THE INFLUENCE OF THE MODE OF

NUTRITION ON THE DIGESTIVE SYSTEM OF

OCHROMONAS MALHAMENSIS

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ABSTRACT

The intracellular distribution and level of acid hydrolases in *Ochromonas malhamensis* were studied in cells grown osmotrophically in a defined medium, in a carbon-free starvation medium, and during phagotrophy in each of these media. By cytochemical techniques, little enzymic reaction product was observed in the vacuoles of osmotrophic cells grown in the defined medium. Starved cells, however, contained autophagic vacuoles and cannibalized other *Ochromonas* cells. Dense enzymic reaction product was observed in the digestive vacuoles and in the Golgi cisternae of these starved cells. Moreover, starved cells and cells grown in a nutritionally complete medium ingested *Escherichia coli* which appeared in digestive vacuoles containing enzymic reaction product. Biochemical assays for lysosomal acid phosphatase (E.C. 3.1.3.2 orthophosphoric monoester phosphohydrolase) and acid ribonuclease (E.C. 2.7.7.16 ribonucleate nucleotido-2'-transferase) were done on *Ochromonas* cultures in the same experimental treatments and under identical assay conditions as the cytochemical study. During starvation, the acid hydrolase specific activities were consistently twice those found in cells grown in an osmotrophic complete medium. *Ochromonas* fed *E. coli* showed no increase in acid hydrolase specific activity as compared to controls not fed *E. coli*. The latency of lysosomal acid hydrolases in cells fixed with glutaraldehyde was reduced, suggesting that this fixative increases lysosomal membrane permeability and may release enzymes or their reaction products into the cytoplasmic matrix during cytochemical analysis. This could explain the cytoplasmic staining artifact sometimes observed with glutaraldehyde-fixed cells when studied by the Gomori technique. This study confirms that *Ochromonas malhamensis*, a phytoflagellate, does produce digestive vacuoles and can ingest bacteria, thereby fulfilling its role as a heterotroph in an aquatic food chain. When *Ochromonas* is grown in a nutritionally complete osmotrophic medium, phagocytosis causes appearance of acid hydrolases in the digestive vacuoles, whereas the total activity of the enzymes remains unchanged. An organic carbon-free medium strongly stimulates acid hydrolase activity and causes these enzymes to appear in the digestive vacuoles whether phagocytosis occurs or not.

INTRODUCTION

The nutritional requirements and ultrastructure and subcellular distribution of several marker enzymes of *Ochromonas malhamensis* have been reported previously.

Aaronson and Baker (1) demonstrated that this chrysomonad flagellate obtains biotin through phagotrophy of *Thiobacillus*.

Anderson and Roels (2) studied the ultrastructure of *Ochromonas malhamensis* and reported that many cells grown in an osmotrophic medium contain a large central vacuole which appears to persist throughout the life of the cell. They found that myelin bodies in the large central vacuole of *O. malhamensis* appear to degenerate into amorphous masses, suggesting that digestive activity occurs in these vacuoles.

In the general framework of studies on marine and fresh water food webs now under way in our laboratories, Lui et al. (28) demonstrated the presence of lysosomes in this organism. These organelles are undoubtedly important for the digestion of intracellular and ingested materials.

This paper reports the effects of osmotrophic nutrition, of phagocytosis, and of starvation on the distribution and the level of lysosomal acid phosphatase (E.C. 3.1.3.2 orthophosphoric monoester phosphohydrolase) and acid ribonuclease (E.C. 2.7.7.16 ribonucleate nucleotido-2'-transferase) in this organism.

MATERIALS AND METHODS

Cell Culture

Ochromonas malhamensis was grown at 24–25°C in Hutner's medium (24) under conditions previously reported from this laboratory (28). Under these conditions, the cells reached the end of the logarithmic growth phase after 150 hr.

Cultures for starvation studies were grown for 3 or 4 days in Hutner's medium (24), collected by centrifugation (36), and transferred to the starvation medium described by Myers and Graham for this organism (32), where they were maintained for 24 hr at 24–25°C at 50 footcandles of illumination from cool white fluorescent tubes. *Escherichia coli* was used for phagocytosis experiments. The bacteria were grown in Difco nutrient broth (Difco Laboratories, Detroit, Mich.) at 25°C for 16–24 hr, centrifuged at 915 g for 15 min, washed with *Ochromonas* starvation medium, and added live to the *Ochromonas* cultures for 2 hr or heat-killed and added for 6, 16, or 22 hr before the cultures were harvested for experimentation. When the *Ochromonas* were subsequently harvested and washed, noningested bacteria remained in the supernatant and were discarded.

In this investigation we used: a) 4 and 5 day old cultures; b) 3 or 4 day old cultures, starved for 6, 16, 22, and 24 hr; c) 4 or 5 day old cultures fed *E. coli* for 2, 6, 16, and 22 hr; and d) 3 or 4 day old cultures, starved for 24 hr, and then fed *E. coli* for 2 hr or

boiled *E. coli* for 6, 16, and 22 hr immediately after the *Ochromonas* was added to the starvation medium. Only those cells fed *E. coli* for 2 and 16 hr were examined with the electron microscope. Light microscopic cytochemistry was done on cells grown for 16 hr in a complete medium, in a starvation medium, or in a starvation medium and fed bacteria.

Cytochemical Procedure — Electron Microscopy

100 ml of cell culture containing 9×10^6 cells per ml were fixed for 2 hr at 0°C with an equal volume of cold 4% (w/v) glutaraldehyde solution (Polysciences, Inc., Rydall, Pa.) prepared in 0.25 M cacodylate buffer, pH 7.1.

The cells were centrifuged at 391 g for 5 min, and the pellet was resuspended in 40 ml of 0.05 M acetate buffer, pH 5.0, and sedimented at 153 g for 4 min. Cells were washed three times by this procedure. Before the final wash, the cell suspension was divided equally into three portions and sedimented separately.

