A STUDY OF PHAGOCYTOSIS IN THE AMEBA *CHAOS CHAOS*

RICHARD G. CHRISTIANSEN, M.D., and JOHN M. MARSHALL, M.D.

From the Department of Anatomy, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania

ABSTRACT

The process of phagocytosis was investigated by observing the interactions between the ameba *Chaos chaos* and its prey (*Paramecium aurelia*), by studying food cup formation in the living cell, and by studying the fine structure of the newly formed cup using electron microscopy of serial sections. The cytoplasm surrounding the food cup was found to contain structures not seen elsewhere in the ameba. The results are discussed in relation to the mechanisms which operate during food cup formation.

INTRODUCTION

One of the most familiar, yet dramatic events in introductory biology is the entrapment of a living celiate by a fresh water ameba. Students of cell physiology have long been interested in the process by which an ameba forms a food cup in response to an appropriate stimulus (Rhumbler, 1898, 1910; Jennings, 1904; Schaeffer, 1912, 1916, 1917; Mast and Root, 1916; Mast and Hahnert 1935), and also in the processes of digestion and assimilation which follow once the cup closes to form a food vacuole within the cytoplasm. The subject was reviewed by Kitching (1956) and Bovee (1960). The early work by light miroscopy was necessarily limited. However, a beginning was made in the experimental analysis of this highly "purposeful" act of feeding, and conflicting ideas regarding the underlying physical mechanisms were advanced by Rhumbler, Jennings, Shaeffer, and Mast.

The subject of food cup formation is of renewed interest at present, partly because electron microscopy makes possible a more detailed analysis but also because feeding in the ameba is a special example of a more general biological problem: that of the movement, transformation, uptake, and turnover of cell membranes. In this sense, the formation of the food cup in response to an external stimulus may be related to the formation of pinocytosis channels and to cytoplasmic streaming in general, subjects already studied extensively in the ameba (Mast and Doyle, 1934; Holter and Marshall, 1954; Chapman-Andresen, 1962; Goldacre, 1964; Wolpert *et al*, 1964; Abé, 1964; Griffin, 1964; Wohlfarth-Bottermann, 1964).

During phagocytosis (as during pinocytosis) large amounts of the plasmalemma are consumed. Recent studies in this laboratory demonstrate that the giant ameba *Chaos chaos*, when grown under optimal conditions on a diet of *Paramecium aurelia*, forms more than 100 food cups during a single 24-hour cycle of growth. The formation of each food cup (which when completed becomes a food vacuole) requires the ingestion of about 10 per cent of the plasmalemma. During a single cycle of growth, therefore, the cell must consume more than 10 times the amount of plasmalemma present at any one time.

This study was designed to investigate the process of food cup formation by observing the initial interactions between the ameba *Chaos chaos* and its prey, by observing the feeding process in the living cell, and by studying the fine structure of the

newly formed food cup using electron microscopy of serial sections.

MATERIALS AND METHODS

(*a*) Experiments were done on specimens of the giant ameba *Chaos chaos* (*Pelomyxa carolinensis*) taken from mass cultures which were maintained in active growth by daily feeding on *Paramecium aurelia* (Var. II). The paramecia were obtained from a tank culture grown continuously in a grass infusion with *Aerobacter aerogenes* as the principal food organism.

The experiments to be described were done with single amebae or small groups of selected organisms, and the paramecia used to induce food cup formation were from the same stock culture used to maintain the mass culture of amebae. The medium consisted of: 5×10^{-4} M $CaCl_2$, 5×10^{-5} M $MgSO_4$, 1.1×10^{-4} M KH_2PO_4, 1.6×10^{-4} M K_2HPO_4. The pH was 6.6 to 7.0.

In order to study efficiently the early formation of the food cup, it was necessary to establish conditions under which the amebae would feed quickly and reproducibly. By selecting amebae of uniform size and by controlling the degree of starvaton of the amebae, this could be assured. Amebae wiere removed from the culture 24 hours before the experiment and placed in clean dishes. They were washed three times to remove food organisms and debrs; they were then agitated gently with air bubbles uintil they rounded up. It was found that amebae 350 to 450 microns in diameter, measured in the rounded state, would often feed within 2 to 3 minutes after the paramecia were added and would always feed within 5 minutes. Amebae of this size were obtained by rejecting the larger and smaller organisms from a randomly selected population of 50 to 100 cells. This technique eliminated the larger cells about to undergo division and the smaller ones which had recently divided.

A concentrated suspension of washed paramecia was prepared by centrifuging, for 2 minutes at 400 *g*, 20 ml taken from the top of a dense culture. The pellet was resuspended in 1 ml of fresh medium, recentrifuged for 2 minutes at 400 *g*, and allowed to stand for 10 minutes before the supernatant medium, containing the paramecia, was pipetted off. The final concentration in the suspension added to the test amebae was approximately 10^4 paramecia per ml.

(*b*) After the paramecium suspension was added to the dish containing the test amebae, feeding was observed a low magnification by use of a dissecting microscotpe. Finer details were studied in some preparations in a shallow chamber under a coverslip by phase-contrast microscopy at 200 or 500 magnification.

Under controlled feeding conditions, the paramecia collect in large numbers immediately around each ameba. It is not unusual to see as many as 10 to 20 of the ciliates arranged in a palisade around the ameba, each making contact intermittently or continuously with the surface of the ameba. Under these conditions, food cups form rapidly and repeatedly until all or most of the paramecia have been ingested.

The local accumulation of paramecia around the ameba is a striking phenomenon which calls for some explanation. Direct observation showed that the ciliates were not simply adherent to the ameba surface, since most were seen to make and break contact repeatedly before being trapped. It seemed possible that the paramecia were attracted to the ameba by some chemotactic or field effect. An experiment was, therefore, designed to determine objectively whether the paramecia were attracted by any type of influence which would lead to initial contacts between paramecia and ameba at a rate higher than could be expected by chance alone.

An ameba starved for 16 hours, having a diameter of 350 to 450 microns, was placed in an embryological watch glass containing fresh medium. In the same microscopic field as the ameba was placed a washed white sand crystal, slightly larger than the ameba. Paramecia were added and the numbers of ciliates striking the sand crystal and striking the ameba were counted in alternating 1-minute time periods for 26 minutes in each run. Only rapid first hits were counted—paramecia circling around the objects to make second contacts were ruled out of the game. The number of paramecia added was such that this could be done easily. Because the influence of the ameba would presumably extend to the crystal within the field tested, the experiment was repeated using separate dishes and equal numbers of ciliates. Amebae in different states were used in the experiment: well fed, starved for 24 hours, 48 hours, and 5 days, and amebae recently divided in half. In each case, the number of first contacts with the ameba was compared with the number of first contacts against the sand crystal.

(*c*) It seemed likely that the structure of the membrane and cytoplasm surrounding the food cup might be altered at a level beyond the limits of resolution of the light microscope. To permit analysis by electron microscopy of food cup formation at the very earliest stages, a technique for rapid fixation by osmium tetroxide was devised.

A moist chamber was constructed around a 20X phase contrast objective and a fine glass capillary tube connected to an OsO_4 source was run into the chamber where it came in close proximity to an ameba-paramecium preparation. At the moment of food cup formation as seen through the microscope, unbuffered 2 per cent OsO_4 (pH 6) was run into the preparation. The technique was designed to stop the process before the food cup had completely closed. The paramecium was alive at the moment of fixation

in all cases, and a fine channel connecting the outside medium with the food cup could be detected in all cases. Cytoplasmic flow and Brownian motion in the ameba, and ciliary movement in the paramecium ceased instantaneously, *i.e.*, in less than 1 second from the time of contact with the fixative.

The fixed amebae were dehydrated through 50 to 100 per cent ethanol solutions and embedded in Araldite. Polymerization took place in gelatin capsules at 40°C for 24 hours followed by 55°C for 48 hours. Serial thin sections of the food cups were cut with a diamond knife. Phosphotungstic acid or lead hydroxide was used to stain the sections before they were examined in an RCA EMU-3C electron microscope.

