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Morphological Studies on Macronucleus of *Spirostomum ambiguum*

I. The Macronuclear Structure in Interphase

By

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Spirostomum ambiguum, a heterotrichous ciliate belonging to the family Spirostomidae, has been studied by some investigators since it was originally described by STEIN (1867). But with regard to the nuclear structure in the interphase and its morphological alterations during dividing stages, few studies have been performed beside those of STEIN and BISHOP ('25).

In the present paper, the macronuclear components and their nature in the interphase are dealt with to contribute to the problems on the macronucleus of the ciliated protozoa which has not clearly been understood as yet.

Best gratitude should be expressed to Prof. K. NAKAMURA for his kind guidance during the course of this study.

Materials and methods

The animals used are obtained from a basin in the botanical garden of our university. Two races are found: one is of a body length of ca. 2500 to 3000 μ at maximum growth, the other less than 1500 μ; no morphological difference can be observed except in the body length.

The culture methods employed are as follows: 1) The animals collected from natural source are centrifuged 3 times with the sterile physiological balanced solution (TAYLER and STRICKLAND, '35, cited by KIRBY). 2) 5 grams of clover hay and 1 gram of dry mud are added to 1 liter of filtrated pond water and then autoclaved for 20 minutes. 3) *Bacillus subtilis* is inoculated into the clover-mud infusion, and after 6 hours, 10 ml of pond water containing *Euglena* sp. or *Pseudomonas* sp. is added to this medium. 4) Animals prepared in (1) are poured into the culture medium (3) and incubated at 18° to 20°C. In this medium the fission rate of *S. ambiguum* is maintained 0.2 to 0.3 times per day. Clover hay can be replaced by strow hay.

For microscopical observation, some different methods are used in this study. Observations *in situ* are carried out under the phase microscope (Olympus: lens system, NH, NM, PM and PL). The direct stainings with acetocarmine and

acetic orcein are tried. To isolate the macronuclear apparatus, detergents, such as 0.5 to 2% solutions of Neugen (NH, ES–160, EA–120 and 80) and Katiogen H (Dai-ichi Chem. Co.) are employed.

Various fixatives are tested and both 10% neutral formalin and Zenker-formol-osmic fluid are found to be most favourable. Heidenhain's haematoxylin, basic dyes, i. e. toluidine blue and safuranine, acidic dyes, i. e. lightgreen and eosin, Feulgen's nuclear reaction and Unna-Pappenheim's methylgreen-pyronine mixture are employed as stains. In order to determine the chemical nature of the macronuclear components, the following reagents are used: trichloroacetic acid, perchloric acid, 0.1 to 2 molar NaCl solutions, DN-ase and RN-ase.

Observations

I. Strucutre of Macronuclear Apparatus

The macronuclear apparatus of *Spirostomum ambiguum* is composed of a number of macronuclear nodes which are arranged in a line mediated by narrow internodal parts like a rosary. The micronuclei of indeterminable number are observed attaching to the macronuclear node in "compact style".

The number of the macronuclear node varies greatly with individuals but there is no difference between the two races. Fig. 1 shows the variation of the number of macronuclear nodes in 355 individuals, which have been cultured in the medium described above (fission rate 0.2/day), collected with centrifugation and suspended in physiological solution for about 48 hours to prevent them from further nuclear alterations proceeding binary fission. From this counting it seems that the macronuclear apparatus of *S. ambiguum* is composed of 21 to 35

