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CORRELATIVE TRANSMISSION ELECTRON MICROSCOPY AND HIGH RESOLUTION SCANNING ELECTRON MICROSCOPY STUDIES ON THE FINE STRUCTURAL ORGANIZATION OF THE CHICKEN PITUITARY GLAND

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Abstract

The present study employs transmission (TEM) and high-resolution scanning electron microscopy (HR-SEM) to examine the inter- and intra-cellular organization of the *pars distalis* of the chicken anterior pituitary gland. The overall view of the cryofractured surface of the *par distalis* illustrates the arrangement of different pituitary cells and tissues in the follicles. Fine structural examination by HR-SEM shows that the membrane of the mitochondria has a similar configuration to that of the rough endoplasmic reticulum. Granule-like structures, on the surface of the mitochondrial membrane, are similar in size to ribosomes. Standard imaging and three-dimensional imaging demonstrated the formation of developing granules inside the Golgi sacs and the release of mature granules from the end of Golgi stacks. The occurrence of granule-granule connections suggests that granules may be released by exocytosis in groups or individually.

Key words: Chicken, pituitary gland, transmission electron microscopy, high resolution scanning electron microscopy.

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Introduction

Cytological and histological studies designed to identify hormones and follow intracellular and transcellular hormone movement have been undertaken on the anterior pituitary gland of many species (1, 3, 7, 20). Based on biochemical and physiological studies, there appear to be at least six hormones produced by the *par distalis* of the adenohypophysis (13). Each of these hormones is biochemically distinct and possesses a separate and unique function (6, 11, 21). A combination of different methods, including histochemical staining (5), immunofluorescence (12), peroxidase-immunocytochemistry (10), immunogold-labeling (16) and autoradiography (9) at light (LM) and transmission electron microscopic (TEM) levels, have been used to establish the cytophysiological relationship between these hormones and the cell types of the *par distalis*. It has been generally concluded that each specific cell type synthesizes and stores a single anterior pituitary hormone. However, recent reports indicate the existence of bihormonal cells in the pituitary gland (2, 4, 8).

Rinehart and Farquhar (14) initially identified rat pituitary cell types using TEM. This instrument offers a great advantage in cell ultrastructural and functional studies. Through such studies, the morphological characteristics for hormone granule size of each cell type has been shown to be altered under different physiological conditions. Thus, identification of a cell type solely by granule size may result in significant confusion.

Tai and Chadwick (17) using LM and TEM combined with the classical staining and immunofluorescent methods, distinguished six different cell types in the pituitary gland of *Gallus domesticus*. In addition, the function of four cell types has been determined by studying the experimentally induced changes in the cell.

The purpose of the present study is to correlate the information obtained on three-dimensional fine structure by high resolution scanning electron microscopy (HR-SEM) with that previously obtained from TEM demonstrating the inter- and intra-intercellular organization, and the intracellular activities associated with hormone

synthesis in the chicken pituitary gland.

Materials and Methods

30 young laying hens (*Gallus domesticus*) between 21 to 25 weeks of age were used, 8 for TEM and 22 for SEM. The pituitary glands were removed within 2 minutes following sacrifice by decapitation. The *par distalis* was dissected from the rest of the pituitary gland and prepared for TEM and HR-SEM studies.

For TEM

The *par distalis* of the chicken pituitary possesses both cephalic and caudal lobes. The two lobes of the *par distalis* were separated and each lobe was divided into two pieces. All the specimen pieces were kept in individual vials and fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH = 7.6) for 3 hours at 4 °C and post-fixed in 2% OsO_4 for an additional 30 minutes at 4 °C. After dehydration, tissues were embedded in Epon 812. Ultrathin sections of 70 nm were obtained using a ultramicrotome (Reichert-Jung Ultracut-E). After double staining with 2% uranyl acetate and lead citrate (20 minutes in each), sections were observed at 75 kV in a Hitachi H-600 TEM.

For HR-SEM

Cryo-fractured surfaces for evaluation by HR-SEM were obtained using the osmium-DMSO-osmium (O-D-O) method (18) with certain modifications. The entire *par distalis* was fixed in 0.5% glutaraldehyde in 0.1 M phosphate buffer (pH = 7.6) at 4 °C for 30 minutes, rinsed with the same buffer and post-fixed in 0.5% OsO_4 for 30 minutes at 4 °C.

Specimens were infiltrated with cryo-protectant (25% and 50% dimethyl sulfoxide solution, DMSO) for 30 minutes each. Each specimen was placed horizontally in a drop of 50% DMSO and rapidly frozen on a pre-chilled aluminum block immersed in liquid nitrogen. After 2 to 3 minutes, specimens were fractured longitudinally with a precooled dissection knife and hammer. In this way, two halves of the cephalic and caudal lobes of the *par distalis* were obtained. The fractured pieces were immediately placed in 50% DMSO solution and thawed at room temperature.

Specimens were rinsed extensively with buffer and post-fixed again in 1% OsO_4 at 4 °C for 1 hour. They were then left in 0.1% OsO_4 for 72 hours at 20°C. After maceration, samples were fixed again in 1% OsO_4 for 1 hour, treated with 2% tannic acid overnight and left once more in 1% OsO_4 for 1 hour. After dehydration through a graded alcohol series, specimens were dried by the critical point procedure. Some specimens were coated with 2 nm chromium film in a Denton DV-602 turbo-pumped system. Specimens were coated with 2 nm platinum in a vacuum evaporator (Polaron 6000). Specimens were examined either in-lens in a Hitachi S-900 Field Emission (FE) SEM or in a below-lens FE-SEM Hitachi S-800 operated at 15 kV.

