INFECTIVE ORGANISMS IN THE CYTOPLASM OF *AMOEBA PROTEUS*

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ABSTRACT

Evidence from electron and phase microscopy is given which shows that infective organisms are present in the cytoplasm of *Amoeba proteus*. Vesicles containing living organisms have been observed after repeated washing and starvation of the amebae for a period of 2 weeks. Exposure to γ-radiation in conjunction with starvation, repeated washing, isolation of single amebae, refeeding with contaminant-free *Tetrahymena*, and clone selection has produced clones with reduced cytoplasmic infection. These findings are discussed in regard to the autoradiographic studies of other investigators on *Amoeba proteus*. The controversies over whether DNA and RNA are synthesized in the cytoplasm may be resolved by the finding of cytoplasmic infection.

INTRODUCTION

Although the synthesis of deoxyribose nucleic acid (DNA) is commonly associated only with the nucleus, there are occasional reports which present evidence for cytoplasmic DNA synthesis in addition to nuclear synthesis. Such a case in *Amoeba proteus* was reported recently by Plaut and Sagan (1) who studied labeling by tritiated thymidine in organisms which had previously been starved for 7 days and washed in a penicillin solution immediately before incubation in the label-containing culture fluid. All of the amebae showed radioactivity in the cytoplasm after 44 hours' incubation, while few showed it in the nucleus; ribonuclease (RNase) treatment of sections before autoradiography had no effect on labeling, but deoxyribonuclease (DNase) or both RNase and DNase treatment resulted in essentially no demonstrable label. Therefore, they suggest that the labeled component is DNA and postulate that it is either synthesized by a normal cytoplasmic process or by a cytoplasmic infective agent. A more recent study indicates similar findings (2).

Similarly, the literature contains conflicting reports on the synthesis of ribose nucleic acid (RNA) in the cytoplasm of *Amoeba proteus*. Prescott (3) studied labeling by C^{14}-uracil and reported that whole and nucleated halves showed cytoplasmic incorporation, while enucleated halves did not. A further report by Prescott (4) shows no incorporation of C^{14}-labeled uracil, orotic acid, or adenine in enucleated halves. However, incorporation of adenine-8-C^{14} (5, 6) and carbon-14-labeled uracil and orotic acid (7) in enucleated halves of amebae has been studied and it has been shown that a cytoplasmic incorporation of precursor(s) operates in the absence of the nucleus. Therefore, it is thought that two processes exist, this and nuclear incorporation; their relative magnitude in intact cells is unknown.

The present report and an earlier oral presentation (8) are the results of a prolonged study of several species of amebae both by electron microscopy of thin sections and by phase microscopy of living organisms. Infective organisms were found repeatedly in the cytoplasm of *Amoeba proteus* by phase microscopy and are related to structures seen in numerous vesicles in virtually every elec-

tron microscope section. Although 4-days' starvation frees the cytoplasm of food vacuoles (4), 13-days' starvation failed to decrease infection. These observations, and also some attempts to rid the cytoplasm of the infective agent, are reported. The presence of such infections may help to resolve certain controversies regarding cytoplasmic sites of nucleic acid synthesis.

METHODS

The amebae used for electron microscopy were obtained from the General Biological Supply House, Inc. in Chicago in about 1957. They were grown in doubly glass-distilled water and fed either axenically grown *Tetrahymena pyriformis* (strain W) which has no infecting organisms or a mixture of *Paramecium caudatum* and *Chilomonas paramecium*. They were fixed in 1 per cent osmium tetroxide which was buffered to pH 8.0 with veronal acetate and which also contained either 0.9 per cent sodium chloride or 0.01 per cent calcium chloride. Graded ethanols were used for dehydration and methacrylate was used for embedding. Sections were cut at 50 to 100 mμ settings of the Porter-Blum microtome and examined in an RCA EMU-3A or 3E electron microscope.

In 1960, amebae were again obtained from the same source on two occasions and also from the Carolina Biological Supply Company, Elon College, North Carolina. These cultures were opened and examined under the light microscope using sterile methods. Phase contrast observations on these stocks and on those obtained in 1957 were made with a Zeiss Winkel microscope at a magnification of 500 diameters both on flattened, living amebae and on amebae immediately after rupturing.

