On the Nature of Cytoplasmic Constituents in *Amoeba diploidea* Examined by Means of Staining and Chemical Reactions[1) 2)]

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(With 3 Tables, 4 Textfigures and 1 Plate)

Concerning the cytoplasmic constituents of amoeba there have appeared many papers reporting studies carried out from different angles of approach. These works can, essentially, be grouped into two categories; those from the viewpoint of morphology and those from physiology.

The investigations from the viewpoint of morphology were initiated by Fauré-Fremiet (1910) on the mitochondria especially in Infusoria and by Hirschler (1914) on the Golgi apparatus in *Monocystis ascidiae* in which he could differentiate Golgi apparatus from mitochondria. In later years the attention of cytologists has been especially focused upon the problem of the Golgi apparatus, because the function of this cell organ was unknown in Metazoan cells despite much effort being made by many cytologists. Above all, the supposed functions of the Golgi apparatus as defined in the so called "Apparatentheorie" by Nassonov (1924) (similarly argued by Bowen, 1924) is most notable. Nassonov extended his hypothesis to the Golgi apparatus of Protozoa (1924 and 1925). He found them in some forms assuming nets or rings surrounding contractile vacuoles, and he claimed that he had established the osmo-regulatory system in the Protozoan cells. This hypothesis attracted the cytologists' interest

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1) Contribution No. 158 from the Zoological Institute, Faculty of Science, Hokkaido Imperial University.

2) The essential points of this study were reported in the 3rd Meeting of the Sapporo Branch of the Zoological Society of Japan held on June 21, 1941.

and many papers dealing with this problem have appeared since then. Another series of works concerning Golgi apparatus in Protozoa, which is worth mentioning, was accomplished by Duboscq and Grassé (1924 and thereafter), who demonstrated their "l'appareil parabasal" in certain Flagellates. They said that these organella correspond exactly to the Golgi apparatus of other Protozoan as well as those of Metazoan cells. "L'appareil parabasal" occurs definitely at the basis of flagellum and consists of a series of vacuoles covered with the osmiophilic cortex. According to their interpretation these bodies can be supposed to be the energy source of the flagellar movement. Concerning the mitochondrial elements in Protozoan cells the homology to those of Metazoan cells has been likewise established. Other cytoplasmic constituents in Protozoan cells have been mainly discussed from the physiological view-point.

Concerning vital staining the pioneer work was done by Vonwiller (1913) on *Amoeba proteus*. Though many works have appeared since then, vital staining has been generally adopted as a method in physiology.

As for the physiology of Protozoa, especially concerning that of amoeba, studies have been carried out for a long time. Such a considerable number of papers have accumulated that it is impossible to refer to every of them. The most excellent investigation in this scope is the series of works on the *Amoeba proteus* by Mast (1926 etc.) and his followers. The details of those works will be discussed in the following paragraphs.

Recently the ultracentrifuging method has been introduced to the field of cytology, by which the specific gravity of cell inclusions can be easily ascertained. This method has been applied in Protozoa by Mast and Doyle (1935b) and Singh (1938); thus it has become easier to homologize the cell inclusion to those which are generally distributed in Metazoan cells.

The present author had an opportunity to culture *Amoeba diploidea* for one year, during which time observations and experiments were carried on both cytologically and histochemically resulting in some interesting new findings. Above all the ordinary cytological and histochemical methods, "Nile blue-sulphate technique" devised by the present author, was successfully applied in obtaining several conspicuous results concerning the nature of the cellular constituents.

The present study was made under the direction of Professor K. Oguma, to whom thanks are due for help and criticism during this work.

Material and Methods

Amoeba diploidea (Hartmann et Nägler)[1] is known as a half parasitic form of amoeba. They occur in various reptiles and mammals. In the present study the material was obtained from the faeces of rabbits.[2] Faeces were arranged in a layer at the bottom of a Petri dish and the tap water was poured upon them so that they were just submerged under the water surface. The dish was left with a cover protecting dust. In Spring it takes about a month till the ammoniac vapour ceases and a white contamination arises. The white contamination after such procedure contains the vegetative forms of *Amoeba diploidea*. These amoeba multiply by binary fission in a short period to a great number. Thus obtained amoeba were transplanted to the following agar culture medium.

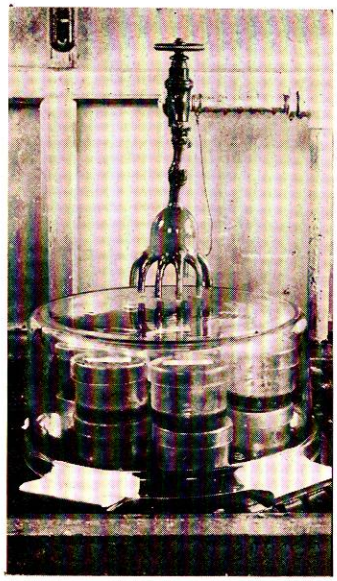
Decoction fluid of timothy hay	1000 c.c.
Agar	5 g.
Teruuchi's pepton	3.5 g.

In the oven kept at 25°C., amoeba and accompanied bacteria multiply rapidly, the former feeding the latter. After successive transplantations to the new culture medium it becomes a pure culture consisting of amoeba and a long slender species of bacteria. Using the cooling apparatus below described the vegetative form of amoeba lasts for eight or nine days and then they enter into the resting stage covered with cyst capsules. As particularly studied by Hartmann and Nägler (1908), before the formation of cyst they perform a kind of conjugation termed "hologamy" which is next followed by the reduction division of nuclei. During this time the pH of the medium changes from 6.8 to 8.5. Before the medium deteriorates and the cyst formation occurs the amoeba must be transplanted to the new medium. The favorite condition for the vegetative form can be prolonged by