One pellet containing approximately 3×10^8 cells was suspended in 30 ml of Gomori acid phosphatase medium containing sodium-β-glycerophosphate (Fisher Scientific Co., Pittsburgh, Pa.) and incubated at 10°C for 30 min (20, 21, 22, 23). The remaining two pellets were used for control experiments. Freshly prepared medium was kept at 37°C for 1 hr and filtered before use (17). In the lead nitrate control, the substrate was omitted from the incubation medium, and in the NaF control, the enzyme was inhibited by adding 0.01 M NaF to the complete incubation medium (19, 22).

The acetate rinse which follows the enzymic incubation originally recommended in the Gomori technique (20) was omitted to avoid the introduction of artifacts (18). Instead, the ochromonads were rinsed after incubation in glass-distilled water and postfixed for 3 hr in 1% osmium tetroxide buffered with 0.05 M cacodylate buffer, pH 7.1. The post-fixed cells were rinsed with glass-distilled water, dehydrated in a graded series of ethanol solutions, cleared in propylene oxide, and embedded in Epon 812 (27).

Thin sections were cut with glass knives or diamond knives on a Porter-Blum MT-2 ultramicrotome and mounted on carbon-coated grids. Some sections were poststained for 10 min with Reynolds' lead citrate (35); other sections were viewed unstained to check if the poststain would interfere with the visualization of the lead phosphate reaction product. Electron micrographs were taken with a Philips EM-200 operating at 60 kv.

Cytochemical Procedure — Light Microscopy

Cell cultures were harvested and fixed as described for electron microscopy. The method of Burstone (7) was used to visualize the acid phosphatase reaction
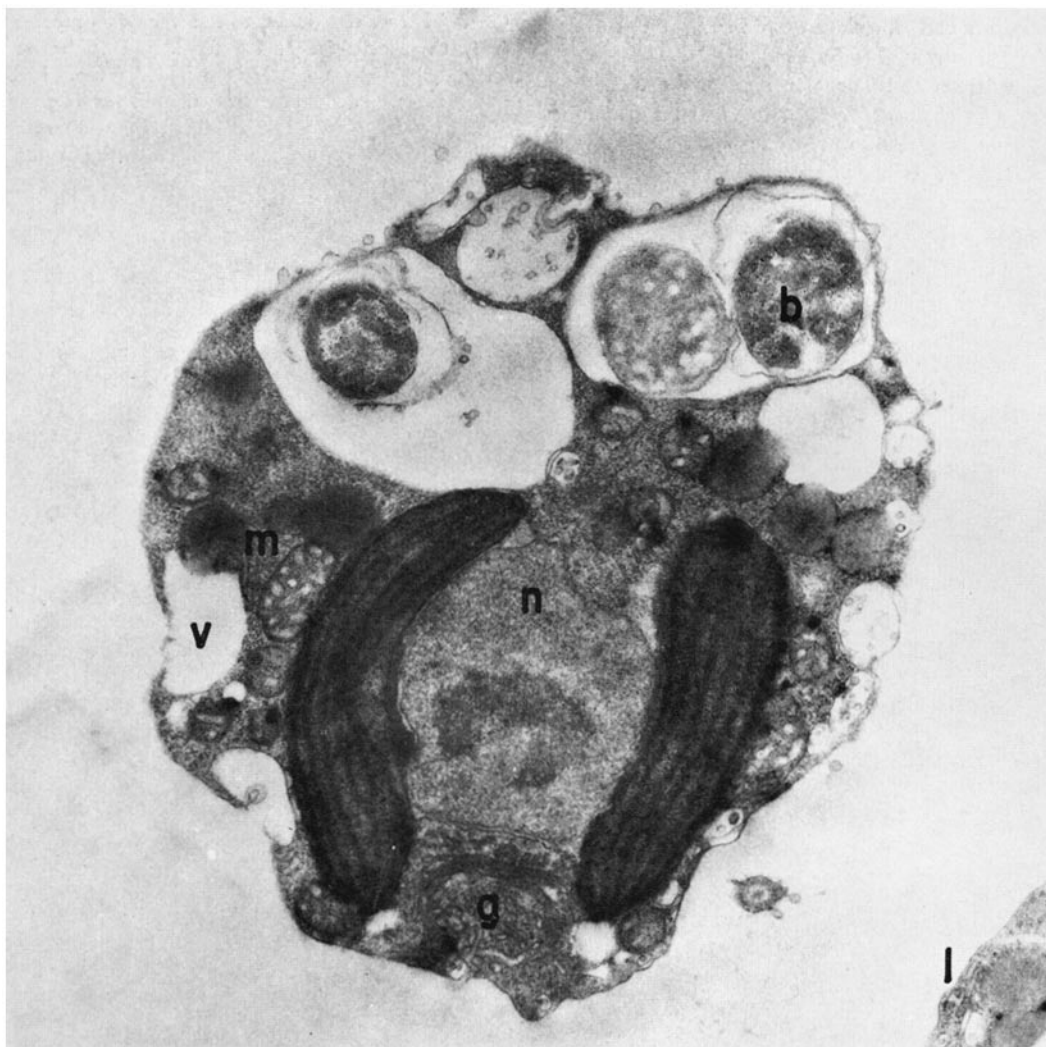
Figure 1 An osmotrophically grown cell containing ingested bacteria. There is no reaction product present in the nucleus (n), Golgi complex (g), mitochondria (m), or bacteria (b) of this sodium fluoride control. × 21,700.

product. Naphthol AS-BI phosphoric acid (Sigma Chemical Co., St. Louis, Mo.) was used as a substrate with Fast Red Violet—LB as a diazonium salt. The cytochemical reaction was done at 25°C for 4 hr in 0.2 M acetate buffer, pH 5.1.

Enzyme Assay

Acid phosphatase (E.C. 3.1.3.2 orthophosphoric monoester phosphohydrolase) was determined with β-glycerophosphate substrate at pH 5, according to the method of Gianetto and de Duve (16), but we incubated at 10°C for 30 min. These conditions were identical to those used in the cytochemical assays.