Figure 1. Most cells of the *par distalis* of the chicken pituitary gland are arranged in follicles that vary in size and shape. Interfollicular space (sp), nerve (ner), capillaries (cap), follicular lumen (*) and epithelial cells (arrowheads) are seen. (Platinum coated).

Figure 2. HR-SEM of three similar cells. They are uniformly oval in shape and contain a centrally located nucleus (Nu). Granules (g) occupy most portion of the cytoplasm. Golgi (G). (Chromium coated).

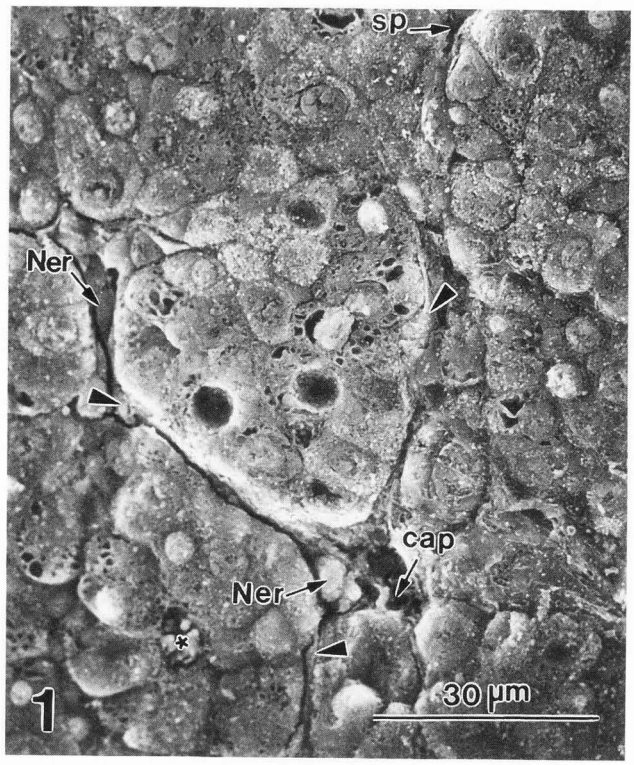
Figure 3. HR-SEM topographic observation showing cells which contain numerous vesicles (v) throughout the cytoplasm. Arrowheads indicate granules forming inside the Golgi vesicles. Nu: nucleus; ep: epithelial cell. (Cr coated).

Figure 4. TEM shows the intercellular organization of several types of pituitary cells (See Tai and Chadwick, ref. 17). Epithelial cells (arrowheads); Ner: nerve; PL: prolactin cells; GT: gonadotropins; sp: interfolicullar space; v: vesicle.

Results - Intercellular and Intracellular Organization

a. General Description

The HR-SEM observation of the sagittally cryo-fractured surface of the *par distalis* of the chicken anterior pituitary gland shows that most pituitary cells are arranged in follicles that vary in size and shape (Fig. 1). Each follicle consists of more than one type of cells. These cells can be distinguished by their structural characteristics such as the shape and the size of cells; the position size and shape of the nucleus and morphological features of the cytoplasm. In some pituitary secretory cells, the hormone granules are tightly packed filling most of the cytoplasm (Fig. 2). In other cells (Fig. 3), the organization of their cytoplasmic constituents is different; organelles are limited to very small areas by numerous irregularly shaped vesicles which occupy a large portion of the cytoplasm. The lumen is seen in the center of some follicles. Blood vessels and nerve tracks are observed in the interfollicular space. Epithelial cells are seen in the intercellular spaces as well as along the periphery of the follicles to form a delimited boundary (Figs. 1 and 4).


