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(Department of Zoology the State College of Washington, Pullman.)

Dedifferentiation and redifferentiation in *Ichthyophthirius*.

II. The origin and function of cytoplasmic granules.

By

Ronald F. Mac Lennan.

(With 55 figures in the text.)

Introduction.

Profound changes in the cytoplasmic granules of ciliates under varied natural and artificial conditions are well known since the thorough series of papers on *Opalina* by KEDROWSKY (1931). These changes show that descriptions of morphology and cytochemistry of cytoplasmic granules are meaningless without reference to the stages of the life cycle and to the previous physiological history of the ciliate. The separation of the life cycle of *Ichthyophthirius* into a parasitic feeding stage and an encysted non-feeding stage free of its host results in the storage of an exceptionally large food reserve and also a complete separation of the processes of storage and those of utilisation. It is thus an unusually favorable form in which to study the formation, function, and changes in the cytoplasmic granules.

The cytoplasmic granules were studied by the classical staining methods and these results were checked and amplified by the use of specific cytochemical methods. This combination of methods makes possible the use of more precise statements as to the nature of the various cytoplasmic inclusions studied. Seven distinct types of cytoplasmic granules were identified and their changes during the various parts of the life cycle studied [1]).

[1]) The author gratefully acknowledges a grant from the ELIZABETH THOMPSON Science Fund which materially aided this investigation.

Material and Methods.

The maintenance of infected fish and the general methods of studying the parasite live and fixed have been described elsewhere (MacLennan, 1935). The cytological methods for Golgi material, vacuome, and chondriome were used as described in MacLennan (1933). The cytochemical methods indicated in the text were used as directed in Gatenby and Cowdry (1928) and Romeis (1932). Smearing the ciliates followed by immediate fixation was found to be the most rapid and the most accurate method of preservation of granules for cytochemical work.

The best method of vital staining is to immerse the infected fish or a whole cyst in 1/5000 to 1/100,000 solutions of dyes in tapwater for four to six hours. The fins or cysts are then mounted in a hanging drop and the ciliates studied as rapidly as possible. This produces a very specific stain without resulting in the clumping of granules or the slowing of ciliary action which usually results from the direct application of dyes to ciliates freed from the fish by scraping. In all cases at least two different brands of vital dyes were used to eliminate any possibility that the results were modified by individualities of manufacture.

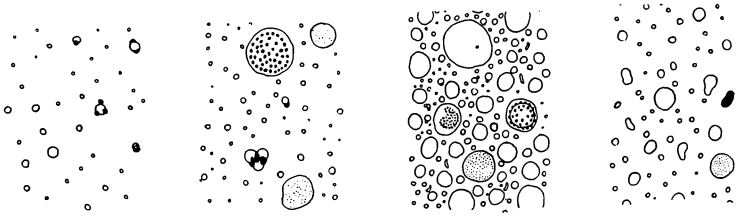
All drawings were made with the aid of a camera lucida. In the case of living animals, outline sketches were made and the details filled in free hand. The number and arrangement of granules present in each stage was studied by means of a complete series of camera lucida drawings for each set of granules. In the case of smear preparations, camera lucida sketches were made before treatment and after each step in the process, so that by a combination of staining reactions and solubility tests, granules of glycogen, lipoids, and proteins were identified in the same preparation and their morphological relationships accurately determined.

Types of cytoplasmic granules.

The cytoplasm of the mature parasite (Fig. 3) is filled with a tremendous number of granules and vacuoles ranging in size from 10—12 μ down to the limits of microscopic visibility. These granules are usually spherical but a few stout rods are also present. During encystment these granules are reduced greatly in number and size but there are still many present in the encysted ciliospore (Fig. 4). The young parasites, 30—45 μ in length, have the fewest granules (Fig. 1). The granules are gradually replaced during the growth

of the maturing parasite (Fig. 2). The cytoplasmic granules thus exhibit as definite a cycle as is shown by the neuromotor system.

Blackened granules are revealed in both ectoplasm and endoplasm after osmic or silver impregnations as used to reveal GOLGI apparatus. The osmicated material was thoroughly bleached with turpentine or hydrogen peroxide to confirm the reaction for GOLGI substance (BOWEN, 1928). The endoplasmic granules range from 0.25—1.0 μ in diameter. Many are found on the surface of fat globules (grey after extraction with turpentine), others on the surface of the food vacuoles and some free in the cytoplasm, particularly in the younger parasites. The ectoplasmic granules are generally smaller ranging from 0.25—0.50 μ in diameter. There are a few free in the ectoplasm, but most of them are concentrated around the diastolic stages of the contractile vacuole.



Figs. 1—4. Endoplasmic granules. Vacuome black, protein granules stippled. $\times$ 780. 1, young parasite 40 μ long; 2, parasite 40 μ long; 3, adult parasite just before encystment, 300 μ long; 4, ciliospore just before excystment, 30 μ long.

Janus green B, used in low concentrations on living parasitized fish for 6—12 hours stains small endoplasmic granules and rods a deep blue-green. These are usually associated with other colorless granules. The stained granules range in diameter from 0.25—0.50 μ, the rods (or stout filaments) may be as long as 1.5—2.0 μ. The ALTMANN acid fuchsin method used after osmic impregnation reveals granuals and rods similar to the above. The granules and rods are interpreted as chondriosomes.

Neutral red, used similarly to the Janus green B, or in greater concentration over a shorter period, stains small granules and rods found in association with food vacuoles. Neutral red granules are not found except in connection with the food vacuoles. The neutral red also stains the food vacuole itself (pink to orange) and the undigested pigment residues (orange to red). Since all the granules found around the food vacuoles stain with neutral red, and since

all granules around the vacuoles are blackened in the preparations for GOLGI material, the two methods show identical granules. These granules constitute the vacuome.

