

OSTEOCLAST CELL-SURFACE CHANGES DURING THE EGG-LAYING CYCLE IN JAPANESE QUAIL

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ABSTRACT

The medullary bone serves as a source of labile calcium mobilized during calcification of the egg shell in birds. Quantitative histological methods demonstrate that the numbers of medullary bone osteoclasts and nuclei per osteoclast remain unchanged during the egg cycle in the Japanese quail (*Coturnix*). Therefore, cyclic changes in bone resorption cannot be explained by modulations of osteoclasts from and into other bone cells, a mechanism previously suggested for certain species of birds. Rather, dramatic changes in osteoclast cell-surface features occur during the egg cycle, which might account for cyclic variations in resorptive activity. During egg shell calcification, osteoclasts with ruffled borders are closely apposed to bone surfaces; the cytoplasm is rich in vacuoles that contain mineral crystals and seem to derive from the ruffled border. At the completion of egg shell calcification, the ruffled borders and vacuoles move away from the bone surface, although the osteoclast remains attached to the bone along the filamentous or "clear" zone. Associated with the disappearance of the ruffled borders is the appearance of extensive interdigitated cell processes along the peripheral surface of the osteoclast away from the bone. These unusual structures, which may serve as a reservoir of membrane, largely disappear when ruffled borders and associated structures reappear. Therefore, in these hens, the osteoclasts modulate their cell surface rather than their population during the egg cycle.

KEY WORDS bone osteoclasts · quail · cell surface · bone resorption

The differentiation of bone cells involved in the periodic resorption of calcium from medullary bone for egg shell calcification in birds has been of interest for many years. Bloom et al. noted that during the egg cycle of pigeons (3) as well as chickens (4), egg shell calcification was accompanied by resorptive activity characterized by numerous osteoclasts lining medullary bone surfaces. Resorption of medullary bone, which fills the marrow cavities of much of the skeleton of egg-laying

birds (22), contributes as much as 40% of the calcium necessary for egg shell formation (11, 32). At the completion of egg shell calcification, Bloom et al. suggested (2, 3) that the multinucleated osteoclasts divide into mononucleated osteoblasts which would rebuild the medullary bone deposits that were previously resorbed. The idea has grown that osteoclasts and osteoblasts are different functional states of the same cell, the so-called osteoprogenitor cell (39), which modulates from one cell type to another, depending upon the current calcium requirements of the bird. This monophyletic theory of bone cells is not supported

by some evidence obtained in other classes of vertebrates, which suggests that osteoclasts mainly arise from hemopoietic cells (37), perhaps monocytes (14, 19).

Attempts have been made to evaluate the relative population changes of osteoclasts during the egg cycle, in the hope of more clearly defining the differentiation and function of this cell in medullary bone resorption. In a later study, Bloom et al. (4) reported that the osteoclast changes in the daily egg cycle of chickens were less distinct than those previously suggested (2), and different from those reported for pigeons (3). Taylor and Belanger (36) also noted the presence of osteoclasts during all phases of the egg-laying cycle in chickens, but drew no conclusions concerning differences in cell populations because of the great variability encountered among individual birds.

We chose the Japanese quail (*Coturnix coturnix japonica*) for a reinvestigation of the origin of osteoclasts in the egg-laying cycle because of the unusual nuclear marker in quail cells which can be used in chick-quail chimeras to trace cell lineage (23). The Japanese quail has a 24-h egg cycle similar to that of the chicken, and it can more easily be bred in and adapt to laboratory conditions where the nutritional status, sexual maturity, and environment can be carefully monitored. We had planned to use transplant chimeras to study the problem of osteoclast lineage. However, it soon became evident that medullary bone osteoclasts in the Japanese quail do not rapidly modulate from and into other bone cells, because their number does not change. Rather, medullary bone osteoclasts undergo profound cell surface changes that are synchronized with different phases of the egg cycle. In this paper, evidence is presented which indicates that medullary bone osteoclasts activate and inactivate depending upon the demand for calcium imposed by egg shell formation.

MATERIALS AND METHODS

Egg-laying Japanese quail (*C. coturnix japonica*, Pharaoh D-1 strain) were obtained from a colony maintained in our laboratory (original stock from Marsh Farms, Garden Grove, Calif.). The breeding and care of these birds was carried out according to established guidelines (17, 35). 4-6 mo-old hens were kept in a light-controlled room (lights on from 7 A.M. to 10 P.M.) and allowed free access to food (Wild Game Bird Starter Mash, Ralston Purina Co., St. Louis, Mo.) and water. Under these conditions, the hens laid eggs from 3 P.M. to 5 P.M. For at least 3 wk before sacrifice, an egg-laying record was kept on all hens to insure consistency. If any irregularities were noted, the hens were not used.

Hens were used at all intervals during the egg cycle, and staged according to the position of the ova in the oviduct, the time since the previous egg was laid (oviposition), and the state of calcification of the egg shell (38).

Microscopy

The distal half of a femur from each bird was trimmed to expose the marrow cavity, and fixed by immersion for 48 h in phosphate-buffered 10% formalin (pH 7.3), decalcified in neutral 10% sodium EDTA, and embedded in paraffin. The 5- μ m paraffin sections were mounted on glass slides and stained with hematoxylin and eosin.

The other distal half of a femur was similarly trimmed to expose the marrow cavity. The medullary bone was gently scraped out and minced into small blocks. Some of this tissue was fixed by immersion in phosphate-buffered 10% formalin for 30 min (10), and then postfixed in phosphate-buffered 1% osmium tetroxide (31). Some of the medullary bone fragments were fixed in osmium tetroxide without prior aldehyde fixation. Other bone fragments were fixed in formalin for 48 h, decalcified in neutral EDTA, and postfixed in phosphate-buffered 1% osmium tetroxide. The tissues were dehydrated through ethanol and embedded in Epon 812, using propylene oxide as an intermediate. All of the dehydration and embedding steps were prolonged to insure proper dehydration and penetration of the embedding medium into the mineralized tissue. Semithin sections were cut and mounted on glass slides and stained with toluidine blue. Thin sections were cut with glass or diamond knives and stained with uranyl acetate followed by lead citrate, and examined on an RCA-G or JEOL 100B electron microscope.

