(Biological Laboratory, University College, New York University.)

The vacuome and the neutral red reaction in *Paramaecium caudatum.*

By

F. W. Dunihue.

(With 7 figures in the text.)

The presence of neutral-red-stainable inclusions throughout the cytoplasm and on the walls of the food vacuoles of *P. caudatum* was reported by Prowazek in 1897. Nirenstein (1905) stated that these inclusions entered the food vacuole during the acid phase of digestion. His work was later corroborated by Rees (1922) in a brief account of micro-injection in *P. caudatum.* Volkonsky (1929) assigned these neutral red bodies to the vacuome, and, on the basis of their supposed penetration into the food vacuole, believed them to be carriers of hydrolytic enzymes. The literature contains no other report of a similar penetration of the food vacuole by the vacuome. In fact, Hall and Dunihue (1930) report that such is not the case in *Vorticella*, while Koehring (1930) states that the neutral red inclusions do not enter the food vacuole in *P. caudatum.* Hall (1930) did not observe a penetration of the food vacuole by the vacuome in *Trichamoeba.* The question naturally arises, are there differences in behavior of the vacuome, with respect to the food vacuoles, among the various groups of the Protozoa and, if so, how are such differences related to the physiological significance?

In view of the differences in the reported behavior of the vacuome it has seemed desirable to investigate its morphology and behavior with respect to the food vacuole in *P. caudatum*, and to correlate the results, in so far as possible, with those obtained in the other groups of the Protozoa.

The writer is greatly indebted to Prof. R. P. Hall for suggesting this problem and for his direction during the course of the investigation.

Material and Methods.

In vital staining, slides free from grease and moisture were filmed with a solution of the dye in absolute alcohol. The dyes were made up in one per cent stock solutions and diluted with absolute alcohol before using. The most favorable dilution for neutral red was found to be 1 : 80, and for Janus green B 1 : 30. For simultaneous demonstration of the chondriome and the vacuome a mixture of Janus green B (1 : 30) and neutral red (1 : 80) was used. After filming, the slides were allowed to dry, and two very thin streaks of a mixture of melted paraffin and vaseline applied one centimeter apart by means of a small camel-hair brush. The streaks of the paraffin-vaseline mixture supported the weight of the cover-slip, thus preventing distortion of the organisms. A drop of the culture medium (hay infusion) was placed on the slide and covered with a cover-slip: then sealed with melted vaseline to prevent evaporation.

In determining the most favorable dilution of the neutral red for use in vital observation organisms were placed on slides filmed with concentrations of the dye ranging from one part of a one per cent solution in ten parts of absolute alcohol to one part in one hundred. It was noted that in the stronger concentrations (1 : 10 to 1 : 60) diffuse staining occurred rather rapidly and that in the lower range red-stained globules appeared on the outer surface of the pellicle. In the more dilute concentrations diffuse staining was rare and the red-stained globules appeared on the pellicle only a short time before the death of the individuals. Aqueous solutions of the dye were prepared in exactly the same manner and added to cultures of the ciliates. It was found that dilutions of 1 : 60 or greater were not toxic, the normal activity of the cultures continuing for several weeks.

Permanent preparations were made by Nassonov's modification of the Kolatchev method of osmic impregnation, the Da Fano silver method, and the Golgi gold chloride method. The material during osmication was kept in a constant temperature oven at 35° C. Samples were removed at intervals of 48, 96, 144, and 288 hours. One half of each sample was mounted without bleaching, and the other half bleached with potassium permanganate and oxalic acid

before mounting. The sectioned material was osmicated in exactly the same manner and at the same time as the whole-mount.

In the starvation experiments the ciliates were removed from the culture medium with a micropipette and placed in tap-water which had previously been boiled for thirty minutes and allowed to cool. They were left in the tap-water until the food vacuoles had disappeared.

The Vacuome.

In *Paramaecium*, stained vitally with neutral red, numerous globular inclusions take the dye within three to six minutes (Fig. 1). These globules, in animated Brownian movement, are swept around in the general cyclosis of the endoplasm without any visible order or definite plan of distribution. There is, however, an apparent localization of the inclusions in the region immediately posterior to and surrounding the forming food vacuole, yet the osmicated material (see later description) would seem to indicate that this distribution is more apparent than real and

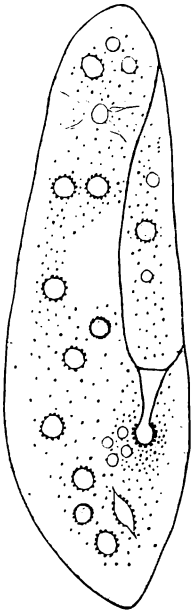

Fig. 1.

Fig. 1. *Paramaecium caudatum*, free-hand drawing, neutral red preparation, ca. 605 : 1. Neutral red globules scattered uniformly throughout endoplasm, clumped in region of forming food vacuole, absent in nuclear area. White vacuoles represent alkaline reaction, stippled vacuoles acid reaction, the more intense stippling indicating greater acidity. Note blurring of globules on walls of extremely acid vacuoles, and that a few globules are present on the alkaline vacuoles. Forming food vacuole colorless; spindle-shaped vacuole, just pinched off from gullet, is faintly stained. Alkaline vacuoles median to gullet circulate in this region for a short time before defecation, and might easily be interpreted as an "alkaline loop-circuit" of newly-formed vacuoles.

that it is probably due to the greater thickness of the organism in the posterior end. Although the globules are seldom found in the ectoplasmic layer they are occasionally caught up in this denser layer and work themselves, by means of Brownian movement, in between the trichocysts which appear as strongly refringent rods. Later, they are caught up again in cyclosis. The outer surface of the forming food vacuole is closely packed with these neutral

red globules, which remain firmly attached throughout most of the digestive period.

