STRUCTURAL AND CYTOCHEMICAL STUDIES OF THE CYTOPLASM IN THE FAMILY AMOEBIDAE[1]

GEORGE D. PAPPAS

Department of Zoology and Entomology, The Ohio State University, Columbus 10

The classification and nomenclature of the cytoplasmic components and inclusions of free-living amebas were systematized by S. O. Mast and his students in a series of papers from 1926 to 1941. However, almost all of their work was done on two rather similar species, *Amoeba proteus* and *Chaos chaos*. In recent years cytochemical and quantitative physiological investigations of these two species, especially *C. chaos*, have been conducted by Holter and Andresen of the Carlsberg Laboratories in Denmark. Such information has been almost entirely lacking about other species of amebas, many of which differ strikingly in their general morphology from the two mentioned. In the present investigation, a study of the cytology and cytochemistry of three very diverse species, *Thecamoeba striata, Mayorella bigemma,* and *Amoeba guttula,* was undertaken, and *Amoeba proteus* and *Chaos chaos* were studied at the same time for purposes of comparison.

Along with the classical cytological techniques, including vital staining, a cytochemical approach has been employed for a better understanding of the nature of the cell components through a knowledge of their chemical composition. Since the majority of present histochemical methods are designed for use with metazoan tissues, special techniques had to be devised for applying these tests to protozoa.

MATERIALS AND METHODS

The Species Studied

Amoeba proteus (Pallas, 1766) Leidy, 1878. Cultures of *Amoeba proteus* have been maintained continuously in the Protozoology Laboratory at The Ohio State University for many years. The culture method, using *Saprolegnia* sp. growing on rice grains, with *Chilomonas paramecium* as the chief food organism, has been described in detail by Handy (1947).

Many synonyms exist for *Amoeba proteus*. (Mast and Johnson, 1931). However, the only other name used today is *Chaos diffluens,* proposed by Schaeffer in 1926. *Amoeba proteus* can be distinguished from two similar species, *Amoeba dubia* and *Amoeba discoides,* by the presence of ridges on one or two of its larger pseudopodia. Also *Amoeba proteus* measures 600 or more microns when elongated, whereas *Amoeba discoides* and *Amoeba dubia* rarely measure more than 400 microns (Kudo, 1946; Jahn and Jahn, 1949).

The cytoplasm of *Amoeba proteus* has undoubtedly been studied more often than that of any other species of ameba. The nomenclature of the various inclusions and structures of the cytoplasm here employed is that proposed by Mast (1926) in his first paper on the structure of this species.

Chaos chaos Linnaeus, 1758. This so-called giant ameba has, along with *Amoeba proteus,* been maintained continuously in the Protozoology Laboratory of The Ohio State University for many years. The culture method employed is similar to that for *Amoeba proteus*. Except for its tremendous size (1–3 mm.) and multinucleate condition, *Chaos chaos* is rather similar to *Amoeba proteus*.

The giant ameba has been designated by several scientific names, the most common being *Chaos chaos* Linnaeus and *Pelomyxa carolinensis* Wilson.

[1]From a dissertation submitted to the Graduate School of The Ohio State University in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

THE OHIO JOURNAL OF SCIENCE 54(3): 195, May, 1954.

Thecamoeba striata (Penard, 1890) Schaeffer, 1926. *Thecamoeba striata* is characterized by the presence of a pellicle which has prominent longitudinal ridges or folds on the upper surface during locomotion. In one respect it differs rather strikingly from all other amebas, both pelliculate and non-pelliculate. It contains a contractile vacuole of irregular and changing shape. Most individuals have a second contractile vacuole which is always spherical.

Thecamoeba striata measures up to 120 μ during locomotion. It is limax shaped, seldom forming any pseudopodia; the whole organism advances with an anterior hyaline cap making up as much as one-third of the entire animal. This anterior hyaline cap is always indicative of the direction of locomotion. A smaller form of *Thecamoeba striata* was described by Penard (1902) and by Pappas (1948). Most of the observations recorded in the present paper were made on the larger of the two varieties.

Clone cultures of *Thecamoeba striata* have been maintained in Reynolds' (1924) strained hay infusion. Ten grams of timothy hay (*Phleum pratense*) are boiled in 250 ml. of distilled water for 15 min. The broth is then strained through cheese cloth and diluted with 2500 ml. of distilled water. Cultures of *Chlamydomonas apiculata* in its passive stage are used as the food organism for the ameba. The cultures are prepared in Syracuse watch glasses.

Mayorella bigemma (Schaeffer, 1918) Schaeffer, 1926. *Mayorella bigemma* is characterized by the presence of small conical pseudopodia which form continuously along the anterior edge and on the upper surface while the animal is in locomotion. These pseudopodia do not determine the direction of locomotion, but are carried along by the ameba as a whole. They are not static, but rather in continuous extension and retraction during locomotion. Both the anterior margin of the ameba and the conical pseudopodia are composed of clear, hyaline cytoplasm. Individuals of *Mayorella bigemma* vary from 60–200 μ in length during locomotion.

Clone cultures of *Mayorella bigemma* were established under the same conditions as those for *Thecamoeba striata*, with *Chlamydomonas apiculata* as the food organism in strained hay infusion. Also *Ochromonas sp.*, *Euglena gracilis*, and bacteria have been used successfully as food organisms in strained hay infusion. The cultures were maintained in short stender dishes. Detailed accounts of culture methods and behavior of *Mayorella bigemma* have been given by Lotze (1934, 1937).

Amoeba guttula Dujardin, 1841. *Amoeba guttula* was found thriving in the top scum formation of hay infusion cultures a week or two after inoculation. The pH of the top scum was 5.0–5.3. When the pH became alkaline, the scum tended to sink to the bottom of the culture. At this time *Amoeba guttula* disappeared completely. Bacteria are the chief food of this organism.

Amoeba guttula is the smallest of the five amebas studied, measuring 20–25 μ during locomotion. This ameba has lobose pseudopodia which are extremely short and composed entirely of hyaline cytoplasm. Because they are both lobose and short, they often appear almost circular. Each pseudopod appears and develops with characteristic abruptness. At the anterior end of the ameba about one-third to one-half of the cytoplasm is hyaline.

Preparation of Amebas

General. The most useful method of investigating the cytoplasm of amebas is undoubtedly by study of the living specimens. Vital stains and phase contrast microscopy have both proven extremely helpful in such study.

However, one cannot avoid the use of killed and fixed material, since most stains and chemicals which are of value for the study of cellular constituents kill the organism. Some reagents, such as Lugol's solution and methyl green, serve as both killing and fixing agents, and are usually employed with simple mounts. However, for most cytochemical methods, more complicated procedures are necessary, including the use of various special reagents. These will be described on a later page.

Many methods have been described for the preliminary handling in preparation for fixing, staining, etc., of protozoa. (See McClung's Handbook of Microscopical Technique, 3rd ed. p. 441 *et seq.*) Most of these methods were tried but with indifferent success and were abandoned in favor of the parlodion trap technique described below.

The Parlodion Trap Technique. The parlodion trap method used in this study was developed by Concannon (1951) for handling small nematodes. With slight modifications, this method was found to be very satisfactory for handling amebas in preparation for subsequent staining, mounting, etc.

The parlodion solution used consists of 3½ g. of parlodion (highly purified cellulose nitrate) dissolved in 100 ml. of absolute alcohol and 100 ml. of ether. The solution should be kept under refrigeration.

The trap itself is a loop made of lacquer coated wire (about #30 B & S gauge) by taking a piece about three inches long, wrapping it once around some cylindrical object, such as glass tubing, and then twisting the free ends together. The circlet is designated as the trap, and the twisted part the handle. The trap and handle are flattened so that both are in the same plane (fig. 1).

FIGURE 1. Diagram of parlodion trap for collecting amebas.

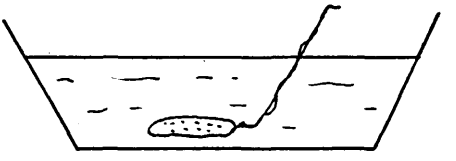


FIGURE 2. Diagram showing position of trap in reagent and staining dishes.

The procedure is as follows. A drop of medium containing amebas is put on a perfectly clean slide and allowed to stand until only a thin wet film remains. A drop or two of absolute alcohol is then added to the middle of the film, which forces the water to the film's periphery. The alcohol fixes the organisms and most of them remain in their original location. The remaining water is then removed with filter paper. If some other fixing reagent is used before the treatment with absolute alcohol, more of the amebas are lost for they do not adhere as well to the glass and are thus forced to the periphery of the film with the water.

When almost all of the absolute alcohol has evaporated from the film, the trap is placed on the slide so that most or all of the film containing the amebas will be inside the loop. One or two drops of parlodion solution are then dropped on the slide within the loop. Care should be taken to see that too much of the solution is not added to the loop, since the parlodion would then be difficult to de-stain. When the parlodion is almost dry, the slide should be tipped so that excess solution will run down the handle from the loop. In this way, a very thin membrane in which the amebas are embedded remains in the loop.

The slide, with the trap in place, is then either flooded or gently immersed in water while being kept horizontal. This is a very important step, as water must penetrate between the parlodion and the glass slide to prevent adhesion between them. After a few minutes, the trap may be removed from the slide by grasping the wire handle with forceps and lifting gently upwards. If the effort has been successful, a very thin membrane of parlodion is left within the wire loop, and in this membrane the amebas are embedded. In a certain percentage of trials, the parlodion film ruptures, but approximately 70–80 percent of the attempts are successful. The diameter of the wire loop should not exceed 12–13 mm., since the membrane ruptures easily on larger loops.

For the subsequent steps (staining, etc.) it is desirable that shallow reagent and staining dishes be used in order that the wire handle of the trap should not be completely immersed in the solutions (fig. 2). If several traps are to be treated

identically, the end of the handle of each may be bent into a hook. Then the traps can be hung from glass rods or thick wire, etc., and in this manner transferred from solution to solution.

The parlodion membrane will take up stain along with the amebas embedded in it, but will lose the stain much more readily than the amebas during washing or passage through alcohol.

Parlodion is soluble in absolute alcohol, so that it is necessary that the final dehydration in the preparation of permanent slides be accomplished by the use of 25 ml. melted carbolic acid crystals to 75 ml. of xylene. When the trap is first placed in this solution from 95 percent alcohol, the parlodion membrane will appear milky, but it usually becomes clear within a few minutes. In any event, the trap should be left in the carbol-xylol long enough for the membrane to appear completely transparent, for it is not until then that the water has been completely removed. The trap is then transferred to xylene.

After a few minutes the trap is taken out of xylene, drained to remove the excess, and placed onto a drop of mounting medium on a clean slide. (Any xylene-soluble mounting medium is satisfactory.) A drop of the mounting medium is then placed on top of the parlodion membrane. The latter is then freed from the sides of the wire loop with a sharp dissecting needle or scalpel, and a very thin coverslip(#0 preferred) is placed on the mounting medium.

The parlodion trap technique is not suitable for some cytochemical tests, because both free lipids and free carbohydrates are removed by the organic solvents. However, for reactions such as Feulgen's and Millon's and for water insoluble polysaccharides visualized by the PAS reaction, material prepared by the parlodion trap method is quite adequate.

Cytochemical Methods

Cytochemistry is concerned with the localization of chemical entities in living animal and plant cells. Lison (1936) first systematized the criteria by which one must evaluate cytochemical technique, and the reader is referred to his discussion of the subject.

Since almost all of the cytochemical methods now employed were originally designed for use with metazoan tissues, it was necessary to devise modifications in applying these tests to protozoa. Such modifications are incorporated into the descriptions of these methods in the following pages.

Reactions Indicating Proteins

Millon's reaction for tyrosine. The cytochemical adaptation of Millon's reaction was accomplished by Bensley and Gersh (1933). The procedure, as outlined in Lison (1936), is modified for this study as follows:

1. Amebas mounted in a parlodion trap are placed in Millon's reagent (at room temperature) for about 5 hr. or until maximum color develops.
2. The trap is then immediately placed in 1 percent nitric acid solution, and left there for 2–3 min.
3. The trap is placed on a slide, and 2–4 drops of absolute alcohol are dropped on the parlodion film, which dissolves almost immediately. The trap is removed and the cover-slip is placed on the alcohol, which now contains all the amebas that were in the film. More absolute alcohol is added at the edge of the cover-slip as needed.

