MICROSCOPICAL STUDY ON THE HYPOBRANCHIAL GLAND OF *HALIOTIS JAPONICA* REEVE WITH A NOTE ON THE RESTITUTION OF THE SECRETION

BY

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(*With 5 Text-figures and 1 Plate*)

The hypobranchial gland is a kind of mucus gland, common to all the prosobranchs, forming the most conspicuous organ in the pallial cavity both for its secretion and for its structure. Several papers have been published in which the gland is dealt with from the histological point of view, but the previous observations still leave incomplete in understanding of the relationship between the various kinds of cells found in the gland. The present study was undertaken with the purpose of making clear how, in the cells of this gland, the secretory granules first appear, then accumulate and are finally discharged. Parallel to the histological observation the frequency of discharge was calculated in every sort of cells constituting the gland.

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Historical

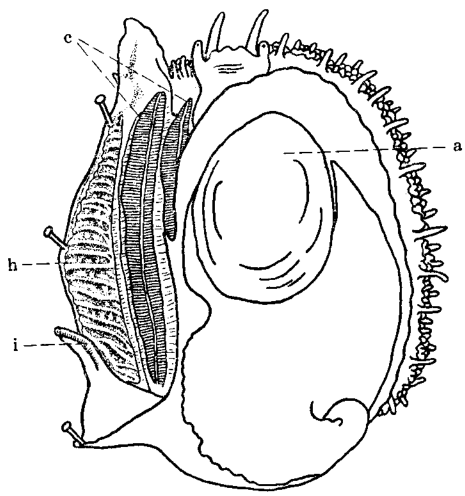
The prosobranchs in general are provided with organs homologous to the hypobranchial gland of *Haliotis*. Many papers have been published dealing with the glands microscopically, among which the following are closely related to the present study. WEGMAN ('84) wrote precisely on the anatomy of *Haliotis*, where the microscopical anatomy of this gland was also described. He classified the glandular cells into three categories: 1) cells composing the cellular membranes, 2) cells with granulous protoplasm and 3) cells with spindle-shaped corpuscles. These spindle-shaped corpuscles seem to be of compact nature and hyaline, but sometimes appear yellowish. On the fresh material he could observe that this hyaline substance was produced directly from the opaque.[1] According to him when the "étranglement circulaire" or "clapet", a cap put on each glandular cell is taken off the mucus can be emitted. Afterwards BERNARD ('90) in his voluminous papers on the homology of the organs of the pallial cavity throughout Gastropoda also observed the hypobranchial gland of *Haliotis*. He made an interesting observation on the mode of emergence of ciliated epithelium cells and mucous cells. In addition to these cells he observed the sustentacular cells which may correspond to WEGMAN's cells composing the membranes. After several years THIELE ('97) offered papers in which he also discussed the homology of the epidermal glands of Mollusca. According to him all the glandular cells in the epidermis of Mollusca can be grouped into two categories, "muköse" and "visköse". The latter is suspected by him to be poisonous in nature. The secretion of the hypobranchial gland of *Haliotis* is said to be "visköser Art", of granulous secretion. DAKIN ('12) on *Buccinum* concluded that the glandular epithelium of the hypobranchial gland is composed of 1) mucous cells, 2) ciliated cells and 3) neuro-epithelial cells. The last correspond to WEGMAN's cells without inclusions and to BERNARD's sustentacular cells.

1) This change is perhaps the postomortem swelling of the mucus in water.

To sum up the data obtained by all these authors the glandular cells of this gland contain fibres or granules which are all considered to be mucous in essential nature.

Material and Method

Haliotis japonica REEVE, a small kind of ear-shell, is widely distributed on the coast of the main land of Japan. They can be easily kept alive in the aquarium by feeding with brown sea weeds, but they are very sensitive to any deficiency of oxygen in the sea water.



