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Studies on *Euglypha.*

I. Cytoplasmic inclusions of *Euglypha alveolata.*

By

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(With 14 figures in the text.)

The present investigation is concerned with the types of cytoplasmic inclusions present in *Euglypha*, with the stages in the development of the reserve shell-plates often seen in the cytoplasm, and with the possible genetic relationship between the anlage of the shell-plates and the formed elements of the cytoplasm — mitochondria and the so-called 'Golgi material', or vacuome. The species under investigation appears to be a spineless variety of *Euglypha alveolata* (see Leidy, 1879), which was maintained in laboratory mass cultures.

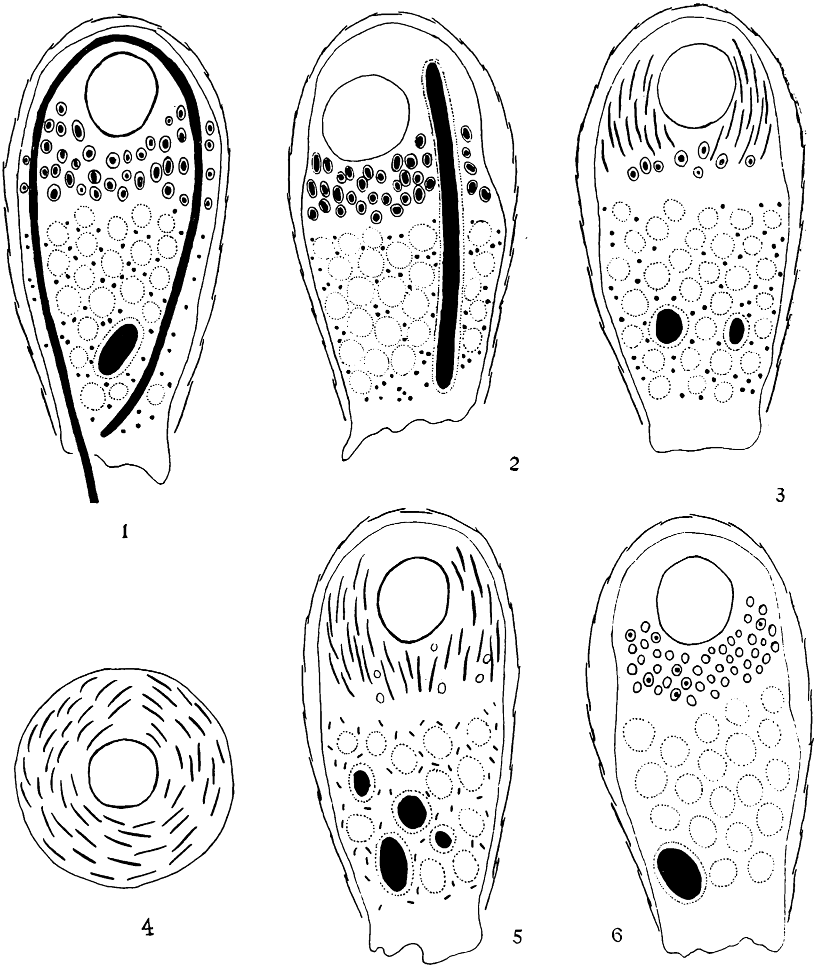
In the use of vital dyes, slides free from grease and moisture were filmed with a solution of the dye in absolute alcohol. One per cent stock solutions of the dye were diluted with absolute alcohol before using. Dilutions of 1 : 20, or higher, have been found satisfactory in the case of neutral red. Janus green was used in 1 % solution as well as in various dilutions, and a mixture of Janus green (1 %) and neutral red (1 : 20) in equal parts was found to be advantageous in distinguishing between mitochondria and neutral-red-stainable inclusions. After filming, the slide is allowed to dry; then a drop of the medium containing the organisms is placed on the film, and a coverglass added. The edges of the coverglass are then sealed with melted vaseline to prevent evaporation and thus permit continuous observation over a period of several hours.

In addition, coverslips were filmed with neutral red, the organisms added, and then the preparations were placed over drops of 2 % osmic solution on slides. With the edges of the coverglass sealed with melted vaseline, the gradual action of the osmic acid on the neutral-red globules and other cell structures could be followed under direct observation with an oil immersion lens. In the concentration of material, the centrifuge method was used (HALL, 1929). Permanent preparations were made by the KOLATCHEV method of osmic impregnation, the DA FANO silver method, the REGAUD mitochondrial method, and by fixation in CHAMPY's fluid and staining in iron-alum hematoxylin.

Inclusions of the living organism.