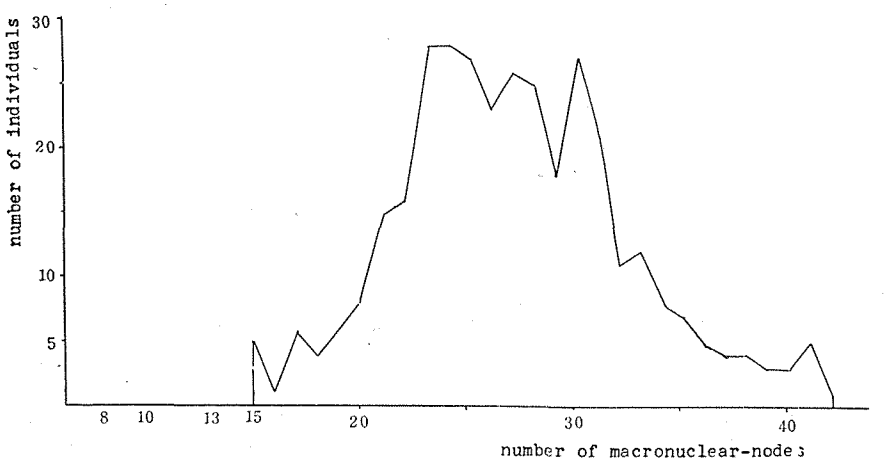


Fig. 1. Diagram of the variation of number of macronuclear nodes in 355 individuals of *Spirostomum ambiguum*.

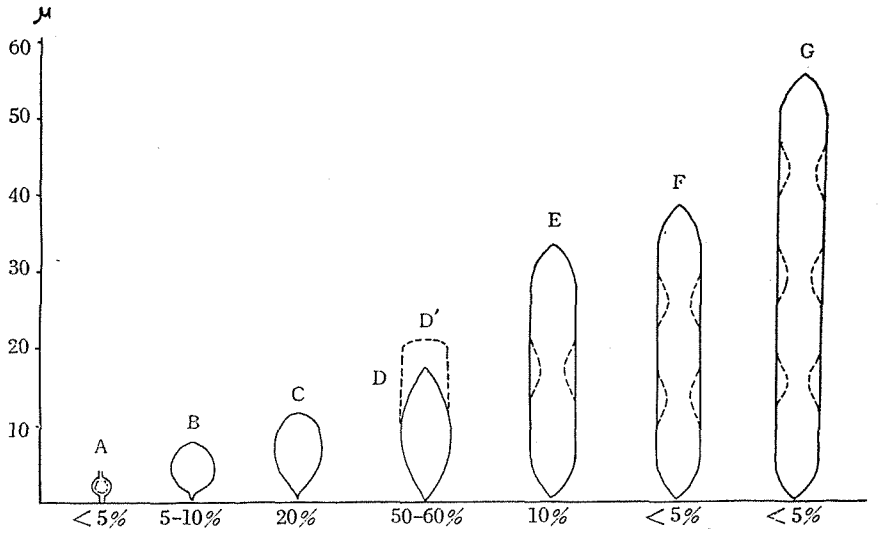


Fig. 2. Various modes of macronuclear nodes and their mean length in *Spirostomum ambiguum*.

macronuclear nodes (80.5%) in most cases. Fig. 2 shows the shape and size varieties of macronuclear nodes and their frequencies occurring in the same materials fixed with 10% neutral formalin. When fixed with Schaudin's fluid, macronuclear nodes have a more round shape. The interphase macronuclear nodes are usually spindle-shaped (D in Fig. 2), but at free ends they are rounded (C and D′ in Fig. 2). The small node (A or B) may probably be still in the course of growth following the multiplication of macronuclear node. On the contrary, the elongate ones (E, F and G) would devide themselves in daughter nodes, from 2 to 4, as shown with dotted line.

Generally, the macronuclear node has a somewhat twisted appearance. Under the phase microscope, the macronuclear surface which is partially wrinkled has elasticity and is resistive to pressure or to the manipulation of glass needle. On the macronuclear node which is isolated under the cover slit after shaking in the 0.5% solution of Neugen EA-120 for 5 minutes, the macronuclear membrane is recognized as a fine film with helicoidal ridge-like thickenings which give a twisted appearance to the whole macronuclear apparatus.

Within the macronuclear membrane, many small bodies which are granules, rods or lines in shape, and smaller spherical microgranules are observed. The former, designated as rod-shaped bodies for convenience, tend to aggregate to form many small masses and the latter are scattered in the node (Fig. 4, 5, 6, 7 and 8).