OBSERVATIONS

1. *Interactions between Ameba and Prey Organism*

The sluggish ameba would seem to be at a distinct disadvantage during any attempt to capture such a rapidly moving organism as a paramecium—yet a single ameba can capture more than 100 paramecia per day. Observation of the feeding process by the methods outlined above suggests the nature of the mechanisms by which the amebae increase the probability of capture.

Within a few minutes after the addition of paramecia to medium containing several amebae, the ciliates are seen by low power microscopy to gather around the ameba much as suckling pigs to a sow. The amebae adhere to the bottom of the dish and assume a shape best described as randomly polypodal. The paramecia that have gathered seem to nuzzle the pseudopods only to dart away, circle around, and then return. Within 2 to 5 minutes paramecia are entrapped within food vacuoles. The trapping continues with more paramecia nuzzling the ameba until all are ingested or until the ameba reaches its capacity. It was found that once feeding begins amebae appropriately starved can take up as many as 10 paramecia in 5 minutes.

The sand crystal experiment was designed to determine whether initial contacts between paramecia and an ameba occurred at a rate higher than could be expected by chance alone. The control contacts with the sand crystal were interpreted as occurring by chance. In no case did the number of initial contacts with the ameba exceed the number of initial contacts with the sand crystal—this was so, no matter what the state of starvation or whether the cell had previously been divided in two. These results show that there is no appreciable chemotactic or field influence drawing the paramecia to the ameba. What then accounts for the ameba's ability to concentrate its prey?

By phase contrast microscopy the feeding process can be observed in greater detail. Some ciliates approach the membrane at high speed, dent it, bend double from the impact, and dart away following a straight line. In most cases they do not return. On the other hand, some paramecia slide gracefully up to the ameba, nuzzle it, back off, circle, and return to repeat the same activity. In this second case, no mechanical denting is noted; rather there is an outsurge of cytoplasm around the point of slow nuzzling, and a food cup begins to form. If the paramecium remains in contact long enough a complete food cup is formed, trapping the ciliate. If the paramecium breaks contact and circles away, the cytoplasmic outsurge around the point of stimulation ceases and the membrane slowly smooths out until no trace of the food cup remains. Attention to the cilia of the slow, nuzzling paramecium, *i.e.* the ciliate which remains in contact with the predator long enough to stimulate the membrane to form a food cup, reveals that clumps of granular material are affixed to one or the other side of the paramecium. The clumps of granules rotate with the moving cilia. The paramecium turns in the direction of the adhering granules much as a row boat turns in the direction of an oar which is fouled by weeds.

The mechanism by which the ameba concentrates its prey is now clear. The paramecia make their initial contacts with the ameba by chance alone, during random movement. As a result of this chance contact, fouling of the cilia occurs. This modifies their "swimming" pattern. Instead of moving in straight, essentially random trajectories, they gyrate about in the immediate vicinity of the ameba. As a result, the probability of repeated contact is greatly increased. With repeated contacts, the stimuli to food cup formation are increased and the probability of capture is enhanced.

2. *Food Cup Formation and the Dynamics of Cytoplasmic Flow*

The formation and closure of a food cup around a food organism can occur within 2 seconds or may take as long as 12 seconds. Formation begins by a doughnut-shaped outsurge of cytoplasm lifting the plasmalemma of the ameba outward

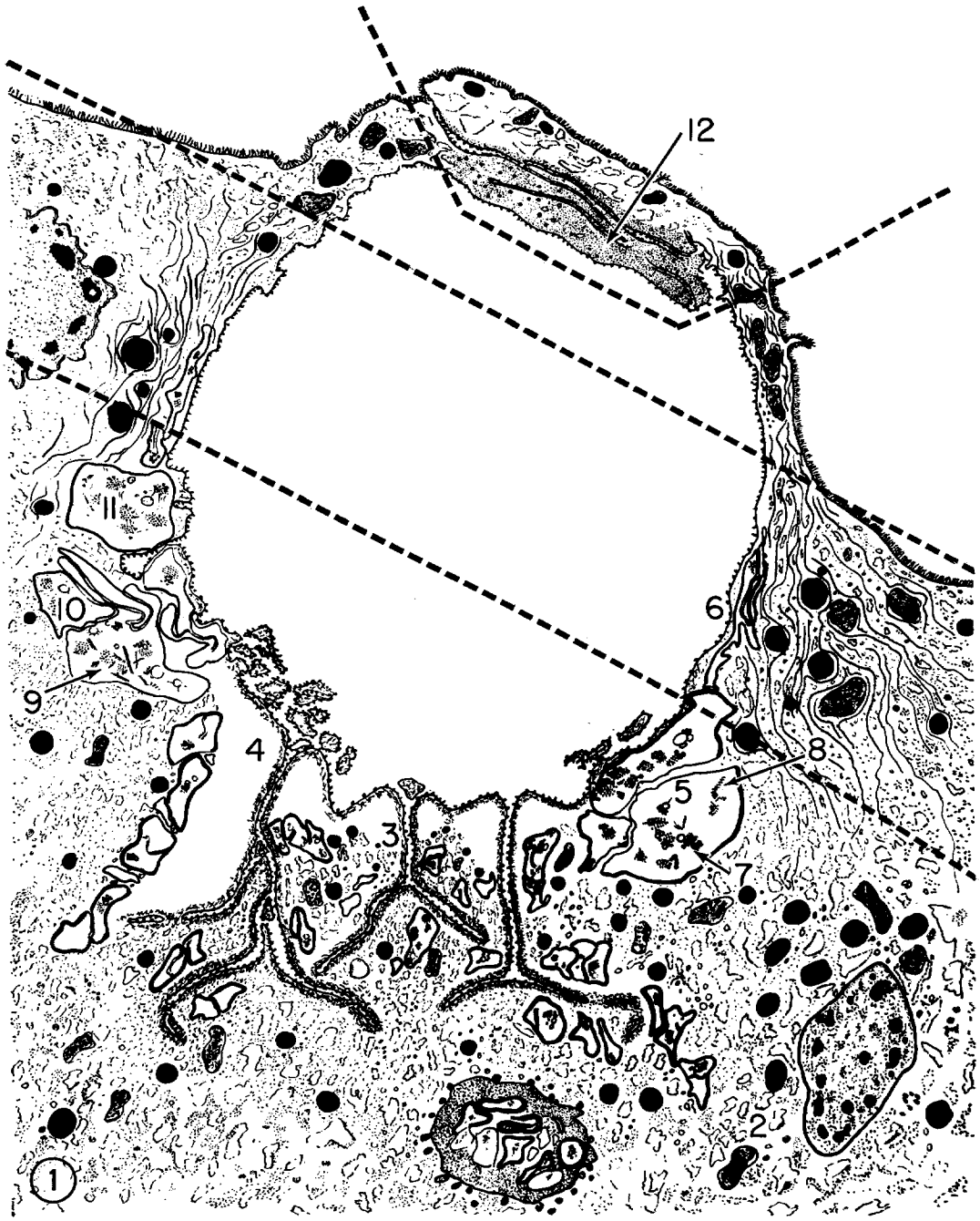

FIGURE 1 Schematic drawing of a food cup taken from electron micrographs of serial sections. The proportions have been altered somewhat and only the principal features are represented. The dotted lines divide the cup into four regions, from top to bottom: the fusing, upper, middle, and base regions. Each number on the drawing corresponds to one of the electron micrographs that follow.