In unstained living specimens of *Euglypha* there is considerable variation in the relative numbers of different types of inclusions, and to some extent the intracellular distribution of these inclusions varies also. DOFLEIN and REICHENOW (1927, p. 38) recognize three zones in the cytoplasm, a 'chromidial zone' surrounding the nucleus, a granular zone lying below the nucleus, and an alveolar zone extending to the mouth of the shell. LEIDY (1879), likewise, mentioned a zone of "oil molecules" lying just below the nucleus, and an alveolar zone extending to the mouth of the shell and containing food-masses and clear fluid-filled vacuoles. In our material such definite zones are often clearly recognizable (Figs. 1, 2, 6); in other instances, however, the central granular zone is not clearly defined (Figs. 3, 5); in such specimens, the number of reserve plates in the cytoplasm is fairly large.

The inclusions of the granular zone are apparently not oil droplets, as was suggested by LEIDY (1879), since they are not dissolved in alcohol and are not stained by methods for demonstration of fatty inclusions. Nor do they appear to be food vacuoles. As previously pointed out by LEIDY, this granular zone presents "by transmitted light a more or less black and punctated appearance". When observed under an oil immersion lens the contents of these vacuoles appear relatively dark and refractile when the diaphragm of the condenser is opened wide; their appearance is strikingly similar to that of the reserve shell-plates in the cytoplasm and the plates making up the shell. Bacteria and other inclusions in food vacuoles do not present such an appearance.



Figs. 1—6. *Euglypha alveolata,* semidiagrammatic sketches from living material; 900 : 1 approximately. Fig. 1. Neutral-red preparation; neutral-red globules scattered among vacuoles of alveolar zone; inclusions of granular zone numerous, but no reserve shell-plates; ingested algal filament and smaller food mass indicated. Fig. 2. Similar neutral-red preparation. Fig. 3. Neutral-red preparation; reserve shell-plates fairly numerous, but only a few inclusions in granular zone. Fig. 4. Optical cross-section at level of nucleus, showing arrangement of reserve shell-plates; outer shell not indicated in drawing. Fig. 5. Janus green preparation showing rod-like mitochondria; numerous shell-plates, and only a very few small, faintly visible spheres in granular zone. Fig. 6. Unstained specimen; granular zone contains many homogeneous, refractile spheres and a few small refractile inclusions in vacuoles.

The lower, or alveolar zone, contains a number of clear fluid-filled vacuoles, usually one or more food vacuoles, and also a number of small inclusions scattered among the vacuoles. The smaller inclusions are of two types: small globules and small rod-shaped inclusions. The food vacuoles are usually confined to this alveolar zone, but some exceptions have been noted, especially in the ingestion of filamentous algae and large diatoms. Such ingested algae may extend into the fundus region (Fig. 2), or may even form a loop completely encircling the nucleus (Fig. 1).

The fundus zone of cytoplasm contains the nucleus and, in many instances, a variable number of reserve shell plates (Figs. 3, 5). These reserve plates lie more or less parallel to the surface of the organism (Fig. 4), and in most cases are limited to the regions lateral to and below the nucleus. One of several exceptions is shown in Figure 8. Relatively few globular or rod-like inclusions are to be observed in the fundus region; these are usually limited to the alveolar zone. The inclusions of the granular zone occasionally extend upward on either side of the nucleus (Figs. 6, 7); such a condition has been noted especially when no recognizable shell-plates are present in this region.

Inclusions stainable with neutral red.

In specimens stained vitally with neutral red (Figs. 1—3) small globules, especially numerous in the alveolar zone, take the dye. These neutral-red globules, in many instances, have not been seen outside this alveolar zone. A few specimens, however, have shown them extending upward almost to the level of the nucleus, especially when a well-defined granular zone is not present, and in rare instances the globules have been seen above the nucleus. These neutral-red globules are more or less uniformly scattered among the vacuoles of the alveolar zone, and they seem to be invariably present in this region. Brownian movement of these inclusions was sometimes observed, for example, near the mouth of the shell during the extension of pseudopodia.

The reaction of the neutral-red globules occasionally varies in different specimens, and sometimes at different periods in the same individual, or even in different regions of the same individual. For instance, the globules are usually a definite pink in color; yet they may, in some cases, appear slightly bluish-red or almost violet, the latter two colors indicating a p_H considerably lower than 6,8. The significance of such differences and changes in reaction of these inclusions is still uncertain.

Osmication of globules stained with neutral red.