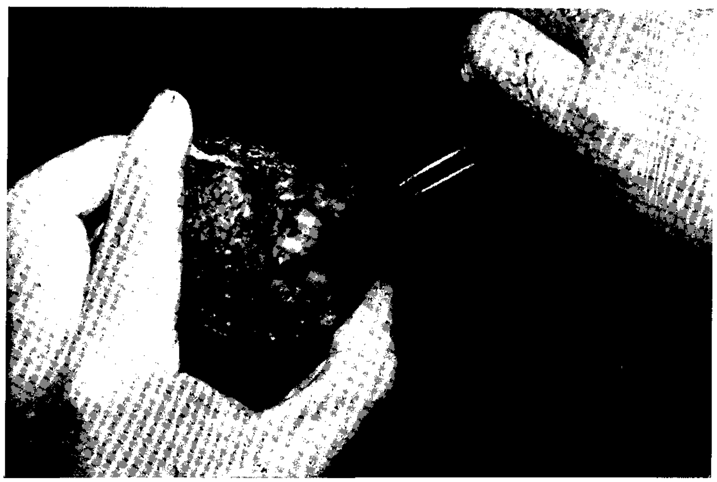
Text-fig. 1. Anatomy of the mantle region to show the hypobranchial gland (h) and ctenidia (c). The shell is removed and the mantle is cut along its upper attachment to the body mass, along the intestine (i). a, aductor muscle. ca×4/5.

The hypobranchial gland of this animal develops very well with its deep folds hanging down into the cavity (Text-fig. 1, h). The secretion is very rich in mucus and it is hyaline or white in colour. It is emitted from the pores arranged in a row along the margin of the shell. As a response to the mechanical stimuli given on the outer surface of the mantle cavity by means of forceps or other utensils the animal discharges the secretion of the gland as shown in Text-fig. 2. If the stimuli be repeated, however, the animal finally ceases the discharge resulting in the condition of the glandular cells free from secretion.[2] In the glands of thus operated animals,

2) As shown later the gland becomes empty only two hours after this operation (Text-fig. 5).

therefore, every step of the whole secreting process can be followed from the commencement.

To expose the gland, the shell should be taken off at first by cutting the large aductor muscle and then the pallial cavity is opened out by incision made along the intestine (Text-fig. 1). In the present study every half hour after the discharge, the glands of the operated animals were cut into pieces and thrown into vials containing fixatives. Various fixatives, such as BOUIN's, BOUIN-ALLEN's,



Text-fig. 2. The discharge of the secretion through the pores of the shell. The mechanical stimuli are given to the out side of the mantle with the tips of a pincette. ca×1/2.

ZENKER's and saturated aqueous solution of sublimate with 1:20 of acetic acid were used, but the best result was obtained by using the "Susa" following M. HEINDENHAIN. After having lain for eight hours in this fluid the material was directly removed into 90% alcohol with a few drops of iodine solution, and then into absolute alcohol, creosote-toluol, toluol, toluol-paraffin successively and at last imbedded in paraffin as usual. Paraffin blocks were cut 10 micra in thickness. The sections were stained with MALLORY's triple stain which was very convenient for differentiating every kind of secretory granulations. Toluidin blue and eosin were also used. For the detection of mucus the author employed 1% aqueous solution of toluidin blue or thionin which stains the mucus metachromasically red, while the other part remains blue. This red colour stands out very conspicuously when the stained sections are mounted with glycerin. But the mucus was also distinguishable by its blue colour with MALLORY's triple stain without difficulty (Fig. 1 and 11, m). To demonstrate the crystals of calcium salts, which are very sparely distributed only in the terminal part of the fold along connective tissue fibres, the material was fixed with absolute alcohol and then KÓSSA's silver nitrate method was applied on the paraffin sections. In this case safranin and light green were used for staining.