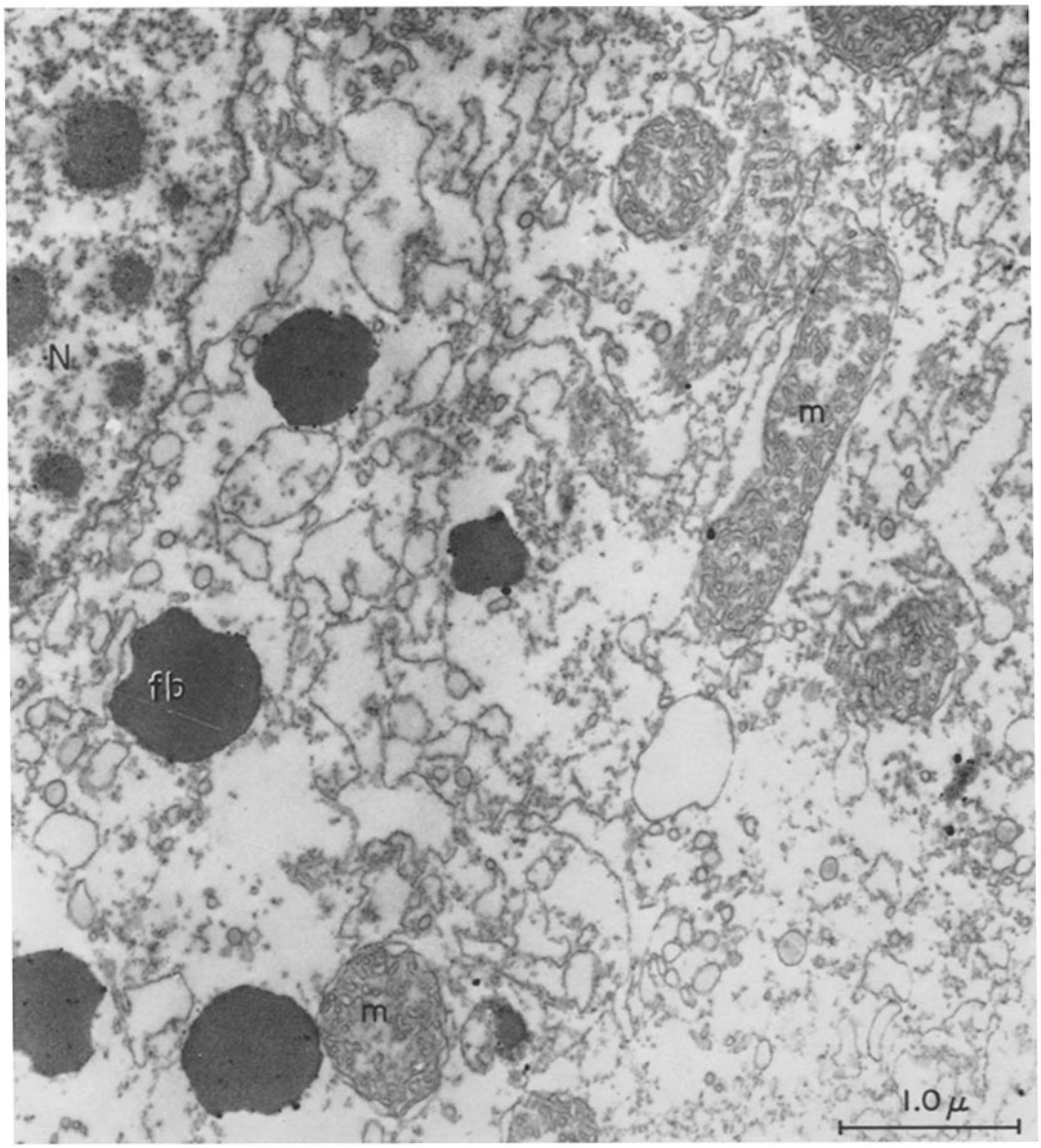

FIGURE 2 Electron micrograph of a typical region of ameba cytoplasm at some distance from the region of food cup formation. It is included for comparison with the micrographs, to follow, of the cytoplasmic structures adjacent to the food cup. The vesicles, membranes, and sparse granules of a typical region of the cytoplasm can be seen in addition to a portion of a nucleus (*N*), fat bodies (*fb*), and mitochondria (*m*). × 24,000.

around a central fixed region of plasmalemma. The fixed region of plasmalemma is that region which received the contact from the paramecium. The cytoplasm beneath the fixed region of plasmalemma shows no cytoplasmic movement and appears to be in what has conventionally been called a gel state. After fixation with OsO_4, this region of cytoplasm at the base of the newly formed food cup is found to be distinctly more osmiophilic than the surrounding cytoplasm. The nature of the structures in the "gelled" region will be considered further in the section dealing with the results of electron microscopy.

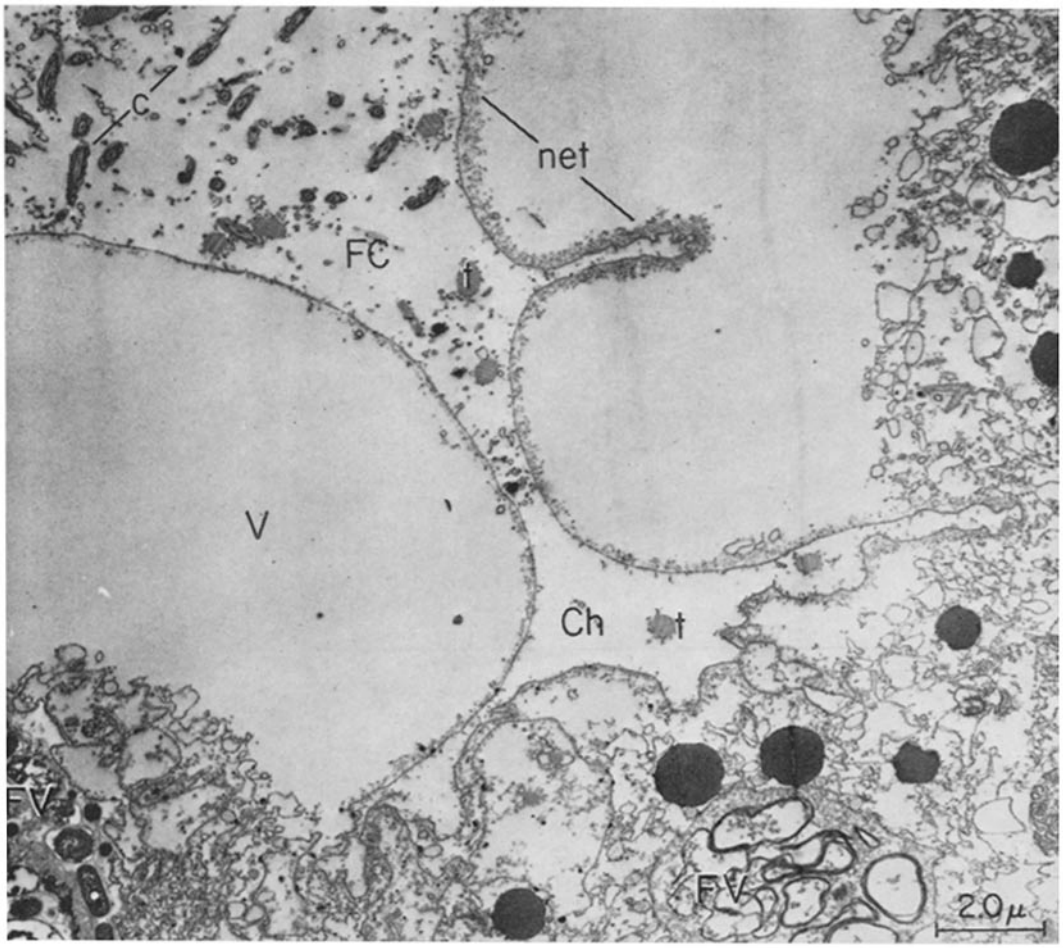

FIGURE 3 Electron micrograph demonstrating the deep folds of the membrane at the base of the food cup, which form channels (*Ch*). In the lumen of the food cup (*FC*) are discharged trichocysts (*t*) and cilia (*c*) of the paramecium. On the cytoplasmic side of the membrane bordering the channels is a fine granular and fibrillar network (*net*). Clear or void regions (*V*) appear between the membrane and the normal cytoplasm. Several old food vacuoles (*FV*) appear in the region of normal cytoplasm. × 7,000.

3. *Closure and Shrinkage of the Food Cup*

The converging lips of the food cup move to overlap one another. Shortly thereafter, their movement stops and fusion of the approximated membranes begins. Fusion begins from the inward or food-vacuole end of the channel and progresses outward. The process may be completed in less than 1 minute, but in other instances has been followed as long as 10 minutes. As will be seen, the fine structure of the cytoplasm surrounding the channels of fusion presents some interesting features.