Following preliminary vital staining with neutral red, specimens of *Euglypha* were treated with 2 $^0/_0$ osmic solution in sealed slide preparations. The gradual action of the osmic acid was then observed under an oil immersion lens. During the first few minutes the principal change noted was the diffuse staining of the cytoplasm

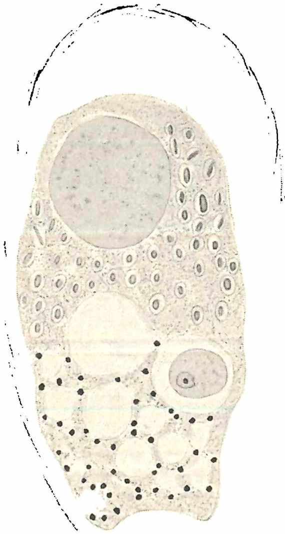


Fig. 7. Kolatchev osmic impregnation, bleached in turpentine 24 hours; organism partly contracted; osmiophilic globules scattered in alveolar zone; granular zone contains numerous refractile inclusions in vacuoles; no reserve plates present. 955 : 1.

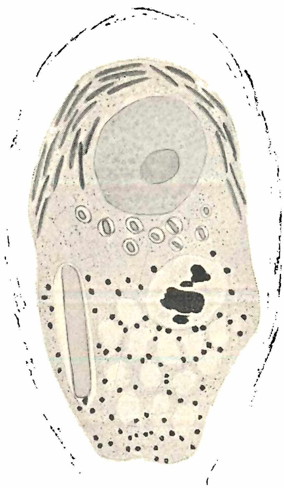


Fig. 8. Kolatchev osmic impregnation, bleached in turpentine 24 hours; numerous osmiophilic globules in alveolar zone; one food vacuole contains blackened material; a number of reserve shell-plates are present, but only a few inclusions are seen in the granular zone. 955 : 1.

with neutral red. As a result, the neutral-red globules stood out less sharply against the cytoplasmic background. After 20—30 minutes, the globules appeared bluish-red in many instances; in other specimens the dye had begun to fade from the globules. After about two hours the dye had faded from most of the globules; they were still distinctly visible, however, and appeared faintly grayish and highly refractile. Eighteen to twenty hours later the globules appeared light gray in color, and after three days they were darkened to a medium shade of gray. At the end of five or six days most of the globules were dark gray, and after 12—14 days they were well blackened.

Inclusions impregnated with osmic acid and silver.

In material impregnated by the KOLATCHEV method and bleached in turpentine, small globules in the alveolar zone are definitely blackened (Figs. 7, 8, 13). These blackened inclusions correspond in size and distribution to the globules stained vitally with neutral

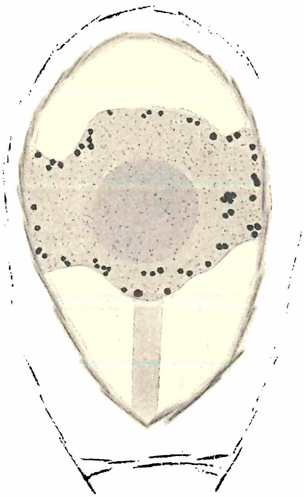


Fig. 9. Immature cyst; DA FANO silver impregnation; argentophilic globules near periphery of cytoplasm. 955 : 1.

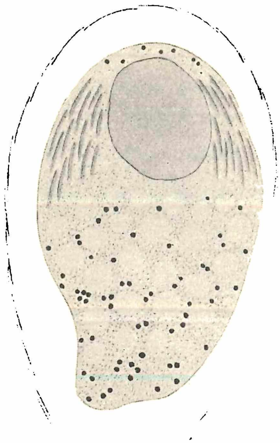


Fig. 10. DA FANO silver impregnation, organism not quite parallel to plane of drawing; argentophilic globules rather numerous in alveolar zone, and a few are seen near the end of the fundus zone; granular zone not evident. 1010 : 1.

red. As a rule, the globules are limited to the alveolar zone, although a few osmiophilic globules were occasionally seen in the lower part of the alveolar zone. Sometimes the contents of one or more food vacuoles may be partly or completely blackened with osmic, but blackening of the wall of vacuoles was not observed.

In encysted stages of *Euglypha* also, small globules are visible in impregnated material. As indicated in the figure of a DA FANO silver preparation (Fig. 9), these inclusions are relatively numerous in the peripheral zone of cytoplasm. Globules of similar appearance and distribution were also seen in KOLATCHEV osmic preparations of encysted stages.