In the macronuclear node there are some clear areas of irregular shapes and various sizes. Occasionally some of these are lacking in microgranules, and in the fixed preparations, they are manifested as spherical vacuolated areas.

Internodal parts, described as filaments by some other authors, range from filament to prolonged spindle in shape. These parts seem to be composed of principally the same components as macronuclear nodes. Structural difference is not found between the macronuclear node and the internodal part, but rod-shaped bodies do not assemble in masses and, in fixed preparations, vacuolated area does not appear in this part.

Sometimes, spherical and homogeneous droplets of various sizes and number are observed in the macronuclear node. In some individuals they are scarcely observed, so they could not be enumerated as one of the constant components of the macronuclear apparatus.

With acetocarmine or acetic orcein, the rod-shaped bodies are stained and appear to be rods or threads in some cases. Microgranules stain, too, while vacuolated areas remain unstained.

These observations suggest that the structural components of macronuclear nodes can be distinguished as follows: nuclear membrane, karyolymph, microgranules, rod-shaped bodies which are variable in shape, vacuolated areas and spherical droplets which are occasionally observed.

The affinities of these structural components to various dyes were tested; the results are listed in Table 1. The rod-shaped bodies are Feulgen-positive, and show strong affinities to methylgreen and other basic dyes. Close examination

Table. 1. Affinities for dyes of the macronuclear components of *Spirostomum ambiguum*.

macronuclear components	affinities for dyes					
	Feulgen's N. R.	Unna-Pappenheim		basic dyes	acidic dyes	Heidenhain's haematoxylin
		methylgreen	pyronine			
nuclear membrane	+～±	+～±	+～±	+～±	±	+
karyolymph	±～−	±	±	±	±～−	−
microgranules	+	+	++	+	±～−	+
rod-shaped bodies	++	++	++～+	++～+	±	+
vacuolated area	−	− −	−	±	±	−
spherical droplets	−	±	++	+	+	+

++ strong, + medium, ± faint, − no affinity.

of the fixed and staind preparation reveals that they are somewhat granular in appearance and are 0.3 to 1μ, rarely exceed 5μ, in length. The microgranules also react considerably to Feulgen's, but they have more affinity for pyronine than for methylgreen. The diameter of this component is less than 0.3μ. The

nuclear membrane faintly stains with basic dyes. Contrary to these, the karyolymph and the vacuolated areas scarcely take any of the dyes used. The spherical droplets are strongly pyronine-positive and Feulgen-negative, as the nucleolus-like bodies in some ciliates are.

The structure of the macronuclear node and internodal part are schematically illustrated in Fig. 3.

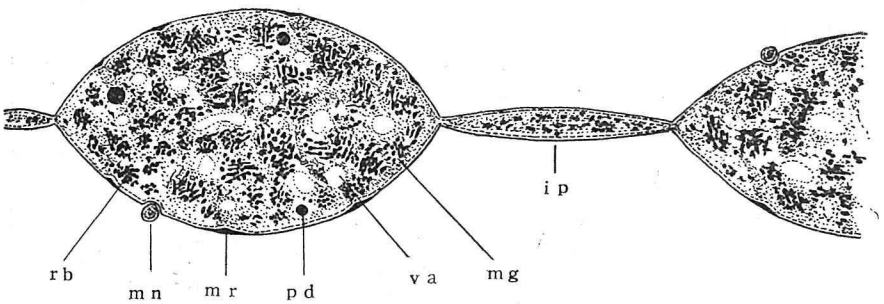


Fig. 3. The general structure of the macronuclear node and the internodal part of *Spirostomum ambiguum*.
rb, rod-shaped bodies. mr, ridge-like part of macronuclear membrane. mg, microgranules. pd, pyronine-positive droplet. va, vacuolated area. ip, internodal part. mn, micronucleus.