The behavior of the food vacuole in relation to shrinkage, pH change, death of the organism, and ultimate digestion was studied extensively by Mast (1942). Reviews of the early work were done by Kitching (1956) and Roth (1960). Roth described the fine structure of food vacuoles at somewhat later stages, well after closure of the food cup. Our observations show that after entrapment the paramecium swims vigorously within the food cup while the vacuole is slowly and continuously shrinking in volume and diameter. At the time of death of the ciliate, which occurs from 5 to 15 minutes after cup formation, the food vacuole closely approximates the contours of the parame-

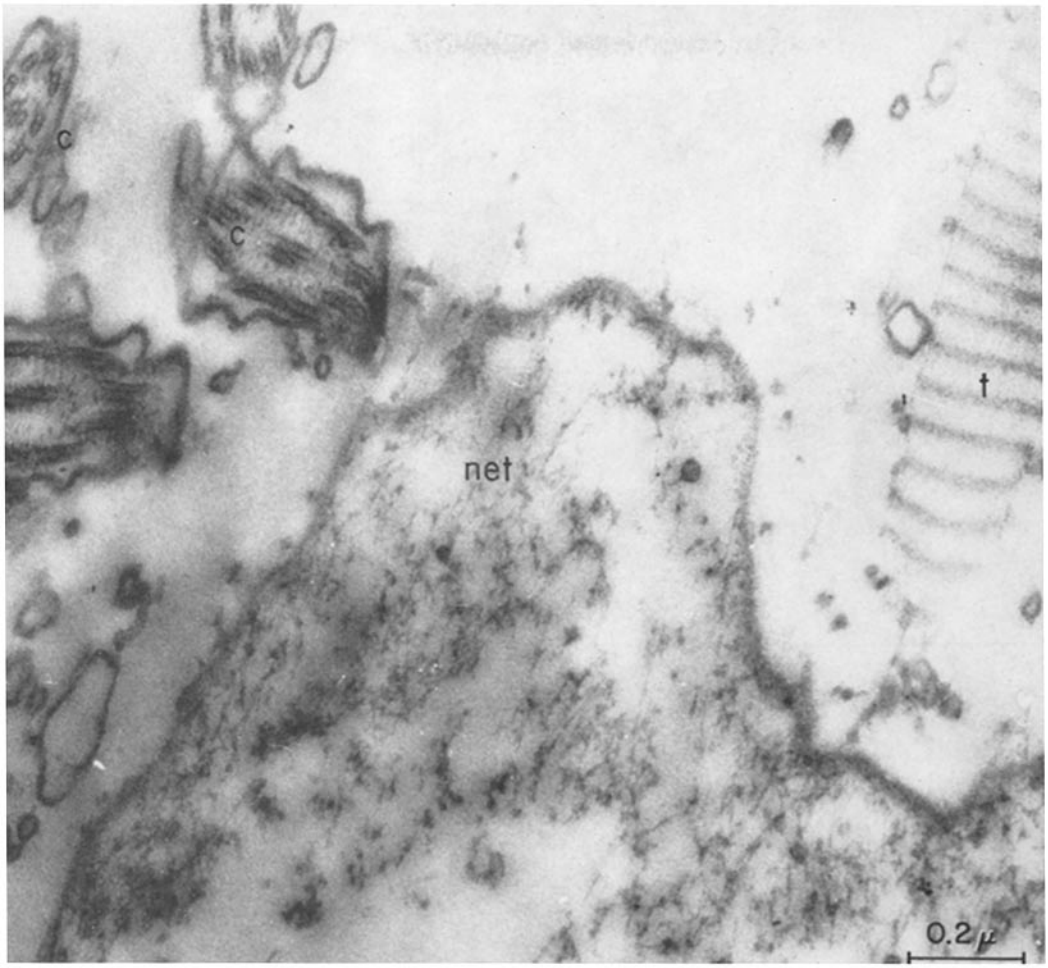

FIGURE 4 Higher magnification micrograph of the granular and fibrillar network that lines the cytoplasmic surface of the membrane along the channels shown in Fig. 3. The diameter of the fibrils is approximately 50 A. Discharged trichocysts (*t*) and cilia (*c*) can be seen within the food cup. × 78,000.

cium. The great reduction in volume and in membrane area during shrinkage of the food vacuole presents an interesting problem. The results obtained by electron microscopy supply information on this question.

4. *Fine Structure of the Food Cup*

Fig. 1 is a schematic drawing of the food cup sectioned in a direction perpendicular to the cell surface. For simplicity of illustration, the proportions have been altered somewhat and only the principal features have been indicated. The food cup has been divided into regions marked by semihorizontal dotted lines—the morphological characteristics differ from region to region. The numbers represent electron micrographs to follow, each of which illustrate the fine structure encountered in the region depicted. A typical region of cytoplasm at a distance away from the food cup is shown in the electron micrograph of Fig. 2. It is included for comparison with the cytoplasmic structure surrounding the food cup.

The region of the food cup which corresponds to that area of the plasmalemma in which the first contact was made with the food organism has been designated as the base. By phase-contrast micrography this region is seen to remain fixed relative to the subjacent plasmagel while the surrounding cytoplasm surges forward to build up the walls of the cup.

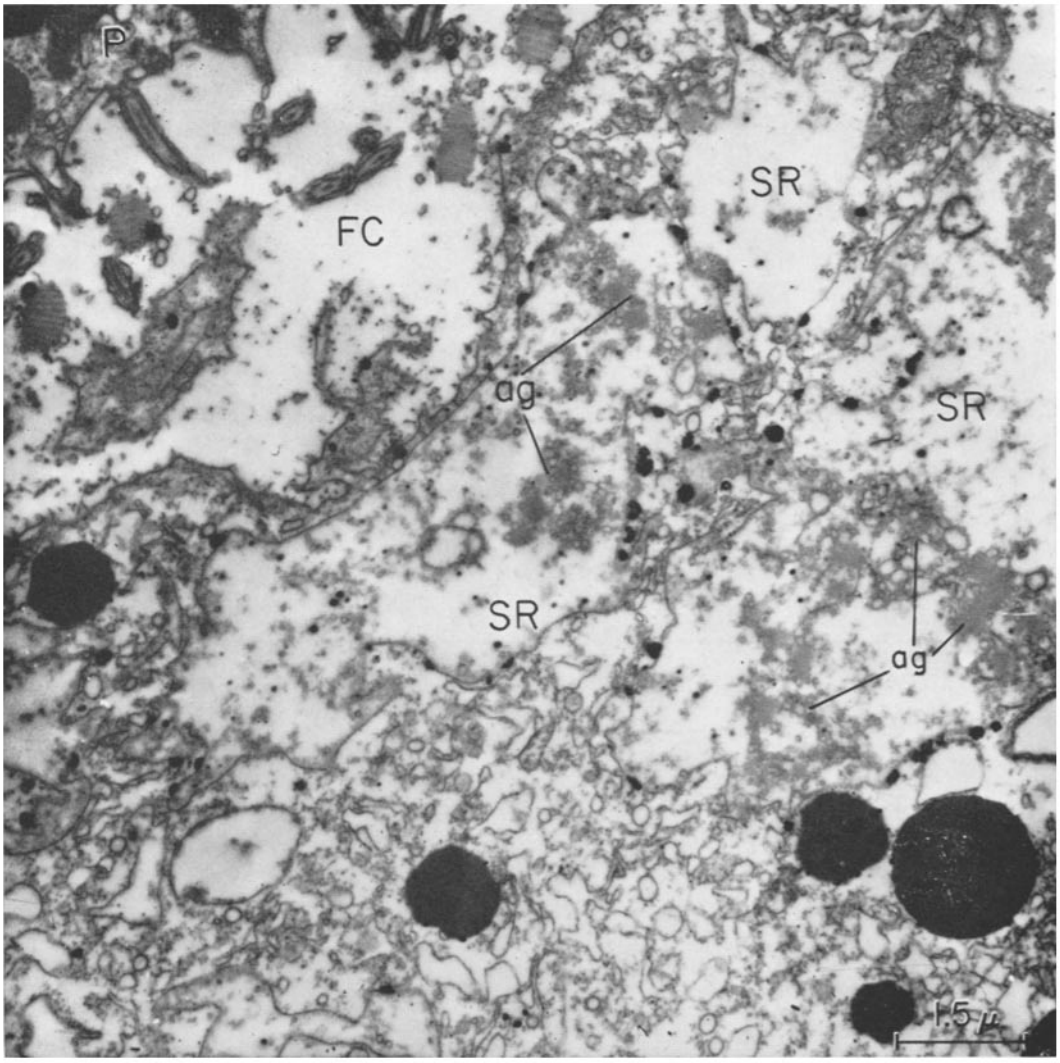

FIGURE 5 Specialized regions (*SR*) of cytoplasm at the base of the food cup. These are loosely contained by unit membrane and have within them aggregates (*ag*) of vesicles and crystalline patches. The various types of fibrils also found in the specialized regions are not in evidence in this field. At the upper left is the food cup lumen (*FC*) containing the paramecium (*P*). × 12,000.

