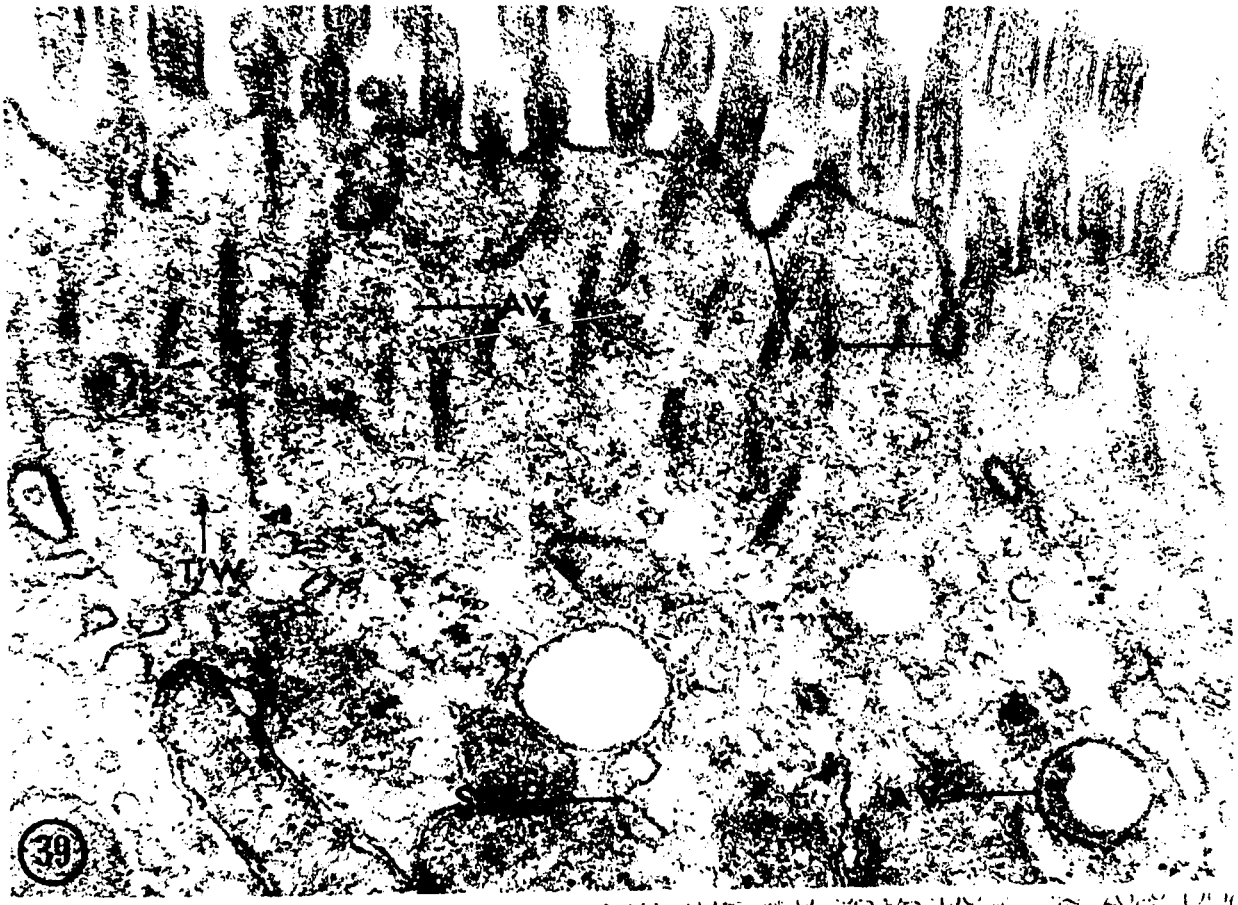


38

Fig. 39. Terminal web region of eighteen hour vitamin D_3-replete. Several apical pits and vesicles are present in this figure; some of these appear to be quite large in size. X 62,700.

Fig. 40. Low-calcium diet chick. Oblique section through terminal web and apical cytoplasm. Many apical pits and vesicles can be seen in the region of the terminal web. Several multivesicular bodies are seen in the apical cytoplasm beneath the terminal web. The mitochondria exhibit prominant electron dense granules within their matrix. X 22,940.


AV
TW
39

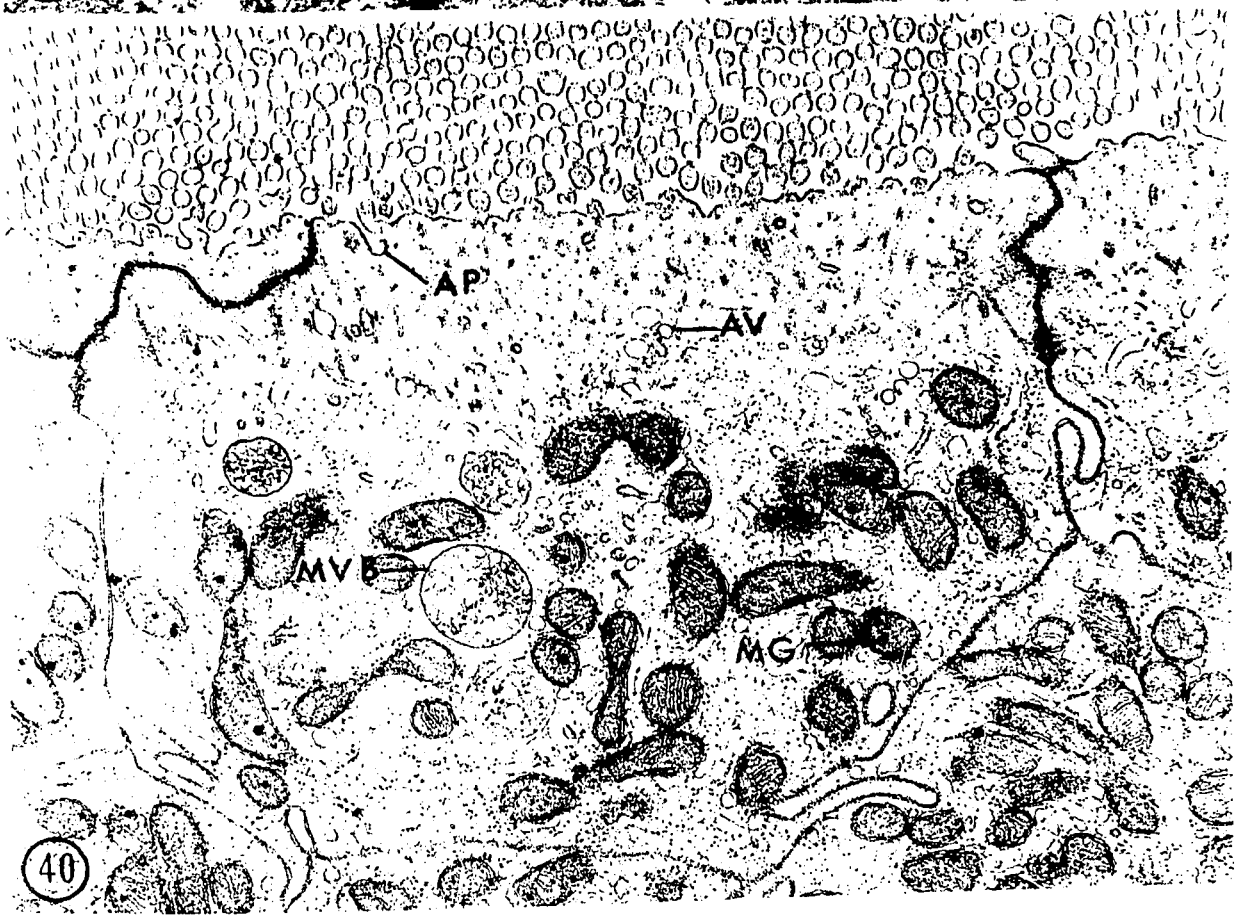
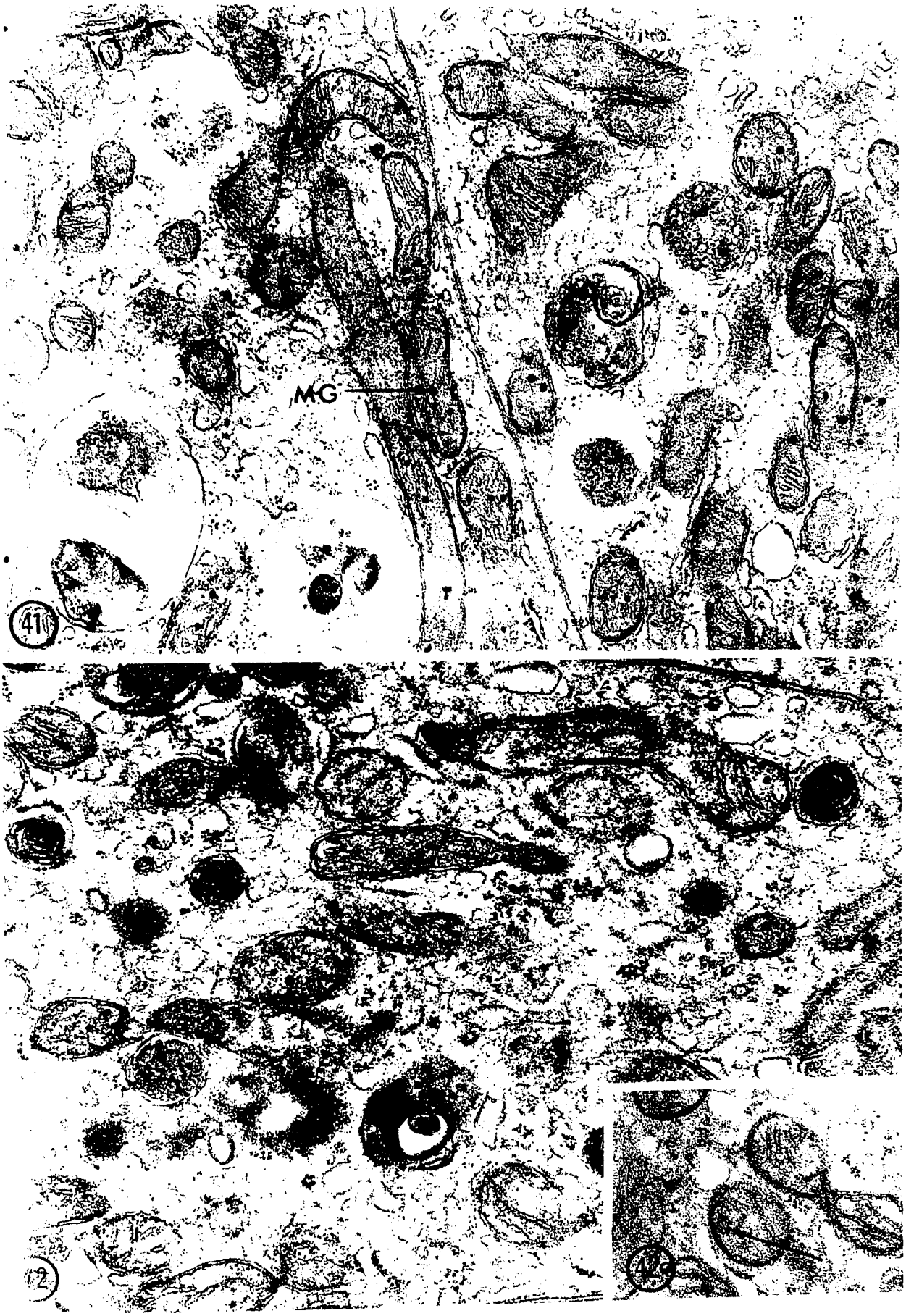

AP
AV
MVB
MG
40

Fig. 41. Mitochondria in the apical cytoplasm of chick fed a low calcium diet. Large electron dense granules appear frequently within the mitochondrial matrix. These are larger and more electron dense than those seen in the other experimental groups and in normal chicks. Unstained preparation. X 39,900.

Fig. 42. Mitochondria of the apical cytoplasm of twenty-four hour vitamin D_3-replete chick. Mitochondrial granules are seen less frequently, and are less electron dense than in the previous figure. Stained with uranyl acetate and lead citrate. X 39,900.

42a. Inset shows mitochondria in an unstained preparation of same group as Fig. 42. These granules appear less electron dense than in stained material or in unstained material from low-calcium diet chicks. (Fig. 41). X 39,900.


MG
41
12
42

Figs. 43 to 46 illustrate material fixed with S-collidine buffered paraformaldehyde.

Fig. 43. Mitochondria of rachitic chick. A few slightly electron dense granules are seen within the mitochondrial matrix (⟶). Unstained preparation. X 39,900.

Fig. 44. Mitochondria of twenty-four hour vitamin D_3-replete chick. Granules appear more frequently within the mitochondrial matrix and are more electron dense than in Fig. 43. Many small electron dense granules appear within the lysosomes (⇨). Unstained preparation. X 39,900.

Fig. 45. Similar to Fig. 44. Stained with uranyl acetate and lead citrate. The granules within the mitochondrial matrix show increased electron density after staining. Note also that individual electron dense granules within the lysosomes become less distinct following staining. X 39,900.

Fig. 46. Mitochondria from twenty-four hour vitamin D_3-replete chick treated with EDTA. The mitochondrial granules are less electron dense than those in Figs. 44 and 45, and resemble more closely those seen in Fig. 43. Unstained preparation. X 39,900.

Figs. 43 to 46 illustrate material fixed with S-collidine buffered paraformaldehyde.

Fig. 43. Mitochondria of rachitic chick. A few slightly electron dense granules are seen within the mitochondrial matrix (⟶). Unstained preparation. X 39,900.

Fig. 44. Mitochondria of twenty-four hour vitamin D_3-replete chick. Granules appear more frequently within the mitochondrial matrix and are more electron dense than in Fig. 43. Many small electron dense granules appear within the lysosomes (⇨). Unstained preparation. X 39,900.

Fig. 45. Similar to Fig. 44. Stained with uranyl acetate and lead citrate. The granules within the mitochondrial matrix show increased electron density after staining. Note also that individual electron dense granules within the lysosomes become less distinct following staining. X 39,900.

Fig. 46. Mitochondria from twenty-four hour vitamin D_3-replete chick treated with EDTA. The mitochondrial granules are less electron dense than those in Figs. 44 and 45, and resemble more closely those seen in Fig. 43. Unstained preparation. X 39,900.

44

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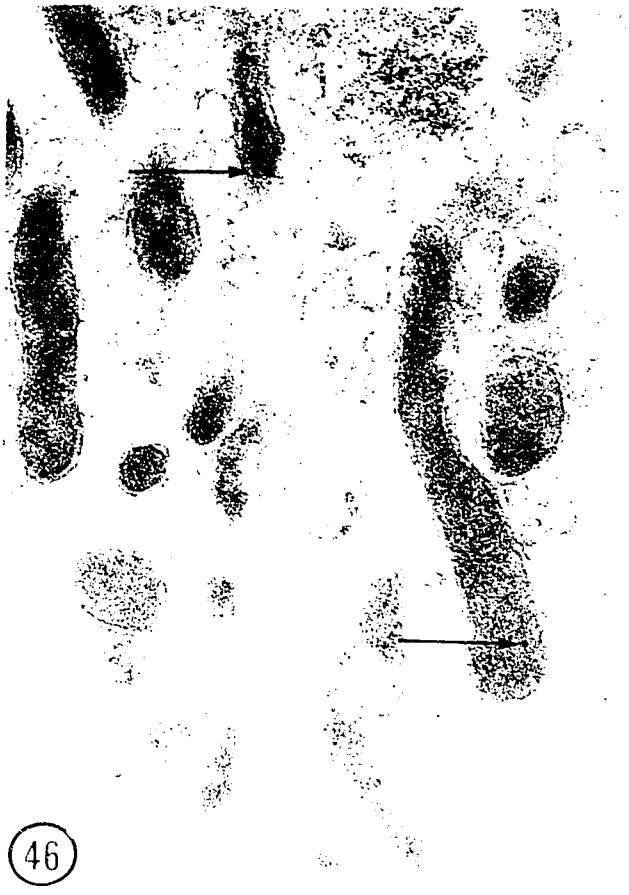

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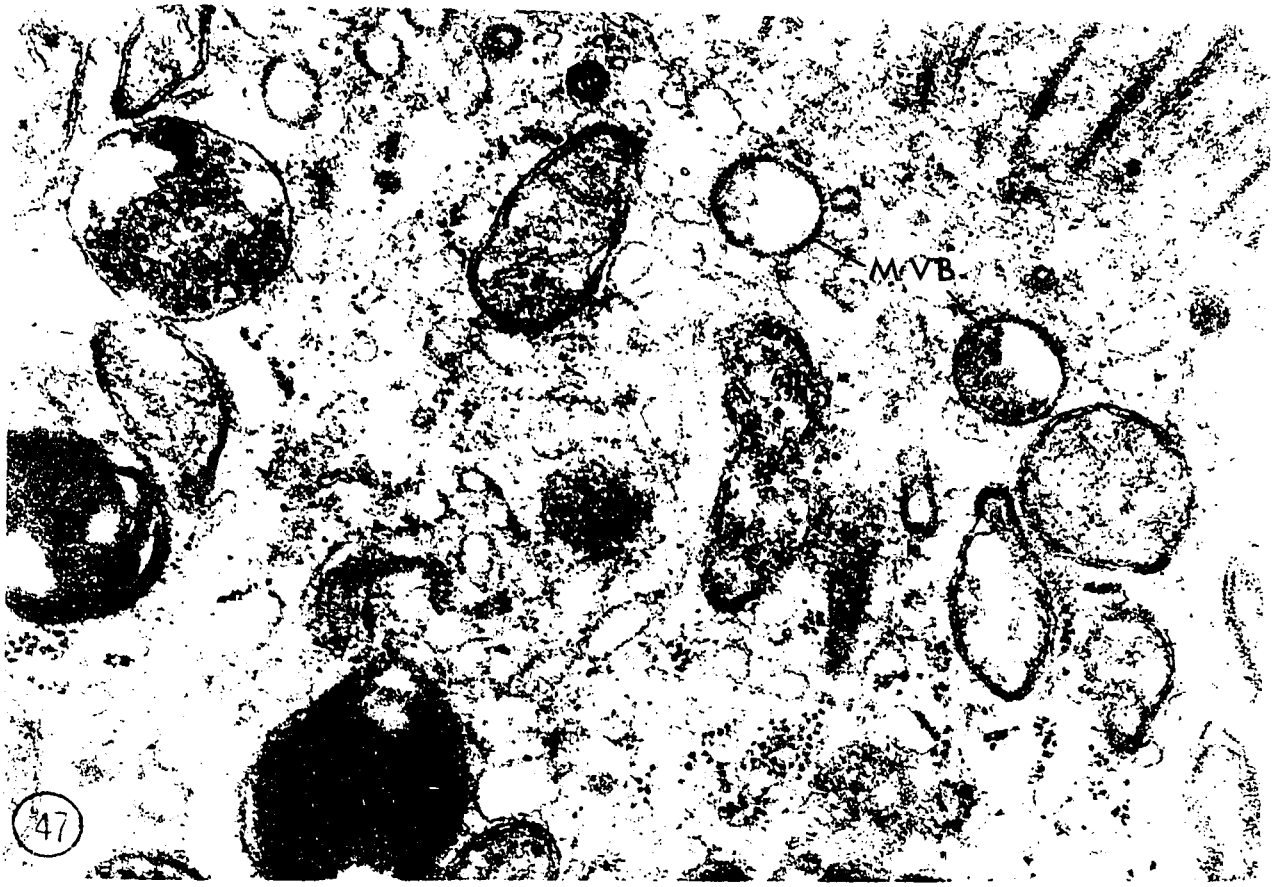
Fig. 47. Apical cytoplasm below the terminal web. Twenty-four hour vitamin D_3-replete chick. Several multivesicular bodies containing vesicles of varying size are seen in this micrograph. X 47,880.

