(The Zoological Laboratory of the Johns Hopkins University.)

Structure, Origin and Function of Cytoplasmic Constituents in *Amoeba proteus*.

I. Structure.

By

S. O. Mast and **W. L. Doyle.**

(With 4 figures in the text.)

Contents.

Introduction.

Numerous observations have been made by various investigators on the structure of cytoplasmic constituents in *Amoeba proteus* and some suggestions have been made as to their origin and function, but there is still great diversity of opinion concerning their structure and the evidence presented in favor of the suggestions concerning their origin and function is very inadequate. This will be demonstrated presently.

Mast (1926, p. 419) maintains that *Amoeba proteus* consists of a central, granular, fluid portion, "plasmasol"; a granular, solid layer surrounding the fluid portion, "plasmagel"; a very thin well differentiated, elastic surface layer or membrane, "plasmalemma" and usually a thin hyaline fluid layer between the plasmalemma and the plasmagel. He says (p. 374): "The plasmasol consists of a fluid in which the following structures are suspended: numerous minute spherical granules (alpha granules); about an equal number of slightly larger irregular granules (beta granules); bipyramidal crystals and rectangular plate-like crystals varying greatly in number and size; refractive spherical bodies which also vary in number and size; various food vacuoles and usually the nucleus and the contractile vacuole."

We have, with various methods, thoroughly investigated all these structures except the nucleus. The results obtained are presented in this series of papers.

Materials and Methods.

Two species were used, *Amoeba proteus* obtained from a strain which has been maintained in the Zoological Laboratory of the Johns Hopkins University for over fifteen years, and *Amoeba dubia* obtained from J. A. Dawson, New York University. Both were cultured on inorganic salt solutions (Hahnert, 1932 and Pace, 1933) plus rice or wheat and food organisms. Cultures were prepared weekly and they were usually used when four weeks old.

The observations on the structure of *Amoeba* were made on living and on fixed specimens. Normal living specimens were put on a slide in culture fluid under a cover-glass supported by a vaseline ring. Then the cover-glass was gently pressed down until they were slightly flattened. Specimens in such preparations remain in active condition for one to three weeks. During this time they

decrease in size and become so translucent that the details of all their constituents can be clearly seen under immersion objectives.

The observations on normal specimens were supplemented by similar observations on specimens treated with the following vital dyes: janus green B (GRÜBLER and General DYESTUFF), bismarck brown, brilliant cresyl blue, chrysoidine, trypan blue, neutral red (VITAL, EHRLICH and COLEMAN and BELL), rhodamine, and benzene-azo-alpha-naphthylamine.

In preparing fixed material which contains chemically diverse structures, it is highly desirable that the techniques used in different methods be as nearly uniform as possible thruout. Otherwise it is difficult to compare the results obtained by the different methods. The cytological effects of most of the modern fixatives have been admirably described in the works of HIRSCH and JACOBS (1925), BĚLAŘ (1927), ZIRKLE (1928—1933), HERTWIG (1930), SCHILLER (1930) and BAUER (1933). These descriptions indicate that for cytoplasmic structures, formalin is an ideal fixative if it can be used in such a way as to avoid shrinking and swelling of the cell as a whole. With this in view many different mixtures were tested. Of these the following was found to be the best: (Culture medium) 8.4 cc.; (Formol C. P. neutralized with $MgCO_3$) 0.6 cc.; (phosphate buffer M/5, p_H 6.2) 1.0 cc.

This mixture does not measurably change the volume of amoebae and it preserves all the constituents of the cytoplasm, except the nuclear granules and the water soluble salts, in a form scarcely distinguishable from the living condition; and it produces comparatively minor chemical changes in these constituents, so that when a second reagent (which may act either like a mordant or a solvent) is applied it acts nearly as it would if it were applied to living cytoplasm except that, owing to the hardening action of the formalin, it produces less distortion.

Fixation with this formalin mixture was therefore a preliminary step in nearly all the cytological techniques used. In this way a high degree of morphological uniformity was obtained which was invaluable in comparing results obtained by the different methods used.

MILLON reagent was used in testing for protein; BENEDICT reagent, NYLANDER reagent, BEST's carmine method and Lugol solution were used in testing for carbohydrates; sudan III and IV dissolved in various mixtures of alcohol acetone and water, FLEMMING fixative (strong) with and without acetic acid, 1 % and 2 % osmic acid, commercial nile blue sulphate and pure nile blue sulphate

oxazone, the Regaud technique, Ciaccio's methods, alcohol, acetone, acetic acid, sodium hydroxide, nitric acid, and turpentine were used in testing for fatty substances; and the following techniques: Altmann's fuchsin, Regaud, Bensley-Cowdry, copper-chrome-haematoxylin, Schridde, Mann-Kopsch, Kolachev, Champy-Kull, Benda, Da Fano, Nassanov and Defrise modification of the Regaud technique were used in testing for mitochondria and Golgi substances.

Numerous specimens were studied by each of the methods used. The results obtained by the different methods were carefully compared. Nearly all of the conclusions reached are based upon results obtained by several different methods. They will be presented under several headings.

Alpha Granules.

Vonwiller (1918) designated the alpha granules 'kleinste Körnchen'. Mast says they are approximately 0.25 micron in diameter. They are so near the limit of microscopical vision that we could see nothing concerning their structure in living specimens, and our efforts to stain them were unsuccessful. It was consequently impossible unequivocally to identify them in fixed preparations. We could therefore ascertain nothing concerning either their structure, origin or function.

Beta Granules.

Mast (1926) maintains that the beta granules are about one micron in diameter and that they are uniformly scattered thru the cytoplasm, except at the surface of the contractile vacuole where they tend to form a layer. They were designated: Elementarkörnern by Greeff (1891), plastiduli fucsinofili by Zoja (1891), Endoplasmakörner by Nirenstein (1905), sphaeroplastes by Fauré-Fremiet (1907), and Vonwiller (1915), excretion granules by Metcalf (1910), and mitochondria by Arndt (1924). They are probably homologous with structures in other organisms called cytomicrosomes and plastosomes by Duesberg (1912) and cytosomes or cytome by Dangeard (1931).

In view of the multiplicity of terms applied to these bodies it is evident that there exists considerable uncertainty as to their attributes.

The outline of the beta granules can be very distinctly seen in living specimens. By observing them from various points of view we found that those which are suspended in the cytoplasm

are nearly spherical or ellipsoidal, but that those which are on the surface of the contractile vacuole are flattened at the surface of contact with the vacuoles and with each other (Fig. 1 a, c). Individual granules were repeatedly selected and studied continuously for several minutes under the oil immersion objective and the following was observed: They slowly change slightly in form. Local protuberances resembling small pseudopodia appear at intervals as short as five minutes but there is no locomotion. The spherical ones sometimes become ellipsoidal or rod-like in form and vice versa. When they are rod-like they look somewhat like the bacteria found in the cytoplasm and in the food vacuoles (Fig. 1 c). In unfavorable culture media, intense light and ultra violet radiation they round up or break up into smaller spheres. This was repeatedly observed under the oil immersion objective.