Acid ribonuclease (E.C. 2.7.7.16 ribonucleate nucleotido-2′-transferase) was assayed according to the method of de Duve et al. (12).

Enzyme activities are expressed in milliunits/mg protein and also in milliunits/cell, one unit being the amount of enzyme necessary to release 1 μmole of product/min under the assay conditions.

To determine the latency of the enzymes, "free" enzyme activity (without Triton X100) and "total" (with 0.1% Triton X100 present) enzyme activity were assayed in 0.25 M sucrose at 10°C for 30 min. Triton X100 (0.1%) was present in all assays, except when "free" activity was measured.
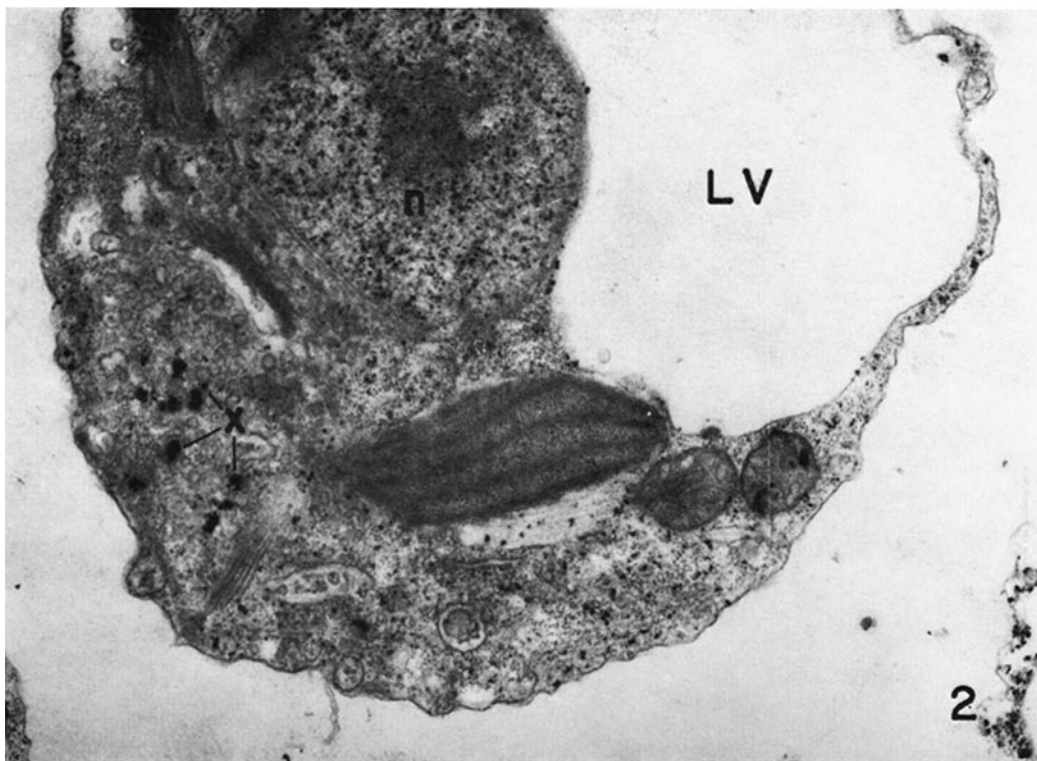
FIGURE 2 A Gomori-stained cell from an osmotrophic culture contains reaction product (x) in clearly defined vesicles and also scattered throughout the cytoplasm and nucleus (n). The vacuole (LV) contains little reaction product. × 24,700.

In the latency experiments with glutaraldehyde, the concentration of glutaraldehyde in the enzymic reaction mixture was 2%; otherwise, the assay conditions were the same. In these experiments, whole cells were used in an attempt to duplicate the conditions used for the cytochemical assay.

In the phagocytosis experiments, all the protein contained in the *E. coli* suspension, added to the *Ochromonas* culture, amounted to 3% of the total protein contained in the *Ochromonas*. After the ingestion period, the *Ochromonas* cells were harvested by centrifugation and washed as described previously (28). In this procedure the noningested bacteria were removed in the supernatants.

RESULTS

Cytochemical Experiments

In this report, large central vacuole (LV) will be used to denote a central massive vacuole as shown in Figs. 2 and 7. Vacuole (v) will be used to describe smaller vacuoles in the peripheral cytoplasm (Fig. 1). When vacuoles contain bacteria or ingested particulate matter, they will be called food vacuoles. Vesicles (gv) appear in the Golgi region (Fig. 4).

CONTROLS To exclude the possibility of nonspecific lead deposition which might be mistaken for enzymic product, two controls were run. No lead precipitate was observed in the lead nitrate controls or in the NaF controls (Fig. 1).

PHOSPHOHYDROLASE IN CELLS GROWN IN A COMPLETE MEDIUM Lead phosphate reaction product was consistently found in the cytoplasmic matrix and nucleus (n) of cells (Fig. 2) grown in Hutner's complete medium (24).

Occasionally, heavy stain (x) was seen in vesicles (Fig. 2) of some sectioned cells.

