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# Polysaccharide Cytochemistry of the Bushy Cells in the Pig *Ascaris* (*Ascaris suum*)

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## Introduction

In the pig *Ascaris*, it has been well established that the bushy cells are giant cells in themselves (Chitwood and Chitwood, 1950; Bolla, *et al.*, 1972). Our previous studies on the bushy cells have revealed that at least two types of polysaccharides are involved in the cytoplasm (Ishikawa and Yamada, 1972). In the present report, therefore, the cytochemical properties of the polysaccharides were studied extensively with a variety of techniques of light and electron microscopic cytochemistry of polysaccharides. The results obtained indicate that the polysaccharides within the cells are mucosaccharide and glycogen in nature. The present results have, in addition, shown that there is an extracellular laminar structure which con tains acid mucosaccharides and is apposed closely to the plasma membrane of the bushy cells. The cytophysiological significance of all these polysaccharides is discussed.

## Materials and Methods

Twenty five female pig *Ascaris* (*Ascaris suum*) were obtained from adult pigs sacrificed in the slaughter house of Nagoya City, Japan. These worms were kept alive in physilogical Ringer solution maintained at 37C, until the time of removal of bushy cells. Bushy cells with and without neighboring tissues were removed from these worms.

1 Preparation of tissues for ligh microscopy

Immediately after the removal from the donors, bushy cell-containing tissues were placed in either of the two fixatives, Bouin's solution or 10% formalin containing 2% calcium acetate (Leppi, 1968). Fixation was performed at room temperature for periods ranging from 24 to 72 hours. The tissues were dehydrated in graded ethanol series and embedded in paraffin. Sections were cut at 6 to 8 $\mu$, deparaffinized, hydrated and subjected to the following staining procedures of light microscopic cytochemistry for polysaccharides and proteins; periodic acid-Schiff (PAS) (McManus, 1946), alcian blue (AB) (pH 1.0) (Lev and Spicer, 1964), AB (pH 2.5) (Pearse, 1968), AB (pH 2.5)-PAS (Mowry, 1963), aldehyde fuchsin (AF) (Pearse, 1968), AF-AB (PH 2.5) (Spicer and Meyer, 1960), high and low iron diamine (HID and LID) (Spicer, 1965), HID-AB (pH 2.5) (Spicer, 1965), LID-AB (pH 2.5) (Spicer, 1965), colloidal iron-ferrocyanide (Pearse, 1968), PAS-acriflavine (Yamada, 1969), coupled tetrazonium (Pearse, 1969), ninhydrin-Schiff (Pearse, 1968), 2-hydroxy-3-naphthoic acid hydrazide (HNAH) diazo blue B (Barrnett and Seligman, 1958) and 2, 2′-dihydroxy-6, 6′-dinaphthyl disulfide (DDD) diazo blue B (Barrnett and Seligman, 1954). Some PAS-stained sections were treated with bacterial amylase (preparation of Ueda Chemical Works, Osaka, Japan; 1.0% solution of the enzyme in buffered neutral saline at 37C for 1 to 2 hours) prior to staining, in order to confirm the presence of glycogen. After staining, sections were dehydrated and mounted in the usual manner.