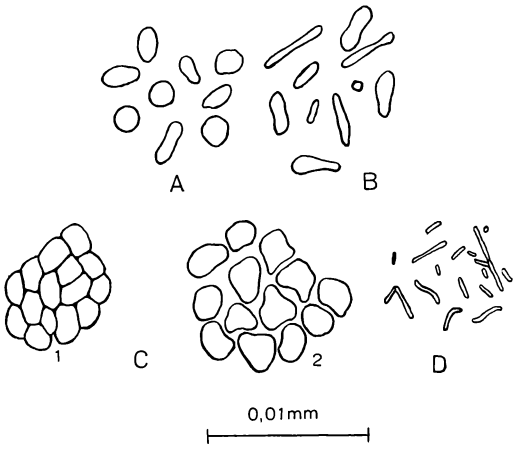

Fig. 1. Camera outline of beta granules (mitochondria) and bacteria in *Amoeba proteus*. A, beta granules found free in the normal living cytoplasm; B, beta granules after fixation with modified Regaud technique; C, beta granules on the surface of the contractile vacuole (1) in close contact with each other (2) with some space between; D, bacteria in the cytoplasm treated with Chrome-Osmic-Haematoxylin technique; mm, projected scale.

The facts that the beta granules become angular when they are in close contact and that they change in form indicate that they are plastic.

If amoebae are put into a 1 : 100.000 solution of janus green in culture fluid and then mounted with air bubbles as previously described, they live for several hours. If they are examined one-half to two hours after the addition of the stain all the beta granules and the bacteria, but no other structures, appear distinctly green. In the beta granules it can be clearly seen in optical section that only the surface is stained. This indicates that they have a differentiated surface layer similar to that in mitochondria.

In specimens treated with the mitochondrial methods given above numerous small bodies were found which have all the staining characteristics of mitochondria. These bodies are point for point like those described and called mitochondria by Fauré-Fremiet, Arndt, Zoja, and Vonwiller. They are, in size, number and distribution, like the beta granules seen in living specimens; but they are in general somewhat more elongated. This is less marked in the specimens treated with the Regaud technique than in those treated with any of the other methods. The elongated form is doubtless due to the action of the chemicals used. These facts therefore seem to demonstrate that the beta granules are mitochondria.

Crystals.

Schaeffer (1916) asserts that plate-like crystals are abundant in *Amoeba dubia* and absent in *Amoeba proteus* and that truncated bipyramidal crystals are very abundant in *Amoeba proteus* and rare in *Amoeba dubia*. Mast (1926) maintains that *Amoeba proteus* contains both types of crystals. Both forms were described in detail by Schewiakoff (1893), Schubotz (1905), Doflein (1907), Schaeffer (1916), Vonwiller (1918), and Ivanic (1924). Vonwiller maintains that they reach a maximum length of ten micra, that many of them are associated with large spheres "Kugeln" filled with fluid and that some of them have small blebs or Knöpfchen at some of their corners or edges. Edwards (1924) maintains that they are in vacuoles and Mast (1926) maintains that they are suspended in fluid in the vacuoles and that this fluid in these vacuoles becomes yellowish in solutions containing neutral red and consequently is alkaline.

Investigations concerning their chemical nature were made by Auerbach (1856), Carter (1856), Wallich (1863), Greeff (1891), Schewiakoff (1893), Stolc (1902), Schubotz (1905) and others.

There is no agreement among these investigators as to the chemical nature of the crystals. Carter (1856), and Schewiakoff (1893) concluded they are calcium oxalate; Wallich (1863) that they are carbonates or calcium salts; Entz (1879) and Rhumbler (1898) that they are excretory products; Stolc (1902) that they are leucine, Schubotz (1905) and Pollack (1928) that they are tribasic calcium phosphate, and it has been verbally suggested that they are cholesterol.

Not all authors distinguish between the two types of crystals found in the cytoplasm and it is not always clear what species

was used in the investigations. From the evidence to be presented it is evident that STOLC and SCHUBOTZ had reference to the plate-like crystals, whereas the other investigators probably dealt with the bipyramidal ones.

We made a thoro study of the crystals in a great many specimens of *Amoeba proteus* under oil immersion objectives, with and without polarized light. The results obtained lead to the following conclusions:

Practically all specimens of *Amoeba proteus* contain both plate-like and bipyramidal crystals. The number of both forms varies

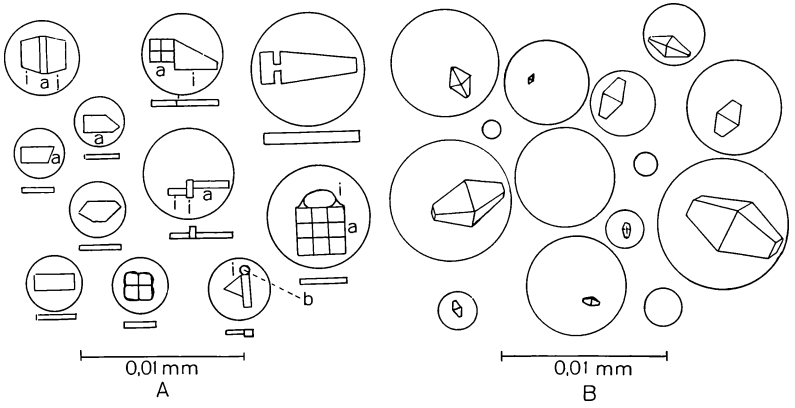

Fig. 2. Camera outlines of crystals in *Amoeba proteus*. A, plate-like crystals; circles, outlines of vacuoles; outlines within the circles, side view of crystals; outlines below circles, edge view of crystals; b, bleb; a, optically active substance; i, optically inactive substance; mm, projected scale. B, bipyramidal crystals, all optically inactive; circles, outlines of vacuoles. Note that the crystals vary greatly in size, that the relation in size between the vacuole and the crystals in it varies greatly and that some vacuoles have no crystals.

greatly in different individuals but the latter are always very much more abundant than the former. They are sometimes so abundant that the cytoplasm appears to consist largely of crystals. Both types are in vacuoles. The vacuoles contain hyaline substance in which the crystals are suspended, usually one in each vacuole. The vacuoles and the crystals vary greatly in size, and the size of the vacuoles in relation to the size of the crystals in them also varies greatly (Fig. 2 a, b).

The hyaline substance in which the crystals are suspended in the vacuoles becomes reddish yellow in neutral red. It is therefore alkaline. Its consistency is under some conditions so fluid that the

crystals move about freely and rapidly, i. e. Brownian movement is marked, and under other conditions so viscous that no Brownian movement whatever can be seen.

1. Plate-like Crystals.

The plate-like crystals are typically orthorhombic but they vary much in form and they frequently occur in hemimorphic combination with other types of crystals (Fig. 2a). They are all optically active but not on all axes and they exhibit parallel extinction of their optical activity. They resemble crystals of leucine in respect to crystal habit and extinction angles and they are insoluble in a saturated solution of leucine. It is therefore highly probable that they are leucine.

2. Bipyramidal Crystals.

The bipyramidal crystals vary in length from the lower limit of visibility to 7 μ, the average being 5 μ (Fig. 2b). No indication whatever of optical activity was observed in any of them. It is therefore obvious that they do not contain any optically active substance, and that since all oxalates, calcium phosphates and carbonates, leucine and cholesterol are optically active they do not contain any of these substances. The conclusions concerning their composition reached by Carter, Schewiakoff, Wallich, Stolc, Schubotz and Pollack are therefore not valid and they are not cholesterol.