These inclusions (vacuome) are divisible into two types on the basis of their size and color reaction to neutral red; small granular inclusions which stain light or dark purple, and larger globular inclusions which stain red to light purple. This difference in size of the constituents of the unstained vacuome was observed by means of the dark field microscope. These two types of inclusions are not to be confused with the "A" and "B" granules of SLONIMSKI and ZWEIBAUM (1922) since their "B" granules are equivalent to the "Exkretperlen" of PROWAZEK (1897) which were found mostly on the exterior of the pellicle. Although these "Exkretperlen" were observed almost simultaneously with the granules present in the endoplasm by SLONIMSKI and ZWEIBAUM and by PROWAZEK, they were observed by the writer only just before the death of the organism or in forms subjected to toxic concentrations of the dye. Consequently, the presence of these "Exkretperlen" is considered to be a definite indication of a pathological condition. In this light they can not be considered as constituents of the normal vacuome or of the normal *Paramaecium*. NIRENSTEIN (1905, 1920) also notes that they occur only after diffuse staining of the *Paramaecium*, but does not suggest that they indicate a pathological condition. In the writer's experience, diffuse staining shortly precedes the death of the ciliates.

HORNING (1926) has demonstrated in *Paramaecium* other vitally staining inclusions, the mitochondria, which differ from the vacuome in size, shape, and staining reaction. These inclusions are rod-like in shape, considerably smaller than the neutral-red-stainable structures, and with careful technique are specifically stained by Janus green B. GUILLIERMOND (1929), from the results of his investigations on plant cells, concludes that the chondriome and the vacuome are separate morphological entities, and that they may be demonstrated with Janus green B and neutral red respectively. Since, in his experiments with plant cells, the vacuome did not ordinarily take mitochondrial stains, he believes, that neutral red may be considered to be "virtually a specific dye for the vacuome"; and he states that only one exception has been noted in which a body not belonging to the vacuome can be stained with neutral red. The chondriome, he reports, takes neutral red only very slowly, if at all.

The presence of mitochondria in *P. caudatum* was demonstrated with Janus green B, and in order to differentiate between the

chondriome and the vacuome both were demonstrated simultaneously by using a mixture of Janus green B and neutral red. The distribution of the chondriome agreed essentially with the description of Horning (1926). The behavior of the chondriome was not studied.

In the light of the above stated evidence that Janus green B is specific for the chondriome it seems necessary to point out that the sweeping generalization, recently made by Koehring (1930),

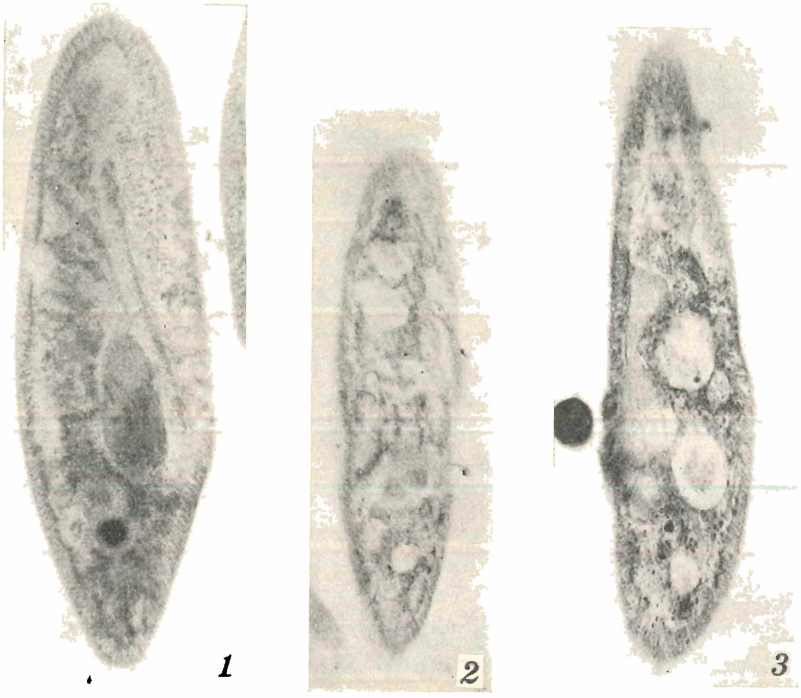

Fig. 2. Photomicrographs. Kolatchev osmic impregnation for forty-eight hours at 35° C without bleaching. 2_1 Whole-mount, 344 : 1. Posterior end is slightly darker than anterior. 2_2 Longitudinal section, 340 : 1. Blackened globules irregularly scattered. 2_3 Longitudinal section, 567 : 1. Inclusions not present in ectoplasmic layer. Distribution is general.

that there is only one "fundamental cytoplasmic inclusion, the mitochondrial complex", is rather premature, since she has not followed the generally accepted technique for the vital demonstration of the chondriome in the Protozoa. In fact, she used only neutral red which ordinarily stains only the vacuome.