Starvation studies were carried out on the Chicago stock of amebae which had been washed repeatedly in sterile distilled water and starved for 7 days. The culture was then separated into two parts, one of which was fed with axenically grown, washed *Tetrahymena* and the other was kept under starvation conditions. Amebae were examined by phase microscopy at 4, 6, 8, 9, 11–13, and 16 days. At 16 days, eleven organisms survived; they were isolated and fed on axenically cultured *Tetrahymena*. Three produced clones which were examined for infection. A second similar starvation experiment lasting 15 days was also performed. A third experiment consisting of washing and starvation for 7 days followed by a 30 minute penicillin treatment (100 units/ml. of culture fluid) was also performed; this method is similar to that used to free amebae of adhering bacteria (1).

For irradiation, amebae were taken from the culture that had been starved for 7 days and then fed for 13 days with *Tetrahymena*. They were divided into four groups of twenty-six each; one served as a non-irradiated control and the others were subjected to gamma irradiation (Co^{60} source) totaling 100, 200, or 300 kr at the rate of 5000 r per minute. Following exposure, each ameba was placed in a separate depression cup and observed for death or establishment of a clone.

Lastly vital dyes were used in an attempt to sele-c tively stain the infective organisms and then kill them by proper irradiation. Neutral red (N.R.) (C.I. No. 825) and acridine orange (A.O.) (C.I. No. 788) were obtained from the pharmaceutical laboratories of the National Aniline Division, Allied Chemical Corporation, New York City, and used in neutral aqueous solutions of low toxicity. The amebae were left in dye solutions a few hours to several days during which they were frequently observed by light microscopy. They were thoroughly washed and then exposed in the Argonne biological spectrograph at the maximum absorption wavelengths of the dyes used (N.R. 526 mμ and A.O. 467 and 497 mμ) for 50 to 60 minutes. The total energy for these positions was 1434 (for N.R.) and 1450 (for A.O.) kiloergs/mm.$^{-2}$ respectively.

OBSERVATIONS

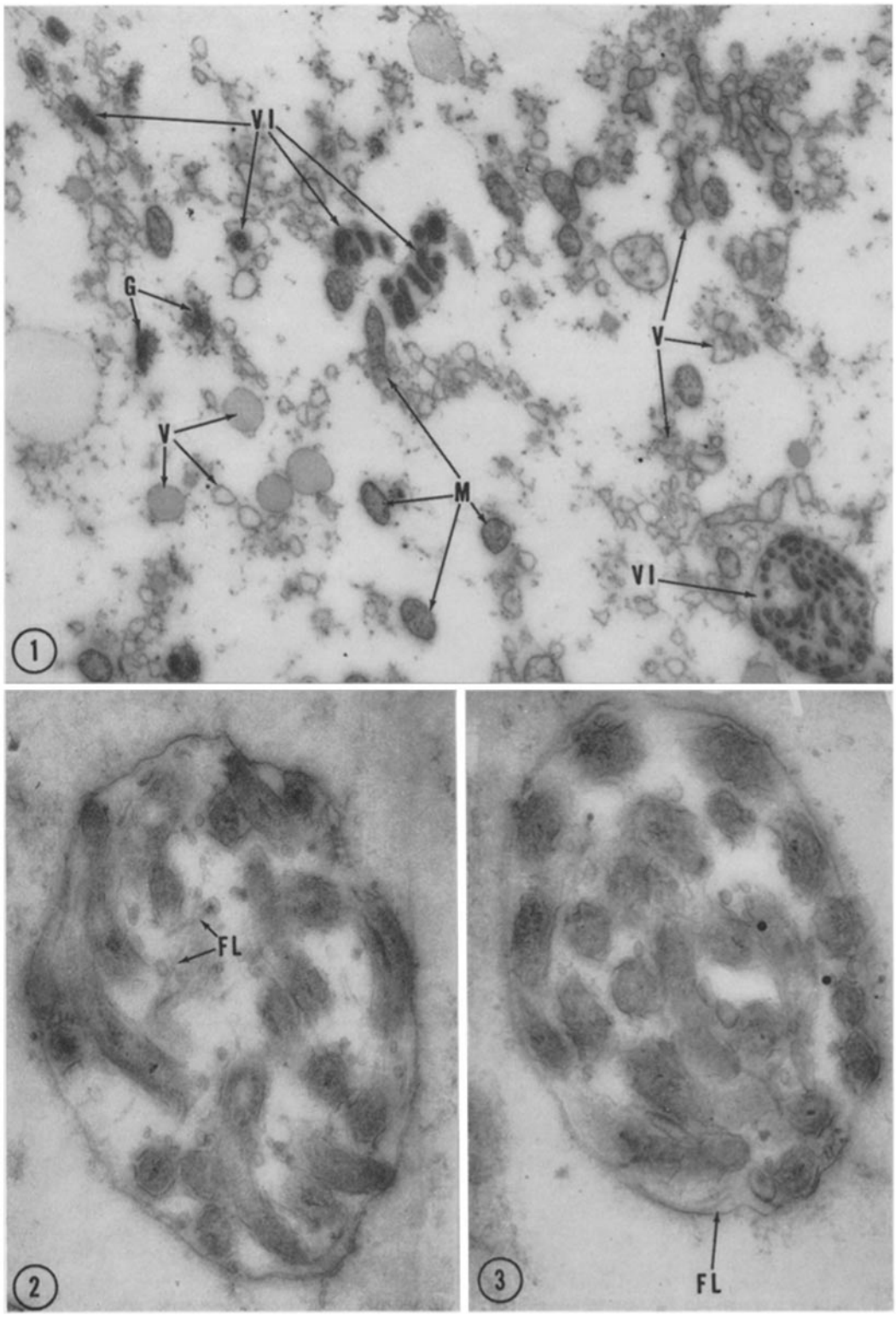
Electron micrographs show that the cytoplasm of *A. proteus* contains many vesicles of several different