PHOSPHOHYDROLASE IN "STARVED" CELLS Cells grown in Hutner's complete medium (24) and subsequently starved for 16 or 24 hr contained lead phosphate reaction product in the vacuoles (v) (Fig. 3) and in the Golgi complex (gc) of most sectioned cells (Fig. 4).
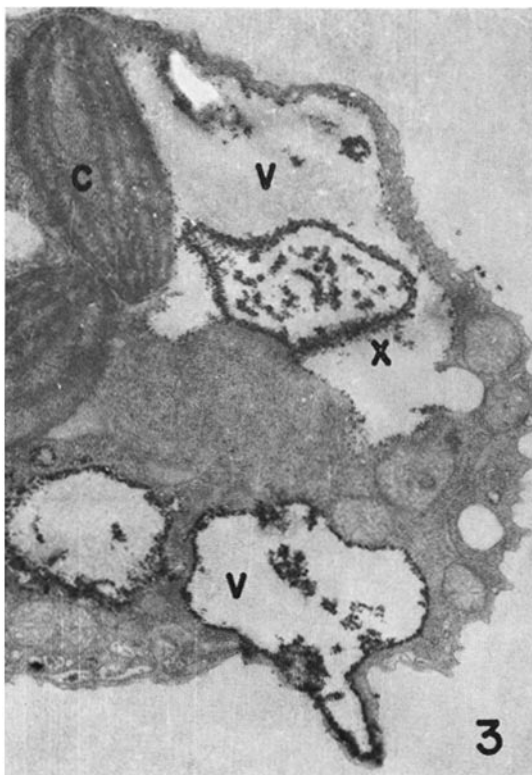
FIGURE 3 The vacuolar region of a starved cell contains reaction product (x) in the vacuoles (v). No reaction product is in the chloroplast (c). Gomori stained. × 18,500.

Cytoplasmic reaction product was seen in some starved cells (Fig. 4) similar to that appearing in cells grown in the complete medium. Some starved cells contained reaction product only in the vacuoles (Fig. 3).

AUTOPHAGY Autophagy was never observed in cells from 4 or 5 day cultures grown in Hutner's complete medium (24). Autophagy appeared in cells kept for 24 hr in the starvation medium.

Fig. 5 shows cytoplasm sequestered in double-membraned, autophagic vacuoles of a cell starved for 24 hr. A lipid droplet (li) is included within one of the vacuoles, whereas the second autophagic vacuole contains only cytoplasmic matrix (h).

The presence of reaction product (x) and degradation of cytoplasm, partially isolated within a vacuole system (Fig. 6) in a cell containing ingested bacteria (b), indicates that autophagy and phagotrophy may occur in the same cell.

PHAGOCYTOSIS Both starved cells and cells

grown in the complete medium ingested particulate food substances.

Cannibalism was observed in cultures starved for 16 and 24 hr. Fig. 7 shows an ingested ochromonad (i) in the large vacuole of a cell containing enzymic reaction product.

A culture of O. malhamensis grown in Hutner's medium (24) and a similar culture grown in starvation medium were allowed to ingest E. coli for 2 hr or 16 hr before being fixed. Some of the food vacuoles (v) formed in normal cells during the ingestion period contain bacteria (b) with enzymic reaction product (x) (Fig. 8).

Fig. 9 shows Gomori stain in the Golgi complex (gc) of cells fed E. coli for 16 hr in Hutner's medium. Starved cells fed E. coli for 2 hr or 16 hr also contained ingested bacteria and Gomori stain in their vacuoles and Golgi complex.

LIGHT MICROSCOPY Light microscopic, cytochemical evidence confirmed that starved cells contained much more azo dye reaction product in their large vacuoles than cells grown in a chemically complete medium. The small size of Ochromonas cells (5 μ) and the great difficulty in distinguishing ingested E. coli made it impossible to identify food vacuoles. Also, it was not possible to localize the Golgi complex.

Biochemical Experiments

Table I shows that the lysosomal phosphatase and ribonuclease activity of cells starved for 24 hr, or starved for 24 hr and then fed E. coli for 2 hr, was twice as high as in cells grown in the complete medium, whether the cells had been fed bacteria or not. In the individual experiments, which were averaged to obtain the data reported in Table I, O. malhamensis from the same culture underwent the four nutritional treatments listed. Although there was some variation in the specific enzyme activity of different cultures grown in the complete medium, starvation for 24 hr always doubled this activity.

