Fine Structural Alterations in Thyroid Parafollicular Cells of Cows in Response to Experimental Hypercalcemia Induced by Vitamin D

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THE THYROID GLANDS of mammals contain an independent endocrine cell population responsible for the synthesis and secretion of thyrocalcitonin (calcitonin), the recently characterized hypocalcemic hormone. Parafollicular (thyroid C) cells are derived embryologically from the ultimobranchial body which is incorporated into the thyroid parenchyma of mammals.[1] The immunofluorescent studies of Bussolati and Pearse [2] localized the production of thyrocalcitonin to the parafollicular cells of the thyroid gland. At the level of ultrastructure the cytoplasm of parafollicular cells in different animal species contains numerous membrane-bound, electron-dense secretory granules.[3-7] Investigations by Bauer and Teitelbaum [8] suggest that the hypocalcemic activity present in the thyroid gland may be associated with these granules. The active particulate fraction of hog thyroid homogenates contained membrane-limited granules resembling morphologically the secretory granules of intact parafollicular cells. The number and distribution of secretory granules within parafollicular cells has been shown to be responsive to changes in the blood concentration of calcium. Hypercalcemic perfusion of the thyroid gland resulted in a rapid degranulation of the cytoplasm.[3,4] In response to dietary hypocalcemia the cytoplasm of parafollicular cells in rats was distended with secretory granules.[9] Cytoplasmic organelles concerned with hormonal synthesis were poorly developed.

In nonmammalian vertebrates ultimobranchial tissue, which appears to be analogous to the parafollicular cell population of mammals, is present in a gland separate and distinct from the thyroid.[10,11] Thyrocalcitonin can be extracted from the ultimobranchial gland but not the thyroid gland in these species.[12] Robertson [13] observed cellular hypertrophy and hyperplasia in the ultimobranchial gland of frogs made

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hypercalcemic by the injection of Vitamin D[2] and maintenance in a 0.8% $CaCl_2$ solution. The initial response to 3 days of hypercalcemia was a depletion of secretory granules from the cytoplasm.[14] Chronic hypercalcemic stimulation of 7 days duration resulted in enlargement of the Golgi apparatus and increased development of ergastoplasm.

The pharmacologic doses of Vitamin D_2 (30 million units/day) administered to cows in this investigation have been shown previously to increase the rate and quantity of calcium absorbed from the intestinal tract [15] and to gradually elevate the blood concentration of calcium.[16] The parathyroid glands after 30 days had ultrastructural evidence of depressed parathyroid hormone synthesis, and contained inactive and atrophic chief cells.[17] The specific objective of this investigation was to determine the time course of the effects exerted by Vitamin D-induced hypercalcemia on the fine structure of parafollicular cells in the thyroid glands of cows. These ultrastructural findings were correlated with the results of thyrocalcitonin biologic assay of the thyroid glands and serum electrolyte concentrations performed on this group of cows and described in detail elsewhere.[18]

Materials and Methods

Nine adult Jersey cows ranging in age from 3 to 9 years were used in this study. Seven cows received 30 million USP units/day of Vitamin D_2* in gelatin capsules as divided doses for 3, 5, 5, 7, 10, 14, and 30 days, respectively. The cows were killed by electrocution after completion of the interval of Vitamin D administration and tissue was collected for electron microscopy. Two additional cows received the same amount of Vitamin D for 5 days followed by an interval of 48 hr prior to necropsy during which time no Vitamin D was administered. All cows were fed a commercial grain mixture and good quality alfalfa or clover hay in amounts adequate to meet daily requirements for calcium and other nutrients. Serum was collected daily prior to and during Vitamin D administration and analyzed for calcium and phosphorus by atomic absorption spectrophotometry (Perkin-Elmer 303) and by the method of Fiske and Subbarow.[19] The ultrastructure of parafollicular cells of control cows has been described in detail in previous reports.[6,20,21]

Multiple blocks of tissue from the thyroid glands of all cows were prepared for study by electron microscopy. The tissue was cut into 0.5-mm cubes immediately following killing and fixed by three different procedures: (1) direct 1% osmium tetroxide in phosphate buffer with 0.1mol calcium chloride, (2) 3% glutaraldehyde with postfixation in 1% osmium tetroxide,[22] and (3) paraformaldehyde-glutaraldehyde according to the method of Karnovsky.[23] After each of the fixation procedures the tissues were dehydrated through ascending concentrations of ethyl alcohol, transferred to propylene oxide, and embedded in Maraglas. Sections were cut at 500 Å on a Porter-Blum ultramicrotome and mounted on 200- and 400-mesh copper grids. The sections were stained with uranyl acetate and lead hydroxide, and examined with a Philips 200 electron microscope. Sections 1-μ thick of Mara-

* QUADRI D "400" (400,000 USP units of Vitamin D_2 per gm in a dry cereal carrier): Nopco Chemical Company.

glas-embedded tissue were stained with toluidine blue. The magnification listed for the electron micrographs represents the final magnification after printing.