FIGURE 1

Survey of cytoplasm in *Amoeba proteus* showing three types of vesicles (*VI*) that contain infective organisms: at upper left, single organisms per vesicle; at center, multiple elements in parallel arrangement; and at lower right, a 4-μ vesicle. Numerous, different vesicles (*V*), mitochondria (*M*), and Golgi bodies (*G*) are also visible. × 5300.

FIGURES 2 AND 3

Enlargements of vesicles that contain numerous organisms. Vesicles of this type show a violent writhing activity when an ameba is ruptured. Phase microscopy further has shown that these freed vesicles "fragment" into individual organisms which may be capable of very rapid locomotion. Those structures indicated (*FL*) may be flagella belonging to the infective organisms. × 28,000.


VI
G
V
V
M
VI
1
FL
2
3
FL

sizes and types (Fig. 1, *V* and *VI*), in addition to mitochondria (Fig. 1, *M*) and Golgi bodies (Fig. 1, *G*). However, a particular type of vesicle is present that is distinguished by relatively dense inclusions that are either circular or elongated when thin sectioned (Fig. 1, *VI*).

In their simplest form, vesicles of this type contain a single inclusion within the vesicle membrane. The inclusion itself has an exterior membrane and a rather homogeneous interior; it measures 0.3 to 0.7 μ in diameter (Fig. 1, upper left, *VI*). A larger, more complex vesicle which measures 3 to 5 μ in diameter is often present (Fig. 1, lower right, *VI*, and Figs. 2 and 3). It has similar inclusions in greater number. A third form is usually elongated rather than spherical and is distinguished by regular interior patterns (Fig. 1, center, *VI*, and Fig. 4). Serial sections demonstrated that this is probably a tightly spirallized structure with as many as twelve turns.

Another vesicle of similar appearance has been observed in thin sections on a few rare occasions. It contains only a few inclusions in a comparatively large volume of fluid (Fig. 5). The vesicle wall is similar to the plasmalemma (Fig. 5, *P*) since its membrane stains with phosphotungstic acid (Fig. 5, *ME*) and has a "fringe" (Fig. 5, *F*).

Two of these forms of vesicles were observed repeatedly by phase contrast microscopy. The coiled form (Fig. 4) appears to be long and narrow and to have cross-striations; it is quiescent and frequent enough to be easily found in most living amebae. The circular vesicles (Figs. 2 and 3) are also quite numerous and are distinguished by a violent internal activity.

In the stock culture fluid, non-motile, rod-shaped, bacterium-like organisms (*ca.* 0.5 x 2 μ) and very rapidly swimming, vibrio-like organisms of about the same size can be seen. Repeated washing frees the fluid from such organisms, so that they do not confuse the observations reported in the following paragraph.