Only temporary mounts are usually attempted with this procedure since the color of the preparation fades within a few days.

Results. A rose or brick color indicates the presence of tyrosine-containing protein in the cell. The coloration is due to the particular aromatic radical found in tyrosine. However, Lison (1936) points out that a certain number of non-protein phenol compounds may also give this reaction. Their distribution in cells, however, is quite limited.

Arginine. Baker's (1947) modification of the Sakaguchi test for arginine was employed. Reagents and procedures were modified in the present study as follows:

Reagents. 1. Absolute alcohol as a fixing agent.

2. Parlodion, 3.5 percent, in a mixture of absolute alcohol and ether in equal volumes.

3. NaOH, 1 percent, aqueous.

4. α-naphthol, 1 percent, in 70 percent alcohol.

5. Hypochlorite solution (2 volumes sodium hypochlorite and 1 volume of 0.05 N NaOH).

6. A mixture of 3 volumes of pyridine with 1 volume of chloroform.

Procedure. 1. Amebas are mounted in a parlodion trap. The trap is then placed in distilled water.

2. Then 2 ml. of 1 percent NaOH are put into an old-fashioned watch glass and 2 drops of α-naphthol solution and 4 drops of hypochlorite solution are added.

3. The parlodion trap is now placed in a depression slide. The mixture just described is then gently shaken and quickly added to the parlodion-embedded amebas on the trap and allowed to remain for about 15 min.

4. The trap is then transferred to a flat slide and pyridine-chloroform mixture is added. This dissolves the film so that the trap may be removed at once, and a cover-slip is placed over the mixture with its contained amebas. More of this mixture is added at the edge of the cover-slip as needed to make up for evaporation.

Results. A pink or red color indicates the presence of arginine (free or combined) or some other positively-reacting guanidine derivative in cells. The test is based on the development of a red color by arginine when α-naphthol and hypochlorite react with it in an alkaline medium.

Feulgen nucleal reaction. This reaction makes possible the visualization of desoxyribonucleic acid (DNA). Indeed, the Feulgen reaction is considered to be one of the most specific cytochemical reactions (Stowell, 1946). The reagents and procedures were modified from those outlined by Glick (1949).

Reagents. 1. Fixing reagent—absolute alcohol or formaldehyde-calcium (10 ml. of full strength formalin, 10 ml. of 10 percent calcium chloride (anhydrous) and 80 ml. of distilled water).

2. HCl, 1 N.

3. Coleman (1938) preparation of Schiff's reagent.

4. Sulphurous acid solution—add 30 ml. of 1 M sodium bisulfate solution and 30 ml. dilute HCl to 600 ml. of tap water.

Procedure. 1. Amebas fixed in either absolute alcohol or formaldehyde-calcium are mounted in parlodion.

2. Parlodion traps containing the amebas are then placed for a minute or two in HCl solution at room temperature.

3. They are then transferred to HCl solution at 60° C for 4–5 min.

4. Then they are treated with Schiff's reagent for 1½–3 hr.

5. The trap is then passed through three separate baths of the sulphurous acid solution and left in each for 1–2 min., agitating frequently.

6. Then the trap is washed for about 5 min. in tap water.

7. The trap is then dehydrated and cleared as has been described for the parlodion trap technique, and mounted in balsam.

Results. A purple color indicates the presence of desoxyribonucleic acid. Acid hydrolysis removes the purine bases and exposes free aldehyde radicals which stain purple when treated with fuchsin-sulfurous acid of Schiff's reagent. The sulphurous acid solution removes the excess Schiff's reagent from the cell.

Detection of ribonucleic acid. Visualization of ribonucleic acid (RNA) in the cell is accomplished cytochemically by the use of subtractive methods, these being either depolymerization of RNA with ribonuclease or extraction with perchloric

acid as compared with non-treated controls. Since nucleoproteins are basophilic, basic dyes such as toluidine blue O will reveal their presence in the cell.

The ribonuclease technique. The technique used in this study was modified from that recommended by Stowell and Zorzoli (1947).

Reagents. 1. Fixing reagent—formaldehyde.

2. Ribonuclease (Worthington Biochemical Company, Freehold, New Jersey) 0.1 mg. per ml. of buffer (McIlvaine's citric acid-disodium phosphate mixture buffered at pH 6.5).

3. Toluidine blue O—0.5 percent in distilled water.

Procedure. 1. Amebas, fixed in formaldehyde, are embedded in parlodion, using the parlodion trap technique.

2. One group of amebas is then put into the ribonuclease solution at 50° C while the other is put into plain McIlvaine buffer at 50° C. Both are kept under these conditions for at least 3 hr.

3. Both groups are quickly washed in separate dishes with distilled water.

4. Then in separate staining dishes, both groups are treated for a few minutes with toluidine blue O. After another quick rinse in distilled water, the amebas are mounted in distilled water.

Results. The ribonuclease-treated group is compared with the non-treated group, on the basis of the presence and distribution of basophilic substances. The absence of basophilia in the treated amebas as compared to the non-treated controls indicates RNA.

Perchloric acid technique. The method used here was modified from those of Seshacher and Flick (1949) and Erickson, Sax, and Ogur (1949).

Reagents. 1. Fixing reagent—acetic acid alcohol mixture (1 volume acetic acid to 3 volumes of 95 percent alcohol).

2. Perchloric acid ($HClO_4$)—5 percent in distilled water.

3. Toluidine blue O—0.5 percent in distilled water.

Procedure. 1. Amebas fixed in acetic acid alcohol mixture are embedded in parlodion, using the parlodion trap technique.

2. One group of amebas is then put into the perchloric acid solution at 70° C while the other is put into plain distilled water also at 70° C. Both groups are kept under these conditions for about 30 min.

3. Both groups are quickly washed in separate dishes with distilled water.

4. Then, in separate staining dishes, both groups are treated for a few minutes with toluidine blue O. After another quick rinse in distilled water, the amebas are mounted in distilled water.

Results. The perchloric acid treated group is compared with the non-treated group as to the presence and distribution of basophilic substances. The absence of basophilia in the treated amebas as compared to the non-treated controls indicates the presence of RNA.

Carbohydrate Reactions

Lugol's solution (4 g. iodine, 6 g. potassium iodide per 100 ml. of solution) was used as fixing reagent and as a stain for starch.

Periodic acid-Schiff (PAS) reaction for polysaccharides. McManus (1946) and Hotchkiss (1948) have formulated a cytochemical reaction for the visualization of polysaccharides such as glycogen, mucin, mucoproteins and presumably hyaluronic acid and chitin. The method of Hotchkiss was modified for this study.

Reagents. 1. Fixing reagent—absolute alcohol.

2. Periodic acid solution (H_2IO_6)—0.9 percent in distilled water. Take 45 ml. of this concentration and add 5 ml. M/5 sodium acetate.

3. Iodide-thiosulfate solution. Dissolve 1 g. potassium iodide and 1 g. sodium thiosulfate ($Na_2S_2O_3 \cdot 5H_2O$) in 20 ml. of distilled water and add with stirring

30 ml. of alcohol followed by 0.5 ml. of 2 N HCl. A sulfur precipitate forms and settles out slowly, although the solution may be used immediately.

4. Schiff's reagent. (For preparation, see Feulgen nucleal reaction.)

5. Sulfite wash solution. Add 0.5 ml. concentrated HCl and 2 ml. of 10 percent potassium metabisulfite to 50 ml. of distilled water.

Procedure. 1. Amebas, fixed in absolute alcohol, are embedded in parlodion, using the parlodion trap technique.

2. The amebas, embedded in parlodion, are placed in the periodic acid solution for about 10 min.

3. Washed with water, they are then transferred to the iodide-thiosulfate solution for about 10 min.

4. Again they are washed with water and placed in Schiff's reagent for about 30–60 min.

5. They are then rinsed in the sulfite wash solution for a few minutes.

6. The amebas are then dehydrated and mounted in balsam in the usual manner for the parlodion trap technique.

Results. A violet color indicates the presence of polysaccharides. Polysaccharides are oxidized under mild conditions by periodic acid to polyaldehydes. Then the iodide thiosulfate solution removes the periodic acid from the cells. The polyaldehydes are then colored by Schiff's reagent. Excess Schiff's reagent is then removed by the sulfite wash solution. The pentoses of nucleic acid are so affected by periodic acid that they will not react.

Lipid Reactions

Lipids may be characterized by their sudanophilic properties, i.e., they become colored by Sudan reagents. The Sudan substances are not dyes because they are not ionized. They color lipids by dissolving in them. (Lison, 1936; Cain, 1950).

Sudan III, IV and black B were prepared as saturated solutions in 70 percent alcohol and filtered twice. Sudan black B, the most powerful of the Sudan reagents, was also prepared in ethylene glycol as suggested by Chiffelle and Putt (1951). According to Cain (1950), coloration with Sudan black B depends ultimately not on unsaturation but on the physical state of the object tested.

A 1 percent solution of nile blue sulfate was used on amebas which usually were fixed previously in formalin. Nile blue sulfate has been used to distinguish neutral fats (triglycerides) from fatty acids, the former being dyed red or pink, the latter blue. Lison (1936), after a detailed study, concluded that the red coloration was characteristic of lipids in general and the blue was merely that of a basic dye and therefore totally unspecific. Cain (1947) maintains that if a substance known to be a lipid colors red with nile blue, it consists of neutral lipids; if it colors blue, it may contain these, but acidic lipids (fatty acids, phospholipids and others) may also be present.

The specificity of the extended procedure required by Baker's (1946, 1947) acid hematin test for the recognition of phospholipids is based on empirical criteria. In this study it was not possible to carry out each step of the test exactly as outlined by Baker. Unless new empirical data were to be collected, results which would have been obtained by modifying the original test would have had no cytochemical meaning.

The Schultz method for the demonstration of cholesterol was used as outlined by Lison (1936). After the amebas have been placed in a 2.5 percent iron alum solution at 37° C. for about 8 hr., they are transferred to a slide. Then they are treated with a few drops of acetic-sulphuric mixture (1 part glacial acetic acid in 1 part concentrated sulfuric acid). If a blue-green color develops, then cholesterol is present. If the color does not develop then no conclusion can be drawn, since negative results in the Schultz test do not mean that cholesterol is absent. (Lison, 1936.) The mordant transforms cholesterol to oxycholesterol,

which in turn becomes a colored substance when acted upon by the acetic-sulfuric mixture.

Plasmal reaction. Feulgen and Voit (1924) have described the demonstration of acetal lipids in the cell. From these acetal lipids ("plasmalogen"), the "plasmal" (free aldehyde) is unmasked. The procedure employed in this study for the visualization of the "plasmal" is that suggested by Hayes (1949).

Reagents. 1. $HgCl_2$, 1 percent aqueous solution.

2. Schiff's reagent. (For the preparation, see Feulgen nucleal reaction.)

3. Sulfurous acid wash solution. (For preparation, see Feulgen nucleal reaction.)

Procedure. 1. One group of amebas is placed in 1 percent $HgCl_2$ in a depression slide for about 3–5 min.

2. This group and a control group are transferred into Schiff's reagent in two separate depression slides and left for about 10 min. The depression slides must be covered during this period.

3. Both groups are then transferred into depression slides containing the sulfurous acid wash solution, and left for 2 min. This step is repeated three times.

4. The amebas are then transferred into depression slides containing distilled water.

5. Temporary mounts are then prepared in distilled water.

Results. The presence of violet fuchsin color indicates acetal lipids. According to Feulgen and Voit (1924), the brief action of $HgCl_2$ on the acetal bond of the bound plasmalogen splits it into a fatty aldehyde (plasmal) and glycero-phosphoro-ethanolamine. The plasmal, being an aldehyde, is then readily colored by Schiff's reagent. Hayes (1949) maintains that the plasmal reaction is highly specific for acetal lipids alone, if the time for the action of the $HgCl_2$ is limited to insure that neither acid hydrolysis nor oxidation takes place, and if controls are carefully checked. As far as is known the only acetal lipids which have been demonstrated as present in cells are phospholopids. However, other, non-phospholipid acetals might exist.