Osteoclast Population Analysis

Osteoclast population parameters were determined in selected medullary bone regions as follows: the total number of osteoclasts and the number of nuclei per osteoclast profile were counted in 32 220- \times 220- μ m fields (total area = 7.04 mm²) in the frontal paraffin sections of the distal femur. These fields were spaced in 880- μ m intervals (Fig. 1). Osteoclasts were identified in these sections as large multinucleated cells near or adjacent to bone surfaces, and having eosinophilic cytoplasm. The osteoclast population was further characterized by relating it to the length of bone surface perimeter (29). Bone surface perimeter was measured in the same areas used for the cell counts, and was accomplished by counting the intersections of the bone surfaces with horizontal grid lines, using an eyepiece ocular grid which was calibrated with a stage micrometer (American Optical Corp., Scientific Instrument Div., Buffalo, N. Y.). The number of bone surface-grid line intersections was determined along the length (220 μ m) of six horizontal grid lines which were spaced at 44- μ m intervals (Fig. 1, inset) in each of the 32 220- \times 220- μ m fields. The length of the bone surface perimeter was estimated by applying

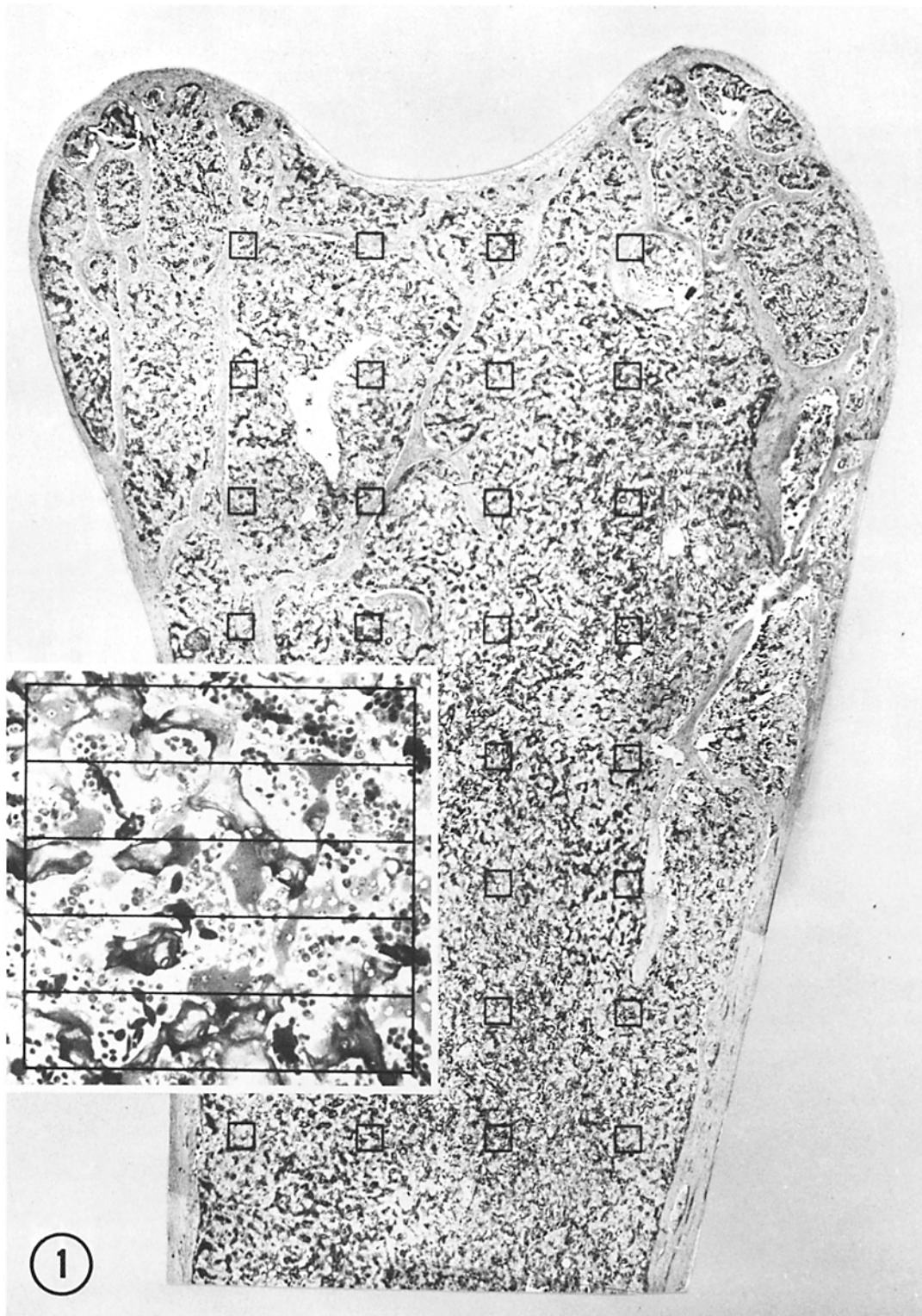


FIGURE 1 Low power micrograph of a frontal section of a distal half of a femur of an egg-laying Japanese quail to illustrate the location of the sampling areas used for determination of quantitative medullary bone osteoclast population parameters. A representative sampling area, overlaid with a grid used for quantitative parameters, is illustrated in the inset. $\times 20$. Inset $\times 300$.

the formula: $Perimeter = I \times D \times \pi/2$, where I is the number of bone surface-grid intersections, D is the distance between the horizontal grid lines (in this case 44 μm), and $\pi/2$ is a shape correction factor (16).

The quantitative osteoclast population parameters were determined in three nonserial frontal sections, at least 100 μm apart, from a femur of each bird. Data were obtained from at least five birds at every staged interval of the egg-laying cycle. Electron microscopy was done on bone samples from all of these birds.

The data are expressed as the mean \pm the standard deviation (\pm SD). The differences in the data obtained at the different intervals of the egg cycle were tested for significance by analysis of variance (1).

RESULTS

Description of the Egg Cycle and Osteoclast Population

In the strain of Japanese quail used in the present study, the hens lay an egg daily for 4–8 days, then stop for several days before resuming. Ovulation usually occurs within 30 min after oviposition (Fig. 2). During the next 4–7 h, the ovum moves through the infundibulum, magnum, and isthmus of the oviduct into the uterus or shell gland. Once in the shell gland, shell calcification progresses slowly for the first 4–6 h, then more rapidly for the next 8–10 h. It is during this 12–15-h period of shell calcification, termed 'active phase' in this paper, that calcium is resorbed from medullary bone and transported to the shell gland. After complete calcification of the shell, usually 2–4 h before oviposition, the coloring pigment is deposited on the shell by the shell gland. The timing and movement of the ovum through the oviduct and deposition of the egg shell in the Japanese quail (38) are illustrated in Fig. 2.

During the egg cycle no significant changes occurred in the total numbers of osteoclasts, nuclei

per osteoclast profile, or osteoclasts per available bone surface perimeter (Table I).

Light Microscope Observations

Considerable variation in the morphology of medullary bone osteoclasts can be seen during the egg cycle, even with the light microscope. During the period of shell calcification, ~8–19 h after oviposition (Fig. 2), the osteoclasts (arrows, Fig. 3) are closely applied to bone surfaces. Their cytoplasm extends along the surface of the bone, often surrounding entire bone spicules. The cytoplasm has a coarse, foamy appearance due to numerous vacuoles. Large vacuoles are especially evident in the region of the cell adjacent to the bone. The nuclei of the osteoclasts during this

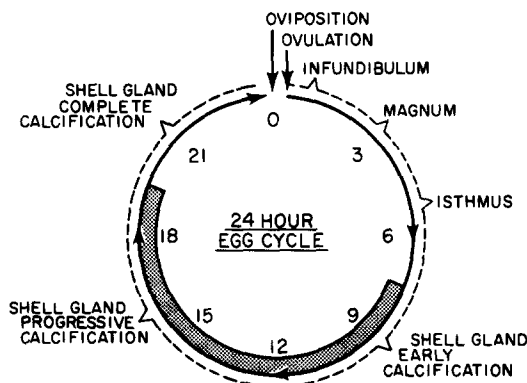


FIGURE 2 The location of the ovum and the state of calcification of the shell during the 24-h egg cycle of the strain of Japanese quail used in this study. The period of egg shell calcification and medullary bone resorption, ~8–20 h after oviposition, is termed active phase in this paper, and is indicated by the shaded region. Egg shell calcification and bone resorption do not occur during the remainder of the egg cycle, termed inactive phase in this paper.