Nile blue sulfate, used according to the method developed by LORRAIN SMITH results in a pink stain specific for neutral fat, and a blue stain specific for fatty acids or other lipoids. Many pink granules ranging in size from 1—10 μ are found in the endoplasm of mature ciliates. The smaller granules are always surrounded by dark blue granules 0.25—0.50 μ in diameter.

Pink centers in blue granules 1 μ in diameter are sometimes found. The blue granules are identical in form, distribution and cycle with the blackened granules surrounding grey fat droplets after the osmification technique. Blue granules are found around food vacuoles and are identical with the neutral red granules. Short stout rods or filaments associated with small non-staining granules are identical with the chondriosomes revealed by the Janus green and ALTMANN'S acid fuchsin. Small blue granules free in the cytoplasm are also present. They probably represent the free granules shown by other techniques but they cannot be identified with these with absolute certainty. All the blue and pink granules of the above techniques are stained a brilliant red with Sudan III, used as a dye for lipoids. By taking the preparations used above, treating them with 100 % alcohol, or ether, and then restaining, it was found that the stainable granules are completely dissolved, thus confirming their general lipoid nature. No ectoplasmic granules are revealed by the lipoid stains used. Since the endoplasmic GOLGI granules give a positive lipoid reaction there is a fundamental difference between the GOLGI granules of the endoplasm and those of the ectoplasm.

Ninhydrin, specific for proteins in general, gives a deep blue color with certain granules 1—10 μ in diameter found in the endoplasm and in vacuoles within the macronucleus. They are also stained orange by MILLON'S reagent, which is specific for tyrosine. These granules may be identified before staining by a faint granular appearance and a slightly more greyish color in transmitted light as compared with the other endoplasmic granules. They are never associated with the other granules to form granule complexes. The granules revealed by specific protein stains are identical with granules revealed by ordinary stains such as haematoxylin or light green.

Smears of ciliates fixed in 100 % alcohol and treated with chlorzinc-iodide or sulfuric-acid-iodine reveal granules of glycogen up to

6 μ in diameter. Prolonged osmification as used in the GOLGI techniques also renders the glycogen insoluble and stainable with Sudan III dissolved in the embedding paraffin. Brown or blackened granules and rods are always associated with the red granules. The red granules will still give a positive iodine reaction for glycogen. The blackened granules correspond in size, shape and relationships to the chondriome granules surrounding colorless vacuoles in other methods.

Table 1.

Reaction of Cytoplasmic Granules of *Ichthyophthirius* with the Main Cytological and Cytochemical Reagents.

	Size (microns)	Osmic Impregnation	Silver Impregnation	Solubility in Ether	Sudan III for Lipoids	Nile Blue Sulphate for Lipoids	Neutral Red	Janus Green	Chlor-Zinc-Iodide	Sulfuric-acid-Iodine	Ninhydrin	Millon's Reagent
Endoplasmic Golgi Bodies	0.25—1.0	+	+	+	+	Blue						
Ectoplasmic Golgi Bodies	0.25—0.50	+	+									
Vacuome	1—4	+	+	+	+	Blue	+					
Chondriome	0.25—0.50	Brown	+	+	+	Blue		+				
Neutral Fat	1—10	Grey		+	+	Pink						
Glycogen	0—6								+	+		
Protein	1—10										+	+

Seven distinct types of cytoplasmic granules are defined when all the reactions used are considered. The endoplasmic GOLGI bodies and ectoplasmic GOLGI bodies respond identically to the metallic impregnation methods. The endoplasmic GOLGI material contains lipoids while the ectoplasmic GOLGI material does not. Vacuome and chondriome are also impregnated by the classical GOLGI methods but are differentiated by their reactions to vital dyes. The vacuome segregates neutral red and allied dyes, while the chondriome segregates Janus green B. Granules of neutral fat, of glycogen, and of protein are also present in the endoplasm. The specificity and interpretation of the tests used are discussed in detail in the appropriate sections.

Vacuome and food vacuoles.

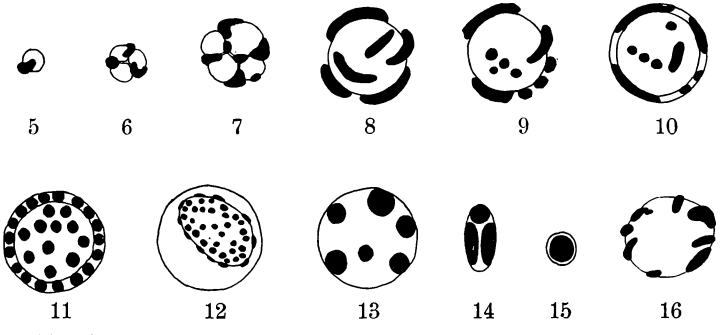
The vacuome, consisting of granules which segregate neutral red from solutions of low concentration, is found in *Ichthyophthirius* only in contact with the food vacuoles. Individual vacuoles and their accompanying granules were followed throughout the digestive cycle, except for the period immediately after ingestion during which the vacuoles are hidden by the complicated oral apparatus. The food vacuoles do not follow a definite path in the endoplasm since the endoplasmic currents constantly change due to the changing shape of the ciliate.

The cycle of the food vacuole in *Ichthyophthirius* is in three distinct parts. The first stage is the formation of a functional food vacuole. This is the equivalent of the pro-gastriole stage of Volkonsky (1929). The second stage, marked by changes in the vacuome and a disappearance of the food granule, is interpreted as the period of digestion. The third stage is interpreted as the period of absorption. These last two stages correspond to the gastriole period of Volkonsky. No granules other than the vacuome could be found in contact with the food vacuoles during any part of the cycle. The conclusion that the vacuome carries the digestive enzymes (Volkonsky, 1929; Koehring, 1930) would seem to be the best explanation in this case.