2 Preparation of tissues for electron microscopy

Immediately after the removal from the donors, bushy cell-containing tissues were cut into tiny pieces and fixed in chilled (4C) phosphate-buffered (pH 7.2) 2% osmium tetroxide or in chilled (4C) cacodylate buffered (pH 7.2) 2.5% glutaraldehyde supplemented with 2% paraformaldehyde for 0.5 to 2 hours. The osmium tetroxide-fixed tissues were dehydrated in ethanol series of ascending concentrations and embedded in Epon 812, as prescribed by Luft (1961). The glutaraldehyde - paraformaldehyde - fixed tissues were rinsed in cacodylate buffer solutions (pH 7.2), sliced into 40 to 60 $\mu$ thick sections with a tissue sectioner (Smith-Farquhar type) and subjected to either periodic acid-thiosemicarbazide-osmium tetroxide (PA-TSC-OS) (Hanker et al., 1964) or colloidal iron (Wetzel, *et al.*, 1966) procedures for elect ron microscopic cytochemical demonstration of neutral or acid polysaccharides. Some of the PA-TSC-OS-stained tissues were digested with bacterial amylase (preparation of Ueda Chemical Works, Osaka, Japan; incubation in the enzyme solution under the same conditions as those employed for specimens for light microscopy) prior to staining. The colloidal iron-stained tissue sections were postfixed with cacodylate-buffered (pH 7.2) 2% osmium tetroxide at room temperature for 1 to 2 hours. All these tissues for electron microscopic cytochemistry were subsequently dehydrated in ethanol series of ascending concentrations and embedded in Epon 812 (Luft, 1961). From materials for both cytology and cytochemistry ultrathin sections were cut on a Porter-Blum microtome (MT-1 type), mounted on copper grids, stained doubly with uranyl acetate (Watson, 1958) and lead citrate (Reynolds, 1963). The stained sections were examined in a Hitachi (HU 11 D or HS 8) electron microscope. Electron microscopic pictures were taken at original magnifications of 2,000 to 10,000 times and photographically enlarged as desired.

## Results

1 Light microscopy

As Bolla *et al.* (1972) reported previously, the bushy cells of the pig ascaris are provided with abundant cytoplasm showing villous configurations in outline and contain a huge oval nucleus.

When sections of calcium acetate formanli-fixed *Ascaris* tissues are stained with the PAS technique, the cytoplasm of the bushy cells is shown to be loaded with a variable amount of PAS-reactive granules of different sizes (Figs. 1–3). These granules exhibit distribution patterns which are variable with locations within the cytoplasm, the granules tend to be condensed right beneath the plasma membrane in the peripheral parts of the cytoplasm forming villous configurations of different sizes (Figs. 1 and 2), while in the cytoplasmic loci close to the nucleus they are accumulated in clusters of different sizes or scattered in variable concentrations in the rest of the cytoplasm (Fig. 3). Minor portions of the PAS-reactive granules of different sizes showing various distribution patterns are susceptible to digestion with amylase and hence are glycogen. In contrast, the majority of the PAS-reactive granules are resistant to digestion with amylase and exhibit positive cytochemical reactions for sulfated mucosaccharides such as AB (pH 1.0) (Fig. 4), AF, AF-AB, HID, HID-AB and PAS-acriflavine. In addition, these granules are found to react positively for AB (pH 2.5) (Fig. 5), AB (pH 2.5)-PAS, LID, LID-AB and colloidal iron-ferrocyanide, which are the reactions for non-sulfated and weakly sulfated acid mucosaccharides. The PAS-reactive granules resistant to digestion with amylase are also found to exhibit positive reactions for proteins such as coupled tetrazonium (Fig. 6), ninhydrin—Schiff, HNAH diazo blue B (Fig. 7) and DDD diazo blue B, even though the staining intensity varies with individual reactions; both the coupled tetrazonium and HNAH diazo blue B reactions of the granules are moderate or strong and

their ninhydrin-Schiff reaction is moderate in intensity, whereas their DDD diazo blue B reaction being exceedingly feeble or doubtful.

2 Electron microscopy

The ultrastructural features of both the cytoplasm and nucleus in the bushy cells were previously recorded in detail by Bolla *et al.* (1972), and all these features have been confirmed in the present observations.

As is apparent in Figure 8, a well developed laminar structure is found to be apposed closely to the plasma membrane of the bushy cells. The average width of this structure ranges from 24 to 100 m$\mu$. When the bushy cells are stained by the colloidal iron procedure, the outer surface of the laminar structure reacts vividly for colloidal iron (Fig. 9), and even in sites where the laminar structures are in direct contact with each other, they are reacted positively for the colloidal iron (Fig. 9).

