CELLULAR ORGANIZATION OF THE PROTOPLASM IN CILIATES

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An understanding of the biological functions of the cell presupposes in an ever-increasing degree of knowledge of the structure and changes of the ultrastructural parts. This follows from the recognized and recently also experimentally proved fact that the energy transformations of living systems take place by structural, subcellular means (GREEN, 1961). The subcellular parts are of universal character. Study of thier structure and functions is therefore just as important in the protozoa as in the higher organisms. But the protozoa are more convenient to use in the investigations. Their life cycle being short they are well suited for the study of cellular organization, particularly in their cryptobiotic state (The term is from KEILIN, 1958, and is used by GROSSOWICZ and associates, 1961).

This state may occur in many protozoa owing to changes in the internal and external factors, which cause reversible cystment and profound changes in the protoplasm, the ultrastructure (DEUTSCH & ZAMAN, 1959, OSADA, 1959, VICKERMAN, 1960, 1962, BICZÓK, 1961), and the biological functions take place (SCHOLANDER at al., 1952, PIGON, 1959). The present study is an attempt at clearing some of the problems of cellular organization through the investigation of these changes.

Materials and Methods

The species *Colpoda fastigata* and *Platyophrya lata*, bred form soil proved very well suited for investigation work because inoculated in sterile soil together with bacteria they encyst well in the course of the drying out of the soil. In the soil the cysts may remain dormant for long years (*Colpoda fastigata* could be activated even after 8 years) and the experimental animals can easily be reactivated from them with the help of a suitable culture medium (chiefly water-diluted extract of roots). It is interesting that the cysts in the Petri-dishes, independently of how long they had been in a dried-out state, could never, with any agent, be excysted after evaporation of the culture medium. Both species are modest, they feed chiefly on bacteria and are easy to breed. *Platyophrya lata* is, however, a better material for investigation than the excellently encysting *Colpoda fastigata* because in the first the protoplasm is of a lighter colour, the wall of the cyst capsula is more translucent and even the protoplasmic particles are more accessible for the light microscope. Besides,

individuals of the species in aging cultures often return to the devouring of cysts and active forms of the same species, that is, they become cannibalistic. The plasmatic structure and behaviour of the cannibalistic organisms differ, according to investigations carried out so far, from the normal. From a knowledge of them some conclusions may be drawn regarding cellular organization of the protoplasm in the normal forms. An average of 90 per cent of the experimental animals encyst at room temperature after 6 to 10 days in an extract prepared with sliced and dried roots of *Daucus carota, Cichorium intybus, Oenothera biennis* in nearly neutral, unbuffered tap water (15 gr of dry root per 1 l of water). Encystment takes place also when the Ciliates are washed and transferred from the root extract into a $CaCl_2$ 0,0500 gr, KCl 0,0350 gr, NaCl 0,1200 gr, $MgCl_2$ 0,0200 gr, KH_2PO_4 0,0150 gr, $FeSO_4$ 0,0020 gr 1000 ml of *aqua bidest.* solution. But in these nearly bacteria-free cultures encystment was long drawn out and the number of cysts formed was comparatively smaller. Cultures of this type were suitable and necessary chiefly for vital staining and electron microscope examinations.

Both species examined, especially *Platyophrya lata,* stain intensively and uniformly in neutral red of 25,000—30,000-fold dilution. Adsorption and desorption of the dye vary considerably in the different stages of active life and encystment. From this fact conclusions may be drawn concerning the relation between the submicroscopical structure and the functional changes. Since the toxicity of the dye was found to be negligeably little, adsorption of it by the protoplasm did not disturb the encystment. On the other hand, observation of the behaviour of the dye during encystment, rest, excystment, and the subsequent active state became possible.

Electron microscope methods were necessary for the sake of completeness. Fixation of the animals was achieved by means of 4 per cent formalin diluted with pH 7,4 acetat-veronal buffer solution, and further after a twofold washing 2 per cent osmiumtetroxid was used. The purpose of the prefixation was getting rid of the great aggregation of the by-products and bacteria resulting of the encystment — which is unavoidable when fixed in osmium. The fixed subject after washing was dehydrated by means of 70, 96- and 100 per cent alcohol at intervals of 30—30 minutes.