If the washed amebae are now compressed until they rupture, the cytoplasm is spewed into the surrounding fluid. The vesicles with coiled structures (as pictured in Fig. 4) show no movement, but the organisms in the spherical vesicles (Figs. 2, 3) show violent activity for several minutes, sometimes with such force that they are freed from the writhing mass and move rapidly away. Some amebae have a single, large, 10- to 12-μ vesicle containing minute motile forms which are liberated as the ameba and the vesicle burst. Observations of the fluid contents now reveal the presence of many similar fast-moving spirallized organisms (*ca.* 0.2 x 1 μ) which appear to have about one turn of spiral structure and a movement clearly different from Brownian motion.

The preceding description applies only to amebae from the Chicago source. The organisms from the cytoplasm of Carolina amebae are very numerous, rod-shaped and motile, and were seen only when the amebae were ruptured. Neither spherical vesicles with obvious internal activity nor vesicles with coiled structures were observed. No electron microscopic observations or starvation experiments were made on this stock, but these cytoplasmic organisms differ morphologically from those of the Chicago stock.

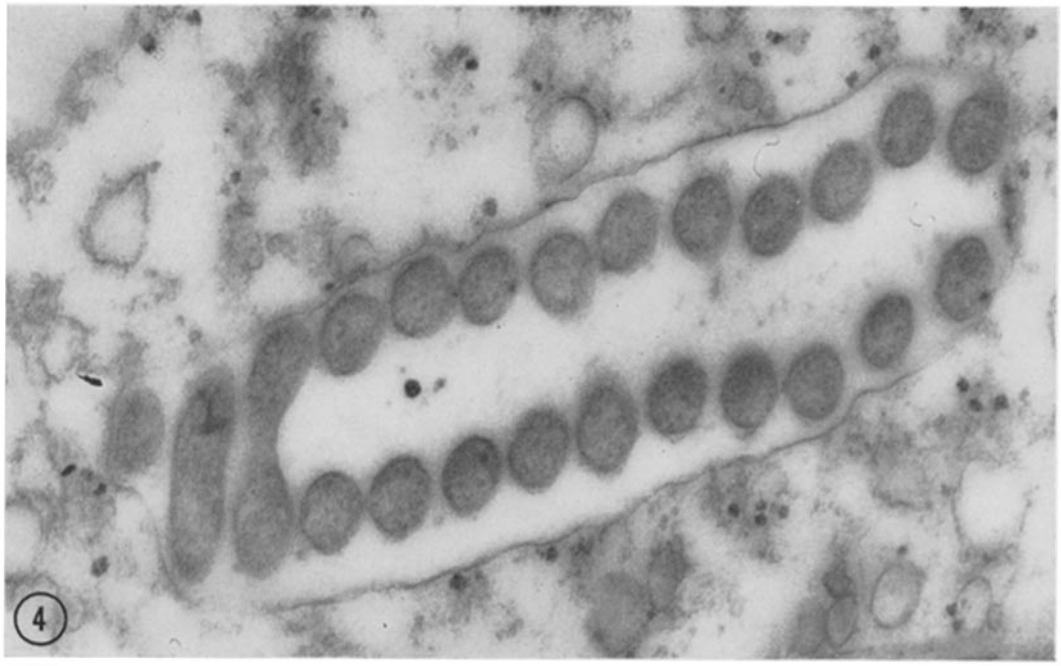
Our attention was turned next to methods of clearing the ameba of the infective organisms; the Chicago stock only was used. Starvation of amebae for 7 days followed by penicillin treatment caused no change in the motility of the infecting organisms. Starvation for 13 days with repeated washing in sterile water failed to produce a significant decrease of infection. At 16 days, although the infection was more difficult to demonstrate