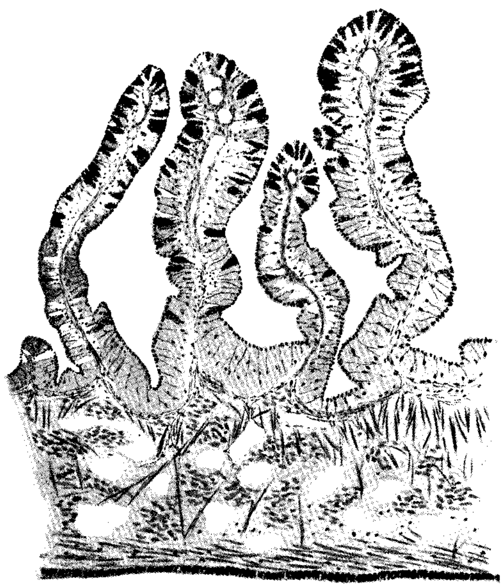
General Structure of the Gland

As already stated, the hypobranchial gland of this species is very well developed with deep folds hanging down into the pallial cavity, forming the modified region of the mantle. The folds are arranged parallel with each other (Text-fig. 1, h), and at a right angle to the median axis of the body. They are greasily white in colour, but their tips sometimes stain brownish.

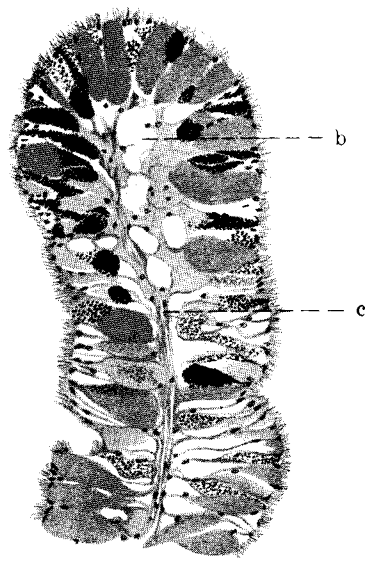
The secretion of the gland contains a great amount of mucus and therefore is abominable to handle. Its colour is either hyaline or slightly white owing to granulous inclusions. Concerning its function the author is of the same opinion as generally held that the secretion is for protection or defence from enemies on the one hand and for clearing dirt, sand grains or other foreign matter from the organs of the pallial cavity on the other hand.

The sections through the mucous gland at a right angle to the folds show that the fold is made up of tall glandular cells in a simple epithelial arrangement (Text-figs. 3 and 4). The glandular cells rest upon the axial connective tissue which is continuous to that of the mantle (Text-fig. 3). The blood vessels penetrate this median axial tissue, especially through the terminal part of the fold (Text-fig. 4, b).

The outer surface of the fold is fringed with ciliated cells (Text-fig. 4, and Fig. 11, c). The nuclei of these ciliated cells are small and ovoidal in shape, each containing nucleoli. They locate, as a rule, at the margin of the fold. WEGMAN's "étranglement circulaire" or "clapet" corresponds certainly to the marginal ciliated cells. We can see tall slender cells among glandular cells containing fluid content (Fig. 11, s). The name "sustentacular cells" of BERNARD ('90) is very fit for these cells because of their connection from ciliated



Text-fig. 3. Transverse section through the folds of the hypobranchial gland. ×40.



Text-fig. 4. Transverse section through a fold. b, blood vessel. c, connective tissue axis. ×360.

epithelium cells to the median connective tissue axis, between which the glandular cells are ordered as a row. The writer cannot consent to the name "neuro-epithelial cells" used by DAKIN ('12). WEGMAN ('84) said "cellule qui n'ont plus que la membrane" concerning these cells.