Results

The most striking alteration in parafollicular cells of cows receiving Vitamin D for 3 days was a reduction in the number of secretory granules in the cytoplasm. The serum calcium was increased to 10.6 mg/100 ml from a baseline level of 9.1 mg/100 ml. Parafollicular cells in various stages of degranulation were present; however, many cells had only a few secretory granules and vesicles situated near the plasma membrane (Fig 1). Parafollicular cells were present in similar numbers as in control cows in an intrafollicular or epifollicular location. The cytoplasmic area was diminished and the intercellular space between adjacent cells was widened and often traversed by cytoplasmic processes. Golgi complexes were small and associated with few vesicles and electron-dense granules. Individual profiles of endoplasmic reticulum, small clusters of ribosomes, and scattered mitochondria were present throughout the cytoplasm. In other degranulated parafollicular cells there was intense aggregation of free ribosomes and long profiles of endoplasmic reticular membranes (Fig 2), suggesting a stimulation of protein synthesis.

Parafollicular cells in 2 cows receiving Vitamin D for 5 days were hypertrophied and appeared to be principally in an "actively synthesizing" stage of their secretory cycle (Fig 3). The mean serum calcium was increased to 12.2 mg/100 ml from a baseline value of 10.9 mg/100 ml. The parafollicular cells were large, irregularly polyhedral, and extended long cytoplasmic projections between adjacent follicular cells but never bordered the luminal colloid directly. An attenuated intervening rim of follicular cell cytoplasm was always interposed between the parafollicular cells and the luminal colloid. The nuclei were large, irregularly indented, and often eccentrically placed within the cell. The nuclear chromatin was aggregated into peripheral clumps and the nucleolus was prominent. Endoplasmic reticular membranes were aggregated into long straight or curved lamellar arrays and had numerous attached ribosomes. Membranes of the Golgi apparatus were extensive and intimately associated with numerous prosecretory granules of variable size and electron density. The number of secretory granules was reduced considerably compared to control cows. In some parafollicular cells free ribosomes were increased markedly in number, aggregated into large clusters, and almost filled the cytoplasm (Fig 4). Secretory granules and organelles were displaced peripherally by the extensive ergastoplasmic development. The limiting membrane of peripherally situated secretory

granules was in close proximity to or fused with the plasma membrane of the parafollicular cells (Fig 5). Scattered large mitochondria and lipofuscin granules were present within the abundant cytoplasmic area. Plasma membranes of parafollicular cells pursued an essentially straight course but had simple interdigitations with adjacent follicular cells.

After 7 and 10 days of Vitamin D administration, the majority of parafollicular cells were hypertrophied and partially granulated (Fig 6). The serum calcium was increased 1.4 and 3.5 mg/100 ml above baseline values to 12.3 and 13.5 mg/100 ml, respectively. Although ergastoplasmic development was prominent, it was consistently less extensive than after 5 days of stimulation. Golgi apparatuses were well developed and associated with many prosecretory granules. Golgi cisternae were moderately distended by a material of low electron density. Large mitochondria were interspersed throughout the cytoplasm. Secretory granules were more numerous than after 5 days but reduced compared to control cows and frequently aggregated into small groups near the periphery of the cell. The number of secretory granules in parafollicular cells was greater in cows receiving Vitamin D for 10 days than for 7 days. Other parafollicular cells were degranulated and were interpreted to be in an "inactive" stage of their secretory cycle (Fig 6). The cytoplasmic area of degranulated parafollicular cells was reduced and contained small Golgi apparatuses, individual profiles of endoplasmic reticulum, dispersed ribosomes, occasional mitochondria and secretory vesicles.

The serum calcium in the cow receiving Vitamin D for 14 days was increased 3.6 mg/100 ml above baseline levels to 13.9 mg/100 ml. Parafollicular cells were present in a similar number as in control cows and were either hypertrophic or degranulated. The hypertrophied parafollicular cells had lamellar arrays of endoplasmic reticulum, aggregated ribosomes, large mitochondria, and small aggregations of electron-dense secretory granules near the Golgi apparatus and plasma membrane. The small cytoplasmic area of the discharged parafollicular cells contained poorly developed organelles and was nearly devoid of secretory granules.

Hyperplasia of parafollicular cells in response to prolonged hypercalcemic stimulation was present in the cow receiving Vitamin D for 30 days. The serum calcium was increased to 13.3 mg/100 ml from a baseline value of 11.0 mg/100 ml. Aggregations of up to eight or ten parafollicular cells were wedged between follicular cells in an intrafollicular location and situated at the periphery of thyroid follicles in an epifollicular position. Adjacent follicular cells were often indented and compressed by the increased numbers of parafollicular cells (Fig 7). The parafollicular cells varied considerably in size and shape, and extended

long cytoplasmic projections between follicular cells. The abundant cytoplasmic area of the hypertrophied parafollicular cells was electron-transparent and contained scattered organelles. The endoplasmic reticulum consisted of straight or circular arrays of rough membranes or small individual profiles with attached ribosomes. Free ribosomes were present individually or in small clusters. Golgi apparatuses of moderate size were observed in the perinuclear region of nearly all parafollicular cells. Numerous prosecretory granules and vacuoles were concentrated in the immediate vicinity of the Golgi apparatus but were present also throughout the cytoplasm and near the plasma membrane. The majority of parafollicular cells were sparsely granulated; however, occasional cells had aggregations of electron-dense secretory granules aligned along the plasma membrane facing the basilar part of the follicle and interfollicular capillary (Fig 8). Scattered mitochondria and lipofuscin droplets were present in the cytoplasm. Other parafollicular cells were small and degranulated with poorly developed cytoplasmic organelles and were interpreted to be in an "inactive" stage of their secretory cycle.