Fig. 48. Apical cytoplasm of rachitic chick. Note that the lysosomal population is composed mainly of dense bodies. X 24,280.


MVB
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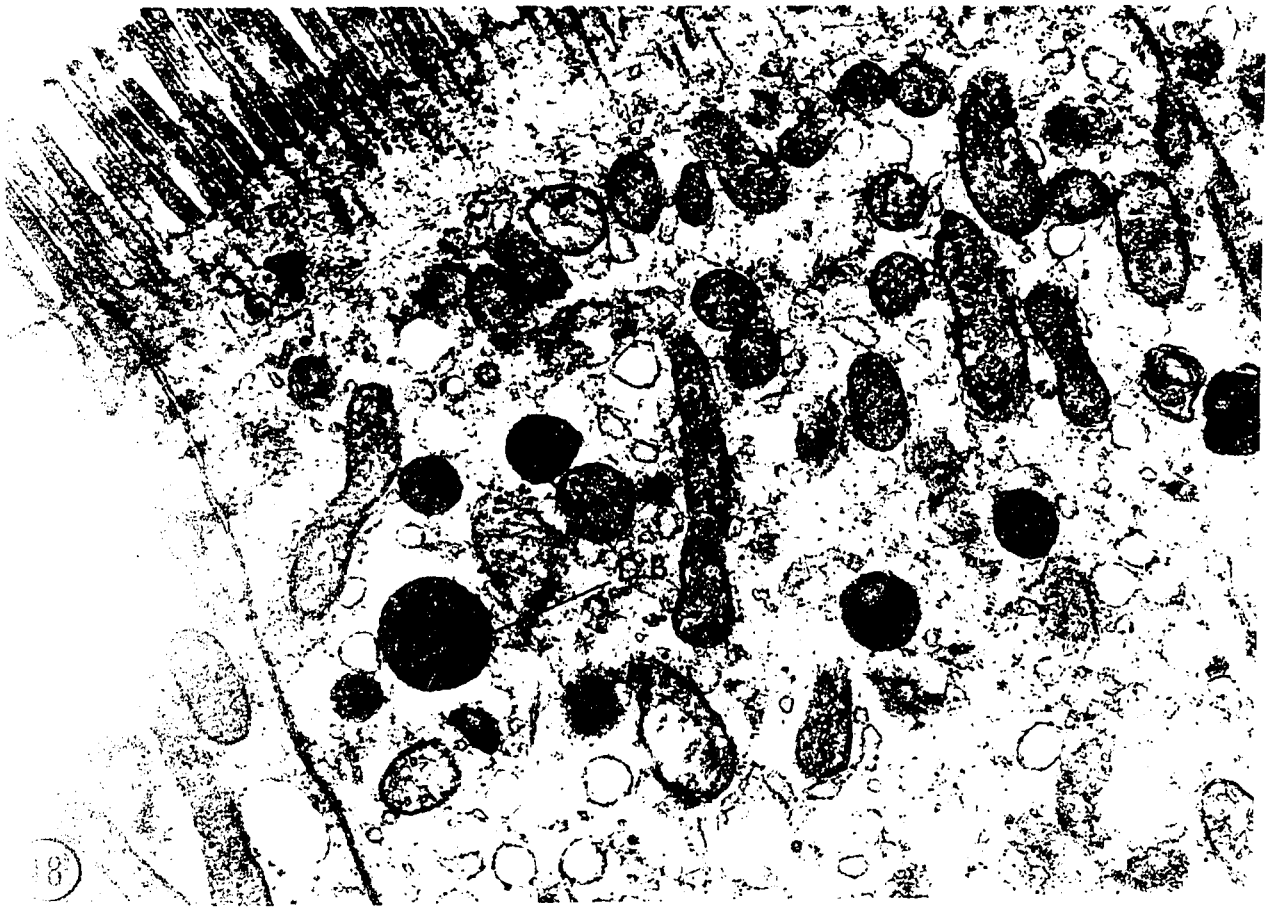
B
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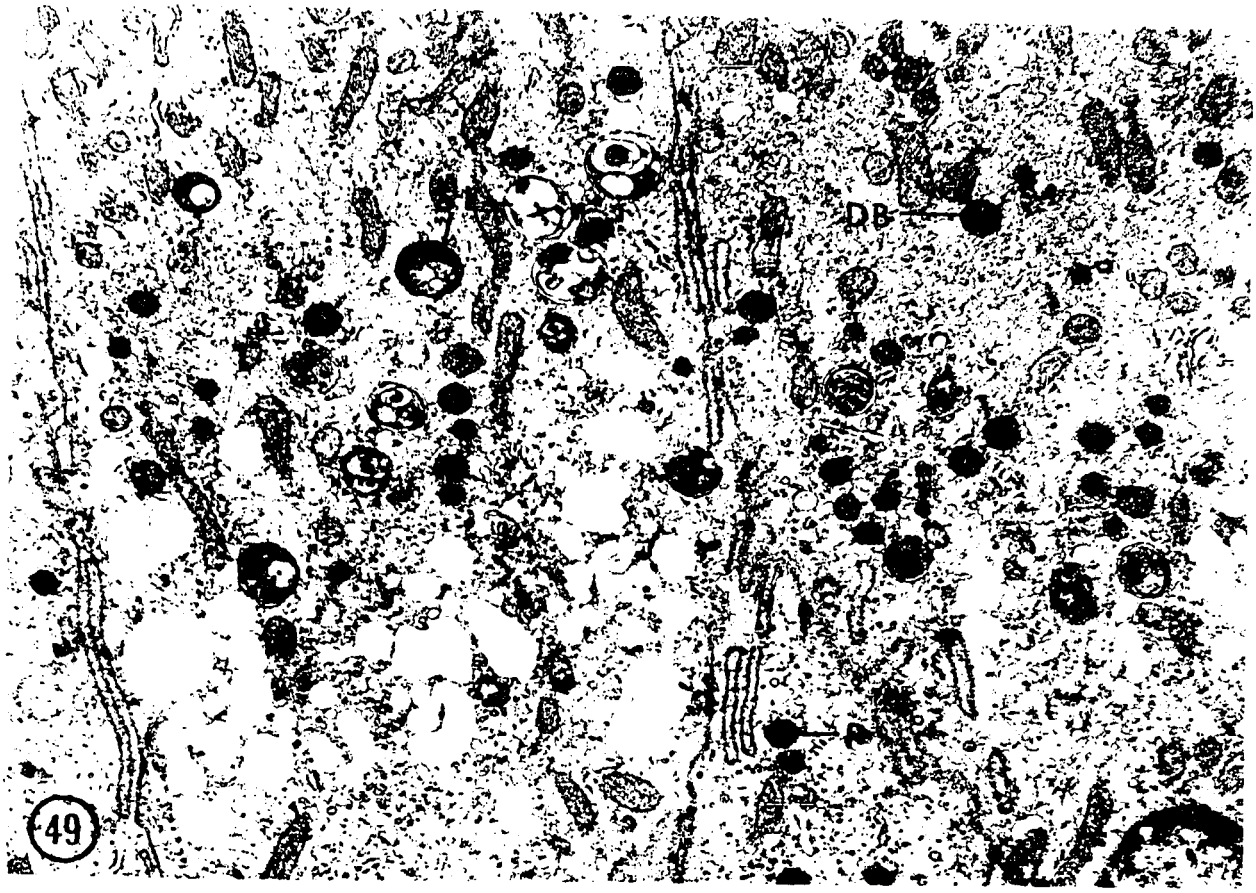
Fig. 49. Apical cytoplasm. Twelve hour vitamin D_3-replete chick. Several dense bodies are seen. Secondary lysosomes appear more frequently here than in cells which show less Golgi activity. X 11,180.

Fig. 50. Apical cytoplasm. Eighteen hour vitamin D_3-replete chick. The lysosomal population is still composed mainly of dense bodies. X 25,650.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50

Fig. 51. Apical cytoplasm. Twenty-four hour vitamin D_3-replete chick. A few secondary lysosomes are seen in this figure. No dense bodies are seen. X 13,680.

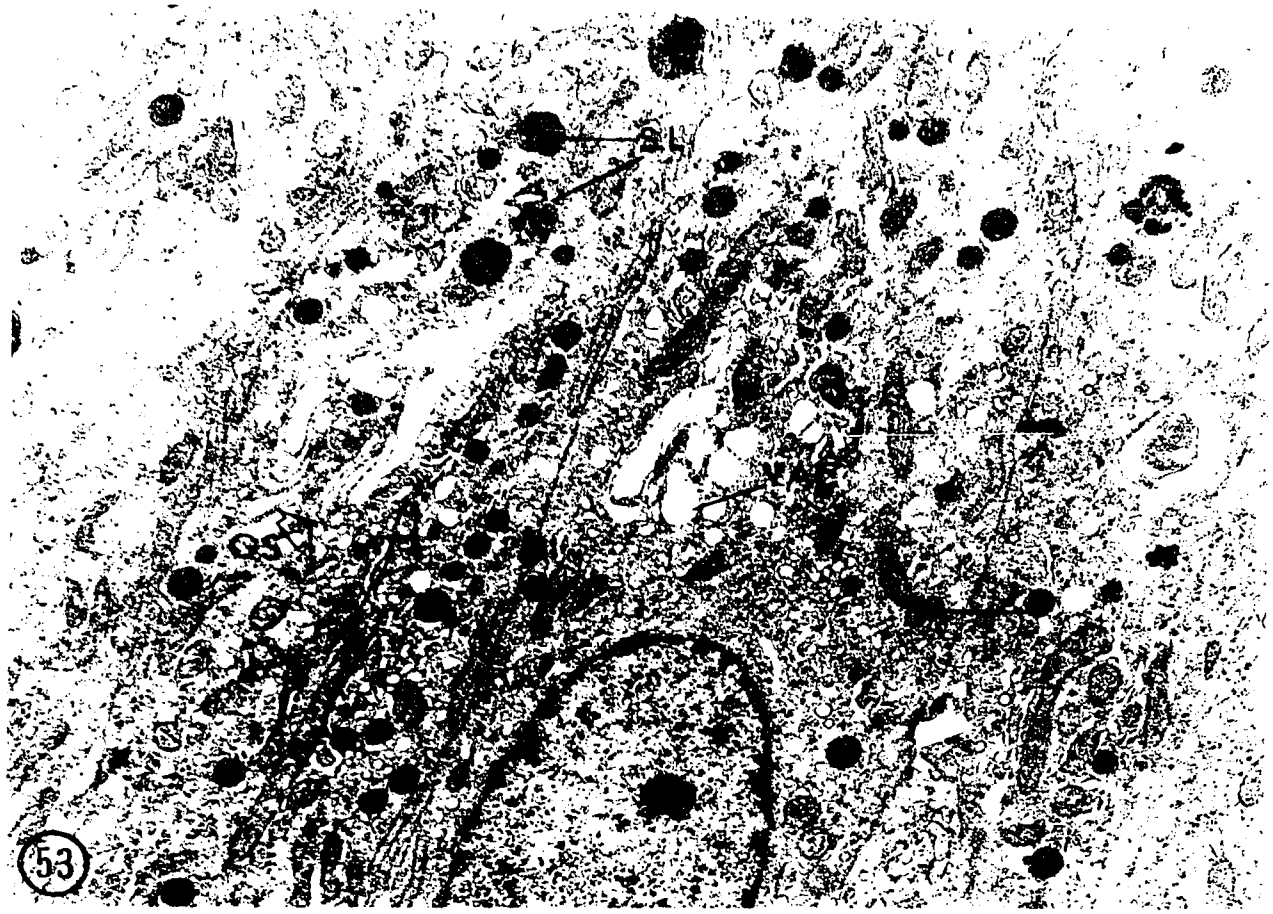
Fig. 52. Apical cytoplasm. Forty-eight hour vitamin D_3-replete. Several secondary lysosomes are seen. A dense body which appears to be transforming into a secondary lysosome is seen (⇨). X 24,280.

51

52

Fig. 53. Apical cytoplasm. Seventy-two hour vitamin D_3-replete chick. Again many secondary lysosomes are seen. X 9,580.

Fig. 54. Apical cytoplasm, oblique section. Low calcium diet chick. Several secondary lysosomes are seen. X 16,100.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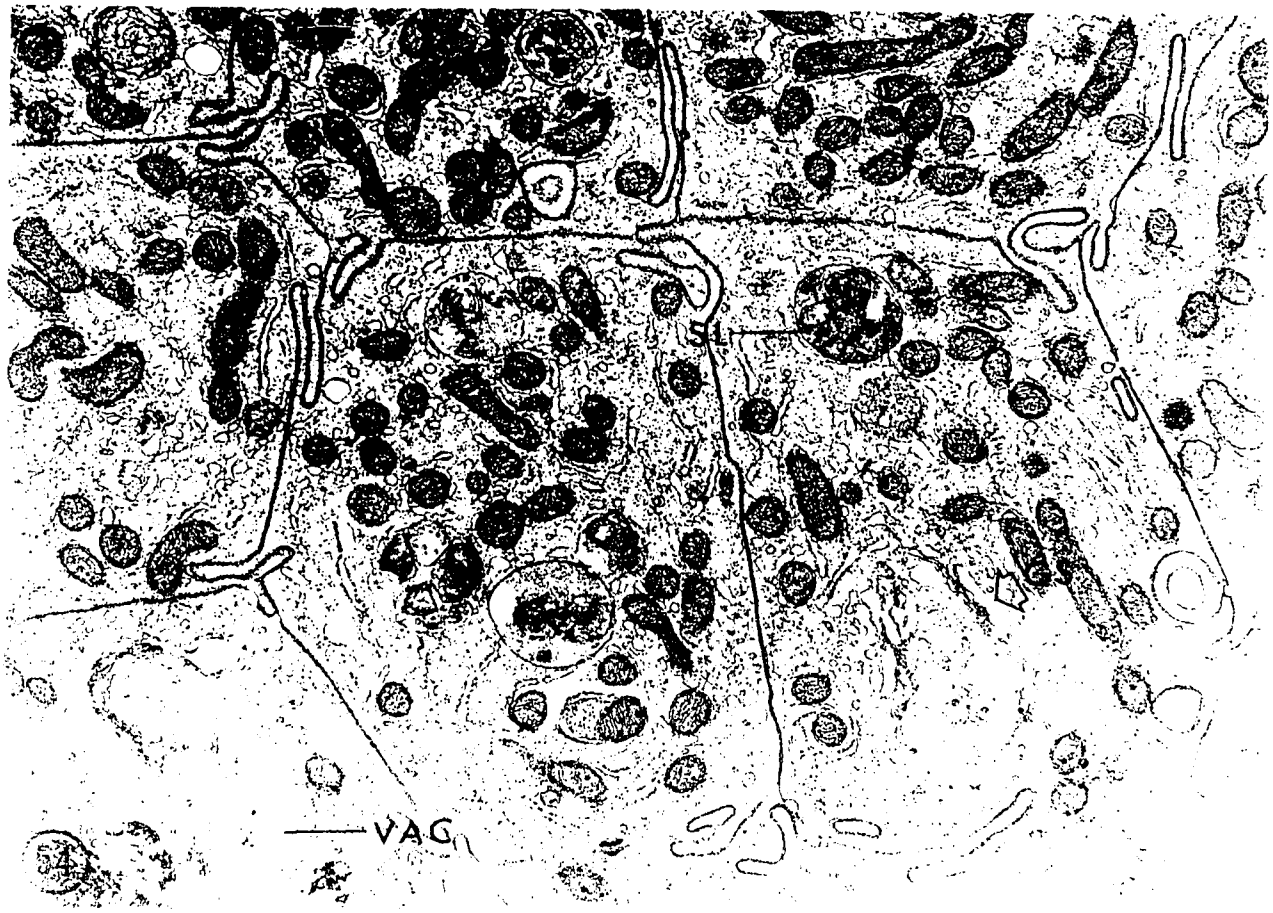
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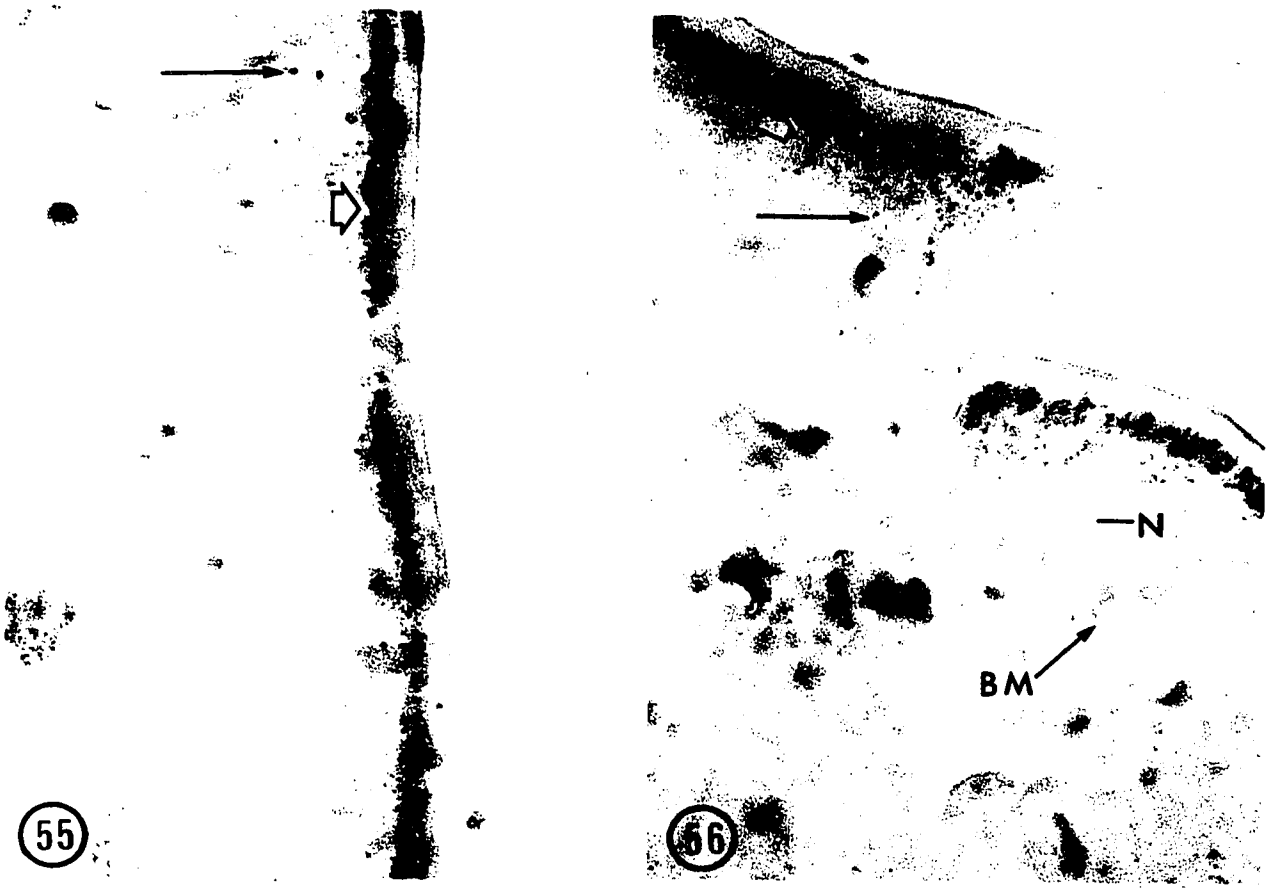
Figs. 55 to 59 illustrate material demonstrating acid phosphatase activity.