II. Results of Cytochemical Procedures on the Macronuclear Components

SCHNEIDER ('45) showed that cold trichloroacetic acid removes both inorganic phosphates and polynucleotides of lower molecular weight from the cells without affecting the nucleic acid. Application of his methods to smears of *Chilodonella uncinatus* was carried out by SESCHACHAR ('50). The same procedures are employed in the present study. As shown by SESCHACHAR, temperature is the factor of the selective action of trichloroacetic acid on nucleotides and nucleic acids. The smears of *S. amibiguum* fixed with 10% neutral formalin are treated with 10 to 15% trichloroacetic acid for 10 to 30 minutes at tempeatures varying from 5° to 90°C. At temperature below 45°C, any significant change is not observed. At higher temperatures, 60° to 75°C, considerable morphological changes are observed in the macronuclear node. By this treatment, microgranules tend to lose reactivity to Feulgen's and in some parts of the macronuclear node they become almost unstainable. Though the rod-shaped bodies turn more granular in appearance, they still give a positive reaction to Feulgen's. The vacuolated areas are somewhat impaired but remain as hollow spheres. For 15 minutes at 90°C, the entire nuclear apparatus becomes Feulgen-negative and unstainable with methylgreen and with other basic dyes. This indicates that the nucleic acid of the macronuclear apparatus is completely obliterated by hot trichloroacetic acid solution. Nevertheless, when residues of the rod-shaped bodies are stained with