In papers hitherto published, the secretions, either mucous or granulous in appearance, have similarly been taken for the mucus. Indeed the gland, especially at the inactive phase is lined mostly by mucous cells between which three kinds of cells with granulations are sparely met with. There is no doubt that WEGMAN's spindle-shaped corpuscles represent merely the coagulations of mucus effected by reagents (Figs. 1 and 11, m), because of their metachromasically red staining by toluidin blue or thionin, while the granules are quite indifferent to this reaction. If the sections are stained with MALLORY's triple stain these secretory granules can be clearly differentiated into four categories: 1, fine granules stained red by acid fuchsin (Figs. 2 and 11, f), 2, large granules also stained red by the same dye (Figs. 3 and 11, l), 3, medium sized granules of brown colour,[3] showing little affinity to dyes (Fig. 4) and 4, minute granules of violet colour in MALLORY's stain (Figs. 5 and 11, u).

Generally speaking, the cells that contain the first three kinds of granules predominate in the terminal part of the folds in an unoperated animal, though they are also found in less number in the middle region of them excepting the cells containing the granules of the third category. The occurrence of the latter kind of cells seems to be limited in the terminal part of the fold both before and after the operation, and they correspond with all probability with WEGMAN's yellow coloured cells.

The cells having granules of the last category are found similarly scattered corresponding to the cells containing the granules of the first two kinds, but they are sharply distinguished from the latter not only by having a smaller wedge-shaped cell-body but also by their characteristic position underneath the cells with other kinds of granulation. As mentioned already, these cells contain minute protoplasmic violet granules as shown by MALLORY's triple staining method, and are considered to be the original and undifferentiated cells from which all other kinds of cells will be derived in course of secretion as described in detail under the next heading.

3) These brown coloured granules are very resistant against both acids and alkalines. So this colour may certainly be due to the presence of melanin in the granules.

The Formation of Secretion

The formation of secretion, either of mucus or secretory granules, could be first found in the preparations of the gland from one and a half to two and a half hours after operation, and again in those about nine and a half hours after operation.

Formation of mucus: The first sign of mucus formation is frequently observed in the cells with fine protoplasmic granulations, located in the basal part of the epithelial arrangement of the cells. The cells are characterized by the presence of a vacuole in which the mucus has already been accumulated (Figs. 6, 10 and 11, u). A nucleus of depressed form is always found attached to the bottom of the vacuole. The mucus seems to be produced in close association with the nucleus, since, during this formation, the latter takes basic dyes with difficulty and becomes to contain several nucleoli. In addition to this, it is always noticed that the nuclear surface where the mucus comes into direct contact shows an irregular contour. If the method of demonstrating thymonucleic acid after FEULGEN is employed, a pitted appearance of the nucleus, which is stained red with basic fuchsin, can be seen (Fig. 10). These tiny pore-like figures correspond to the nucleoli mentioned above.

This formation of mucus is thought to be carried on very rapidly, if it be recollected that such cells are very few in occurrence as compared with those in the course of the formation of other secretions.

Formation of fine granules: In other cells, considered to be the same kind as those in which the mucus is produced, a number of fine granules, stained red with acid fuchsin, are very often found (Fig. 7). It is these cells that contain ultimately the granules of the first category. Differently to the mucus cells, in the cells of this kind no conspicuous changes of the nucleus are observed. The granules gradually increase in number in parallel to the growth of the cell-body and finally occupy the greater part of the cytosome where a certain number of protoplasmic granules still remain. In brief, this kind of granules is produced directly from the minute protoplasmic granules, as every kind of granules of intermediate colour can be found from original violet to the ultimate red.

Formation of large granules: The large granules apparently much resemble the fine granules mentioned above in respect to the staining affinity to acid fuchsin, as well as in the mode of their formation. But in this case the granules grow markedly in course of time and their number is considerably less than in the preceding case. On the other hand, the nucleus increases in diameter ana fluidal vacuoles of large size appear in the protoplasm (Fig. 8). There are found no granules to be considered as an intermediate colour between violet and red. This compels the supposition that the granules of this kind are essentially different in genesis from the fine granules in spite of similar affinity to acid fuchsin.