To obtain further information concerning the chemical composition of the bipyramidal crystals we attempted to analyze them spectrographically and to ascertain their melting point and their solubility in various solutions.

a) *Solubility.*

Numerous observations were made under high power objectives on amoebae, some intact and some crushed, in various solutions. The results obtained are presented in table 1. By referring to this table it will be seen that the crystals are very soluble in all aqueous solutions, except concentrated ammonia and ammonium sulphate; that they are soluble in all mineral acids except concentrated sulphuric acid in which they decompose; that they are insoluble in concentrated organic acids, organic fat solvents and carbon disulphide; but that they are soluble in carbon disulphide saturated with ammonia. Their solubility is consequently like that of amino-acids. This therefore indicates that the crystals consist

Table 1.

Table showing solubility of the crystals of *Amoeba proteus* in various reagents. Dil, dilute; conc, concentrated; sat, saturated; aq, aqueous; alc, alcoholic; dec, decomposes; xxx, immediately soluble; ---, insoluble after several days; x, slowly soluble; -, soluble in one to two days. For solvents immiscible with water the specimens were dried by fixation in absolute alcohol.

Solution	Solubility	Solution	Solubility
H_2O	xxx	LiCl	xx
HCl (conc)	xx	$(NH_4)_2SO_4$	-
HCl (dil)	xxx	CH_3COOH (dil)	x
HNO_3 (conc dil)	xxx	CH_3COOH (conc)	---
H_2CrO_4 (dil)	x	HCOOH (conc)	---
H_3PO_4	x	$(COOH)_2$	-
H_2SO_4 (conc)	dec	Carnoy's	---
H_2SO_4 (dil)	xx	CS_2	---
NaOH (conc dil)	xxx	CS_2NH_3	xx
KOH (conc dil)	xxx	Alcohol (95%)	--
NH_4OH (conc)	-	Alcohol (70%)	x
NH_4OH (dil)	xxx	Ether	---
NaCl (sat)	x	Chloroform	---
$AgNO_3$	xx	Benzene	---
$Na_2S_2O_5$ (sat)	x	Toluene	---
KI (aq)	x	Glycerine	---
KI (alc)	--	Balsam	---
KNO_3	xxx	Xylol	---
K_2SO_4	xx		

of amino-acid. But if they do they must consist of glycine for, as demonstrated above, they are not optically active and glycine is the only amino-acid which is not optically active. The form of the crystals and the fact that solutions in which crystals have been dissolved give positive chemical tests for glycine also indicate that they are glycine. These tests were made as follows:

Approximately 5000 amoebae were put into 1 cc. of each of the solutions in which it was found that the crystals are readily soluble (Table 1) and left for several hours; the solutions were then filtered thru glass wool after which the filtrate of each was divided into two portions and sodium hypochlorite added to one and ferric chloride to the other. Positive tests for glycine were obtained.

If the crystals are glycine they must be insoluble in a saturated solution of glycine. It was found however that if amoebae are mounted in a saturated solution of glycine and torn open the crystals rapidly dissolve even when they are in close contact with crystals of glycine which, owing to evaporation, are increasing in size. It is consequently evident that the bipyramidal crystals are not glycine.

The facts presented clearly demonstrate, however, that they have a number of properties which are like those found in glycine. It is probable therefore that they consist of a compound of glycine.

b) *Melting Point.*

Ten to twenty amoebae were washed in distilled water and placed on a slide in a small drop of water. The slide was then put into a drying oven at 50° C and left eight to twelve hours. As a result the amoebae were dried and spread out in a very thin layer in which the crystals could be distinctly seen.

Minute crystals of a salt with a known melting point were now prepared by grinding some of the salt in an agate mortar and drying to constant weight at 110° C. A few of these minute crystals were then placed on the slide so that they and the crystals in the amoebae were visible in the same field under the low power of the microscope. The slide was now warmed from beneath by means of a micro burner attached to the stage of the microscope. This was continued until the crystals of the salt or those in the amoebae melted, sublimed or decomposed. The whole process was then repeated with other salts until one was obtained which melted at the same time that the crystals in the amoebae underwent a marked change.

It was found that when stannous chloride was used the crystals in the amoebae darkened a few seconds before the crystals of stannous chloride melted, that shortly after this they appeared to give off a small puff of smoke or vapor and that there remained a dark residue which became white when heated until the slide began to bend. It was concluded that the crystals in *Amoeba* decompose rather than melt and that this decomposition occurs at a temperature a little lower than the melting point of crystals of stannous chloride (246° C), i. e. that they decompose at about 240° C. Glycine crystals decompose at 275° C. The results obtained therefore support the conclusion presented above, namely, that the bipyramidal crystals in *Amoeba* are not glycine, but the facts that they decompose and leave a dark residue which becomes white with heat indicate that they consist of a combination of organic and inorganic substances, and the fact that the temperature required for decomposition does not greatly differ from that required for decomposition of crystals of glycine indicates that they probably consist of a salt of glycine.

c) *Spectrographic Analysis.*

To analyze a substance spectrographically it is highly desirable to have it pure. We therefore attempted to separate the bipyramidal crystals from the other substances in the amoebae. Two methods were used.

1. Large numbers of amoebae were killed in absolute alcohol and then put into each of various solutions in which the crystals are soluble and left 24 hours, after which the solutions were slowly evaporated. No crystals were obtained in any of them.

2. Numerous specimens were washed in distilled water then put into culture fluid which contained no calcium or magnesium, and left 48 hours with transfer to fresh culture fluid every 12 hours. Two hundred of these specimens were suspended in centrifuge tubes and rotated until they separated into two parts. The lighter parts were then collected and washed in two changes of distilled water, after which a drop of the last wash water was put into the depression of a clean graphite electrode in a spark apparatus aligned with a medium sized quartz spectograph. The circuit was now closed and the spectrum produced photographed. This was repeated with respectively the lighter and the heavier portions of the amoebae in the electrode which had contained the wash water.

Magnesium lines were found in the photograph of the spectrum produced by the heavier parts but in none of the others. In this spectrum the 279.5 mμ, the 280.2 mμ, the 285.2 mμ, the 292.8 mμ, and the 293.6 mμ, sensitive lines of the magnesium spark spectrum were fairly distinct; numerous lines representing other elements were found but the magnesium lines were the only ones not found in all of the spectra. During the process of centrifuging, nearly all the crystals and all the refractive bodies aggregated at the heavier end of the amoebae. The fact that the magnesium lines were found only in the spectrum of the heavier parts of the amoebae therefore indicates that either the crystals or the refractive bodies or both contain magnesium.

The experiment was consequently repeated with specimens containing many crystals but only very few refractive bodies. The results obtained were the same as those obtained in the preceding experiment. The magnesium is therefore probably in the crystals.

If the crystals contain magnesium and have properties similar to those of glycine, as demonstrated above, they probably are a magnesium compound of glycine. But if this is so, they are insoluble

in a saturated solution of this compound. We consequently made observations with methods described above, on the relative solubility of the crystals in saturated solutions of leucine, glycine, methyl glycine (sarcosine), trimethyl glycine (betaine) and methyl glycine plus magnesium hydrate respectively.

We found that they are soluble in all these solutions, but that the time required to dissolve in the solutions was much greater for methyl glycine plus magnesium hydrate than for any of the other substances tested. Consequently, while we have no conclusive proof as to what the crystals are, the facts that they contain magnesium, have several important properties of glycine, and are not readily soluble in a solution saturated with magnesium hydrate and methyl glycine indicate that they consist of a substance closely related to a magnesium salt of a substituted glycine.