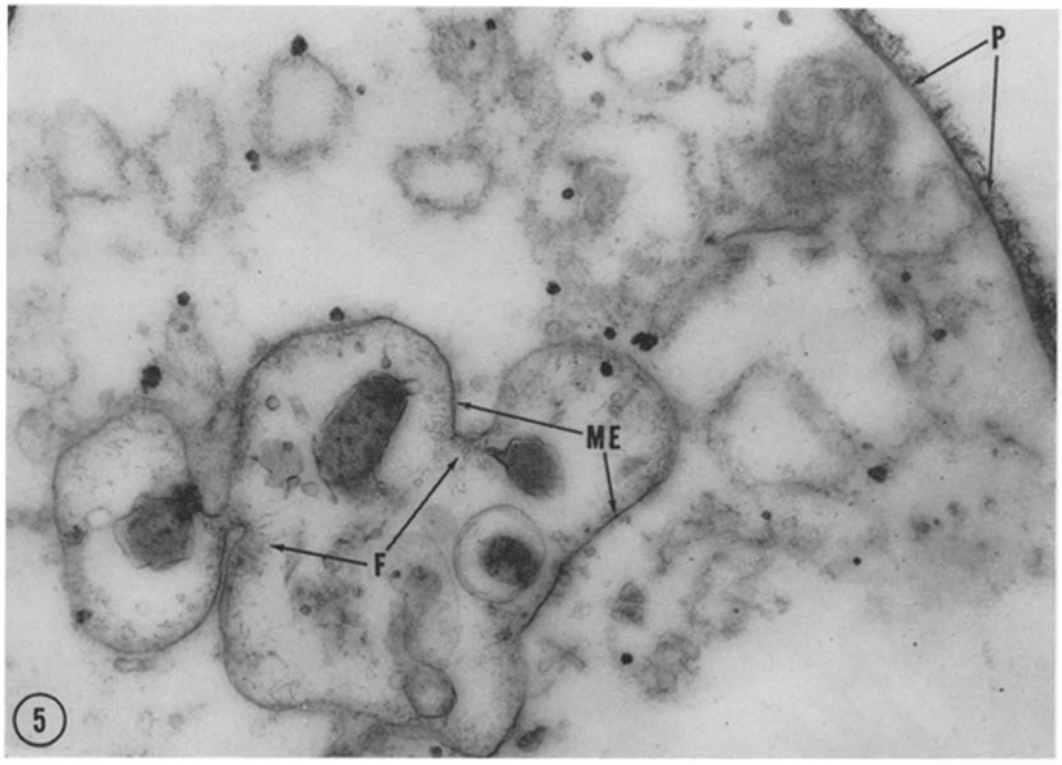
FIGURE 4

Enlargement of a vesicle that contains parallel profiles or paired rows of circular profiles; serial sections show this to be a spiral structure. By phase microscopy, these vesicles usually show no activity when released from ruptured amebae. × 23,000.

FIGURE 5

Sections of four structures which have similar morphology to that of Figs. 2 and 3. However, the vesicle wall has a fringe (*F*) and a membrane which is stained by phosphotungstic acid (*ME*) in a manner similar to the plasmalemma (*P*). This may be an indication that organisms enter the ameba by being included in a pinocytosis vesicle. × 24,000.


4


P
ME
F
5

by phase microscopy, refeeding with washed, axenically grown *Tetrahymena* and the establishment of clones from the survivors, re-established typical infection in all instances. A second group of amebae were repeatedly washed in sterile water at the beginning and at 2-day periods during starvation for 15 days, but this also failed to free the cytoplasm of the infective agents. *Thus, starvation with penicillin treatment did not eliminate the infection.*

Since *Amoeba proteus* is relatively resistant to ionizing radiation (9), this method was used in an attempt to free the cytoplasm of the contaminating organisms. Doses of 300 kr γ-radiation caused no deaths during exposure, although no amebae divided or survived longer than 3 weeks; thus none were examined for infection. Following 200 kr γ-radiation, all except one ameba died within 3 weeks. This survivor divided several times, but the daughter cells that were examined contained typical infections. Of the amebae irradiated with 100 kr γ-rays, fifteen of twenty-six gave rise to clones (58 per cent) as compared to fourteen of twenty-six (54 per cent) in the non-irradiated control group. Phase microscopic examination showed that all clones of the control group were infected; however, a definite reduction of infection was observed in three surviving clones in the 100 kr group. These clones died after several weeks.

Finally, an approach was used which offered the possibility of killing the infective organisms by photodynamic action. Different amebae were placed in separate solutions of neutral red and acridine orange dyes, exposed in the Argonne biological spectrograph at the maximum absorption wavelengths of the dyes used, and then examined with the phase microscope. There was no obvious change in the motility or numbers of infective organisms in the cytoplasm as compared with non-irradiated controls.