Formation of brown granules: The production of granules of this kind is confined only to cells composing the terminal part, and unfortunately the author failed to observe the earliest stage at which such granules first appeared. Different from granules of the preceding two kinds, the brown granules, so far as the present observation shows, are not found uniformly scattered as red-stained ones, but aggregate into small masses, enclosed within vacuoles (Fig. 9). The fully developed gland cells contain granules from brown to hyaline in colour, the latter being stained orange or green with MALLORY's triple stain.

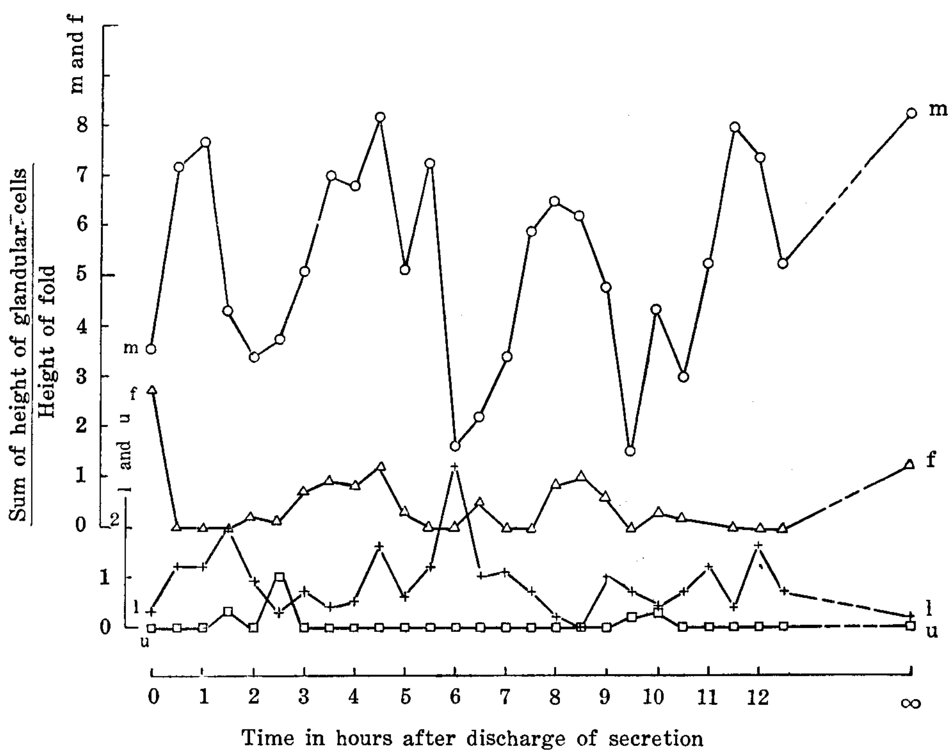
The Restitution of Glandular Cells

As a rule, every kind of the glandular cells varies in size according to the advance of the secretion. At least in the present case the height of the secreting cells evidently indicates the grade of secretion. Making use of this point an attempt was made to learn how the restitution is carried on in the glandular cells after artificial discharge of their secretion. In order to obtain the relative amount of secretion of the same kind, at first, animals of equal size (ca. 6 cm. in length) were selected, as it is expected that in animals of the same size the size of cells of the same kind should be approximately similar so far as they are in the same stage in secretion; then the cells were measured in paraffin sections. The folds for measurement in this way were taken from the sections through the middle region of the glandular lamellae. Consequently the value, sum of the heights of each kind of glandular cells divided by the height of the fold, may give the relative amounts of every secretion.[4)]

As the graph shows the volume of mucus and fine granules attain to their minima at about two, six and ten hours after the discharge of secretion, i.e. at four hours' interval (Text-fig. 5). And their maxima occur at about four, eight and twelve hours after the discharge, also having four hours' interval. Very interesting to say, the undifferentiated cell can be seen at from one and a half to two and a half and again at from nine and a half to ten hours after the operation. This frequency of the appearance of undifferentiated cells directly before the maximal volume of mucus and fine granules favours the writer's opinion that the latter are directly derived from the former. Theoretically there must be another appearance of the undifferentiated cells at about six hours after the discharge of the secretion. The large granules always appear when mucus and small granules are at their minima. In the glands of the unoperated animals the mucous cells take the greater part and the cells with fine granules also amount to a pretty large volume, while that of the large granules is very small. This rhythmical restitution continues for a while and gradually the curves become chaotic till they attain to the equilibrium (Text-fig. 5, ∞).