The smallest food granules, 2—4 μ in size are accompanied by a small hemispherical or rod-like vacuome granule adhering to the surface (Fig. 5). Several of these food granules soon collect in a single mass (Fig. 6). The various food granules fuse together, as do the vacuome granules, until large complexes 8—12 μ in diameter are formed (Fig. 7). The granules in this mass then fuse to form a single vacuole with eight or ten large vacuome rods adhering to its surface (Fig. 8). The rods almost immediately begin to break up into small spherical bodies (Fig. 9).

There is no demonstrable fluid vacuole surrounding the food granules up to this time and both the vacuome and the food body is in direct contact with the endoplasm. The next step is the rapid differentiation of a membrane around both the food body and the vacuome granules (Fig. 10). The boundary of the inner food body is still clearly visible. The vacuome granules thus do not migrate through a membrane, but the membrane is formed around them. This process of the development of a mature food vacuole occupies forty minutes to one hour.

The vacuome rods next completely break up into small spherules about 1 μ in diameter which form a shell around the central food granule (Fig. 11). These spherules break up into smaller granules which stain less intensely than in the pro-gastriole stage and the food mass itself begins to stain a faint pink. This latter color reaction changes soon after to orange and then to yellow. The food mass shrinks rapidly (Fig. 12) and finally becomes indistinguishable except for occasional residues from the pigment cells of the fish. Along with this change in the food body, the small vacuome granules again fuse and form 4—6 large intensely red bodies (Fig. 13) 2—3 μ in diameter. Throughout this period the diameter of the vacuole has not changed appreciably, the only changes being the solution of the food body and the changes in the vacuome. The second part of the cycle occupies thirty minutes to one hour.



Figs. 5—15. Changes in a food vacuole traced throughout the digestive cycle. Vitally stained with neutral red. $\times$ 1560. — Fig. 16. Food vacuole. KOLATCHEW-NASSONOW impregnation for GOLGI material. $\times$ 1560.

The third period of the vacuole is marked by a rapid diminution in the fluid resulting in the formation of a large, solid vacuome granule 2—4 μ in diameter lying in a very narrow vacuole which may be clear or may contain the residue of pigment from the fish (Fig. 14). The vacuome residue is slowly resorbed (Fig. 16) and finally disappears leaving the granular pigment residue in small masses throughout the cytoplasm. No defecation was observed, the waste pigment usually being discarded in the cyst (MACLENNAN, 1935). The absorption of the vacuolar fluid occupies only five or ten minutes, while the absorption of the neutral red residue may take twenty-four to thirty-six hours.

The vacuome is not demonstrable with neutral red or the other stains in the late stages of encystment and in the ciliospores. It

is therefore probable that it is formed de novo in the cytoplasm upon contact with the food particles. This de novo origin of the vacuome in *Ichthyophthirius* agrees with the findings of KEDROWSKY (1931) with respect to the ectosomes of *Opalina.*

The details of morphology and topography of the vacuome vary widely even within such a relatively restricted group as the infusoria. It may consist of uniform granules scattered throughout the cytoplasm and showing no obvious relationship with any of the structures or activities of the ciliate. DUNIHUE (1931) found this in *Paramecium caudatum* and HALL (1931) in *Stylonynchia.* LYNCH (1930) found in *Lechriopyla* three such sets, differing in depth of staining reaction, position and size. He accepted only the smaller granules as true vacuome. BUSH (1934) described two sets of vacuome in *Haptophrya.*

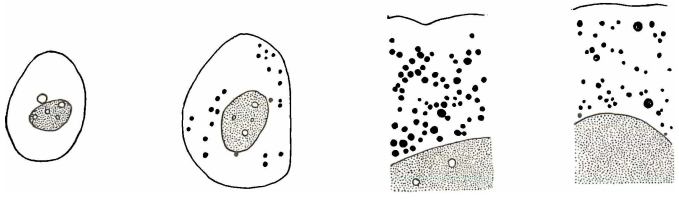
Identification of the vacuome as GOLGI material in protozoa depends largely upon similarities in reaction to the methods of metallic impregnation. HALL and his associates in a series of papers (1929—31), VOLKONSKY (1929), LYNCH (1930), and the present paper show that the vacuome in the ciliates studied may be specifically impregnated with osmium and silver compounds. In other ciliates, MACLENNAN (1933) and BUSH (1934), using the methods recommended by HALL (1929), demonstrated that the vacuome is not impregnated by the metallic GOLGI methods. Thus within the infusoria there have been demonstrated ordinary neutral red granules, neutral red granules which may be impregnated by GOLGI methods, and GOLGI granules which do not segregate neutral red. In addition to this complexity, there may be two or more sets of ordinary neutral red granules within the same individual distinguished by variations in their reactions to neutral red, by constant differences in size and by differences in localization.

The relatively limited information on the function of the vacuome in ciliates gives final evidence of the heterogeneity of granules grouped under the vacuome hypothesis. The association of one type of vacuome with the food vacuole and the correlated changes during digestion shows clearly a digestive function. On the basis of this type of evidence NIERENSTEIN (1905), VOLKONSKY (1929), KOEHRING (1929) and others have interpreted these granules as enzymatic centers. The vacuome of *Ichthyophthirius* is most highly specialised in that it is found only in association with the food vacuoles. The ectosomes of *Opalina* segregate neutral red and are thus vacuome. KEDROWSKY (1931) proved that these granules are synthetic and storage centers for a variety of materials. Both the cytological

and morphological evidence show that the granules of the so-called vacuome are not a homogeneous group but in reality are a combination of several different groups differing in distribution, staining reactions and in their specific functions. Vacuome has no meaning except as a convenient term for the group of granules which segregate neutral red.

Neutral fat granules and endoplasmic GOLGI bodies.