DISCUSSION

Infection Related to Studies of Synthetic Processes: There is no doubt that, in the amebae examined, the cytoplasm contains one or more types of infective organisms. Plaut and Sagan (1) suggested that such a possibility exists from their studies of thymidine incorporation, and Brachet (10) has said the same in relation to studies of RNA synthesis. Furthermore, Mercer (11) also pictures electron-dense structures which he calls unknown bodies in his electron microscopic study of another stock of *A. proteus*. We have observed similar structures in an electron microscope study of the giant ameba, *Pelomyxa illinoisensis* (12). The evidence indicates that infection of ameba cytoplasm is probably rather widespread.

Amoeba proteus has been widely used in biochemical cytology. The literature contains accounts of certain controversies that may be resolved by the knowledge that the cytoplasm of at least some *A. proteus* stocks contains living, bacterium-like organisms. The interpretations of experiments that reputedly show incorporation of RNA and DNA precursors in ameba cytoplasm must be regarded with skepticism unless it can be established that the clone used was free of infection. Comparative studies of nucleated and non-nucleated halves (*e.g.*, 13, 14) and the micrurigical studies of transplanted, labeled nuclei (15, 16) are still useful, but would be more decisive if the effect of the infective organisms could be eliminated.

The finding of amebae infections demonstrates that microscopic studies are a necessary prerequisite to studies of synthesis. A stock of cells, preferably a clone, should be investigated carefully and perhaps periodically for infection, and stock designations or sources of supply should be listed in publications. Incorporation studies on organisms other than amebae, *e.g. Acetabularia* (6, 10), should also be more firmly established by the demonstration that no infection is present.

Uninfected Amebae: *A. proteus* stocks which are free of infection are needed. Attempts to this end have been made using five methods: repeated washing in sterile water, starvation, γ-irradiation, clone selection, and photodynamic action. Our results thus far show that no one of these is sufficient alone; in fact, it is improbable that even combinations are effective in eliminating infection.

The survey of existing stocks is another method which should be considered as a practical and promising approach. From our evidence indicating stock variations it may be possible to find organisms which are free of infection or have an infection amenable to treatment. The organisms used by Prescott (3, 4) which show no cytoplasmic incorporation of labeled RNA precursors may be an uninfected stock.[1] Once established, freedom from infection can undoubtedly be maintained by feeding only axenically grown *Tetrahymena* and *Chilomonas*.

[1] A recent communication from Dr. Prescott informs us that this stock is no longer in existence.

The determination of freedom from infection should rest at least on the following three methods: (*a*) phase microscopy of living and recently ruptured organisms, (*b*) electron microscopy at a survey level using 200 mμ sections, and (*c*) autoradiography with tritiated thymidine.[2] One or two methods alone cannot, if negative, be regarded as establishing freedom from infection; all three are subject to limitations.

Mode of Entry of Infective Organisms: Although the infection could be maintained by inclusion of infective organisms in each daughter at division, our electron microscope observations suggest that pinocytosis or phagocytosis is a means of entry. In a study of pinocytosis and food vacuoles in amebae (17), it was shown that the plasmalemma retained its fringe and phosphotungstic acid (PTA) stainability for only a short time after it had been changed into a vacuole membrane. The observation of vacuoles that contain structures similar to those of the 3- to 5-μ vesicles, which have a fringe and which are stainable with PTA, may indicate entry by inclusion in pinocytosis or phagocytosis vesicles. However, this does not exclude the possibility that the infective organism forces its way into the cytoplasm and is walled off into a vesicle which appears as if produced by pinocytosis or that some vesicles may burst inside of the cytoplasm.

Relation of Infective Organisms to Host Amebae: Although infective agent(s) found in the cytoplasm of amebae can explain certain autoradiographic findings, their effect on and their total relationship to host amebae are not clear. Whether the different forms found in the cytoplasm are separate species of various stages in the life cycle of a single species has likewise not been determined. For further information on this problem, the reader is referred to a stimulating discussion of bacterial parasitism and symbiosis in protozoa (18). Several species of bacteria are reported to be present in the giant ameba, *Pelomyxa palustris*, and other species that are coccus-, rod-, or spiral-shaped are reported in other protozoa.

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[2] A positive finding of thymidine incorporation in the cytoplasm could not be considered conclusive evidence for infection, since final evidence against normal DNA synthesis in the cytoplasm is lacking.