In DA FANO silver preparations of vegetative forms (Fig. 10) small blackened globules are scattered through the alveolar zone. In a few instances, as indicated in Fig. 10, impregnated globules were observed in the upper part of the fundus zone. In size and distribution these argentophilic globules resemble the inclusions stained vitally with neutral red and the osmiophilic globules seen in KOLATCHEV preparations.

Mitochondria.

Short, rod-like inclusions take the dye in preparations stained vitally with Janus green (Fig. 5). These inclusions are rather

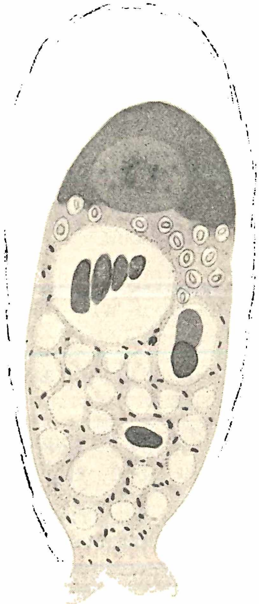


Fig. 11. Champy fixation followed by iron-hematoxylin; the rod-like inclusions of alveolar zone assumed to be mitochondria; granular zone displaced somewhat by a large food vacuole. 955 : 1.

Fig. 12. A smaller specimen showing fewer mitochondria; REGAUD'S chondriosome method. 1350 : 1.

numerous, as a rule, and are scattered throughout the alveolar zone, although they often appear to be somewhat more numerous near the periphery of the cell. In Janus green preparations, the vacuolar inclusions of the granular zone remained unstained.

Preparations fixed in CHAMPY'S fluid and stained in hematoxylin show numerous rod-like inclusions scattered among the vacuoles of the alveolar zone (Fig. 11). These inclusions are similar in size and distribution to the ones observed in material stained vitally with Janus green. Similar inclusions were observed in preparations by REGAUD's method for demonstration of chrondriosomes (Fig. 12). In both types of material, the inclusions of the granular zone were not stained.

Origin of reserve shell-plates.

In observations on both living specimens and permanent preparations of *Euglypha* extensive variations have been noted in the number of reserve shell-plates in the fundus zone. Some specimens show no reserve plates, and when this is the case the granular zone invariably contains a large number of inclusions (Figs. 1, 2, 6, 7). Specimens containing a few reserve plates show a smaller number of inclusions in the granular zone (Figs. 3, 8), while those with many reserve plates show few or no inclusions in the granular zone (Figs. 5, 10). In other words, the number of inclusions in the granular zone is, in a general way, inversely proportional to the number of recognizable shell-plates in the cytoplasm.

These inclusions are apparently not oil droplets, as was suggested by LEIDY (1879). They are not blackened with osmic acid, and they are not stained by the CIACCIO method for demonstration of lipoids; nor are they soluble in alcohol. Furthermore, these inclusions do not appear to be food vacuoles, since they do not show the pink or yellow color with neutral red which is so characteristic of those vacuoles definitely recognizable as food vacuoles. As mentioned above, these inclusions also differ in optical appearance from food vacuoles of comparable size in unstained specimens.

In both permanent preparations and living specimens, the inclusions are usually seen as vacuoles containing homogeneous refractile bodies, varying in size and to some extent in shape. Occasionally, however, the inclusions seem to be dense, refractile homogeneous spheres (Fig. 6). In other instances the inclusions appear as vacuoles containing very small refractile bodies; these are often found associated with the homogeneous spheres just mentioned. In other specimens the vacuoles contain somewhat larger inclusions (Figs. 1—3, 11—12). In many cases the inclusions are elongated, and appear to be definitely flattened (Figs. 1, 2, 7, 13); the vacuoles which contain them are correspondingly elongated. These elongated inclusions are of different sizes, the large ones approaching closely, in both size and appearance, the definitive reserve shell-plates (Fig. 13).

Thus it is obvious that these inclusions of the granular zone may be arranged in a series (Fig. 14), extending from homogeneous spherical bodies on the one hand to elongated plate-like structures which seem to differ from definitive reserve plates only in that the former lie in vacuoles and are somewhat smaller in size. The optical appearance of the inclusions — from the small, almost granular inclusions to the elongated plates — is the same. They are refractile, and resemble in appearance the material of which the shell plates and reserve plates is composed.

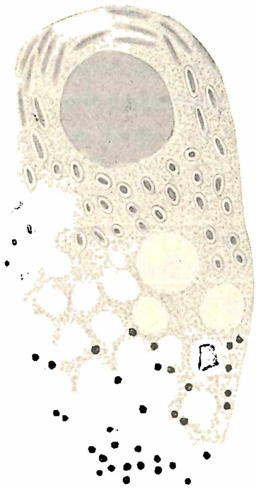


Fig. 13. Kolatchev osmic impregnation, bleached in turpentine; this specimen shows a number of elongated and flattened inclusions in the granular zone, some of which are seen in edge view, and a few plate-like inclusions in vacuoles lateral to the nucleus; in the upper part of the fundus zone a few definitive reserve plates are seen; relatively few osmiophilic globules in alveolar zone. 1145 : 1.