In the cytoplasm of the bushy cells, membrane-limited granules of different densities and diameters (0.5 to 1.0 $\mu$) are scattered above all in the vicinity of the Golgi apparatus and in the peripheral parts of the cytoplasm (Figs. 8 and 9). The electron opacity of the granular inclusions gradually declines, as they come to the peripheral cytoplasm right beneath the plasma membrane. Hence, these granules are considered to be secretory in nature. The inclusions of these granules are reacted positively for PA-TSC-OS (Fig. 10). In the cytoplasm other elements exhibiting positive PA-TSC-OS reaction are seen which are particles ranging in diameter from 10 to 90 m$\mu$ (Fig. 10). If the bushy cells are subjected to digestion with amylase, these particles disappear (Fig. 11), indicating that they are composed of glycogen. The two types of PA-TSC-OS-reactive elements, secretory granules and glycogen particles do not acquire electron density due to reaction product, if prior treatment of periodic acid (PA) is omitted (Fig. 12).

## Discussion

From not only the previous cytological data reported by Bolla *et al.* (1972) but the results obtained in the present study, it is apparent that the bushy cells of the pig ascarsis contain varying amounts of secretory granules and glycogen particles. In view of their positive PAS, AB (pH 1.0), AF, AF-AB, HID, HID-AB, PAS-acriflavine, AB (pH 2.5), AB (pH 2.5)-PAS, LID, LID-AB and colloidal iron-ferrocyanide reactions, the secretory granules are conceived to contain a substance which is a neutral and acid (both sulfated and carboxylated) mucosaccharide (Spicer *et al.*, 1967). In addition, the positive reactions of the granules for proteins such as coupled tetrazonium, ninhydrin-Schiff, HNAH diazo blue B and DDD diazo blue B are believed to substantiate that the secretory substances contain protein. The positive PA-TSC-OS reaction of the inclusions of secretory granules as revealed by electron microscopy is well correlated with the positive PAS reaction of the granules seen in light microscopic preparations. Taken altogether, therefore, the secretory substances are conceived to be a neutral and acid (both sulfated and carboxylated) mucosaccharide protein complex.

The cytophysiological functions of the bushy cells can not now be precisely determined. However, certain topographic morphology of the bushy cells and the results of previous experimental studies on them (Ishikawa, 1960) appear to suggest ideas about the possible functions of the bushy cells. In the pig *Ascaris*, the bushy cells are exposed to coelomic fluid between the lateral lines and intestine of the worm. Such topographic situation of the cells is taken to imply that the exchange of substances between the bushy cells and coelomic fluid easily takes place. According to the results of previous experimental studies on the bushy cells of the pig *Ascaris* (Ishikawa, 1960), moreover, pilocarpine and adrenalin administrations result in apparent swelling and shrinkage of the bushy cells respectively,

and the body color of the worm is changed from yellow pink to red, if the bushy cells are burnt by means of electrocautery. These morphological situations and experimental evidences are provocative of the concept that the bushy cells may be comparable in function to endocrine cells in higher animals and are releasing an active principle which regulates metabolic processes in the *Ascaris*. Such concept is consistent with the present result that the secretory substances of the bushy cells are composed of a mucosaccharide protein complex, inasmuch as in vertebrates glycoproteins and related substances are known to be a hormonal substance (Pearse, 1968). In line with this concept, furthermore, Chitwood and Chitwood (1950) suggested the possibility of an endocrine function for the bushy cells of nematodes.

In the cytoplasm of the bushy cells examined in the present study, glycogen partlcies are detected which exhibit positive PAS and PA-TSC-OS reactions. The cytophysiological significance of this polysaccharide in the bushy cells remains to be elucidated. Kemnitz (1972) advanced the view that the bushy cells have the ability to break down glycogen very rapidly and thus contribute to the general metabolism, since after injecting ascarids with glycogen, he found none in the cells. If the cells are endocrine in function, on the other hand, the polysaccharide may supposedly be a form of energy storage, inasmuch as in certain endocrine cells of vertebrates such as parathyroid glands (Yamada, 1965) this cytophysiological significance of the substance is substantiated.