Fig. 55. Light micrograph of an intestinal villus rachitic chick. Dense precipitate indicative of acid phosphatase activity is seen below the brush border and over rounded vesicles which form a prominant band across the apical cytoplasm. A few dense vesicles also appear scattered through the cytoplasm below this band. Unstained preparation. X 40.

Fig. 56. Light micrograph of an intestinal villus of twenty-four hour vitamin D_3-replete chick. Again acid phosphatase activity appears as a band of vesicles below the terminal web, and some dense vesicles appear scattered in the cytoplasm below this band. Unstained preparation. X 40.

Fig. 57. Apical cytoplasm of rachitic chick. Electron dense precipitate indicative of acid phosphatase activity is seen over lysosomes, most of them occurring in the cytoplasm below the terminal web region. Most of the lysosomes seen here appear to be dense bodies. Unstained preparation. X 16,100.



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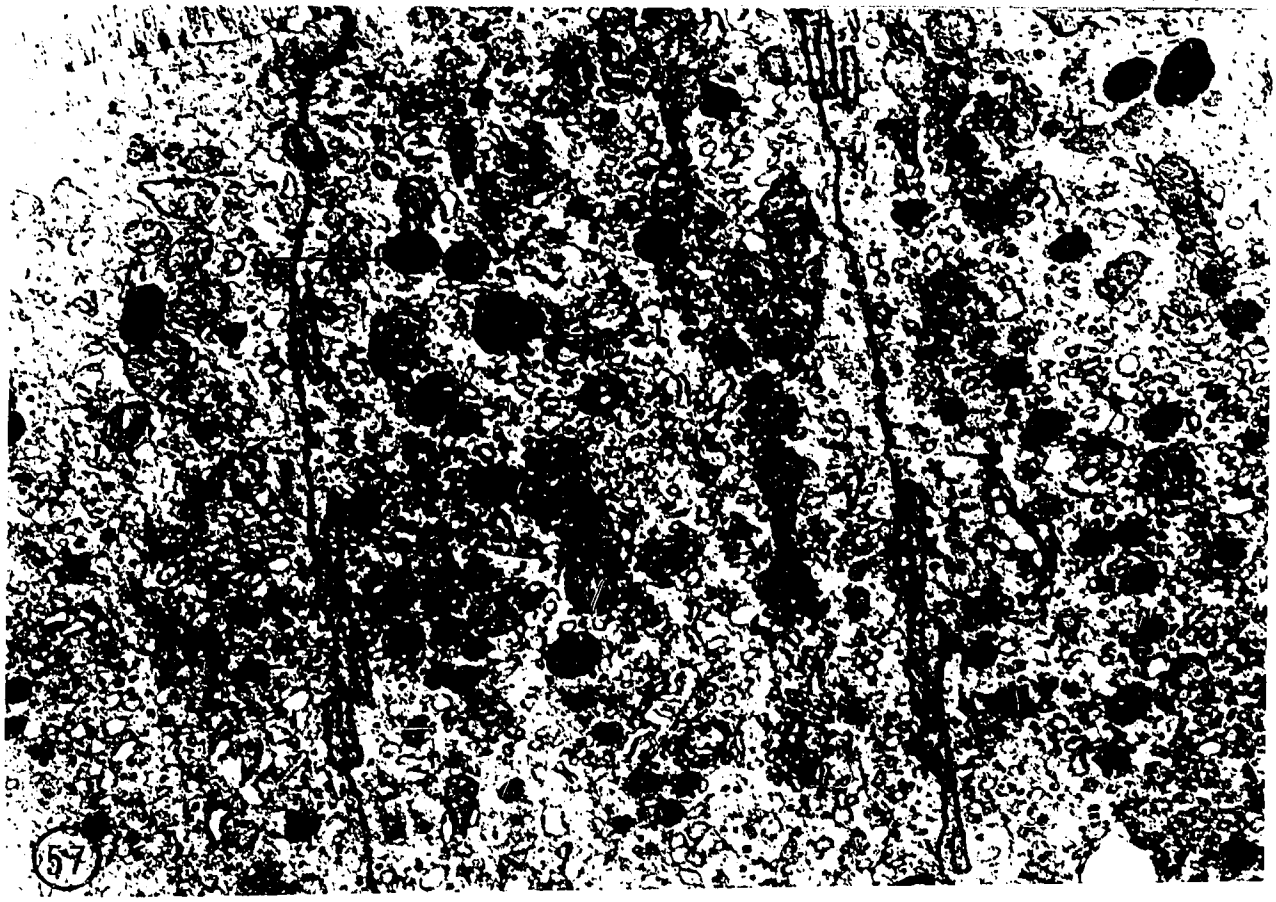


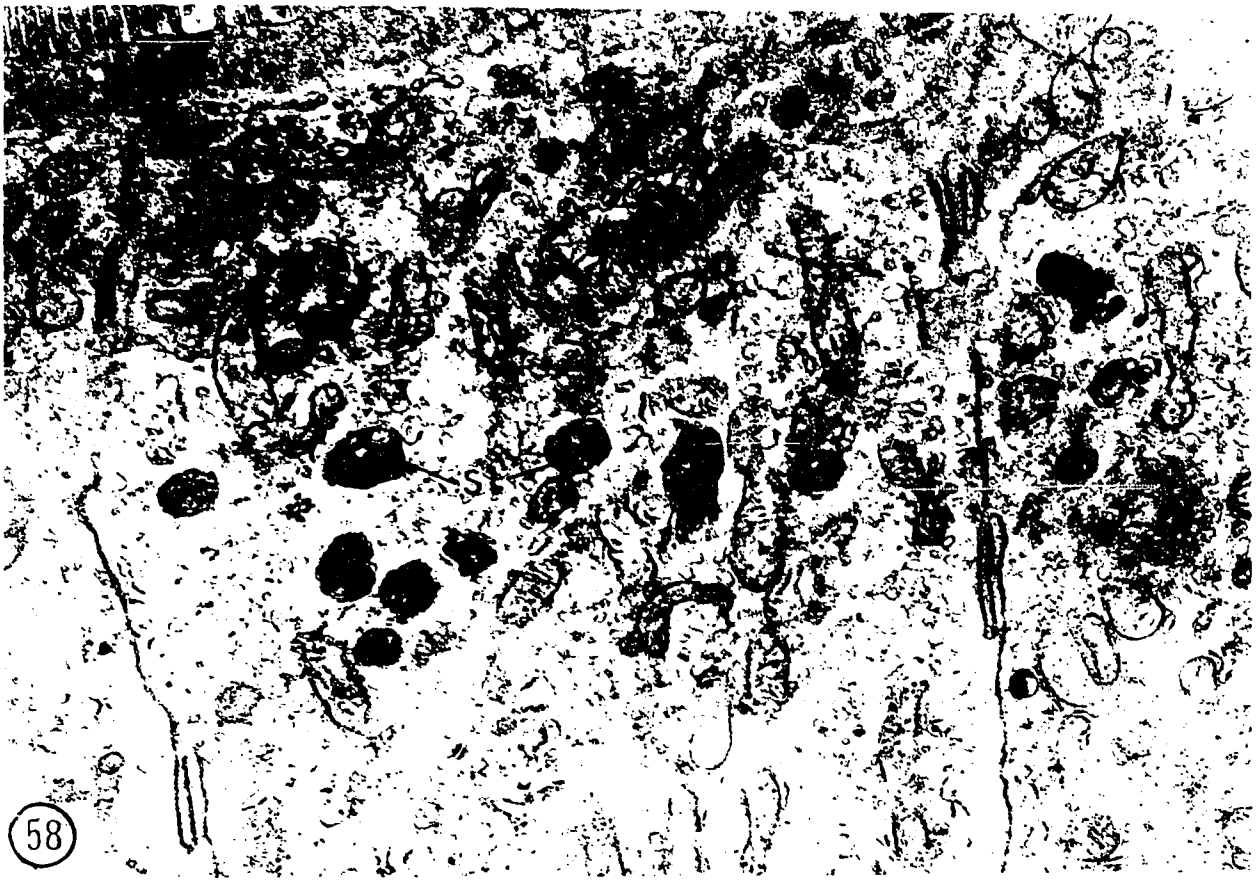
57

Fig. 58. Apical cytoplasm of twenty-four hour vitamin D_3-replete chick. Electron dense precipitate is seen over lysosomes in a similar region of the ctyoplasm to those in Fig. 57. Most of these appear to be secondary lysosomes. Unstained preparation. X 16,100.

Fig. 59. Basal cytoplasm of rachitic chick. A few lysosomes covered by electron dense precipitate are seen in the basal cytoplasm. This situation is similar to that seen in vitamin D_3-replete chicks. Unstained preparation. X 7,980.

59a. Dense body. Electron dense precipitate completely covers the dense body. Next to it is a portion of a secondary lysosome, which is partially covered by electron dense precipitate. Unstained preparation. X 47,880.

59b. Two secondary lysosomes. The secondary lysosomes are partially covered by electron dense precipitate. Note the absence of precipitate over the adjacent peroxisome. Unstained preparation. X 47,880.



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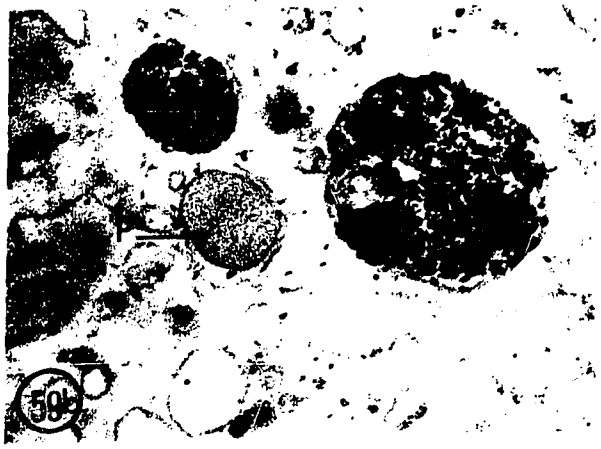
59b



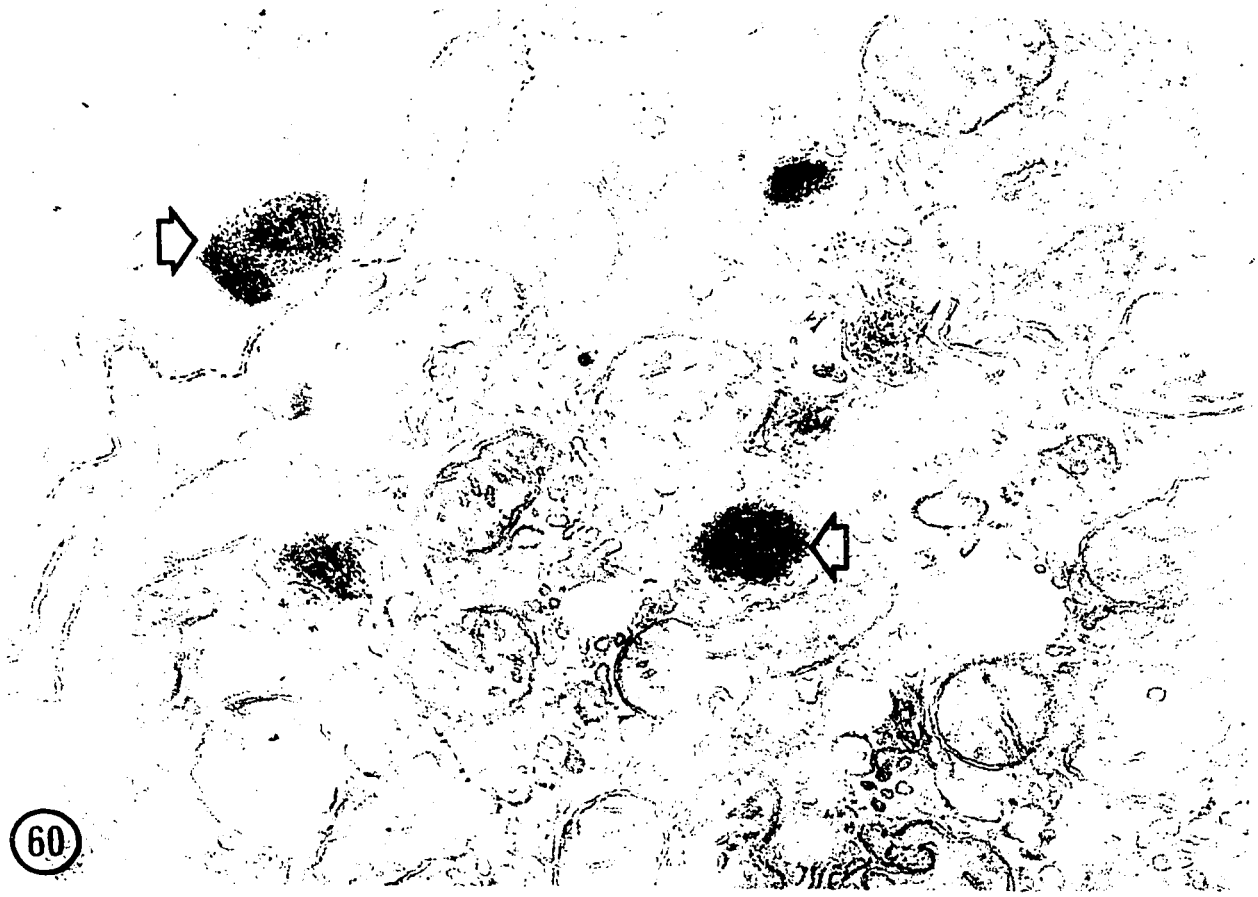
59c

Figs. 60 to 65 illustrate material fixed with S-collidine buffered paraformaldehyde.

Fig. 60. Apical cytoplasm of a rachitic chick. Small electron dense deposits are seen in two lysosomes which appear to be dense bodies (⇨). Groups of similar deposits are occasionally seen in the cytoplasm without any apparent relationship to the lysosomes. Unstained preparation. X 39,900.

Fig. 61. Apical cytoplasm of a twenty-four hour vitamin D_3-replete chick. Electron dense granules similar in appearance to thos seen in Fig. 60 appear in secondary lysosomes. Sometimes the granules are arranged in concentric lamellae (⇨). Unstained preparation. X 39,900.

61a. Inset shows these granules at higher magnification. Note that the peroxisome near the lysosome contains no granules. X 62,700.



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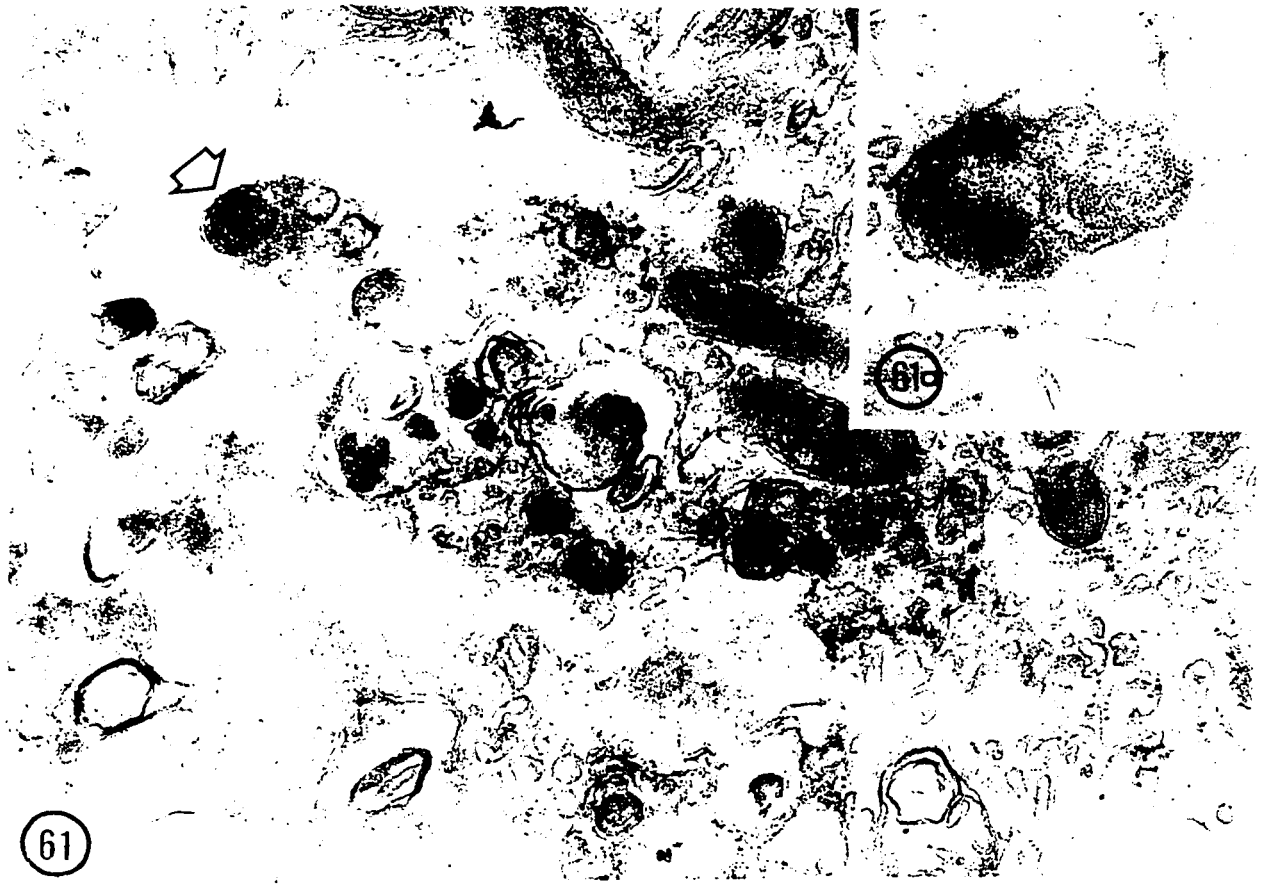