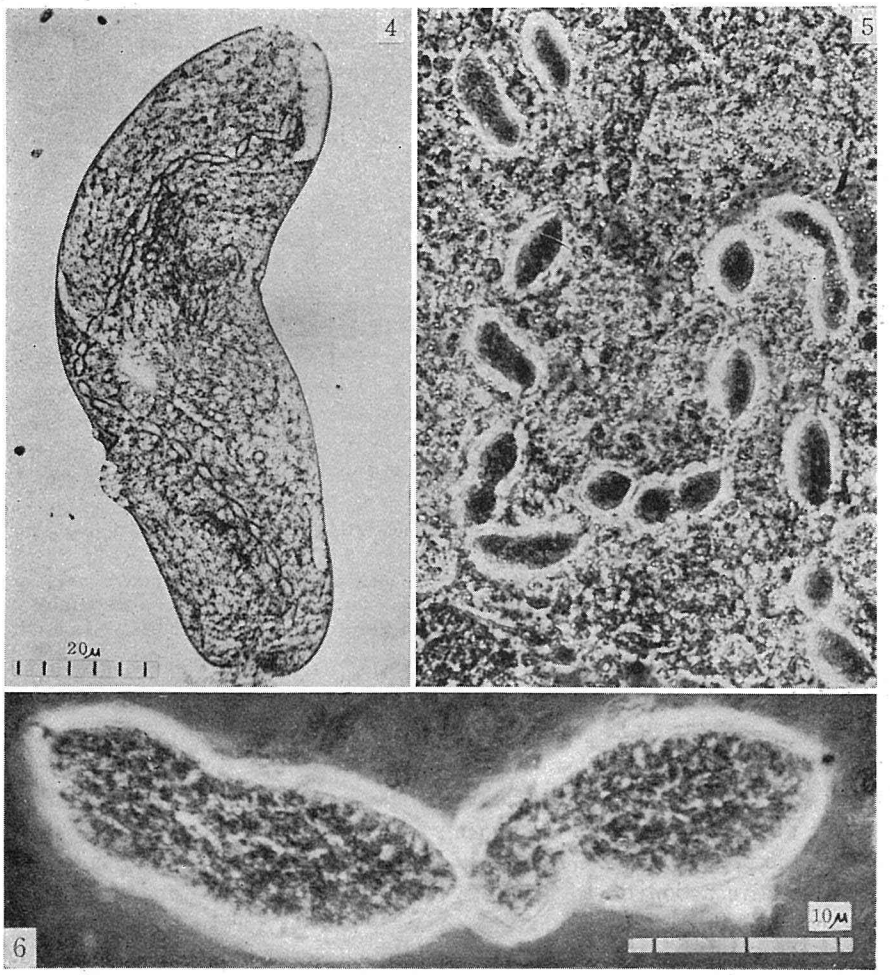


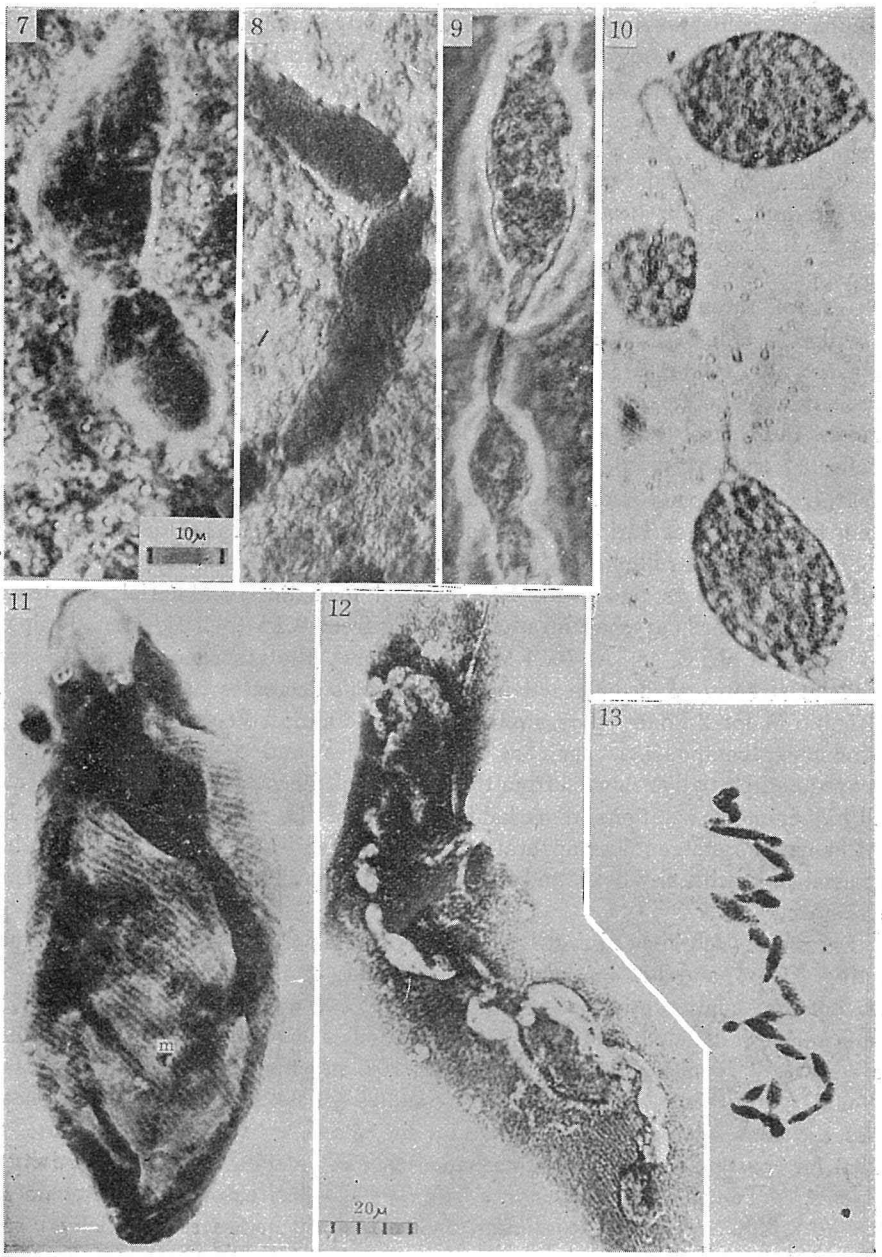
Fig. 4. The total view of *Spirostomum ambiguum*, somewhat contracted. The rosary-like macronuclear apparatus is shown.

Fig. 5. The interphase macronuclear apparatus, phase microscope (Olympus; PM)

Figs. 6–10. Isolated macronuclear nodes showing the structural components. Fig. 6, fixed with 10 % neutral formalin, phase microscope (PL). Fig. 7, unfixed, phase microscope (PM). Fig. 8, directly stained with acetic orcein, usual microscope. Fig. 9, with aceto-carmine, phase microscope (PM). Fig. 10, directly stained with acetic orcein, usual microscope (Zeiss, Apo. 1.5 mm. Obj.).