sp
Ner
cap
Ner
*
1
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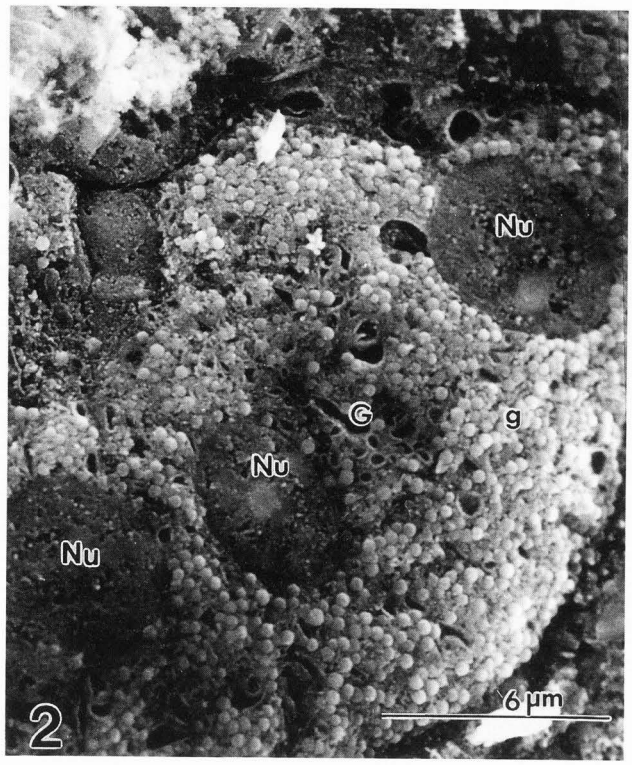
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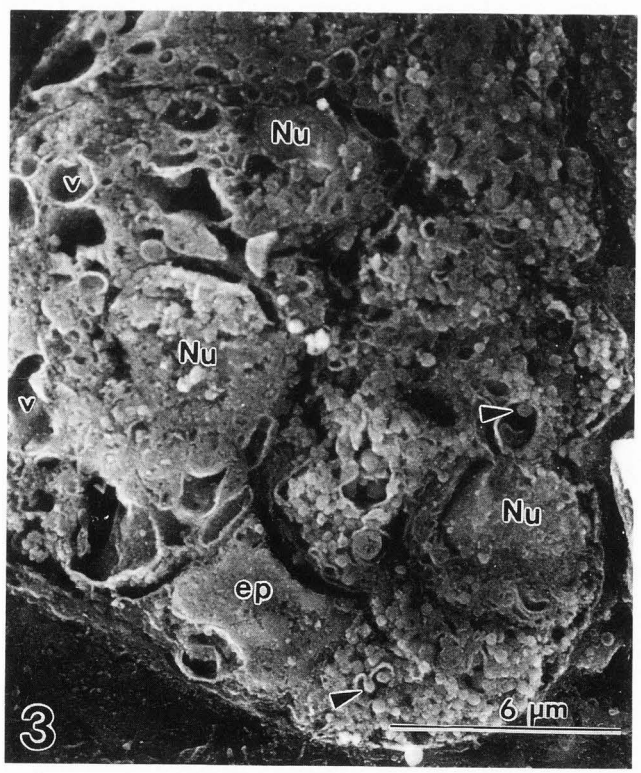
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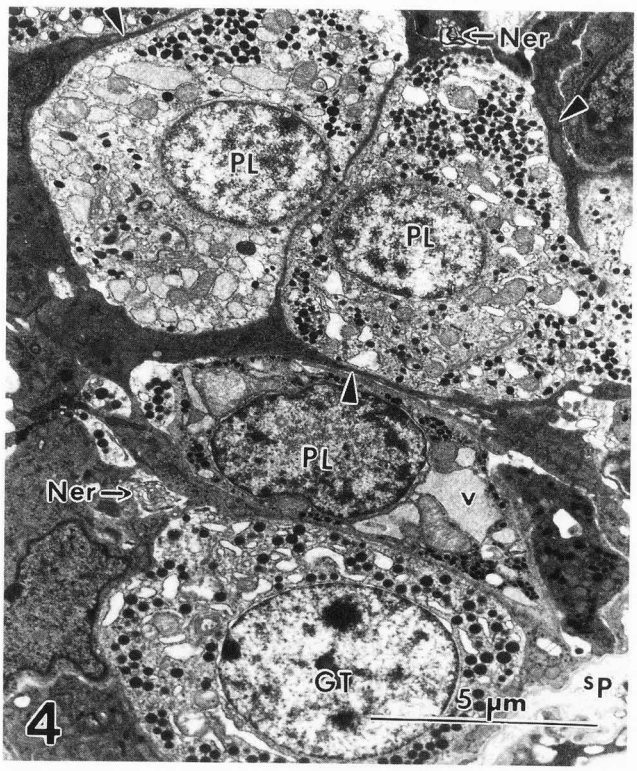
Ner
PL
PL
PL
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5 µm
sp
4

b. Endoplasmic Reticulum and Golgi Apparatus

Cells actively engaged in synthesizing hormones show abundant cisternae of rough endoplasmic reticulum (rER) and a very extensive Golgi apparatus. In Fig. 5, it is difficult to separate these two organelles; tightly packed cisternae of the rER are continued to the Golgi stacks. Considerable granular material covers the inner membrane of the Golgi sacs. A granule is seen budding from the end of a Golgi stack (Fig. 5). Under TEM observation, a thin section of a pituitary cell (Fig. 6) also shows the rER extends throughout the cytoplasm from the cell membrane to the margin of the nuclear envelope. Many transfer vesicles can be observed along the cis-face of the Golgi stack. The TEM two-dimensional image of an extremely active Golgi apparatus (Fig. 7) revealed that hormone granules in different stages of formation were seen in different portions of the Golgi apparatus. Fenestrations are also seen in this cell. HR-SEM can provide vivid images showing the process of synthesis of hormone granules. In Fig. 8, different sized granules appear to be developing in different Golgi vesicles, while several hormone granules are seen forming in a single Golgi sac.

c. Mitochondria

The cross-section images of mitochondria under HR-SEM (Fig. 9) and TEM (Fig. 10) are very similar. The mitochondria is surrounded by rER and delimited by the bilayered membranes. The inner membrane is developed into many cristae. The space between the inner and outer membrane is about 5 nm (Fig. 10).

The HR-SEM of mitochondria (Fig. 9) illustrates that the mitochondrial membranes have a very similar configuration to that of the rER. Granule-like material, similar in size to that of the ribosomes, is attached to the surface of both outer and inner membranes. However, utilizing TEM (Fig. 10) it was difficult to see granules on the mitochondrial membrane.

d. Granulation

In TEM micrographs, pituitary tissue shows differences in the size and shape of granules in a single cell. This can be due to off-equatorial sections through any portion of the granules. Therefore, using standard thin sectioning for TEM, it is difficult to determine the actual size and shape of secretory granules for any type of pituitary cell. By using HR-SEM to observe a cryo-fractured and well-macerated surface of a pituitary cell, granules can be viewed in their entirety and stalk-like structures which are single or double (Figs. 11 and 12) can be observed between granules. The width of this structure is about 30 to 40 nm. With TEM (Fig. 13), electron-dense structures are also seen in the same relative positions between granules.

Figure 5. HR-SEM shows tightly packed rER cisternae in association with Golgi stacks. A granule is budding from a Golgi stack (arrowhead). G: Golgi apparatus; g: granule; M: mitochondria; rER: rough endoplasmic reticulum. (Pt coated).

Figure 6. An illustration similar to Fig. 5 as shown by TEM. Abundant rough endoplasmic reticulum (rER) and an extended Golgi complex (G) are seen. Transfer vesicles (arrows) are along the cis-face of the Golgi stack. Nucleus (Nu); arrowhead: a granule in the cisternae.