TABLE I
Medullary Bone Osteoclast Population Parameters during the Egg-Laying Cycle of Japanese Quail

Location and calcification of egg	Approximate hours since previous oviposition	Total osteoclasts	Nuclei per osteoclast profile	Osteoclasts/mm bone surface
		no./7.04 mm ² \pm SD	no. \pm SD	
Infundibulum magnum, or isthmus	0–6	267 \pm 30	4.0 \pm 0.4	4.5 \pm 1.0
Shell gland early calcification	6–11	249 \pm 27	4.1 \pm 0.4	4.1 \pm 0.7
progressive calcification	11–20	238 \pm 19	3.6 \pm 0.4	4.6 \pm 0.7
complete calcification, colored egg	20–24	253 \pm 29	3.8 \pm 0.6	4.3 \pm 0.3

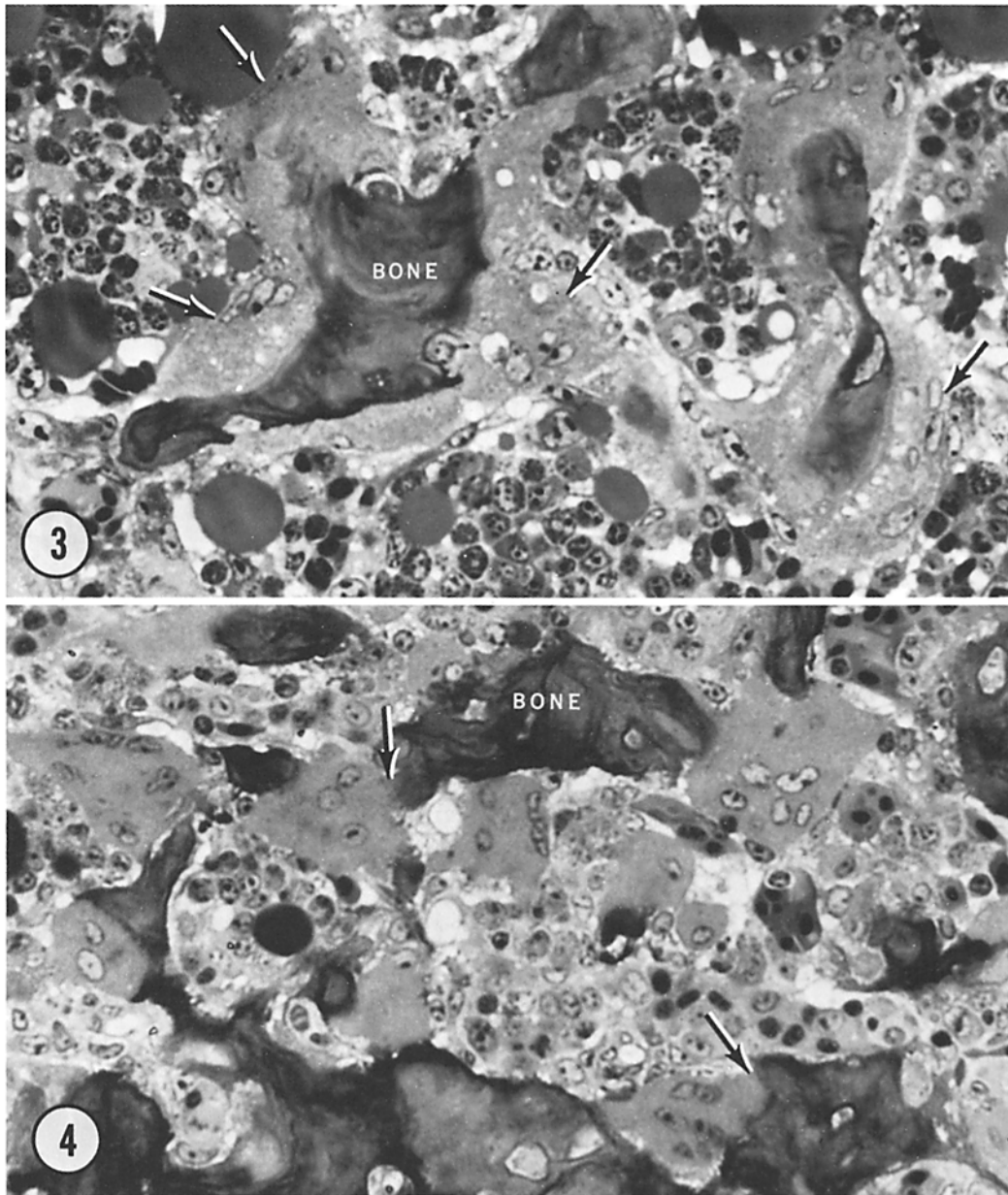


FIGURE 3 Light micrograph of an Epon section of decalcified medullary bone taken from a bird during the active phase of the egg cycle, 15 h after oviposition. Numerous osteoclasts (arrows) with vacuoles in their cytoplasm surround bone spicules. Toluidine blue stain. $\times 800$.

FIGURE 4 A section of decalcified medullary bone taken from a bird during the inactive phase of the egg cycle, 4 h after oviposition. There are numerous rounded osteoclasts with homogeneous cytoplasm which make small areas of contact (arrows) with bone. Toluidine blue stain. $\times 800$.

active phase are generally peripherally located, opposite the bone surface, and have the large heterochromatic masses associated with nucleoli characteristic of interphase quail nuclei (23).

During the period when an egg shell is not being calcified, at 0–8 and 20–24 h after oviposition (Fig. 2), the appearance of the medullary bone osteoclasts is quite different (Fig. 4). The osteo-

clasts, although just as numerous with a comparable number of nuclei (Table I), are not as extended along the bone surfaces, and each osteoclast appears to be slightly smaller (Fig. 4) than its counterpart in the resorbing bone (Fig. 3). During this inactive phase of the egg cycle, the osteoclasts are generally rounded in shape, but they maintain small areas of close contact (arrows, Fig. 4) with bone surfaces. The cytoplasm has a compact homogeneous appearance, and large vacuoles are absent.

Electron Microscope Observations

Numerous mitochondria dispersed throughout the cytoplasm of osteoclasts are visible by electron microscopy during the active phase (Figs. 5, 6) and inactive phase (Figs. 7, 8) of the egg cycle. Rough endoplasmic reticulum is most conspicuous in the peripheral regions of the cell away from the bone surface, whereas the Golgi profiles are generally found near the nuclei. Polyribosomes are dispersed throughout the cytoplasm. As in osteoclasts in other species, the multiple centrioles are clustered together in a centrosphere (28).