The embedding was made — after ROTH- into 60 per cent n-butyl- and 40 per cent methyl-metacrylate. The substance was kept in this solution, below 0° C, for 24 hours. Then the preparation treated with 0,5 per cent 2—4 dichlorobenzoil peroxid was polymerized in a thermostate, at 60° C.

Cystment

The Ciliates *Colpoda fastigata* and *Platyophrya lata* -like most soil-inhabiting protozoa encyst excellently. The pellicle which in the *Platyophrya* is soft anyway, loses, under certain conditions, its comparative rigidness. The animals which usually congregate in larger or smaller groups take on roundish shapes close near each other and while in rotating movement changing direction they secrete a protective capsule. Among the cysts thus formed all of the three known types may be found: the resting (protective) type, the thick-walled stable and the thin walled unstable types and the thinner-walled dividing

(reproductive) type. Besides these peculiar forms could be observed occasionally; in the *Colpoda fastigata* sculptured, thick-walled cysts in which the animal, dividing itself into 2-, 4- or occasionally 8 parts, had produced daughter cells which again secreted thick, sculptured capsules. On other occasions the animal, shrunk to 1/5 of its original size within the rough, thick cyst wall, was wrapped in a thinner-walled capsule.

Results

Changes during encystment: The cytoplasm of *Colpoda* and *Platyophrya* that have begun encystment was markedly solified. With the help of the micrometer placed in the ocular the increase in the intensity of flow could be easily seen from the movement of the plasmic parts and granules. But the size of the contractile vacuole and the frequency of its function also increase (Biczók, 1959). As a consequence of this, some animals empty nearly eight times as much water as before encystment. Considering that the vacuole that has grown from 7 μ to 12 μ works at a frequency of 15 sec. instead of the original 23, it is easy to see that in 9 minutes a rounded-off animal of 40 μ diameter presses out through the excretory pore of the vacuole an amount of water equal to the animal's own volume. The rhythm of this work often does not change essentially for more than an hour! This goes to show that the increased vacuole activity during encystment cannot be regarded as a physiological process that leads to „active dehydration", to the shrinking of the cytoplasm.

The fine, diffuse staining of the animals does not show any essential change during encystment. The *Platyophrya lata* cultures in which cannibalism has arisen are exceptions. The cannibalistic forms very often reach the size of 70—85 μ exceeding the average size of 55—60 μ. The changing of their forms is more pronounced. Their cytoplasm is often to a great extent vacuolized. Such active animals adsorb neutral red much more slowly and imperfectly than usual. Their staining is not the normal pale red but yellowish. While in the normal forms at the beginning of encystment or immediately before it dye clots occur but occasionally in the cannibalistic *Platyophryae* partial desorption of the dye, the formation of aggregates or clots usually begins already before encystment and becomes more intense during encystment. In the electronmicrograph these fine aggregates can well be seen in different areas of the cytoplasm, among others directly under the pellicle (Fig. 7).

Changes in the rest period of the cyst: During cyst forming the flow movements of the cytoplasm gradually cease. The activity of the contractile vacuole diminishes and finally ceases completely. In unstable protective cysts the condition of the animal shows no essential change for weeks. In thicker-walled protective cysts, occasionally also in reproductive ones, the phenomenon of syneresis well-known in colloid gels, appears the spontaneous loss of water and consequently shrinking of the plasm occur. The syneresis is sometimes strikingly rapid. Certain *Platyophryae* shrank to 1/10 or 1/15 of their original volumes after a week without losing their vitality. The animals shrunk to 1/2 or 1/4 usually formed new cyst-capsules and within them went on reducing their volumes at the initial rate. Such intracapsularly re-encysted small forms

could be activated or excited with artifical light of 20,000—30,000 lux intensity to perform limited rotating movement. The unstable protective cysts responded very sensitively to light of such intensity or even excysted. The gradual desorption of neutral red adsorbed in the active state is undoubtedly one of the most interesting phenomena of the resting state of the cyst. First submicroscopic clots appear which unite later into larger aggregates. These clots of dye complexes form a ring round the nearly centrally located nucleus. By the way, there are considerable differences in the formation of the dye aggregates. Their genesis is connected with the very finely dispersed dye ring which forms a wide bandlike ring round the nucleus. The cannibalistic animals which possess a protoplasm of coarse consistency and in which this ring is much coarser and much more irregular, form clotlike aggregates not only here, but before their development in other parts of the cytoplasm, too (Fig. 1-, 2-, 7.).