4) Though the cells with brown granules predominate in the unoperated animals they show very indefinite frequency after the operation. So it is unnecessary to try the graphical analysis; yet they must be derived directly from the undifferentiated cells with protoplasmic granules as described in the foregoing paragraph.

Recently HIRSCH ('31) advocated his "Theory of fields of restitution" where he insisted upon the presence of rhythmical restitution in many phenomena in the animal bodies. He and his co-workers have presented many papers where the rhythmical restitution, especially in the gland, is proved by means of HIRSCH's "Stufenmethodik"[5]. Here the writer can offer another favourable instance of the rhythmical restitution of the gland on the hypobranchial gland of the ear-shell.



Text-fig. 5. Graphical analysis of the restitution of the secretions after their discharge. Detailed explanations in the text. m, mucus. f, fine granules. l, large granules. u, undifferentiated protoplasmic granulations.

5) The "Stufenmethodik" was invented first by HIRSCH ('15) on the livers of flesh feeding gastropods, where he counted the number of cells of every stage during the formation of the secretion after feeding. Together with this experiment he also measured the strength of enzyme after meals.

Summary

1. The secretory cells of the hypobranchial gland of *Haliotis japonica* REEVE which have been thought to consist only of mucous cells prove to be grouped into four categories; mucous cells, cells with fine oxyphilic granules, cells with large oxyphilic granules and cells with brown granules.

2. These terminal stages of the secreting cells are all derived from the undifferentiated cells with violet granules in MALLORY's stain. In the production of mucus their nuclei seem to be closely associated.

3. By dint of graphical analysis this genetical relationship between every kind of secreting cells can be verified and the rhythmical restitution of glandular cells after the discharge of secretion is also detected.

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Plate VI

Explanation of Plate VI

All the figures of the plate were drawn at the level of the stage of the microscope, with the aid of ABBE's drawing apparatus. Figs. 1 to 10 were drawn with LEITZ aplanatic objective apart. 1.30 and ocular 6, t. l. 200 mm. The magnification is about 1800 times. Fig. 11 was drawn with LEITZ objective 8 and ocular 10, t. l. 200 mm., the magnification being 1200 times.

Figs. 1 to 9 are drawings from the sections of the material fixed with "Susa" after M. HEIDENHAIN and stained with MALLORY's triple stain. Fig. 10 is a drawing from the section of the material fixed with the same fixative as above. Red stain is the reaction of thymonucleic acid after FEULGEN and the counter stain is with trypan blue. Fig. 11 is a drawing from the section fixed as above and stained with toluidin blue and eosin.

Fig. 1. Mucous cell.

Fig. 2. Cell with fine secretory granules.

Fig. 3. Cell with large secretory granules.

Fig. 4. Cell with brown secretory granules.

Fig. 5. Undifferentiated glandular cell.

Fig. 6. Undifferentiated glandular cell in the course of its production of mucus.