In osmic impregnation of *Paramaecium* those organisms osmicated for forty-eight hours appeared, in toto, and without bleaching, to

be slightly dark at the posterior end and yellow, or olive, in color at the anterior end (Fig. 2_1). Observation with oil immersion revealed blackened globules distributed throughout the cytoplasm in essentially the same order as the globules in the neutral red preparations. In a study of longitudinal (Fig. $2_{2,3}$ and Fig. 3) and cross sections of the forty-eight hour osmicated individuals the distribution of the blackened globules appeared to be approximately the same per unit volume, with occasional points of localization. Distribution of the globules did not indicate a definite morphological pattern, or a "well-defined osmic gradient" as described by PARK (1929). Since the pattern of the vacuome in these sections agrees rather closely with that demonstrated by neutral red it is assumed that only the vacuome was impregnated. The contractile vacuole was not blackened in these forty-eight hour stages.

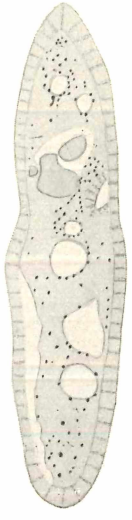

Fig. 3. KOLATCHEV osmic impregnation for forty-eight hours at 35° C without bleaching. Camera-lucida drawing of longitudinal section, 405 : 1. Distribution of inclusions approximately the same per unit area.

Preparations following the techniques of DA FANO, GOLGI, and CAJAL gave superimposable pictures.

In order that the direct action of osmic acid on the vacuome might be observed, a cover-slip was filmed with neutral red and the *Paramaecium* placed upon it. After the globules were stained the coverslip was inverted and sealed over a welled-slide containing a drop of two per cent osmic acid. The organisms were killed immediately and within five minutes the cytoplasm had become pink; twenty-five minutes later the globules were slightly gray and the cytoplasm light brown; within two days the globules were black and the cytoplasm dark brown; and at the end of a week the entire organism was black.

A great number of the organisms osmicated for ninety-six hours were blacker than the forty-eight hour individuals and were slightly darker in the posterior than the anterior region (Fig. 4_1). Others (Fig. 5_1) were still darker with uniform blackening at the posterior and anterior ends, and a few were entirely blackened. The blackened material was more abundant, and in sections (Fig. 4_2) different types of inclusions could be identified; one the globules

or the vacuome, and the other rod-like forms or the chondriome. In many cases the inclusions were clumped in solid black masses and seemed somewhat larger than those demonstrated vitally. This clumping and increasing in size may possibly have been due to a deposition of osmium on the walls of, or between, the inclusions as suggested by Parat and Painleve (1925).

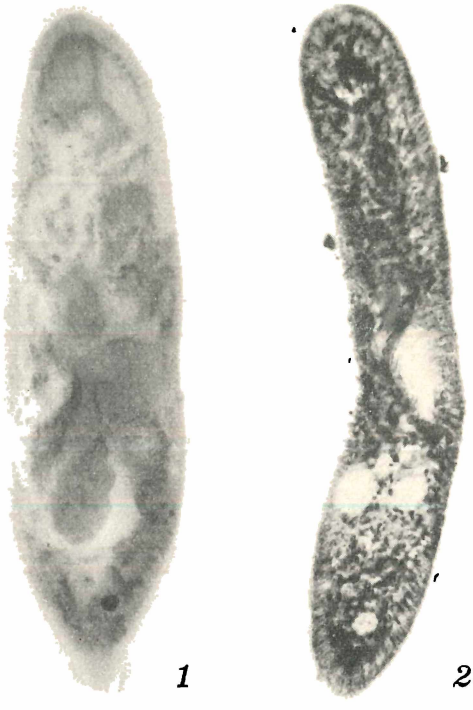

Fig. 4. Photomicrographs. Kolatchev osmic impregnation for ninety-six hours at 35° C without bleaching. 4_1 Whole-mount, 430 : 1. Slightly darker than Fig. 2_1. No increase in size of inclusions. 4_2 Longitudinal section, 637 : 1. Note increased amount of blackened material and the variation in size of the inclusions. Compare Fig. 2_3.

The contractile vacuoles were blackened in these and succeeding stages of osmication. Thus, in reaction to osmic acid, the contractile vacuole is more like the mitochondria than the vacuome (Golgi apparatus); this does not support Nassonov's (1924) idea that the contractile vacuole is the homologue of the Golgi apparatus. Lynch (1930) also reports that treatment of *Lechriopyla mystax* with osmic acid did not result in a blackening of the walls of the contractile vacuole, and points out that "the belief of Nassonov (1924, 1925) and Kraschenninkow (1929) that the contractile vacuole of ciliates is the homologue of the metazoan Golgi apparatus is not universally valid, if indeed it is valid at all".

Osmication for a longer period of time usually resulted in completely blackened, opaque individuals (Fig. 5_2). Bleaching these completely osmicated forms with potassium permanganate and oxalic

acid gave practically the same cytoplasmic picture as in the case of the forty-eight hour osmication without bleaching.