Figure 7. TEM shows a very active Golgi apparatus containing granules of different sizes (arrowheads). Fenestrations (Fen) are also seen. M: mitochondria.

Figure 8. Several granules of different sizes are seen forming in a single Golgi sac; other granules occur individually in different Golgi vesicles. Arrowheads indicate the developing granules. (Cr coated).

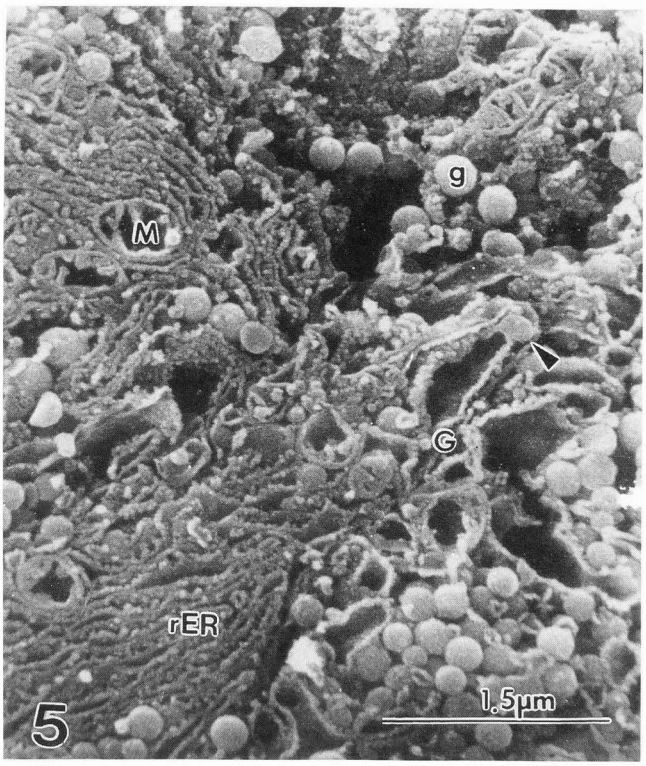
All pituitary hormone granules are formed in the Golgi apparatus (Figs. 3, 6, 7, and 8). The mature granules are released from the Golgi sacs and enter the cytoplasm (Fig. 5). In some cells, it appears that granules tend to group in distinct compartments situated in different parts of the cytoplasm. It is observed that some granules are connected to others and it is possible that these clusters of granules are released jointly rather than as individual granules (Fig. 14).

e. Microfilaments

The present HR-SEM work demonstrated the presence of cytoplasmic filaments. These are present as aggregates of elongated protein molecules forming a framework for the attachment of the cytoplasmic structure. Microfilaments 7 nm in diameter were seen between the cisternae of the rER, Golgi and mitochondria. Ten nm diameter filaments with intermittent 25 nm expansions along their length are also seen between subcellular organelles (Figs. 15 and 16).

Discussion

One of the most important factors for obtaining optimal results for TEM and HR-SEM is the specimen preparation technique (9). For routine TEM studies, standard fixation methods and stained 70 nm sections can be used. For evaluation by HR-SEM, freshly-extracted pituitary specimens can be cryo-fractured, fixed and then macerated to reveal the intracellular details. The pituitary gland is a complex structure consisting of several kinds of tissues. Preliminary experiments examining the effects of different fixations and the duration