In the bushy cells of the pig *Ascaris*, the laminar structure apposed to the plasma membrane reacts positively for colloidal iron. This is interpreted to indicate that acid mucosaccharides are involved in the laminar structure and appear to play a significant role for supporting the bushy cells in the coelomic cavity of the ascaris.

## Summary

In the bushy cells of the pig *Ascaris* (*Ascaris suum*) polysaccharides were studied by means of light and electron microscopic cytochmistry. In the cytoplasm of the cells mucous granules and glycogen particles are visualized. The mucous granules are composed of a neutral and acid mucosaccharide protein complex, in view of their positive reactions for periodic acid-Schiff (PAS), alcian blue, aldehyde fuchsin, iron diamines, acriflavine, colloidal iron-ferrocyanide, reagents for proteins and periodic acid-thiosemicarbazide-osmium tetroxide (PA-TSC-OS). The glycogen particles react positively for PAS and PA-TSC-OS, but are digested with amylase. The mucous granules are conceived to be secretory in nature, whereas the cytophysiological function of glycogen particles remains to be precisely determined. In addition to these intracellular polysaccharides, an extracellular laminar structure is found which contains colloidal iron reactive acid mucosaccharides and is apposed closely to the plasma membrane of the bushy cells.

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## ブタ回虫の花房状細胞における多糖類の細胞化学的研究