Other Methods, Reagents, and Stains

Sulfhydryl Test. The nitroprusside test for sulfhydryl groups as outlined by Chalkley (1937) was employed. One drop of 20 percent zinc acetate was placed on a clean depression slide. By means of a micropipette, amebas were transferred into the zinc acetate solution. Care was taken so that only a minimum of culture medium was transferred with the amebas. One drop of a 1 percent sodium nitroprusside solution was then added. A rose red color results where sulfhydryl is present. Since the color fades in a few hours, observations on the amebas in the depression slide must be made immediately. The nitroprusside test has been severely criticized by Lison (1936) and Hammett and Chapman (1939) as being unreliable as a quantitative reaction and also because the resulting coloration may diffuse throughout the cell. Bennett (1948) has developed a more accurate method for the cytochemical determination of sulfhydryl, employing a compound which was synthesized specifically for this purpose. The compound was not available, however, for this study.

Miscellaneous. A 2 percent solution of osmium tetroxide (0.5 g. osmic acid crystals in 25 ml. distilled water) was employed as a fixing reagent. Exposure to the OsO_4 vapors of this solution for 30 sec. to 2 min. was quite satisfactory.

Commercial formalin (40 percent formaldehyde) vapors were employed for fixing, as well as 10 percent solutions of formalin. Formalin-calcium solution (10 ml. full strength formalin, 10 ml. calcium chloride 10 percent, anhydrous, and 80 ml. distilled water) was also used. Champy's fluid (70 ml. of 3 percent potassium bichromate, 70 ml. of 1 percent chromic acid, 40 ml. of 2 percent osmium tetroxide) was used as a fixing agent for mitochondria. Transeau's fixative (H_2O—6 parts, 95 percent alcohol—3 parts, formalin—1 part) was used as a

general fixing reagent. Schaudinn's fluid (saturated aqueous solution $HgCl_2$—2 parts, 95 percent alcohol—1 part) was also used as a routine fixing agent.

A 0.02 percent solution of ruthenium red (ruthenium oxychloride, ammoniated, 1 : 5,000) was employed. Neutral red was used in the following concentrations: 1 : 75,000 (0.00133 percent), 1 : 100,000 (0.001 percent), and 1 : 150,000 (0.00077 percent). Janus green B was used in a concentration of 1 : 100,000 (0.001 percent). A 0.2 percent solution of methyl green was prepared in 1 percent acetic acid. Aniline fuchsin for staining mitochondria was prepared from 10 g. acid fuchsin in 100 ml. of aniline water (8 ml. aniline oil in 180 ml. of distilled water). Of the hematoxylin stains, Delafield's and Heidenhain's iron hematoxylin were used. A 2 percent iron alum solution was used as a mordant when staining with Heidenhain's iron hematoxylin. Toluidine blue O was prepared as a 0.5 percent solution in distilled water as recommended by Lison (1936). A stock solution of 3 percent H_2O_2 was also used.

Doyle's (1933) preparation for the simultaneous demonstration of fat and starch was used.

Microscopes and Accessories

A Spencer (American Optical Company) binocular research compound microscope with apochromatic objectives (16, 4, and 2 mm.) and compensating oculars (10, 15, and 20×) was used throughout this study. A phase-difference microscope (American Optical Company) equipped with a 97× B minus contrast (low) objective as well as 10, 43, and 97× dark contrast (medium) objectives was also used. A stereoscopic binocular microscope (American Optical Company) was used as an aid in manipulating and transferring amebas. A polarizing microscope (American Optical Company) was used to determine optical activity of crystals.

Measurements were made with the aid of ocular micrometers. Each microscope was calibrated for use with a specific ocular micrometer. All drawings were made with the aid of a camera lucida.

RESULTS

The Cell Membrane

The cell membrane or outermost limiting layer is a seemingly unspecialized plasma membrane in *Amoeba guttula* and *Mayorella bigemma*. In the genus *Thecamoeba*, there is formed an outer relatively thick covering called a pellicle. Howland (1924c) removed the pellicle from *Thecamoeba verrucosa*, leaving the plasma membrane intact. Apparently intermediate between a thickened pellicle and an unspecialized plasma membrane is the outer covering, the plasmalemma, found in *Amoeba proteus* and *Chaos chaos*.

Thecamoeba striata is characterized by the presence of a number of delicate longitudinal lines (usually 4 to 6) on the surface. These longitudinal lines are present during locomotion or whenever the ameba is elongate. When it rounds up, there are many folds and indentations on the surface. The width of the longitudinal lines is about 0.75 μ. The thickness of the pellicle is around 0.33 μ if we assume that each longitudinal fold is composed of two layers of pellicle with little, if any, material between them. Measurements of the pellicle made at the edge of the ameba, whether living or fixed, were approximately the same.

Unless an extreme amount of pressure is applied to the coverslip, *Thecamoeba striata* will not rupture even in the event of evaporation of the surrounding medium. The pellicle does not stain with neutral red, nor does it hamper the entrance of this stain into the cytoplasm to any great degree. The time required for given concentrations (1 : 75,000, 1 : 100,000 and 1 : 150,000) of neutral red to appear within the cytoplasm of this ameba is about the same as in the non-pelliculate amebas studied.

When *Amoeba proteus* and *Chaos chaos* are active and have extended pseudopodia, the plasmalemma is generally smooth and without folds. Wrinkling

of the plasmalemma occurs only when the cells are rounded up. The thickness of the plasmalemma was found to be about 0.25 μ. It should be noted that the plasmalemma of these amebas ruptures much more easily than the pellicle of *Thecamoeba striata*.

In the other amebas studied, *Amoeba guttula* and *Mayorella bigemma*, the thickness of the cell membrane was not ascertained since it is extremely thin and much less than 0.25 μ.

Cytochemistry of the cell membrane. The cytochemical reactions hereinafter described were very easily visualized in the cell membranes of all the amebas studied, both pelliculate and non-pelliculate. In the extremely thin plasma membranes of *Mayorella bigemma* and *Amoeba guttula*, the results were not as pronounced as in the other species, but were definite nevertheless.

The cell membrane in all the amebas stained red with 1 : 5000 ruthenium red (ruthenium oxychloride, ammoniated). This metallic pigment has been employed in microscopic work as a test for pectin in plant cells (Bonner, 1936, 1946, 1950). Its specificity for pectin alone, however, is greatly in doubt. Hence no definite conclusions as to the chemical nature of the cell membrane can be drawn from the use of ruthenian red.

The cell membrane appeared violet after being treated by the PAS reaction. As was stated earlier, this reaction involves the oxidation of adjacent hydroxyl groups to aldehydes with Schiff's reagent. Thus the presence of the violet fuchsin color indicated that the pellicle of *Thecamoeba striata* as well as the cell membranes of all the other species contain polysaccharides.

Since non-figured (free) glycogen is water-soluble to some degree, and of course the cell membrane is not, it cannot be present as an important constituent of the cell membrane. However, glycogen could be present as a figured substance, i.e., combined with or bound to some other substance and thus not necessarily water soluble. By the application of saliva, the pellicle was found to be ptyalin-resistant, thus showing that it does not contain figured glycogen.

The Millon reaction (Bensley and Gersh, 1933) for tyrosine and the Sakaguchi test (Baker, 1947) for arginine were employed. Since the vast majority of proteins contain tyrosine and arginine, these two cytochemical tests are actually good protein indicators. The cell membrane stained red with both tests, indicating the presence of protein. Thus the cell membrane of all the amebas tested contains protein and polysaccharides. The saliva-resistant, PAS positive, protein-combined carbohydrates belong to the muco-polysaccharide group. A muco-polysaccharide (mucin) is a compound containing protein and polysaccharide in which the polysaccharide is predominant.

Some basic dyes stain certain cell components the same color as that of the dye itself, but stain certain other elements with a different color. This is called metachromatic staining. It has been explained (Lison, 1936) that muco-polysaccharides which stain metachromatically, with toluidine blue, for instance, (usually pink or red) are acid polysaccharides and usually contain sulfate. Hyaluronic acid is the common acid polysaccharide found to be present intercellularly in metazoan tissues, although it does not contain sulfate. The cell membrane in the amebas was found not to be metachromatic. That is, it stained blue with toluidine blue, the same color as the dye. As a further check, several amebas were treated with hyaluronidase before staining with toluidine blue. Those treated with hyaluronidase did not stain any differently than the non-treated controls. Therefore it may be concluded from these tests that the cell membrane is composed in part of a neutral mucopolysaccharide or mucin.

Cytoplasmic Components and Inclusions

The Alpha Granules. The alpha granules are rather abundantly represented in all the amebas studied. In the unstained living cells they are easily seen with the phase-difference microscope. The size of these granules, about 0.25 μ, appears

to be the same in all the species studied. Due to the fact that the staining reactions were inconclusive, nothing could be ascertained concerning the nature of these granules.

The Beta Granules. The beta granules in the five species of amebas studied are very similar in size and shape. They are predominantly spherical and measure about 1 μ in diameter. Although Mast and Doyle (1935a) claim that the beta granules change their shape in *Amoeba proteus*, no evidence for this was obtained in the present study. However, in *Thecamoeba striata* (and in no other species studied) coalescence of beta granules has been observed several times both in stained and unstained amebas. The coalesced granules appear as homogeneous spheres, measuring up to 2.6 μ in diameter. Six to ten such spheres have been found in some individuals. Clumps of beta granules have also been observed in this species, but these granules retain their individual shapes in the clump. No direct observations were made as to whether this clumping of granules precedes coalescence; but this seems to be probable. Coalescence and clumping of beta granules have not been reported previously in amebas. Bourne (1951) reports that clumping and coalescence of mitochondria in certain mammalian cells often occurs in cases of scurvy. The significance of this phenomenon in *Thecamoeba striata* is not known.

The beta granules may be considered under two headings, those that surround the contractile vacuole and those that do not. The observations made in this study corroborate the conclusions of Mast and of Andresen that this division is purely an artificial one, since the only difference in the granules surrounding the contractile vacuole is that they are more or less flattened as they lie on the surface of this vacuole. The staining qualities, plasticity, and composition appear to be the same in all the beta granules, regardless of their distribution in the cell.

Janus green B has been found to be a specific stain for beta granules. (Mast and Doyle, 1935a, 1935b; Andresen, 1942.) Cytologists have long considered Janus green B a specific stain for metazoan mitochondria. (McClung, Handbook of Microscopical Technique, 3rd ed., p. 140.) When used in optimum concentration (1 : 100,000) the beta granules, and no other structures, appear distinctly green after about 3 or 4 hr. Mast and Doyle (1935a) declared that in *Amoeba proteus* only the outer layer of the beta granules stained with Janus green B, indicating a differentiated surface layer. In the present investigation the same results were at first obtained with *Amoeba proteus* and *Thecamoeba striata*. However, when the amebas were left in the Janus green solution for more than 24 hr., the granules appeared homogeneous. Observations made with the phase-difference microscope on unstained amebas also showed no differentiation of parts.

Mast and Doyle (1935a) applied classical metazoan mitochondrial staining methods to *Amoeba proteus* and found that the beta granules behaved like the mitochondria of metazoa. Andresen (1942) used aniline fuchsin after Champy-Kull fixation and successfully stained the beta granules of *Chaos chaos*. These methods were repeated in the present study and similar results were obtained. Since Janus green B and the classical mitochondrial staining methods definitely stain these granules, it seems very probable that the beta granules are actually the mitochondria of amebas.

Mast and Doyle (1935b) state that the beta granules are much more numerous at the surface of the food vacuoles during the initiation of digestion and toward the end of this process. Today, observations on this point are made much easier and more definite by the use of a phase-difference microscope, which was not available to Mast and Doyle. Such observations at different phases of digestion revealed no beta granules definitely associated with food vacuoles at any time.

Cytochemistry of beta granules. The beta granules gave a positive reaction when treated with Millon's reagent for tyrosine and Sakaguchi test for arginine. In order to test for the presence of lipids in the beta granules, amebas were treated

with Sudan III and also Sudan IV in 70 percent alcohol. The results were negative. When Sudan black B was employed, however, the beta granules did show a faint bluish-black color. The affinity of the Sudan black B for the beta granules was greatly enhanced when ethylene glycol (Chiffelle and Putt, 1951) was used as a solvent instead of 70 percent alcohol. The PAS reaction for polysaccharides gave negative results. Ribonuclease and perchloric acid techniques for the presence of ribonucleic acid also gave negative results. It seems, therefore, that if ribonucleic acid is present in these granules it is in minute quantities. Using the nitroprusside test, negative results were also obtained for sulfhydryl groups. The cytochemical tests described above suggest that the beta granules of the amebas studied are lipid and protein in composition, as are metazoan mitochondria.