When interculture differences were neglected, the increase in specific enzyme activity due to starvation was always very close to 100%. Phagocytosis of bacteria for 2, 6, 16, 22, or 24 hr, in either the normal or the starvation medium, did not significantly alter the level of acid phosphatase in the cells (Tables I and II). However, starvation caused a gradual increase in acid phosphatase, which reached 100% increase after 24 hr, whether the cells were phagocytizing or not.
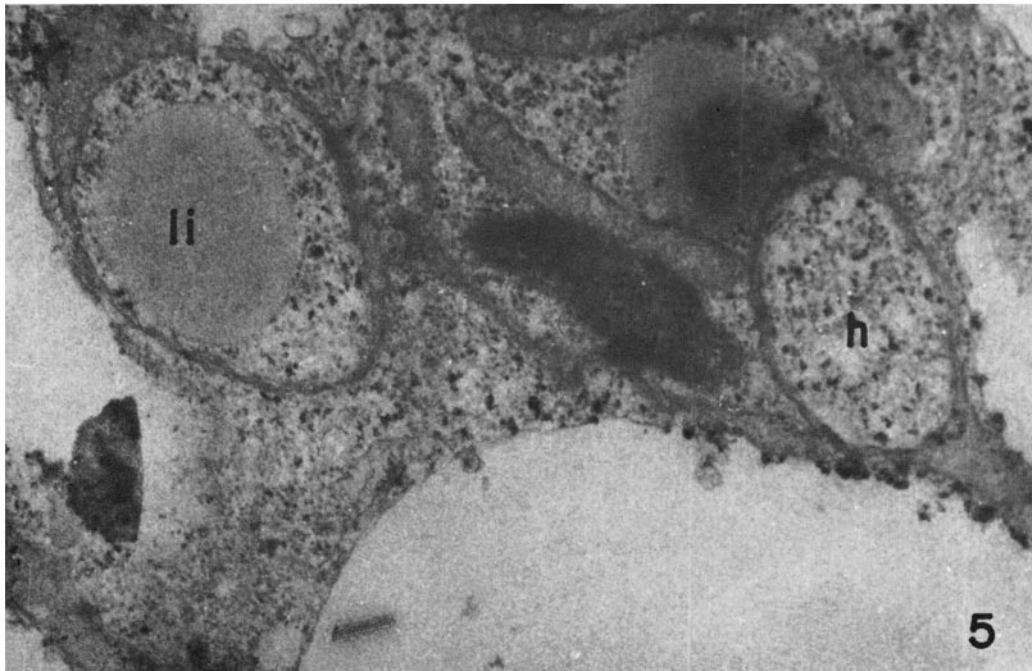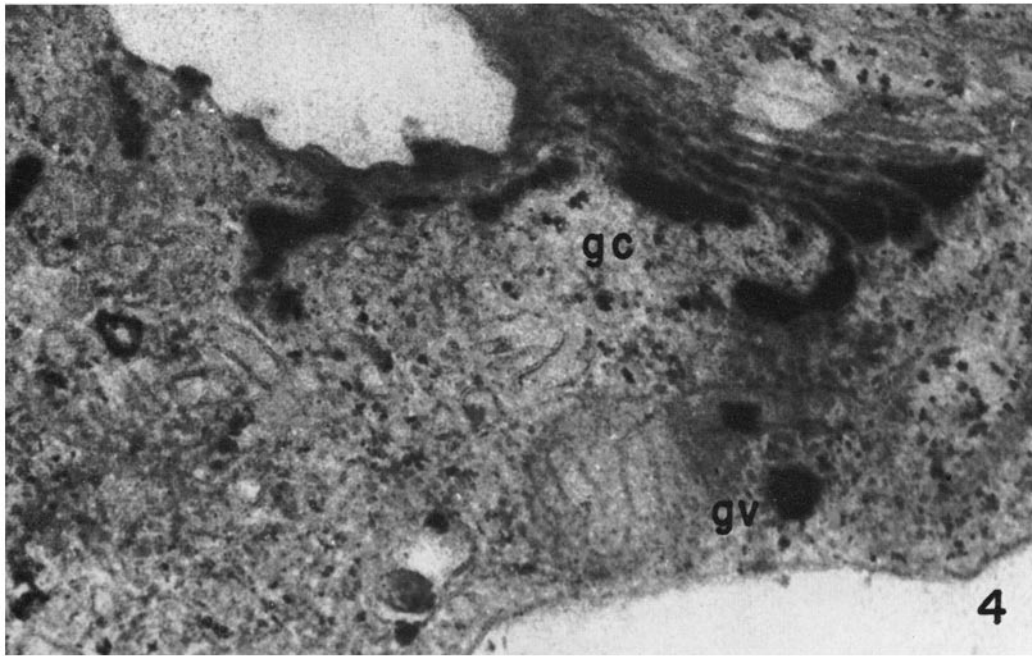
FIGURE 4 A cell starved for 24 hr contains Gomori reaction product in the Golgi complex (gc) and Golgi vesicle (gv). × 81,900.

FIGURE 5 A lipid inclusion (li) and cytoplasm (h) are sequestered within starvation-induced autophagic vacuoles surrounded by double membranes in this Gomori-stained cell. × 43,500.
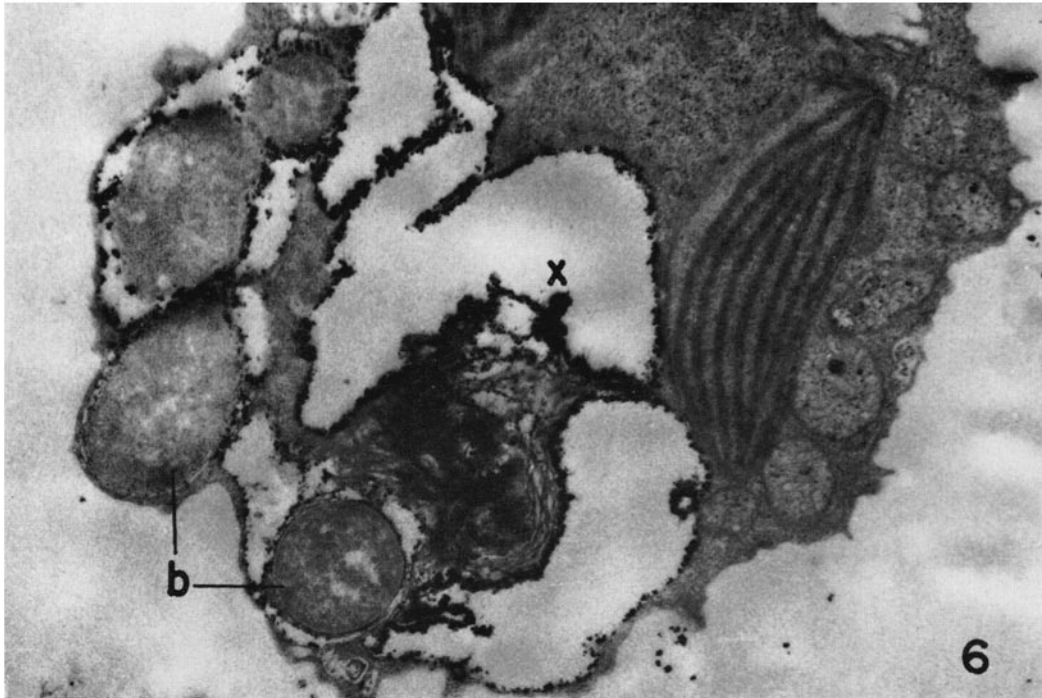
FIGURE 6 A starved cell fed *E. coli* for 2 hr contains ingested bacteria (*b*). Gomori reaction product (*x*) is abundant in the digestive vacuoles. Little reaction product is in the cytoplasm or chloroplast. × 28,800.

Table III shows that glutaraldehyde lowered the specific activity of acid phosphatase in *O. malhamensis.*

The addition of glutaraldehyde also decreased the latency of this enzyme in the whole cells, which indicates that the substrate was more easily accessible to the enzyme in both normal and starved cells.

The standard deviations for the latency of the enzyme are large due to differences among cultures.

When the latency of the enzyme in the glutaraldehyde-treated cells was compared to the latency of the enzyme in untreated cells from the same culture, glutaraldehyde treatment consistently increased the "free" activity two- to threefold.