Two cows received Vitamin D for 5 days followed by an interval of 48 hr during which no Vitamin D was administered. The mean serum calcium was increased 1.9 mg/100 ml above the baseline level to 12.8 mg/100 ml at the end of Day 5 but decreased only to 12.2 mg/100 ml after the 48-hr recovery period. Parafollicular cells were present in a similar number as control cows and many were interpreted to be in a "storage" phase of their secretory cycle. The large, irregularly shaped cytoplasmic area of the parafollicular cell contained numerous electron-dense secretory granules (Fig 9). Long cytoplasmic projections packed with secretory granules extended between adjacent follicular cells. Organellar development was not extensive and consisted of large mitochondria, individual profiles or small lamellar arrays of endoplasmic reticulum, and clusters of free ribosomes. Other parafollicular cells were hypertrophied and had extensive lamellar arrays of endoplasmic reticulum, prominent Golgi apparatuses with prosecretory granules, and clusters of secretory granules near the plasma membrane adjacent to the basilar portion of the follicle. They resembled the most frequently encountered type of parafollicular cell in the thyroid glands of cows evaluated after completion of Day 5 of Vitamin D. Ultrastructural changes were not present in follicular cells of cows fed Vitamin D.

Discussion

The parafollicular cell population in the thyroid glands of cows was responsive to an increase in the serum concentration of endogenous

calcium induced by the administration of pharmacologic doses of Vitamin D. A reduction in the number of secretory granules and aggregation of free ribosomes was the initial response observed after 3 days. The cytoplasmic area was diminished and Golgi apparatuses were small. Parallel studies on this group of cows in which the thyrocalcitonin content of the thyroid glands was determined by biologic assay add support to the ultrastructural observation that the parafollicular cells contained a reduced amount of secretory material.[18] The thyroid gland of the cow fed Vitamin D for 3 days contained only 6% as much thyrocalcitonin activity as control cows. Thyroid perfusion studies reported by others have demonstrated that parafollicular cells respond to experimental hypercalcemia by a rapid degranulation within 2–4 hr.[3,4] In addition, Robertson [14] observed secretory granule depletion in analogous cells of the amphibian ultimobranchial gland as the initial response at 3 days to hypercalcemia induced by Vitamin D.

The extensive membranous arrays of endoplasmic reticulum and large aggregations of free ribosomes in hypertrophied parafollicular cells after 5 days suggested a stimulation of protein synthesis in response to hypercalcemia. Golgi apparatuses were prominent in almost every cell and associated with many prosecretory granules. Secretory granules were not numerous and were situated peripherally in the cells. Although parafollicular cells were interpreted to be primarily in an actively synthesizing phase of their secretory cycle, little hypocalcemic activity was stored since the gland-content of thyrocalcitonin was only 4% that of control cows.[18]

The parafollicular population in cows receiving Vitamin D for 7 and 10 days consisted of hypertrophied and small degranulated cells. Secretory granules were more numerous in hypertrophied parafollicular cells than after 5 days but reduced compared to control cows. It appeared that the increased organellar development had partially fulfilled the demands of secretion and permitted some hormonal storage as secretory granules since the gland-content of thyrocalcitonin in the cow receiving Vitamin D for 10 days was 43% that of control cows.[18] The degranulated parafollicular cells appeared to have discharged their secretory products and entered an "inactive" or "resting" phase of their secretory cycle. The cytoplasmic area was diminished and organelles concerned with protein synthesis and packaging of secretory products were inconspicuous.

Hyperplasia of parafollicular cells was observed after chronic hypercalcemic stimulation of 30 days duration. Large groups of hypertrophied and degranulated parafollicular cells were wedged between follicular cells. In control cows parafollicular cells were present individually be-

tween follicular cells or in small groups of two or three adjoining cells at the periphery of thyroid follicles.[6,20,21] The majority of parafollicular cells after long-term hypercalcemic stimulation were sparsely granulated and the thyroid gland contained only 10% as much thyrocalcitonin activity as control cows.[18] These findings suggested that the persistent hypercalcemia did not permit significant hormonal storage in the thyroid gland as secretory granules despite the presence of increased numbers of hypertrophied parafollicular cells.

The hypertrophy and hyperplasia of parafollicular cells and release of stored thyrocalcitonin were unable to overcome the hypercalcemic effects of Vitamin D and return the serum calcium level to the normal range. Although thyrocalcitonin has been reported to be active in animals with hypervitaminosis D,[24,25] the serum calcium of cows in the present investigation remained elevated during long-term Vitamin D administration reaching a maximum of 14.6 mg/100 ml on Day 23 in the cow receiving Vitamin D for 30 days.