Refractive Bodies.

Various investigators have observed relatively large, spherical, highly refractive bodies in the cytoplasm and in the food vacuoles in amoebae and they found that these bodies become deep red if the amoebae are stained with neutral red. Mast (1926) calls all these bodies refractive spherical bodies. Other investigators have used various other terms.

Those free in the cytoplasm were designated discoid ovules by Carter (1856), nucleated corpuscles by Wallich (1863), Glanzkörper by Greeff (1874), Glanzkügelchen by Stolc (1902), secondary nuclei by Calkins (1905), Eiweißkügeln by Vonwiller (1918), parasites by Mattes (1924), cysts by Taylor (1924), Golgi bodies by Brown (1930), nutritive spheres by Taylor (1932), and globules oleagineuse, spherules chromatiques, liposomes, and ergastome by Dangeard (1931, 1932, 1933). Bodies similar to those in the food vacuoles were designated Ballen and Körnerkonglomerate by Nirenstein (1905), Reservestoffe by Heironymus (Lafar, 1907), mitochondria by Horning (1925—1928), chromies and spherules metachromatiques by Dangeard (1931, 1932, 1933), vacuome by Volkonsky (1933), and spherical bodies by Mast and Hahnert (1935).

The extraordinary variety of names for the bodies clearly shows that the authors held widely divergent views as to their origin, structure and function. Thus Wallich, Calkins and Taylor (1924) considered them reproductive bodies; Carter, Greeff, Stolc, Vonwiller, Taylor (1932) and Dangeard, deutoplasmic bodies; and

Brown, Golgi bodies. Greeff, Stolc, Taylor (1932) and others held that they are homogeneous in structure and Carter, Wallich, Calkins, Vonwiller, Taylor (1924) and Brown held that they are not.

Despite evidence of occasional critical examination of the literature few authors have mentioned the views of previous investigators. This was probably due chiefly to the fact that different investigators used such widely different techniques that they did not recognize that the different terms used referred to the same structures.

We found that the refractive bodies in the cytoplasm differ radically from those in the food vacuoles. The former stain less quickly in solutions of neutral red, they are more complicated in structure and they are larger than the latter (the diameter of the largest being fifteen micra for the former and only ten for the latter). We will refer to those in the cytoplasm as refractive bodies and to those in the food vacuoles as vacuole refractive bodies.

1. Refractive Bodies.

Amoebae were put into a dilute solution of neutral red (approximately 1 : 50,000) and left until the refractive bodies were stained deep crimson. A specimen was then mounted in culture fluid under a cover-glass supported by a vaseline ring and greatly flattened by applying pressure to the cover-glass. The specimen was then studied under low and high power. It was seen that the central portion of the refractive bodies was definitely lighter than the outer portion and that the lighter portion was eccentric in some of the bodies. While being observed under moderate magnification (8 mm objective) pressure was applied to the cover-glass with a needle directly over the specimen. The refractive bodies spread out and as they spread the central portion became lighter until it was precisely differentiated from the outer portion which now consisted of a thick dark crimson layer. The pressure was now decreased, the diameter of the refractive bodies immediately decreased, and the crimson layer became thinner. This shows that the refractive bodies are highly elastic. The pressure was then gradually increased. The crimson layer became thicker and the central portion spread out until it had increased by about one-fifth in diameter, then it suddenly cracked into several pieces and a clear fluid flowed out of it (Fig. 3c). This shows that the central portion consists of a spherical fragile shell which surrounds a fluid substance.

In other preparations, the cover-glass was pressed down and released several times in rapid succession with the needle over a

region containing several refractive bodies in close contact with each other. The crimson layers of these refractive bodies fused and formed a continuous matrix thru which the central colorless masses were scattered (Fig. 3 b). It is therefore evident that the outer layer is definitely fluid but rather oily and viscous and that this is the only portion of the refractive body that stains with neutral red.

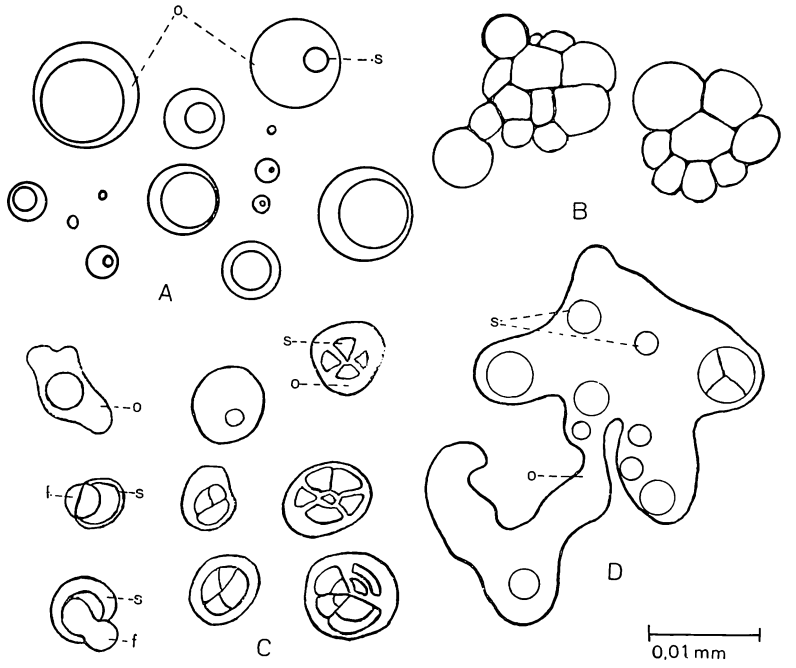

Fig. 3. Camera drawings of refractive bodies in the cytoplasm of *Amoeba proteus*. A, specimens free in the cytoplasm; o, outer layer (this stains crimson with neutral red); s, outline of fragile shell (this contains a fluid substance). Note that the refractive bodies vary greatly in size and that the outer layers vary greatly in thickness. B, specimens in contact with each other. Note that the outlines are angular indicating plasticity. C, specimens which had been subjected to pressure; o, outer layer; s, shell; f, fluid. Note that the shells have broken in some and cracked in others and that the central fluid was partly forced out of some which were cracked. D, outlines showing the effect of successive increase and decrease in pressure on a group of specimens in contact with each other. O, fused outer layers; s, shells; mm, projected scale. Note that one of the shells is cracked.

The refractive bodies consequently consist of an outer relatively fluid layer which stains crimson in neutral red and a central globular shell filled with a fluid which does not stain with neutral red.

Figure 3 shows that the size of the refractive bodies varies greatly and that the size of the shell in relation to the thickness of the outer layer also varies greatly.

Vonwiller (1918) maintains that the refractive bodies sometimes contain three or four differentiated masses. We observed this only in specimens which had been subjected to pressure. It is therefore probable that it occurs only in specimens in which the outer layers of refractive bodies have fused.

Taylor (1924) sectioned and stained what she called encysted amoebae and she maintains that she found in them cysts composed of a thin outer layer ("cyst wall") surrounding a droplet of "hatching ferment" in which are embedded a varying number of "chromosomes" and that these are "young amoebae". It is quite apparent from her description and more apparent after examination of her plates that the structures she observed were refractive bodies with cracked shells (probably broken by sectioning), that what she called chromosomes were fragments of the shell, that what she called hatching ferment was the fluid contained in the shell and that what she called cyst wall was the outer oily layer.