The Neutral Red Reaction and the Behavior of the Vacuome.

Following vital staining the ciliates were kept under continuous observation, in sealed preparations, for periods ranging from two to three hours, and later were examined at intervals of six hours until all of the organisms were dead. The food vacuoles and the vacuome take the stain completely within six minutes, the posterior region staining shortly before the anterior end. The forming food vacuole, although colorless, is sharply outlined by the red globule sadhering to its walls, as noted by NIRENSTEIN (1905); in fact, this and the following description will be found to agree in general with that of NIRENSTEIN, although some additional information is presented and one or two points of difference are to be found. Quite often a few small bodies within the forming food vacuole are stained with the neutral red. These bodies are probably stained bacteria, rather than constituents of the vacuome as suggested by VOLKONSKY (1929), since similar stained bodies have been observed to enter the vacuole through the gullet, and since there is no visible decrease of the globules on the walls of the vacuole. A light pink

Fig. 5. Photomicrographs. KOLATCHEV osmic impregnation without bleaching. 5_1 Whole-mount, 96 hours osmication, 390 : 1. Increased amount of blackened material and variation in size of granules. No "osmic gradient" indicated. 5_2 Whole-mount, osmicated for 288 hours, 390 : 1. Completely blackened, partially due to deposition of osmium on the surface of the pellicle.

color begins to invade the vacuole soon after it has pinched off from the gullet, indicating the production of acid. The vacuome at this stage exhibits two phases, as previously noted, the larger globules staining pink with a tinge of purple and the smaller ones staining reddish purple. With the continued production of acid the vacuole becomes successively dark pink, rich red, and Tyrian purple, reaching this latter stage within thirty to forty-five minutes. The globules on the walls of the vacuole have darkened considerably during this time, the purple color appearing in them shortly before the vacuole reaches the Tyrian purple phase. This latter stage of the food vacuole is the one of greatest acidity, and as a result of the blurring of the globules it is almost impossible to determine their relative numbers and size. It should be noted that the globules have not entered the vacuole during this acid phase, as was described by other investigators (Nirenstein, 1905; Volkonsky, 1929). Within the next fifteen to thirty minutes after the Tyrian purple stage the color of the vacuole gradually shades off into orange-green, and finally into yellow-green, just before defecation. In this, the alkaline period, the globules are not universally present upon the wall of the vacuole; when present, they are seen only in greatly diminished numbers (twelve at the most). Their absence from the wall of the vacuole is first noted in the early orange-green stage, although they occasionally disappear earlier. It appears that these globules simply break loose from the wall of the vacuole, since this phenomenon has been observed to occur in the case of a few individual globules remaining on the walls of the vacuoles in the later orange-green and early yellow-green stages. The complete process has, however, not been observed due to the difficulty of resolving the vacuome into separate globules during the Tyrian purple phase.

The organisms thus observed fed actively for the first hour, but feeding activities were soon brought to an end. Although not feeding, the ciliates were normal in shape, locomotion, and general appearance. It is assumed that this cessation in feeding activities is due to the depletion of the food supply and perhaps also to the accumulation of waste products, since the organisms continued to live for a period of twenty-four to seventy-two hours in these sealed preparations, and since they resumed active feeding when transferred from the sealed slide to fresh culture medium. Further evidence that this cessation in feeding activities is due to a depletion of the food supply and not to an injury resulting from

the dye is furnished by the fact that the ciliates were able to live several weeks in a rich culture medium containing approximately the same concentration of the dye. As a control, unstained organisms were handled in exactly the same manner as the stained ones, and, with the exception of slight variations in the time periods, their reaction was the same.

Observations made six hours later, or nine hours after the application of the dye, found the ciliates apparently normal with respect to locomotion and behavior, no food vacuoles present, a few excretory crystals in the anterior end, and the vacuome appearing more uniformly purple than at first, possibly as a result of a decrease in number of the larger type of globules. However, the total volume of the vacuome did not seem to be reduced.

Successive observations at fifteen, twenty, and twenty-five hours revealed that the constituents of the vacuome were becoming more uniform in size, tending towards the small purple granules, and that the total volume was decreasing. This decrease was very marked at twenty-five hours and did not seem to vary, remaining about the same until the death of the *Paramaecium*. In order to determine whether this decrease was real or merely the result of a fading of the dye, organisms were transferred after twenty-five hours to a slide with fresh dye and again sealed. There was no increase in the number of globules or granules, and at the end of an hour or more these individuals began to stain diffusely making it difficult to observe the vacuome. The basal granules became intensely stained and the nucleus became visible as a clear area. The "Exkretperlen" of PROWAZEK (1897) were observed in a few forms at this stage (Fig. 6_1). Death usually followed within an hour. This diffuse staining upon a second application of the dye would seem to indicate that starvation increases the susceptibility of *Paramaecium* to neutral red. This is borne out in a later series of experiments.