Numerous dense bodies are present in the cytoplasm of medullary bone osteoclasts during all phases of the egg cycle. These dense bodies are membrane-bounded and composed of heterogeneous electron-dense material. During the phase of active medullary bone resorption in osteoclasts (Fig. 2), the larger of these dense bodies are more peripherally located (arrows, Fig. 5). During the inactive phase in osteoclasts, these dense bodies are generally smaller and distributed throughout the cytoplasm (Figs. 7, 8).

During the period of shell calcification, ~8–19 h after oviposition (Fig. 2), the majority of osteoclast profiles have well-developed, ruffled-border regions adjacent to bone surfaces (*RB*, Fig. 5). Over 300 separate, complete osteoclast profiles were examined in this phase of the egg cycle. Ruffled borders (25, 33) are characterized by numerous folds and finger-like cell processes bordering channels that extend into the cytoplasm, and seem to connect with or give rise to vacuoles of various sizes near the ruffled border (*VAC*, Figs. 5, 6). The larger of these vacuoles are visible in sections viewed in the light microscope (Fig. 3). The extracellular spaces between the cell processes comprising the ruffled border may end in dilations termed channel expansions (24). The channel expansions often seem to connect with vacuoles deep in the cytoplasm. Small dissociated

mineral crystals are found between the membrane folds and within the channel expansions of the ruffled border (Fig. 6). Similar appearing material can also be found in the vacuoles deeper in the cytoplasm (*VAC*, Fig. 6). Collagen fibers (arrows, Fig. 6) are found entangled in the processes of the ruffled border. These are fibers from which the bone mineral has been previously removed (7), or, less likely, fibers which were never mineralized (5).

As in osteoclasts observed in other species, the ruffled border present on the osteoclasts during the active phase of the egg cycle is enclosed by an area devoid of organelles in close contact with the bone, which is called an ectoplasmic zone (6), modified cytoplasmic zone (27), or clear zone (34, [*CZ*, Fig. 5]). The clear zone is actually rich in cytoplasmic filaments, some of which have been identified as actin (21). The bone directly adjacent to the ruffled border of the osteoclast appears loose, disorganized, and of lower electron density than the remainder of the mineralized tissue, whereas the bone adjacent to the clear zone is very dense (Fig. 5). The peripheral surface of the osteoclast away from the ruffled border region during the active phase of the egg cycle is generally smooth, and contains a few narrow cell processes (Fig. 5).

There are profound changes in the morphology of the cell surface of the osteoclast during the period when an egg shell is not being calcified. During this inactive phase of the egg cycle, ~0–8 and 20–24 h after oviposition (Fig. 2), osteoclasts lack the ruffled borders and associated vacuoles (Fig. 7) characteristic of osteoclasts during the active phase of the egg cycle (Figs. 5, 6). Therefore, the osteoclast cytoplasm appears homogeneous as observed in the light microscope at this time (Fig. 4). Several hundred osteoclast profiles were examined with the electron microscope during the interval from oviposition to 8 h after oviposition, during which time an egg shell was not being calcified. Ruffled borders were not observed on any of these profiles. In addition, many more osteoclasts were examined during this period with the light microscope, and cells with a foamy appearance and large vacuoles, like those illustrated in Fig. 3, were rarely, if ever, seen.

Despite the disappearance of ruffled borders on the osteoclast during the period from ~2 h before oviposition to ~9 h after oviposition (Fig. 2), small clear zones abutting bone surfaces remained on the osteoclasts (*CZ*, Figs. 7, 8). These clear

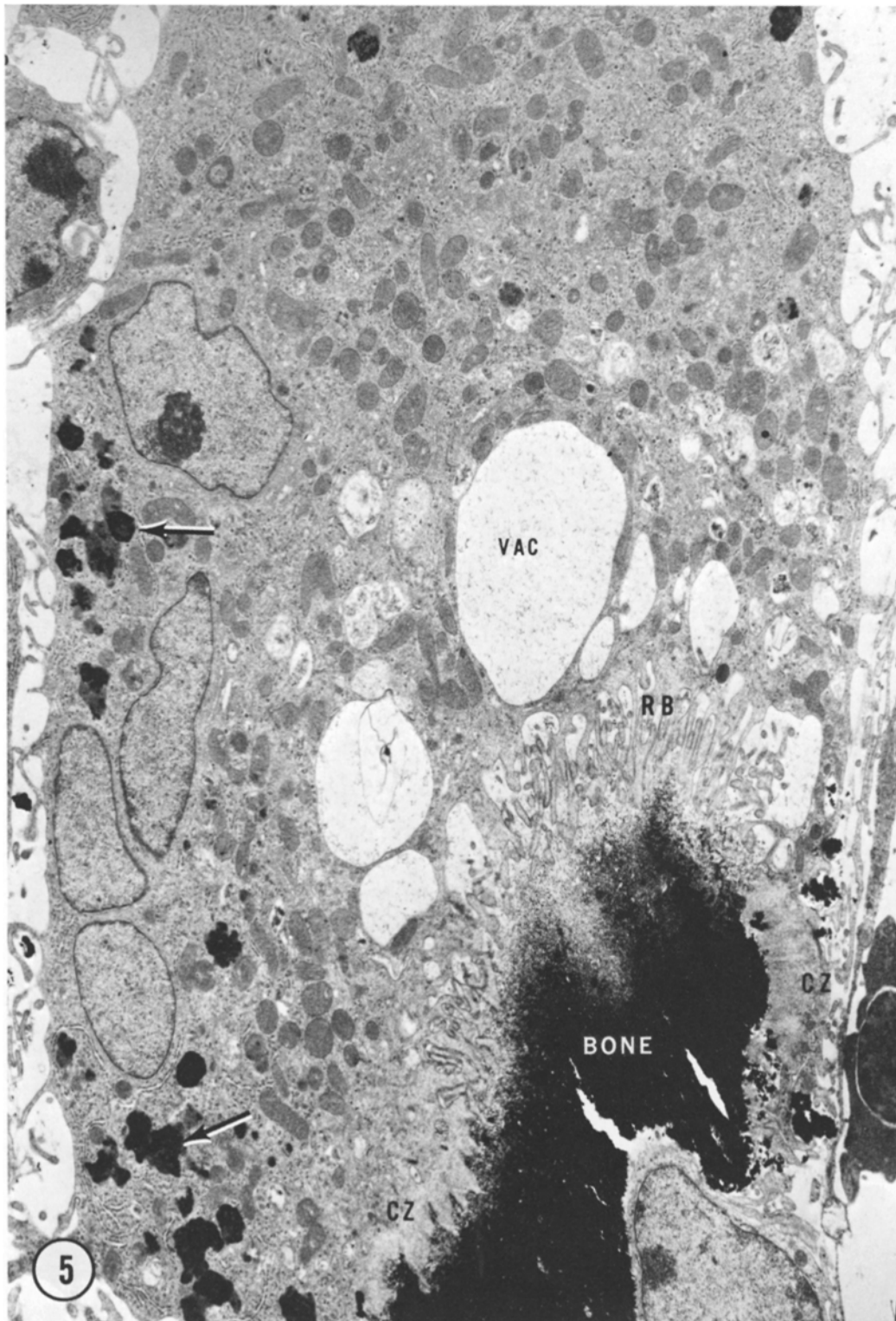


FIGURE 5 Low power electron micrograph of an osteoclast taken from a bird during the active phase of the egg cycle, 16 h after oviposition. The osteoclast has a ruffled border region (*RB*) and numerous associated vacuoles (*VAC*). The bone beneath the clear zones (*CZ*) is more compact than that beneath the ruffled border. Numerous, large, irregular electron-dense bodies (arrows) are present in the outer parts of the cell away from bone. The cell surface, peripheral to the ruffled border region, is smooth with a few narrow cell processes. $\times 5,500$.