We found that glutaraldehyde by itself gives color in the Lowry and Lopez reaction (26) which was used for our phosphate determinations. However, we compensated for this increased color intensity due to the added glutaraldehyde by subtracting the appropriate blank values.

DISCUSSION

Lui et al. (28) found that β-glycerophosphate phosphatase in *O. malhamensis* has a broad pH curve with two peaks at pH 5.4 and pH 6.3, respectively. These investigators believe that the same lysosomal phosphatase can react with either glucose-6-phosphate or β-glycerophosphate as substrate. The subcellular distribution and the latency of all acid hydrolases they tested (cathepsin, ribonuclease, acid phosphatase) were similar, which clearly indicates the presence of lysosomes in *O. malhamensis* that are grown osmotrophically in Hutner's complete medium (24). Lui et al. (28) used the same substrate, buffer, incubation temperature, and pH for some of their phosphatase assays as were used in the cytochemical and biochemical studies reported here, except, of course, for the glutaraldehyde fixation of the cells and the presence of lead nitrate in the incubation mixture in the cytochemical assays.

In *O. malhamensis,* lead phosphate reaction product was found in the nucleus and in the cyto-
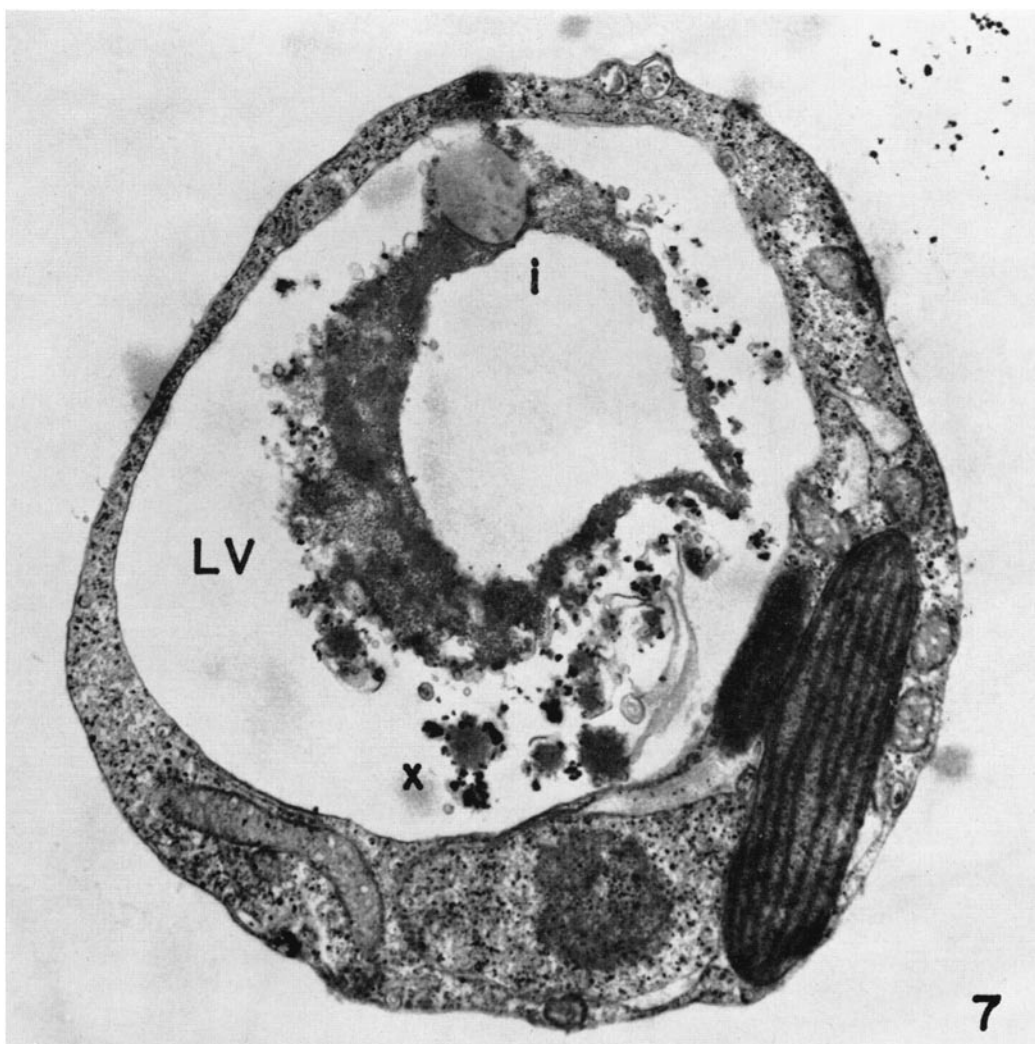
Figure 7 A starved cell contains a partly degraded mass of cytoplasm (*i*) in the large vacuole (*LV*). The ingested matter is probably a small ochromonad. Gomori reaction product (*x*) clearly demonstrates enzymic activity in the vacuole. × 22,100.

plasmic matrix of all cells. The absence of reaction product in these locations in the lead nitrate and sodium fluoride controls clearly indicates that the nuclear and cytoplasmic reaction products were of enzymic origin. Most cytochemists regard nuclear localization of acid phosphatase by the Gomori technique (β-glycerophosphate substrate, pH 5.0) as nonspecific staining or a diffusion artifact (23), because nuclear staining cannot be demonstrated with any of the azo dye methods (7). De Jong et al. (14), however, using both the