The granules of neutral fat first begin to appear in young ciliates about 45 μ long (Fig. 17, 18) and steadily increase in number and size to maturity (Fig. 19). Their diameter ranges from the limits of visibility up to 6 μ. They decrease slowly both in number and size during encystment (Fig. 20), but there are usually a few left in the ciliospore after excystment. The fat granules are the commonest of the three types of storage granules and make up at least half of the total number in each ciliate.



17 18 19 20

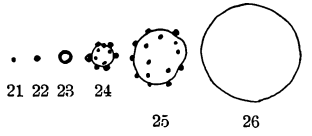
Figs. 17—20. Variation in granules of fat during the life-cycle. KOLATCHEW-NASSONOW impregnation, bleached with turpentine. $\times$ 370. 17, young parasite; 18, growing parasite; 19, adult parasite; 20, encysted parasite after the second division.

The neutral fat granules are associated with a set of smaller satellite granules 0.25—1.0 μ in diameter which fulfil the criteria for GOLGI material. These granules give a positive test for fatty acids with the LORRAIN SMITH method. These endoplasmic GOLGI bodies are few or entirely lacking in the very young parasite (30—40 μ in length). They appear only as fairly numerous single bodies 0.25—0.50 μ in diameter in the 40—45 μ stage. A few have the appearance of a hollow sphere the center of which is shown to be neutral fat with the LORRAIN SMITH method (Fig. 23). As the parasite grows these bodies become more numerous and additional stages appear. The GOLGI shell breaks up into spherules (Fig. 24) surrounding a core 1—3 μ in diameter. When the core is 4—5 μ

in diameter, the GOLGI bodies decrease in number (Fig. 25). The GOLGI bodies are not found around the large fat granules (Fig. 26).

A few of these formative stages of fat droplets are found in the early stages of encystment while the remaining food vacuoles go through the digestive and absorptive cycles. After this, during the period of active utilisation of reserves, the granules of fatty acids disappear and the fat droplets show no association which other granules.

The cytochemical tests of the fat droplets and the GOLGI are suggestive in regard to the synthesis of the fat. TENNENT, GARDINER and SMITH (1931) find that the presence of very small quantities of fatty acids dissolved in fats gives an intense blue coloration. The fat granules give no indication at all of the presence of fatty acids, as would be expected if the fats are synthesized on the surface of the fat granules themselves. On the other hand the morphology of the GOLGI bodies, their hypertrophy during the storage periods and absence during the encysted and early parasitic stages suggest that they are concerned with the synthesis of the fat. The cytochemical tests support this hypothesis by the indication of free fatty acids in these bodies. The fatty acids and glycerine dissolved in the endoplasm are thus segregated and concentrated in the GOLGI bodies and are there synthesized into tri-glycerides which are then added to the mass of neutral fat in the center of the GOLGI complex.

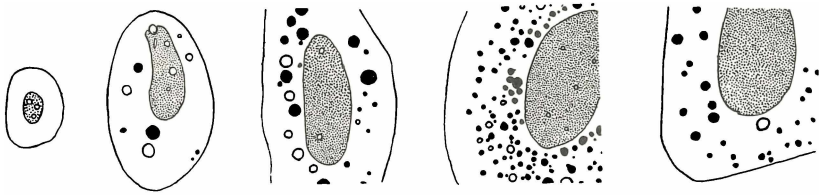


Figs. 21—26. Formation of granules of neutral fat. LORRAIN-SMITH's nile blue sulfate method. Blue granules in the original preparations are represented as black, pink granules represented in outline. × 1560.

The synthesis and storage of fat is the only function which has thus far been definitely ascribed to the endoplasmic GOLGI apparatus. (The granules of fatty acid described by KEDROWSKY as precursors of the granules of neutral fat obviously correspond to the endoplasmic GOLGI granules of *Ichthyophthirius.*) The accumulation of fatty acids necessitated by this process would account for the GOLGI reactions (TENNENT, GARDINER and SMITH). In *Ichthyophthirius* it is probable that there is no other function since these granules are demonstrable only during fat synthesis and storage. The use of neutral fat as a reserve food is now well known in the infusoria. ZWEIBAUM (1922) showed that neutral fat is abundant during active feeding periods, but disappears during conjugation. POLJANSKY (1934) found a similar condition.

Glycogen granules and the chondriome.

The glycogen granules first appear in the young parasites 45—50 μ in diameter, along with the first protein and fat granules (Fig. 27). They rapidly increase in number (Fig. 29) and in the adult (Fig. 30) are almost as numerous as the fat granules. They decrease in number and in size in the encysted stages faster than any of the other components. Even as early as the third division in the cyst (Fig. 31) the number of glycogen granules is markedly small.



27 28 29 30 31

Figs. 27—31. Variations in granules of glycogen during the life cycle. Osmic impregnation followed by Sudan III in paraffin. Glycogen granules represented as black, protein granules in outline. $\times$ 370. 27, young parasite; 28, growing parasite; 29, growing parasite; 30, adult parasite; 31, encysted parasite after the third division.

The small growing glycogen granules always appear in conjunction with mitochondria. After GOLGI preparations stained with ALTMANN's aniline acid fuchsin method, mitochondria surround vacuoles from which the glycogen is dissolved. After the GOLGI-Sudan III technique, the red glycogen granules are surrounded by mitochondria which impregnate with osmic acid. After the LORRAIN SMITH method (Fig. 38) the mitochondria may be recognized as stout blue filaments associated with empty vacuoles. This agrees with the results of BENSLEY and HOERR (1935) who found fatty substances in mitochondria of the liver. After vital staining with JANUS green B (Fig. 32—37), the mitochondria are found surrounding clear granules.

32 33 34 35 36 37 38

Figs. 32—37. Changes in chondriosomes and growth of secretion granule. Stained vitally with Janus green B. $\times$ 1560. — Fig. 38. Chondriosome stained dark blue by LORRAIN-SMITH's nile blue sulfate method. $\times$ 1560.