Whether these stages represent a genetic series in the development of reserve shell-plates is, of course, not entirely certain. It would be difficult to prove any direct connection between the homogeneous refractile spheres and the vacuoles with definite refractile inclusions, although it is not impossible that a condensation of the contents of such a spherical body might result in formation of a concrete mass enclosed in a vacuole. From the very small inclusions to the elongated plate-like bodies, however, the continuous series of gradations in size, as well as the striking similarity in optical appearance throughout the series, points to the plausibility of such an explanation of the origin of reserve shell-

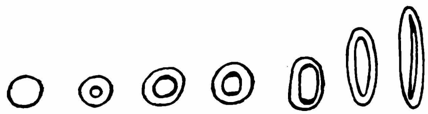


Fig. 14. Diagrammatic sketches showing range in shape and relative size of inclusions seen in granular zone.

plates. Furthermore, the number of such inclusions is in a general way inversely proportional to the number of definitive plates present in the cytoplasm.

Granting the derivation of the reserve plates from these inclusions of the granular zone, there still remains the question of a possible genetic relationship between such plate anlage and the mitochondria or the neutral-red globules. It has been stated by JONES (1929) that shell plates are formed from mitochondria in *Euglypha alveolata*; the evidence for this statement was not presented in his paper, however, and we are thus unable to compare his observations with our own.

In our material there appears to be no evidence which suggests the origin of reserve plates from either the neutral-red globules or the mitochondria. The definitive shell-plates of the cytoplasm can be traced with some degree of probability to the small refractile inclusions of vacuoles in the granular zone; and it is possible that the latter may be derived from the small, homogeneous refractile spheres occasionally seen in this region. We have been unable, however, to find any reason for relating such anlage to the vacuome or to the mitochondria. There are no similarities in reaction to vital dyes, to osmic or silver impregnation, to methods for demonstration of mitochondria, or in optical appearance, which might support such an hypothesis. Although extensive synthetic powers have often been attributed to the mitochondria in both *Protozoa* and *Metazoa*, the writers believe that there is no sound basis for adding the shell-plates of *Euglypha* to the already long list of mitochondrial products.

Discussion.

Neutral-red-stainable inclusions of *Protozoa* have been observed by a number of investigators, but JOYET-LAVERGNE (1926) was apparently the first to identify these bodies with similar inclusions which may be impregnated with osmic acid. Among the *Sarcodina*, such osmiophilic, neutral-red-stainable inclusions have been described previously in *Trichamoeba* sp. (HALL, 1930) and in *Arcella vulgaris* (NIGRELLI and HALL, 1930). The neutral-red-stainable inclusions of *Euglypha alveolata* appear to be quite similar in form and in reactions to vital dyes and osmic and silver impregnation. As in *Arcella* and *Trichamoeba*, these inclusions of *Euglypha* are visible in the living organism, are stainable vitally with neutral red, and may be impregnated by osmic or silver methods without previous treatment with vital dyes. Furthermore, material has been stained vitally with neutral red and then blackened with osmic under direct observation in sealed slide preparations; the inclusions stained with neutral red are gradually impregnated by this method. Hence, there appears to be no probability that these osmiophilic inclusions of *Euglypha* are in any sense 'artefacts', or that their appearance is induced by the action of vital dyes. The similarity of these inclusions of *Euglypha* to the vacuome (see PARAT, 1928) is obvious. They also resemble, in their reactions to osmic and silver impregnation, the dispersed phase ('GOLGI bodies') of the metazoan GOLGI apparatus.

BEAMS (1929) has stated that, in mammalian pancreatic cells, the vacuome "does not blacken with the osmic acid methods commonly used to demonstrate the GOLGI apparatus . . . unless previously vitally stained with neutral red". He concludes, therefore, that in his material the vacuome and the GOLGI apparatus are separate and distinct cell constituents. In *Euglypha*, as well as in *Arcella* and *Trichamoeba*, the neutral-red-stainable inclusions (vacuome) may be impregnated by osmic and silver methods without previous exposure to vital dyes. And, as indicated by vital staining followed by osmic impregnation under direct observation, the neutral-red globules and the osmiophilic globules are one and the same type of inclusions.

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