Fig. 11. An animal treated with 10% trichloroacetic acid for 15 minutes at 90°C. Residues of macronuclear nodes are shown (m). Total preparation: fixed with 10% neutral formalin, stained with Heidenhain's haematoxylin.

Fig. 12. Macronuclear apparatus treated with molar NaCl for 4 hours at 15°C. The entire macronuclear apparatus is removed from its sites. Total preparation: fixed with 10%



neutral formalin, stained with Heidenhain's haematoxylin.

Fig. 13. Macronuclear apparatus. Vacuolated areas are observed. Total preparation: fixed with Zenker-formol-osmic, stained with Feulgen-lightgreen.

Heidenhain's haematoxylin, it is shown that they are not damaged completely but in a loosely bound state, while the microgranules almost lost.

The action of nucleases, both DN-ase and RN-ase, on the constituents of macronucleus are examined. All the procedures are carried out following those described by SESCHACHAR '50) and MOSES ('50); tests are performed on the smears fixed with 10% neutral formalin. The action of DN-ase, 100γ/ml at 37°C, is rather gradual. The structure of the rod-shaped bodies turn incompact and the microgranules are gradually removed from peripheral parts of the node; thus the macronuclear basophility seems to decrease as a whole. An ability to react to Feulgen's of these components decreases during the digestion, but the affinities for Feulgen's and basic dyes still faintly persist. On the contrary, the action of RN-ase, 20γ/ml for 1 to 3 hours at 56°C, is not so remarkable except partial disappearance of microgranules. The affinity of the rod-shaped bodies for pyronine decreases and that of the microgronules is almost lost. The reactivities to Feulgen's are affected and decreased, too.

MIRSKY ('43) pointed out that cold molar NaCl extracts desoxypentose nucleoprotein from nuclei of various cells, and he also mentioned that the concentration is the factor in controlling the action of NaCl. In this study, his method is applied to the smears fixed with 10% neutral formalin. Materials are treated with NaCl solutions, at concentrations varying from 0.14 to 2 M, for 30 minutes to 4 hours at 15°C. At 0.14 M for 4 hours, the macronuclear nodes show a granular structure and the reactivity to Feulgen's decreases to some extent. At 0.4 to 0.6 M for 2 hours, they swell and become almost Feulgen-negative. The macronuclear components, such as microgranules and rod-shaped bodies, are destroyed and partially removed from the nodes, and the vacuolated areas obliterated. With higher molar solutions, remarkable changes of the structure occur. At 1 M for 4 hours, the macronulear apparatus becomes a series of hollow spheres, which are unstainable with any dyes, and it is indicated that all of the nuclear substances are removed.

ERICKSON ('49) showed that cold perchloric acid solution removes RNA without affecting DNA, and hot one removes both kinds of nucleic acids. Employing these hot and cold solutions, SESCHACHAR ('49, '50) discriminated DNA and RNA in the nuclei of ciliated protozoa. His method is applied to the present study. The smears of *S. ambiguum* are treated by 10% perchloric acid at 5°C for about 17 hours. By this treatment, the microgranules are destroyed and the rod-shaped bodies are somewhat changed to granules in shape. Both of them lose the affinity for pyronine, and the rod-shaped bodies maintain slight reactivity to Feulgen's. The same phenomenon is observed by the treatment with hot normal HCl. At 60°C for 15 to 20 minutes, the rod-shaped bodies are partially turned to granulated bodies, the microgranules and the vacuolated areas almost disappear. The affinities for Feulgen's reaction, methylgreen-pyronine and other basic dyes are decreased.