The Spherical Refractive Bodies. Numerous spherical refractive bodies varying in size and number were found in three of the five species studied, (*Amoeba proteus*, *Chaos chaos*, and *Thecamoeba striata*). They vary from 1.4 to 7 μ in diameter. At times it was estimated that as many as 200 were present in *Chaos chaos*, while *Amoeba proteus* was found to have as many as 40 and occasionally as few as 4 of these bodies. The number in *Thecamoeba striata* varies from as many as 20 to as few as 6.

In *Amoeba proteus* and *Thecamoeba striata* these spherical refractive bodies appear homogeneous in unstained individuals, and stain red, vitally, with neutral red. However, when various concentrations of neutral red (1 : 75,000, 1 : 100,000 and 1 : 150,000) were used, it was found that only the outer cortical layer of the larger bodies was stained. In many of the smallest of these bodies (those measuring 1.4–1.8 μ) no such differentiation between inner and outer portions was shown, and neutral red stained them homogeneously in the living ameba. In the larger spherical refractive bodies, the differentiation of cortical and inner regions was also observed in untreated living amebas with the aid of the phase-difference microscope.

The spherical refractive bodies in *Chaos chaos* did not in most cases stain vitally with neutral red. This is in accord with the observations of Andresen (1942). In one or two living individuals, however, a few of the larger bodies did stain. In every other respect, the spherical refractive bodies were found to be alike qualitatively in the three species of amebas in which they were present.

Osmium tetroxide vapors blacken the outer portion of the spherical refractive bodies. Subsequent treatment with 3 percent hydrogen peroxide did not bleach them. The outer portion also stained a faint grey-blue with Sudan black B, indicating the presence of lipid material. Both Millon's reagent for tyrosine and the Sakaguchi test (Baker's 1947 modification) for arginine gave a pink coloration of the same intensity as the cytoplasmic ground substance indicating the presence of protein in the cortex of the refractive bodies. Mast and Doyle (1935a) claim that between the inner and outer portion of these refractive bodies a layer of carbohydrate is present. However, in the present study only negative results were obtained when the PAS reaction was used to test for the presence of polysaccharides in them, and Lugol's solution did not demonstrate any starch.

Thus it was found that the outer cortical layer of the spherical refractive bodies is composed of lipid and protein material, while the inner, medullary portion contains a fluid of unknown composition. The reason for assuming that it is fluid in nature is its eccentric position in the majority of cases. The ratio of the outer lipo-protein layer to the inner portion varies greatly. When the body is larger there is proportionally more of the central fluid present. It seems probable that these bodies grow in size by the increase of the inner fluid portion.

The general staining properties and cytochemical reactions of the spherical refractive bodies are very similar to those of metazoan Golgi substance. The probable mode of increase in size of these bodies, by the progressive accumulation of the central fluid, may be similar to the condensation function of the metazoan Golgi substance. The observations here recorded lend support to the ideas

of previous workers, who claim that the spherical refractive bodies are the Golgi bodies of amebas (MacLennan, 1941).

It has been suggested (Wilber, 1942, 1945b) that the refractive bodies merely represent reserve food or volutin. However, as was first shown by Andresen (1942) and substantiated in the present study, they do not diminish greatly in number under starvation conditions. When they do diminish in number, the remaining bodies become larger, indicating coalescence rather than utilization as reserve food.

The Contractile Vacuoles. The number of contractile vacuoles varies, depending on the species. *Amoeba proteus* and *Amoeba guttula* have only one contractile vacuole. The contractile vacuole of *Amoeba proteus* and related species has undergone a thorough investigation by Mast (1938) and a few other authors, and there is general agreement as to the facts. *Chaos chaos* generally has about 7–10 contractile vacuoles. Each of these vacoules is rather similar in size (80–120 μ before systole) and in behavior to that of *Amoeba proteus.*

Mayorella bigemma has 4–9 contractile vacuoles, with a diameter of about 15 μ before systole. Schaeffer (1918) states that the contractile vacuoles of *Mayorella bigemma* never coalesce. However in the present study of this species it was found that among the smaller, "growing" vacuoles coalescence was fairly common. In the larger vacuoles no coalescence was observed.

There are usually two contractile vacuoles in *Thecamoeba striata.* The outstanding and unique characteristic of this ameba is the curiously shaped larger contractile vacuole. This vacuole is almost never spherical; it is irregularly lobed and constantly changing in shape during locomotion. When the ameba is rounded up and stationary, the contractile vacuole assumes a more regular, rather oval form. Penard (1902) suggested that the curiously lobed appearance of this vacuole is due to the constant formation of smaller new vacuoles adjacent to it, which coalesce with it. During the present study, no evidence was obtained for such an interpretation.

As stated earlier, there are two varieties of *Thecamoeba striata.* In the larger variety, the two contractile vacuoles present include a smaller, spherical vacuole at the posterior and anterior to it, the larger amorphic vacuole which may at times, move forward, carried by the streaming protoplasm. The smaller spherical vacuole sometimes develops into and assumes the position of the larger, lobed vacuole which then disappears. A new spherical vacuole then develops at the posterior end. This, however, is not the general rule. The vacuoles function independently, both emptying their contents to the outside of the cell.

The smaller variety of *T. striata* usually possesses only the amorphic contractile vacuole.

The larger vacuole, in individuals whose length is 80 μ, sometimes reaches a length of 30 μ, but the spherical smaller vacuole is never larger than 15 μ in diameter.

In the past, investigations have ignored or minimzed the possible effects of metabolic activity on the rate of contraction of the contractile vacuole. (See Weatherby, 1941.) Rudzinska and Chambers (1951) report that the pulsation rate of the contractile vacuole in the suctorian *Tokophrya infusionum* accelerated greatly during an increase in metabolic activity of the organism. In *Thecamoeba striata* during locomotion, the time between contractions of the larger lobed vacuole averages 2.5–3 min. The smaller, posterior vacuole takes almost the same length of time. They usually do not contract simultaneously but rather, alternately. In individuals that are not moving but are rounded up and with little or no internal protoplasmic streaming, the time between contractions is about 8 or 10 min.

Howland (1924a) reported that the contractile vacuole in *Thecamoeba* (*Amoeba*) *verrucosa* is not surrounded by beta granules. In *Thecamoeba striata,* however, such a layer of beta granules is definitely present. These granules are not densely packed and occasionally gaps without granules can be found. These granules are

more or less flattened on the surface of the contractile vacuole; upon the contraction of the vacuole, they become spherical. Mast (1938) showed that in *Amoeba proteus* these granules do not determine the site of formation of the new vacuole. In the present study, the same results were obtained with *Thecamoeba striata*. It was also observed, using the phase-difference microscope, that the layer of cytoplasm about 2 μ thick, surrounding the contractile vacuole and containing the beta granules, appears to be in the gel state, and differs in this respect from the cytoplasm surrounding it.

It was found that the beta granules which surround the larger, amorphic vacuole of *Thecamoeba striata* do not in any given period between contractions interchange position with other granules either during the vacuole's rapid changes of shape or during its movement with the streaming protoplasm.

The origin of the contractile vacuoles was investigated in most of the species studied. The evidence indicates that the new vacuole is usually formed *de novo*.

The site of origin can not be predicted by the position of the beta granules that surrounded the old vacuole. However, the new vacuole does form in the approximate position where the earlier vacuole had undergone systole. In *Mayorella bigemma* the formation of a new contractile vacuole is accomplished by the coalescence of at least 6 or 8 very small vacuoles.

It was found on careful observation of the vacuoles of *Amoeba proteus*, *Chaos chaos*, and *Thecamoeba striata* that occasionally the vacuole does not contract completely and a minute vacuole, more oval than spherical, measuring 2 to 2.5 μ, remains. The growth of the new vacuole is then merely the enlargement of this minute vacuole. But in a few cases, it was observed that a new vacuole formed adjacent to the minute residual one, and upon subsequent enlargement incorporated the latter.

Crytsals and Crystal Vacuoles. Crystal inclusions were found in *Amoeba proteus*, *Chaos chaos*, and *Mayorella bigemma*. These found in *Amoeba proteus* and *Chaos chaos* are similar and of two types, plate-like and bipyramidal. Their size varies from 2–7 μ and they are always found in vacuoles, which vary in size from 2.5–9 μ. The crystal vacuoles show a great affinity for neutral red, varying from an orange to a light red and deep red color. Some were found to be more alkaline than others, as demonstrated by the color of the neutral red stain. After 24–36 hr., however, most of the vacuoles were stained a deep red.

The ratio of the crystal to the vacuole size varies greatly. Often a small crystal was found in a large vacuole and vice versa. The crystals were usually found to lie eccentrically in the vacuoles. Not more than one crystal was found in any given crystal vacuole.

According to Mast and Doyle (1935a) the plate-like crystals are probably composed of leucine and the bipyramidal ones probably consist of a magnesium salt of a substituted glycine.

Mast and Doyle (1935a) described "blebs" adherent to the surface of some of the crystals, and suggested that these blebs represent the beginnings of the spherical refractive bodies. In the present study such "blebs" were seen only occasionally. It seems very unlikely that such crystals would give rise to the lipid- and protein-composed spherical refractive bodies. Furthermore, it was found that the blebs do not reduce OsO_4, as do the spherical refractive bodies.

In no case did the cytochemical tests indicate the nature of the fluid within the crystal vacuoles. Coalescence of crystal vacuoles was observed only under starvation conditions.

Schaeffer (1918) described in *Mayorella bigemma* small dumb-bell shaped, or hour-glass shaped and occasionally club-shaped crystals as diagnostic for this species. With the use of the polarizing microscope these crystals were easily seen to be anisotropic and strongly birefringent. Schaeffer reported that these crystals have a close affinity to "excretion spheres" which are never larger than 3 μ. Such so-called "excretion spheres" were not found during the present study. These

crystals of *Mayorella bigemma*, unlike those in *Amoeba proteus* and *Chaos chaos*, lie free in the cytoplasm and were not found at any time to be enclosed in vacuoles. These observations were repeatedly checked with vital staining and with the aid of the phase-difference microscope. Their size was 1 to 2 μ through their longest axis.

The Food Vacuoles. The breakdown of food vacuoles into smaller vacuoles was traced in all of the species studied except *Amoeba guttula*. In *Thecamoeba striata* and *Mayorella bigemma* it was found that a newly formed food vacuole very soon (1 to 2 hr.) breaks down into two vacuoles. Within this short time the food organism, *Chlamydomonas*, loses its definite outlines, and the two food vacuoles are colored uniformly green by the presence of chlorophyll. Also desoxyribonucleic acid (DNA), demonstrable by the Feulgen test in the nucleus of the food organism, is found diffused in the resulting two vacuoles. After subsequent breakdown of the secondary food vacuoles into still smaller vacuoles, DNA can only rarely be demonstrated within them, and chlorophyll is no longer present. However, the occasional presence of the DNA might be accounted for in another way which will be discussed later on.

It was not possible in any of the species studied to determine the exact number of times that a newly-formed food vacuole divides into smaller vacuoles. It is probably variable from species to species, and probably to a lesser degree from individual to individual.

In attempting to study the frequency of division of food vacuoles, amebas were placed in a medium containing no food organisms, to avoid confusion arising from the formation of new food vacuoles. It was found that within 24 hr. most of the larger food vacuoles had disappeared and in 36 hr. all of the food vacuoles had disappeared.

During starvation, in those species possessing spherical refractive bodies, some of the latter apparently coalesced. At about this time, numerous non-contractile vacuoles appeared, some of which coalesced. In those species which normally have non-contractile vacuoles (*Chaos chaos* and *Mayorella bigemma*) some of these vacuoles also coalesced and more appeared. Previous investigators (Andresen and Holter, 1945, and Andresen, 1945) who studied the changes in *Chaos chaos* and *Amoeba proteus* during starvation, recorded similar results. The significance of these changes is not understood.

No spherical refractive bodies, beta granules, or crystals were found inside the food vacuoles at any time. This result is supported by observations made earlier on *Chaos chaos* by Andresen and Holter (1942). In contrast, Mast and Doyle (1935a, 1935b) reported crystals and spherical refractive bodies in the food vacuoles of *Amoeba proteus*, and concluded that these inclusions are formed directly by the food vacuole

In all the species studied, small food vacuoles containing undigested food residues accumulate in the posterior end of the ameba and are subsequently egested. It was observed that two or three waste food vacuoles were sometimes egested at one time as if they were one mass, but after their release into the surrounding medium it was found that they had not coalesced but were still separate. The process of egestion was observed in *Amoeba proteus*, *Thecamoeba striata*, and *Mayorella bigemma*.