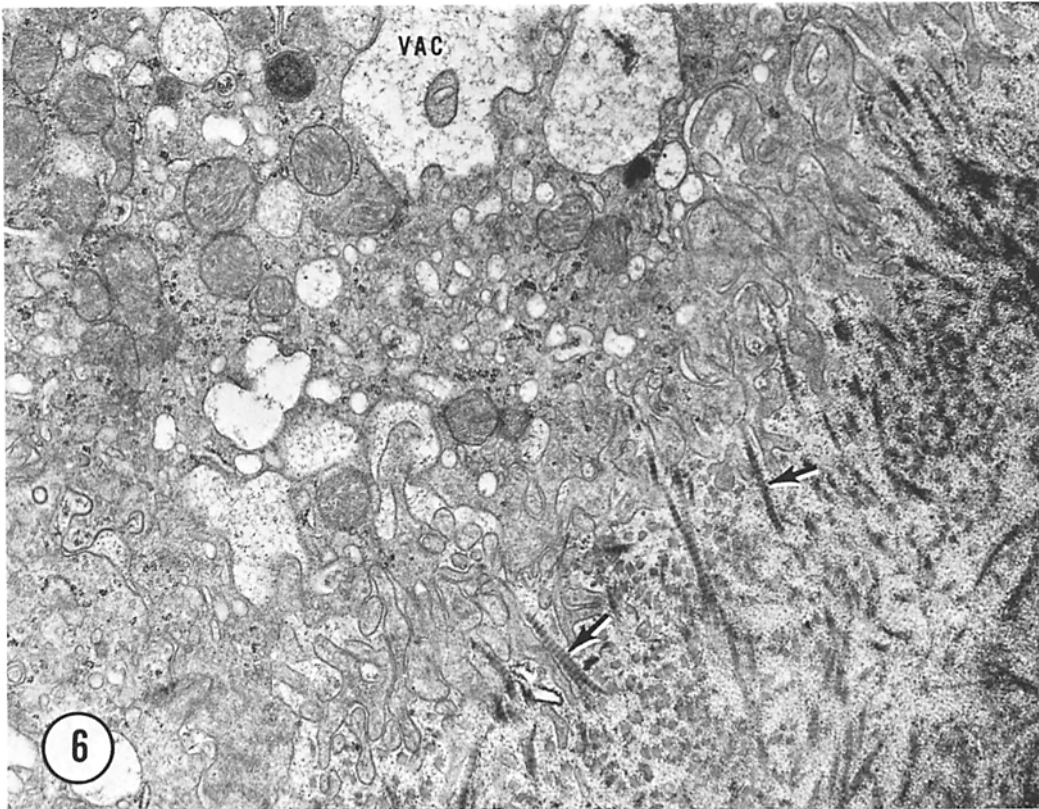


FIGURE 6 Greater detail of a ruffled border of an osteoclast, taken from a bird during the active phase of the egg cycle, 11 h after oviposition. Collagen fibers (arrows) are present between projections of the ruffled border. Mineral crystals are present in the ruffled border and in vacuoles (VAC) deeper in the cytoplasm. $\times 12,500$.

zones seem identical in fine structure to those which surround ruffled borders on osteoclasts during the period of shell calcification (CZ, Fig. 5), and like these zones, they are closely apposed to the bone surface, whereas the remainder of the cell surface is not (Fig. 8). The clear zones are located in areas of close contact between osteoclasts and bone surfaces as observed in sections of these cells with the light microscope (Fig. 4). They are clearly an area of cell attachment to the bone (18).

Another characteristic and unusual feature of the osteoclasts during the inactive phase of the egg cycle, from ~ 2 h before oviposition to ~ 9 h after oviposition, is the presence of numerous, interdigitated cell surface projections on their peripheral surface (arrows, Fig. 7). These unusual structures are seldom found on osteoclast surfaces during the active phase of the egg cycle when ruffled borders are present, ~ 9 – 19 h after oviposition. The inter-

digitated cell processes can appear as irregular folds (Fig. 9), or as stacks of fairly uniform, finger-like projections (Figs. 10, 11). Small clumps of mineral crystals are often found between these cell surface projections (arrows, Figs. 10, 11).

Because of the dramatic difference in cell surface morphology between osteoclasts in the active and inactive phases of the egg cycle, it was of interest to examine intervals of the egg cycle, where transitions from one morphology to the other might be rapidly occurring. From ~ 19 – 23 h after the previous oviposition, calcification of the egg shell and bone resorption are completed. It is during this period that ruffled borders of the osteoclast adjacent to bone disappear. During this time, ruffled border-like surfaces (RB, Fig. 12) with channel expansions and cytoplasmic vacuoles (VAC, Fig. 12) could be found away from the bone and peripheral to the CZ regions (Fig. 12). The channel expansions and vacuoles contain crys-