Gomori technique and the Burstone azo dye method, claimed that glutaraldehyde activates acid phosphatase in the nuclei of cultured tobacco cells. Our quantitative biochemical results (Table III) indicate that glutaraldehyde lowered the specific activity of acid phosphatase and decreased the latency of this enzyme when whole cells were assayed under conditions similar to those used in the cytochemical procedure. We therefore believe that the cytochemical reaction product observed in the nucleus and cytoplasmic matrix may well
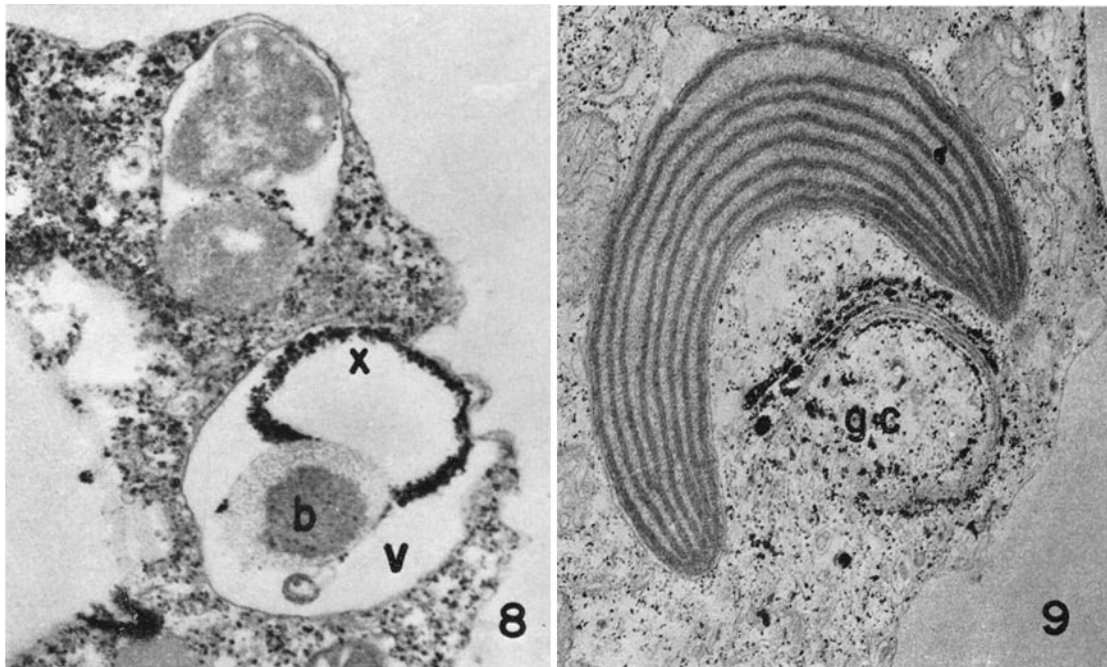
FIGURE 8 Bacteria (b) after 2 hr of feeding have been ingested in vacuoles (v) of a cell grown in Hutner's medium. Gomori reaction product (x) is adjacent to a bacterium in one of the vacuoles. Light Gomori stain is present in the cytoplasm. × 27,000.

FIGURE 9 Gomori stain fills the Golgi cisternae (gc) of a cell grown in Hutner's medium and fed E. coli for 16 hr. × 25,000.

TABLE I

Effect of Different Nutritional Treatments on the Specific Activity of Acid Phosphatase and Ribonuclease in Ochromonas malhamensis

Nutritional condition of the cells	Acid phosphatase activity		Acid ribonuclease activity	
	milliunits/mg protein	milliunits/cell	milliunits/mg protein	milliunits/cell
Grown in the complete medium	$11.5 \pm 2.2^*(6)$†	$2.1 \times 10^{-7}(2)$	$2.3 \pm 0.3(2)$	$3.1 \times 10^{-8}(1)$
Grown in the complete medium and fed E. coli	$12.9 \pm 3.9(3)$	$2.4 \times 10^{-7}(1)$	$2.5 \pm 0.3(2)$	$2.6 \times 10^{-8}(1)$
Starved	$24.9 \pm 4.7(6)$	$4.1 \times 10^{-7}(2)$	$5.3 \pm 0.6(2)$	$8.5 \times 10^{-8}(1)$
Starved for 24 hr and fed E. coli for 2 hr	$25.6 \pm 5.9(5)$	$4.0 \times 10^{-7}(1)$	$5.0 \pm 0.5(2)$	$6.1 \times 10^{-8}(1)$

* Standard deviation.
† The figures in brackets indicate the number of times the experiments were repeated with different cultures.

TABLE II

Effect of Phagocytosis and Starvation on the Acid Phosphatase Level of Ochromonas malhamensis at Different Time Intervals

Cells were grown in the complete medium for 4 days. Half of them were harvested and washed once with starvation medium and then transferred to the starvation medium at a concentration of 5×10^6 cells per ml. Boiled *E. coli* was then fed to the cells both in the complete medium and in the starvation medium. 6, 16, and 22 hr after the addition of bacteria, acid phosphatase activity was determined in the cultures. Controls were part of the same cultures, grown under exactly the same conditions without added bacteria

Nutritional condition of the cells	Acid phosphatase activity		
	6 hr	16 hr	22 hr
	milliunits/mg protein		
Grown in the complete medium	12.6 ± 0.6*(2)†	12.4 ± 0.2(2)	13.6 ± 1.8(2)
Grown in the complete medium and fed *E. coli*	13.4 ± 0.2(2)	13.1 ± 0.4(2)	14.3 ± 2.9(2)
Starved	15.2 ± 0.7(2)	19.1 ± 1.3(2)	22.4 ± 3.2(2)
Starved and fed *E. coli*	15.6 ± 0.4(2)	18.9 ± 0.3(2)	22.1 ± 4.0(2)

* Standard deviation.

† The figures in brackets indicate the number of times the experiments were repeated with different cultures.

TABLE III

Effect of Glutaraldehyde on the Specific Activity and Latency of Acid Phosphatase in Ochromonas malhamensis

Nutritional condition of the cells	Glutaraldehyde concentration in the enzymic assay	Acid phosphatase specific activity	Latency of the enzyme*
	%	*milliunits/mg protein*	
Grown in the complete medium	0	11.5 ± 2.2‡(6)§	13.1 ± 3.8(5)
	2	10.0 ± 3.1(6)	36.1 ± 12.1(5)
Grown in the complete medium and fed *E. coli*	0	13.0 ± 3.9(3)	
	2	8.9 ± 2.0(3)	
Starved	0	24.9 ± 4.7(6)	17.8 ± 12.4(5)
	2	17.9 ± 5.0(6)	34.6 ± 9.5(5)
Starved and fed *E. coli*	0	25.6 ± 5.9(5)	17.4 ± 6.2(4)
	2	16.4 ± 5.8(5)	46.4 ± 14.5(2)

* $\dfrac{\text{Free activity}}{\text{Total activity}} \times 100.$

‡ Standard deviation.