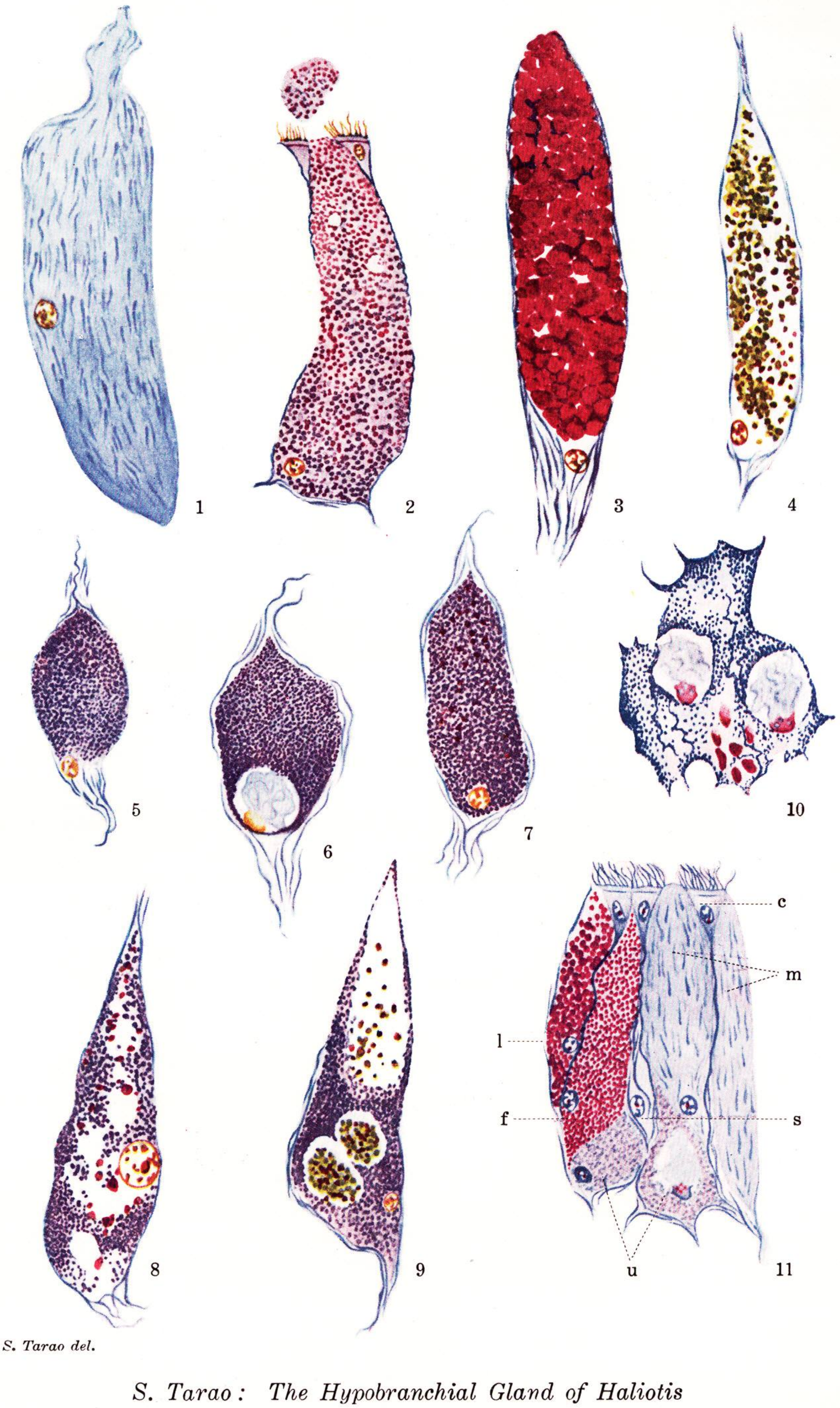
Fig. 7. Undifferentiated cell in the course of its production of fine secretory granules.

Fig. 8. Undifferentiated cell in the course of its production of large secretory granules.

Fig. 9. Undifferentiated cell in the course of its production of brown secretory granules.

Fig. 10. Undifferentiated cell in the course of its production of mucus. The presence of thymonucleic acid is showed as red stain with basic fuchsin after FEULGEN.

Fig. 11. A clump of glandular cells. c, ciliated epithelium cells. s, sustentacular cells. m, mucous cell. f, cell with fine secretory granules. l, cell with large secretory granules. u, undifferentiated cells; one of which is in the process of mucus production.



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S. Tarao: The Hypobranchial Gland of Haliotis