The Fat Globules. Relatively large numbers of fat globules were found in all of the species of amebas studied. Fat globules were easily seen with the phase-difference microscope; but in the unstained ameba they could not always be differentiated from the smallest spherical refractive bodies, in those species possessing the latter. They measured 1.2–2.5 μ in diameter, being somewhat smaller in the smaller amebas. They colored red with Sudan III and Sudan IV and blackish blue with Sudan black B. The mixture of Doyle (1933) for the simultaneous visualization of starch and fat proved unsatisfactory, since some of the fat globules present in the cytoplasm did not react. The fat globules stained red with Nile

blue sulfate, indicating that they are composed of neutral fats. Negative results were obtained with the Schultz test for cholesterol.

The number and size of the fat globules varied greatly from individual to individual in a given culture. Generally speaking, however, when an ameba appears to be well fed, large numbers of these globules are found, varying in size and scattered throughout most of the cytoplasm. These globules were never within vacuoles of any kind. Under starvation conditions, the number of fat globules decreases. When death from starvation occurs, some fat globules are still present. An estimate of the amount of fat present after death showed that there was great variation from cell to cell. In the majority of the individuals, around one-third of the original total still remained, while in some, very little fat was left. Contrary to the above results, Andresen (1945) found that in *Amoeba proteus* all microscopically visible fat had disappeared by the time death occurred from starvation. However, the present experiments were conducted on *Chaos chaos*, *Thecamoeba striata*, and *Mayorella proteus*, as well as *Amoeba proteus*, and the results obtained were identical in all four species.

Glycogen. Although glycogen was not visible in the untreated cells, it was easily demonstrated in all species studied with the use of Lugol's solution and alcoholic iodine solution. When the amebas had been starved for 36 hr. or more, the results obtained with these iodine solutions were entirely negative.

Permanent vacuoles. Various vacuoles have already been discussed. However, under normal conditions, clear permanent vacuoles are also present in *Chaos chaos* and in *Mayorella bigemma*. These vacuoles differ from contractile vacuoles in that they do not undergo systole and are not surrounded by beta granules. They are readily distinguished from empty crystal vacuoles in *Chaos chaos* because the permanent vacuoles do not show any affinity for neutral red. In *Chaos chaos* permanent vacuoles vary from 2 to 10 μ in diameter. In *Mayorella bigemma* they are usually around 5 μ, although a few have been found as large as 15 μ in diameter. These permanent vacuoles never contain any granules or other particulate matter. Under normal conditions, they never coalesce. During starvation, however, coalescence is common and the size of the vacuoles increases greatly. Also, many new non-contractile vacuoles are formed at this time, and even in *Amoeba proteus* and *Thecamoeba striata* where they are not ordinarily found, similar vacuoles frequently form under starvation conditions.

Neutral red granules. Many observations in this study have been made with the aid of the vital stain neutral red. When amebas are stained with neutral red (Vonwiller, 1913, and many later authors), some of the cytoplasmic inclusions show an affinity for it while others do not. However, certain granules appear which were not visible previous to the neutral red staining. These granules are generally known as neutral red granules. A tremendous amount of confusion concerning them has accumulated in the literature (MacLennon, 1941).

The facts obtained concerning the neutral red granules were about the same in all the species studied. A dilute solution (1:150,000) of neutral red was found to be the most satisfactory for this purpose. About a half hour after this solution was added, numerous small deep red granules about 0.5 to 1 μ in diameter appeared. These granules could not be confused with the alpha and beta granules, which do not take up neutral red. In about 8 hours, more of these deep red granules appeared and some had enlarged to about 2.5 or 3 μ in diameter. Intermediate sizes were also found. In 24 to 48 hr. a few granules measured as much as 6 μ in diameter, and no granules smaller than 1.8 μ were found after a day or two. The color of all these granules, whether large or small was the same deep red. It might be pointed out that the maximum sizes reached by these granules were less in the smaller species than in the larger, being about 4 μ in *Mayorella bigemma* and *Amoeba guttula*.

Extended observations showed no coalescence of neutral red granules in any of the species studied. Up to about 24 hr., the neutral red granules were found free in the cytoplasm. Later, however, these granules were found in vacuoles, usually

one granule, 3 to 6 microns in diameter, per vacuole. The diameter of the vacuoles was found to be 5 to 10 microns. A few vacuoles contained 2 to 3 granules, but these were not common. These intravacuolar neutral red granules accumulate in the posterior portion of the cell during locomotion. Three days after the neutral red solution had been added, the situation had not changed in regard to the number, size, and color of the neutral red granules. However, the number of granules per vacuole had increased from one to as many as 12. The diameter of the vacuoles had increased from 10 to 20 μ, but the number of vacuoles had decreased.

While coalescence of the vacuoles containing neutral red granules was observed a few times, coalescence of the granules themselves was not observed at any time. Therefore the observed increase in size of these granules may be explained as a result of condensation of material but not as the staining of preformed inclusion bodies.

The Cytoplasmic Ground Substance. No exhaustive study was made especially on the cytoplasmic ground substance of the amebas. However, when cytochemical tests were used to determine the distribution of certain compounds in the cell inclusions, it was observed that some of these compounds were found only in the cytoplasmic ground substance or hyaloplasm. Ribonucleic acid (RNA) was found in this ground substance and in the nuclei, but not in any of the cytoplasmic inclusions. Similar results were obtained by Roskin and Ginsburg (1944a, 1944b). Desoxyribonucleic acid (DNA) was demonstrated only in the nucleus and some food vacuoles (Lucas, 1930, and Chalkley, 1936). The results of Chalkley (1937, 1951) showing that sulfhydryl material is found diffuse in the ground substance, were confirmed in the present investigation in *Amoeba proteus*, *Chaos chaos*, and *Thecamoeba striata*. With the aid of the plasmal reaction, plasmalogen was found diffuse in the ground substance, and not in any cell inclusion. Only the three larger species (*Amoeba proteus*, *Chaos chaos*, and *Thecamoeba striata*) were tested, as it was not possible to follow the necessary procedure with the smaller amebas. Brachet (1950a) was not able to demonstrate plasmalogens in amebas. However, it is not possible to evaluate these contrary results since he does not tell us what species of amebas he tested or what procedure he followed.

Andresen (1945) and Andresen and Holter (1945) state that during starvation in *Amoeba proteus* and *Chaos chaos* the viscosity of the cytoplasm decreases. Their observations were corroborated in this study. It was found that the heaviest inclusions, the spherical refractive bodies, settled to the bottom of the ameba during starvation, indicating decrease in cytoplasmic viscosity.

DISCUSSION

The criteria for separating genera of the Family Amoebidae are, in general, such gross morphological features as size, number and shape of pseudopodia, number and form of nuclei, etc. (Schaeffer, 1926). The five species here studied represent at least four different genera. They vary in size from 20 microns to 3 millimeters, and the number of nuclei varies from one to several hundred. In these and other respects, these species show great diversity in their gross structure. It is therefore surprising to find a remarkable degree of similarity in their finer structure and cytochemistry. The differences noted are chiefly in the presence or absence of certain components rather than differences in the components themselves. That is, a given cell component shows only slight differences in the various species. This will be brought out in the subsequent discussion.

Cytochemical tests indicate that the outermost layer of all the amebas studied (the "cell membrane"), regardless of thickness, contains neutral mucopolysaccharide material. The tests upon which this finding is based have apparently not been applied to other protozoa. Carbohydrates and proteins in various relationships, are known to be essential constituents of the external membranes of metazoan cells (DeRobertis, *et.al.*, 1948).

Alpha granules (about 0.25 μ in diameter) were found to be present in all the

species studied. Nothing new was ascertained concerning their function, structure, and origin.

Beta granules, predominantly spherical and measuring about one micron in diameter, were found to be present in all the species studied. In the present study, cytochemical methods were applied for the first time to these granules, and confirmed that they are lipid and protein in composition, as are metazon mitochondria. Their staining vitally with Janus green B, as first reported by Mast and Doyle (1935a), was also confirmed. Hence the conclusion of these and other authors that the beta granules represent mitochondria seems to be justified.

It was found with the aid of the phase-contrast microscope that the beta granules are not directly involved in food vacuole digestion, as suggested by Horning and others.

Recent investigations (Bourne, 1950) on liver and other metazon tissues indicate that mitochondria contain a high percentage of those enzymes which play an important part in the aerobic respiratory metabolism of the cell. According to Bourne, many of the enzymes found in mitochondria are constituents of the Krebs tricarboxylic acid cycle, the cycle which has been described as a meeting point of protein, fat, and carbohydrate metabolisms. This indicates that mitochondria may be concerning with breakdown of protein, fat and carbohydrate, as well as with the synthesis of these three substances. Wilber (1945) states that in *Chaos chaos*, after the removal of most of the beta granules, the giant ameba appeared normal. This would seem to indicate that the Krebs cycle enzymes may not be found exclusively in the beta granules, but may be present in the cytoplasmic ground substance as well.

Spherical refractive bodies (present in three of the five species studied) are generally considered to be the Golgi sustance, as first proposed by Brown (1930), because their staining reactions are similar to those of metazoan Golgi material. In general the Golgi elements of metazoan cells are osmiophilic; they stain as a rule with neutral red; and they are believed to act as secretory centers. They are believed to be primarily spheres containing lipid and protein, from which a variety of substances segregate or condense out (Baker, 1944). As already pointed out, the spherical refractive bodies of amebas are always osmiophilic and generally stain vitally with neutral red. The interpretation of Brown would thus seem to be a reasonable one.

It has been shown in the present paper that at least the larger of the spherical refractive bodies in the amebas consist of an outer osmiophilic portion staining with neutral red and an inner portion which shows these reactions very little or not at all. Although the composition of this inner portion was not determined, it might be a condensation substance. It may be further pointed out that the larger sized bodies contain a proportionally greater amount of this inner material, indicating a possible condensation of the outer, corical lipid-protein layer. As in the case of Golgi material of the metazoan cell, the amount of spherical refractive material present in amebas varies from cell to cell and from time to time. It has been shown in this and previous (Andresen and Holter, 1945) studies that the variation in numbers of spherical refractive bodies is not due to their action as reserve food materials, but rather to some other, as yet unknown, physiological condition arising in the ameba. Wilber (1945b) claims that these bodies consist of volutin and contain free aldehydes because they stain with Schiff's reagent without prior treatment. His methods, however, are open to serious criticism. First, the use of Schiff's reagent on cells without prior treatment does not give specific results, and second, the presence in the cell of free aldehydes which have not been unmasked is highly improbable (Hayes, 1949).

Mast and Doyle (1935a) suggested that the origin of the spherical refractive bodies may be directly from the food vacuoles. The present observations do not substantiate such an origin since no spherical refractive bodies were ever found in or

near food vacuoles. Further it seems highly unlikely that both the process of break-down of nutrient material and its resynthesis into cell components should occur in the food vacuole. Extended observations on the food vacuole showed a progressive disintegration of particulate food organisms (digestion), and never a differentiation of this material into anything that would resemble spherical refractive bodies, crystals, or any other type of cell inclusion. Therefore, since there is no definite evidence that these bodies arise from the food vacuoles directly, it may be postulated that they arise from the cytoplasmic ground substance.

Spherical refractive bodies are not present in *Mayorella bigemma* or *Amoeba guttula*, and no other Golgi-like material has been demonstrated in these amebas. This obviously somewhat weakens the interpretation of the refractive bodies as Golgi elements. Another possible difficulty lies in the high refractive index of these bodies; the Golgi material of metazoan cells is notoriously difficult to differentiate from the surrounding cytoplasm. It is of course, conceivable that the function of Golgi bodies may be carried out in some species without the agency of any demonstrable specialized inclusion bodies.

The number of contractile vacuoles present in different species of amebas varies from one to about twelve. However, for a given species the number is fairly constant. It has been found in this study that the rate of pulsation of the contractile vacuole is directly related to the degree of activity of the organism. Similar observations have been made on the contractile vacuole of a suctorian (Rudzinska and Chambers, 1951). It is now generally agreed that the contractile vacuole is primarily a hydrostatic organelle, equalizing the intake and outgo of water. It is conceivable that minute quantities of nitrogenous waste products from the cytoplasmic ground substance may also be excreted (Weatherby, 1941).