The initial discharge of secretory granules, subsequent hypertrophy and hyperplasia of parafollicular cells, and reduction of gland-content of thyrocalcitonin in cows with Vitamin D-induced hypercalcemia with an absence of changes in follicular cells support the present concepts that: (1) parafollicular cells are responsible for the secretion of thyrocalcitonin, and (2) thyrocalcitonin is stored within secretory granules.[4,7-9,26] The reduction in number of secretory granules and stimulation of organellar development in parafollicular cells was interpreted as a response to the hypercalcemia induced by Vitamin D. Although a direct effect exerted by Vitamin D on parafollicular cells cannot be excluded, an elevation of the blood concentration of calcium has been reported as the major physiologic stimulus for thyrocalcitonin release.[27,28] Gittes, Toverud, and Cooper [29] did not observe an additive effect on the stimulation of thyrocalcitonin secretion when high doses of Vitamin D were added to high calcium diets fed to rats for 11 days. In addition, other investigations have shown that Vitamin D does not directly affect the secretory activity of the parathyroid gland but rather exerts an effect by virtue of its ability to influence the serum concentration of calcium.[30,31]

Since the most intensive stimulation of organellar development in parafollicular cells was observed after 5 days, 2 cows received Vitamin D for 5 days followed by a recovery period of 48 hr during which no Vitamin D was administered. The serum calcium decreased 0.6 mg/100 ml after the 2 day interval and many parafollicular cells were interpreted to be in a "storage" phase of their secretory cycle. The cytoplasm was

packed with secretory granules, but organelles concerned with protein synthesis and packaging of secretory products were poorly developed by comparison to cows evaluated after completion of Day 5 of Vitamin D. The gland content of thyrocalcitonin was greatly increased compared to cows after 5 days of Vitamin D administration and had increased (127%) above control cows.[18] Gittes, Toverud, and Cooper [29] reported the thyroid content of thyrocalcitonin in rats made hypercalcemic by the intraperitoneal injection of $CaCl_2$ was decreased significantly after 2 hr. However, when rats were allowed to recover for 16 hr the gland content had returned to slightly above the level in control rats.

Summary

Hypercalcemia was induced in cows by the daily oral administration of pharmacologic doses of Vitamin D_2 (30 million units/day) for intervals from 3 to 30 days. Parafollicular cells in the thyroid glands, which are responsible for the synthesis and secretion of thyrocalcitonin, responded to the hypercalcemia by a reduction in the number of secretory granules and aggregation of ribosomes after 3 days. Parafollicular cells were hypertrophied after 5 days. The abundant cytoplasmic area had extensive lamellar arrays of endoplasmic reticulum, aggregations of free ribosomes, and prominent Golgi apparatuses associated with prosecretory granules. Hyperplasia of parafollicular cells was present after chronic hypercalcemic stimulation of 30 days duration. The thyrocalcitonin content of the thyroid glands measured by biologic assay was consistently decreased following hypercalcemic stimulation compared to control cows. Parafollicular cells in cows fed Vitamin D for 5 days followed by a recovery period of 48 hr accumulated secretory granules and the gland content of thyrocalcitonin was increased slightly above control cows. The hypertrophy and hyperplasia of parafollicular cells and release of thyrocalcitonin from the thyroid gland appeared to be unable to overcome the hypercalcemic effects of Vitamin D and maintain a normal serum concentration of calcium.

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Legends for Figures

Fig 1. Two degranulated parafollicular cells within thyroid follicular wall of a cow receiving Vitamin D for 3 days. Small cytoplasmic area contains a few peripherally situated secretory granules *(S)*, individual profiles of endoplasmic reticulum *(E)*, small clusters of ribosomes, and scattered mitochondria. Intercellular space is widened *(arrow)*. Paraformaldehyde-glutaraldehyde. × 20,750.

Fig 2. Degranulated parafollicular cell interposed between follicular cells within wall of thyroid follicle in a cow receiving Vitamin D for 3 days. There is intense aggregation of free ribosomes and long profiles of endoplasmic reticular membranes *(arrow)* in cytoplasm. Intervening rim of follicular cell-cytoplasm with prominent terminal bars *(T)* is interposed between the parafollicular cell and luminal colloid *(C)*. *N*, nuclei of follicular cells. Osmium tetroxide. × 12,700.