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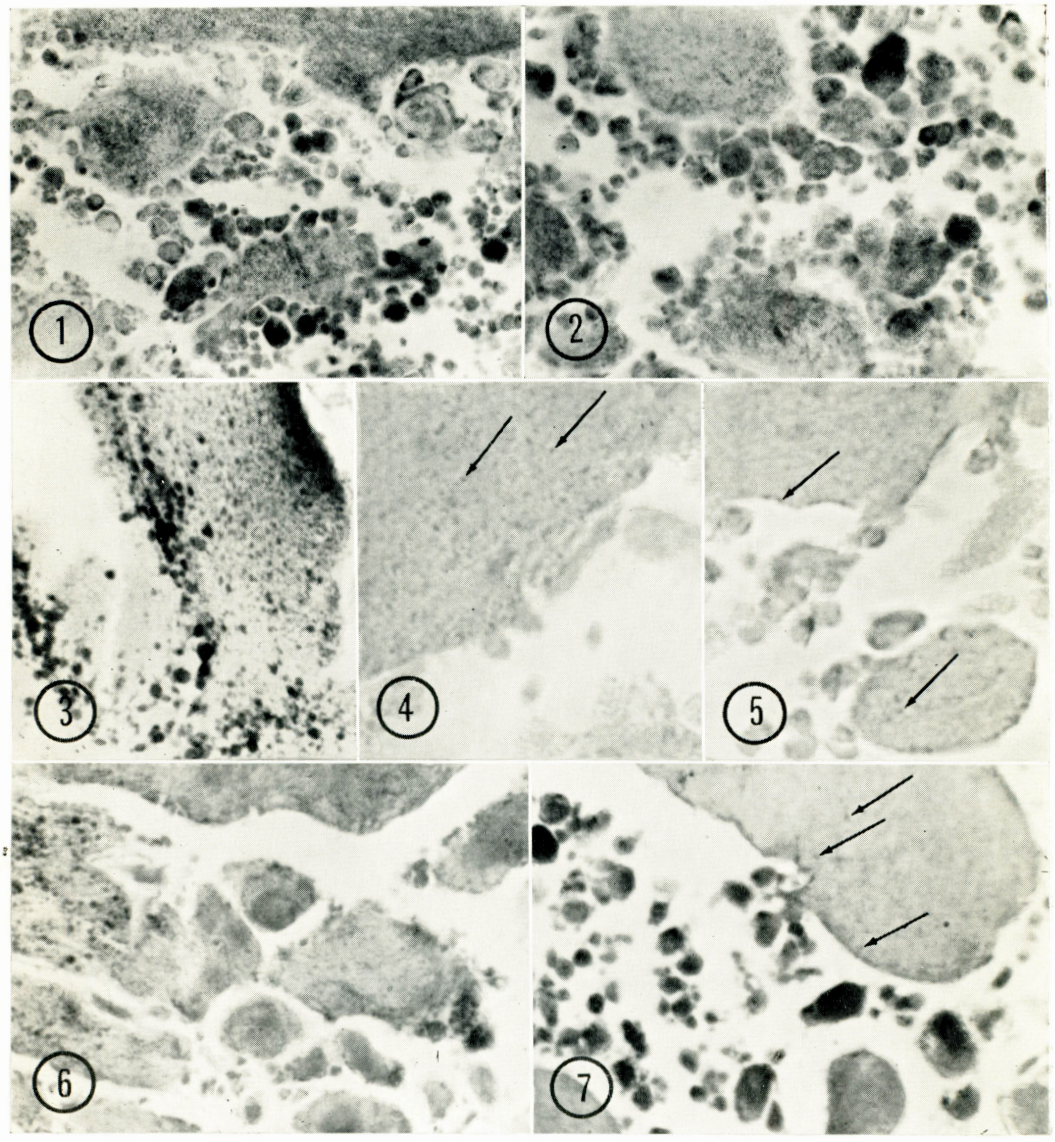
ブタ回虫の花房状細胞における多糖類を光学ならびに電子顕微鏡細胞化学の方法によつて研究した．ブタ回虫の花房状細胞の細胞質には粘液顆粒とグリコーゲン粒子が認められる．粘液顆粒は過沃素酸-Schiff，アルシアン青，アルデヒドフクシン，鉄ジアミン，アクリフラビン，コロイド鉄-フエロシアニド，蛋白質，過沃素酸-チオセミカルバジド-オスミウム酸などの諸反応陽性であるので中性ならびに酸性ムコ糖蛋白質複合体よりなる．グリコーゲン粒子は過沃素酸-Schiff および過沃素酸-チオセミカルバジド-オスミウム酸反応陽性でありアミラーゼによつて消化される．