Crystals were found in *Amoeba proteus*, *Chaos chaos*, and *Mayorella bigemma*. Those found in *A. proteus* and *C. chaos* were similar and of two types, plate-like and bipyramidal. Their composition was determined by Mast and Doyle (1935a) to be probably leucine and a magnesium salt of a substituted glycine, respectively. These crystals are always found enclosed in vacuoles, which show a great affnity for neutral red. The crystals found in *Mayorella bigemma*, however, are usually dumb-bell shaped and lie free in the cytoplasm, never enclosed in vacuoles.

In a few instances certain "blebs" (first described by Mast and Doyle, 1935a) were observed on the crystals of *Amoeba proteus* and *Chaos chaos*. According to Mast and Doyle these "blebs" represent the beginning of the formation of spherical refractive bodies. To the present writer they appear to be merely imperfections or blemishes on the surface of the crystals. They do not reduce osmium tetroxide as do the refractive bodies.

Mast and Doyle (1935a) postulated that the crystals originate directly from the food vacuoles. No evidence has been found to support this.

In the study of food vacuoles, it was found that the process of digestion is usually accompanied by the division of a vacuole into two or several smaller vacuoles. The undigested residues tend to be massed or packed in the posterior of the organism. Subsequent egestion of these waste vacuoles was observed. These observations approximate those made earlier by Mast (1942).

In a few cases, it was noted that in *Thecamoeba striata* very small food vacuoles (about 3 μ in diameter), which were considered to be waste vacuoles containing food residues, contained desoxyribonucleic acid. It may be postulated that the presence of DNA in these waste vacuoles indicates that at times not all of this compound is broken down during digestion. It is possible, however, that these vacuoles were not waste vacuoles but rather newly formed, small food vacuoles containing bacteria. Ingestion of bacteria alone has not been observed in this species, however.

Egestion of food vacuoles under starvation conditions has been observed in the present study in all the species except *Amoeba guttula*. Andresen (1942) reported this phenomenon in *Chaos chaos*. Mast and Hahnert (1935) state that food

vacuoles in *Amoeba proteus* coalesce during starvation conditions. This was not observed in the present work.

The fat globules were found to be composed of neutral lipids. It was found further that when death from starvation occurred, approximately one-third of the original number of fat globules still remained. The phenomena which occur when amebas are placed under starvation conditions show that starvation does not result in a simple diminution of available food until none exists. The non-availability of food initiates certain phenomena, such as egestion of food vacuoles, vacuolization of the cytoplasm, coalescence and vacuolization of refractive bodies, etc. The significance of these changes is not known. The process of starvation is a very complex one and the end result of that process, death, does not come about simply by the lack of available food. Rather, it probably occurs by the derangement of certain processes, caused by a deficiency of essential compounds.

The permanent vacuoles, normally present only in *Mayorella bigemma* and *Chaos chaos*, are distinguished from contractile vacuoles not only by their non-contractility, but also because they are not surrounded by a layer of beta granules. They are differentiated from empty crystal vacuoles in *Chaos chaos* by the fact that they do not stain with neutral red. These vacuoles normally do not coalesce. Nassonov (1924) thought that the permanent vacuoles together with the contractile vacuoles make up the Golgi complex of protozoa. However, staining properties and behavior of these permanent vacuoles lend no support to this theory. Their origin and function is not known. It is interesting to note that under starvation conditions coalescence and the appearance of new permanent vacuoles occur not only in *Mayorella bigemma* and *Chaos chaos*, where they are normal components, but also in *Amoeba proteus* and *Thecamoeba striata*, where they are not ordinarily found.

On an earlier page we have described the appearance and enlargement of neutral red granules during staining with neutral red, and the subsequent formation of vacuoles around them. Similar observations were made by Andresen (1945). Since the the enlargement of neutral red granules is not brought about by coalescence, and also since these granules have not been demonstrated in the cytoplasm prior to the introduction of neutral red, it is concluded that these granules are not preformed. Probably the action of neutral red on the cytoplasmic ground substance causes certain elements which show a great affinity for the dye to condense or precipitate out and to appear as discrete bodies.

The results obtained in the present study on the cytochemistry of the cytoplasmic ground substance (hyaloplasm) lead to the interpretation that this cytoplasmic ground substance is more than a matrix in which the various inclusion bodies are found. Indeed, it has been suggested that the inclusion bodies to a great degree are merely evidences of the physiological activity of this ground substance.

In metazoan cells (Lazarow, 1943; Claude, 1943; Brachet, 1950) two types of submicroscopic particles have been separated by means of ultracentrifugation from the ground substance: particulate glycogen and the "microsomes." In the present study glycogen was found to be microscopically visible only when treated with an iodine solution. It seems quite possible, therefore, that it is normally present as submicroscopic particles. The action of iodine probably causes these particles to precipitate and to stain reddish brown.

According to Brachet (1950b), the "microsomes" isolated by means of ultracentrifugation were found to contain most of the cytoplasmic RNA, sulfhydrhyl, and plasmalogen. Bayliss (1920) with the aid of the ultramicroscope described certain particles in ameba which are not ordinarily microscopically visible. According to Lazarow (1943) these particles of Bayliss were the equivalent of the "microsomes" which have been subsequently isolated from metazoan cells. However, it is not clear exactly what Bayliss did see as "shimmering points of light." Until all the techniques which have been applied to metazoan cells are adapted and applied to the amebas, no definite statement can be made as to the presence or absence of "microsomes."

SUMMARY

1. A comparative study has been made of the structure and cytochemistry of the cytoplasm of five species in the Family Amoebidae: *Amoeba proteus* (Pallas) Leidy, *Chaos chaos* Linnaeus, *Thecamoeba striata* (Penard) Schaeffer, *Mayorella bigemma* Schaeffer, and *Amoeba guttula* Dujardin.

2. A description is given of the various techniques employed, including the parlodion trap technique, various kinds of staining, and cytochemical methods.

3. The cytoplasmic components studied were: the cell membrane, the alpha and beta granules, the spherical refractive bodies, the contractile vacuoles, the crystals and crystal vacuoles, the food vacuoles, the fat globules, the permanent vacuoles, the neutral red granules, and the cytoplasmic ground substance or hyaloplasm.

4. The five species of amebas studied show great diversity in their size and gross structure. However, on the whole, a remarkable degree of similarity was found in the finer structure and cytochemistry of their cell components.

5. It was found that in all the species studied the outermost layer, or cell membrane, contains neutral mucopolysaccharides. Whether this layer was the seemingly unspecialized plasma membrane of *Mayorella bigemma* and *Amoeba guttula*, or the much thicker, more or less rigid covering (pellicle) of *Thecamoeba striata*, the cytochemical results were the same. The thickness of the pellicle of *Thecamoeba striata* was found to be about 0.33 micron. In both *Amoeba proteus* and *Chaos chaos* the thickness of the plasmalemma is somewhat less, or about 0.25 μ. The cell membrane of *Mayorella bigemma* and *Amoeba guttula* is very much thinner, and it was not possible to measure it.

6. The alpha granules (0.25 μ in diameter) were found to be present in all the species studied.

7. The beta granules in all the species studied are very similar in size (about 1 micron in diameter) and in shape (usually round). In *Thecamoeba striata*, and in no other species studied, coalescence of beta granules was observed. In all cases the beta granules were found to stain with Janus green B. When cytochemical tests were applied to them, it was found that these granules are lipid and protein in composition, as are metazoan mitochondria. No evidence was found that they are involved directly with food vacuole digestion or with the functioning of the contractile vacuole.

8. The spherical refractive bodies were found in only three species (*Amoeba proteus*, *Chaos chaos*, and *Thecamoeba striata*) of the five studied. They vary in number and size. The larger bodies are not homogeneous, but are divided into an outer cortical layer which stains with neutral red and an inner unstained portion, which is often eccentric in position. These bodies measure from 1.4 to 7 μ in diameter. The ratio of the outer layer to the inner, eccentric portion varies greatly. When the body is larger, there is proportionally more of the central portion present. Cytochemical tests visualize the presence of lipid and protein material in the outer portion. The composition of the inner portion was not ascertained. It seems probable that these bodies represent Golgi material.

9. The number of contractile vacuoles present in amebas is variable. However, for a given species the number is more or less constant. The shape of these vacuoles is almost always spherical. The only exception is the larger of the two vacuoles in *Thecamoeba striata*. This vacuole is irregularly lobed and constantly changing in shape.

10 It has been found in this study that the rate of pulsation of the contractile vacuole is directly related to the metabolic activity of the organism. Further, it was observed that the beta granules surrounding the contractile vacuoles do not determine the site of formation of the new vacuole. It was found that in a few instances the vacuole does not contract completely; a minute vacuole (2–2.5 microns) remains and this is usually the beginning of the next vacuole.

11. Crystal inclusions were found in *Amoeba proteus*, *Chaos chaos*, and *Mayorella bigemma*. Those found in *Amoeba proteus* and *Chaos chaos* are similar, 2 to 7 μ in diameter, and of two types, plate-like and bipyramidal. These crystals are found in small vacuoles (2.5 to 9 μ in diameter). The crystal vacuoles stain readily with neutral red. In a few instances tiny "blebs" were seen on crystal surfaces. It is suggested that these "blebs" do not represent the formation of new refractive bodies as suggested by Mast and Doyle (1935), but rather crystal imperfections.

The crystals of *Mayorella bigemma* are usually dumb-bell shaped and rather small, measuring 1 to 2 μ through their longest axis. Unlike the crystals of *Amoeba proteus* and *Chaos chaos* they are not enclosed in vacuoles, but lie free in the cytoplasm.

12. In the study of food vacuoles, it was found that at least in the four larger species the process of digestion is accompanied by the breakdown of the vacuoles into smaller ones. Egestion of waste food vacuoles was observed. However, under starvation conditions, egestion of any food vacuole may occur.

13. Fat globules were found in all the species studied. They are composed of neutral fats. Usually about one-third of the fat globules are still present when death from starvation occurs. During starvation, all the free glycogen disappears.

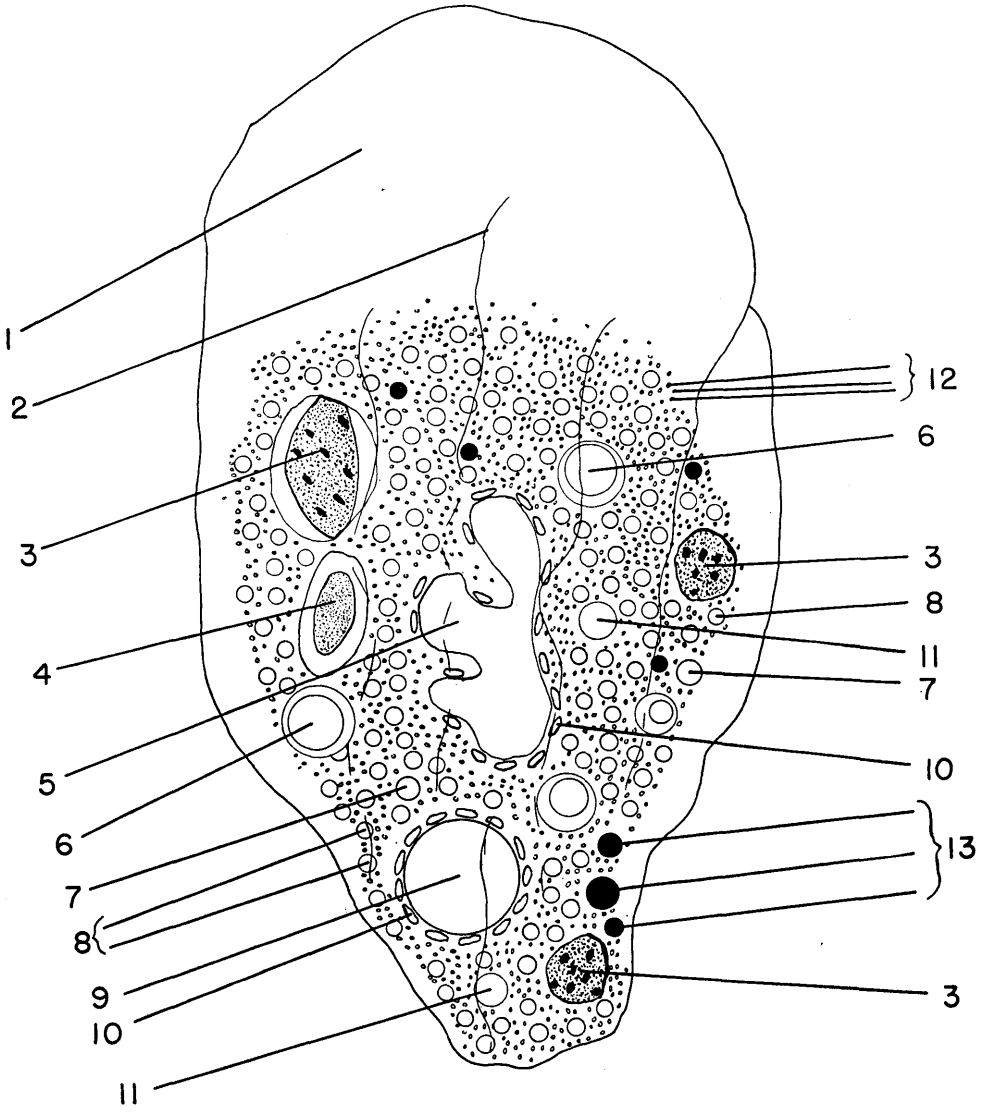
14. Permanent non-contractile vacuoles are normally present in *Mayorella bigemma* and *Chaos chaos*. These vacuoles differ from contractile vacuoles in that they do not contract, and are not surrounded by beta granules. They are readily distinguished from empty crystal vacuoles because they do not stain with neutral red. These permanent vacuoles normally do not coalesce, but under starvation conditions coalescence is common, and both size and number of the vacuoles increases. In *Thecamoeba striata* and *Amoeba proteus*, permanent vacuoles appear in the cytoplasm under starvation conditions only.