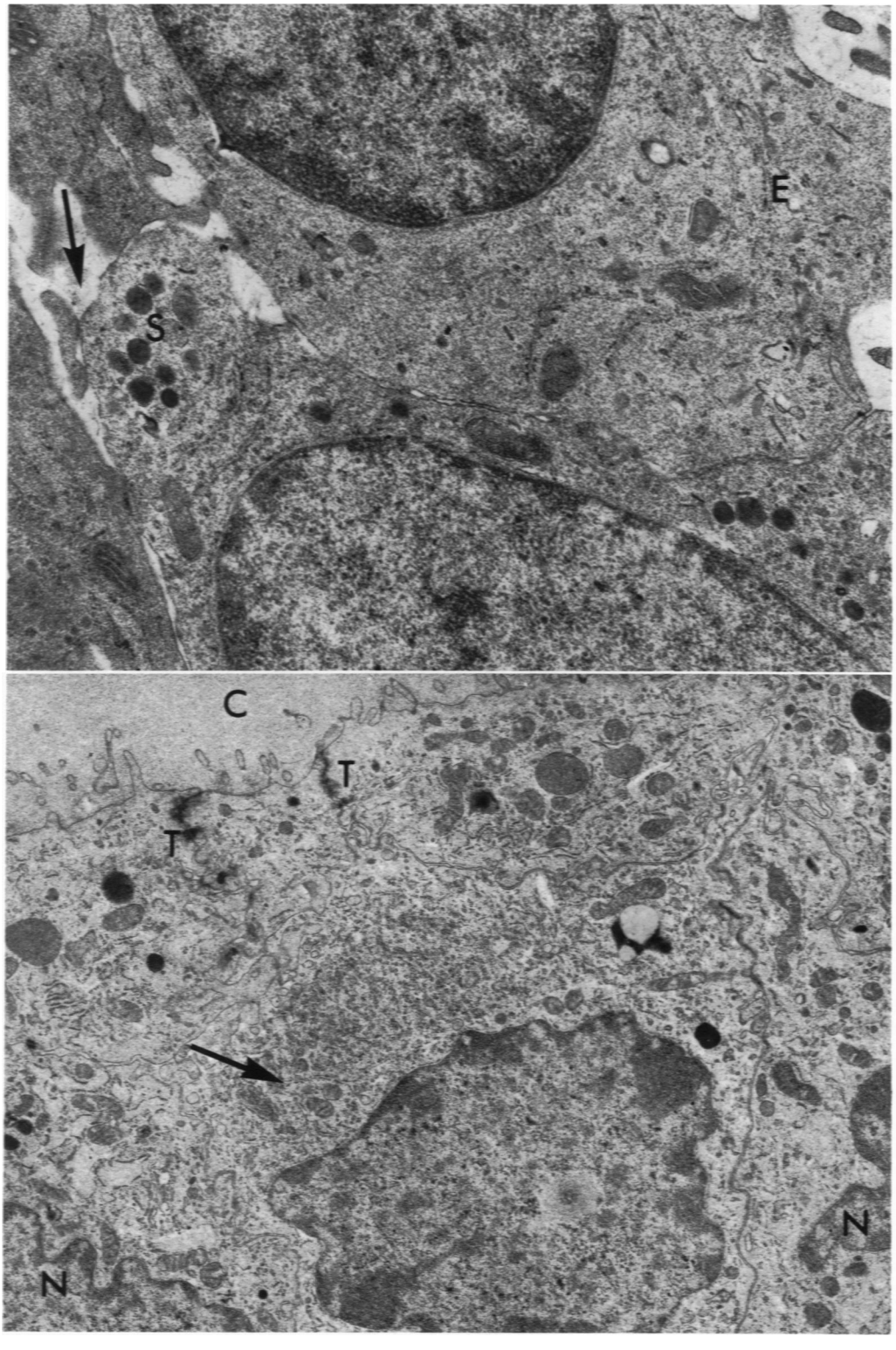

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Fig 3. Hypertrophied parafollicular cell extending from basement membrane (*B*) of follicle to near the luminal colloid (*C*) in a cow administered Vitamin D for 5 days. Endoplasmic reticular membranes (*E*) are aggregated into large lamellar arrays and ribosomes (*R*) are present in clusters. Golgi apparatus (*G*) is prominent and associated with prosecretory granules (*arrow*). Secretory granules (*S*) are present near Golgi apparatus and at cell periphery. Paraformaldehyde-glutaraldehyde. × 11,200.

Fig 4. Hypertrophied parafollicular cell with extensive ribosomal development in cow receiving Vitamin D for 5 days. Secretory granules (*S*), mitochondria, and profiles of endoplasmic reticulum (*E*) appear to be displaced peripherally by numerous clusters of ribosomes. *N*, nucleus of parafollicular cell. Paraformaldehyde-glutaraldehyde. × 18,250.