This decrease of the constituents of the vacuome in sealed preparations made it extremely desirable to determine the specific effect of starvation. Accordingly, the *Paramaecium* were washed several times in distilled water and transferred to tap-water which had previously been boiled for thirty minutes and allowed to cool. The p_H of the tap-water was approximately the same as that of the culture medium. At the end of twenty-four hours most of the organisms were free of food vacuoles, and upon staining with neutral red a very slight, if any, decrease in the number of globules was

noted. The color of the vacuome was more uniformly purple in these animals than in those from a rich culture medium. As starvation progressed the vacuome decreased in volume and was composed mostly of the small purplish granules. After seventy-two hours of starvation there was no further decrease, the volume remaining rather constant; diffuse staining was more pronounced, and the organisms were becoming very sluggish. At the end of ninety-six hours of starvation the animals were beginning to die; those which were still living stained so diffusely that the vacuome could not be observed with any degree of accuracy. The time of starvation as given is only an approximation, since the time required

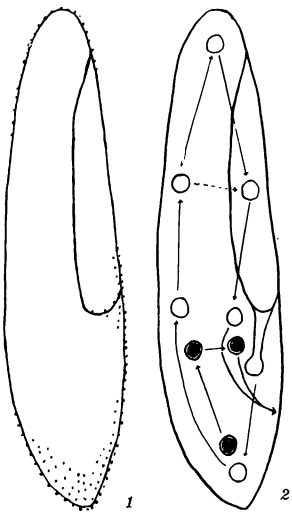

Fig. 6.

Fig. 6. 6_1 Sketch of *Paramaecium* showing "Exkretperlen" on the surface of the pellicle; these indicate a pathological condition which results in the death of the ciliate. 6_2 Composite diagram of the course of the food vacuoles in *Paramaecium*. During digestion the food vacuoles may traverse the path indicated by the colorless vacuoles, passing to the extreme anterior end or crossing immediately anterior to the nucleus, or they may follow the course as shown by the black vacuoles. In either case they may make only one or several circuits, and often a food vacuole follows one path for one circuit and another path for the next.

depended upon many factors, such as the physiological condition of the ciliates, and the number of bacteria transferred with them. It was concluded from this series of experiments that starvation causes a decrease in the total volume of the vacuome, within limits; that the neutral red globules become smaller and more uniform in size; and that the susceptibility of the *Paramaecium* to neutral red is greatly increased. A somewhat similar condition was observed by Calkins and Bowling (1929) in *Dallasia frontata*. They found that the neutral red staining granules were much fewer and smaller in size in the starved individuals.

It was noted that the *Paramaecium* from well-fed cultures, two to four weeks old, usually have more globules present and are not as sensitive to the dye as those from cultures two to three months old.

In dividing forms, in which the gullet is very immature and in which no food vacuoles are being formed, vital staining presents essentially the same cytoplasmic picture as in the vegetative forms, the distribution and the number of globules per unit volume being practically the same. Vital staining of conjugating forms likewise shows the vacuome and the neutral red reaction to be similar to that of the vegetative forms. Since in both of these stages the neutral red reaction occurs when there are no food vacuoles being formed and when the gullet is very immature, it seems more logical to suppose that the dye enters the organism by diffusion through the pellicle rather than by means of a "complete digestive system" as postulated by KOEHRING (1930).

The Formation and Behavior of the Food Vacuole.

Although the writer's observations on the formation and behavior of the food vacuole agree in many particulars with NIRENSTEIN's (1905) description, they will be included in their entirety for completeness and to facilitate a more accurate comparison with the recent work of SHAPIRO (1927) and KOEHRING (1930).

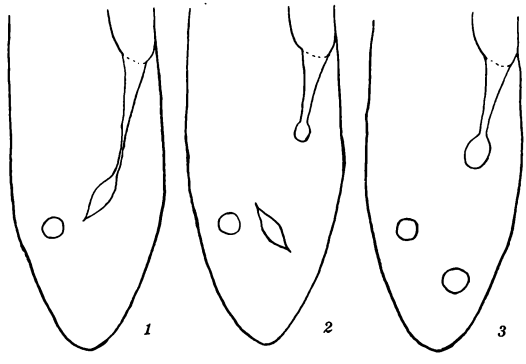

Fig. $7_{1, 2, 3}$. Successive stages in food vacuole formation.

The food vacuole forms as an enlarging bulb on the lower end of the gullet and, after attaining a fairly definite size, pinches off, becomes pointed at both ends, and "plows" through the surrounding globules directly to the posterior end of the organism (Fig. $7_{1, 2, 3}$). From the time of its formation until it reaches the posterior end of the animal the food vacuole is rarely stained by the neutral red, and then only a very light pink. In no case was SHAPIRO's (1927) intervening alkaline loop-circuit observed. At the posterior end of the organism the vacuole becomes rounded and, after a short interval during which there is little or no movement, it is caught up in the current of cyclosis. Henceforth the course of the vacuole during the digestive period is very irregular and uncertain (Fig. 6_2).

It may slowly circle the cell, stopping intermittently; or it may remain stationary in the region above the gullet, rotating occasionally upon its vertical axis. It is obvious that in such an irregular course the vacuoles formed at one time may, and often do, overtake and pass ones previously formed. As new vacuoles are formed and break away from the gullet they often collide with previously formed vacuoles causing a certain amount of movement in them; however, movement of the vacuoles which were not directly concerned in this collision was not observed. Indeed, the course of the vacuole seems to depend entirely upon chance, with no evidence for a "complete digestive system with orderly progression of vacuoles from ingestion to defecation" as has been described by Koehring (1930). There is, however, considerable similarity between the initial and final stages of the food vacuole in different specimens. The alkaline vacuoles, just before defecation, collect with great regularity in the region median to the gullet (Fig. 1), and, in the opinion of the writer, this phenomenon accounts for the so-called "alkaline loop-circuit" of the newly formed food vacuoles as described by Shapiro (1927).