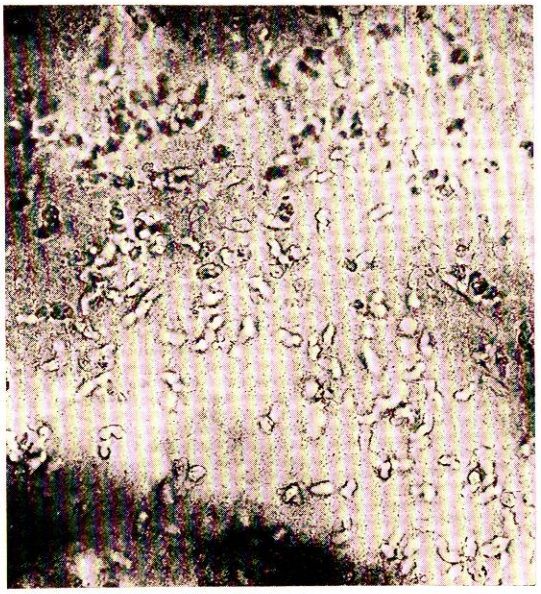
1) According to Wenyon (1926) it is better to term it *Sappinia diploidea* (Hartmann et Nägler). But *Amoeba diploidea* has been commonly used heading the work of Hartmann and Nägler, so this original terminology will be retained in the present paper.

2) It was personally informed by Kodera (1937) that they also inhabit the mucus of the oral cavity of pigeon.

keeping the amoeba in a cooling apparatus as indicated in Textfig. 1. The water runs uniformly over the outer surface of the jar covering the culture pots, and it enters into the inside of the jar through the interstice between the margin of the jar and the basal glass plate. Then it flows over the latter. This cooling apparatus is very useful in keeping the cultures free from harmful bacteria and dust, because those which happen to enter into this cooling chamber will be washed out by the water running over the floor glass.



Textfig. 1. The cooling apparatus for the cultivation of amoebae.



Textfig. 2. A colony of amoebae reaching to the maximal number. ca. ×150.

By means of this cooling apparatus the temperature of the culture pots could be kept at 10–13°C. almost throughout the year. Amoebae reached their maximum number in the fourth day from the transplantation (Textfig. 2). Copulation began on the eighth almost simultaneously. This prevalency may be comparable with the "conjugation epidemy" of *Paramoecium*. The cysts were formed about in the ninth or the tenth day.

Amoebae were plunged in the fixing fluid with their culture agar, as they creep underneath the agar surface. The fixed material was

cut employing the paraffine method. To demonstrate the Golgi bodies the material was fixed and impregnated after Kolatschev's osmium impregnating technique. For the mitochondria the combination of Champy's fluid accompanied by Benda's chromierung and Heidenhain's hematoxylin staining was employed. Formol fixation followed by various stainings was also tried.

The vital staining of amoeba was performed adopting the hanging drop method under the cover slip. The solutions of vital stains were all prepared as 1:10000 in concentration, dissolving each in the tap water. In special cases when the intensity of the staining was very weak, the dyes were dissolved in the culture medium. In order to study the behaviour of cell inclusions and other cellular structures to certain chemical substances, the latter were dissolved in the vital dye solutions, their concentrations being respectively mentioned in the later paragraph.

The histochemical methods for detecting lipoids, glycogen and oxydase were attempted. The concentrations and other special cares will be detailed in the paragraph dealing with histochemistry.

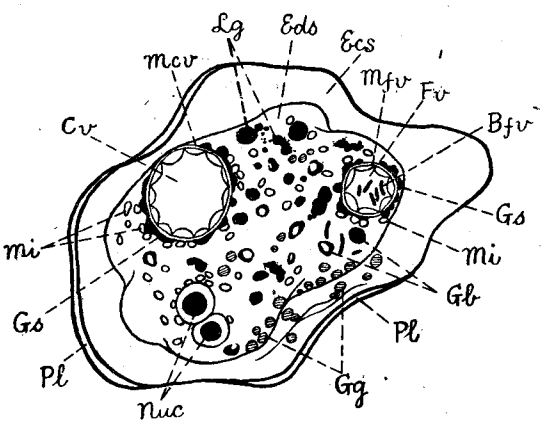
In order to obtain an accurate knowledge on the nature of the cellular constituents the author observed their behavior in the solutions of acids, alkalis and salts. The physiological meaning of their behavior to these chemicals are not discussed in this work, because it is aimed to know the nature of the constituents identified by the morphological technique.

Designation of Cytoplasmic Constituents

Before proceeding further it seems better for the readers' understanding to give a schematic figure which is summarized from the data appearing in the following paragraphs (Textfig. 3). The detailed morphological and physiological properties of these constituents shall not be given here.

The diameter of the amoeba ranges between 35 and 20 micron according to the frequency of the division. They are well known for the characteristic double nuclei attaching closely to one another. Usually each amoeba contains one large contractile vacuole and several food vacuoles of smaller sizes. The body can be divided into two regions; the inner endosarc and the outer ectosarc. The endosarc comprises the whole formative inclusions as seen in the schematic figure.

The "ectosarc" and "endosarc" are designated from the classical nomenclature only by their topographical relation. The former is made up of a hyaline outer area, the latter the granular inner area which comprises all the cellular inclusions. Other than the illustrated elements the ground matrix of the endosarc contains many minute



Textfig. 3. A schematic figure of an *Amoeba diploidea* summarizing the data obtained in the present study. *Lg*, Lipoid granules; *Eds*, endosarc; *Mfv*, membrane of a food vacuole; *Fv*, food vacuole; *Bfv*, bacteria in food vacuole; *Gs*, Golgi substance; *Mi*, mitochondria; *Gb*, Golgi bodies; *Pl*, plasmalemma; *Gg*, glycogen granules; *Nuc*, nuclei; *Cv*, contractile vacuole; *Mcv*, membrane of contractile vacuole.

granules. These granules correspond fully to Vonwiller's (1913) "kleinste Körnchen" and Mast's "alpha granules." Concerning their properties no special data could be ascertained, because their size is beyond the capacity of close microscopical observation.

The architecture of the nuclei is not discussed in the present paper, as nothing interesting could be observed. The cytoplasmic constituents in the cysts could not be well differentiated owing to the obscurity in staining reactions.

Another constituent, "refringent granules" which are described in the paragraph on vital staining, are not shown in this schematic figure, because they are surmised to be the same elements as Golgi bodies in all respects.