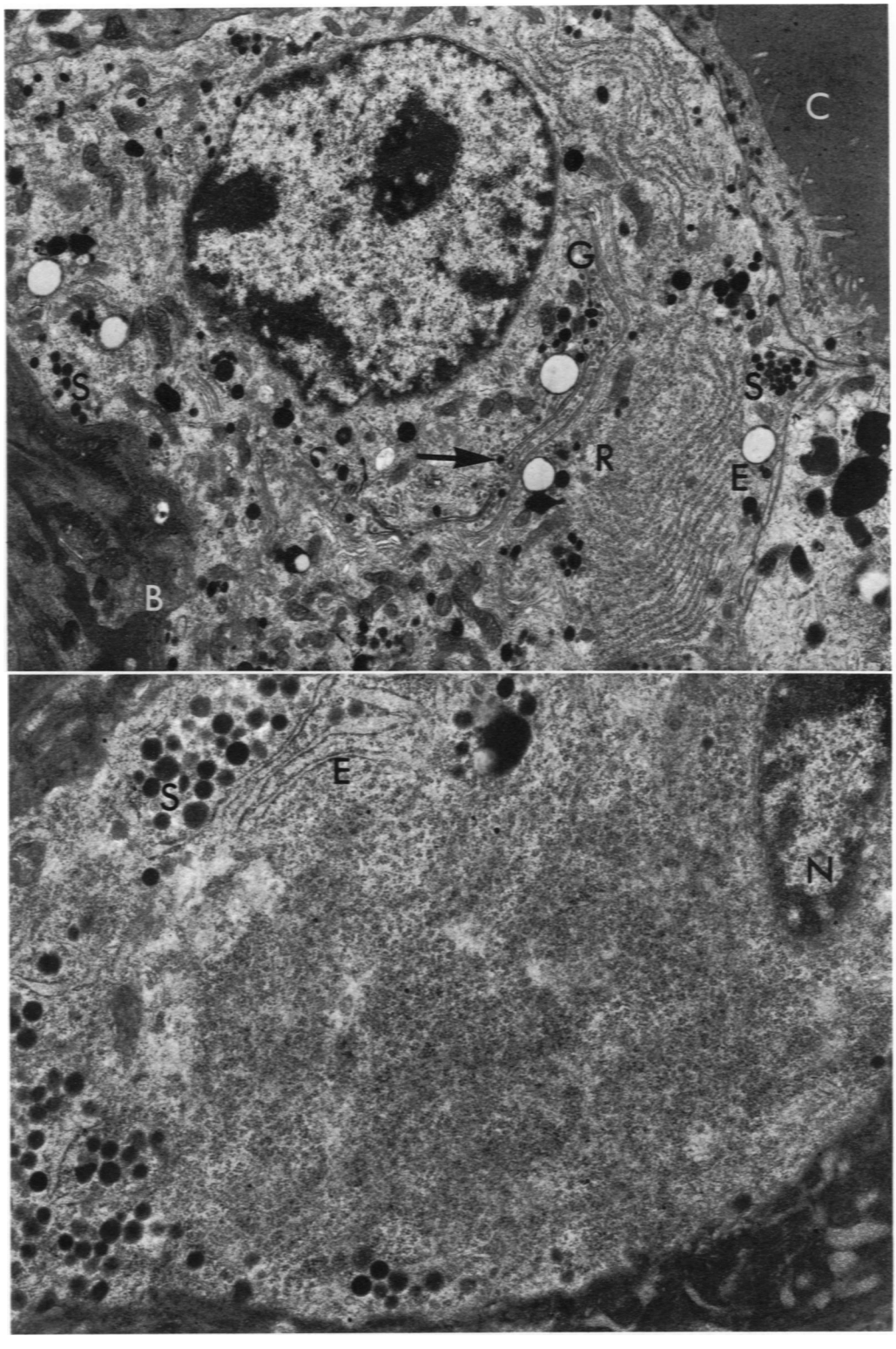

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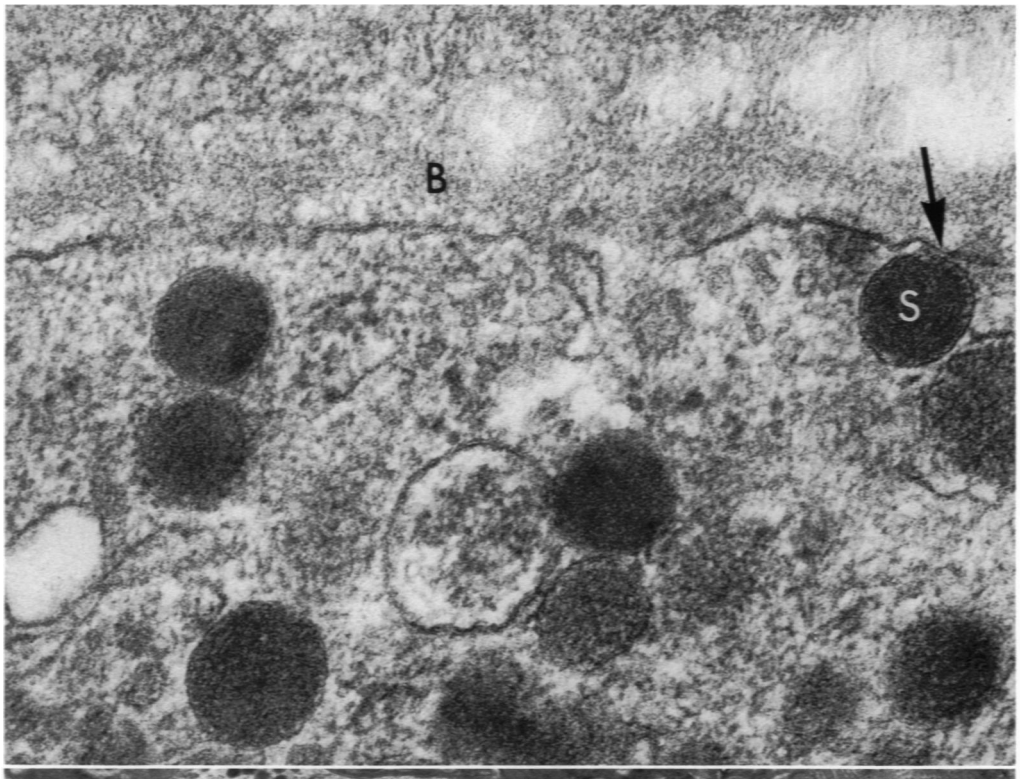
Fig 5. Secretory granules (*S*) near plasma membrane of parafollicular cell from cow receiving Vitamin D for 5 days. Limiting membrane of secretory granule is in close proximity to plasma membrane (*arrow*). *B*, basement membrane of follicle. Paraformaldehyde-glutaraldehyde. × 97,600.

Fig 6. Partially granulated (*left*) and degranulated (*right*) parafollicular cells in cow receiving Vitamin D for 7 days. Golgi apparatuses (*G*) with prosecretory granules, mitochondria, and aggregations of secretory granules (*S*) are prominent in partially granulated parafollicular cell. Degranulated parafollicular cell is devoid of secretory granules and contains individual profiles of endoplasmic reticulum (*E*), mitochondria, and small Golgi apparatus. Paraformaldehyde-glutaraldehyde. × 13,100.