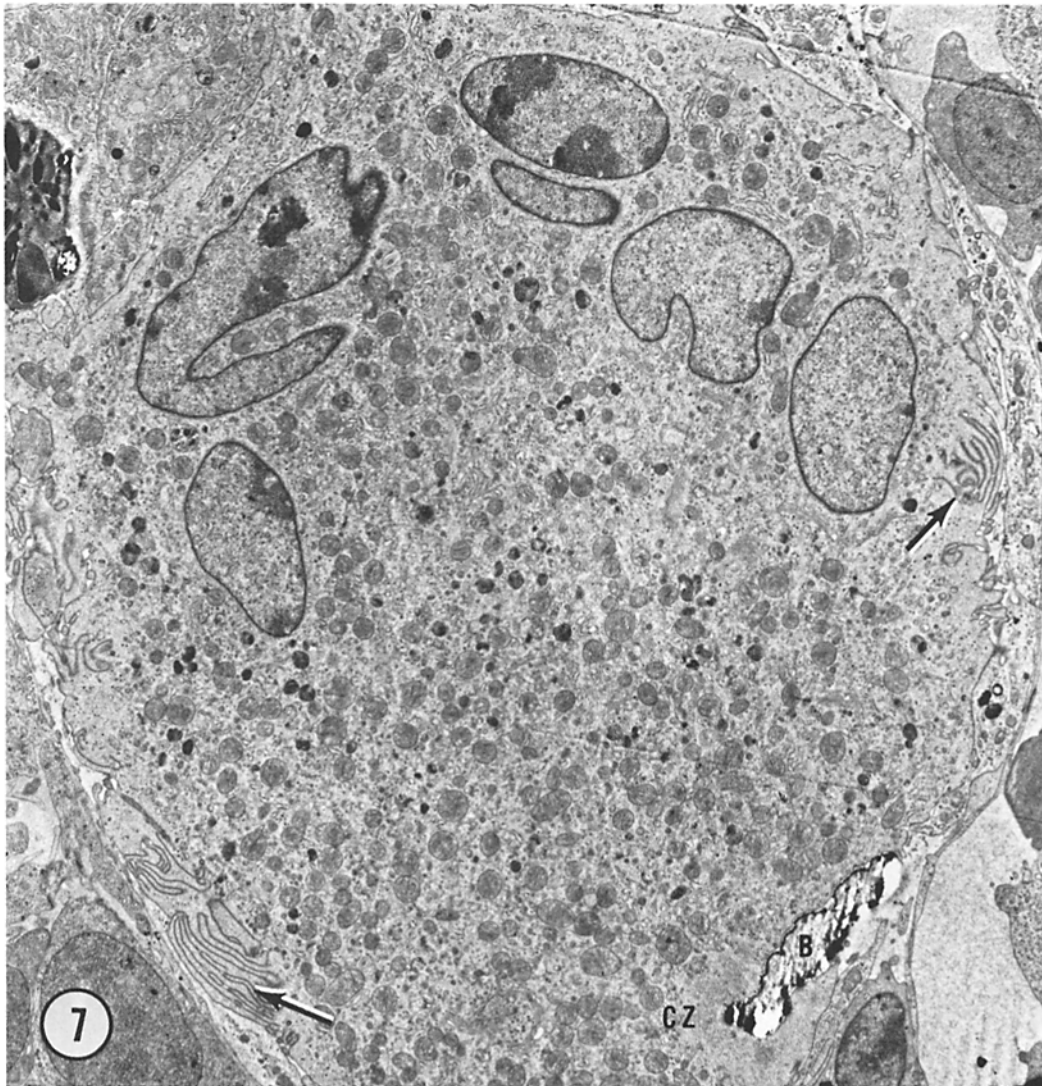


FIGURE 7 Low power electron micrograph of an osteoclast taken from a bird during the inactive phase of the egg cycle, 4 h after oviposition. The osteoclast is attached by a clear zone (CZ) to a small bone spicule (B). Ruffled borders are absent on the osteoclast during this period of the egg cycle, however, interdigitated cell surface projections (arrows) are common on the peripheral parts of the cell away from bone. $\times 4,000$.

talline and amorphous material like that found in ruffled borders during the period of egg shell calcification (Figs. 5, 6). At the same time that ruffled borders move peripherally away from bone surfaces, interdigitated stacks of cell surface projections appear distal to them (arrows, Fig. 12). Virtually the entire osteoclast surface, except that part occupied by the clear zone adjacent to bone, is soon covered with layers of the interdigitated microprojections. The interdigitated peripheral

cell processes are commonly found on osteoclast surfaces from ~ 2 -3 h before oviposition to ~ 8 -11 h after oviposition; they largely disappear from osteoclast surfaces ~ 8 -11 h after oviposition when the next egg shell commences calcification, and ruffled borders reappear.

DISCUSSION

This study demonstrates that during the egg-laying cycle of the Japanese quail there is no significant

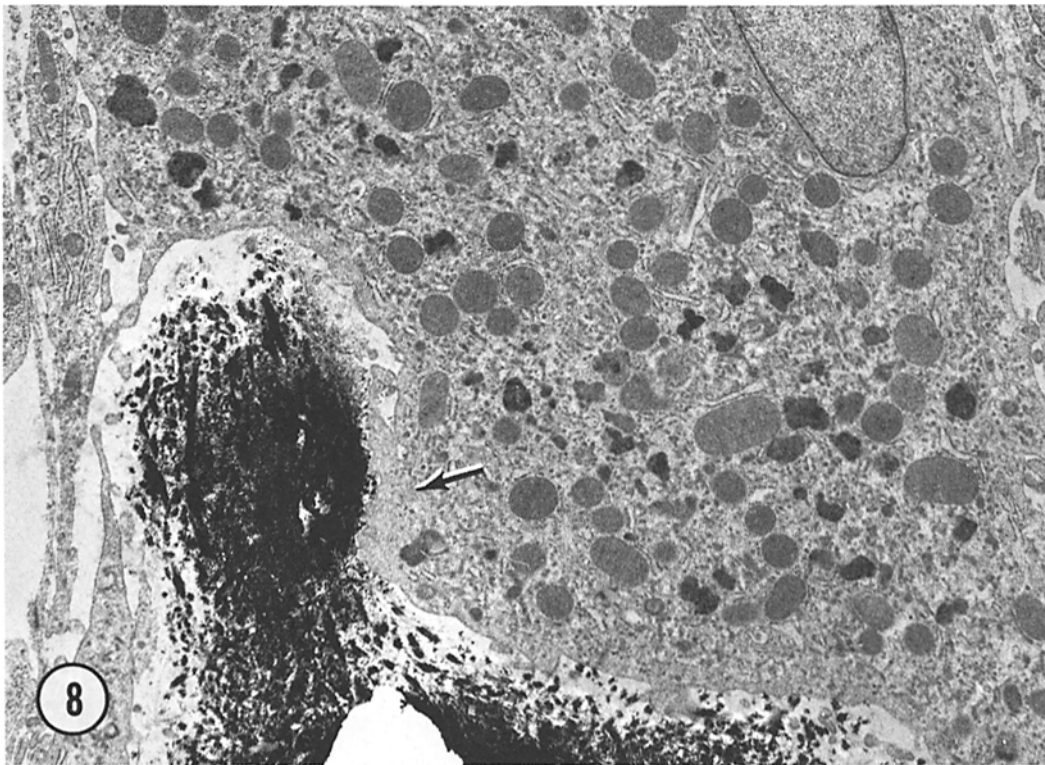


FIGURE 8 Greater detail of the attachment of an osteoclast by a clear zone (arrow) to a bone surface during the inactive phase of the egg cycle, 5 h after oviposition. The clear zone is closely apposed to the bone, whereas the remainder of the cell surface is not. Numerous electron-dense bodies are present in the cytoplasm. $\times 8,000$.

relative change in medullary bone osteoclast populations. Although the osteoclast population probably has a certain amount of normal turnover (30, 39), the state of calcification of the egg shell has no detectable influence on population parameters. These findings do not support observations made in pigeons (3) and chickens (2, 4), that osteoclast populations differentiate from and into other bone cells during the egg-laying cycle.

However, there were profound changes in osteoclast morphology during the egg-laying cycle, summarized diagrammatically in Fig. 13. These findings suggest that these cells are going through cyclic functional modifications, rather than population transformations, which may explain their changing role in medullary bone resorption. During the period when medullary bone reserves of calcium are being used for egg shell formation, the osteoclasts are spread along bone surfaces and contain well-developed ruffled borders with numerous associated vacuoles. The bone beneath

these ruffled borders is loose and frayed, as if it were undergoing dissolution. Contrarily, the osteoclasts are partly removed from bone surfaces, and lack ruffled borders and associated vacuoles during the remainder of the egg cycle when medullary bone calcium is not needed for egg shell formation. These osteoclasts appeared to be slightly smaller than those observed during the active phase, probably due to the absence of vacuoles and distended channels in their cytoplasm.