The secretion cycle of the glycogen is as definite as in the case of neutral fat. Granular mitochondria 0.25—0.50 μ in diameter

are found in the smallest parasites (Fig. 32) along with hollow spheres up to 1.0 μ in diameter (Fig. 33). In slightly older parasites there also appears heavy mitochondrial caps on one side of the glycogen sphere 1.0—2.0 μ in diameter (Fig. 34). As the spheres enlarge (Fig. 35—37) more mitochondria are added and they become larger and markedly rod-like. After the glycogen spheres reach a diameter of 5 or 6 μ the mitochondria disappear rapidly and are absent from the large mature granules. During the absorption of the glycogen in encystment no other granules come into direct contact with the glycogen. It seems probable therefore that the mitochondria are instrumental in the synthesis of the glycogen, but not in the utilization process.

Glycogen, or the nearly related substance paraglycogen, has been found in many ciliates. Studitsky (1930) found that it increases in amount in preparation for encystment. Zhinkin (1930) found it was used during starvation. It is a real food reserve in many ciliates.

The cytology of the formation of these glycogen reserves is little known. Alexeieff (1929) considered that the glycogen reserves of several mastigophora are segregated by the chondriome. Mac Lennan (1934) found that the highly specialised glycogen reserves of *Polyplastron* arise independently of other cytoplasmic components.

The presence of chondriosomes in protozoa has been accepted since the classical paper of Fauré-Fremiet (1910). The function of the chondriome is less well explored. Horning (1926) described chondriome in close association with food vacuoles and thus concerned with digestion. Hall and Nigrelli (1930) dispute this theory. Joyet-Lavergne (1928) presented evidence showing that in the chondriome of gregarines the oxydation-reduction reactions of the cell are localized. This reaction was not studied in *Ichthyophthirius*, so no comparison can be made on this point.

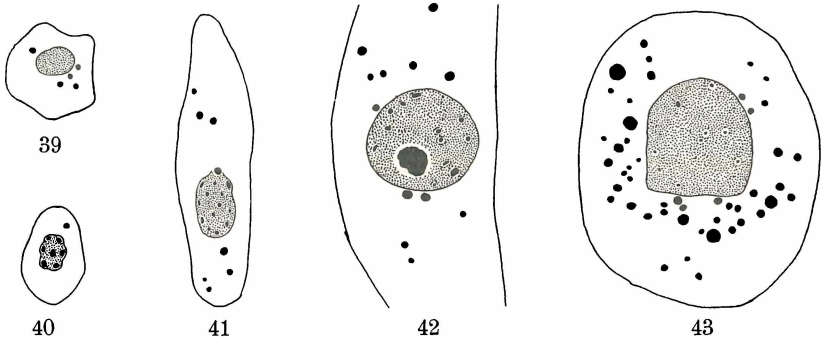
No chondriome can be demonstrated by any of the methods used in the ciliospore or very young parasite. The chondriosomes as well as the Golgi bodies and vacuome are formed de novo during the feeding stage. Horning (1929) found a similar situation in a sporozoan. Genetic continuity is not an essential feature of the chondriome.

Protein Granules.

The protein granules show a cyclic change in number. They are not found in the cytoplasm of the youngest parasites, but are found first in growing forms 30—50 μ long (Fig. 40). As the para-

site feeds and grows in the fish more and more granules appear (Fig. 41—43), but at no time do they approach in number either the granules of neutral fat or of those of glycogen. Soon after encystment the granules become smaller and fewer in number (Fig. 39), until by the end of encystment there are none left.

The formation of the protein granules may be observed clearly in living, normal ciliates. They are seen as large very faintly granular masses in the more finely granular chromatin of the macronucleus. The larger granules slowly migrate to the border of the macronucleus and finally raise the nuclear membrane above the surface. Sooner or later this ruptures, the granules are expelled, and the break in the membrane repaired.



Figs. 39—43. Variations in granules of protein during the life cycle. Protein granules in solid black. FEULGEN-light green stain. ×370. 39, ciliospore before completion of last division stages; 40, young parasite, 30 μ long; 41, growing parasite, 70 μ long; 42, mature parasite, 250 μ long; 43, encysted, just before division.

Thin sections show profound changes in the macronucleus during the formation and extrusion of the protein granules. These changes are best shown in preparations stained according to FEULGENS method and counterstained with light green. During the nuclear reorganization found near the end of encystment, the macronucleus is apparently pure chromatin, all of the granules being stained with the FEULGEN reagent. As soon as reorganization is complete, a few minute granules of protein, not reactive to the chromatin stain are found in the macronucleus (Fig. 45). These remain few in number during the rest of encystment and during the free swimming stage. Soon after the parasitic feeding stage commences, the nonchromatin protein increases greatly (Fig. 46, 47). A few mature granules are expelled from the macronucleus by the time the ciliate is 40—50 μ

in length. The chromatin matrix is filled with irregular protein granules ranging from the limits of visibility up to two or three microns in diameter. The chromatin granules themselves appear less sharply marked off than in the earlier stages and are clearly connected by strands of less deeply staining chromatin (Fig. 48). As the protein granules enlarge, the adjacent chromatin gradually disappears until the granules come to lie free in vacuoles (Fig. 49). These vacuoles can be seen in living ciliates and are not the result of shrinkage during the fixation process. The nuclear changes reach