Changes during excystment: The comparative state of rest of the cytoplasm and its gel structure may be disturbed by certain factors (vegetable extracts, mechanical, and light stimuli, ultrasound etc.) and under favourable conditions, so far as these effects do not endanger fitness for life, they may start excystment. This is first of all introduced by the appearance of the contractile vacuole of a few μ size which becomes wider and wider, which indicates that the cytoplasm has taken up a considerable amount of water from the surrounding medium. Simultaneously with this the following changes may be observed: locomotive movement of granules and mitochondria, start of plasm stream, then rotation of the whole animal. The spontaneous movements of the mitochondria that can then be seen are very characteristic and instructive. In the protoplasm of the unstable protective cyst of *Platyophrya lata* induced by artifical light these movements take place chiefly along straight lines in different directions to a distance of 20—25 μ with a speed of 9—12 μ/sec. When plasm streams can hardly be observed granular locomotion is also very limited; this is the most intensive movement in the cytoplasm.

During excystment the size of the contractile vacuole increases considerably, its lumen is usually widely gaping not infrequently attaining $^3/_4$ of the diameter of the still roundish intracapsular animal. The large vacuole contracts slowly only after some minutes and continues to pulsate arhythmically for a longer time. All this points to a comparatively insufficient function of the cytoplasm against the in-streaming water.

During excystment the contraction of the neutral red clots continues. Sometimes a uniform aggregate forms, other times 2-, 3 or more smaller clots form from whose movements or relative positions to each other the varied, multidirectional streaming of the cytoplasm solified before excystment can well be seen. By the way, the time of excystment is dependent on the thickness of the cyst wall.

After excystment the function of the contractile vacuole and the locomotion of the animals are uncoordinated for about 15—20 minutes. Around the centre in the cytoplasm which has become colourless 1—2 dye clot complexes are visible that cannot be dispersed even by ultra sound.

Discussion

Cystment consists of a succession of complex organizational and disorganizational processes which are partly of colloid chemical, partly of biochemical character. To a fuller understanding of these processes an insight into the changes of ultrastructures is more and more necessary.

The most conspicuous change accompanying encystment is the solgel transformation. As to the colloidal chemistry of this phenomenon, we might mention thixotropy or synaeresis. It was ANGERER who called attention to thixotropy (1936) and who observed that the frame structure of the *Amoeba* plasm cortex breaks up when shaken, becomes fluid, solification sets in, then a new frame structure forms. Thixotropy-like reversible solification (often excysment) takes place in unstable, protective cysts when gently shaken; the same occurs also in thinner-walled stable cysts under the effect of more violent mechanical shocks (BICZÓK, 1963). In most cysts synaeresis appears which deprives the cytoplasm of a considerable amount of water. A stable enough gel structure forms. The question of the relation between colloid chemical changes and metabolism may be raised here.

It is known that after encystment there is a great decrease in the metabolic processes (SCHOLANDER and associates, 1952, PIGON, 1959, 1960, 1961). PIGON's thorough and comprehensive investigations in connection with this call attention to reversible enzyme inhibition. Besides this it is an important fact that in the synaeresis of the cytoplasm part of one of the indispensable media of the enzymatic processes, water, gets lost. In this way, but also through the development of the gel structure, the conditions may come into being that lead to the decrease of metabolism. Of course in this case, too, we ought to look for a factor, a substance X with enzyme-like effects which is primarily responsible for the starting of encystment and indirectly for the synaeresis, too. In connection with this supposition it must be taken into consideration that the activization of the cyst itself invariably begins with uptake of water. In thixotropy, simultaneously with solification a small widening vacuola indicates the uptake of water and subsequently the plasm inclusions show the increase of the metabolic activity. However, we must take into consideration that the mechanical effect causing thixotropy not only destroys the gel structure but also the connection of certain enzymes with an inhibiting substance or inhibiting substances.