The question now arises as to what the composition of the different parts in the refractive bodies is. To answer this question numerous amoebae were treated with the reagents listed below and the effects on the various parts of the refractive bodies observed.

a) *Outer Layer.*

Specimens were treated with various fat stains. The outer layer was not stained by sudan dyes unless the amoebae were treated with phenol in accord with the method of Ciaccio (1925) before they were put into the dyes, then it became red. This layer became intensely blue in nile blue; and it became black in osmic acid, after which it did not bleach in hydrogen peroxide or turpentine. The fact that it does not stain with sudan dyes shows that it is not fat; but the fact that it stains in these dyes subsequent to treatment with phenol, and that it blackens with osmic acid and is not readily bleached, indicates that it is lipoidal; and the fact that it stains blue in nile blue shows that it contains fatty acid.

Amoebae were immersed in alcohol and thirty-five percent acetic acid respectively and left in each for twelve hours and then examined. The refractive bodies did not dissolve nor did they noticeably decrease in size. They were however less refringent after the treatment and they no longer stained with nile blue, osmic acid or

sudan dyes used in accord with Ciaccio's method. This indicates that the outer layer consists of a substance, which is soluble in fat solvents, dispersed in a substance which is not soluble in fat solvents, i. e. a lipid dispersed in a substance which is not lipid.

Numerous amoebae were either fixed in formalin and then put into methylene blue in sulphuric acid or put directly into methylene blue in sulphuric acid. In the latter, the refractive bodies aggregated and formed several different masses and the outer layers of the bodies fused and became blue-black. In those previously fixed in formalin they did not fuse but the outer layer became blue-black. This staining shows that the outer layer of the refractive bodies contains metachromatin, i. e. a substance composed of nucleic acid and an unknown base. Nucleic acids are frequently associated with proteins, the outer layer therefore probably contains protein.

Some specimens were put into acetone and then treated with Millon's reagent and others were put into acetic acid of various concentrations. In the Millon's reagent the outer layer of the refractive bodies gave a faint positive protein reaction. This shows that it contains protein. In acetic acid in concentrations up to thirty percent the outer layer of the refractive bodies dissolved. In higher concentrations it did not. This is precisely what would be expected if this layer consists of lipid substance and protein, for if it does the lipid would dissolve in the acetic acid in all concentrations used but the proteins would coagulate in the higher concentrations and disintegrate in the lower, and this would cause the outer layer to disappear in the weaker solutions but not in the stronger which is in accord with what was observed.

All of the results presented consequently indicate that the outer layer of the refractive bodies consists of a protein stroma which is impregnated with a lipid substance.

The fact that the outer layer contains protein lends some support to Vonwiller's contention that the refractive bodies are „Eiweißkügeln".

b) *Shell.*

The shell in the refractive bodies was tested for proteins, lipids and carbohydrates and it was studied in polarized light. No proteins, lipids or starches were found in it, but it was found that it is faintly optically active, that it reduces Nylander's reagent and that it dissolves in concentrated sodium hydrate. These reactions indicate that altho the shell is not starch, it is a carbohydrate.

c) *Fluid.*

Nothing was ascertained concerning the chemical composition of the fluid within the shell.

d) *Discussion.*

Brown (1930) as previously stated, concluded that the refractive bodies are Golgi bodies.

There is much controversy concerning the origin, structure, function and composition of the Golgi bodies. They are consequently not well defined. A considerable number of methods have been devised to test for them. Among the more important are those of Nassonov, Bensley-Cowdry, Champy-Kull, da Fano, Kolachev, Mann-Kopsch and Holmgren. All of these tests are based upon the chemical properties of substances in the Golgi bodies. The results obtained by these methods indicate that the Golgi bodies contain two substances. One which becomes black in osmic acid and does not readily bleach in turpentine, hydrogen peroxide or chlorine water but dissolves in fat solvents, and one which does not dissolve in fat solvents, e. g. trichloracetic acid. The former substance is often designated "O" and the latter "T". Beams (1931) and Tanaka (1932) have demonstrated that the "O" substance becomes red in solutions of neutral red and blue in nile blue and that the "T" substance does not stain in these solutions.

von Berger (1904) contends that the "O" substance is myelinoid or lecithin-like, Sjovall (1905) designates it myelinogen; Weigl (1911, 1912) says that is is lecithin; Rauber and Kopsch (1926) conclude that it is an albuminose or a peptone; and Tennent, Gardiner and Smith (1931) demonstrated in experiments on eggs of echinometra that it is a lipid characterized by the presence of unsaturated fatty acid. Holmgren (1902) concludes that the "T" substance is not fatty, and Bensley (1910) and Guilliermond (1933) come to the same conclusion.

Numerous amoebae were treated in accord with each of the methods named. In all, the outer layer of the refractive bodies give positive Golgi tests. In other experiments, described above, it was demonstrated that this layer contains fatty acid and protein. The outer layer of the refractive bodies therefore has the characteristics usually ascribed to Golgi bodies. Moreover, there are no other structures in the cytoplasm which have these characteristics. It is therefore highly probable that if *Amoeba proteus* possesses Golgi

substance it is in the refractive bodies. These bodies however contain structures which are not ordinarily found in Golgi bodies, namely, the shell and the central fluid. However, globules or vacuoles (which are sometimes themselves internally differentiated) are frequently found in Golgi bodies, e. g. the acroblast; and the structures in plant tissues most frequently considered to be the homolog of the Golgi bodies in animal tissues, viz. the plastids, quite commonly have an internal structure not unlike the shell and central fluid in the refractive bodies.

Zirkle (1926) says: "the ground substance of the chloroplast is in the form of a hollow, flattened, prolate spheroid surrounding a large central vacuole," and he holds that one or more vacuoles are present in the ground substance and that starch is formed in these vacuoles. Sharp (1934, p. 67) says: "A prevalent view regarding the interior of the chloroplast is that it consists of a somewhat denser cytoplasm, the stroma, in which there is a lipide carrying chlorophyll"; and he says that different types of plastids are "characterized by different principal products of metabolic activity. In the higher plants and green algae this product is usually starch".

The facts that the central portion of the refractive bodies contain a carbohydrate and that the outer layer consists of a stroma which is impregnated with fatty substance, therefore indicate that they are in some respects related to plastids.

2. Vacuole Refractive Bodies.

The vacuole refractive bodies were studied in specimens treated in the same way as those in which the refractive bodies were studied. The following results were obtained:

In solutions containing neutral red the vacuole refractive bodies become crimson in color just as the refractive bodies do, but they stain much more rapidly and considerably more densely and all the substance in them stains, not merely a layer at the surface as in the refractive bodies. If pressure is applied to the cover-glass they spread and if it is released they round up like the refractive bodies, but there is no indication of any differentiation in the structure of different portions, i. e. they contain nothing in the nature of a shell. If pressure is applied and released several times in rapid succession vacuole refractive bodies which are in contact with each other fuse and form a large undifferentiated homogeneous mass which, when the pressure is released, assumes a globular form. The vacuole refractive bodies are therefore semi-fluid and elastic but homogeneous

in structure. They consequently differ radically in structure from the refractive bodies.