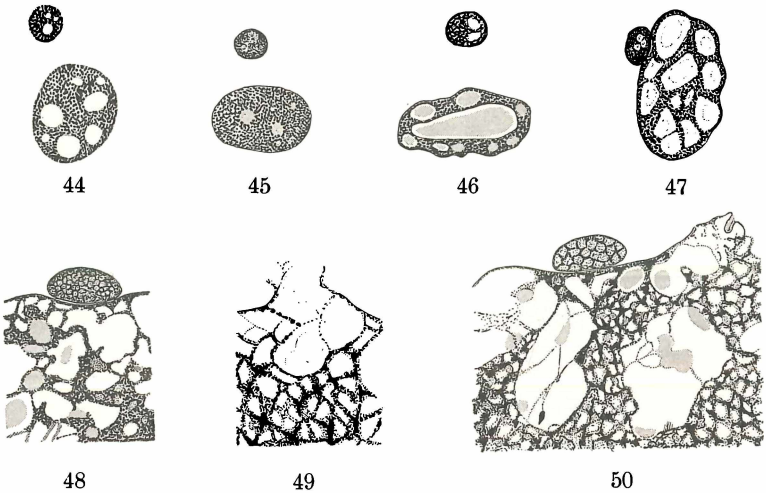


Fig. 44. Nuclei of young parasite. Macallum's Prussian Blue technique for iron. × 1560. — Figs. 45—50. Formation of protein granules in the macronucleus. Feulgen stain represented as black, light green stain as gray. × 1560. 46, ciliospore at time of excystment; 47, young parasite; 48, young parasite; 49, growing parasite; 50, mature parasite, portion of macronucleus showing extrusion of granules; 51, same parasite as in 50, showing large vacuoles in chromatin net.

their height in parasites 300—800 μ in length (Fig. 50): The whole macronucleus then has a fine reticular chromatin structure outlining large vacuoles. The looseness of this net makes it particularly favorable for detailed observation of the formation of the various granules. The smallest nonchromatin protein granules are embedded in the reticulum in intimate contact with the chromatin. As the granules become larger they are gradually freed from the reticulum. They are often irregular in shape but as they come to lie near the periphery of the macronucleus they assume a spherical shape. The nuclear membrane is raised over the granule and begins to fray

out (Fig. 49) thus releasing the granule which is soon swept away by the cytoplasmic currents. The nuclear membrane is continous with the chromatin reticulum and reacts to FEULGENS stain.

The formation of the protein granules continues at a decreasing rate after encystment while the food vacuoles already present are digested. By the third or fourth division the food vacuoles have disappeared and the macronucleus shows a more evenly granular chromatin structure with relatively few protein granules. By the time of micronuclear reorganization in the latter part of encystment, only granules giving a positive chromatin reaction can be discerned.

Spherical chromatin masses are extruded into the cytoplasm during encystment. This process has often been mistaken for the formation and extrusion of micronuclei from the macronucleus (PEARSON, 1933). This process of chromatin extrusion has been described thoroughly by HAAS (1933) and is clearly a part of macronuclear reorganization in connection with the division and conjugation processes. This extrusion of chromatin and the formation and extrusion of non-chromatin protein occur at different stages of the life cycle and are entirely separate processes. The formation of protein granules is not a process of reorganization but has the characteristics of a secretory cycle.

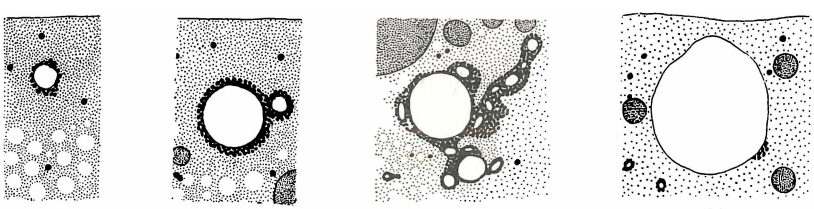
This secretory cycle is susceptible of at least two explanations. The organized chromatin may act as a type of segregation apparatus, merely concentrating the protein and not in itself being used up by the process. On the other hand, the marked diminution in the concentration of chromatin during the secretion phases suggests that the chromatin itself is broken down to form this protein. A positive test with FEULGENS reagent, used without a preliminary hydrolysis is usually considered to be a safe test for free thymonucleic acid (REICHENOW, 1928). This test was applied to all stages of *Ichthyophthirius* and a positive reaction was given in the parasitic stages in which the protein granules are being actively formed. No reaction was given in the stages of encystment in which the protein granules are not being formed. It is considered most likely that the non-chromatin granules are formed by a splitting of the chromatin into the protein group and a group containing the thymonucleic acid and iron (Fig. 44).

Definite cases of the use of granules of protein as reserves have been described far less frequently than those of fat and glycogen. ILOWAISKY (1926) described reserve granules in *Stylonychia* which are probably protein. These are used up during encystment. KAZANCEW

(1928) found accumulations of protein, or "nucleoli" in the macronucleus which showed indications of being reserve material. KEDROWSKY (1931) found protein is stored in the ectosomes of *Opalina*. LUCAS (1932) describes two types of protein granules. One, reacting to FEULGENS stain, is formed in the macronucleus and expelled into the cytoplasm. Another, larger type, non-reactive to FEULGENS stain is found in the cytoplasm. These granules are resorbed during redifferentiation stages during which no food is absorbed. The storage of reserve protein is thus found in both parasitic and free-living ciliates of different orders and will probably be found to be as common as the case of other types of food reserves.

Contractile vacuoles and ectoplasmic Golgi granules.

Contractile vacuoles are found in all stages of the life cycles. The number varies with the size of the ciliate — the ciliospore has one, the adult has many. The size of the fully expanded vacuole is approximately the same in all stages, averaging about 10 μ in diameter. Each vacuole lies close to the pellicle in the ectoplasm and is fixed in position. Since the constant motion of the thick-set cilia in the normal ciliate prevent any detailed observations being made on the pulsatory cycle in living forms, the description below is made entirely from material impregnated by the osmic or silver methods for Golgi material.