g
M
G
rER
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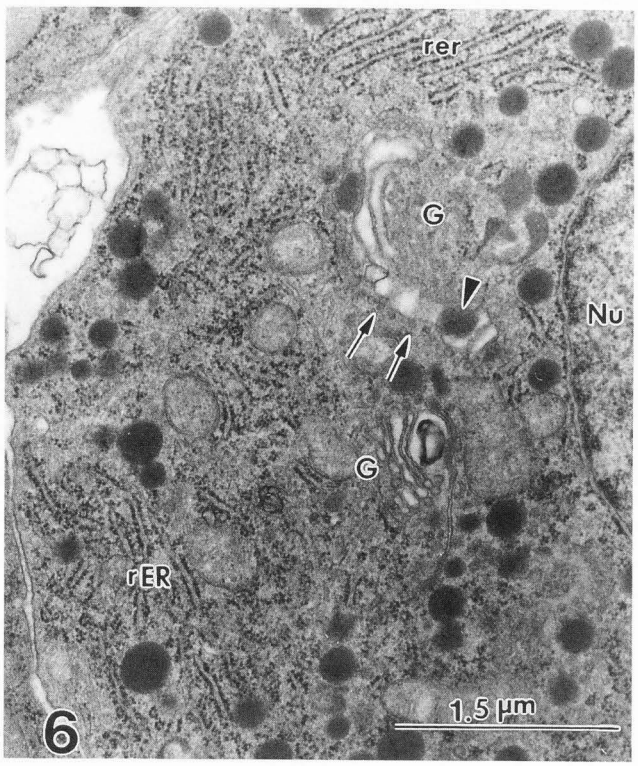
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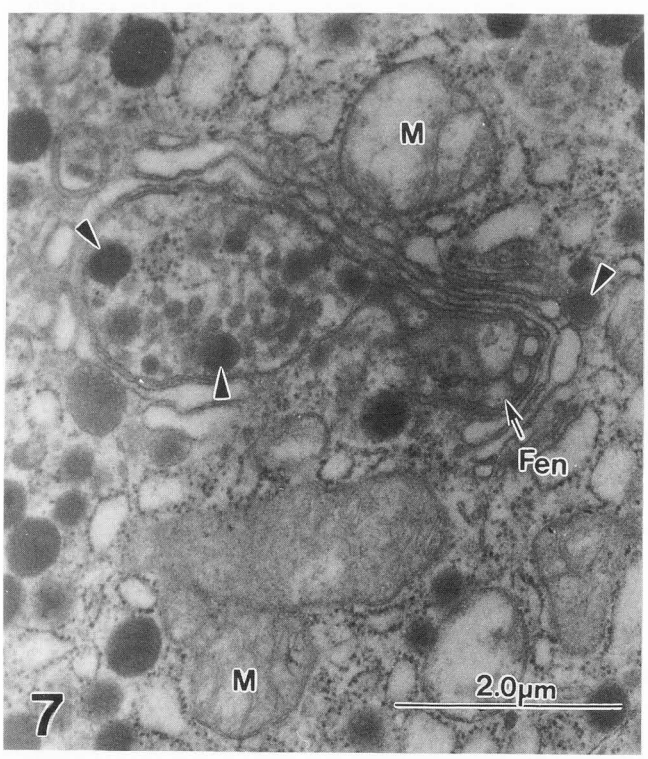
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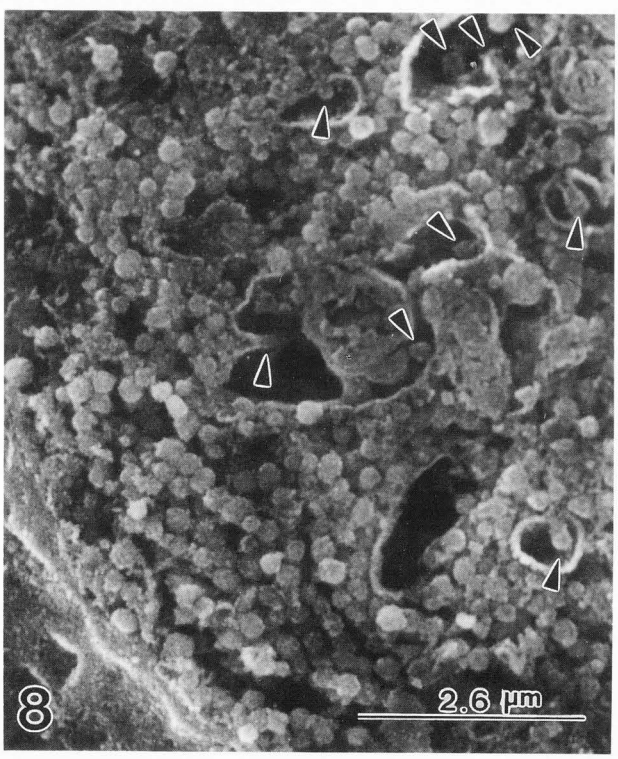
7


2.6 μm
8

and temperature at which maceration is carried out were performed. Best results were obtained when the specimen was allowed to freeze for 2 to 3 minutes before cryo-fracturing and when maceration was carried out over a 72 hour period at 20 °C, which agrees with the report of Tanaka (18) and Tanaka and Mitsuchima (19).

The sagittally-fractured surface of an entire *par distalis*, including cephalic and caudal lobes, permits overall observation of pituitary cell arrangement at low magnification. The distribution of each cell type as well as the relationship between different types of cells and tissues in the pituitary gland can be visualized. This is an advantage of HR-SEM, as TEM observation is limited to a smaller and thin sectioned specimen.

The exact shape and size of hormone granules in any pituitary cell is difficult to assess by TEM, because ultra-thin sections can only provide two-dimensional images. The chance for any granule being sectioned through the equator is minimal. However, this is not a problem for the HR-SEM, because all granules in the specimen were preserved in their entirety. Thus, the actual size and shape of hormone granules for each cell type can be seen. It is noted using these methods that granules of different sizes and shapes coexist in the same cell.

The HR-SEM demonstrated stalk-like connections between hormone granules. Prior examination of TEM micrographs had noted electron dense structures located between granules. However, prior to examination with HR-SEM, they were not recognized as granule-granule connections. Senda *et al.* (15), using TEM and SEM, identified these connections as fine cytoplasmic filaments which connected directly between the limiting membrane of all secretory granules in the rat pituitary cells. Due to the existence of these granule-granule links, and the accumulation of granules in groups along the cell membrane, it is possible that the secretion of hormone granules during exocytosis occurs in groups, since most granules appear to be connected to one another.

The cryo-fractured surfaces of a very extended Golgi apparatus including the Golgi cisternae, vesicles and fenestrations were observed. Many different sized developing granules were being formed in different Golgi cisternae. Granules were shown to be budding from Golgi stack. These three-dimensional images provided a more complete picture of the formation of hormone granules in the Golgi apparatus.

HR-SEM at low and high voltage has become a powerful tool for cell ultrastructural studies since it can contribute complimentary information to verify TEM results, or to add novel ideas and concepts. For example, granule-granule connections and cytoplasmic microfilaments whose diameters range from 5 to 50 nm are seen in relation to each other and to larger defined structures.

Figure 9. A HR-SEM micrograph of mitochondria illustrates that the inner and outer membranes (arrowheads). Granules are seen on the surface of both membranes. rER: rough endoplasmic reticulum. (Cr coated).

Figure 10. High-magnification TEM image shows that the mitochondria is surrounded by a rough endoplasmic reticulum (rER), and delimited by a bilayer membrane (arrow).

Figures 11 and 12. HR-SEM illustrates the granule-granule connections in single or double forms (arrowheads). (Cr coated).

TEM study of ultrathin tissue sections shows these structures only in cross section and without their connections to other structures.