Discussion

One of the most interesting problems in the field of protozoan cytology is that the nuclear apparatus in the ciliates is differentiated into two elements, namely macro- and micronucleus, which can easily be distinguished one from the other by the size, dividing behavior and prospective function. The micronucleus is believed to be a germline nucleus which divides mitotically, whereas the macronucleus, a somatic nucleus, divides amitotically. Notwithstanding this, the macronucleus is usually derived irreversibly from a synkaryon of the micronuclei in the course of sexual reproduction. These differences between the both types of nuclei provided many conceptions in this field (BĚLAŘ, '26, BAKER, '48).

In this paper, studies are confined to the components of macronuclear apparatus. Beside the common morphological methods which have been employed by many authors, cytochemical methods are applied with regard to the recent advances in cytochemistry.

The macronuclear apparatus of *Spirostomum ambiguum* is of a rosary-like appearance in the interphase. Based on the affinities for the different stainings and consequences of the cytochemical treatments, the following elements can be distinguished as the constituents of macronucleus: nuclear membrane, karyolymph, rod-shaped bodies, microgranules and vacuolated areas. Other than these, there are nucleolus-like droplets which appear in some cases.

In the fixed preparations, the rod-shaped bodies show various figures, such as granules, rods and fibres, their variability in shapes stands in contrast with other components. They are Feulgen-positive, specifically stained by methylgreen, and can be removed by trichloroacetic acid and digested by DN-ase. The microgranules, on the other hand, show strong affinity for pyronine but are easily be affected by both perchloric acid and RN-ase as compared with the rod-shaped bodies, and can be removed from their sites by these treatments. The action of NaCl solution on these components are remarkable. With 0.4 M NaCl for 4 hours at 15°C, the microgranules are almost dissolved and the rod-shaped bodies are partially removed, so that the whole macronucleus appears to be more granulous. With molar NaCl, most of the nuclear components are removed from the macronuclear apparatus. These facts seem to reveal that the greater part of rod-shaped bodies consist of DNA-rich protein, and the microgranules RNA-rich protein.

The vacuolated areas which correspond to the clear spaces of macronuclear nodes under the phase microscope are scarcely stained with any dyes. Any difference does not exist to distinguish them from karyolymph.

The pyronine-positive droplets which appear occaionally in the node may be compared with the nucleolus-like bodies found in macronuclei of some ciliates. Their variabilities would be correlated with physiological conditions of the animal, and further consideration on this will be given in a later paper.

As the rod-shaped bodies are the most prominent structures and composed of DNA-rich protein, they are regarded as the main component of the macronucleus in *S. ambiguum*. From the genetical point of view, the existence of the physiologically functional unit nuclei in the macronucleus of ciliates has been presumed by SONNEBORN ('49). However, there is no ample evidence to recognize them as such unit nuclei or to identify them as individual chromosomes. The microgranules are also one of the main components but they contain far less of DNA than the rod-shaped bodies in proportion.

Such macronuclear components are also observed in the macronucleus of *Paramecium caudatum* (SATO and SAITO, unpublished). KIMBALL ('49) has recognized the rod-shaped macrogranules and the microgranules in *P. caudatum*, those granules would correspond to the rod-shaped bodies and the microgranules of *S. ambiguum*. Morphological difference between the rod-shaped bodies of this animal and the macrogranules found in *Chilodonella uncinatus* (SESCHACHAR, '50) does not depend upon the effects of cytochemical techniques used but on structural difference; the latter would correspond to the same macronuclear component found in *Tetrahymena geleii*.

The behaviors of macronuclear components in the macronuclear morphogenesis during the "binary fission" will be described in the following paper.

Summary

The macronuclear components of the macronucleus of *Spirostomum ambiguum* are dealt with. They are distinguished as follows: rod-shaped bodies, microgranules, karyolymph, vacuolated areas, nuclear membrane and nucleolus-like droplets which appear occasionally and are strongly pyronine-positive. The rod-shaped bodies are not of a definite shape but range from granules to fibres. This variability in shapes stands in contrast with other components.

By means of treatments with trichloroacetic acid and DN-ase, it is pointed out that the rod-shaped bodies are mainly composed of DNA-rich protein, and with perchloric acid and RN-ase, it is also revealed that the microgranules are rich of RNA-protein. The nature of these components is discussed.

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