As noted by Nirenstein (1905), the food vacuoles undergo an increase in volume during the acid phase and a decrease in volume during the alkaline phase. The writer observed that the vacuole increased in volume in the early acid stage and decreased in the late acid stage, remaining approximately the same size throughout the alkaline phase.

The So-called "Capillary Irrigation System" of Ciliates.

Cosmovici (1931) has recently described a "preexistent capillary irrigation system" in *Colpidium colpoda* which, according to him, is easily and rapidly demonstrated with iodine after feeding with soluble starch. This system, he believes, represents the vacuome of ciliates. The following experiments were performed in order to determine if such a capillary system exists in *Paramaecium caudatum*.

Actively feeding ciliates were passed through several washes in tap-water by means of a micropipette and then placed in a saturated solution of soluble starch. Some of the organisms were removed at the end of an hour and stained with an iodine solution which was strong enough to kill them but caused little distortion. The cytoplasm was colored light brown or yellow and the vacuoles blue-black or black. Staining these organisms at hourly intervals

over a period of eight hours gave exactly the same results as obtained at the end of the first hour. The capillary canals of COSMOVICI were not present at any time nor was there any indication of a hydrolytic action on the starch. The medium failed to give the characteristic blue iodine-starch reaction after twenty-four hours. The cytoplasm of the control animals from the hay infusions was colored light brown when stained with iodine and the vacuoles a darker brown. The only perceptible difference in the staining reaction of the control and the starch-fed organisms was that the vacuoles of the starch-fed ciliates were colored blue-black due to the presence of the starch.

Organisms from the hay infusion were transferred after several washes in tap-water to a medium consisting of two-thirds hay broth and one-third saturated starch solution. Upon staining with iodine, after forty-five minutes, the ciliates were killed, the cytoplasm stained light brown and the vacuoles blue-black. In some cases the cytoplasm became lightly vacuolated. The culture medium gave the characteristic iodine-starch reaction. Successive tests with iodine at hourly intervals revealed no change except that the number of vacuoles decreased considerably. The medium failed to give a color reaction with iodine after eight hours. Most of the organisms were free of vacuoles at the end of forty-eight hours, and staining with iodine was the same as in the earlier tests.

Starved ciliates (no food vacuoles) when placed in a medium of two-thirds hay broth and one-third saturated starch solution for forty-five minutes did not differ in any respect, when stained with iodine, from those organisms starved but not placed in starch medium. At the end of three hours in this medium they became very sluggish and were usually dead at the end of the fourth hour. When these starved ciliates were placed in a saturated starch solution they usually died within two and one-half hours.

This series of experiments seems to support METALNIKOFF's (1912) contention that *Paramaecium caudatum* does not use starch as food. Further, it makes it seem very unlikely that there is a "preexistent capillary irrigation system" present in all ciliates as suggested by COSMOVICI (1931).

Discussion.

A survey of the literature reveals that the chondriome and the vacuome are universal constituents of the cell. GUILLIERMOND (1929), in his review of the cytoplasmic constituents of plant cells,

states that the chondriome, staining vitally with Janus green B, and the vacuome, staining vitally with neutral red, are distinct morphological entities, occurring universally in plant cells. And Parat (1929), working with metazoan cells, has concluded that Guilliermond's theory is applicable to metazoan cells. The vacuome, he believes, is comparable with the chondriome and the nucleus in importance. Many other investigators (Joyet-Lavergne, 1926; Volkonsky, 1929; Hall, 1930; Dawson, 1931) [1]) have demonstrated the existence of two distinctly different cytoplasmic inclusions by the use of the vital dyes, Janus green B and neutral red. Volkonsky (1929) states that there are two cytoplasmic constituents in *Paramaecium caudatum*, the chondriome and the vacuome, which may be demonstrated with Janus green B and neutral red respectively. The results of this investigation are in complete agreement with this statement of Volkonsky, the chondriome and the vacuome having been demonstrated simultaneously with a mixture of Janus green B and neutral red. Koehring (1930), using neutral red which is virtually a specific due for the vacuome, has demonstrated a system of inclusions in *Paramaecium caudatum* which she terms the "mitochondrial complex", and has compared this complex with the mitochondrial pattern of *Paramaecium caudatum* as described by Horning (1926). Since Horning used Janus green B, which rarely stains the vacuome, and Koehring used neutral red, which ordinarily stains only the vacuome, it is obvious that there is no common basis for this comparison. Furthermore, Koehring did not use the cytological method in general use for the vital demonstration of the chondriome, but rather the method for the demonstration of the vacuome. Consequently, her postulation of one fundamental cytoplasmic constituent in *Paramaecium caudatum* is not warranted by the results of her investigation, and the "mitochondrial complex", which she describes, is unquestionably the vacuome.