61a

61

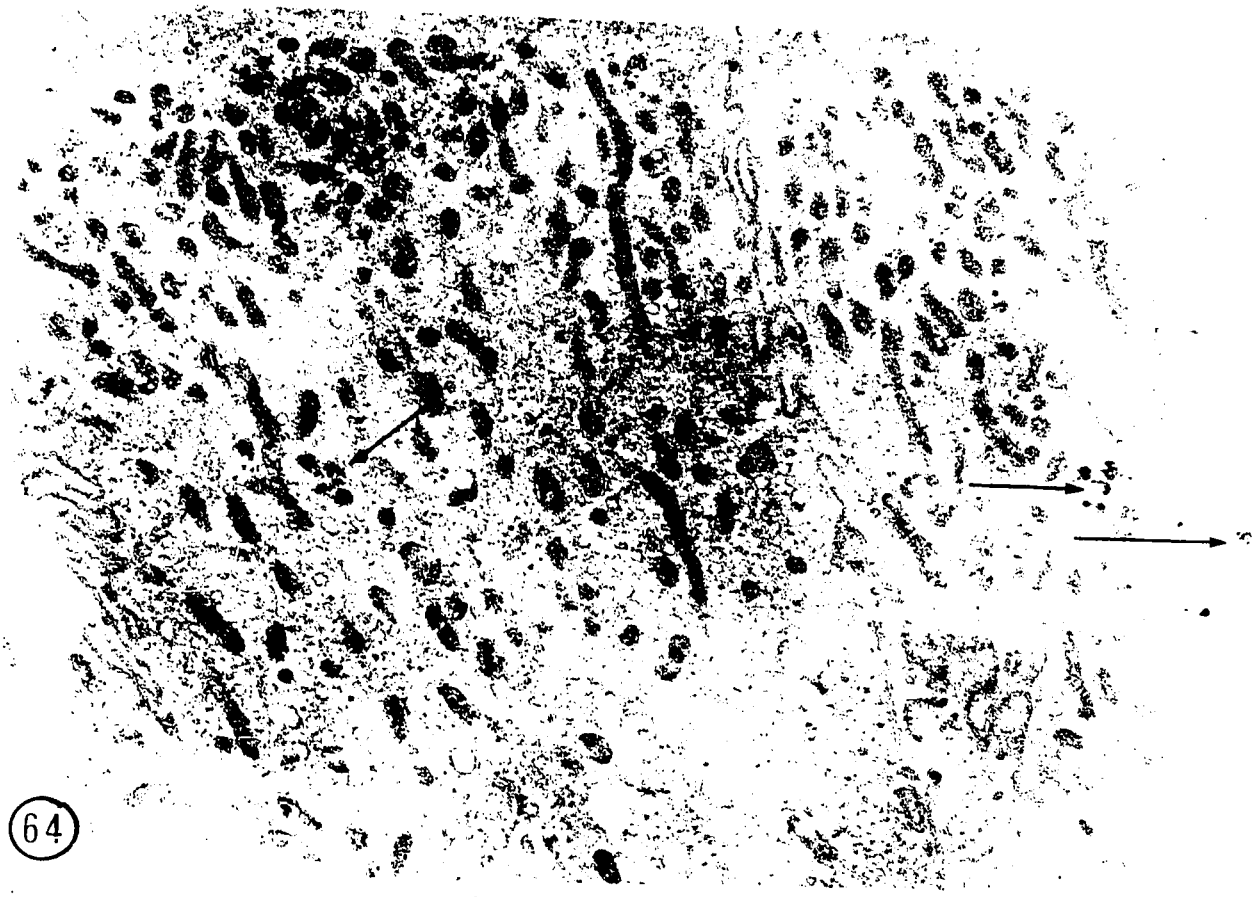
Fig. 62. Apical cytoplasm of a rachitic chick. Lysosomes containing electron dense granules stand out (⟶). Most of these appear to be dense bodies and are uniformly filled with granules. Unstained preparation. X 11,400.

Fig. 63. Apical Cytoplasm of a twenty-four hour vitamin D_3-replete chick. Many lysosomes containing electron dense granules are seen (⟶). Most of these appear to be secondary lysosomes. Unstained preparation. X 9,600.

62

Fig. 64. Apical cytoplasm of twenty-four hour vitamin D_3-replete chick, treated with EDTA. Lysosomes containing electron dense granules are again seen (⟶). These do not appear as numerous as in Fig. 63. Unstained preparation. X 9,600.

Fig. 65. A higher magnification of material similar to that seen in Fig. 64. Electron dense granules appear diffusely scattered within the secondary lysosomes (⇨). Unstained preparation. X 39,900.



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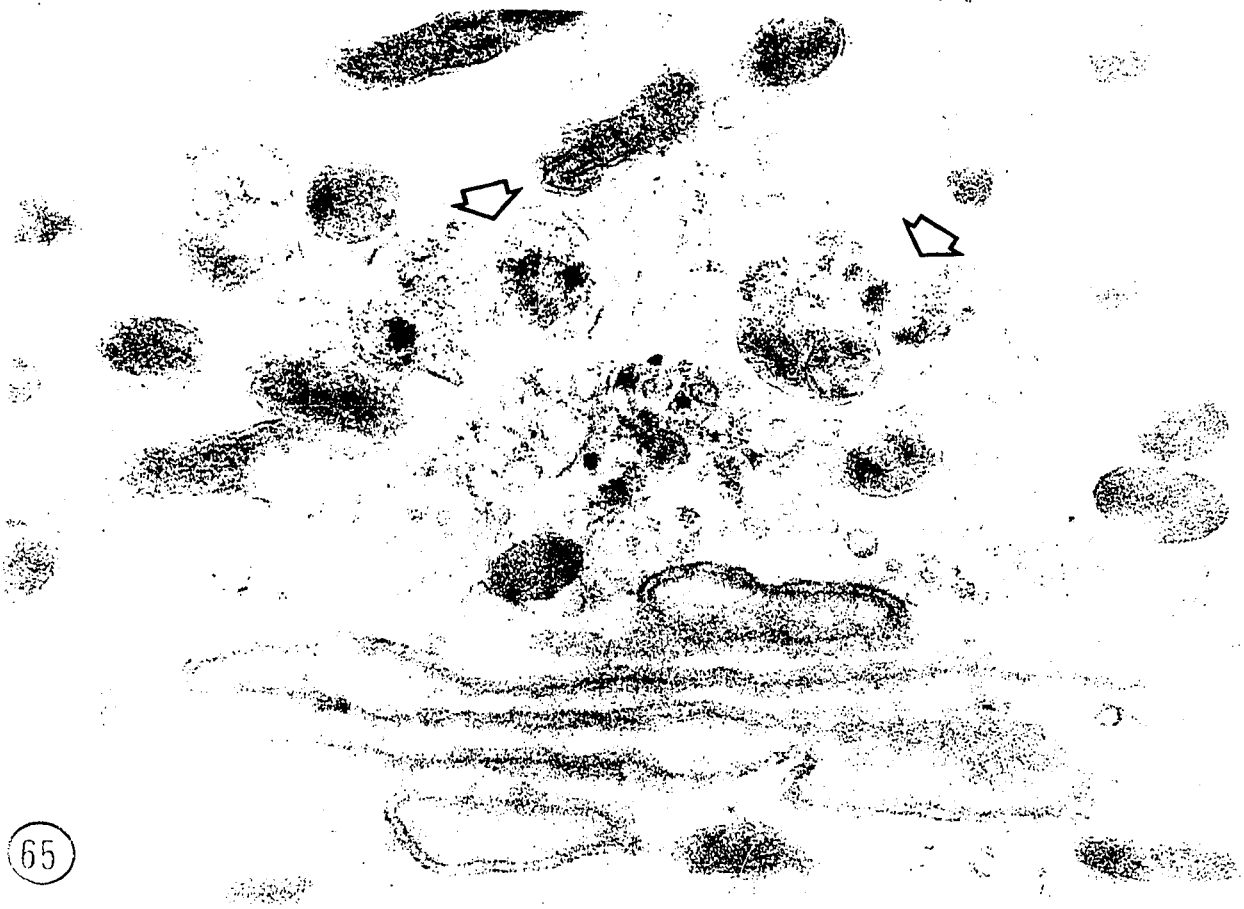
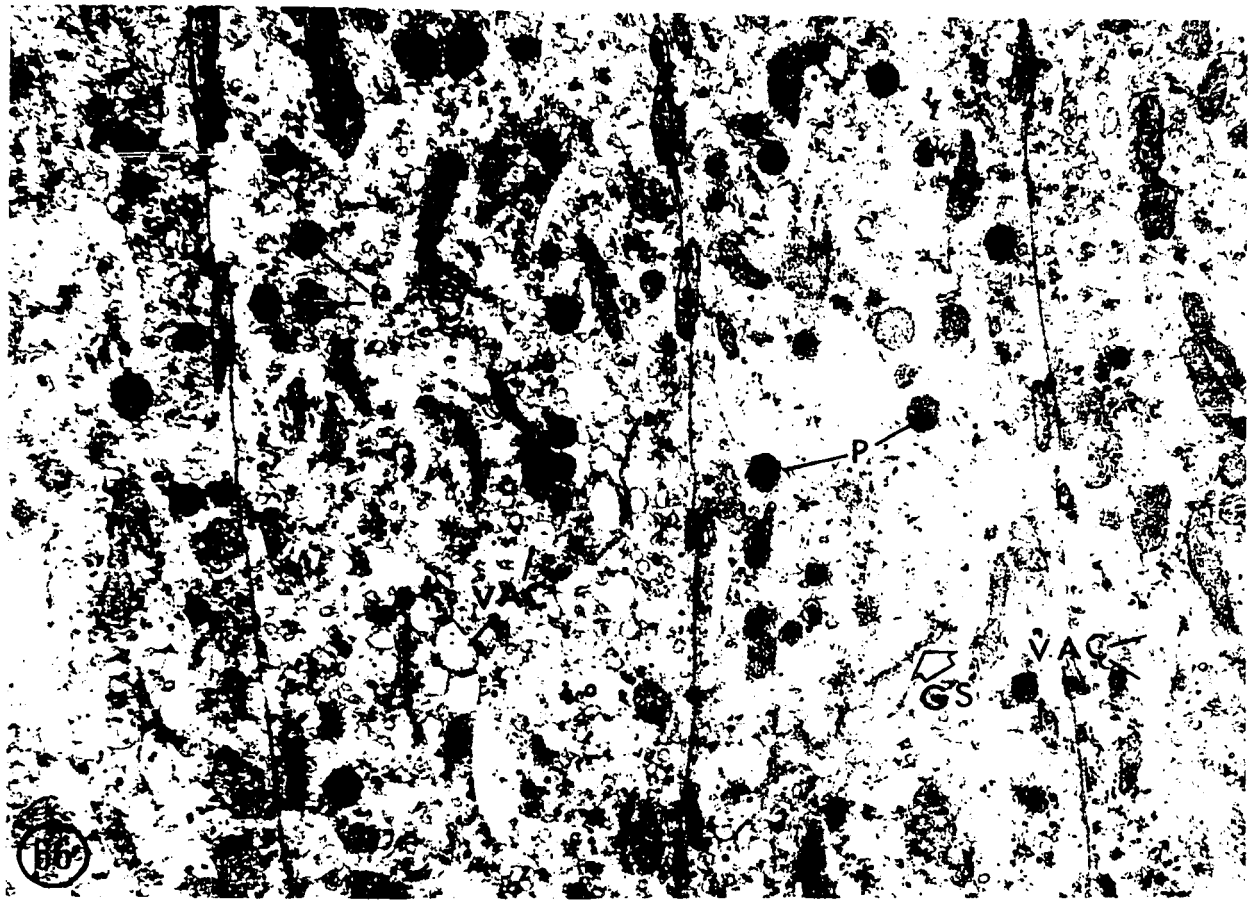


65

Fig. 66. Apical cytoplasm of a rachitic chick. The Golgi apparatus shows some activity. Golgi saccules (GS), small vesicles, and Golgi vacuoles (GV) are visible. Many peroxisomes are also visible in this region. X 11,180.

Fig. 67. Apical cytoplasm of a twelve hour vitamin D_3-replete chick. The Golgi apparatus appears more active than in the previous figure. Many large Golgi vacuoles are seen, and these form a prominant band across the apical cytoplasm. Many peroxisomes are again visible. Note the cisternae of granular endoplasmic reticulum arranged in parallel rows in the supranuclear region (⇨). X 11,180.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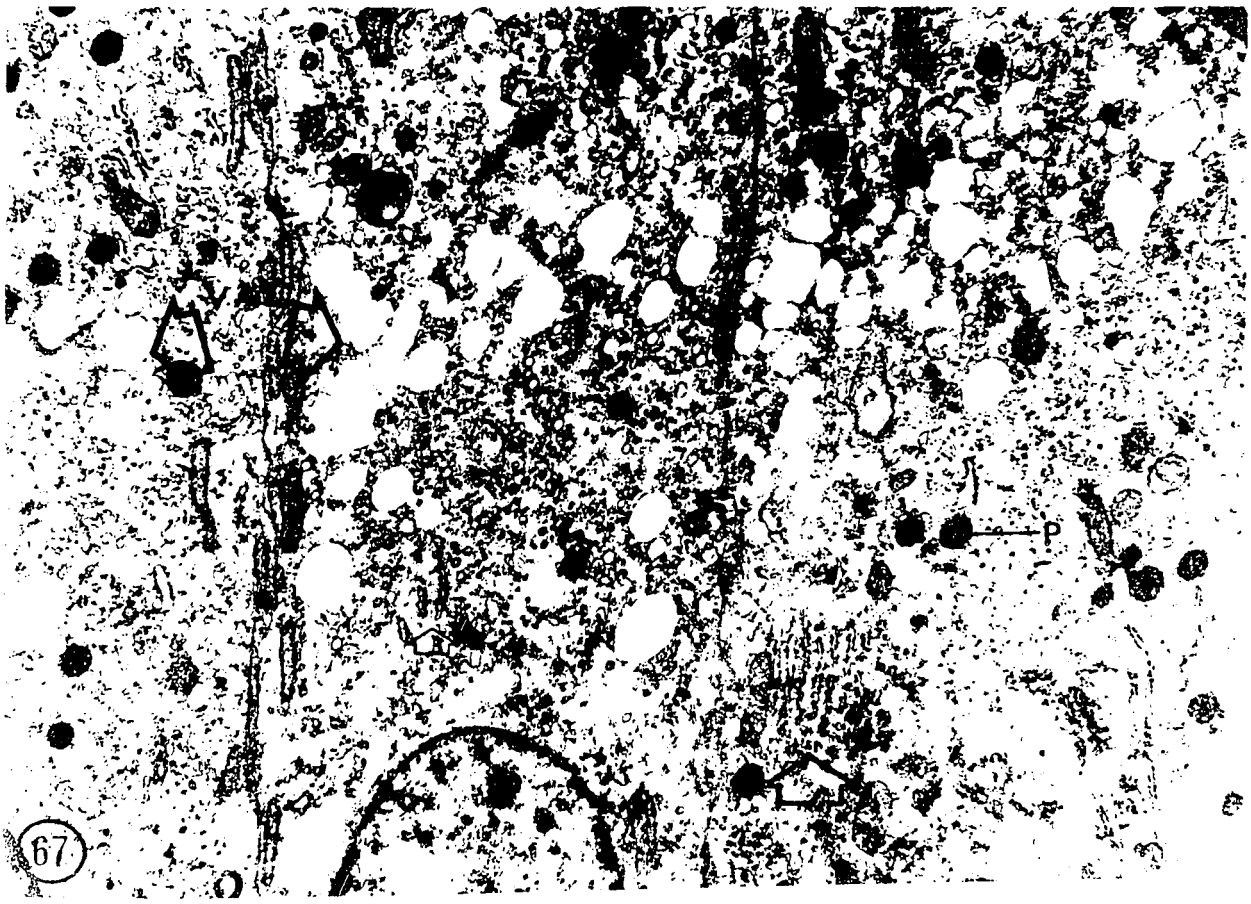
P
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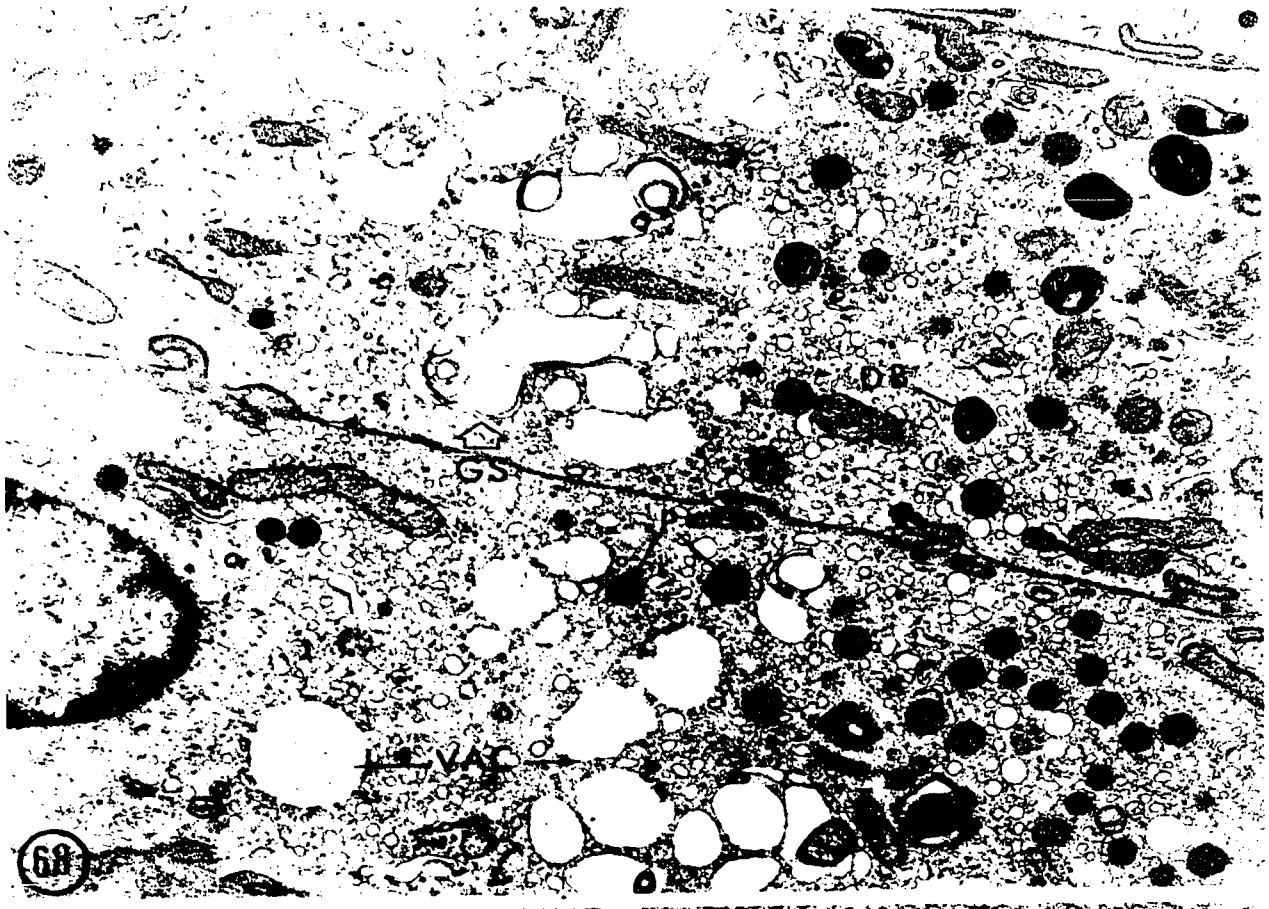
Fig. 68. Apical cytoplasm of an eighteen hour vitamin D_3-replete chick. The Golgi apparatus still is very active. The large band of vacuoles is not quite as prominant as at twelve hours. The large vacuoles are clearly seen to be composed of smaller vesicles, giving them a globular appearance. The outer saccules of the Golgi apparatus are greatly distended by slightly electron dense material. Many peroxisomes, and several dense bodies and small secondary lysosomes are also visible. The cytoplasm is full of small rounded vesicles. Some of these may be agranular endoplasmic reticulum. X 13,680.