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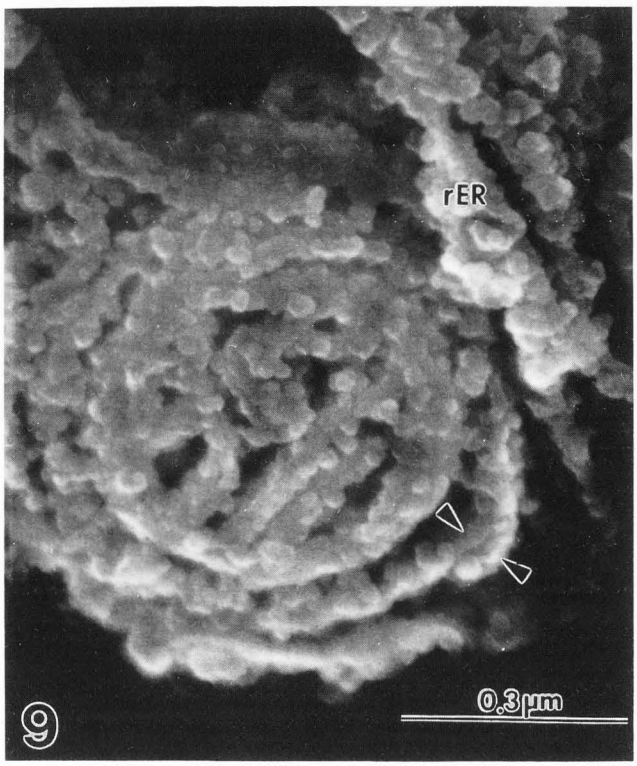
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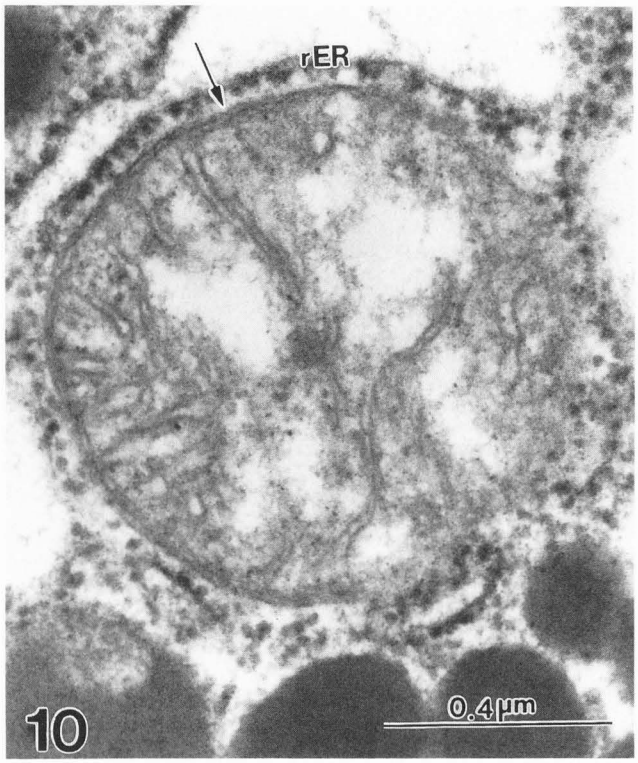
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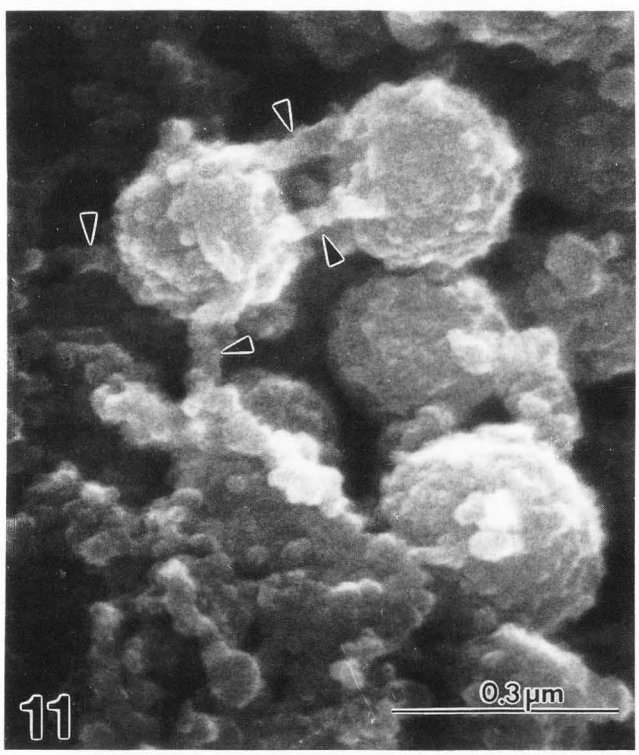
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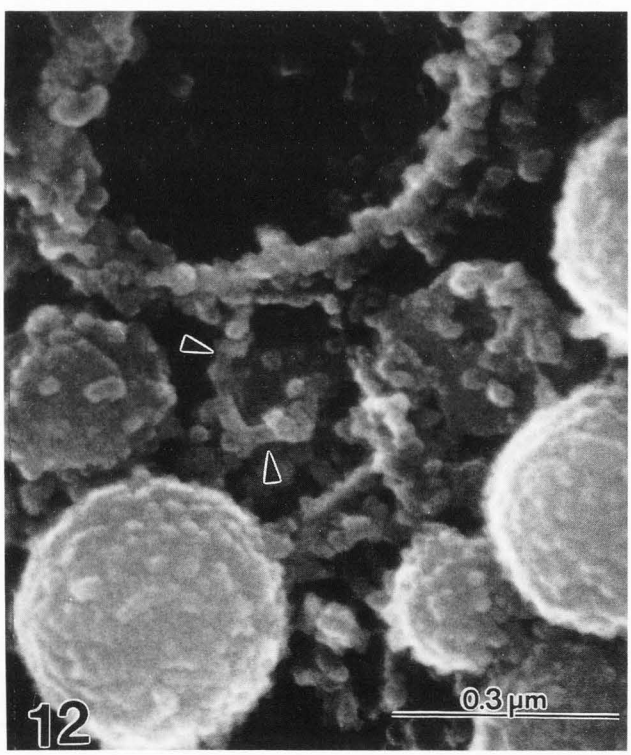
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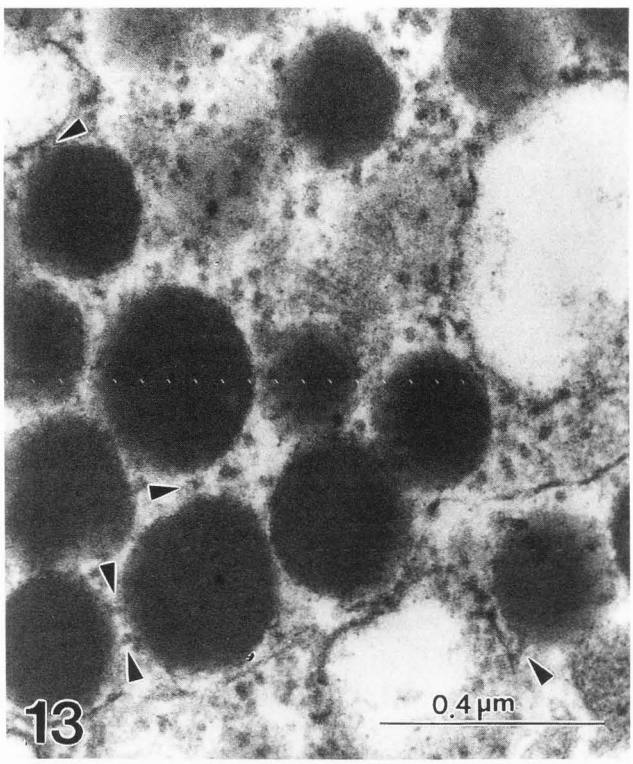