From the point of view of a further approach to this problem the results obtained in the study of sol-gel transformation accompanying the plasm movements are worth mentioning. According to several authors (GOLDACRE, 1952, KAMIYA and associates, 1957, KAMIYA, 1959) the motor of these changes is the ATP and their basis the folding and unfolding of the polypeptid chains of the plasm (GOLDACRE—LORCH, 1950, GOLDACRE, 1952). Unfortunately the role of ATP in the process of cystment is not yet cleared, although it may be important. This seems to be indicated by the fast movement of the mitochondria in the solifying protoplasm of the excysting animal before the starting of more lively plasm streams. Since there is often a correlation between the location of the mitochondria and the form of the plasm movement (KAMIYA, 1951), we may be led to think that the diffusible ATP obtained by respiration and penetrating into the basic plasm from the mitochondrium may play an important role in the formation or organization of plasm movements. On the

other hand, the disorganization occuring in the mitochondria may exclude the forming of ATP in the mitochondria, which again may play a role in the gradual ceasing of the plasm streams and in the fact that after the contractile vacuole has ceased working a comparatively rigid plasm gel forms with simultaneously accompanying spontaneous loss of water.

We have reliable enough results concerning the slackening in the respiration of the encysting organism and the change of the structure of the mitochondrium (VICKERMAN, 1960, 1962). I myself have called attention, on the basis of photos of ultra thin sections made of *Colpoda fastigata* immediately after excystment, to the reorganization of the ultrastructure and especially of the mitochondria as it may be of some importance in cystment (1961). In fact, besides the nearly round, on an average $^1/_3$ μ large but not numerous normal forms there were many transitory mitochondrium forms of different sizes that were of conspicuously osmiophil character and which distinguished themselves by their three- or fourfold elongated hot dog forms, fine granules of high electron density and in less easily distinguishable, somewhat lighter-coloured and lower crests (Fig. 6). It seems quite evident that the inertia of the protoplasm against the inpouring water, the appearance of the giant contractile vacuole, then its irregular function, the uncoordinated behaviour immediately after excystment are the consequence of the fact that the reorganization of the ultrastructures is not yet completed.

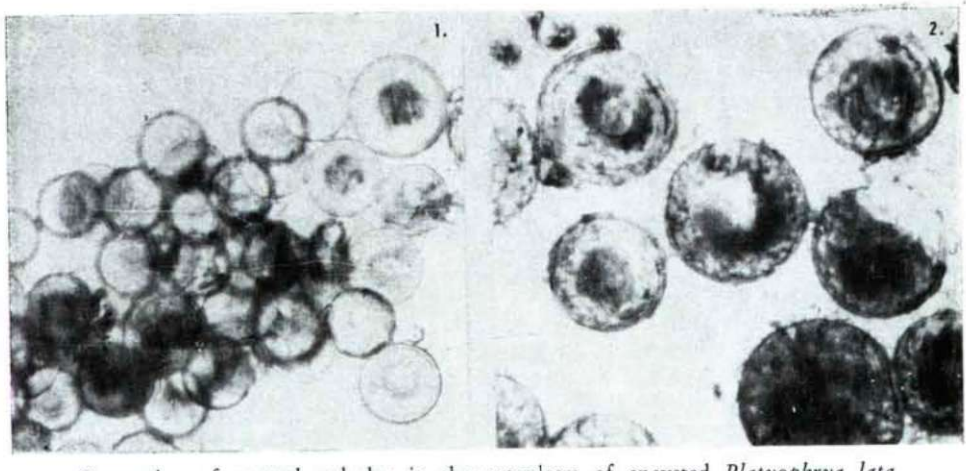
The investigations of the spatial and functional changes of sol- gel polypeptid chains in the plasm carried out with neutral red dye in the *Amoeba* (GOLDACRE—LORCH, 1950, GOLDACRE, 1952) directed my attention not only to the fact that these chains are in an unfolded state in the gel but also to the fact that they adsorb substances more intensively. The sol is the produce of their contraction their folding and this state leads to the desorbtion of the substances in question, the latest experiments also made with *Amoeba* (ALLEN et al. 1960, ALLEN, in the work of BRACHET, 1961) seem to contradict the extremely fruitful and multilaterally supported hypothesis. Although in the question of cystment we, too, hold that the forming of gels is accompanied not by the uptake of neutral red dye but by its giving off, we attribute very great importance to GOLDACRE's conception in the interpretation of certain changes in the plasm and in the elucidation of the sol — gel state.