Observations

Results through cytological methods

I. *Golgi element.* In this kind of amoeba the Golgi element is represented as rings or dots scattered uniformly in the cytoplasm (Textfig. 1, *Gb*). By means of the classical impregnation technique, the analysis of their architecture is rather difficult. The Golgi bodies appear only as dots in a diffusely impregnated ground cytoplasm which makes observation difficult (Pl. I, Figs. 1 and 2). This defect is to some extent avoidable when the present author's protease-Nile blue sulphate technique is applied. For this material digestion by trypsin (Pl. I, fig. 6), or better by the combinations of pepsin-trypsin (Pl. I, figs. 7–10) and pepsin-papain (Pl. I, fig. 11), gave excellent results. The staining by an aniline dye, Nile blue sulphate, never exaggerates in revealing their contour as the impregnation technique does. Though not clear in the photographs of the plate, the perforation of tiny pores upon the Golgi bodies is easily observable. The diffuse impregnation by osmium upon the ground cytoplasm, sometimes exceeding the limit of observation (Pl. I, fig. 3), is indicative of the presence of an abundant quantity of lipoid and it is not an artifact brought about by the undue impregnation. This fact is clearly proven by the digestion method which yields an diffuse blue staining of cytoplasm (Pl. I, figs. 6–11).

The Golgi substance, on the other hand, adheres closely to the surface membrane covering the vacuole. In this connection, Nassonov (1924 and 1925) could demonstrate the Golgi net or ring surrounding the contractile vacuole in several species of Ciliates, and he imagined that he could establish the osmo-regulatory system. It seems to the present author that this association has resulted purely from the adsorption phenomenon and does not possess such an important physiological meaning as suggested by Nassonov, because it varies considerably in quantity.

Aside from certain kinds of Ciliates (viz. *Chilodon* and *Chilomonas* by Nassonov in 1924 and 1925), in which the Golgi apparatus is seen to be constructed of nets surrounding the contractile vacuole, it seems to be a general rule that the Golgi apparatus in the Protozoan cells assume the form of rings scattered throughout the cytoplasma. Good examples can be seen in *Adelea* (King and Gatenby 1923) and some species of Gregarina (Joyet-Lavergne 1924, Daniels

1938, and Subramaniam 1938). In *Chilomonas paramoecium* and *Chilomonas subtilis* Gatenby and Smyth (1940) could demonstrate small discrete rings other than nets surrounding the contractile vacuoles which were first discovered by Nassonov.

Concerning the Golgi apparatus of amoeba several accounts have been reported. Mast (1926) described accurately the cellular constituents of *Amoeba proteus* among which small bodies, which he termed "refractive bodies," attract our interest. He and one of his followers identified them as the Golgi bodies afterward (Mast and Doyle 1935a). According to them these bodies are blackened by osmium tetraoxyde and are not bleached in terpentine oil. For these reasons they identified them to the Golgi bodies found in the same species by Brown a little previously (1930). On the other hand, in the same paper, they observed that these granules were stained intensely by neutral red and consequently they claimed that these granules were at the same time "vacuome" of Metazoan cells. Directly after this in their second paper (1935b) they attempted the ultra centrifuging experiment. By this violent centrifugal force the cell inclusions move apart to the centrifugal and centripetal poles. The refractive bodies are displaced to the heavier pole, the centrifugal pole. This significant nature of specific gravity found in this experiment betrays apparently the data obtained generally in the Metazoan cells using the same tool (cf. Brambell 1924, Beanms & King 1934, Muliyil 1935, Dornfeld 1936, Hellbaum 1936, Norminton 1937 etc.). These workers reported likewise that the Golgi elements removed to the lighter pole, the centripetal pole. The present author considers that Mast and Doyle correctly identified the Golgi bodies in the fixed and impregnated preparations, and made a mistake in identifying another cellular element for them in the living material. As for the Golgi bodies in the living state reference should be made to the paragraph on vital staining of the present paper.

II. *Mitochondria.* Parallel to the ordinary cytological technique on the fixed material, vital staining with Janus green B was also employed. Mitochondria, which assumed granular shape (*Mi* in Textfig. 3; Pl. I, figs. 4 and 5), could be detected by both methods. They have never been observed to be filamentous. They are uniformly distributed throughout the cytoplasm. Some of them adhere to the contractile vacuoles and the food vacuoles. In the latter case the mitochondria are located upon the wavy surface of the Golgi

substance attached to the vacuole membrane. This location may not be of such important meaning as having excretory function which Metcalf (1910) suggested. Probably this association is a purely physical phenomenon, because their number varies considerably irrespective of the state of the vacuoles. Moreover, mitochondria frequently adhere also to the surface of nuclei (Pl. I, fig. 5).

III. *Membrane of contractile vacuole and food vacuole.* The existence of the membrane surrounding the contractile vacuole was maintained first by Metcalf (1910) who observed small round granules called "microsomes" or "cytomicrosomes" gathering upon this membrane. Mast (1938) also observed a membrane about 0.5 micron thick which is optically well differentiated from the adjoining substance. He supposed that this membrane is probably due to the action of the fluid in the vacuole on the adjoining cytoplasm. On the surface of this membrane the beta granules or Metcalf's miscrosomes, better called mitochondria, are arranged.

Apart from the contractile vacuoles in amoeba, a kind of membrane covering the vacuome in plant cells has been observed by many investigators headed by de Vries (1885). He made this membrane clear by causing plasmolyse to appear using 10% KNO_3. This elastic membrane, "Tonoplast" or "Turgorbilder" after him, is fixed in osmium tetraoxyde and becomes brown in potassium bichromate. Chambers and Höfler (1931) separated the membrane in the epidermic cells of onion by means of a micromaniplator. They said that this membrane is immiscible in water and cytoplasm and is broken when it comes in contact with chloroform. Mothes (1933) supposed that this membrane is of fluid and protein nature. The latter property was deduced from the fact that the membrane was digested in papain solution with the addition of HCl-cystein.