15. The appearance, color, and growth of neutral red granules have been described, as well as the development of vacuoles around them. Since the growth in size of neutral red granules is not brought about by coalescence, and also since these granules are not found in the cytoplasm prior to the introduction of neutral red, it is concluded that these granules are not preformed. It is thought that they arise through the precipitation of certain material from the cytoplasmic ground substance by the action of the neutral red.

16. Diffused in the cytoplasmic ground substance or hyaloplasm were found ribonucleic acid, plasmalogen, and sulfhydryl. These compounds were not found in any of the cytoplasmic inclusions. The ground substance, it is concluded, is not merely a matrix in which various inclusions are suspended. On the contrary, the inclusion bodies may be looked upon as evidence of the physiological activity of the ground substance itself.

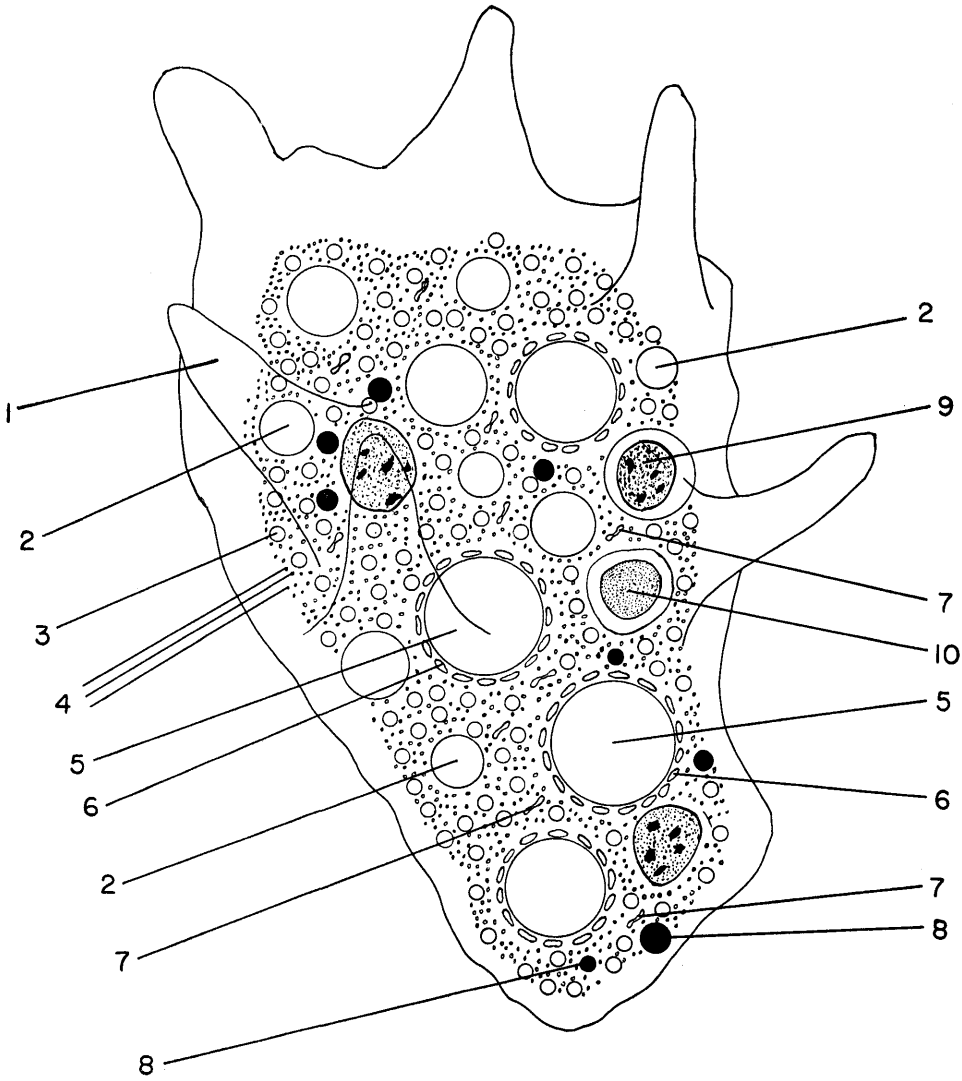
ACKNOWLEDGMENTS

I wish to express my sincere thanks to Professor W. J. Kostir, who suggested and supervised this investigation, and to Dr. E. R. Hayes and Dr. H. Hoffman for their help with the technique of cytochemistry.



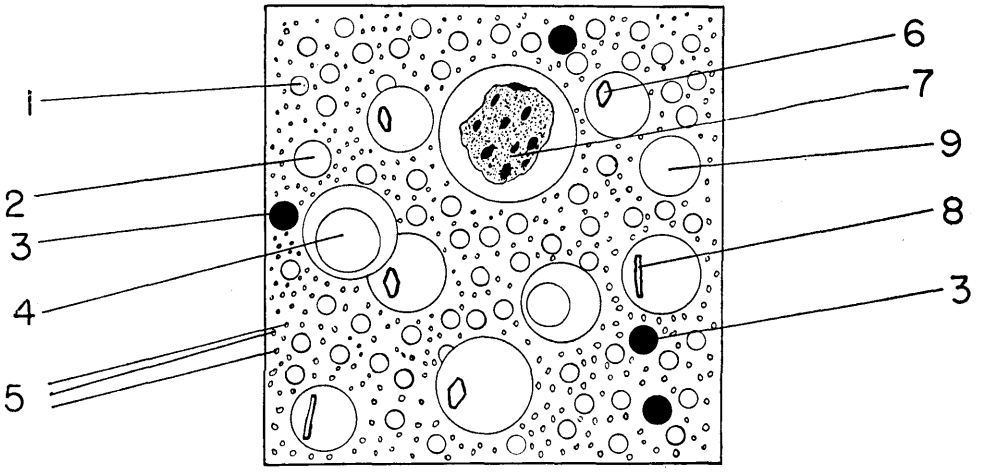
Thecamoeba striata (Penard) Schaeffer. Length, 67.0 μ. Semidiagrammatic.

1—hyaline cap. 2—longitudinal striation or fold. 3—food vacuole. 4—nucleus. 5—amorphic contractile vacuole. 6—larger spherical refractive body. 7—smaller spherical refractive body. 8—beta granules. 9—posterior spherical contractile vacuole. 10—layer of beta granules surrounding the contractile vacuole. 11—coalesced homogeneous sphere of beta granules. 12—alpha granules. 13—fat globules.

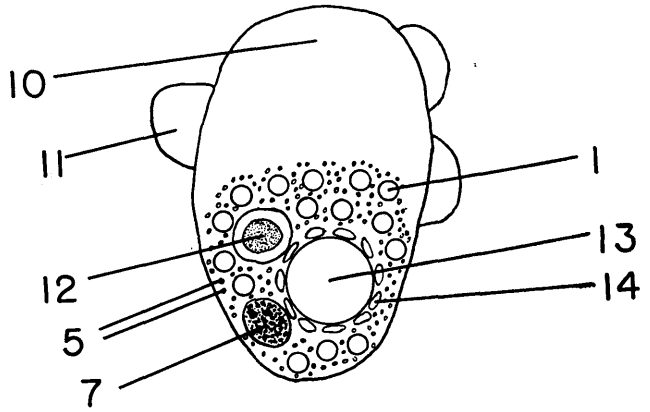


Mayorella bigemma (Schaeffer) Schaeffer. Length, 75.0 μ. Semidiagrammatic.

1—pseudopodium containing hyaloplasm. 2—permanent vacuole. 3—beta granule. 4—alpha granules. 5—contractile vacuole. 6—layer of beta granules surrounding the contractile vacuole. 7—crystal inclusion. 8—fat globules. 9—food vacuole. 10—nucleus.



A



B

A

Diagram showing an area of cytoplasm of *Amoeba proteus* (Pallas) Leidy. Semidiagrammatic

B

Amoeba guttula Dujardin. Length, 20.5 μ. Semidiagrammatic.

1—beta granule. 2—smaller spherical refractive body. 3—fat globule. 4—larger spherical refractive body. 5—alpha granules. 6—bipyramidal crystal in vacuole. 7—food vacuole. 8—plate-like crystal in vacuole. 9—empty crystal vacuole. 10—hyaline cap. 11—pseudopodium containing hyaloplasm. 12—nucleus. 13—contractile vacuole. 14—layer of beta granules surrounding the contractile vacuole.