Cosmovici (1931) described a system of preexistent canals in *Colpidium colpoda* which is easily and rapidly demonstrated with iodine after feeding with starch. This system of canals, he believes, represents the vacuome (Golgi apparatus, or the canalicular apparatus of Holmgren) of the Infusoria, and justifies Holmgren's hypothesis of a canalicular state of the Golgi apparatus. The writer, using Cosmovici's procedure, has been unable to demonstrate such a system of canals in *Paramaecium caudatum*. In fact, the vacuome

[1]) The papers cited contain reviews of the literature on the vacuome.

of *Paramaecium* in vital staining and fixed material has been found to be in a dispersed state, which is in agreement with the description of the vacuome in other Protozoa (see JOYET-LAVERGNE, 1926; HALL, 1931). Thus the present investigation, since it is concerned with a fairly representative ciliate, does not permit the application of COSMOVICI'S hypothesis to the Infusoria as a group.

As early as 1897 PROWAZEK, using neutral red among other dyes, demonstrated a system of granules in *Paramaecium caudatum*. These granules were of two types; the "Plasma Körnchen" present in the plasma, and the "Exkretperlen" present on the surface of the pellicle. The "Plasma Körnchen" PROWAZEK believed to be concerned with digestion and assimilation, and the "Exkretperlen" with excretion. NIRENSTEIN (1905) observed that the "Exkretperlen" were present in concentrations of the dye which produced diffuse staining, and in weaker concentrations only after a considerable period of time. Neither PROWAZEK nor NIRENSTEIN gave these "Exkretperlen" much consideration, other than of a morphological nature; however, two later workers, SLONIMSKI and ZWEIBAUM (1922), assigned to them the particular function of the excretion of the dye. Other investigators (REES, 1922; VOLKONSKY, 1929; KOEHRING, 1930) did not observe these "Exkretperlen", probably because they used non-toxic concentrations of the dye or did not observe the animal until its death. In the writer's preliminary experiments, to determine the proper concentration of neutral red to be used, the "Exkretperlen" were always present in toxic concentrations of the dye, but in the more dilute solutions they appeared a short time before the death of the animal. Since the "Exkretperlen" are found only when the organism is undergoing pathological changes they probably should not be considered constituents of the normal *Paramaecium* or of the normal vacuome.

NIRENSTEIN (1905) described the "Fermentträgen" ("Plasma Körnchen" of PROWAZEK) in *Paramaecium caudatum* as globules, in animated Brownian movement, swept around by the current of cyclosis, packed closely on the walls of the forming food vacuole, and penetrating the food vacuole during the early acid phase. These "Fermentträgen", he believed, were necessary in initiating the digestive processes. Their penetration into the food vacuole was, according to NIRENSTEIN, supported by the appearance of neutral-red-staining bodies within the vacuoles during the early acid phase of digestion. The actual penetration of the neutral red globules was apparently not observed by him. REES (1922) reported that his results are in

complete agreement with those of Nirenstein on the morphology and behavior of the "Fermentträgen". It is to the description of Nirenstein, says Volkonsky (1929), that we owe our exact knowledge of the "Fermentträgen", which he (Volkonsky) considers to be the vacuome. However, Volkonsky adds, in support of Nirenstein's contention that the "Fermentträgen" penetrate the food vacuoles, that there is a decrease in the number of the globules on the walls of the vacuole during the early acid stage and correlates this decrease with the appearance of neutral-red-staining globules within the vacuole. That the neutral-red-staining bodies within the vacuole are bacteria, which have taken the dye, rather than the globules of the vacuome is substantiated by the writer's results. In many cases a very few bodies within the forming food vacuole were seen to take the stain, and often the vacuole, immediately after its separation from the gullet, contained several stained bodies. This appearance of stained bodies within the vacuole before any decrease in the volume of the vacuome on its walls lends further support to the idea that the stained bodies seen by Volkonsky were bacteria. In fact, this same phenomenon was noted by Volkonsky in his experiments with Janus green B and was advanced by him as an argument against the penetration of the food vacuole by the mitochondria as described by Horning (1926). A decrease in the number of globules on the vacuole in the early acid phase was never noted by the writer, and in the late acid phase accurate determination of the volume of the vacuome on the walls of the vacuole is extremely difficult, since the globules are packed closely together as the size of the vacuole decreases. Furthermore, the presence of these globules, although in decreased numbers, on the walls of the vacuole in the late acid and early alkaline stages argues against penetration in the early acid phase. Koehring (1930) also failed to find any evidence for the penetration of the food vacuole by these neutral-red-stainable inclusions.

The distribution of the vacuome as noted by Nirenstein (1905), Volkonsky (1929), and the writer, is more or less equal throughout the cytoplasm, with occasional points of localization, such as around the forming food vacuole. Park (1929), using osmic acid which demonstrates the vacuome, has postulated an axial gradient in *Paramaecium caudatum* based upon a graded difference in the amount of blackened material in the ectoplasmic layer between the posterior and anterior ends. In vital preparations, however, the vacuome is

not present with any degree of constancy in the ectoplasmic layer, and in sections of osmicated material such distribution is rarely observed. However, observation of whole-mounts osmicated for forty-eight hours does give the impression that more material has been blackened in the posterior than in the anterior end, but this difference may be explained on the basis of the greater thickness of the posterior half of the organism. This explanation is borne out by the examination of sectioned material, which shows an equal distribution of the vacuome per unit volume. Continued osmication generally resulted in completely blackened individuals, perhaps partially due to the deposition of osmium on the surface of the organism. Sections of this completely blackened material reveals that both the chondriome and the vacuome have been blackened, yet there is little difference between the amount of blackened material per unit area in the posterior and anterior ends. While this evidence in no manner refutes the presence of an axial gradient in *Paramaecium*, it does seem to leave no basis for a "well-defined osmic acid differential" as described by PARK (1929).