When serial sections of the basal region were examined by electron microscopy, structures were seen which were not found elsewhere in the cytoplasm. One group of structures unique to the food cup have been called "specialized regions." These are circumscribed regions adjacent to the wall of the food cup (Fig. 5). Serial sections reveal that the specialized regions are generally elongated or tubular in form, and are coiled or wound around the base of the food cup. Unit membrane completely encases each such region, in sectioned profiles, and it was, therefore, thought at first that they were large vacuoles pinched off from the plasmalemma lining the food cup. However, some hundreds of serial sections failed to demonstrate continuity between the membrane of the cup and that of any such region. The space within the regions was never found to communicate with the space within the cup. Cilia and discharged trichocysts from the paramecium, which were commonly found in the cup space and which should appear in any large vacuoles pinched off the cup, were

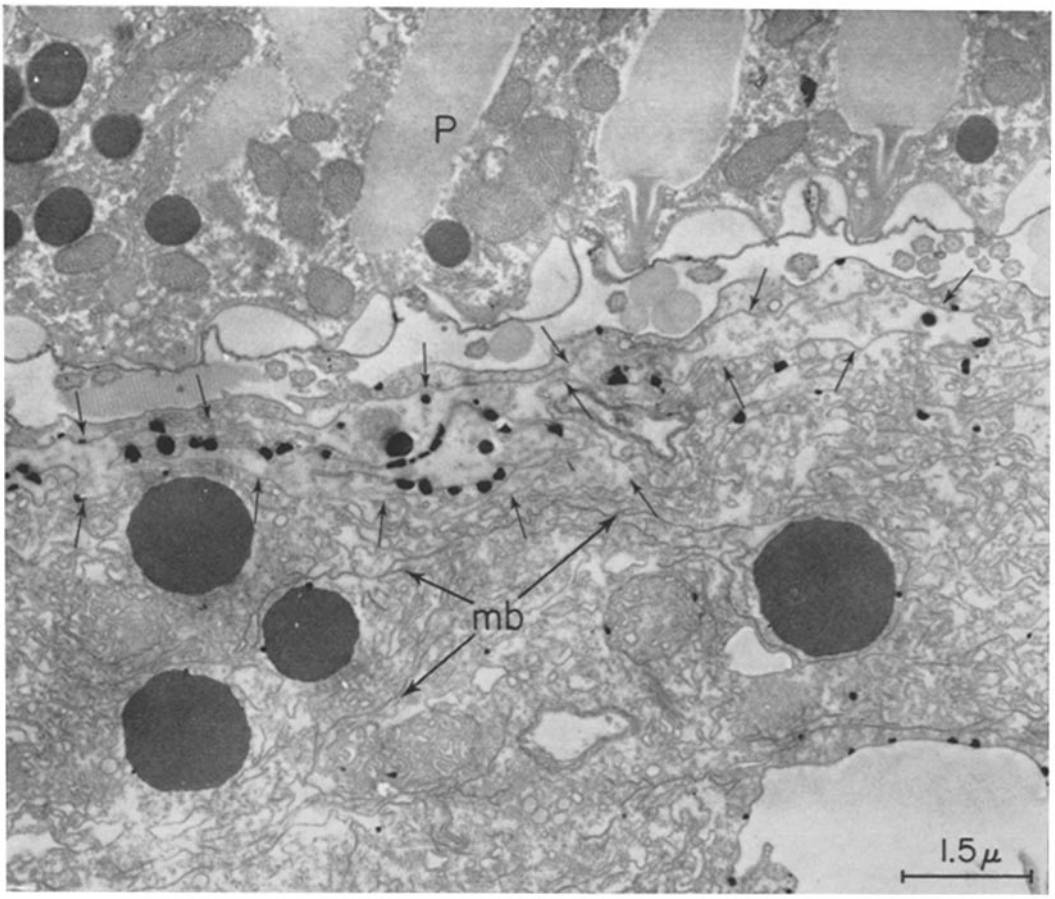

FIGURE 6 Electron micrograph showing the form of the specialized regions at the middle portion of the food cup. A region is defined here by the small arrows. It is more flattened than the specialized regions at the base, and in close proximity to the food cup membrane. Deeper in the cytoplasm are seen the long membranous structures (*mb*) which are peculiar to the middle portion of the cup. The paramecium (*P*) within the food cup is at the top of the field. × 12,000.

never observed within the specialized regions. Although the regions appear vacuolar, in the sense that the background density after staining is low and in the sense that they are demarcated from the remainder of the cytoplasm by membrane, their content is distinctly different from that of the void space within the food cup. They must, therefore, be formed rapidly from components present in the cytoplasm.

Within the specialized regions are clusters of small vesicles (Fig. 7) some of which are enclosed by a single membrane and some by double or unit membrane, patches of material with a repeating pattern suggesting a paracrystalline order (Fig. 8), and two types of fibrillar elements (Figs. 9 to 11). One type of fibril (Fig. 9) has an irregular contour and a diameter ranging from 400 to 450 A, and appears to be an aggregate of smaller fibrils. Figs. 10 and 11 show smaller fibrils, 175 to 225 A in diameter. These thinner fibrils are spread randomly throughout the region, as in Fig. 10, or are seen to connect the patches of crystalline material (Fig. 11).

Although the specialized regions are similar in structure and location, different combinations of the fibrillar, vesicular, and crystalline elements are seen within different regions. One interpretation is that fixation has caught the elements of a contractile system in various states of aggregation and dispersal.

In close association with the specialized regions are numerous deep folds in the plasmalemma at