In the present material the membrane surrounding the contractile vacuole and food vacuole is blackened slightly by osmium impregnation (Pl. I, figs. 2 and 3). By the shrinkage caused by fixation, it is sometimes torn (Fig. 3). Using protease-Nile blue sulphate technique it remains undigested staining blue with Nile blue sulphate. In Fig. 9. the vacuole membrane is differentiated as a wrinkled wall by pepsin-trypsin, while in Fig. 11, subjected to pepsin-papain, the vacuoles are in full expansion and consequently their membranes have a smooth contour. In the amoeba fixed in formol, soaked in alcohol and then digested by trypsin the dissolution of

membrane of vacuoles is observed (Fig. 12). The above mentioned observations indicate that this delicate membrane surrounding the vacuole has properties similar to those of the Golgi substance. In other words it contains a protein destroyed by trypsin and a lipoid which is dissolved in alcohol. Other than their morphology, the only difference between this membrance and the Golgi substance is that the former react to the lipoid tests considerably weakly compared with the latter as in the cases of osmium reduction and Nile blue sulphate staining. It may be safe to suppose at present that both the elements have the same chemical substances but in different quantities.

To sum up the observations described hitherto, the contractile vacuole and the food vacuole are, from inside to outside, covered by a thin elastic membrane, by the irregular mass of Golgi substance and by a layer of mitochondria. The mutual association of these three elements is shown schematically in Textfig. 3.

The fluid of the contractile vacuole, and also that of the food vacuoles in a certain stage of digestion, is segregated into two parts; the outer circularly arranged blebs and the inner multiangular parts (Textfig. 3). The tips of the angles of the latter part are always in contact with the membrane of the vacuoles. The festoon-like bodering line between these two parts is probably due to the difference of their surface tension. This segregation of fluid is rather well distinguished in the living condition, especially by the vital staining, but it is obscure in the fixed material. Data on the nature of these two phases of fluid is lacking at present, and further investigation is required.

IV. *Plasmalemma.* There have been many authors who claimed the existence of a membrane covering the amoeba. Especially Mast (1926) described clearly the plasmalemma in *Amoeba proteus* as a fairly tough elastic membrane approximately 0.25 micron in thickness. If it was torn the broken edges were clearly observed. In the present species it is very difficult to differentiate by the ordinary cytological preparations, to say nothing of observation upon the living animals. This difficulty is overcome by the application of the present author's digesting method. As shown in the figures in the Plate (Figs. 6, 9, 10, 11 and 12) the plasmalemma remains undigested after the trypsin (Fig. 6), pepsin-trypsin (Figs. 9 and 10), or pepsin-papain digestion (Fig. 11). This undigested membrane be-

comes clearly visible when stained with Nile blue sulphate. Upon close examination this membrane is seen not to have an equal thickness everywhere on the cell surface, but to vary irregularly. It may be surmised from the reaction to the Nile blue sulphate after the protein digestion that it contains a lipoidal substance. One thing quite strange is that the plasmalemma is not dissolved even after soaking in alcohol and subsequent digestion by trypsin (Fig. 12). It is not yet understood whether the substnce here remaining is lipoidal in nature or is some other undigestible substance.

Staining reactions after formol fixation

The material fixed with formal was washed thoroughly with distilled water and was stained by vital staining dyes. Preparations were mounted in pure glycerin. The results will be seen in the following Table I.

TABLE I. Formol fixation

	Nile b. s.	Neut. r.	Jan. g. B.	Cres. e. v.	Sud. III
Lipoid g.	++	+	+?	+	+₊
Refr. g.	+!	−	+₊!	−	−
Mitoch.	++!	−	++	++	−
Cont. vac.	+red!	+	+purp.!	+purp.	+
Mem. c. v.	++!	−	++!	++!	−
Bac. f. v.	++	++	++	++	−
Food v.	+	+	−	+	+
Endosc.	+	+	+	+purp.	−
Ectosc.	+	−	−	−	−
Bac. cyt.	+	++	++	++	−

Remarks to Table I. Abbreviations of uppermost row: Nile b. s., Nile blue sulphate; Neut. r., neutral red; Jan. g. B., Janus green B; Cres. e. v., cresyl echt violet; Sud. III, Sudan III. Abbreviations of left column: Lipoid g., lipoid granules; Refr. g., refringent granules; Mitoch., mitochondria; Cont. vac., contractile vacuole; Mem. c. v., membrane of contractile vacuole; Bac. f. v., bacteria in food vacuole; Food v., food vacuole; Endosc., endosarc; Ectosc., ectosarc; Bac. cyt., bacteria in cytoplasm. ++ indicates an intense staining; +₊ a relatively strong staining; + staining visibly enough; ₊ weak staining; − negative to staining.

It is quite natural that the lipoid granules are stained intensely by these lipoid staining dyes, especially by Nile blue sulphate and Sudan III. The colour tone of Nile blue sulphate of the lipoid granules in amoeba is absolutely blue, and never has been red. The refringent granules, probably the Golgi bodies, are stained blue by Nile blue sulphate and by Janus green B. It comes to notice that they are quite uneffected by neutral red. Mitochondria, as was expected, were stained intensely by Janus green B. Nile blue sulphate and cresyl echt violet stain them markedly, while neutral red and Sudan III do not. The fluid portions of both contractile vacuole and food vacuole are stained in a similar manner. But the former stains always metachromasically, while the latter only becomes metachromasically stained as the digestion advances. The membrane of a contractile vacuole is reactive to the dyes which stain the refringent granules and mitochondria. This fact is strongly suggestive that, as it is seen in the foregoing paragraph, the membrane has the double nature of a combination of Golgi substance and mitochondria. Bacteria which are fixed in formol are unexceptionably stainable in all dye solutions saving Sudan III. Similarly the endosarc is stained by all these dyes except Sudan III. These staining properties may surely be indicative of the alpha granules after Mast (1926). Contrariwise to the endosarc the ectosarc is entirely negative to the dyes except Nile blue sulphate. It is interesting that the Nile blue sulphate stains exceptionally the ectosarc especially strongly at the periphery of the zone. This fact apparently indicates the presence of the plasmalemma which is evidently demonstrated by the cytological and enzymological techniques, and it would be quite natural to say that the plasmalemma is of lipoidal nature.

Vital staining

For vital staining, dyes were usually dissolved in tap water. The staining became almost complete after ten minutes. In some cases, divergent modes of staining resulted according to the duration of the stay in the dye solution. The staining of longer period was performed on the culture medium to which the dye solutions were added. The data obtained by this study are summarized in the following table (Table II).