粘液顆粒は分泌物であると考えられるが，グリコーゲン粒子の正確な細胞生理学的機能は不明である．これら細胞内多糖類のほかに細胞外に，コロイド鉄反応陽性の酸性ムコ糖を含む板状構造が認められ花房状細胞の形質膜に沿つて存在する．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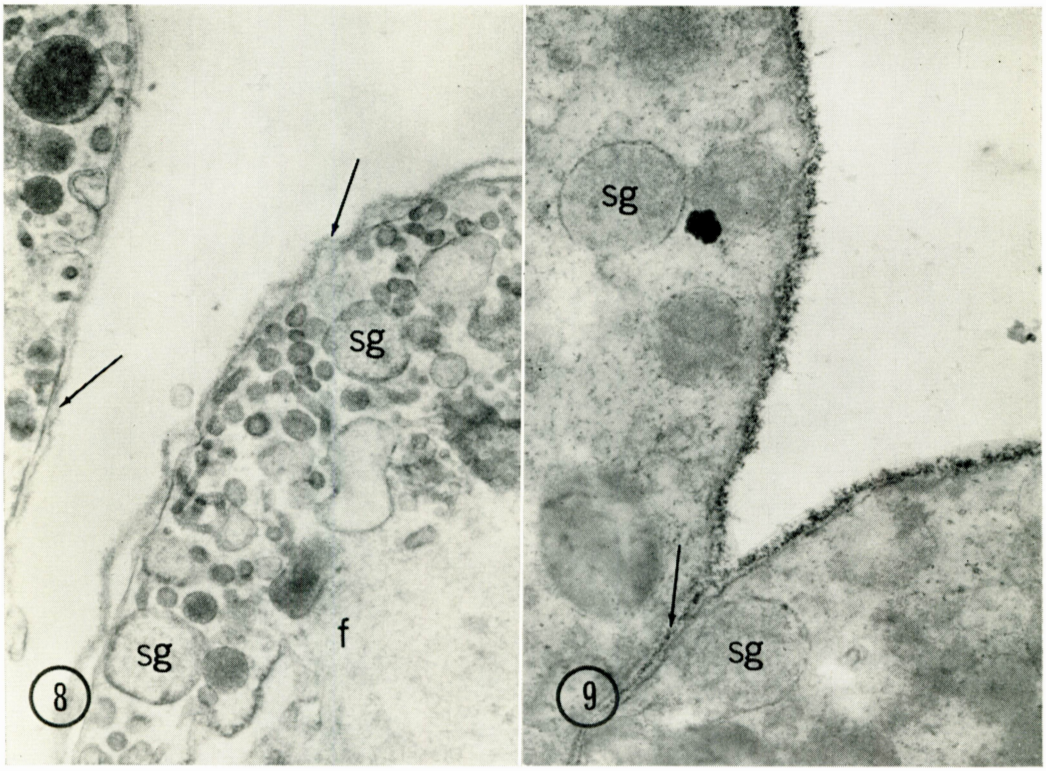
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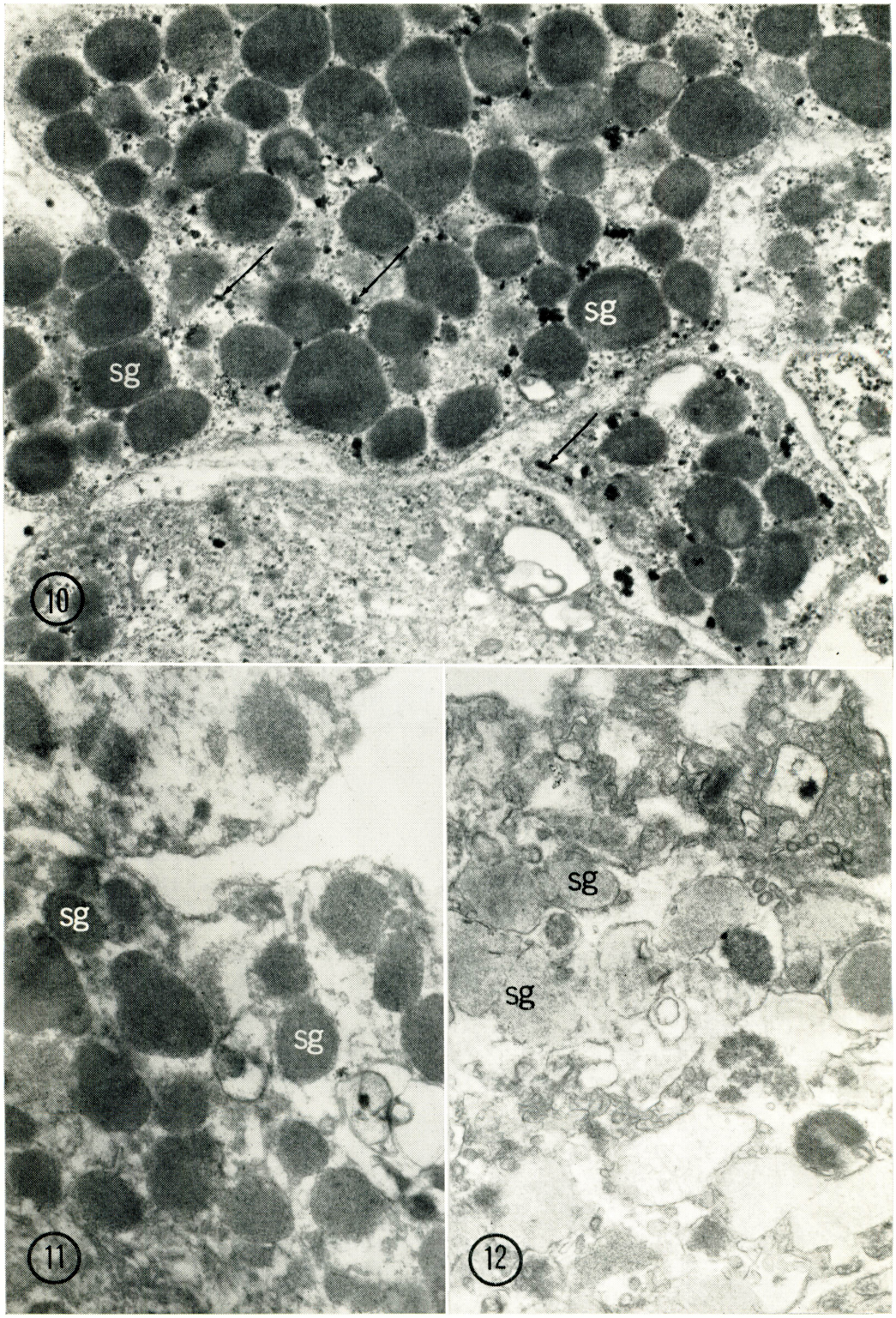
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## Legends of Figures