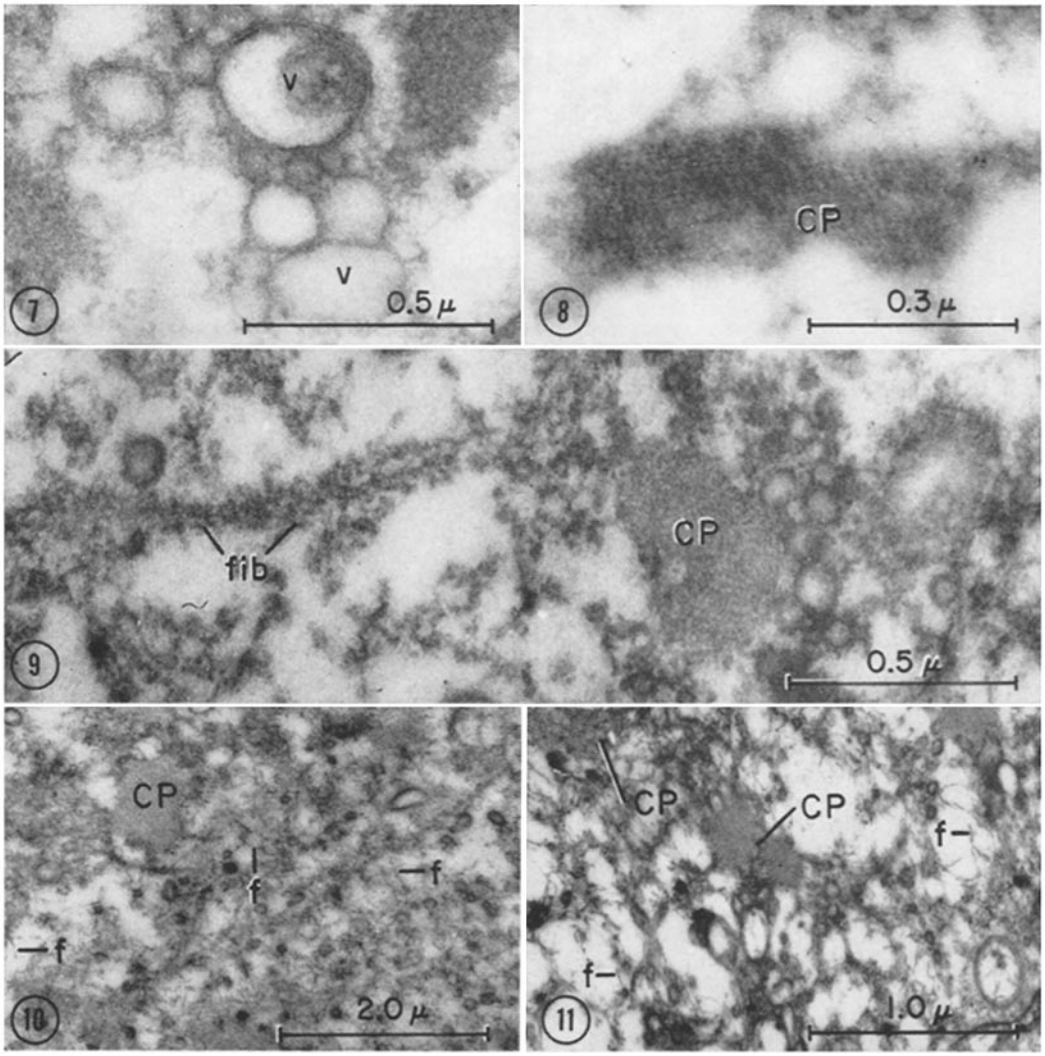

FIGURE 7 The vesicles (*v*) which occur in clusters within the specialized regions. × 62,000.

FIGURE 8 Micrograph showing the periodicity of structure within the crystalline patches (*cp*) of the specialized regions. The distance between the repeating elements comprising the pattern is 200 to 250 A. × 90,000.

FIGURE 9 A large fibril (*fib*) located within a specialized region. A crystalline patch (*cp*) is also present. The fibril diameter is 400 to 450 A. × 62,000.

FIGURE 10 Smaller fibrils (*f*) randomly distributed within a specialized region. The diameters are approximately 150 to 250 A. *cp*, crystalline patch. × 16,000.

FIGURE 11 Smaller fibrils (*f*) within another specialized region, in a somewhat less random array. Here they appear to connect the crystalline patches (*cp*) within the region. × 18,000.

the base of the food cup. These folds or invaginations appear in profile as long channels coursing deep into the cytoplasm (Fig. 3). Bordering the channels on the cytoplasmic side of the plasmalemma is a network of fine fibrils and granules (Fig. 4). This network appears somewhat like the dense material described by Peachey and Rasmussen (1961) at the free surfaces of epithelial

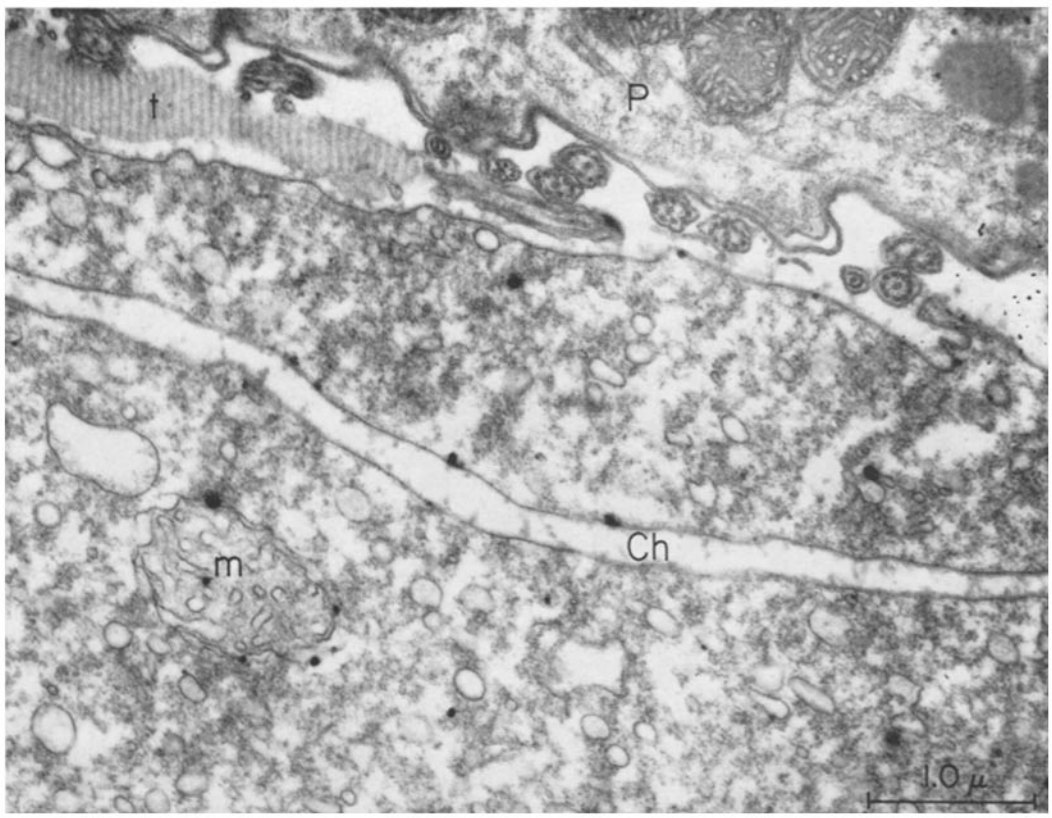

FIGURE 12 The fusing region of the food cup. The channel (*Ch*) is formed by the apposition of the overlapping parts of the lip or rim of the food cup. Only a part of the thickness of the region of fusion is included in this field. The cytoplasm contains granules and small vesicles. Mitochondria (*m*) are very rare in this region as are the other larger organelles of the normal cytoplasm. Note the absence of the long membranous structures and the specialized regions which were seen elsewhere around the food cup. *P*, paramecium; *t*, trichocyst. × 21,000.

cells bordering on the lumen of the toad bladder, and like the material described by Hayward (1963) along pinocytosis channels in *Amoeba proteus*. Between the dense network and the adjacent cytoplasm is a zone devoid of all structured osmiophilic material and of very low density after phosphotungstic acid or lead staining (Fig. 3). Such void regions are clearly defined and are bordered by regions of cytoplasm which contain the normal array of particulate and membranous structures encountered throughout the rest of the cell, but there is no limiting membrane between the void area and the normal cytoplasm. These relationships suggest that the void regions are formed by the passage of water through the membrane of the food cup.

The middle portion of the food cup also has "specialized regions" in the cytoplasm within the wall (Fig. 6), but here the regions are more elongated and are found in closer apposition to the plasmalemma. Also characteristic of the middle portion are long membranous sheets (Fig. 6) coursing through the cytoplasm. They are oriented along an axis corresponding to the direction of cytoplasmic flow within the advancing pseudopod which makes up the walls of the food cup.

During closure of the food cup the lip of the pseudopodal process which forms the walls of the cup converges opposite the base. Different parts of the converging lip or rim overlap each other and begin the process of fusion. Sections through this region of fusion (Fig. 12) demonstrate a cytoplasmic arrangement different from those types already described. Long folds in the cell mem-

brane or plasmalemma appear as channels in thin sections cut through the region. The cytoplasm appears denser than normal cytoplasm and is nearly devoid of all regular cytoplasmic organelles such as nuclei, food vacuoles, endoplasmic reticulum, etc. There are vesicles spread throughout and clumps of granular aggregates. There is a suggestion of a repeating or crystalline pattern in the granular arrangement of the ground cytoplasm, but the region has not been studied extensively enough to permit a decision on this point.