Fig. 69. Apical cytoplasm of a twenty-four hour vitamin D_3-replete chick. The Golgi apparatus shows less activity than at twelve and eighteen hours. It is composed mainly of stacks of saccules and small vesicles. X 13,680.


GS
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VA
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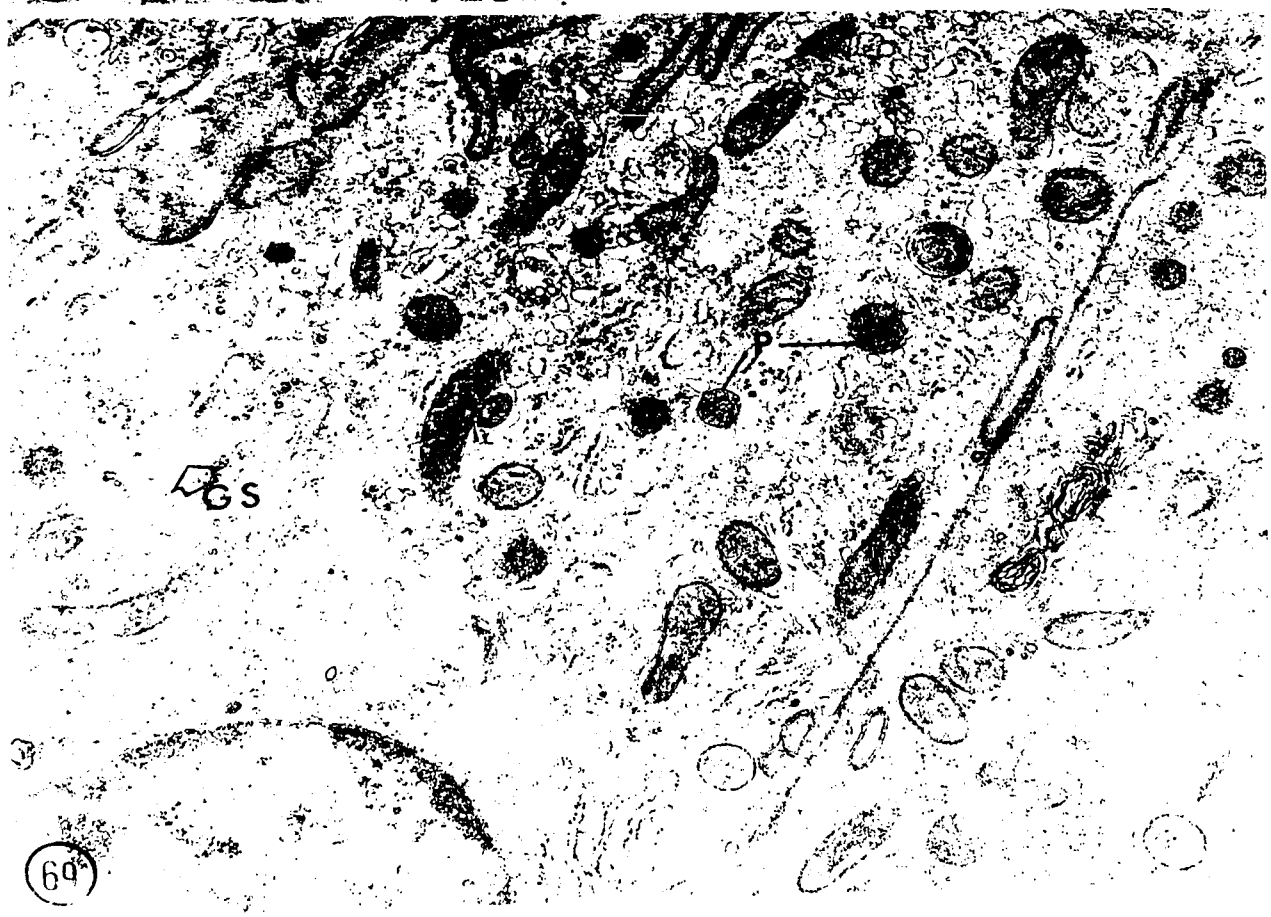
P
GS
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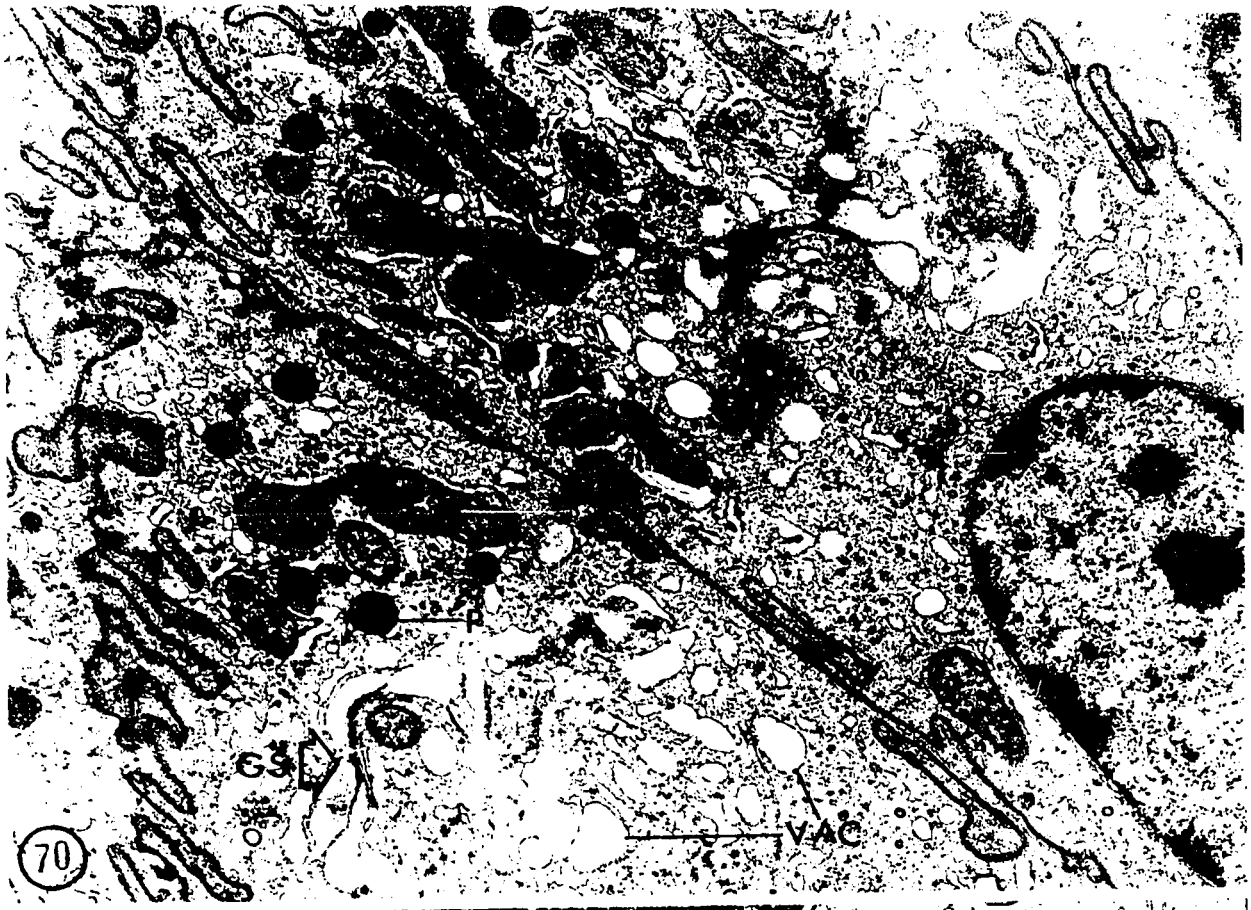
Fig. 70. Apical cytoplasm of a forty-eight hour vitamin D_3-replete chick. The Golgi apparatus appears active. X 16,180.

Fig. 71. Apical cytoplasm supranuclear region of a seventy-two hour vitamin D_3-replete chick. Part of the Golgi apparatus is seen, and is similar to forty eight hours in activity. (See also Fig. 53). A few peroxisomes are fisible. Note the proliferation on granular endoplasmic reticulum in this region. X 16,100.



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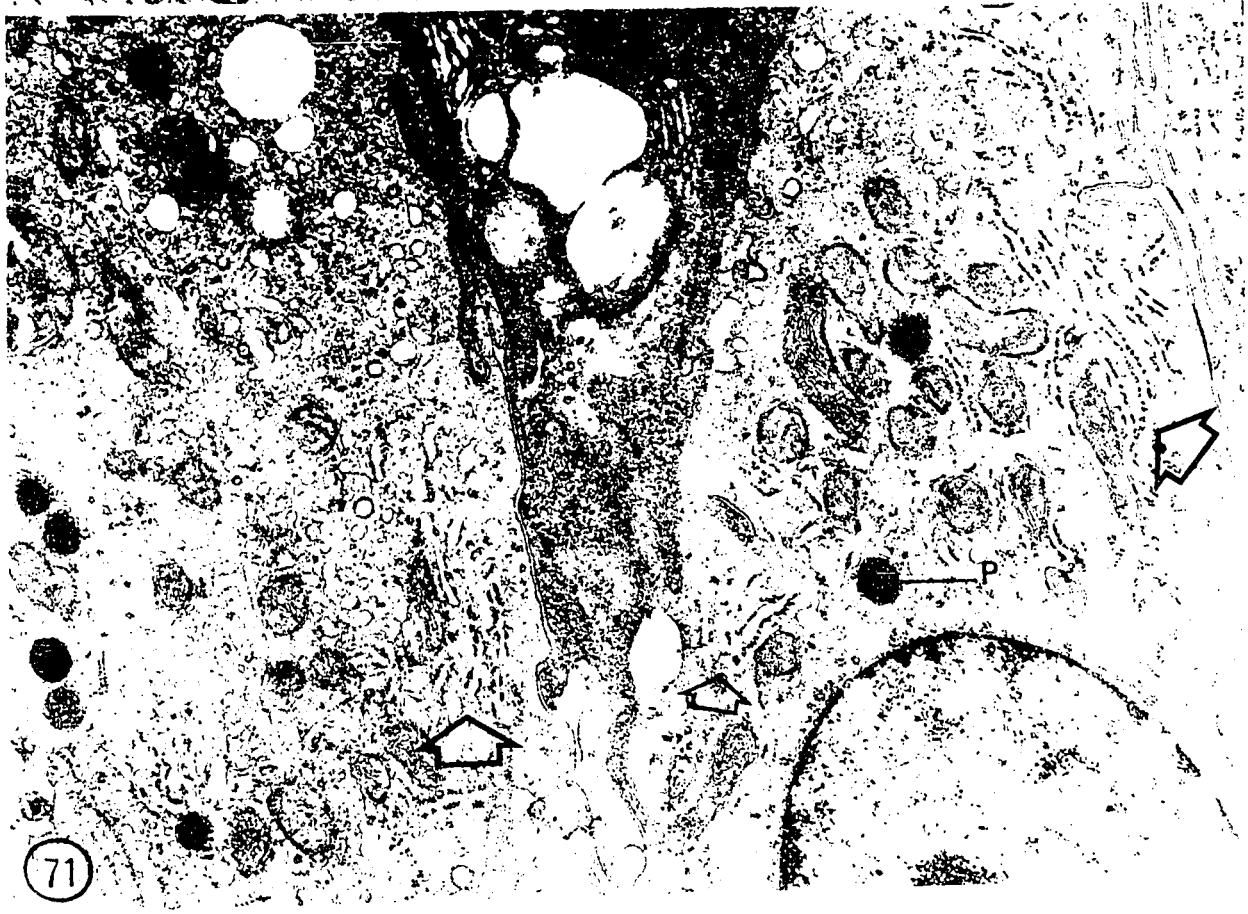

71

Fig. 72. Basal cytoplasm. Low-calcium diet chick. Note the large electron dense granules within the mitochondrial matrix, similar to those seen in the apical cytoplasm of these animals. X 39,900.

Fig. 73. Basal cytoplasm of rachitic chick. In this low power micrograph many mitochondria, some granular endoplasmic reticulum, a few dense bodies, and some peroxisomes are seen. X 9,580.

M.C.
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Fig. 74. Basal cytoplasm of a twelve hour vitamin D_3-replete chick. Note the increased amount of granular endoplasmic reticulum. Many of the cisternae appear distended by slightly electron dense material. (See also Fig. 26). X 18,810.

Fig. 75. Basal cytoplasm of an eighteen hour vitamin D_3-replete chick. Many dilated cisternae of granular endoplasmic reticulum appear, most of these in close proximity to mitochondria. Many free polyribosomes can be seen. Note the globular material within dilations of the intercellular space. Similar material also appears below the basal margins of the cells. X 20,520.

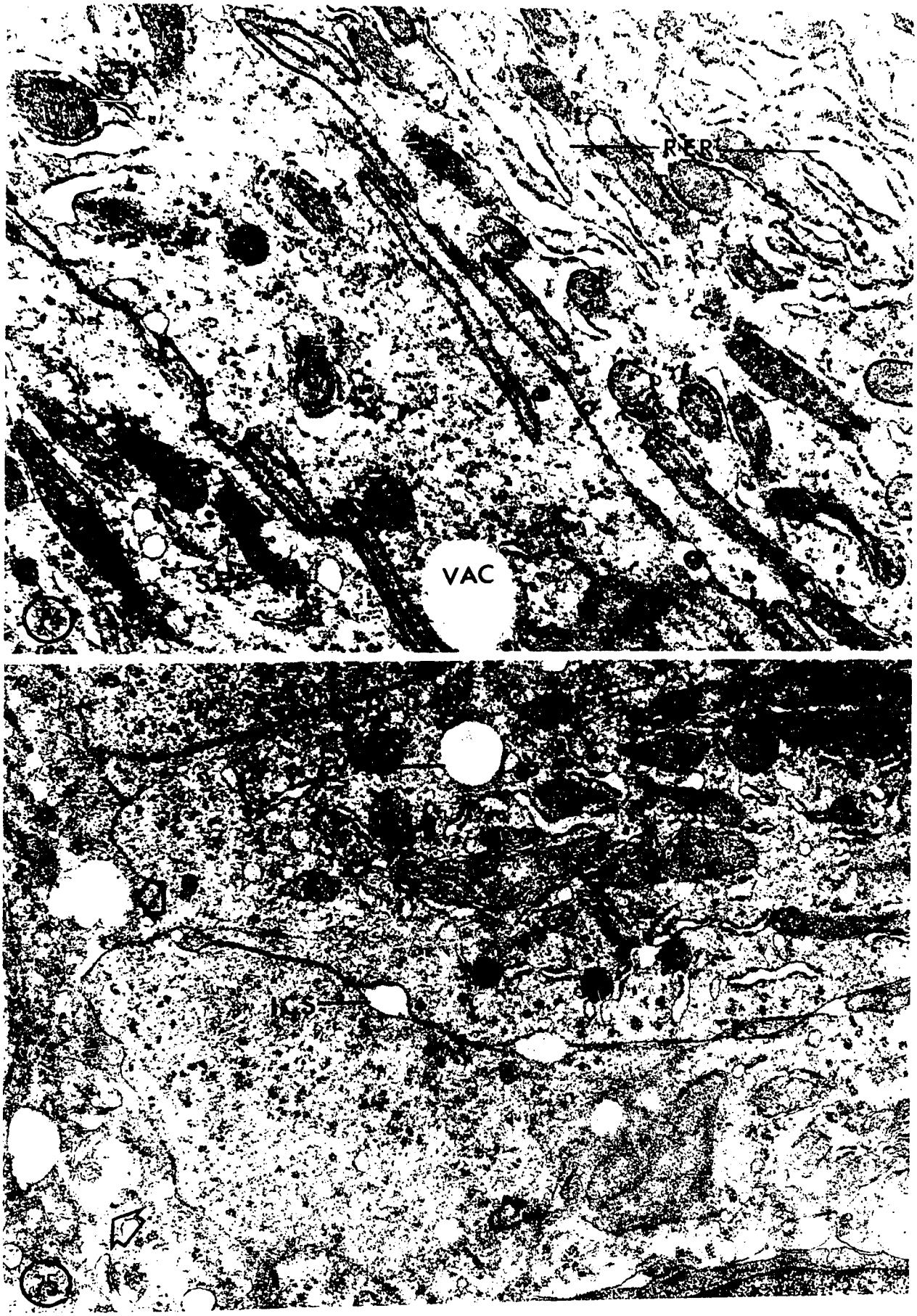
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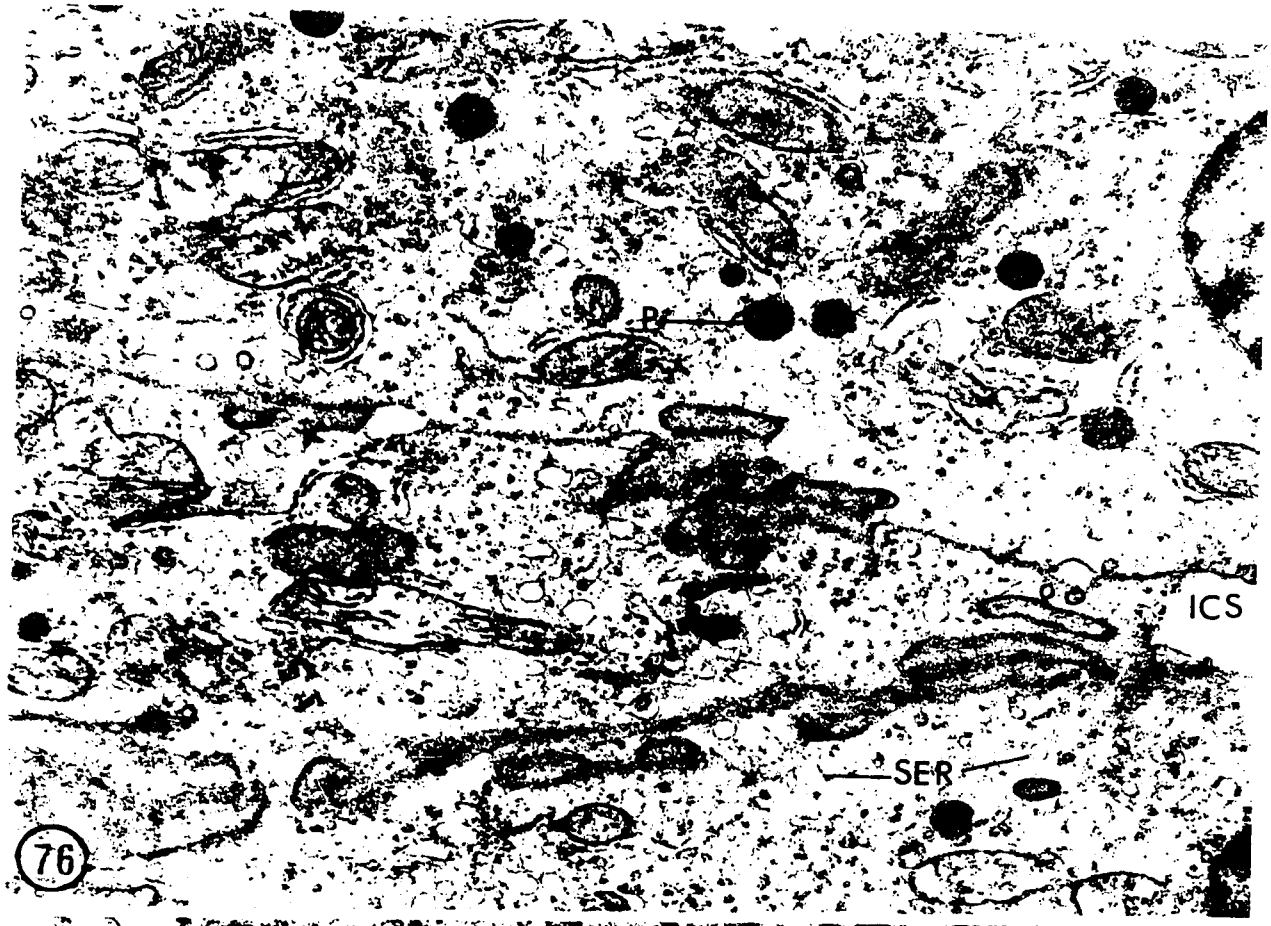
Fig. 76. Basal cytoplasm of a twenty-four hour vitamin D_3-replete chick. The basal cytoplasm appears similar to that seen at eighteen hours. X 16,100.