In the foregoing we have pointed out that the neutral red dye bound by the polypeptids remains in an adsorbed state only in the period of pre — cystment; indeed, in the cannibalistic forms partial desorption of the dye, the formation of small irregular dye clots,* begins already at this stage (Fig. 7). The highly vacuolized coarse plasm structure containing submicroscopic associations and the decrease of the adsorptive capacity which goes together with the decrease of dispersion must have something to do with the desorption of the dye (Fig. 3). Besides, it cannot be left out of consideration that some of the mitochondria which are much larger than normal show signs of serious degeneration (Figs. 4. and 5). (Unfortunately it is not yet decided how far this phenomenon is a cause and how far a consequence just as we do

* It is desirable to call *clots* the coarse, irregular, inhomogeneous aggregates of the plasm and *granules* the regular preformed formations occasionally having a membrane structure. The aggregates formed from desorbed dye during cystmnet — Fig 7 — are clots, while the subpellicular neutral red stained plasm parts that cold be seen arraging themselves side by side at the beginning of encystment are granules (Fig. 8).

not know to what extent this degeneration affects metabolism and respiration). This phenomenon may, with reservations, be paralleled with the plasmic changes and the formation of dye clots in the cysting normal forms, although here the mitochondria are not degenerated but as a link in a chain of successive complex changes a part of them is partially or completely demolished. The stopping of plasm streams is followed by reversible denaturation and unfolding of the polypeptid chains and synaeresis (with smaller water blobs in the cytoplasm). At the same time very feeble double refraction may be observed with the help of the polarization microscope on the outer edge of the slightly shrunken cyst which suggests orientation and association of the polypeptid chains. The plasm that becomes gradually coarser and coarser gives off its dye which forms around the nucleus an ionotropy- like ring that can be produced with a polycation of increasing intensity (BICZÓK, 1963). The greatest part of the neutral red clots accumulates here.

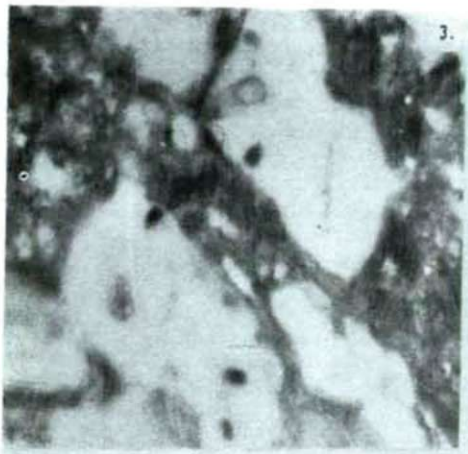
In the above we have tried to clear a few general problems of cellular organization by the investigation of cysting protozoa. We have left out of consideration many important influencing factors such as the pH, the role of ions, especially of the Ca^{**} ions which are so important in the organization of the plasm, the cooperative effects (KLOTZ, 1958), etc. The work of many researchers is needed if we are to make a synthesis based on the consideration of these effects which should give a true picture and an all-round explanation of the organization of cystment. Nevertheless we have come nearer to certain problems, e. g. the organization of the gel, the coarsening of the plasm which in many respects reminds of the phenomenon of aging with the difference that this one is reversible. The investigation of their relations with metabolism help largely in the understanding of why the neutral red dye clots are missing from the young fibroblasts of the chicken and why LEWIS 1919, (in the work of CRAWFORD, 1957) saw them compact in the fibroblasts of old cultures.



Desorption of neutral red dye in the cytoplasm of encysted *Platyophrya lata*

Fig. 1. In normally feeding individuals a finer, more regular dye ring can be seen here and there with aggregates in formation.