VOLKONSKY (1929), on the basis of the supposed penetration of the food vacuoles, considers the vacuome to be the carrier of hydrolytic enzymes, while LYNCH (1930) reports that he finds no evidence that the neutral red granules play any part in intracellular digestion in *Lechriopyla mystax*. KOEHRING (1930), although she has erroneously called it the "mitochondrial complex", believes that the vacuome is the center of both hydrolytic and synthetic enzymes, differing only in the free water content. The present investigation offers no solution as to the function of the vacuome, nor does it present convincing evidence for or against any of the prevailing theories. While the writer's findings contradict VOLKONSKY's belief that the vacuome enters the food vacuole, and hence weaken his theory that the vacuome is the carrier of hydrolytic enzymes, they do not rule out such a function since diffusion of the enzymes might occur. On the other hand, the decrease in the volume of the vacuome during starvation lends support to the theory of GUILLIERMOND (1929), that "the vacuome is a reservoir of important metabolic products".

The course of the food vacuole during digestion in *Paramaecium caudatum*, according to NIRENSTEIN (1905), REES (1922), and KOEHRING (1930), is regular and uniform. However, the path as described by each author differs from that described by the others. NIRENSTEIN

(1905) pictures the vacuole as sinking to the posterior end of the organism immediately after pinching off from the gullet. It is then swept up the left side in the forward streaming of the cytoplasm to the level of the gullet where it performs a loop-circuit and, then continues to make regular and complete circuits of the cell until digestion is completed. Metalnikoff (1912) points out that although the path traversed by the vacuole is regular within limits it is by no means as regular as Nirenstein supposed. The course as described by Rees (1922) does not contain a loop-circuit and the entire path is in the posterior half of the organism, the vacuole never advancing anteriad past the macronucleus. Koehring (1930) describes the path as being very regular, and making a complete circuit of the organism. She believes that this regularity could not result from the streaming of the cytoplasm but only from the presence of a "complete digestive system". As further evidence for this "complete digestive system", she states that a slender, invisible canal connects the newly formed vacuole with the gullet and all other vacuoles; that each vacuole is emptied in the same order as its formation; and that the staining of vacuoles formed previous to the application of the dye probably depends upon the passage of the dye through these slender connecting canals. If such an invisible canal does exist between the food vacuoles it seems perfectly obvious that a regular path would be an absolute necessity for the perfect functioning of the vacuolar system, and further evidence superfluous. Conversely, if an irregular behavior of the vacuoles could be established such a complete digestive system would be extremely improbable. That such an irregular behavior of the food vacuoles does exist in *Paramaecium caudatum* has been demonstrated by the writer. The staining of *Paramaecium* with neutral red, as demonstrated in dividing and conjugating forms, takes place during a cessation of feeding activities and hence does not depend upon the presence of a complete digestive system. Thus in *Paramaecium* neutral red apparently diffuses through the cell wall, as is the case in astomatous ciliates (unpublished observations of R. F. Nigrelli).

The staining reaction of the newly-formed food vacuole indicated, in every case, an acid reaction, contrary to the description of an initial alkaline stage by Shapiro (1927). This initial acid reaction of the newly-formed vacuole has been noted by several other investigators (Metalnikoff, 1912; Rees, 1922; Nirenstein, 1925; Volkonsky, 1929; Koehring, 1930) and unquestionably occurs under ordinary culture conditions.

Summary.

Two distinct cytoplasmic elements, the chondriome and the vacuome, have been demonstrated vitally in *Paramaecium caudatum*. The morphology and behavior of the vacuome has been studied vitally with neutral red and in fixed material prepared by the KOLATCHEV method of osmic impregnation, GOLGI's gold chloride, and DA FANO's silver technique.

The vacuome of *Paramaecium caudatum* was not found to consist of a "capillary irrigation system" as suggested by COSMOVICI (1931) but of a system of dispersed globules.

The distribution of the vacuome was found to be approximately the same per unit area along the anterio-posterior axis, with no evidence for a "well-defined osmic acid differential". The globules of the vacuome were never observed to penetrate the food vacuoles, and evidence has been presented which indicates that such a phenomenon is extremely unlikely. The reduction in the volume of the vacuome during starvation tends to support the theory that it is the center of "important metabolic products" rather than the center of hydrolytic and synthetic enzymes.

It has been shown that the paths of the food vacuoles during digestion are very irregular and subject to variation, and that in view of such irregular behavior the existence of a "complete digestive system" seems very improbable.

The initial reaction of the food vacuoles was found to be acid as has been reported by the majority of investigators. The volume of the vacuoles increased in the early stages of acidity and decreased before reaching the most acid phase.

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