In nile blue, they become intensely blue; in neutral red, crimson; in brilliant cresyl blue, blue; and in osmic acid, black. In sudan dyes they do not stain unless they are first treated with phenol; and in fat solvents they disappear entirely. This shows that they contain fatty acid and other lipids, but that they do not contain neutral fat, protein or a carbohydrate which is insoluble in water. They are therefore like the outer layer of the refractive bodies in that they contain fatty acid and other lipids which stain crimson with neutral red, blue with brilliant cresyl blue, and black with osmic acid, and do not readily bleach with turpentine, hydrogen peroxide and chlorine water; but they differ from this layer in that they do not contain protein and are entirely soluble in fat solvents, e. g. trichloracetic acid.

In osmic acid the vacuole refractive bodies become black more rapidly than the refractive bodies and although they remain black much longer than the fat globules in the cytoplasm they bleach more rapidly in hydrogen peroxide and turpentine. This is what would be expected if the protein in the outer layer of the refractive bodies is in the form of a stroma and the lipids are imbedded in the interstices of this stroma, for this would retard the entrance of solutions which act on the lipids.

The facts that the vacuole refractive bodies become red in neutral red and black in osmic acid indicate they contain substance which is like that which Parat (1928), Hall (1929, 1930), Volkowsky (1933) and Finley (1934) call vacuome.

Blebs on the Crystals.

About five percent of the crystals have blebs attached. The blebs are globular in form with flat surfaces where they are in contact with other bodies. This shows that they are plastic. They vary greatly in size. The largest found were nearly as large as the largest crystals and the smallest were barely visible. They are usually on the under surface of the crystals, and they are consequently more readily seen if the main axis of the microscope is horizontal. This indicates that their specific gravity is greater than that of the crystals.

The blebs become intensely blue in nile blue and crimson in neutral red. They stain red considerably more rapidly than the

refractive bodies but not so rapidly as the vacuole refractive bodies. They become black in osmic acid and do not readily bleach in turpentine, hydrogen peroxide or chlorine water. The small ones become definitely dark in thirty minutes; the large ones require much longer. The former are soluble in trichloracetic acid, the latter are not. These facts indicate that the blebs change in composition as they develop and that the small ones have properties somewhat similar to those of the vacuole refractive bodies, and the large ones properties somewhat similar to those of the outer layer of the refractive bodies; and they suggest that the blebs form from the vacuole refractive bodies and give rise to the outer layer of the refractive bodies.

Fat.

Various investigators have observed that well fed specimens of *Amoeba proteus* usually contain in the food vacuoles and scattered thruout the cytoplasm, numerous globules which become black in osmic acid and red in sudan dyes; and they have found that in the absence of food these globules decrease greatly in number; but they gave no description of the globules other than the sui generis implication of the terms fat and oil. No observations have been reported on the origin of the fat beyond the fact that it in some way comes from the food in the food vacuoles; and no chemical changes have been observed in the process of digestion or synthesis of fat (Greenwood, 1886; Dawson and Belkin, 1928 and 1929; Mast and Hahnert, 1935).

With a view to obtaining more information concerning the nature of the fat we made the following experiments:

Some amoebae were fixed respectively in osmic acid, formalin and mercuric chloride and treated with various different fat stains. Others were put into culture fluid containing vital dye chrysoidine, and still others were subjected to low temperatures.

The following was found: The fat globules in the cytoplasm stained orange with sudan III in alcohol, red with sudan III in alcohol-acetone mixtures, red with sudan IV in alcohol, maroon with sudan IV in alcohol-acetone mixture, pink with nile blue sulphate, red with pure nile blue sulphate oxazone, brown to black with osmic acid (brown in 15 minutes and black in several hours), and yellowish with chrysoidine. The fat globules in the food vacuoles like those in the cytoplasm, stained with the sudan dyes, osmic acid and chrysoidine, but they stained blue instead of pink with nile blue sulphate.

In specimens subjected to a temperature of 5° C for several hours the globules in the cytoplasm crystallized but those in the food vacuole did not (Fig. 4).

The facts that the fat globules in the cytoplasm stain pink with nile blue sulphate and crystallize at 5° C and that those in the food vacuoles stain blue with nile blue sulphate and do not crystallize at 5° C indicate that the former are composed largely of neutral fat and the latter largely of fatty acids.

In staining solutions containing less than thirty percent water the fat globules dissolved. In fixatives containing chromates as the only oxidizing agent they were poorly preserved against dissolution in the higher alcohols. The significance of these reactions is not known.

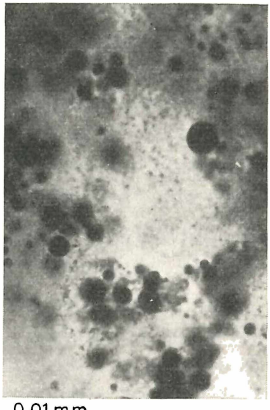

Fig. 4. A, photomicrograph showing the fat globules in a small region in a specimen of *Amoeba proteus* fixed in formalin stained with sudan IV and mounted in glycerine. The black circular areas represent fat globules. Note that they differ greatly in size and are very numerous. (Sudan IV, glycerine mount, 2 mm obj.-Zeiss Phoku camera). B, camera outlines of fat bodies after subjecting the organism to 5° C for twelve hours; mm, projected scale. Note the angular outlines indicative of crystallization.

Contractile Vacuole.

Metcalf (1910) demonstrated that the surface of the contractile vacuoles is well covered with a layer of granules and he concluded that the granules are involved in excretion. Nassonov (1924, 1925) using cytological methods studied the contractile vacuole in several protozoa and concluded that it is the homolog of the metazoan Golgi apparatus. There has been considerable controversy concerning the validity of these conclusions (Hall, 1929; Brown, 1930; Moore, 1934).

We made observations on *Amoeba proteus* with reference to these conclusions and found it to be an ideal organism for the investigation. The results obtained will be considered in a subsequent paper.

Organisms.

Numerous small rod shaped structures were found scattered fairly uniformly thru the cytoplasm in all specimens of *Amoeba proteus* examined. These structures look and stain like bacteria and are definitely distinguishable from the mitochondria. They are therefore probably bacteria. No attempt was made to culture them.

Mold spores of various kinds were frequently found in *Amoeba proteus*. These are usually engulfed like food but there is no evidence indicating digestion. They are usually retained for considerable periods of time and they often form in masses containing a hundred or more before they are voided. In a number of specimens spores were found with hyphae growing from them. Some of these hyphae branched and were in length equal to the diameter of the amoebae in which they were found. These hyphae were repeatedly seen to increase in length and in a few amoebae spores were found in all stages of development. Some spores obviously grow and fruit in the cytoplasm.

During several weeks a large percentage of specimens collected in a small portion of a permanent pond near Bar Harbor, Maine, had marked tufts of hyphae projecting from the posterior end. These hyphae were attached to groups of spores imbedded in the cytoplasm at the posterior end of the amoebae. The spores probably germinated in the amoebae and the hyphae formed probably broke thru the surface and then grew outside forming the tufts observed [1]).

The portion containing the spores to which these hyphae were attached was cut off in a number of specimens. This did not in any way interfere with locomotion and all the operated specimens lived for a number of days but they did not feed. However none of those with tufts attached had any food vacuoles or were observed to feed. It is therefore probable that they were in a pathological condition.