Fig. 2. In cannibalistic individuals the coarser, more irregular dye ring and the more intensive aggregation are remarkable.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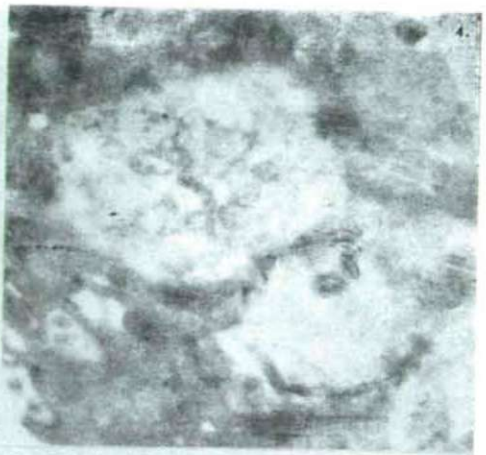
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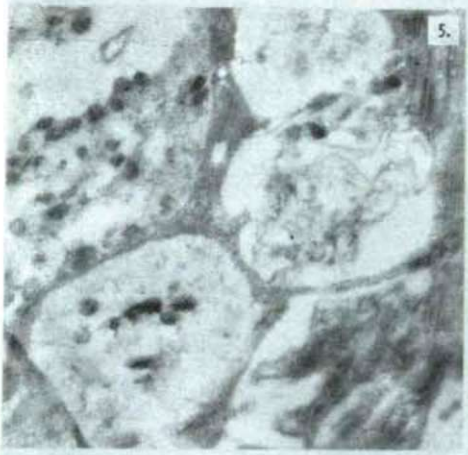
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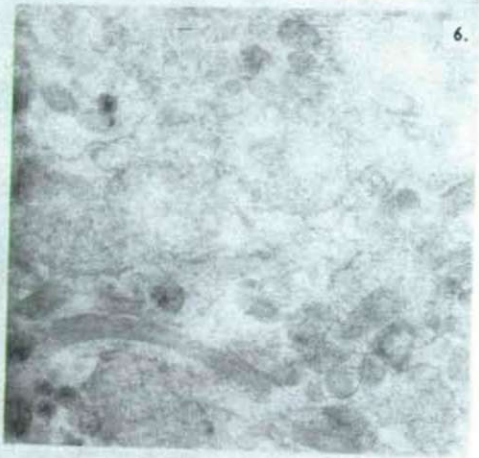
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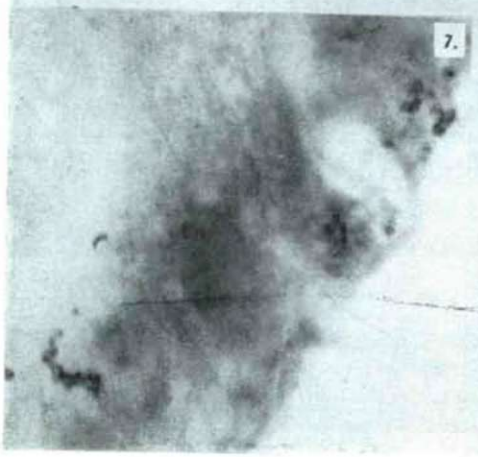
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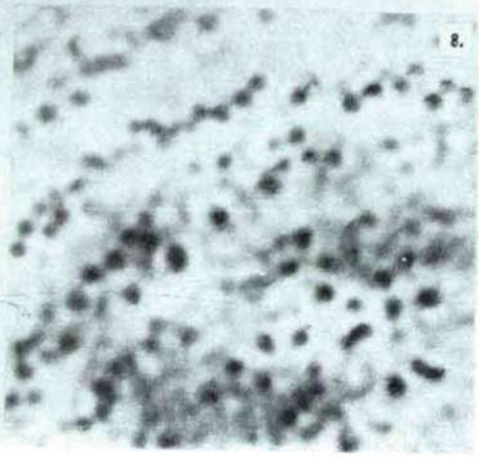
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Fig. 3. A part of the highly vacuolized, coarse-structured cytoplasm of a cannibalistic *Platyophrya lata* (14,000×).
Fig. 4. and 5. Degenerated mitochondria from a cannibalistic *Platyophrya lata* (26,000× and 14,000×).
Fig. 6. Part of the cytoplasm of a *Colpoda fastigata* after excystment with fully developed and developing, elongated mitochondria (34,000×).
Fig. 7. Submicroscopic neutral red aggregates in the cytoplasm of *Platyophrya lata* near the surface (50,000×).
Fig. 8. Subpellicular granules stained with neutral red in encysting *Platyophrya lata* (3,600×).