The lipoid granules reveal uniformly positive reactions to the dyes employed, except Sudan III[1] and tropaeolin. Toluidin blue stains the lipoid granules positively. This fact reminds one of the same property of "Lipochondria" of Ries (1935), who supposed these

TABLE II. Vital staining

	Nile b. s.	Neut. r.	Jan. g. B.	Cres. e. v.	Sud. III.	Bril. c. b.	Chry.	Trop.	Tol. b.	etc.
Lipoid g.	++	++	+	→++ blue	−!	++	++	−	+!+	
Ref. g.	+!	−	−	−	+! surface	−	−	+!+	−	Tryp. b.+! Methyl. b.+!
Mitoch.	−	−!	++	−	−	−	−	→+?	−	
Cont. vac.	+! viol.	+	−	+ viol.	+!	→−	−	−	+ viol.	Jan. g.−
Mem. c. v.	−	−	−	−	−	−	−	+!	−	Methyl. b.+!
Bac. f. v.	− ↘ +	− ↘ ++	− ↘ +	− ↘ + viol.	−	− ↘ ++	− ↘ ++	↘ +	↘ +	
Food. v.	+ viol. ↓ blue	∓*	−	+ viol. ↓ blue	∓	→ + viol. ↓ blue	−	+	∓	Jan. g.+
Endosc.	∓*	−	+	−	−	∓*	−	→+?	+	
Ectosc.	∓+*!	−	−	−	−	∓*	−	−!	+! viol.	
Bac. cyt.	∓*	∓*	−	→ + viol.	−	∓*	−	−	∓*	

Remarks to Table II. Abbreviations of uppermost row: Bril. c. b., brillant cresyl blue; Trop., tropaeolin; Tol. b., toluidin blue; Tryp. b., trypan blue; Methyl. b., methylen blue; Jan. g., Janus green. The abbreviations of other dyes are the same as Table I. The notations of the intensity of staining are also the same as Table I. ! calls attention to important data. * shows the behaviour of the constituents to the long period of staining. ? indicates obscure data. The long arrows running from one row to another denote the necessity to compare one with another. The short arrows in the row of Bac. f. v. indicate conversion from one to the other. The suffixing small letters remark the colour tones specially noted.

1) The aqueous solution of Sudan III was made after the following procedure. Sudan III dissolved in aceton of stronger per cent was mixed in the tap water. The aceton was driven off from this mixture by means of the vacuum pump. This condenced dye solution was diluted with water to the certain per cent.

bodies to be capable of producing the Golgi bodies. The present material afforded nothing concerning the relationship between the two elements, but it is highly probable that the lipoid granules are nothing but one of the end products of the digestion of food. This process was roughly pursued by the vital staining with cresyl echt violet.

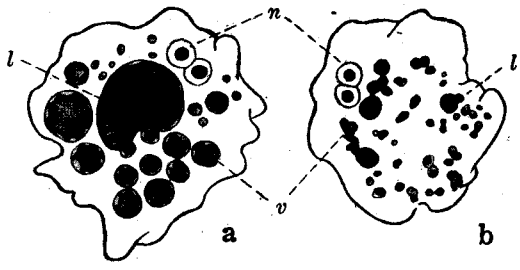
The refringent granules, probably the Golgi bodies, are small spherules of a high refractive index. They are stainable only with Nile blue sulphate, tropaeolin, trypan blue and methylen blue. Sudan III stains only the cortex of them. The possibility of the vital staining of the Golgi apparatus with Nile blue sulphate, though not intense, was claimed by the present author on the Golgi apparatus of pancreatic cells of the mouse (1940). Neutral red does not stain at all, neither does Janus green B. It is very interesting that tropaeolin stains them a little heavier. In accordance with the data obtained by the cytological and histochemical methods, the Golgi substance attached to the membrane of the contractile vacuoles, sometimes to that of food vacuoles, was also found stained positively by the tropaeolin. Trypan blue when applied for a long duration, stains the Golgi bodies in a blue tone. One can refer this phenomenon to the study of vital staining made by Glasunow (1928) on the several kinds of epithelial cells of the Guinea pig. It is also noteworthy that methylen blue usually stains positively, though not intense.

Mitochondria behave indifferently to almost all the vital staining dyes, except Janus green B. It is worth mentioning that they are never stained by neutral red. "Chondriomes actifs" as supposed by Parat (1929) do not exist in the present material. Tropaeolin stains the ground cytoplasm of endosarc to a yellow tone. It is not yet made clear whether this colour is due to the mitochondria or to the alpha granules after Mast, if present.

The excreted fluid in the contractile vacuole possesses lipoid nature from the staining properties with vital dyes, notably with Nile blue sulphate, neutral red, cresylecht violet, toluidin blue, and with Sudan III. Another characteristic feature is that it always expresses the staining reactions inclining to the alkali side, the staining expressing so-called "metachromasy". Here, it is necessary to describe as a comment the difference between the nature of the contractile vacuoles and food vacuoles. The architecture of the membranes covering them resemble one another from the vital

staining reaction as well as from the cytological point of view. Nevertheless, their contained fluids differ considerably as observed by means of vital staining in every respect. Aside from the presence of bacteria fed by the animal, which disappear upon digestion, the food vacuoles can be identified by their positive staining with Janus green[1] in bluish tinge; the contractile vacuole does not stain. The food vacuoles undergo marked change from the alkali side to the acid side as the digestion proceeds, while the contractile vacuole retains always the alkali colour. Another remarkable difference is that a long application of neutral red solution upon the food vacuoles makes their colour very intense, on the other hand the excretion fluid in the contractile vacuole is discharged before it attains to an intense coloration.