DISCUSSION

1. *Prey-Predator Interaction*

Feeding by *Chaos chaos* on *Paramecium aurelia* depends upon a definite interaction between the two organisms. Both are adapted in a way which aids the feeding process.

Many studies on fresh water amebae have shown that the organisms will ingest a variety of food objects (Mast and Hahnert, 1935; Schaeffer, 1916; Mast and Root, 1916; Bovee, 1960). Therefore, the stimulus to food cup formation is apparently not highly specific in some species. Some studies, however, have shown amebae to be capable of selecting one prey species in greater quantity than another from the same medium (Mast and Hahnert, 1935). This indicates that the nature or behavior of the food organism itself must play a role in the feeding process.

From our observations on *Chaos chaos*, there is no evidence that the behavior of the paramecia is influenced before the first contact with the ameba. The paramecia make the first contact with the ameba by chance during random movement. Further contacts with the ameba, however, do not occur by chance. They are influenced by the fouling of the cilia which leads to restricted movements of the prey around the ameba. The cilia are fouled by a sticky granular material from the surface coat of the ameba adhering at the time of the first contact. Even though contact does not result in adherence in the strict sense, it does lead to an increased probability of repeated or prolonged contact which provides the stimulus to food cup formation. As a result, the over-all process of feeding is facilitated. It is possible that this mechanism is adaptive and that it underlies the preferential uptake of certain prey species. Its value should be greatest in situations of sparse food supply, when it would serve to concentrate "preferred" prey in the immediate vicinity of the ameba.

The adhesive properties of cells have been studied widely and in many different contexts. What is relevant here is the concept of a "sticky surface" on the ameba during feeding. Ray (1959) described the feeding of *Hartmanella* sp., a soil ameba, on various types of bacteria. She described the adherence or agglutination of motile bacteria on the surface of the ameba before pseudopod extension pinches off the food cup. Ray also demonstrated in the same study that motility of the bacteria is necessary in order to have agglutination occur. It appears that the bacterial flagella are "fouled" in a manner analogous to that of the paramecial cilia. References to further examples of prefeeding adherence are cited by Ray, including the adherence of bacteria to leukocytes and of erythrocytes to *Entamoeba histolytica*. Bovee (1960) reported that *Thecamoeba sphaeronucleolus* extends a long pseudopod which adheres to the shell of the prey as a necessary first step in a complex ingestion process. Prey adherence has been examined in the suctorian *Podophyra collini* (Hull, 1961). The interaction between prey and predator in the suctorian feeding system has been found to be more complex than that described here in the ameba. It is interesting, however, that mechanisms of contact and adherence appear to be involved in both systems.

2. *Stimulation of Food Cup Formation*

Jennings in 1904 stated without detailed evidence that the extension of a psuedopod toward a food particle should be attributed partly to chemical stimulation. Schaeffer (1916) believed that, to induce food cup formation, a substance must be soluble and actively diffusing from a definitely localized region next to the ameba. Edwards (1925) concluded that the pH of the medium and its effect on the surface of the ameba was an important factor in food cup formation. Mast and Root (1916) showed that changes in surface tension, as had been proposed by Rhumbler (1898) and others, could not account for the force involved in the formation of a food cup. Schaeffer (1912) attached importance to movement or vibration of an object for the induction of a food cup. He believed that contact was not necessary.

Different species of ameba and various food organisms were used in the studies cited above.

In this study on *Chaos chaos* and *Paramecium aurelia*, it was observed that direct contact with the prey is necessary and that the duration of contact must be longer than that provided by a simple collision. Paramecia in contact for less than a second do not induce cup formation, but prolonged or repeated contact for 2 seconds or more causes a rapid response. Dead paramecia are usually not taken up even after prolonged contact with the ameba, but it has been found that paramecia immobilized or killed by brief heating will still induce cup formation if used immediately. This indicates that ciliary movement is not essential although direct contact is and that only living cells or cells freshly killed can induce cup formation.

The evidence available is not adequate to define the inducing stimulus more precisely, but it suggests that a labile substance on the cilia or pellicle of the paramecium is responsible for inducing food cup formation. From work done on the induction of pinocytosis channels in ameba (Schumaker, 1958; Brandt, 1958; Marshall, Schumaker, and Brandt, 1959; Rustad, 1959; Nachmias and Marshall, 1961; Chapman-Andresen, 1962) it seems reasonable to postulate that the inducer substance acts by binding to specific charged sites on the mucopolysaccharide surface coat of the ameba. It would be desirable to demonstrate this more directly as has been done in studies on pinocytosis, since the two processes of food cup formation and pinocytosis channel formation appear to involve the same fundamental mechanisms.

The surface properties of the ameba depend upon the characteristics of the slime coat. The filamentous or fibrillar structure of the coat in *Chaos chaos* was described by Pappas (1959) and Brandt and Pappas (1960). Studies in this laboratory indicate that the surface coat has several functions. It contains the anionic sites which are important in the induction of pinocytosis. It functions in the regulation of water and ion permeability (Bruce and Marshall), and it participates in the dynamic cycle of membrane turnover.

One question of some interest is that of the linkage between the external stimulus and the internal response. The problem is analogous to that of excitation-contraction coupling in muscle. Although there is as yet no direct evidence bearing on this problem, it is possible that coupling depends on the displacement of calcium ions from binding sites on the surface and on their movement through the membrane to activate a cytoplasmic contractile system.

3. *The Response to the Stimulus*

The formation of a food cup requires the transformation of an apparently quiescent portion of ameba membrane and cytoplasm into an actively moving region which surrounds the prey organism. The stimulus of the paramecium is required not only for the initiation of this process, but for its normal progression as well. The paramecium is not an externally fixed or resistant object about which the ameba flows. It is, in fact, often quite free in the medium, making no contact with the substratum, and intermittently breaking and remaking contact with the ameba. Furthermore, the membrane of the cup as it forms is not closely fitted to the surface of the prey. Measurements of the dimensions of the cup at the instant of closure show that the volume enclosed is usually about 10 times that of the paramecium. It is clear, therefore, that surface wetting forces between the two cells cannot adequately explain food cup formation in *Chaos chaos*. The process corresponds rather to what Rhumbler (1910) called "circumvallation," which Jennings (1904) perceived must depend upon ameboid movement rather than on simple surface forces.

In the living ameba, the cytoplasm underlying the region of the plasmalemma that received the stimulus is observed to be in a non-moving or gelled state. Around this zone, the cytoplasm surges outward to form the walls of the food cup. After fixation with OsO_4, the gelled region appears in phase contrast to be much darker than any other part of the cell, indicating either that the material at that site has been altered to become more osmiophilic or that a considerable amount of already osmiophilic material has been concentrated in that region.

Serial sections of the fixed ameba studied by electron microscopy reveal that there are distinctive structures in the dense region at the base of the cup. The base is distinguished by folds of the cup membrane running deep into the cytoplasm and also by unique specialized regions of cytoplasm containing aggregates of small vesicles, crystalline structures, and fibrillar material. The intricately crumpled or folded membrane along with the newly formed specialized regions must account for the increased osmiophilia seen by light microscopy at the base. Thus, new osmio-

philic structures have formed (the specialized regions) and there has also occurred a concentration of material which was already osmiophilic (the folded membrane).

The evidence obtained by electron microscopy, considered with that derived from direct observation of food cup formation in the living cells, makes possible an integrating hypothesis concerning the response to the stimulus. It is proposed that the membrane area directly stimulated by the prey organism becomes attached to the underlying cortical "gel." With that region held fast, the surrounding cytoplasm flows outward around the food object. The force responsible for cytoplasmic flow need not be generated locally, but may be provided by the same system which operates in normal psuedopod formation. The food cup is, therefore, a single pseudopod built up around a central cavity, the base of that cavity being the region of attachment of the plasmalemma to the cortical "plasmagel." It is proposed that the specialized regions seen in fixed material by electron microscopy represent the structures which are responsible for that attachment and for the subsequent contraction in volume and membrane area of the food vacuole.