Leidy (1874) found in the neighborhood of Philadelphia a considerable number of amoebae which had at the posterior end long filamentous projections, forming a sort of caudal appendage. This appendage was essentially the same in all the amoebae which had it. In other respects some of the amoebae werelike *Amoeba proteus*; the rest were considerably smaller and differed in other respects. Owing to the presence of the caudal appendage Leidy held that

[1]) These observations were made jointly by Percy L. Johnson and S. O. Mast (Johnson, 1935).

the amoebae which have it are generically distinct and he consequently grouped them together under the generic name Ouramoebae, i. e. amoebae with tails (1879). Our observations demonstrate fairly conclusively that the caudal appendages observed by LEIDY were tufts of fungous hyphae and that there consequently is no foundation for the genus Ouramoeba.

No zoochlorellae were found either in *Amoeba proteus* or *Amoeba dubia.*

Summary.

1. The cytoplasm of *Amoeba proteus* contains numerous granules, some .25 $\pm \mu$ in diameter (alpha granules, and others 1 $\pm \mu$ in diameter (beta granules), plate-like and bipyramidal crystals, highly refractive, spherical bodies, some in the cytoplasm (refractive bodies) and others in the food vacuoles (vacuole refractive bodies).

2. Nothing is known concerning the structure and the composition of the alpha granules.

3. The beta granules vary in shape from nearly spherical to rod-like. They are elastic and they stain like the granules in other cells known as mitochondria. They tend to aggregate at the surface of the contractile vacuole.

4. There are two kinds of crystals, plate-like and bipyramidal. They are in vacuoles which contain fluid substance in which they are suspended. They vary in length from a maximum of 7 $\pm \mu$ nearly to zero. The vacuoles vary in diameter from a maximum of 9 $\pm \mu$ nearly to zero. The relation between the size of the vacuole and that of the crystal in it varies greatly. The substance in the vacuole is alkaline and it is under some conditions so fluid that the crystals are in violent Brownian movement and under others so viscous that no movement can be detected. Some have attached to them one or more blebs which are not optically active.

5. The plate-like crystals are all optically active but not on all axes. They are probably leucine.

6. The bipyramidal crystals are not optically active. Results obtained in spectroscopic analysis and observations made on their solubility and on their form show that they contain magnesium and have properties of glycine. They probably consist of a magnesium salt of a substituted glycine.

7. The refractive bodies are spherical in form. They vary in diameter from 15 μ nearly to zero. They consist of a hollow fragile shell filled with a fluid substance and a semi-fluid outer layer. The

outer layer stains crimson with neutral red. It is composed of a protein stroma impregnated with lipid containing fatty acid. It gives positive reactions to all important tests for Golgi substance. The shell is composed of a substance which has carbohydrate properties, but is not starch or glycogen. The composition of the central fluid is not known.

8. The vacuole refractive bodies vary in diameter from 10 μ nearly to zero. They stain crimson with neutral red like the outer layer of the refractive bodies. They are semi-fluid and elastic and homogeneous in structure. They are composed of lipids containing fatty acid. They do not contain neutral fats, proteins or carbohydrates.

9. About five percent of the crystals have blebs attached to their surface. These blebs change in composition as they develop. The smaller ones are somewhat similar to the vacuole refractive bodies and the larger ones somewhat similar to the refractive bodies. They probably form from the vacuole refractive bodies and give rise to the refractive bodies.

10. The fat globules in the cytoplasm stain pink with nile blue sulphate and crystallize at 5° C. The fat globules in the food vacuoles stain blue with nile blue sulphate and do not crystallize at 5° C. The former are composed largely of neutral fat and the latter largely of fatty acid.

Bibliography.

Arndt, A. (1924): Rhizopodenstudien. I. Arch. f. Protistenk. Bd. 49 p. 1—83.
Auerbach, L. (1856): Über die Einzelligkeit der Amöben. Zeitschr. f. wiss. Zool. Bd. 7 p. 365—430.
Bauer, H. (1933): Mikroskopisch-chemischer Nachweis von Glycogen und einigen anderen Polysacchariden. Zeitschr. f. Mikr. Anat. Forsch. Bd. 33 p. 143—160.
Belar, K. (1927): Über die Naturtreue des fixierten Präparats. Zeitschr. f. Indukt. Abst. u. Vererb. Suppl.-Bd. 1 p. 402—407.
Beams, H. W. (1931): A cytological study of the spinal ganglion cells of the rat with special reference to the Golgi apparatus, vacuome, canalicular apparatus, mitochondria, clear canals of Penfield and Nissl bodies. Anat. Rec. Vol. 49 p. 309—344.
Brown, V. E. (1930): The Golgi apparatus of Amoeba proteus Pallas. Biol. Bull. Vol. 59 p. 240—246.
Calkins, G. N. (1905): Evidences of a sexual cycle in the life history of Amoeba proteus. Arch. f. Protistenk. Bd. 5 p. 1—16.
Carter, H. J. (1856): Notes on the freshwater Infusoria of the Island of Bombay. No. 1. Organization (Continued). Ann. of Nat. Hist. Ser. 2 Vol. 18 p. 221—249.
Ciaccio, C. (1926): I lipoidi considerati come constituenti essenziali della cellula. Boll. Soc. Biol. Sperim. Vol. 1 p. 47—50, 144—147.
Dangeard, P. A. (1931): Memoire sur la terminologie des éléments cellulaires et son application a l'étude des Champignons. Le Botaniste Ser. 22 T. 6 p. 325—490.
— (1932): Note sur la formation de granules chromatiques dans le cytoplasm de quelques algues sous le influence des colorants vitaux et en particulier du rouge neutre. Ibid. Ser. 24 T. 1—2 p. 157—171.