Figures 1–7. Bushy cells of *Ascaris suum*.

1. Peripheral parts of the cytoplasm. PAS-reactive granules of different sizes are visualized. PAS stained. × 400.
2. Peripheral cytoplasm. PAS-reactive granules of different sizes are distributed, above all in parts exhibiting villous configurations. PAS stained. × 560.
3. Juxtanuclear parts of the cytoplasm. Varying concentrations of PAS-reactive granules of different sizes are distributed in various loci. PAS stained. × 560.
4. Parts of the cytoplasm. Granules of different sizes (arrows) are detected which exhibit positive alcian blue reaction. Alcian blue (pH 1.0) stained. × 680.
5. Parts of the cytoplasm. Granules of different sizes (arrows) are seen which exhibit positive alcian blue reaction. Alcian blue (pH 2.5) stained. × 680.
6. Parts of the cytoplasm. Granules of different sizes are observed which exhibit positive coupled tetrazonium reaction. Coupled tetrazonium stained. × 600.
7. Parts of the cytoplasm. Granules of different sizes (arrows) are visualized which exhibit positive HNAH diazo blue B reaction. HNAH diazo blue B stained. × 600.

Figures 8–12. Peripheral parts of the cytoplasm in bushy cells of *Ascaris suum*.

8. A laminar structure (arrows) is apposed closely to the plasma membrane enclosing the cytoplasm, in which secretory granules (sg) with inclusions of declining electron opacity are discerned. Filaments (f). Uranyl acetate and lead citrate stained. × 21,000.
9. The laminar structure apposed to the plasma membrane is reacted positively for colloidal iron. An arrow indicates the positive colloidal iron reaction of the laminar structures in direct contact with each other. Secretory granules (sg). Colloidal iron reacted and uranyl acetate and lead citrate stained. × 23,600.
10. Secretory granules (sg) and glycogen particles (arrows) are noted, both of which exhibit positive PA-TSC-OS reaction. PA-TSC-OS reacted and uranyl acetate and lead citrate stained. × 13,200.
11. Secretory granules (sg) exhibiting positive PA-TSC-OS reaction are noticed, whereas no glycogen particles are detected at all. Amylase digested, PA-TSC-OS reacted and uranyl acetate and lead citrate stained. × 14,500.
12. Neither secretory granules (sg) nor glycogen particles acquire electron opacity. TSC-OS reacted and uranyl acetate and lead citrate stained. × 13,200.