The ruffled border, only present on medullary bone osteoclasts during the period of shell calcification, is a surface modification of the cell which appears to facilitate bone resorption (13, 15, 25). Although the fundamental role of the osteoclast in medullary bone resorption in birds has been questioned (36), the observation of the presence of ruffled borders and associated structures on osteoclasts only during the period of egg shell calcification suggests that osteoclasts are indeed resorbing bone during this period of the egg cycle, and

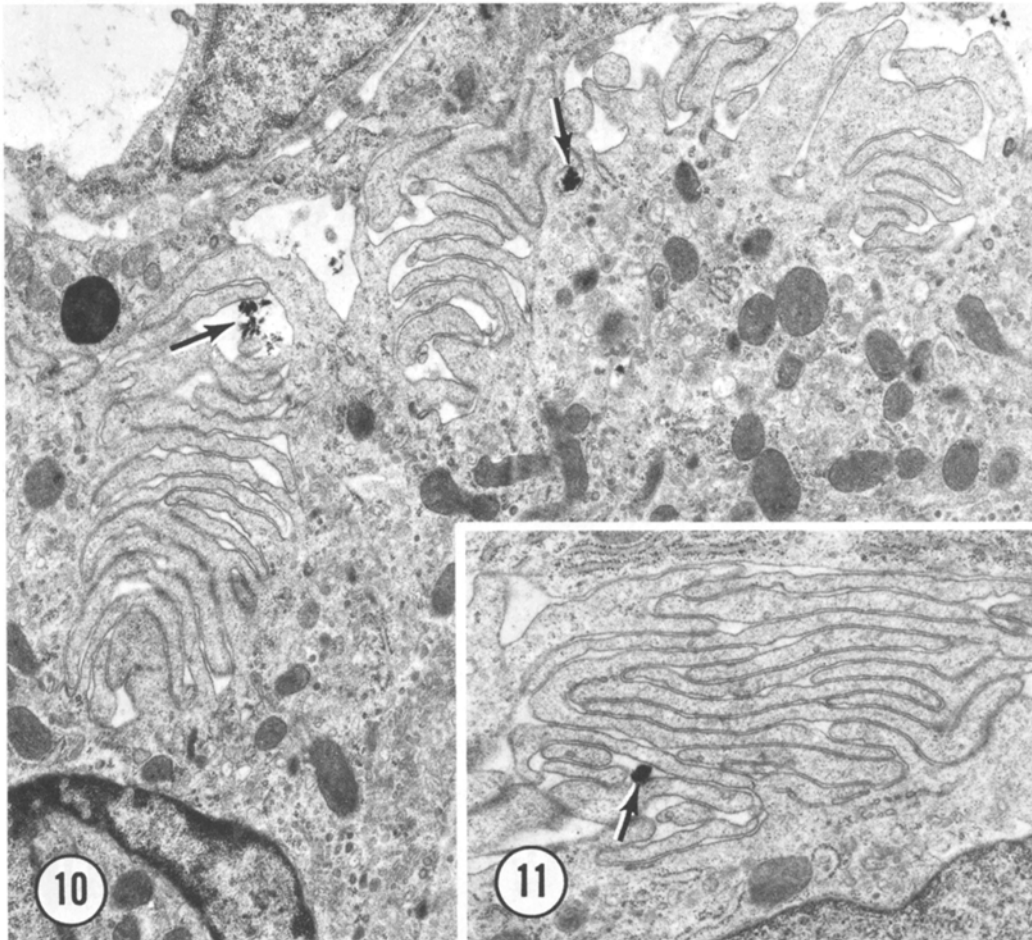
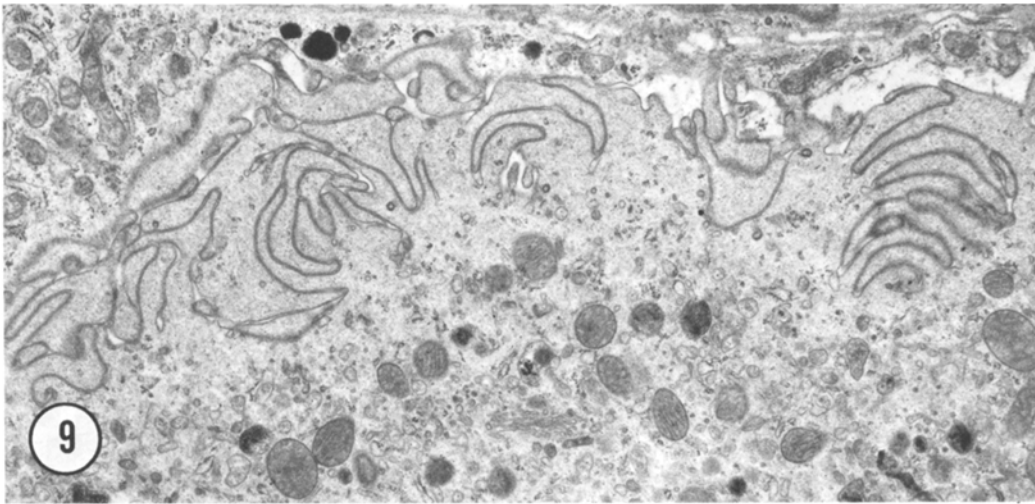


FIGURE 9 Electron micrograph of a peripheral osteoclast surface away from bone, taken during the inactive phase of the egg cycle, 3 h after oviposition. Numerous interdigitated cell surface folds are characteristic of osteoclast surfaces during this phase of the egg cycle when ruffled borders adjacent to bone are lacking. $\times 8,000$.

FIGURE 10 The interdigitated cell-surface folds present on osteoclasts during the inactive phase of the egg cycle often appear as stacks of fairly uniform, finger-like projections. Small clumps of mineral crystals (arrows) are commonly found between these membrane folds. $\times 12,500$.

FIGURE 11 An extensive array of long, interwoven cell processes present on an osteoclast surface, away from bone, taken from a bird 4 h after oviposition. A small clump of mineral (arrow) is present between these processes. $\times 13,000$.