51 52 53 54

Figs 51—54. Pulsatory cycle of the contractile vacuole in the parasitic stages. KOLATCHEW-NASSONOW impregnation for GOLGI material. × 1560.

Granules blackened by the osmic or silver methods show cyclic aggregations around the contractile vacuoles in the parasitic stages of *Ichthyophthirius* similar to the cycle described in *Epidinium* (MAC LENNAN, 1933). The small vacuoles (Fig. 51) forming after systole are almost obscured by these granules. As the accessory vacuoles coalesce, the larger ones thus formed are likewise covered by blackened granules (Figs. 52, 53). However, as the vacuole grows the layer

of granules becomes thinner until they are almost or completely absent at the end of diastole (Fig. 54). Small blackened granules similar to those around the contractile vacuoles are also found in the ectoplasm. No blackened granules were found in the ectoplasm or around the contractile vacuoles of encysted forms.

These granules are indistinguishable in impregnation reactions from the Golgi bodies of the epithelium of the fish (Fig. 55), vacuome granules, and the endoplasmic Golgi granules of the ciliate. They can be distinguished only by position or in some cases by shape. The Golgi granules of the ectoplasm cannot be stained with vital dyes, nor with methods specific for lipoids. They are therefore fundamentally distinct from the endoplasmic Golgi granules and from the vacuome.

NASSONOW (1924) was the first to show adequately the homologies between the osmiophilic material around the contractile vacuoles and Golgi material. Subsequent work by many authors has shown that such a concentration is found in many, but not all ciliates. It was shown in a previous paper (1933) that such differences are correlated with different degrees of concentration in the formation of the contractile vacuole. Thus in some forms the formation of the vacuole is concentrated in a limited region and there is a corresponding concentration of osmiophilic material, in others the accessory vacuoles form throughout the ectoplasm with a corresponding scattering of the osmiophilic materials. The changes during the life cycle of *Ichthyophthirius* reveal an additional factor determining the presence or absence of osmiophilic aggregations. There are undoubtedly more wastes produced during the active feeding and storage stage than during the relatively quiescent encysted stage. During the feeding stage the vacuoles will excrete water plus large amounts of other wastes. while during the encysted stage water plus only small amounts of other wastes will be excreted. It is probable that some of these wastes are capable of reducing OsO_4 and when segregated in the granules of the contractile vacuoles form typical Golgi bodies. Where these wastes are not excreted no osmiophilic granules are present. The ectoplasmic Golgi bodies arise de novo in each feeding stage. This explanation is not presented as a proven fact, but as a tentative correlation of the known cytochemical and physiological facts.

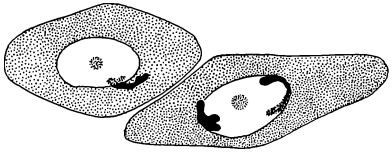


Fig. 55. Epithelial cells from tail of fish. KOLATCHEW-NASSONOW impregnation for GOLGI material. × 1560.

Discussion.

The identification and classification of cytoplasmic granules in ciliates has proceeded since the days of EHRENBERG. Renewed interest has been aroused recently by attempts to extend the results of metazoan cytology and to identify various granules or organelles of ciliates as the GOLGI material, vacuome or chondriome. The identification of mitochondria in ciliates has resulted in practically unanimous acceptance of granules, rods, or platelets as homologus structures, although apparent discrepancies have been noted from time to time. The GOLGI and vacuome hypotheses, however, have produced conflicting evidence and interpretations. HILL (1933) after reviewing the literature on GOLGI apparatus in protozoa, closed with the remark, "It remains then for future workers to elucidate further the nature of the GOLGI apparatus in protozoa".

The GOLGI apparatus of over thirty genera of ciliates, distributed in all the major groups have been studied by various authors. The new evidence afforded by these studies has not cleared up the inconsistences of fact and of interpretation but has intensified them. One group finds the GOLGI apparatus associated chiefly with the contractile vacuoles. In some cases it is also present as scattered cytoplasmic granules (NASSONOW, 1924; MAC LENNAN, 1933; etc.). Another group holds that since not all the contractile vacuoles show osmiophilic materials, when such materials are present they cannot be considered to be true GOLGI material. The scattered granules only are the true material (HILL, 1933; LYNCH, 1930). A third major group holds that the true GOLGI bodies are stained by neutral red as well as by the methods of metallic impregnation — an extension of the vacuome hypothesis of Parat (HALL, 1929; DUNIHUE, 1931; etc.). In some forms the presence of any true GOLGI material has been questioned or denied (PESHKOWSKAYA, 1928).

The specificity and therefore the validity of metallic impregnation methods, the chief criterion of GOLGI material, has been questioned for a long time. Recently TENNENT, GARDINER, and SMITH (1931) have shown by the use of known compounds that many otherwise unrelated reducing substances will produce typical GOLGI bodies. More recently evidence has been accumulating that neutral red is not specific in all cases for particular types of granules. LYNCH (1930) found two types of granules or vacuoles, but accepted only one as true "vacuome". FINLEY (1934), BUSH (1934) and others have also found more than one type of granule stainable with neutral

red. These and other discrepancies are too wide and too consistent to be entirely accounted for on the basis of inaccuracies in observational technique. The evidence presented by KEDROWSKY (1931) and in this paper demonstrates that many of the special tests are actually based upon incidental properties of the materials which are segregated. Although these various methods give sharp and reproducible results in the same type of cell under the same physiological conditions, much evidence is against the hypothesis that results of the classical GOLGI methods on one type of cell can be compared directly with results in an entirely different type of cell.