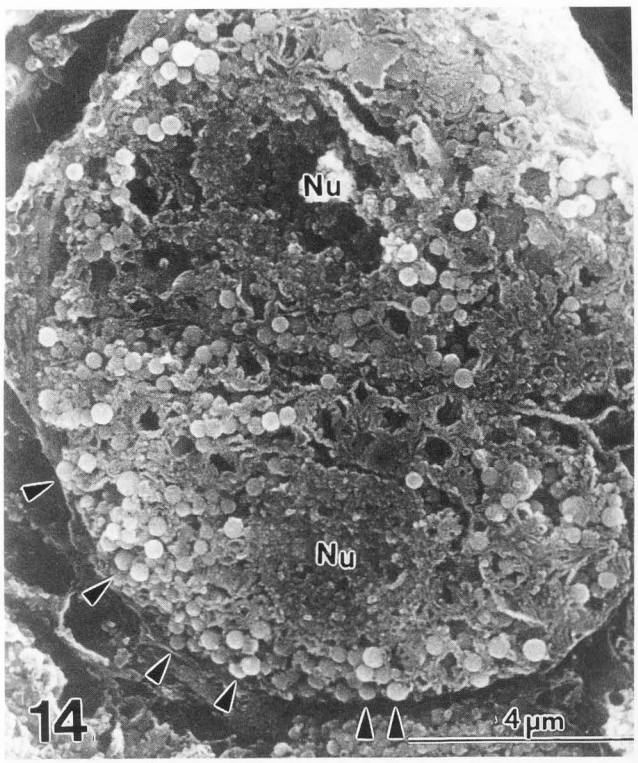

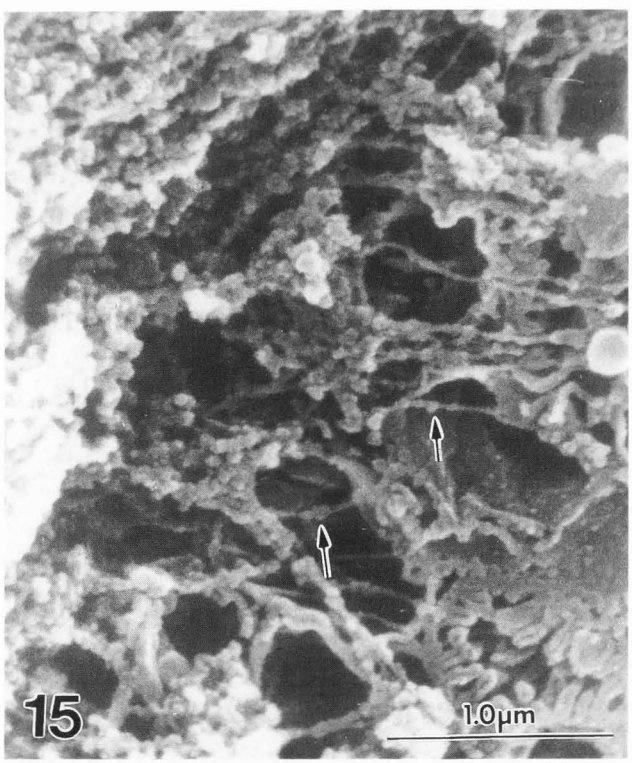

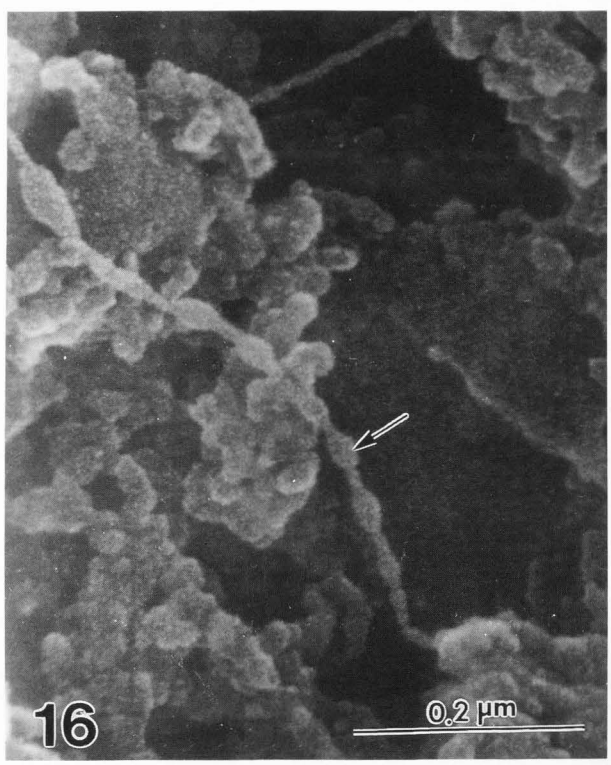

Figure 13. TEM shows the spot-like granule-granule connections (arrowheads).

Figure 14. Granules (arrowheads) close to the cell membrane appear ready for release. Nu: Nucleus. (Pt coated).

Figures 15 and 16. Microfilaments with intermittent expansions (arrow). (Pt coated).

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Discussion with Reviewers

R.O. Kelley: What predictions can be made about structures which might correlate to functional activities of these cells? What do immunocytochemical localization of marker molecules tell us about organelles and structures within these cells?

Author: Functional activities of pituitary secretory cells can be described by their ultrastructural appearance. For instance synthetic activity is indicated by the presence of rough endoplasmic reticulum, the complex structure of the Golgi apparatus, the formation of granules inside Golgi and the amount of mature granules in the cytoplasm. Whereas exocytosis may be observed when the cell is active in secretion.

Immunocytochemical methods not only can tell the distribution of the specific cell type, but also show localization of large granules in the trans-most cisternae of the Golgi apparatus (Hashimoto *et al.*, 1987). Therefore we can use immunolabeling technique to study the Golgi structure and its association with large hormone granules.

R.O. Kelley: What structural features distinguish individual cell types in this organ?

Author: The structural features used for the identification of the cell types are: the size and shape of the cell, and the size and shape of hormone granules. However, these elements were previously studied under several different physiological conditions, so that the morphological classification could be verified by immunofluorescent, immunocytochemical or classical staining techniques.

R.O. Kelley: Do fixation artifacts confuse interpretation of high resolution SEM images, and if so, what might be factors which lead to that confusion?

Author: Yes, unsatisfactory fixation may cause a number of morphological alterations, including changes in size, shape and surface appearance, which can confuse interpretation regarding the structure and function of any subcellular organelle.

R.O. Kelley: What other techniques might be employed which could produce similar images to provide structural correlation for purposes of credibility?

Author: There are two techniques which may be useful:

a. The method reported by Scala *et al.* (1990). Tissues were subjected to the osmium maceration proce-

dure and then embedded in acrylic resin. Semi-thin sections of the tissues blocks were first provided for LM and then examined by SEM.

b. The reconstruction of scanned images by confocal scanning microscopy and computerized imaging system (Tai and Albrecht, 1992).