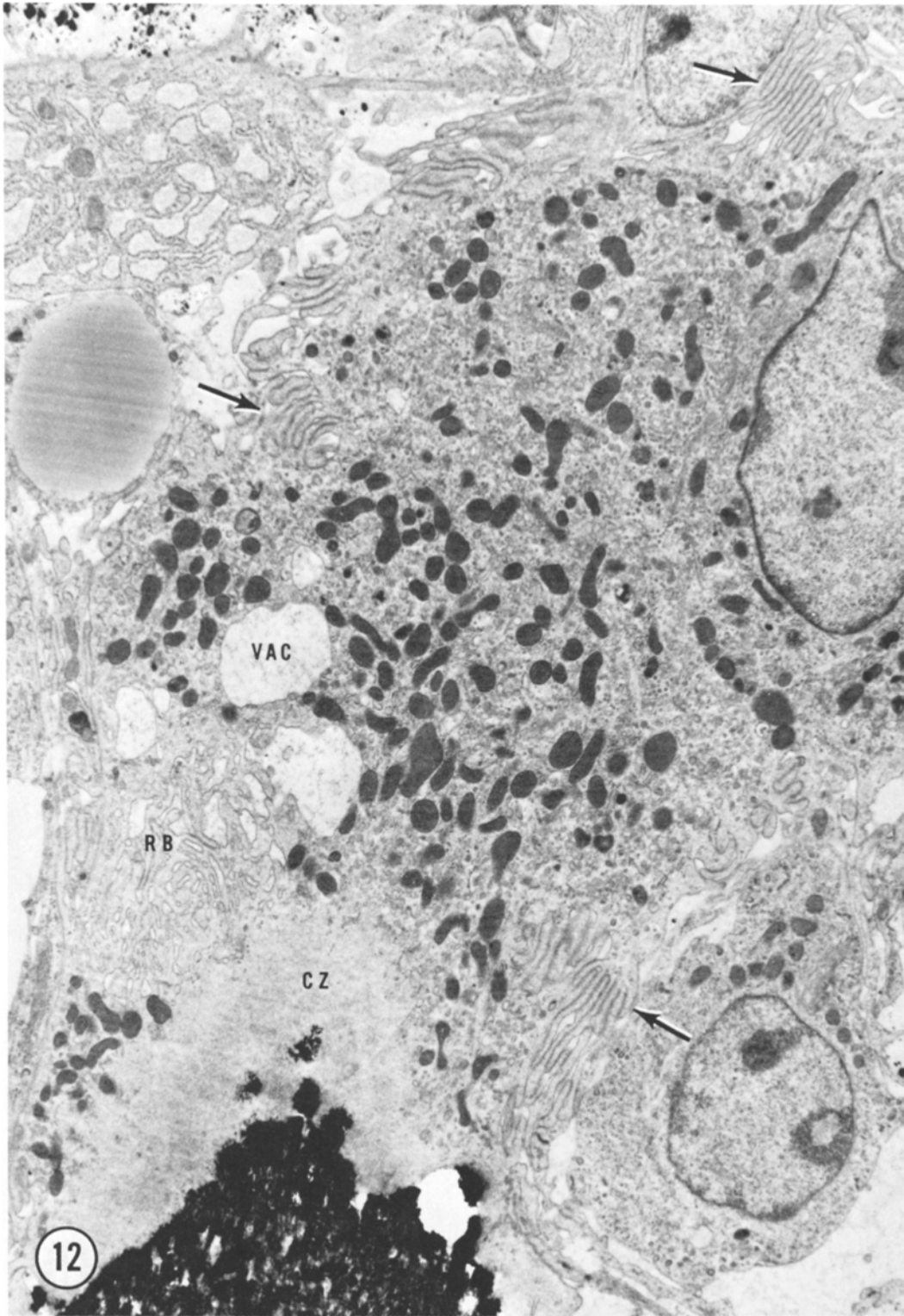


FIGURE 12 Low power electron micrograph of an osteoclast taken at 23 h after oviposition during the period when egg shell calcification is completed, medullary bone resorption ceases, and osteoclasts lose their ruffled borders. The ruffled border (*RB*), channel expansions, and vacuoles (*VAC*) which contain mineral crystals are away from the bone surface peripheral to the clear zone (*CZ*). During this period of the egg cycle, interdigitated stacks of cell processes (arrows) cover the remainder of the osteoclast surface. These stacks of cell processes remain in large quantity on the osteoclast surface until an egg shell begins to calcify and ruffled borders next to bone reappear. $\times 4,500$.

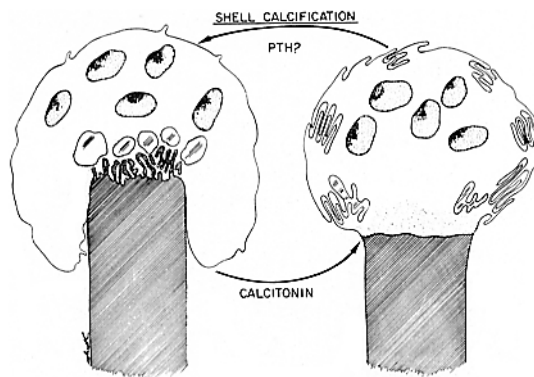


FIGURE 13 Diagram summarizing the changes in osteoclast morphology observed in this study during the egg-laying cycle of Japanese quail. During egg shell calcification, osteoclasts have extensive ruffled borders with numerous associated vacuoles adjacent to bone surfaces. At the completion of egg shell calcification, ruffled borders next to bone disappear, and numerous interdigitated cell surface folds appear on the peripheral parts of the cell. The number of osteoclasts or the number of nuclei per osteoclast do not significantly change during the egg cycle.

therefore contributing to calcium homeostasis of the animal.

When ruffled borders and cytoplasmic vacuoles disappear at the completion of egg shell calcification, large numbers of interdigitated stacks of surface projections appear on the side of the osteoclast away from the bone. These structures remain in large quantity on the osteoclast until later in the egg cycle, when ruffled borders and associated structures reappear. Although the function of these structures is not known, it seems likely that they serve as reservoirs of membrane which may have been previously invested in the ruffled borders, channel expansions, and cytoplasmic vacuoles. These structures have not been previously described on osteoclasts, but somewhat similar appearing structures, of unknown function, have been observed on multinucleated giant cells derived from monocytes and macrophages in granuloma lesions (9).

It is interesting to note that the osteoclasts did maintain clear zones adjacent to bone surfaces during the periods when ruffled borders were lacking. Clear zones, which contain actin-like filaments and surround the ruffled borders of actively resorbing cells (21), may serve as the sites of adhesion of the osteoclast to the bone surface (18). The persisting clear zones on osteoclasts may serve to keep the osteoclasts attached to and per-

haps oriented with bone surfaces during the period of resorptive quiescence, when medullary bone calcium is not needed for egg shell calcification. The clear zone of each cell appears to contract into one confluent zone when the ruffled border disappears.

It would be of interest to define the regulatory factors responsible for the dramatic changes in osteoclast cell surface features during the egg-laying cycle. The synchronous disappearance of ruffled borders and cytoplasmic vacuoles observed in this study at the completion of egg shell calcification is reminiscent of the reported changes observed in osteoclasts treated with calcitonin in vitro (18, 20) and in vivo (26). Japanese quail, like other birds and lower vertebrates, have naturally high levels of circulating calcitonin which, in the Japanese quail, fluctuates during the egg cycle (12). The lowest levels of calcitonin were reported to occur during the period of egg shell calcification, when ruffled borders and associated structures were observed on osteoclasts in the present study. However, calcitonin levels were found to return to their naturally high level (12) during the period when ruffled borders on osteoclasts were found to be absent in the present study. Parathyroid hormone may also be involved in the regulation of calcium metabolism during the egg-laying cycle (8, 36). Parathyroid hormone stimulates bone resorption and causes an increase in the area occupied by ruffled borders on osteoclasts in vitro (18). These correlations make it tempting to speculate that the decreases in calcitonin during egg shell formation, and perhaps also a rise in parathyroid hormone, stimulate the osteoclast differentiation involved in the periodic resorption of medullary bone.

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