Here, some further remarks may well be made upon some data from the experiments employing vital staining. The most interesting effect of salts upon the amoeba is that of lithium salts. They have an effect of producing numerous abnormal vacuoles, probably food vacuoles. As seen in Text-fig. 4, the lithium carbonate brings about the appearance of comparatively large vacuoles with or without bacteria and lipoid granules (Textfig. 4, a). These vacuoles stain



Textfig. 4. a. The effect of the lithium carbonate dissolved in neutral red solution, the concentration of the salt being one-fifth of the saturation. b, The effect of the lithium chloride dissolved in the neutral red solution. The concentration is the same as above. *l*, lipoid granules; *n*. nuclei; *v*, vacuoles. The explanation is in the text. ca. ×1200.

more or less red by the neutral red. The vacant vacuoles thus produced are judged to be food vacuoles from the fact seen in the case of the effect of lithium chloride. In the latter case, the concrete bodies are all naked, driven from the vacuoles which are as a result

1) Needless to say, Janus green must not be taken for Janus green B.

scatered as numerous irregular small vacuoles (Textfig. 4, b). In this connection, the writer is reminded of the work of Kiyono and others (1938) who employed the "Lithionkarmin" technique in the demonstration of histiocytes and the phagocytic leucocytes. By means of this technique these cell species appear loaded with carmin vacuoles. Their figures strikingly resemble the amoeba subjected to the present lithium carbonate-neutral red experiment. As a matter of fact, the "Lithionkarmin" yields on amoeba absolutely the same result as that obtained by the lithium carbonate-neutral red experiment. Another marked fact is that the sodium chloride of hypertonic concentration has an effect of diminishing the vacuolar system, sometimes even destroying it, as mentioned by Day (1927).

The free swimming bacteria are almost non-reactive to the vital staining, as are also the freshly fed bacteria in the food vacuoles. As the digestion advances they change exceedingly in colour as indicated by arrows in Table II. The bacteria fed by the amoeba are sometimes found grouped in a small clump, without forming a food vacuole, in direct contact with the cytoplasm. They are probably freshly taken ones and are aggulutinated in a clump in the cytoplasm. They are gradually stained by certain dyes making their appearance with a clear contour.

The endosarc is stained to a certain degree by long duration of staining in several dye solutions which are specific to the albumen-lipoid system. It is uncertain whether this fact is due to the property of mitochondria or to the alpha granules claimed by Mast (1926) which are supposed to be present in the endosarc.

The ectosarc, especially the plasmalemma, behaves quite similarly to the refringent granules. This fact accords with the data obtained by the cytological methods. Against all these favourable data, two important betraying facts must be mentioned. These are the staining properties to tropaeolin and to toluidin blue. The refringent granules are stainable by tropaeolin but not by toluidin blue; in the case of the plasmalemma they react contrariwise. This discrepancy may be caused by the difference in their chemical constituents as may also be possibly supposed from their behavior to the treatment with the alcohol-trypsin (see p. 19).

Histochemical reactions

The staining reactions are not sufficient for ascertaining the distribution of chemical substances in the amoeba. Several lipoid

reactions, oxydase reactions, and a glycogen reaction were tried, which are reported in the following table (Table III).

The cytoplasmic constituents which are supposed to possess lopoidal substance react uniformly to the lipoid tests in more or less degree. The mitocondria are apt to stain with basic dyes rather than lipoid staining dyes as compared with other elements. This

TABLE III. Histochemical Reactions

	Ciaccio S. H.	Cia. Ess. S. H.	Sm.-Di.	Cia. Kul.	Cia. Nil.
Ref. g.	++red	++red	++	++	+
Mitoch.	+viol.	++red viol.	++	++	+
Mem. c. v.	+	+	++	++	+₊

	L. Br. c. b.	A. pyrog. Dia. Hyd.	Iod. vap.
Lipoid g.	++	++	+
Ref. g.	+?	+	+₊!
Mem. c. v.	×	++!	+
Mitoch.	+	+	+₊
Glycog.	—	—	++

Remarks to Table III. Ciaccio S. H., Ciaccio's method stained with Sudan III and Delafield's hematoxylin. Cia. Ess. S. H., Ciaccio's modification as combined with acetic acid, subsequently stnined as above. Sm.-Di., Smith-Dietrich's method, the staining being Kultschitzky's hematoxylin. Cia. Nil., Ciaccio's chromierung and staining with Nile blue sulphate. L. Br. c. b., leuco brillant cresyl blue as prepared in the text. A. pyrog., 2% aqueous solution of pyrogallic acid. Dia., 2% aqueous solution of diamidophenol. Hyd., 2% aqueous solution of hydroquinon. Iod. vap., iodine vapour applied as described in the text. × means destruction by the reagent. Other abbreviations as already employed in the other tables.

fact indicates the content of a considerabel amount of basophilic protein in them. The membrane of the contractile vacuole, or that of food vacuole sometimes, varies somewhat in staining intensity from others. Reactions of Ciaccio or Ciaccio-essig accompanied with the staining of Sudan III-Delafield's hematoxylin and Ciaccio followed by Nile blue sulphate stain it far more weakly, while other reac-

tions reveal equal intensity as other elements. This apparently unreasonable behaviour comes surely from the complexiety of the architecture of the contractile vacuole as suggested in the cytological section. The plasmalemma, which does not appear in this table, becomes clearly stained bluish red by the method of Ciaccio-essig followed by staining with Sudan III and Delafield's hematoxylin.

Leuco brillant cresyl blue was prepared using rongalit and hydrochloric acid quite similar to the leuco methylen blue, "Rongalitweiss", after Unna. Lipoid granules become intensely blue by this reaction. The identification of refringent granules is difficult due to distortion by the reagent. The vacuoles swell up and are torn respectively into several irregular ones, and consequently it is impossible to make a precise study. Mitochondria appear as blue flocculent images on the back ground of cytoplasm stained faintly blue. Glycogen granules do not react at all. Against the easily oxydizable reagents, such as pyrogallic acid, diamidophenol, and hydroquinon, the elements which contain lipoids react positively. It deserves mention that the membrane surrounding the contractile vacuole is seen reactive to these reagents appearing as a black wrinkled membrane. This blackening is due for the most part to the Golgi substance existing there, the lipoid fraction of which oxydizes the reagents, perhaps catalytically.