Reviewer III: Considering resolving power of the methods used in this investigation, why does the author feel that HR-SEM "correlates" with the finding of TEM?
Author: Conventional SEM cannot produce high resolution images for intracellular studies. HR-SEM provides 3-dimensional images of the cryofractured surface of intracellular features which in TEM would require serial reconstruction methodology using sections. It is important to combine both HR-SEM and TEM in order to obtain complimentary information from the same cell with similar ultimate levels of resolution.

Reviewer III: Please expand on the significance of joint rather than single granule release from the pituitary cells.
Author: There are many instances when the body urgently requires a considerable amount of a certain hormone. Radioimmunoassay studies have indicated that hormone levels can increase several hundred fold within very short time after receiving a stimulus. When HR-SEM reveals the existence of granule-granule connections and clusters of granules in distinct cytoplasmic compartments, this leads us to think that the hormone secretory cell may be structured to permit combined granule release in order to rapidly meet body requirement.

J.C. Curtis: First, and foremost, immersion fixation of labile tissues, such as the brain and pituitary, fell into disrepute 20 years ago (Hayat, 1980). It makes little sense to apply high resolution SEM to a study in which immersion fixation has been employed with all the attendant problems resulting from the variations in the quality of fixation. This deficiency in this manuscript is readily demonstrated by comparing Figures 2 and 3. While the secretory granules and cell nuclei appear well-preserved in Figure 2, subcellular details are poorly preserved and the cell nuclei appear to have shrunk considerably (note that these two figures have the same magnification).

Authors: There is no doubt, that fixation for any animal tissue can be performed previous to immersion using perfusion techniques. However, it is not always possible to use this technique. There are many factors such as the time, or the effects of anesthesia that should be considered. For TEM pituitary samples, we have practiced for years with both immersion and perfusion methods for fixation and compared the results through measuring of the size of different organelles. We, sometimes, do not want to risk anesthetizing and perfusing an experimental animal after a certain period of treatment because of undesirable physiological changes associated with the required anesthetic agent. While the present procedure is somewhat of a compromise, it is, based on our experience to date, the most reliable and practical approach. We are however continuing to evaluate techniques in order to: 1. minimize various sources of artifact including pre- and post-mortem changes and 2. develop procedures which are compatible with preparative procedures for SEM, TEM, and which allow immunolabeling of the tissue.

Additional References

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