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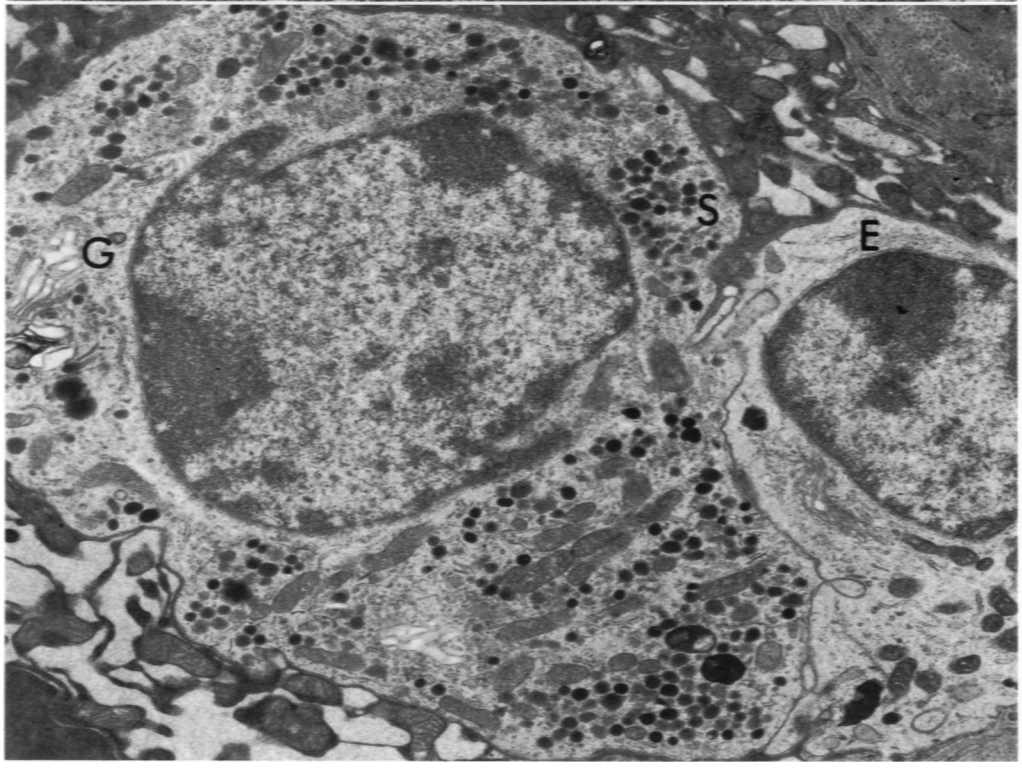

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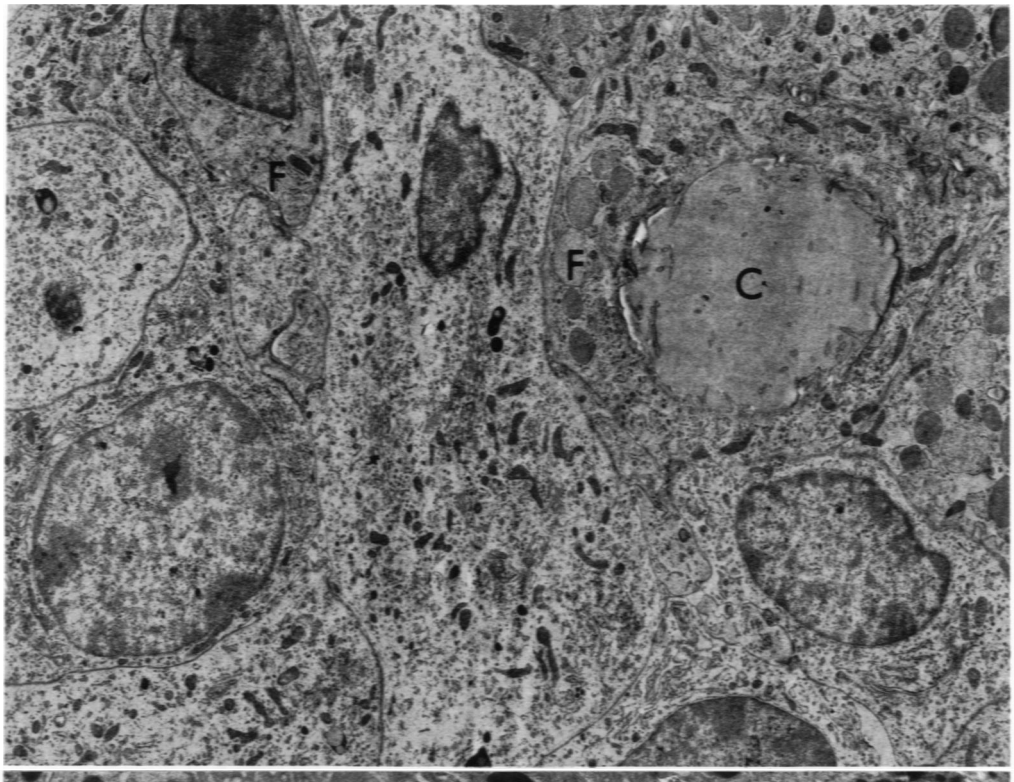
Fig 7. Hyperplasia of parafollicular cells in response to prolonged hypercalcemic stimulation in a cow administered Vitamin D for 30 days. Portions of seven irregularly shaped parafollicular cells are wedged between follicular cells (*F*) surrounding luminal colloid (*C*). Osmium tetroxide. × 5600.