Dangeard, P. A. (1933): L'agonie d'un Infusoire, le Prorodon teres, et considerations générales sur le vacuome et les autres formations cellulaires des Infusoires. Ibid. Ser. 24 T. 5—6 p. 281—332.
Dawson, J. A. and M. Belkin (1928): Digestion of oils by Amoeba dubia. Proc. Soc. Exp. Biol. and Med. Vol. 25 p. 790—793.
— (1929): Digestion of oils by Amoeba proteus. Biol. Bull. Vol. 56 p. 80—86.
Doflein, F. (1907): Studien zur Naturgeschichte der Protozoen. V. Amöbenstudien. Arch. f. Protistenk. Suppl.-Bd. 1 p. 250—294.
Duesberg, J. (1912): Plastosomen usw. Zeitschr. f. d. ges. Anat. Abt. 3, Ergebn. d. Anat. u. Entwges. Bd. 20 p. 567—916.
Edwards, J. G. (1924): The action of certain reagents on amoeboid movement. II. Physiological and locomotor reactions. Brit. Journ. Exp. Biol. Vol. 1 p. 571—595.
Entz, G. (1879): Über einige Infusorien des Salzteiches Szamosfalva Termesyetsajze Fuzeteke. Bd. 3 p. 10—11.
Faure-Fremiet, E. (1907): Mitochondries et spheroplastes chez les infusoires cilies. Compt. Rend. Soc. de Biol. T. 62 p. 523—525.
Greeff, R. (1874): Pelomyxa palustris (Pelobius), ein amöbenartiger Organismus des süßen Wassers. Arch. f. mikr. Anat. Bd. 10 p. 51—74.
— (1891): Über den Organismus der Amöben. Biol. Zentralbl. Bd. 11 p. 599—608.
Greenwood, M. (1886): On the digestive process in some rhizopods. Journ. Physiol. Vol. 7 p. 253—273.
Guillermond, A. (1933): Traité de cytologie vegetale. Paris.
Hahnert, W. F. (1931): A quantitative study of reactions to electricity in Amoeba proteus. Physiol. Zool. Vol. 5 p. 491—526.
Hall, R. P. (1929): Reaction of certain cytoplasmic inclusions to vital dyes and their relation to mitochondria and Golgi elements in the flagellate Peranema trichophorum. Journ. Morph. Bd. 48 p. 103—122.
— (1930): Osmiphilic inclusions similar to the Golgi apparatus in the flagellates, Chomulina, Chilomonas, and Astasia. Arch. f. Protistenk. Bd. 69 p. 7—22.
Hertwig, G. (1930): Der Einfluß der Fixierung auf das Kern- und Zellvolumen. Ztschr. f. mikr. Anat. Forsch. Bd 23 p. 484—509.
Hirsch, G. C. and W. Jacobs (1925): Experimentelle Untersuchungen über das Wesen der Fixierung, Teil I. Ztschr. f. Zellf. u. mikr. Anat. Bd. 3 p. 198—228.
Horning, E. S. (1925): The mitochondria of a protozoan (Opalina) and their behavior during the life cycle. Austral. Journ. Exp. Biol. and Med. Vol. 2 p. 167—171.
— (1928): Studies on the behavior of mitochondria within the living cell. Ibid. Vol. 5 p. 143—148.
Ivanic, M. (1924): Fortpflanzungserscheinungen einiger Süßwasseramöben. Arch. f. Protistenk. Bd. 50 p. 113—134.
Johnson, P. L. (1930): Reproduction in Amoeba proteus. Arch. f. Protistenk. Bd. 71 p. 463—498.
Lafar, F. (1907): Technische Mykologie. XII, 871 p. Jena.
Leidy, J. (1879): Fresh water rhizopods of North America. U. S. Geol. Surv. Vol. 12 XI + 324 p. 48 plates.
Mast, S. O. (1926): Structure, movement, locomotion and stimulation in Amoeba. Journ. Morph. Vol. 41 p. 347—425.
Mast, S. O. and P. L. Johnson (1931): Concerning the scientific name of the common large amoeba usually designated Amoeba proteus (Leidy). Arch. f. Protistenk. Bd. 75 p. 14—30.
Mast, S. O. and W. F. Hahnert (1935): Feeding, Digestion and Starvation in Amoeba proteus (Leidy) Physiol. Zool. In press.
Mattes, O. (1924): Über Chytridineen in Plasma und Kern von Amoeba sphaeronucleolus und Amoeba terricola. Arch. f. Protistenk. Bd. 47 p. 413—430.
Metcalf, M. M. (1910): Studies upon Amoeba. Journ. Exp. Zool. Vol. 9 p. 301—331.
Moore, I. (1934): Morphology of the contractile vacuole and cloacal region of Blepharisma undulans. Journ. Exp. Zool. Bd. 69 p. 59—104.
Nassonov, D. (1924): Der Exkretionsapparat (Kontraktile Vakuole) der Protozoa als Homologen des Golgi'schen Apparatus der Metozoazellen. Arch. f. mikr. Anat. u. Entw. Bd. 103, p. 437—482.

Nassonov, D. (1925): Zur Frage über den Bau und die Bedeutung des lipoiden Exkretions- apparats bei Protozoa. Zeitschr. f. Zellf. u. mikr. Anat. Bd. 3 p. 52—79.
Nirenstein, E. (1905): Beiträge zur Ernährungsphysiologie der Protisten. Ztschr. f. allg. Physiol. Bd. 5 p. 435—484.
Pace, D. M. (1933): The relation of inorganic salts to growth and reproduction in Amoeba proteus. Arch. f. Protistenk. Bd. 79 p. 133—145.
Pollack, H. (1928): Micrurgical Studies in Cell Physiology. VI. Calcium Ions in Living Protoplasm. Journ. Gen. Physiol. Vol. 11 p. 539—545.
Rhumbler (1898): Physikalische Analyse von Lebenserscheinungen der Zelle. I. Arch. Entw. d. Org. Bd. 7 p. 103—350.
Schaeffer, A. A. (1916): Note on specific and other characters of A. proteus Pall. (Leidy), A. discoides spec. nov., and A. dubia spec. nov. Arch. f. Protistenk. Bd. 37 p. 204—228.
Schewiakoff, W. (1893): Über die Natur der sogenannten Exkretkristalle der Infusorien. Ztschr. f. wiss. Zool. Bd. 57 p. 32—56.
Schiller, W. (1930): Gewebsfixierung unter Erhaltung der Basischen Kernfärbung. Ztschr. f. Zellf. u. mikr. Anat. Bd. 11 p. 63—177.
Schubotz, H. (1905): Beiträge auf Kenntnis der A. blattae und A. proteus. Arch. f. Protistenk. Bd. 6 p. 1—46.
Sharp, L W. (1934): Introduction to Cytology. N. Y.
Stolc, A. (1902): Über das Verhalten des Neutralrots in lebendigen Protoplasm. Ztschr. f. allg. Physiol. Bd. 1 p. 209—219.
Tanaka, H. (1932): Vital staining of the Golgi apparatus. Arch. f. Exp. Zellf. Bd. 13 p. 47—52.
Taylor, H. (1924): Amoeba proteus: Some new observations on its nucleus, life history and culture. Quart. Journ. Micr. Sci. Vol. 69 p. 119—143.
— (1932): Proper name of the Ameba. Nature Vol. 130 p. 168.
Tennent, D. H., M. S. Gardiner and D. E. Smith (1931): A cytological and biochemical study of the ovaries of the sea-urchin, Echinometra lucunter. Carn. Inst. Wash. Pub. 413. Papers from the Tortugas Lab. Vol. 27 p. 1—40.
Volkonsky, M. (1933): Digestion intracellulaire et accumulation des colorants acides. Bull. Biol. France et Belg. T. 67 p. 135—275.
Vonwiller, P. (1915): Die Sphaeroplasten von Amoeba proteus. Anat. Anz. Bd. 48, p. 485—488.
— (1918): Über den Bau des Plasmas der niedersten Tiere. Arch. f. Prot. Bd. 38 p. 279—323.
Wallich, G. C. (1863): Further observations on an undescribed indigenous Amoeba, with notes on remarkable forms of Actinophrys and Difflugia. Ann. & Mag. Nat. Hist. Ser. 3 Vol. 12 p. 329—337, 448—468.
Zirkle, C. (1928): The effect of hydrogen in concentration upon the fixation image of various salts of chromium. Protoplasma Bd 4 p. 201—227.
— (1928): Nucleolus in root tip mitosis in Zea Mays. Bot. Gaz. Vol. 86 p. 402—418.
— (1929): Fixation images with chromatin and acetates. Protoplasma Bd. 5 p. 511—534.
— (1932): Vacuoles in primary meristens. Ztschr. f. Zellf. u. mikr. Anat. Bd. 18 p. 26—47.
— (1933): Cytological fixation with the lower fatty acids, their compounds and derivatives. Protoplasma Bd. 18 p. 90—111.
Zoja, L. e R. (1891): Intorno di plastiduli fucsinofili. Mem. del Reale Inst. Lombarde di Acienze e Lettere Vol. 1 p. 237—270.