Iodine reaction was attempted applying the following method. A small piece of iodine crystal was set at the bottom of a hole in a hole glass, which was covered by a coverglass with a hanging drop containing amoebae. Beside the glycogen granules those having lipoid substance were all tinged a brown colour. The refringent granules stain much less faintly than the lipoid granules and glycogen granules. The lipoid granules are distinguishable from the glycogen granules in the following experiment. The amoeba is first stained vitally with neutral red, which stains the lipoid granules intensely but does not stain the glycogen granules. Then the stained amoeba is exposed to the iodine vapour. The red colour of the lipoid granules become garnet, while the glycogen granules appear possessing a brown colour. Setting aside from this experiment one can easily become accustomed to differentiation of these two elements by the difference in their size and in their distribution. The glycogen granules are usually larger occupying mostly a peripheral position in the endosarc, sometimes invading the ectosarc (see Textfig. 3).

By the iodine vapour mitochondria are caused to exhibit a flocculent feature found throughout the endosarc.

Summary

1. *Amoeba diploidea* was obtained from the faeces of rabbits and was cultured upon agar medium with pepton. The cytoplasmic constituents were studied employing cytological methods both on fixed and on living material. In addition to the ordinary methods for the demonstration of the mitochondria and the Golgi apparatus, staining with various dyes upon the formol fixed material was also carried out. The results are summarized in Table 1. The vital staining of amoebae using various dye solutions was tried for comparison with the data concerning the fixed material. The results are also shown briefly in Table II. The histochemical reactions, chiefly on lipoid, oxydase, and on glycogen, were tried. The results are given in Table III.

2. The Golgi apparatus assume annular or granular shape uniformly distributed in the endosarc. The Golgi substance is observed adhering on the surface membrane of contractile vacuole and food vacuole. Golgi bodies remain undigested after trypsin or pepsin-trypsin digestion. In the living material they appear as granules with high refractive index, hence they are temporary termed "refringent bodies" in the section on vital staining. They are stainable in living condition with Nile blue sulphate, tropaeolin, trypan blue and methylen blue. They are positive to the lipoid and oxydase reactions and are caused to become brown by iodine vapour.

3. The mitochondria are of granular type scattered throughout the cytoplasm. They attach on the Golgi substance adhering to the membrane which covers the contractile vacuole or the food vacuole. They react positively to the lipoid reactions and to some of the oxydase reactions.

4. The existence of the membranes of the contractile vacuoles and of the food vacuoles is established from their affinity to osmium tetraoxyde and from their resistance to trypsin and to pepsin-trypsin. The membrane after digestion with protease is stained blue by Nile blue sulphate. The affinity to osmic acid and to Nile blue sulphate after the action with the proteases leads one to suppose that this membrane contains lipoidal substance.

5. The plasmalemma bordering the cell surface is distinguishable by subjection to the present author's protease-Nile blue sulphate technique. Plasmalemma remains unaffected by digestion with trypsin, pepsin-trypsin, or pepsin-papain. After these proteolytic digestions the plasmalemma is stained by Nile blue sulphate. It is supposed from this fact that this membrane has lipoidal substance amongst its constituents. Why this membrane still remains after soaking in alcohol and digesting subsequently with trypsin is yet uncertain. The thickness of the plasmalemma is not uniform throughout the cell surface, but seems to vary at random without rule. Owing to its lipoidal nature it stains vitally in the solutions of Nile blue sulphate, brillant cresyl blue, and toluidin blue.

6. The mode of staining with various dyes after fixation with formol conforms roughly to the mode of vital staining.

7. Salts of lithium, especially lithium carbonate and lithium chloride, brought about the appearance of many food vacuoles with or without inclusions. Sodium chloride of hypertonic concentration has an effect of diminishing the vacuolar system as mentioned by Day (1927).

8. Glycogen granules were demonstrated by the iodine vapour to appear both in the endosarc and in the ectosarc.

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Explanation of Plate I

Photomicrographs of *Amoeba diploidea*, all being taken under the same magnification of ×1000.

LETTERING

Gb., Golgi bodies; *Gs.*, Golgi substance; *Mcv.*, membrane of contractile vacuole; *Mi.*, mitochondria; *Pl.*, plasmalemma.

Figs. 1–3. Kolatschev material.

Figs. 4 and 5. Champy-Benda's chromierung-Heidenhain hematoxylin material.

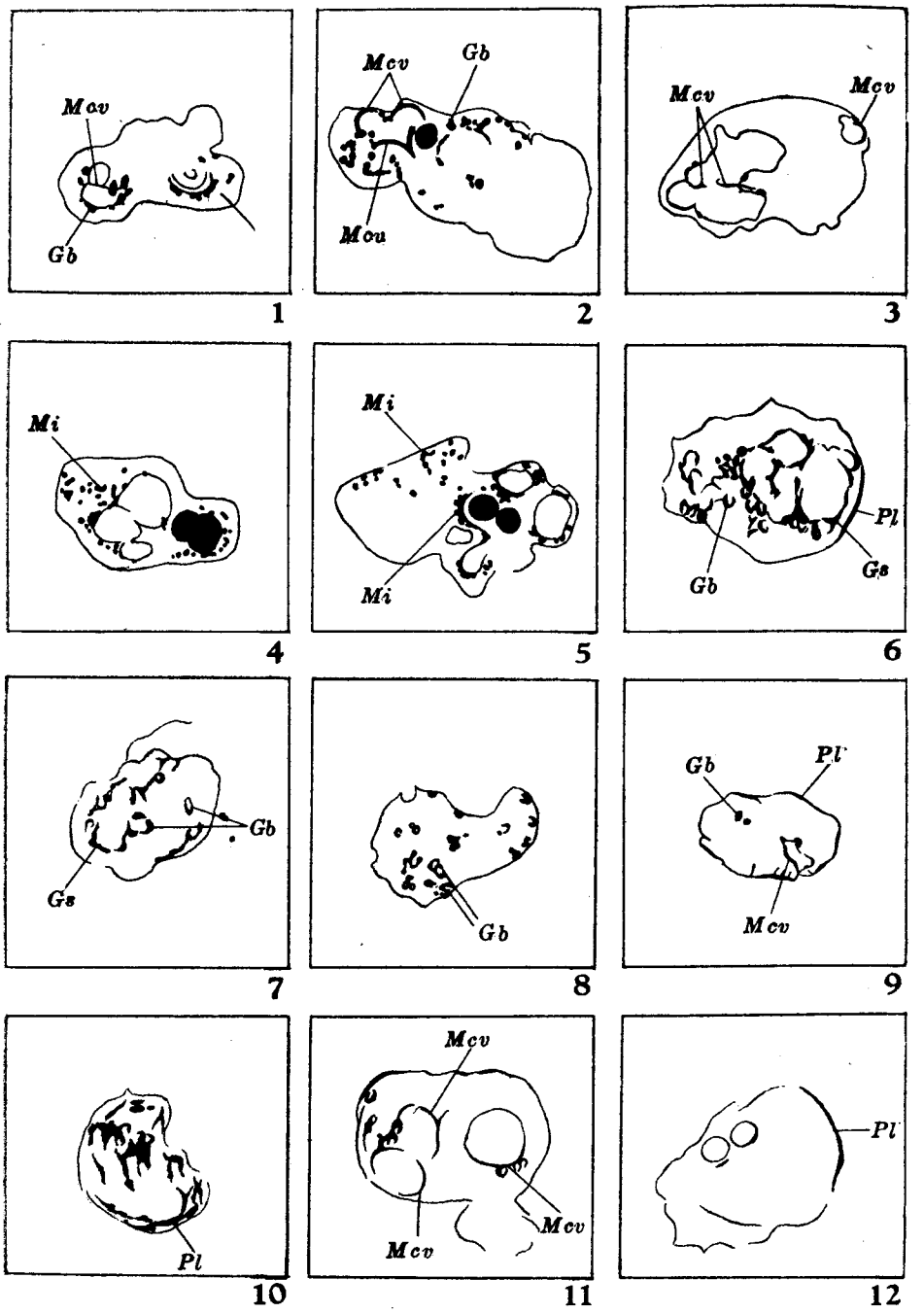
Fig. 6. Prepared with trypsin-Nile blue sulphate technique.