BIBLIOGRAPHY

Andresen, N., 1942. Cytoplasmic components in the amoeba *Chaos chaos* Linné. Compt. Rend. Trav. Lab. Carlsberg, Ser. Chim., 24: 139–184.
———. 1945. Cytoplasmic changes during starvation and during neutral red staining of the amoeba *Chaos diffluens* (*A. proteus*). Compt. Rend. Trav. Lab. Carlsberg, Ser. Chim., 25: 169–189.
———. and **H. Holter.** 1945. Cytoplasmic changes during starvation of the amoeba *Chaos chaos* L. Compt. Rend. Trav. Lab. Carlsberg, Ser. Chim., 25: 107–146.
Baker, J. R. 1944. The structure and chemical composition of the Golgi element. Quart. Jour. Microsc. Sci., 85: 1–71.
———. 1946. The histochemical recognition of lipine. Quart. Jour. Microsc. Sci., 87: 441–470.
———. 1947a. The histochemical recognition of certain guanidine derivatives. Quart. Jour. Microsc. Sci., 88: 115–121.
———. 1947b. Further remarks on the histochemical recognition of lipine. Quart. Jour. Microsc. Sci., 88: 463–465.
———. 1950. Cytological Techniques. 3rd. ed. Methuen Co., London.
Bayliss, W. M. 1920. The properties of colloidal systems. IV. Reversible gelation in living protoplasm. Proc. Roy. Soc. (B), 91: 196–201.
Bennet, H. S. 1948. Cytochemical evidence for sulfhydryl in nerve axoplasm, retinal rods, and capillary endothelium. Anat. Rec., 100: 640. (abs.)
Bensley, R. R., and **I. Gersh.** 1933. Studies on cell structures by the freezing-drying method. II. Anat. Rec., 57: 217–233.
Bernheimer, A. 1938a. A comparative study of the crystalline inclusions of Protozoa. Trans. Amer. Microsc. Soc., 57: 336–343.
———. 1938b. Fate of the crystals in amebas. Arch. Protistenk., 90: 366–368.
Bonner, J. 1936. The chemistry and the physiology of pectins. I. Bot. Rev., 2: 479–497.
———. 1946. The chemistry and the physiology of pectins. II. Bot. Rev., 12: 535–537.
———. 1950. Plant Biochemistry. Academic Press, New York, N. Y.
Bourne, G. H. 1950. Recent discoveries concerning mitochondria and Golgi apparatus and their significance in cellular biology. Jour. Roy. Microsc. Soc., 70: 367–380.
———. 1951. Cytology and Cell Physiology. 2nd ed., Chap. 6. Oxford Univ. Press, London.
Brachet, J. 1950a. Une étude cytochimique des fragments nucléés et énucléés d'amibes. Experientia, 6: 294–295.
———. 1950b. The localization and the role of ribonucleic acid in the cell. Ann. N. Y. Acad. Sci., 50: 861–869.
Brown, V. E. 1930. The Golgi apparatus of *Amoeba proteus*. Biol. Bull., 59: 240–246.
Cain, A. J. 1947. The use of nile blue in the examinations of lipoids. Quart. Jour. Microsc. Sci., 88: 383–394.
———. 1950. The histochemistry of lipoids in animals. Biol. Rev., 25: 73–112.
Chalkley, H. W. 1936. The behavior of the karyosome and the "peripheral chromatin" during mitosis and interkinesis in *Amoeba proteus* with particular reference to the morphologic distribution of nucleic acid as indicated by the Feulgen reaction. Jour. Morph., 60: 13–29.
———. 1937. The chemistry of cell division. VII. The distribution of sulfhydryl in *Amoeba proteus* in interkinesis and mitosis, as shown by the nitroprusside test. Protoplasma, 28: 489–497.
———. 1951. Control of fission in *Amoeba proteus* as related to the mechanism of cell division. Ann. N. Y. Acad. Sci., 51: 1303–1310.
Chambers, R. 1924. The physical structure of protoplasm as determined by microdissection and injection. Sec. V, General Cytology, 234–309, (edited by E. V. Cowdry). Chicago.
Chiffelle, T. L., and **F. A. Putt.** 1951. Propylene and ethylene glycol as solvents for Sudan IV and Sudan black B. Stain Technol., 26: 51–56.
Claude, A. 1943. Distribution of nucleic acids in the cell and the morphological constituents of cytoplasm. Biol. Symposia, 10: 111–129.
Concannon, J. N. 1951. A study of the free-living nematode *Panagrellus silusiae* (deMan, 1913). M.Sc. Thesis. The Ohio State University, Columbus.
Coleman, L. C. 1938. Preparation of leuco-basic fuchsin for use in the Feulgen reaction. Stain Technol., 13: 123–124.
DeRobertis, E. D. P., W. W. Nowinski, and **F. A. Saez.** 1948. General Cytology. W. B. Saunders, Co., Philadelphia.
Doyle, W. L. 1933. Method for simultaneous demonstration of starch and fat in protozoa. Stain Technol., 8: 117.
Edwards, J. G. 1925. Formation of food cups in *Amoeba proteus* induced by chemicals. Biol. Bull., 48: 236–246.
Erickson, R. O., K. B. Sax, and **M. Ogur.** 1949. Perchloric acid in the cytochemistry of pentose nucleic acid. Science, 110: 472–473.
Feulgen, R., and **Voit, K.** 1924. Über einen weitverbreiteten festen Aldehyd. Arch. ges. Physiol., 206: 389–410.

Glick, D. 1949. Techniques of Histo- and Cytochemistry. Inter-science Publishers, Inc., N. Y.
Handy, C. C. 1947. Cultivation of *Amoeba proteus* with *Saprolegnia* and *Chilomonas paramecium*. M.Sc. Thesis. The Ohio State University, Columbus.
Hayes, E. R. 1949. A rigorous re-definition of the plasmal reaction. Stain Technol., 24: 19–23.
Holter, H. 1936. Studies on enzymatic histochemistry. XVIII. Location of peptidase in marine ova. Jour. Cell. and Comp. Physiol., 8: 179–200.
———, and **W. L. Doyle.** 1938. Über die Lökalisation der Amylase in der Amoben. Compt. Rend. Trav. Lab. Carlsberg, Ser. Chim., 22: 219–225.
———, and **M. J. Kopac.** 1937. Studies on enzymatic histochemistry. XXIV. Location of peptidase in the ameba. Jour. Cell. and Comp. Physiol., 10: 423–437.
Hopkins, D. L. 1938. The vacuoles and vacuolar activity in the marine amoeba *Flabellula mira* Schaeffer and the nature of the neutral red system in Protozoa. Biodyn., no. 34, 22 pp.
———. 1941. The cytology of *Amoeba verrucosa*. Biol. Bull., 81: 299–300.
———. 1946. The contractile vacuole and the adjustment to changing concentration in fresh water amebae. Biol. Bull. 90: 158–176.
Horning, E. S. 1933. The enzymatic function of mitochondria. Ergebn. der Enzymforsch., II, 336.
Hotchkiss, R. D. 1948. A microchemical reaction resulting in the staining of polysaccharide structures in fixed tissue preparations. Arch. Biochem., 16: 131–141.
Howland, R. B. 1924a. On the excretion of nitrogenous waste as a function of the contractile vacuoles. Jour. Exp. Zool., 40: 231–250.
———. 1924b. Experiments on the contractile vacuole of *Amoeba verrucosa* and *Paramecium caudatum*. Jour. Exp. Zool., 40: 251–262.
———. 1924c. Dissection of the pellicle of *Amoeba verrucosa*. Jour. Exp. Zool., 40: 263–270.
Hyman, L. 1936. Observations on Protozoa; The impermanence of the contractile vacuole in *Amoeba vespertilio*. Quart. Jour. Microsc. Sci., 79: 43–56.
Jahn, T. L., and **F. F. Jahn.** 1949. How to Know the Protozoa. W. C. Brown Co., Dubuque, Iowa.
Kepner, W. A., and **J. G. Edwards.** 1917. Food reactions of *Pelomyxa carolinensis* Wilson. Jour. Exp. Zool., 24: 381–407.
Kudo, R. R. 1946. Protozoology. 3rd ed. C. C. Thomas, Springfield, Ill.
Lazarow, A. 1943. The chemical structure of cytoplasm as investigated in Professor Bensley's laboratory during the past ten years. Biol. Symposia, 10: 9–26.
Lehmann, F. E. 1950. Globäre Partikel als submikroscopische Elemente des tierischen Zytoplasmas. Experientia, 6: 382–384.
Lison, L. 1936. Histochimie Animale. Gauthier-Villers, Paris.
Lotze, J. C. 1934. Observations on the physiology of the ameba *Mayorella bigemma* (Schaeffer). Ph.D. Diss. The Ohio State University, Columbus.
———. 1937. In *Culture Methods for Invertebrate Animals*. P. S. Galtsoff and J. G. Needham, eds. p. 81. Comstock Pub. Co., Ithaca, N. Y.
Lucas, M. S. 1930. Results obtained from applying the Feulgen reaction to protozoa. Proc. Soc. Exp. Biol. and Med., 27: 258–260.
Luce, R. W., and **A. W. Pohl.** 1935. Nature of crystals found in Amoeba. Science, 82: 595.
MacLennan, R. F. 1941. In *Protozoa in Biological Research*. G. N. Calkins and F. M. Summers, eds. Chap. III. Columbia Univ. Press, New York.
Mast, S. O. 1926. Structure, movement, locomotion and stimulation in Amoeba. Jour. Morph., 41: 347–425.
———. 1932. Locomotion in *Amoeba proteus* (Leidy). Protoplasma, 14: 321.
———. 1938. The contractile vacuole in *Amoeba proteus* (Leidy). Biol. Bull., 74: 306–313.
———. 1942. The hydrogen-ion concentration of the contents of the food vacuoles and the cytoplasm and other phenomena concerning the vacuoles. Biol. Bull. 83: 173–204.
———, and **W. L. Doyle.** 1935a. Structure, origin and function of cytoplasmic constituents in *Amoeba proteus*. I. Structure. Arch. Protistenk., 86: 155–180.
———, and ———. 1935b. Structure, origin, and lunction of cytoplasmic constituents in *Amoeba proteus*, with special reference to mitochondria and Golgi substance. II. Origin and function based on experimental evidence; effect of centrifugation on *Amoeba proteus*. Arch. Protistenk., 86: 276–306.
———, and **W. F. Hahnert.** 1935. Feeding, digestion, and starvation in *Amoeba proteus* (Leidy). Physiol. Zool., 8: 255.
———, and **P. L. Johnson.** 1931. Concerning the scientific name of the common large amoeba, usually designated *Amoeba proteus* (Leidy). Arch. Protistenk., 75: 14–30.
McClung, C. E. 1950. Handbook of Microscopical Technique. 3rd. ed. R. M. Jones, editor. Paul B. Hoeber, Inc., New York.
McManus, J. F. A. 1946. Histochemical demonstration of mucin after periodic acid. Nature, 158: 202.
Metcalf, M. M. 1910. Studies upon Amoeba. Jour. Exp. Zool., 9: 301–331.
———. 1926. The contractile vacuole granules in *Amoeba proteus*. Science, 63: 523–524.

Nassonov, D. 1924. Der Exkretionsapparat (Kontractile Vakuole) der Protozoa als Homologe des Golgischen Apparats der Metazoazellen. Arch. Mikr. Anat. Entw., 103: 437–448.
Pappas, G. D. 1948. Observations and study of five species in the Family Amoebidae. M.Sc. Thesis. The Ohio State University, Columbus.
Penard, E. 1902. Faune Rhizopodique du Bassin du Leman. Henry Kündig. Geneva.
Reynolds, B. D. 1924. Interactions of protoplasmic masses in relation to the study of heredity and environment in *Arcella polypora*. Biol. Bull., 46: 106–142.
Roskin, G. I., and **A. S. Ginsburg.** 1944a. Zymonucleic acid of the protozoic cell. Compt. Rend. Acad. Sci. U.R.S.S., 42: 348–351.
———. 1944b. Basophily of protoplasm in protozoa as connected with the occurrence in the cell of zymonucleic acid. Compt. Rend. Acad. Sci. U.R.S.S., 43: 122–125.
Rudzinska, M. A., and **R. Chambers.** 1951. The activity of the contractile vacuole in a suctorian (*Tokophrya infusionum*). Biol. Bull., 100: 49–58.
Schaeffer, A. A. 1918. Three new species of amebas; *Amoeba bigemma* nov. spec., *Pelomyxa lentissima* nov. spec., and *P. schiedti* nov. spec. Trans. Amer. Microsc. Soc., 37: 79–96.
———. 1926. The Taxonomy of the amebas with descriptions of thirty-nine new marine and fresh water species. Papers Dept. Marine Biol., Carnegie Inst., Washington, 24: 1–116.
Seifriz, W. 1936. Protoplasm. McGraw-Hill, New York.
Seshacher, B. R., and **E. W. Flick.** 1949. Application of perchloric acid technique to protozoa. Science, 110: 659.
Singh, B. N. 1938. The cytology of *Amoeba proteus* "Y" and the effects of large and small centrifugal forces. Quart. Jour. Microsc. Sci., 80: 601–635.
Stowell, R. E. 1946. The specificity of the Feulgen reaction for thymonucleic acid. Stain Technol., 21: 123–124.
———, and **A. Zorzoli.** 1947. The action of ribonuclease on fixed tissues. Stain Technol., 22: 51–61.
Vonwiller, P. 1913. Über den Bau der Amöben. Arch. Protistenk., 28: 387–410.
Weatherby, J. H. 1927. The function of the contractile vacuole in *Paramecium caudatum:* with special reference to the excretion of nitrogenous compounds. Biol. Bull., 52: 208–218.
———. 1929. Excretion of nitrogenous substances in Protozoa. Physiol. Zool., 2: 375–394.
———. 1941. In *Protozoa in Biological Research.* G. N. Calkins and F. M. Summers, eds. Chap. VII. Columbia Univ. Press, New York.
Wilber, C. G. 1942. The cytology of *Pelomyxa carolinensis.* Trans. Amer. Microsc. Soc., 61: 227–235.
———. 1945a. Origin and function of the protoplasmic constituents in *Pelomyxa carolinensis.* Biol. Bull., 88: 207–219.
———. 1945b. The composition of the refractive bodies in the rhizopod *Pelomyxa carolinensis.* Trans. Amer. Microsc. Soc., 64: 289–292.