Fig. 77. Basal cytoplasm of a forty-eight hour vitamin D_3-replete chick. Again this region appears similar to the situations at eighteen and twenty-four hours. X 16,100.



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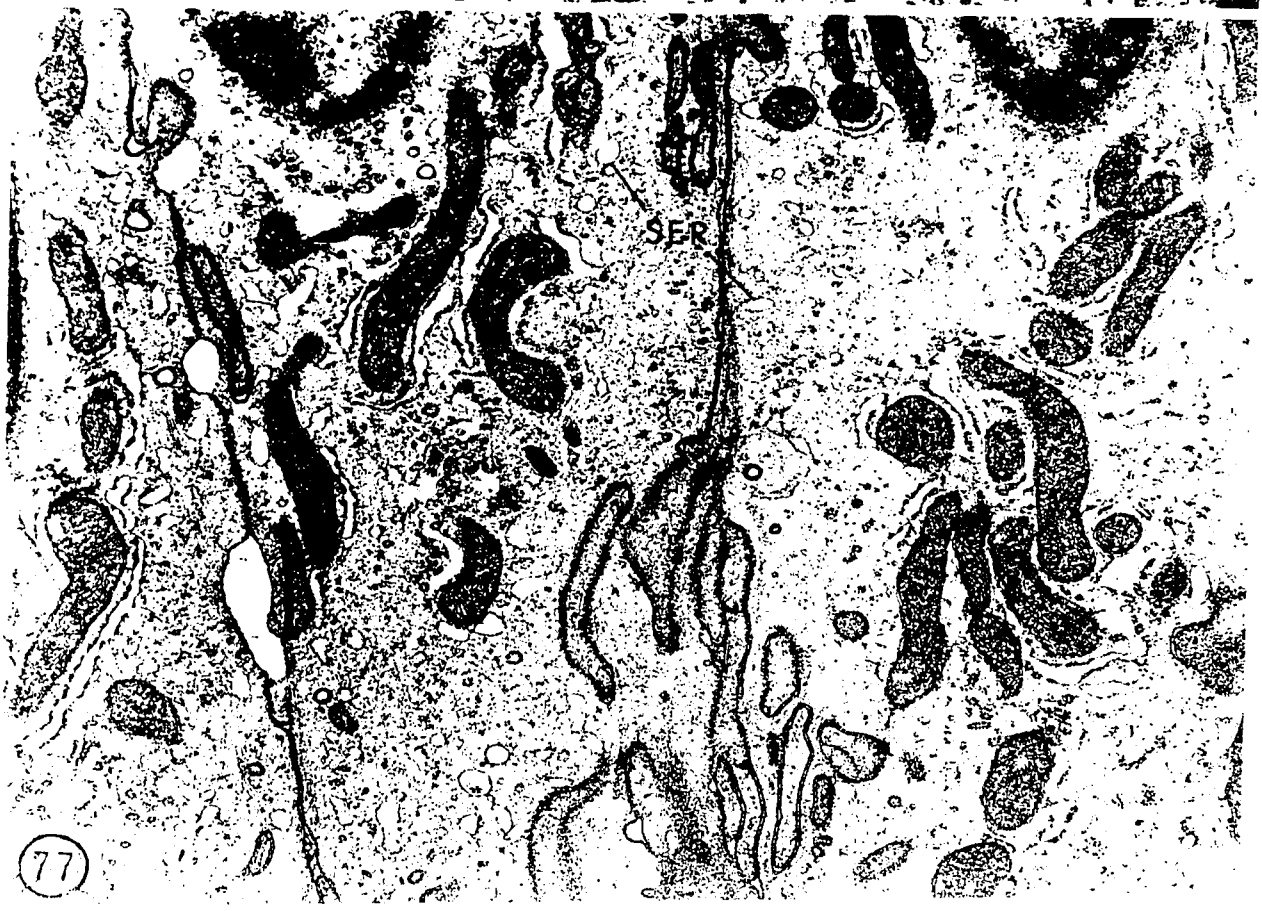

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Fig. 78. Basal cytoplasm of a seventy-two hour vitamin D_3-replete chick. Note large amount of granular endoplasmic reticulum. Many cisternae are arranged in parallel rows (⇨). X 16,260.

Fig. 79. Basal cytoplasm of a low calcium diet chick. This region is quite similar to that seen at eighteen hours. Dilated cisternae of granular endoplasmic reticulum appear, mainly in close proximity to mitochondria. X 13,680.

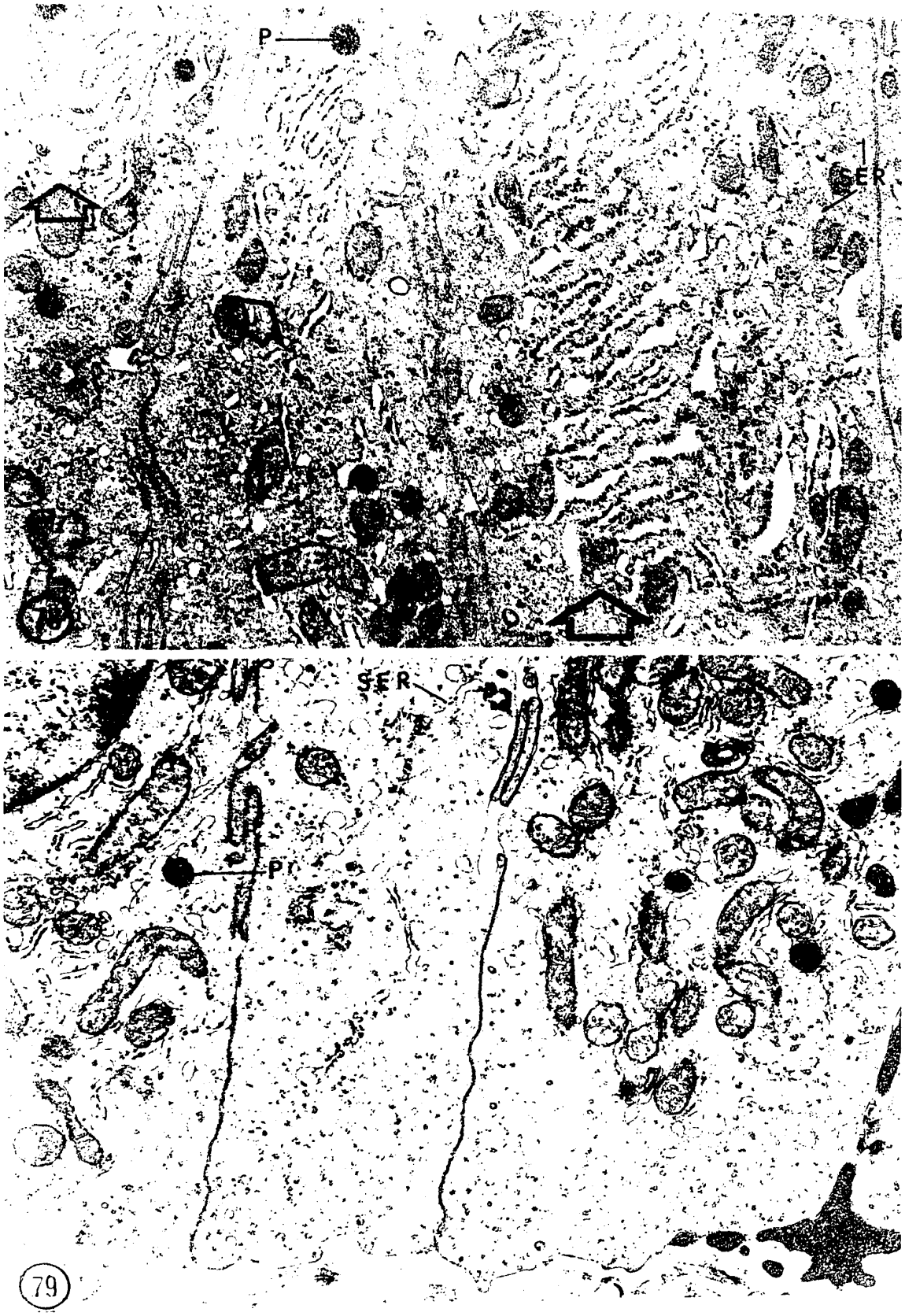
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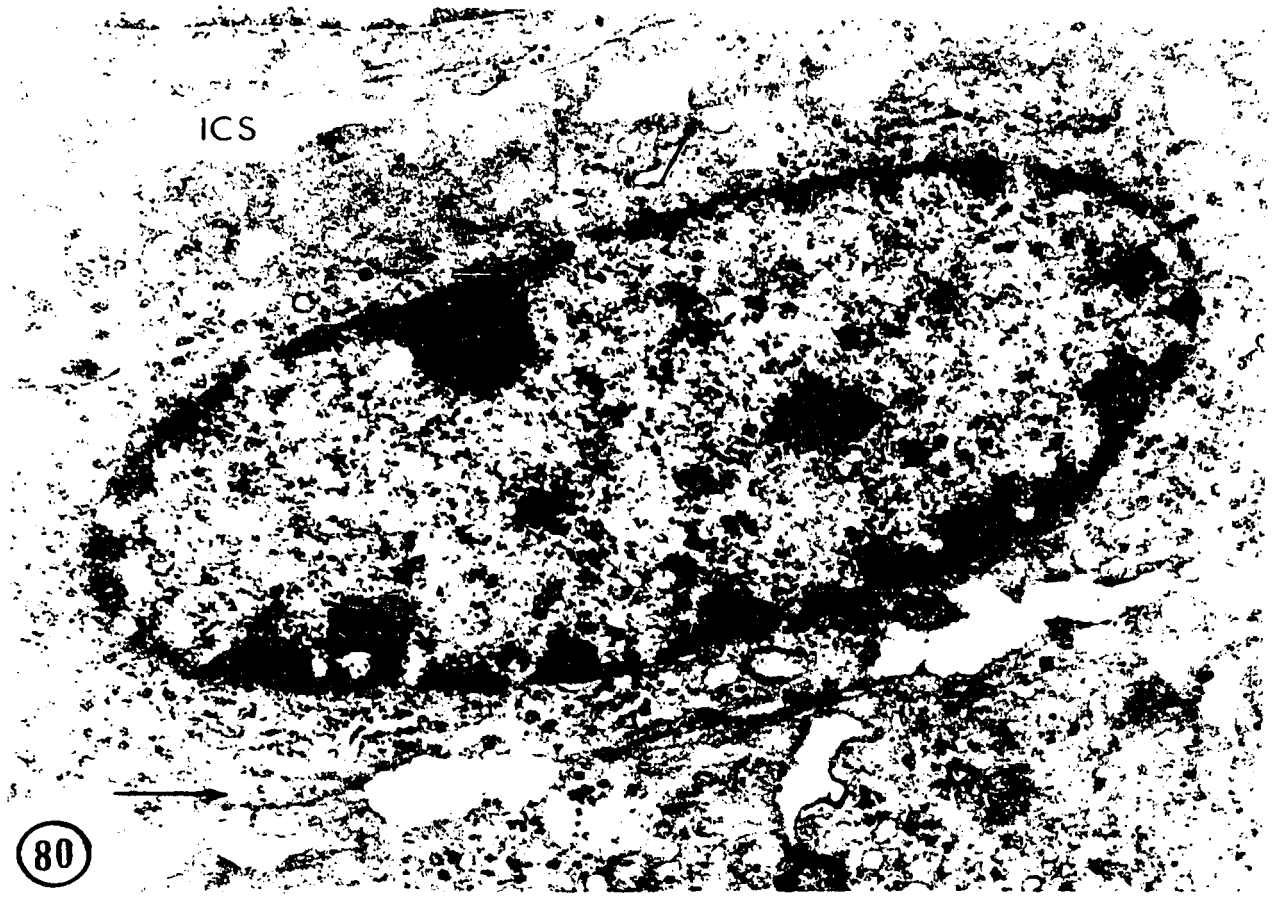
Fig. 80. Nuclear region of an eighteen hour vitamin D_3-replete chick. Note the large amount of globular material within the intercellular spaces, and the similarity of this material to that contained within the Golgi vacuoles (Fig. 68). At the arrows vesicles are seen which may be fusing with the plasma membrane and discharging their contents into the intercellular spaces. X 20,520.

Fig. 81. Basal region of an eighteen hour vitamin D_3-replete chick. Note the large amount of globular material below the basal margins of the cells. Some of it appears below the plasma membrane, and some below the basal lamina. Also note the similarity of this material to that seen in the intercellular spaces and in the Golgi vacuoles (Fig. 68). X 16,100.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Figs. 82 to 87 illustrate material fixed with S-collidine buffered paraformaldehyde.

Fig. 82. Apical cytoplasm. Rachitic chick. Small electron dense deposits are seen adhering to the lateral membrane (⟶) and within the intercellular space (⇨). The electron dense deposits adhering to the plasma membrane appear similar to those seen previously on the microvillar membrane (Figs. 35, 36). Unstained preparation. X 51,080.

Fig. 83. Apical cytoplasm. Twenty-four hour vitamin D_3-replete chick. Small electron dense deposits are again seen adhering to the lateral plasma membranes and within the intercellular space. Note the similarity in distribution and appearance of these deposits to those in the previous figure. Unstained preparation. X 62,700.

82

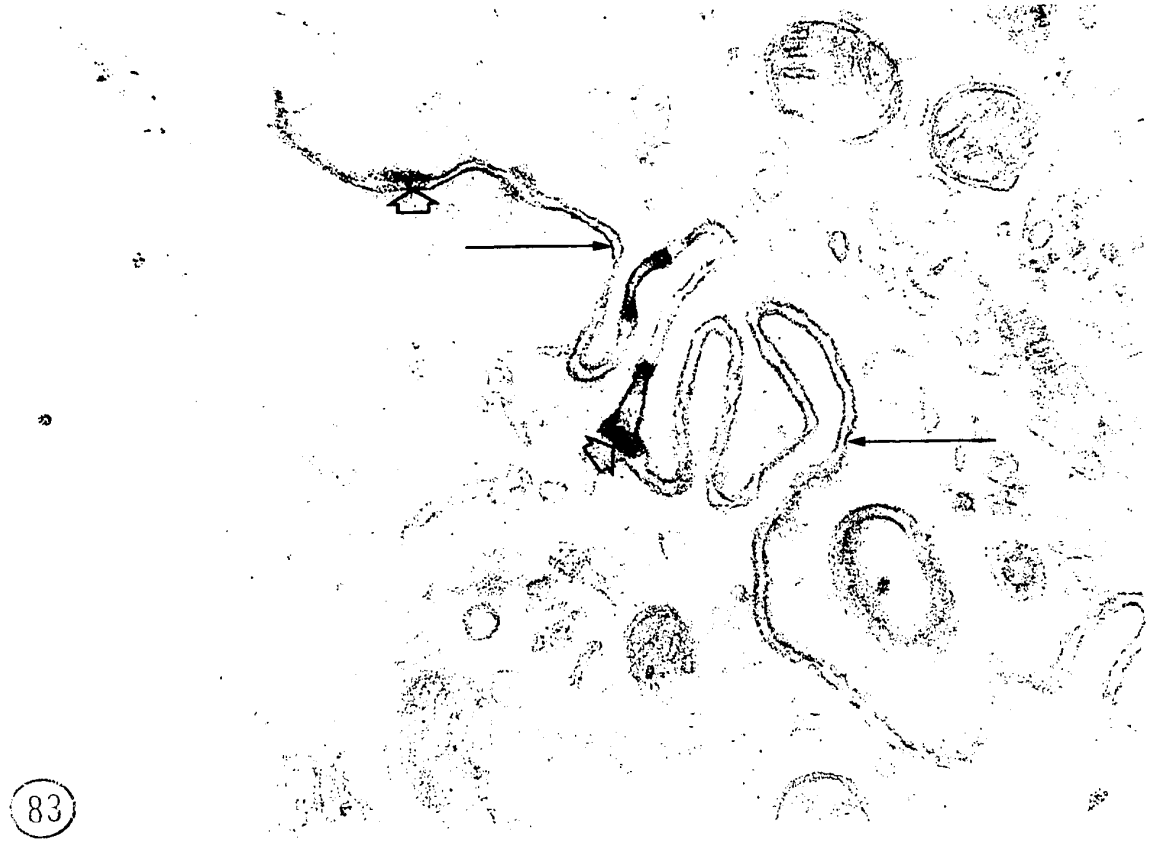

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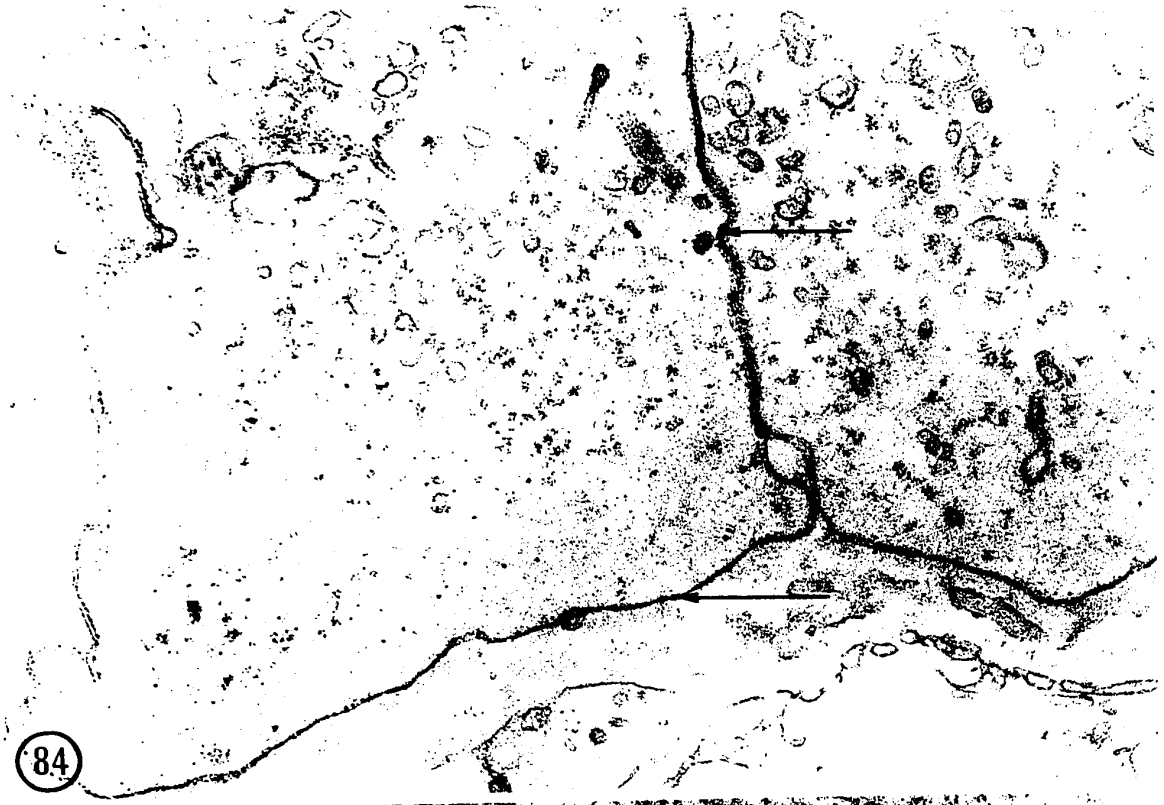
Fig. 84. Basal region of a rachitic chick. Note the electron dense deposits adhering to the lateral and basal plasma membranes. These appear similar to electron dense deposits on the plasma membrane in the previous two figures. Note the absence of deposits on the endothelium of the capillary seen at lower right. Unstained preparation. X 25,650.