Figs. 7–10. Prepared with pepsin-trypsin-Nile blue sulphate technique.

Fig. 11. Prepared with pepsin-papain-Nile blue sulphate technique.

Fig. 12. Showing the effect of the treatment with alcohol and trypsin, the staining being Nile blue sulphate.

Superimposed drawings on figures of Pl. I.



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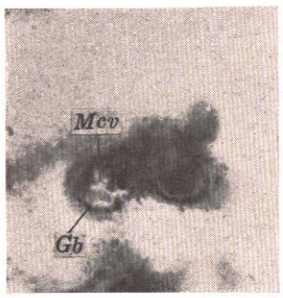

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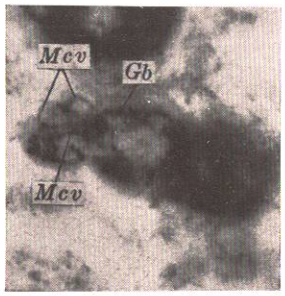

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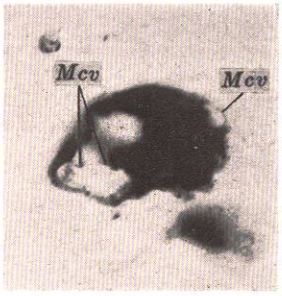

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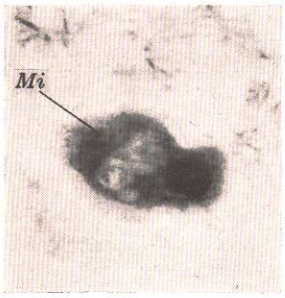

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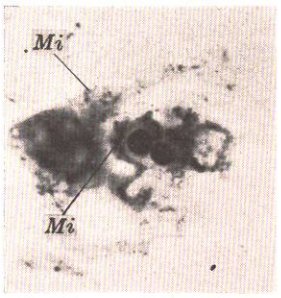

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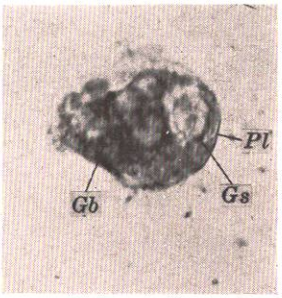

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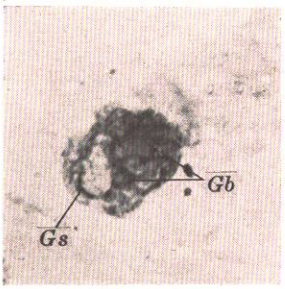

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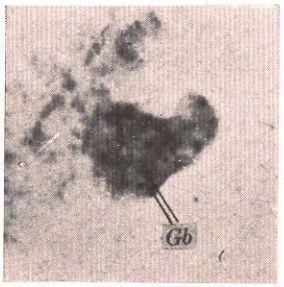

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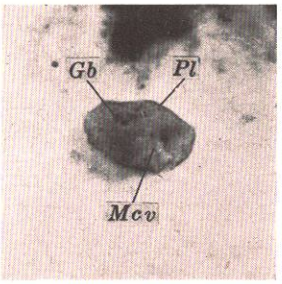

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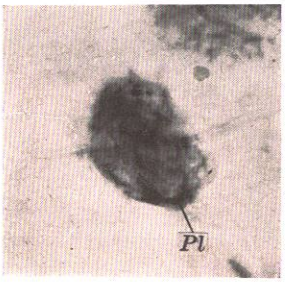

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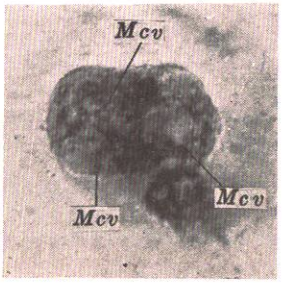

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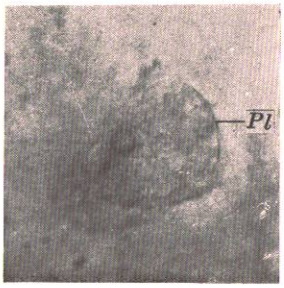

12

S. Tarao photo.

S. Tarao : Cytoplasmic constituents in Amoeba diploidea