In an ameba fixed at the instant of closure of the food cup, the structures seen in serial sections at different levels, going from the base to the outer region of fusion, represent successive stages in a dynamic process. Considered in this light, the specialized regions at the base which represent the structures by which the membrane was initially fixed to the underlying plasmagel are further differentiated than are the structures which were formed later in the process. As the food cup is built up and as the lip converges towards closure, the paramecium continues to stimulate the membrane, often circling within the cup and making contact with the entire inner surface. This causes new regions of attachment to form as cytoplasmic structure builds up from the base to encircle the cup. The relative complexity of the cytoplasmic structures at the base and the greater complexity of folding of the membrane there as compared to the middle portion of the cup may be explained in this way.

4. *Closure of the Food Cup and Subsequent Events*

This study deals primarily with the interactions between the ameba and its prey, the characteristics of the stimulus to food cup formation, and the dynamic changes in structure which are produced by the stimulus. Its scope is limited somewhat arbitrarily to that point in the process at which the cup closes, the rim becomes intimately apposed, and membrane fusion begins. The evidence available regarding the sequence of changes during convergence of the lip and during the breakage and refusion of the membrane is not adequate to explain in any detail these events, but it suggests that the dynamic process of structural transformation in the cytoplasm, which began at the base and spread outward around the forming cup, is responsible.

Similarly, the complex changes which occur following the separation of the food vacuole from the plasmalemma have not been treated. These include the shrinkage in volume and membrane area of the vacuole, the death and digestion of the prey, and ultimately the assimilation of some materials and the defecation of the residuum. These steps may well involve other processes, but the evidence suggests that some of the mechanisms responsible for these events are already in operation during the formation of the food cup.

The authors gratefully acknowledge the technical assistance of Miss Marianne Pieren, Mrs. Ann Garvin, and Mrs. Christine Kennedy.

This investigation was supported by Public Health Service Research Grant No. CA-01957 from the National Cancer Institute and by Research Career Development Award No. 5-K3-GM-477 from the National Institute of General Medical Sciences.

Received for publication, May 5, 1964.

REFERENCES

1. ABÉ, T. H., Mechanisms of ameboid movement based on dynamic organization: Morphophysiological study of ameboid movement. IV, *in* Primitive Motile Systems in Cell Biology, (R. D. Allen and N. Kamiya, editors), New York, Academic Press, Inc., 1964, **4**, 221.
2. BOVEE, E. C., Studies of feeding behavior of amebas. I. Ingestion of thecate rhizopods and flagellates by verrucosid amebas, particularly *Thecamoeba sphaeronucleolus*, *J. Protozool.*, 1960, **7**, 55.
3. BRANDT, P. W., A study of the mechanism of pinocytosis, *Exp. Cell Research*, 1958, **15**, 300.
4. BRANDT, P. W., and PAPPAS, G. D., An electron

microscopic study of pinocytosis in ameba. I. The surface attachment phase, *J. Biophysic. and Biochem. Cytol.*, 1960, **8**, 675.

5. BRUCE, D. L., and MARSHALL, J. M., Some ionic and bioelectric properties of the ameba *Chaos chaos*, in preparation.
6. CHAPMAN-ANDRESEN, C., Studies on pinocytosis in amoebae, *Compt. rend. trav. Lab. Carlsberg*, 1962, **33**, 73.
7. EDWARDS, J. G., Formation of food-cups in *Amoeba* induced by chemicals, *Biol. Bull.*, 1925, **48**, 236.
8. GOLDACRE, R. J., On the mechanism and control of ameboid movement, *in* Primitive Motile Systems in Cell, Biology, (R. D. Allen and N. Kamiya, editors), New York, Academic Press, Inc., 1964, 237.
9. GRIFFIN, J. L., The comparative physiology of movement in the giant, multinucleate amebae, *in* Primitive Motile Systems in Cell Biology, (R. D. Allen and N. Kamiya, editors), New York, Academic Press, Inc., 1964, 303.
10. HAYWARD, A. F., Electron microscopy of induced pinocytosis in *Amoeba proteus*, *Compt. rend. trav. Lab. Carlsberg*, 1963, **33**, 535.
11. HOLTER, H., and MARSHALL, J. M., Studies on pinocytosis in the amoeba *Chaos chaos*, *Compt. rend. trav. Lab. Carlsberg, Ser. chim.*, 1954, **29**, 7.
12. HULL, R. W., Studies on suctorian protozoa: The mechanism of ingestion of prey cytoplasm, *J. Protozool.*, 1961, **8**, 351.
13. JENNINGS, H. S., Contributions to the study of the behavior of lower organisms, *The Carnegie Institution of Washington, Pub. No. 16*, 1904.
14. KITCHING, J. A., Food vacuoles, *Protoplasmatologia*, III D3b, 1956, 1.
15. MARSHALL, J. M., SCHUMAKER, V. N., and BRANDT, P. W., Pinocytosis in amoebae, *Ann. New York Acad. Sc.*, 1959, **78**, 515.
16. MAST, S. O., and ROOT, F. M., Observations on ameba feeding on rotifers, nematodes, and ciliates, and their bearing on the surface-tension theory, *J. Exp. Zool.*, 1916, **21**, 33.
17. MAST, S. O., and DOYLE, W. L., Ingestion of fluid by amoeba, *Protoplasma*, 1934, **20**, 555.
18. MAST, S. O., and HAHNERT, W. F., Feeding, digestion, and starvation in *Amoeba proteus* (Leidy), *Physiol. Zool.*, 1935, **8**, 255.
19. MAST, S. O., The hydrogen ion concentration of the content of the food vacuoles and the cytoplasm in *Amoeba* and other phenomena concerning the food vacuoles, *Biol. Bull.*, 1942, **83**, 173.
20. NACHMIAS, V. T., and MARSHALL, J. M., Protein uptake by pinocytosis in amoebae: Studies on ferritin and methylated ferritin, *in* Biological Structure and Function, Proceedings of the First IUB/IUBS International Symposium, (T. W. Goodwin and O. Lindberg, editors), New York, Academic Press, Inc., 1961, **2**, 605.
21. PAPPAS, G. D., Electron microscope studies on amoebae, *Ann. New York Acad. Sc.*, 1959, **78**, 448.
22. PEACHEY, L. D., and RASMUSSEN, H., Structure of the toad's urinary bladder as related to its physiology, *J. Biophysic. and Biochem. Cytol.*, 1961, **10**, 529.
23. RAY, D. L., Agglutination of bacteria: A feeding method in the soil ameba *Hartmanella* sp., *J. Exp. Zool.*, 1959, **118**, 443.
24. RHUMBLER, L., Physikalische Analyse von Lebenserscheinungen der Zelle, *Arch. Entwcklngsmech. Organ.*, 1898, **7**, 103.
25. RHUMBLER, L., Die verschiedenartigen Nahrungsaufnahmen bei Amöben als Folge verschiedener Colloidalzustände ihrer Oberflächen, *Arch. Entwcklngsmech. Organ.*, 1910, **30**, 194.
26. ROTH, L. E., Electron microscopy of pinocytosis and food vacuoles in *Pelomyxa*, *J. Protozool.*, 1960, **7**, 176.
27. RUSTAD, R. C., Molecular orientation at the surface of amoebae during pinocytosis, *Nature*, 1959, **183**, 1058.
28. SCHAEFFER, A. A., Contributions on the feeding habits of ameba, *Tr. Tennessee Acad. Sc.*, 1912, **1**, 59.
29. SCHAEFFER, A. A., On the feeding habits of ameba, *J. Exp. Zool.*, 1916, **20**, 529.
30. SCHAEFFER, A. A., Choice of food in ameba, *J. Animal Behavior*, 1917, **7**, 220.
31. SCHUMAKER, V. N., Uptake of protein from solution by *Amoeba proteus*, *Exp. Cell Research*, 1958, **15**, 314.
32. WOHLFARTH-BOTTERMANN, K. E., Cell structures and their significance for ameboid movement, *Internat. Rev. Cytol.*, 1964, **16**, 61.
33. WOLPERT, L., THOMPSON, C. M., and O'NEILL, C. H., Studies on the isolated membrane and cytoplasm of *Amoeba proteus* in relation to ameboid movement, *in* Primitive Motile Systems in Cell Biology, (R. D. Allen and N. Kamiya, editors), New York, Academic Press, Inc., 1964, 143.