Fig. 85. Basal region of a twenty-four hour vitamin D_3-replete chick. Note the similarity in appearance and abundance of electron dense deposits on the lateral and basal membranes to those seen in the previous figure. Unstained preparation. X 25,650.



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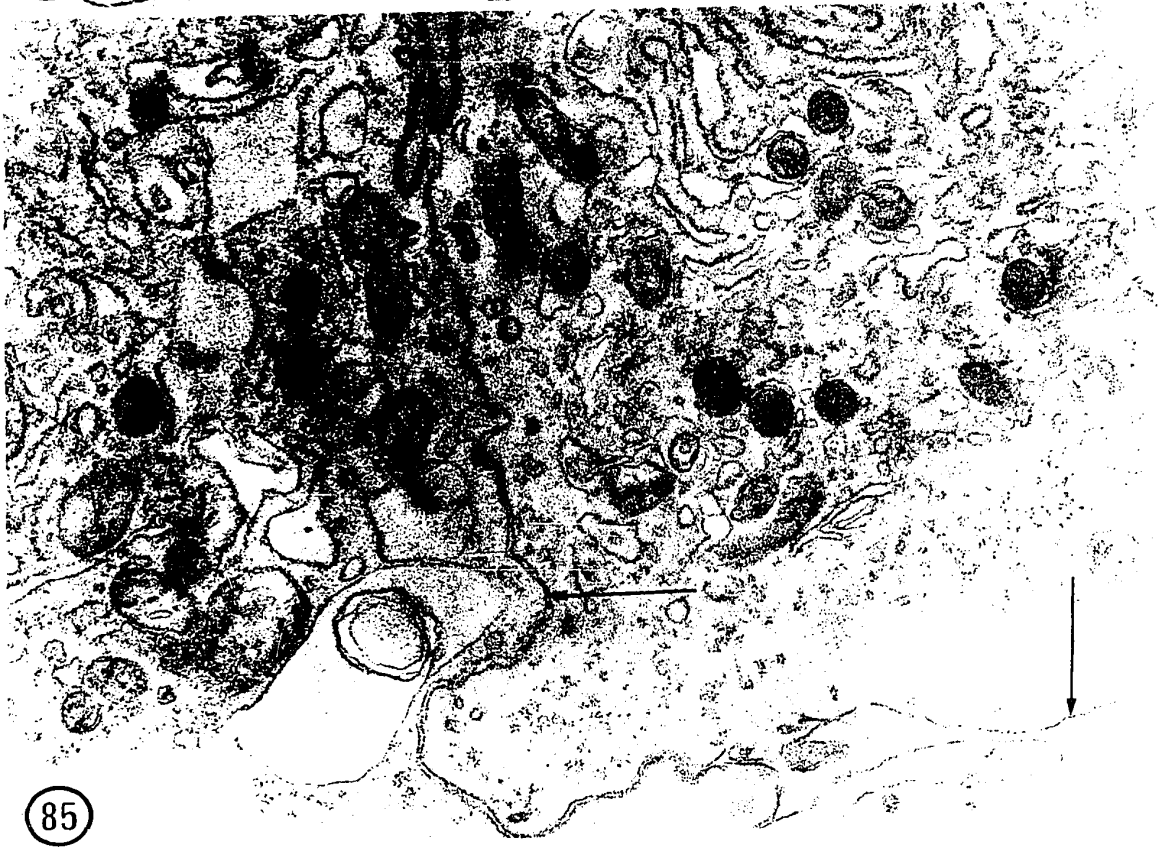

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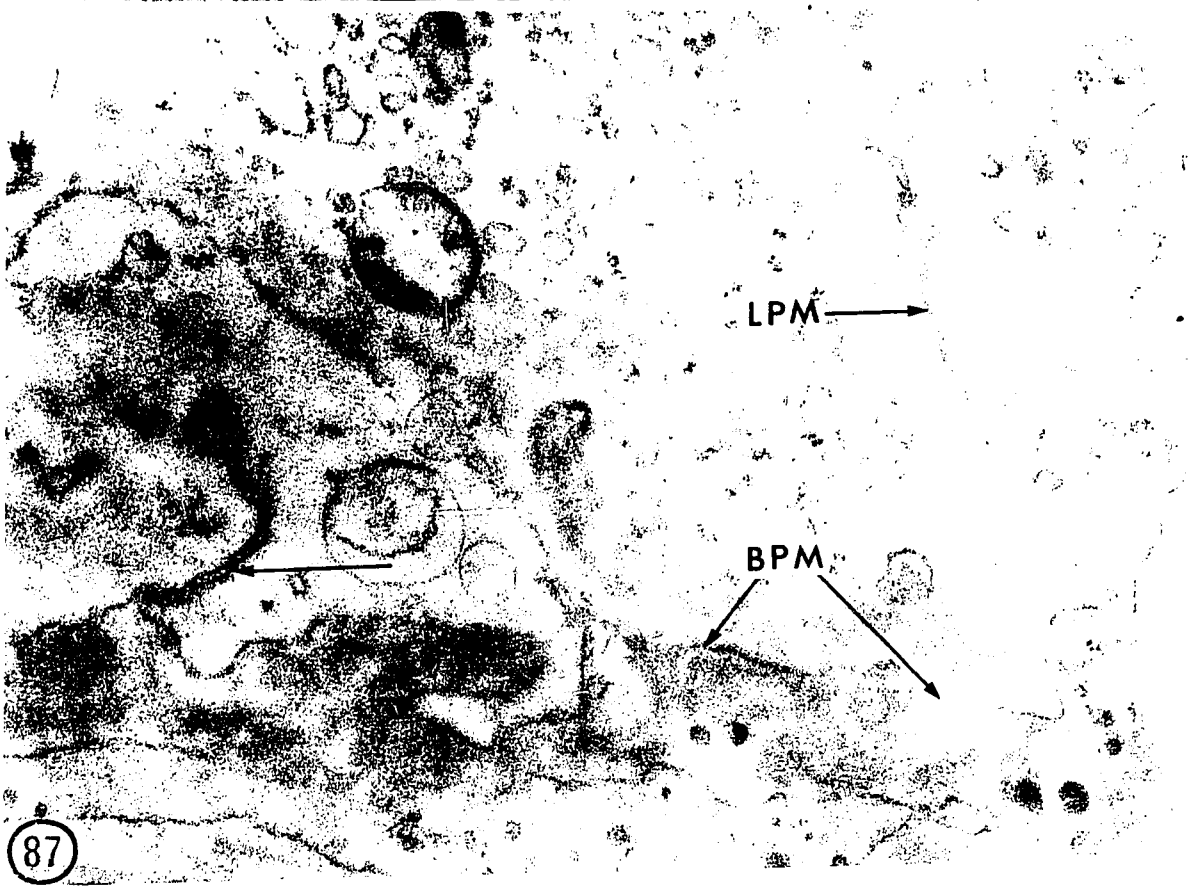
Fig. 86. Apical cytoplasm of a twenty-four hour vitamin D_3-replete chick, treated with EDTA. Little evidence of electron dense deposits can be seen on the plasma membranes or within the intercellular space. Some electron dense material is seen at arrow. Unstained preparation. X 47,880.

Fig. 87. Basal cytoplasm of a twenty-four hour vitamin D_3-replete chick. Note the absence of electron dense deposits on the lateral and basal plasma membranes. Arrow at left indicates lateral plasma membrane of the cell which occupies the centre of the figure. Unstained preparation. X 25,650.

86

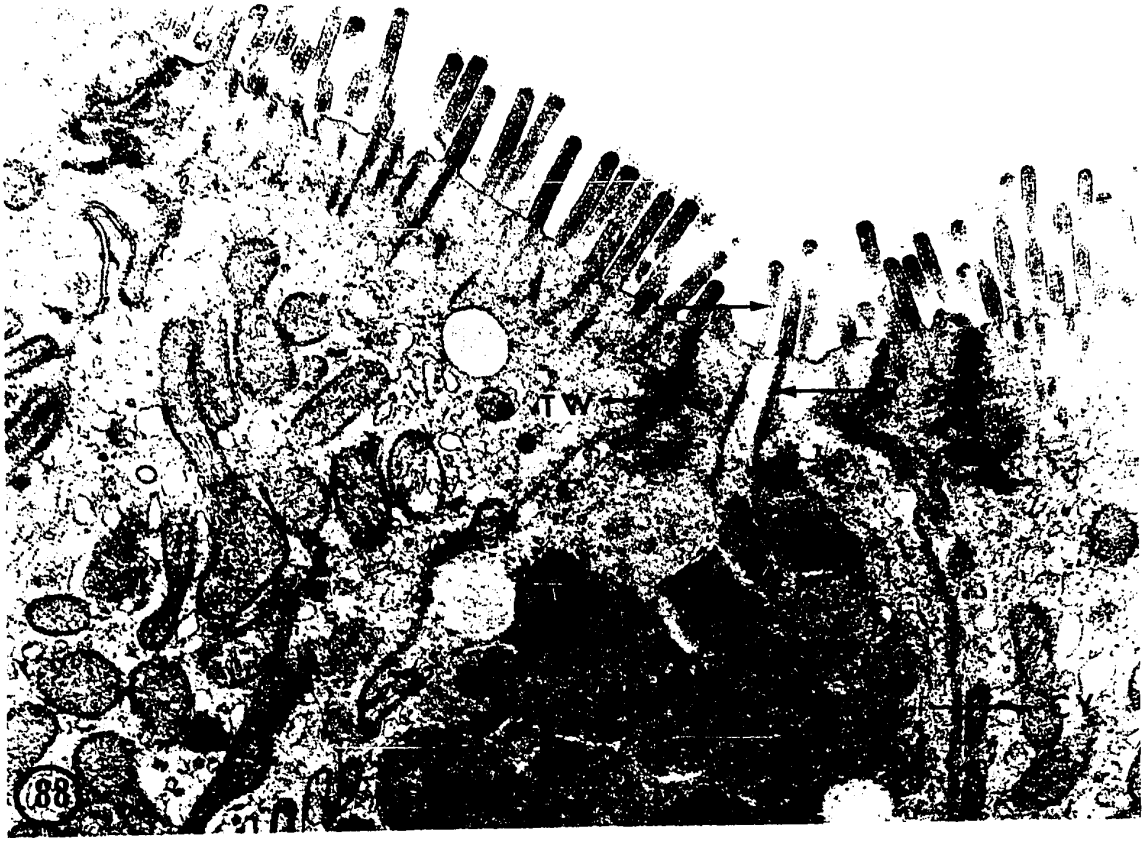


87

Figs. 88 to 98 illustrate ultrastructural aspects of the goblet cells.

Fig. 88. Apical region of a goblet cell which is not in the process of expelling. Eighteen hour vitamin D_3-replete chick. The microvilli resemble those of the absorptive cells. The terminal web appears incomplete. The juxtaluminal junctional complexes can be seen between the goblet cell and the adjacent absorptive cells. The layer of compressed cytoplasm (CY) is visible adjacent to the lateral margins of the mucous mass.

Fig. 89. Apical region of goblet cell. Low-calcium diet chick. This cell appears to be about to expel. The microvilli and terminal web show increased electron density and evidence of disrutpion. Within the mucous small areas of greater electron density than the rest of the mucous mass can be seen (*). X 20,240.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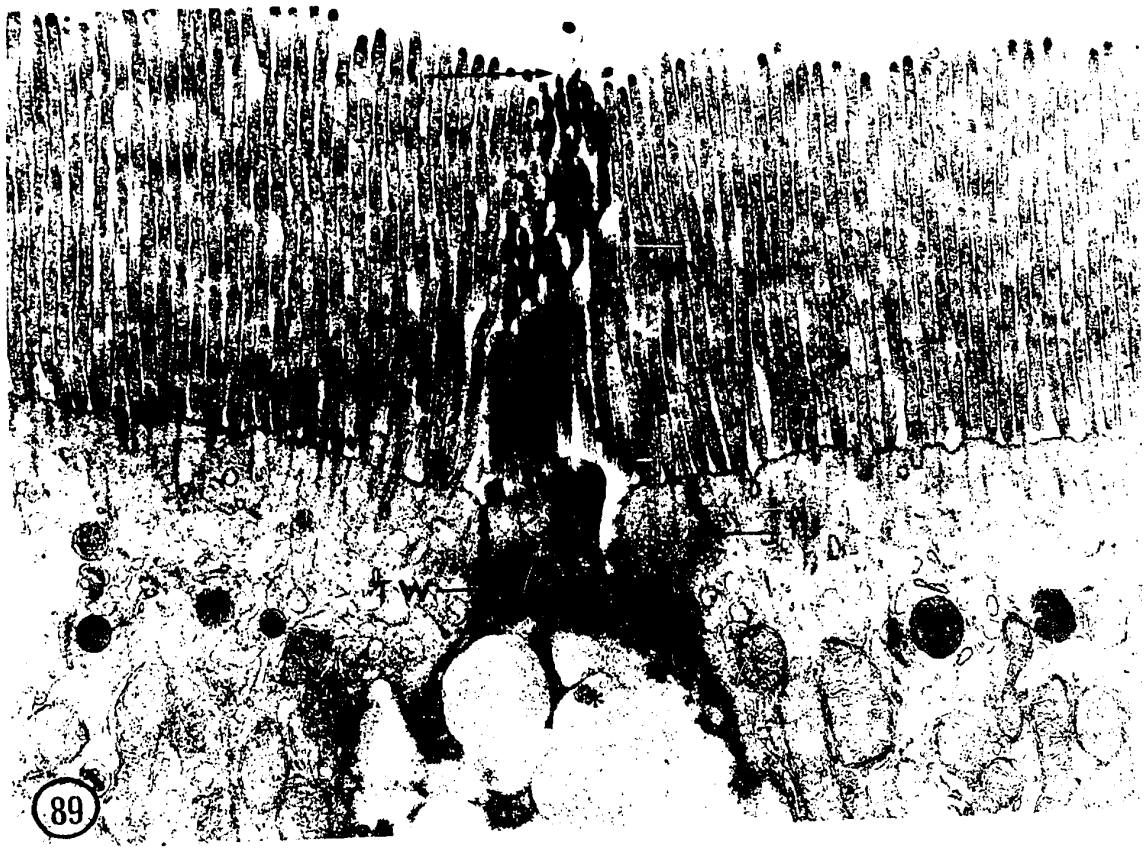
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Fig. 90. Goblet cell in the process of expelling mucous. Low calcium diet chick. The varying electron density of the mucous droplets is clearly visible. Portions of the cytoplasm are seen projecting between the mucous droplets at the lateral and basal portions of the mucous mass, and trapped within the mucous mass (→). In some areas the mucous appears to be in direct contact with the lateral plasma membrane (⇨). Most of the droplets appear coalesced although retaining their varying electron densities. X 7,980.