The function of the cytoplasmic granules rather than their form or staining reactions should be the fundamental distinction used in any attempt at classification. Metallic impregnation fails as a criterion because of lack of specificity. Morphological distinctions fail because of startling differences in differentiation even within such a restricted group as the infusoria. Function is the only criterion which will allow fundamental comparisons. Any functional classification made at the present time can be at best only tentative and can serve only as a rough approximation since the function (or more probably functions) of the intracellular organelles is far less known than their morphology and their staining reactions. Functional classification of these granules is not of course new. METCALF (1910), NASSONOW (1924), FAURÉ-FREMIET (1925) and others have pointed out ths relationship between plasma or granules and the excretion of water and other associated materials by the contractile vacuole. KEDROWSKY (1931) after a careful physiological and cytochemical study of the granules of OPALINA classified them on a functional basis.

The functions of a sufficient number of granules in *Opalina* (KEDROWSKY, 1931), the *Ophryoscolecidae* (MACLENNAN 1933, 1934), and *Ichthyophthirius*, and in part in many other ciliates have been described clearly enough to afford a comparative view. The functions ascribed to these granules may be grouped as follows: fat storage, protein storage, carbohydrate storage, digestion and excretion. This classification is not meant to imply that these are the only functions for these granules or that they are the only granules, but that these granules and these functions of these granules are the ones that have been actually demonstrated. The varied degree of differentiation in these ciliates is reflected as clearly by their granules as by their locomotor structures, nuclei, etc. In *Opalina* a single class of granules, the ectosomes, perform three of the functions outlined above. Fat storage alone is idependent. In *Ichthyophthirius*, a

separate type of granule exists for each for four functions. In addition the assembly of stores of protein is transferred to the macronucleus and the cytoplasm merely receives it and resorbs it in times of necessity. In the *Ophryoscolecidae* no significant stores of either protein or fat can be demonstrated, and thus the granules differentiated for these functions are not apparent. Excretion and digestion are each taken care of by separate granules. Carbohydrate storage is accomplished by highly specialised granules and plates.

These varied functions may be included under one head as the synthesis or segregation and storage of various types of materials. Each type of granule is more or less specialised for a particular part of this general function of segregation. The varied staining reactions are a direct result of the varied materials segregated. Since the physiological problems of each group of ciliates are somewhat different, their granules show differing degrees of specialisation resulting in differing details of morphology, staining reactions, and localisation. For example, fat storage is eliminated as an important cytological factor in the *Ophryoscolecidae*. As a result this family has no granules directly comparable with the fat storage granules of *Opalina* and *Ichthyophthirius*. The transfer of the formation of protein reserves from the cytoplasm to the macronucleus in *Ichthyophthirius* emphasizes the fact that the cytoplasmic granules of ciliates cannot be compared in fine detail.

The term segregation apparatus, borrowed from metazoan cytology, has been used by Kedrowsky (1931) to summarize the activity of the ectosomes which segregate vital dyes and other materials. It seems more useful to the present author to extend the use of this term to include all of the normal segregating activities of the granules of the cytoplasm and that is the sense in which it has been used in this paper. The granules included in this single functional concept can be compared easily in ciliates. They include a varied number of types of granules which can be differentiated by the use of a combination of various methods including metallic impregnation, vital staining, microchemical reactions, and history during the life cycle. The different types of granules within the general group of segregation apparatus are not homologous in detail from one ciliate to another and such terms as Golgi apparatus, vacuome, and chondriome do not have fundamentally equivalent meanings in all ciliates. However, it has been found to be most convenient to retain these terms as being a brief and accurate way of designating granules according to the cytological procedure used. Thus Golgi

apparatus refers to granules impregnated by specific metallic methods used as outlined by BOWEN, NASSONOW, HIRSCHLER, and others. These terms are specific for a given animal under given conditions and thus have a clear meaning of practical used. The functional method of classification, however, is the only one offering a fundamental basis for differentiating the varied types of granules.

The granules of the segregation apparatus of *Ichthyophthirius* are recognizable only during the feeding phase of the life cycle. Since the characteristic reactions by which these granules are recognized are due, at least in large part, to the characteristics of materials segregated, the apparent disappearance of this apparatus during the encysted stage may be only a loss of specific staining reactions rather than a true dissappearance. However, lack of division stages in granules during periods of rapid increase in the active granules indicates a true de novo origin of the segregation apparatus. Since KEDROWSKY (1931) found that the ectosomes of *Opalina* also have a de nove origin, it is possible that this is a general rule in all ciliates. The segregation apparatus in ciliates is a specific differentiation of the underlying hyaloplasm rather than an autonomous self-perpetuating set of granules.

Dedifferentiation and redifferentiation in *Ichthyophthirius* involves all visibly differentiated parts of the cell-all cytoplasmic granules, the neuromotor system, and the nuclei (HAAS, 1933). The neuromotor system and the nuclei are reorganized from preexisting elements, but the cytoplasmic granules show no such genetic continuity.

Conclusions.

1. Seven types of cytoplasmic granules are distinguishable by their reactions to cytological and cytochemical methods: vacuome, chondriome, ectoplasmic GOLGI bodies, endoplasmic GOLGI bodies, and reserve granules of neutral fat, of glycogen, and of protein. Vacuome, chondriome, and GOLGI bodies cannot be identified in the late stages of encystment, thus indicating a de novo origin for these components.

2. The vacuome granules are concerned with digestion, and the ectoplasmic GOLGI bodies with excretion. Neutral fat granules are formed by the endoplasmic GOLGI bodies, the glycogen by the chondriosomes. The protein granules are formed within the macronucleus.

3. The cytoplasmic granules of *Ichthyophthirius* are more highly differentiated in function than those of *Opalina* and in turn less highly differentiated than those of the *Ophryoscolecidae*. The cytoplasmic granules of ciliates are thus not comparable in detail. All

these granules, however, are comparable in their function of synthesis and concentration of materials. From this standpoint, the two types of GOLGI bodies, vacuome, and chondriome, may be considered to be a single functional group—the segregation apparatus.

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