Fig 8. Parafollicular cell containing secretory granules (*S*) aggregated along plasma membrane facing basement membrane (*B*) of thyroid follicle. Arrays of endoplasmic reticulum (*E*) and membranes of Golgi apparatus are present in opposite pole of cell. Microvilli of follicular cells (*arrow*) extend into luminal colloid. Cow administered Vitamin D for 30 days. Glutaraldehyde. × 11,000.



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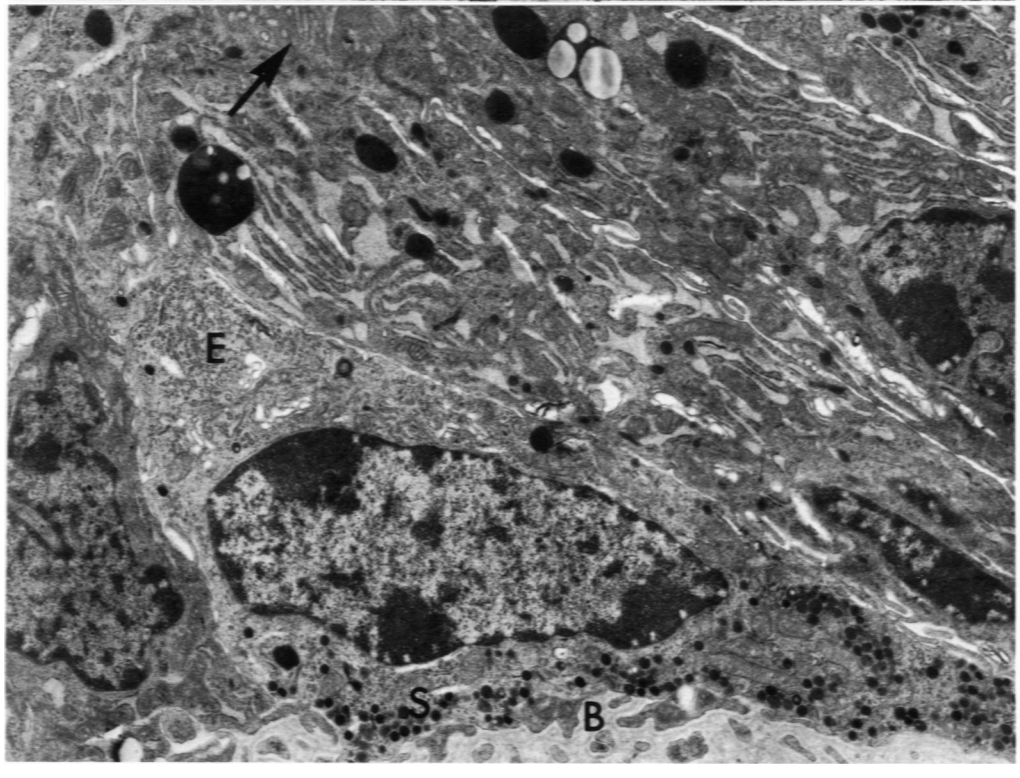

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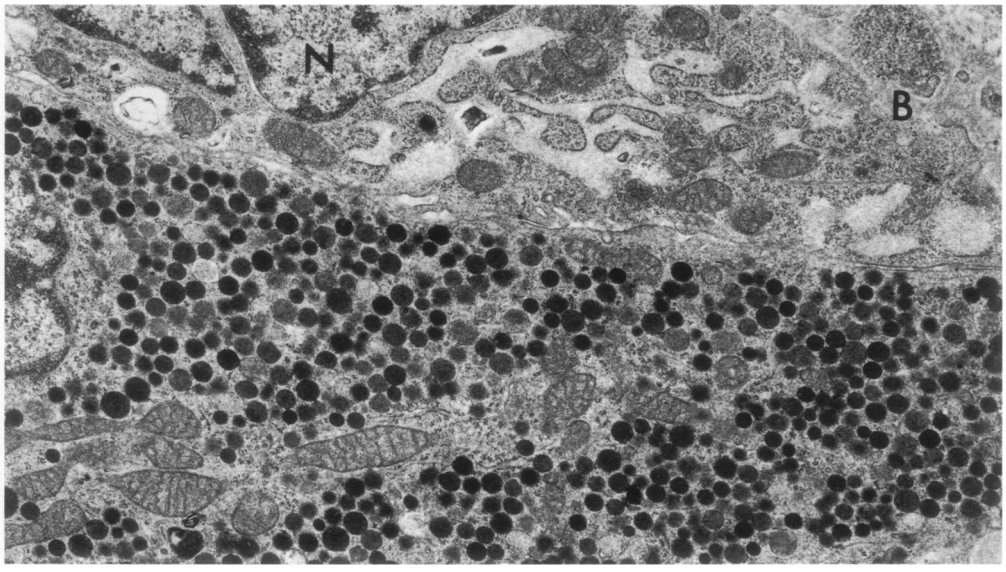

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Fig 9. Densely granulated parafollicular cell (*bottom*) from a cow receiving Vitamin D for 5 days followed by recovery period of 48 hr. Cytoplasm is packed with secretory granules but organelles other than large mitochondria are sparse. *N*, nucleus of follicular cell; *B*, basement membrane of thyroid follicle. Glutaraldehyde. × 14,400.