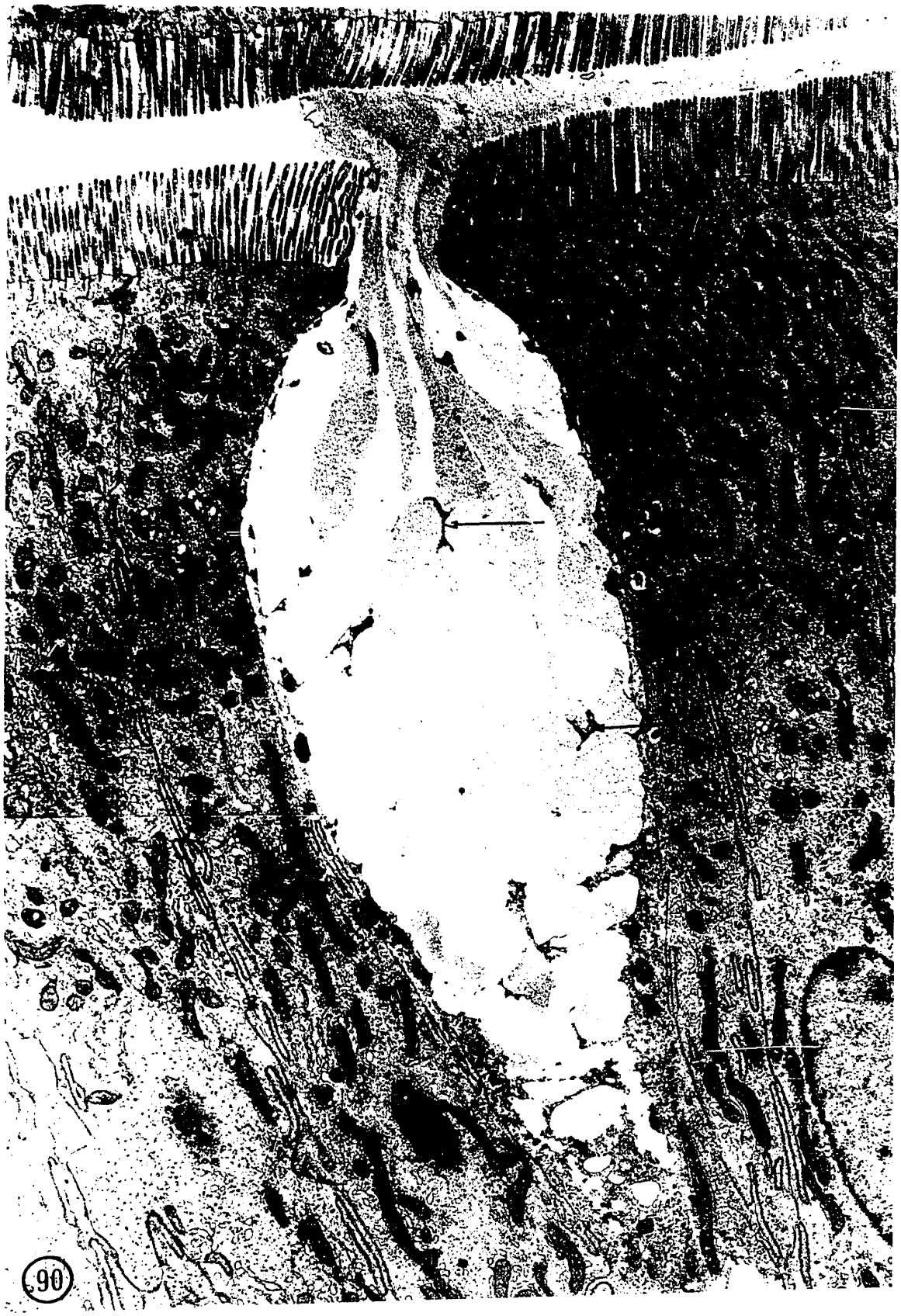
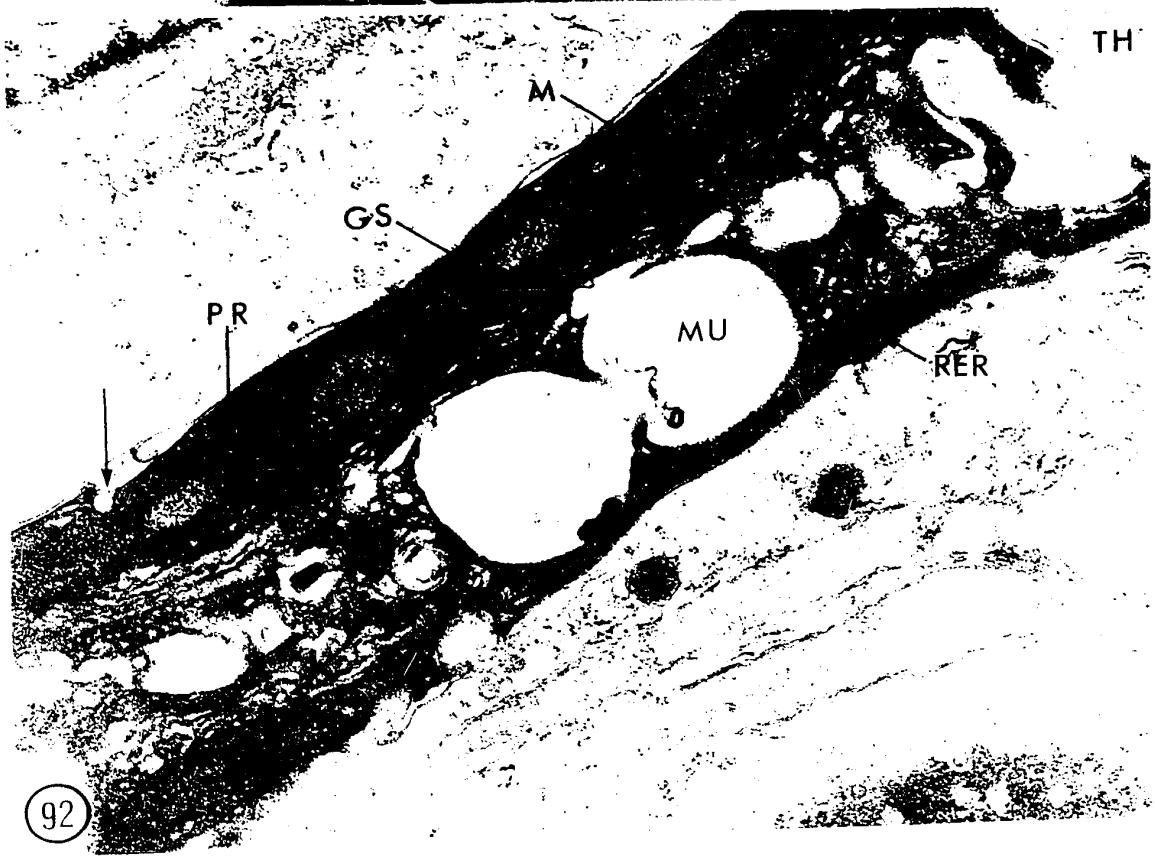

90

Fig. 91. Higher magnification of apical portion of goblet cell seen in Fig. 90. The fibrillar composition of the mucous is visible, especially in those droplets which are the least electron dense. Cytoplasmic remnants (⟶) are clearly visible in the expelling mucous. X 20,235.

Fig. 92. The upper region of a goblet cell foot. Low calcium diet chick. The organelles in this region are seen to be closely packed and oriented parallel to the cell's long axis. Long Golgi saccules arranged in lamellae are visible, as are mucous droplets of varying sizes (MU). The largest droplets can be seen to be fusing. Longitudinally oriented mitochondria and granular endoplasmic reticulum are visible. Many free polyribosomes are seen in the lateral portions of the cytoplasm. At arrow a vesicle is seen in contact with the lateral plasma membrane. X 25,080.

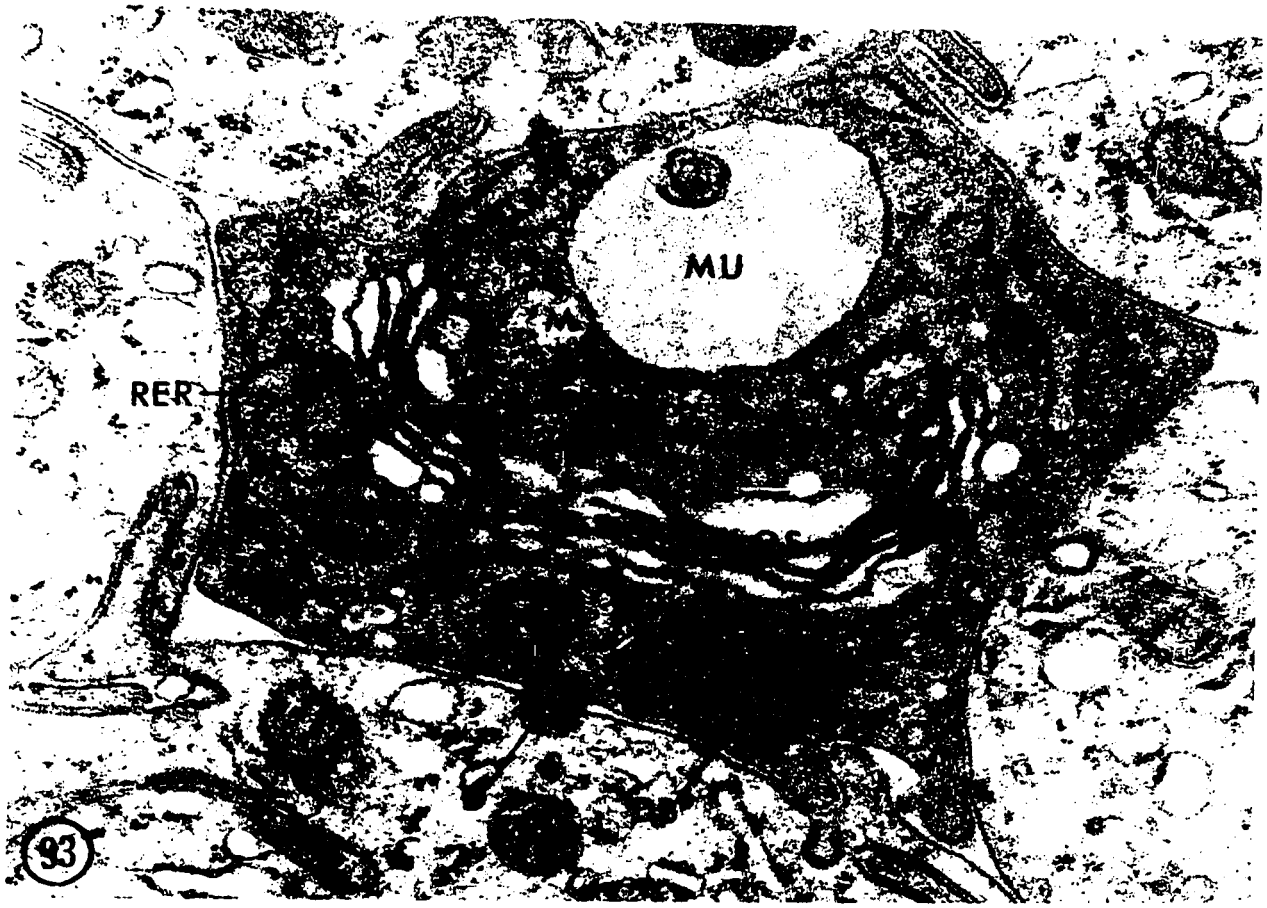
91



92

Fig. 93. Cross section through foot. Twelve hour vitamin D_3-replete chick. Most of the Golgi saccules appear distended by slightly electron dense contents. Note the distension of of the cisternae of granular endoplasmic reticulum by material which is more electron dense than that within the Golgi saccules. Several mitochondria and a lysosome are visible in section. Desmosomes are seen holding the lateral membranes of the goblet and absorptive cells in close apposition. X 39,900.

Fig. 94. Basal portion of foot region of a low calcium chick. The nucleus is seen to be more irregular in shape than that of the absorptive cells. Basal to it are seen granular endoplasmic reticulum, sections of mitochondria, and free polyribosomes. The basal membrane forms several small foot-like projections (seen at right). X 25,650.

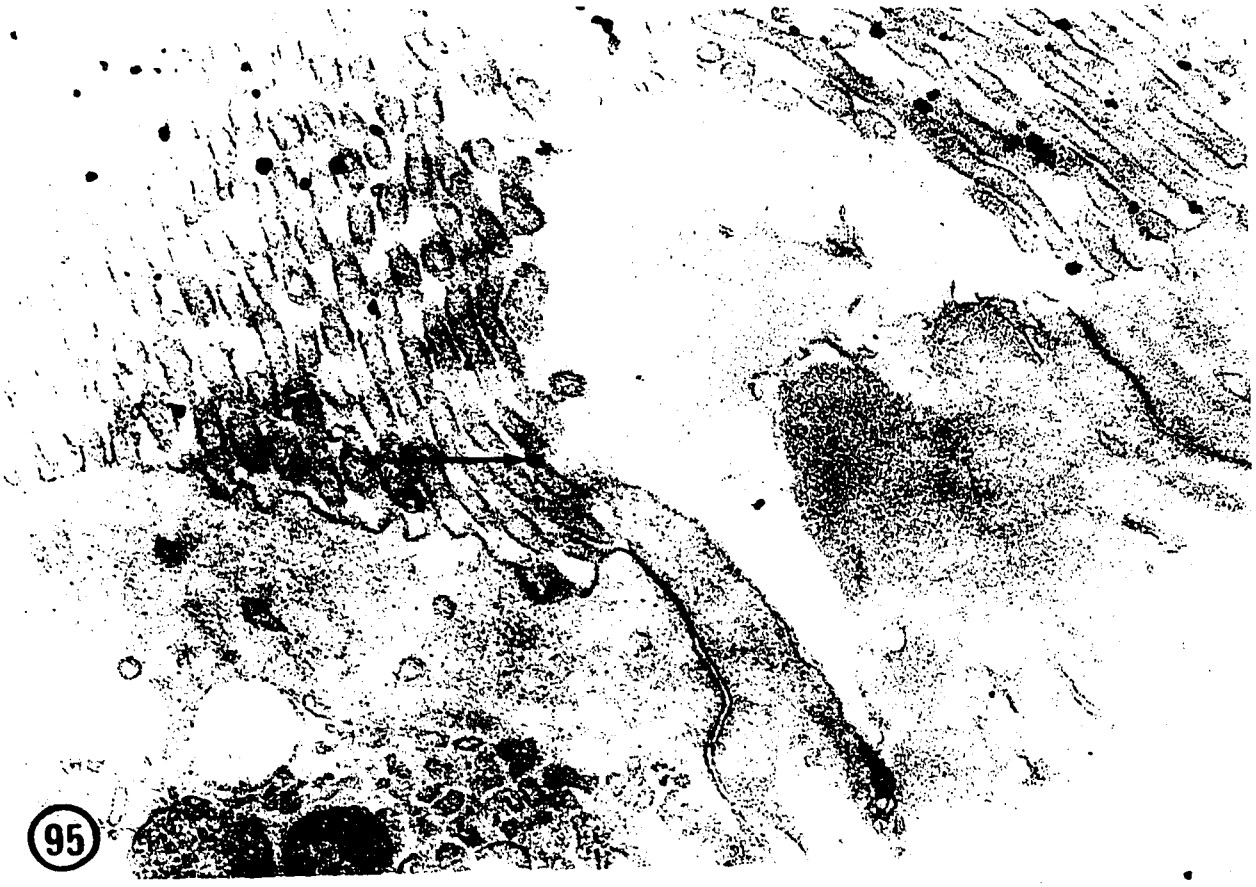

MU
RER
93


N
94

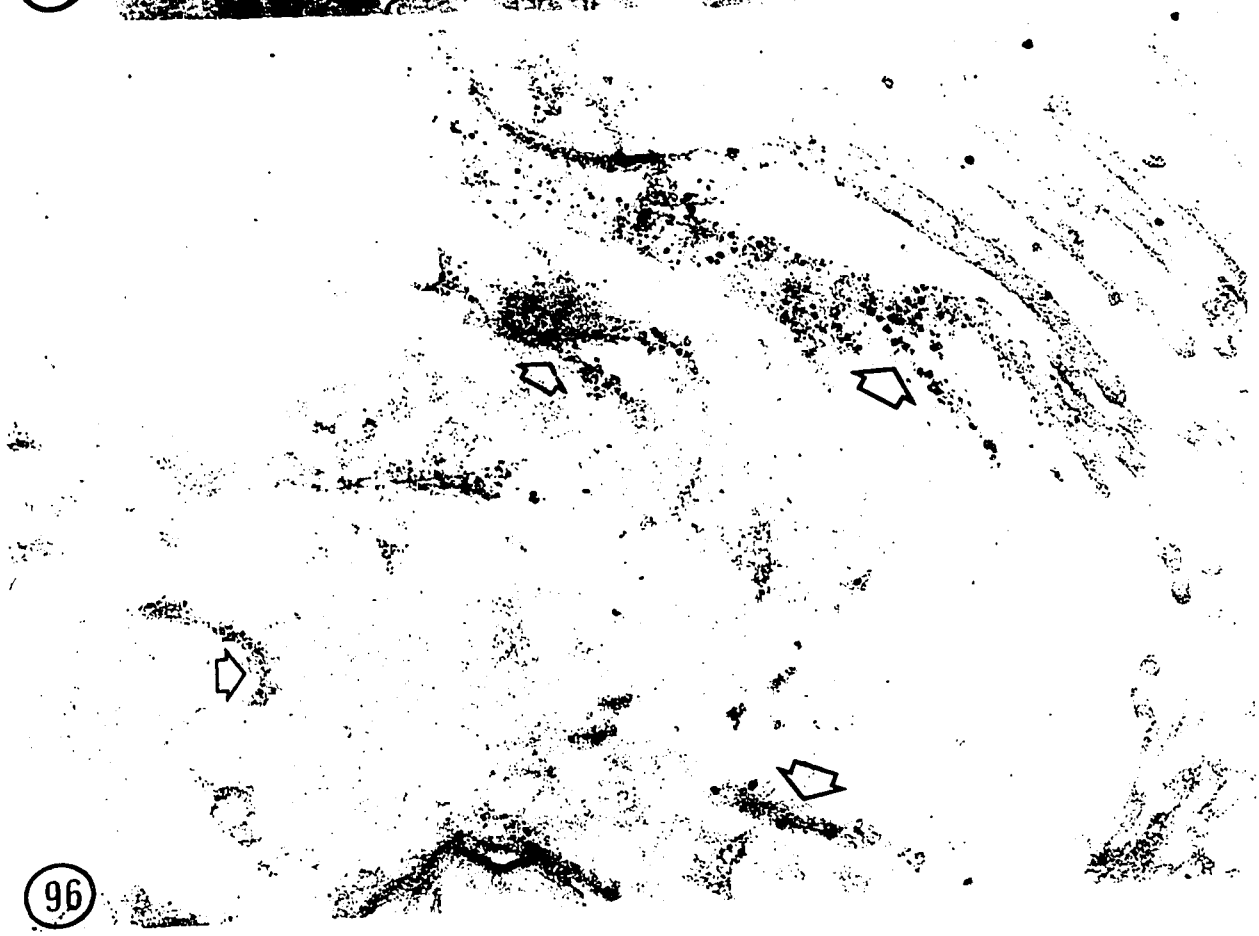
Figs. 95 to 98 illustrate goblet cells from material fixed with S-collidine buffered paraformaldehyde.

Fig. 95. Apical portion of expelling goblet cell. Rachitic chick. Electron dense granules (→) are seen adhering to the goblet cell microvilli. These are similar to those seen on the absorptive cell microvilli. Unstained preparation. X 39,900.

Fig. 96. Apical portion of expelling goblet cell. Twenty-four hour vitamin D_3-replete chick. Many small electron dense granules (⇨) are seen on the microvilli and cytoplasmic areas adjacent to the mucous, and over electron dense areas within the mucous. X 39,900.



95



96

Fig. 97. Similar to Fig. 96. Stained, uranyl acetate and lead citrate. The electron dense granules (⇨) are seen to be adhering to the cytoplasmic remnants within the mucous, but not to the small areas of mucous which show the greatest electron density (*). X 20,520.

Fig. 98. Apical portion of twenty-four hour vitamin D_3-replete goblet cell treated with EDTA. Note the absence of electron dense deposits from the microvilli, lateral cytoplasm and cytoplasmic remnants. Unstained preparation. X 31, 920.

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APPENDIX

1. All groups examined were composed of three chicks. Rachitic chicks were sacrificed at the same time intervals (three chicks at each time) as were the vitamin D_3 replete chicks. As all of the rachitic chicks appeared similar when examined electron microscopically they were treated as a single experimental group for descriptive purposes. No animals died while being raised on experimental diet or following injection.

2. From each animal at least three blocks were cut and examined electron microscopically.

3. In each case the upper third of an intestinal villus, excluding the tip of the villus, was selected for electron microscope examination.

4. Microvillar measurements were made on electron micrographs which represented a magnification of 62,700 X. At least ten microvilli were measured in five different areas from the same group, and the average of these was taken. No statistical analysis was done of these measurements.

5. The microvillar measurements showed the following dispersions:

	Microvillar Length (μ)
Normal	1.8 ± 0.12
Rachitic	1.1 ± 0.15
Vitamin D_3 Replete	
12 Hours	1.3 ± 0.20
18 Hours	1.9 ± 0.10
24 Hours	1.5 ± 0.20
48 Hours	1.7 ± 0.15
72 Hours	1.7 ± 0.13
Low Calcium Diet	1.8 ± 0.20

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