§ The figures in brackets indicate the number of times the experiment was repeated with different cultures.

be due to diffusion of enzyme or of enzymic reaction product caused by glutaraldehyde-induced changes in lysosomal membrane permeability. However, the presence of phosphatases in these regions of the cells cannot be excluded. The lowering of the specific activity in the presence of glutaraldehyde may be due to the release of proteolytic enzymes caused by these changes in membrane stability.

We found no cytochemically-detectable acid phosphatase activity by both the Gomori and azo dye methods in the vacuoles of most cells

grown osmotrophically in a synthetic medium. Similarly, no stain was observed in the Golgi complex of osmotrophic cells by the Gomori technique; the azo dye method was inconclusive in this respect because of the lack of resolution of the light microscope. Heavy acid phosphatase activity was detected in vesicles of a few cells (Fig. 2, x). These vesicles are probably primary lysosomes; their small size and low numbers make their cytochemical detection difficult. De Duve and Wattiaux (13) remark that "primary lysosomes, if they exist, must be too small or too rare for easy cytochemical detection in many cell types."

Phagocytosis

Protozoan phagocytosis and metazoan phagocytosis are remarkably similar. Mouse mononuclear phagocytes growing in tissue culture (8, 9, 10) and protozoa growing in nutritionally complete osmotrophic media (38, 39) form few cytoplasmic vacuoles, and acid hydrolases are localized in vesicles. Upon phagocytic stimulation, however, cytoplasmic vacuoles increase in number and acquire lysosomal enzyme activity as demonstrated by Gomori stain (8, 9, 10, 15, 29, 38, 39).

Müller et al. (29) found that *Paramecium multimicronucleatum* forms acid phosphatase-positive vacuoles when polystyrene latex beads, bacteria, or proteins are ingested. They employed both the azo dye method and the Gomori method (30). The occurrence of acid phosphatase in digestive vacuoles has been demonstrated in many different types of protozoa (31). *Tetrahymena pyriformis* forms few food vacuoles when cultured in an axenic, chemically-defined medium (38, 39), whereas phagocytosis increases vacuolization (15).

Ochromonas malhamensis responds in a similar manner, i.e., the vacuolar reaction product was intensified when *O. malhamensis* was stimulated by phagocytosis. The absence of cytochemically-detectable acid phosphatase activity in the vacuoles of cells in the osmotrophic medium is understandable since the cells obtain all essential nutrients by absorption and there is less requirement for digestive activity. As in *T. pyriformis* (15), the ingestion of particulate food results in increased acid phosphatase-staining in the food vacuoles. This clearly confirms the catabolic role of the large vacuoles in *O. malhamensis*, as suggested by our previous observations that myelin-like bodies are degraded in the large central vacuole of this organism (2).

Food vacuoles in protozoa are formed by membrane invagination which engulfs food particles and a small amount of surrounding medium (31, 38, 39). It is not known whether food vacuoles in *O. malhamensis* can merge with the preexisting vacuoles in the cell or whether ingested materials go directly into the preexisting vacuoles. It seems most likely that ingested particulate food substances may go into newly-formed vacuoles in the peripheral cytoplasm of the cell and that these vacuoles subsequently merge with preexisting vacuoles. The membranous elements surrounding two of the bacteria in Fig. 1 may be remnants of the phagocytic vacuole which has become engulfed in a digestive vacuole. The merging of primary and secondary lysosomes with phagocytic vacuoles has been inferred from observations of *T. pyriformis*, kidney cells, and L fibroblast cells (15, 22, 40).

The biochemical data, reported in Tables I and II, show clearly that phagocytosis alone does *not* significantly increase the specific activity of the acid hydrolases, whether the cells were grown in the complete or in the starvation medium.

Autophagy

The induction of autophagy in the flagellate, *Euglena gracilis*, was demonstrated by Brandes et al. (6), who showed that cells grown in a carbon-free medium produced many vacuoles and segregated portions of their cytoplasm in cytolysosomes, containing dense deposits of Gomori reaction product. A fivefold increase in acid phosphatase activity was observed in starved *Euglena* when compared to cells grown in a complete medium containing acetate (3). In our study, *O. malhamensis*, grown in Hutner's medium (24) and subsequently starved for 24 hr in a sucrose-free medium, showed a twofold increase in acid phosphatase activity, and heavy acid phosphatase activity was seen in the large and small vacuoles. These vacuoles, tentatively identified as secondary lysosomes, were devoid of particulate substances as reported in metazoan cells (11, 13, 33).

It was demonstrated by the Burstone azo dye and Gomori techniques that starvation induces an increase in vacuolar reaction product, coupled with a doubling of the specific activity of acid hydrolases (Table I). This is obviously the cells' response mechanism to the changing environment. Heavy reaction product within the Golgi cisternae and Golgi vesicles of starved cells further

confirms that the Golgi complex may be a site of primary lysosome formation in *O. malhamensis*.

Novikoff (33, 34) believes that the Golgi complex is involved in the transfer of acid phosphatase from the ribosomes to vacuoles where autophagy or intracellular digestion of food occurs. The Golgi complex and perinuclear region have been shown to be possible sites of primary lysosome formation in over 100 cell types, including *Euglena*, mouse mononuclear phagocytes, and kidney cells (5, 8, 9, 33, 34).

Under ordinary conditions, acid phosphatase is not detected in the Golgi complex of human liver cells. Bertolini and Hassan (4) observed the presence of acid phosphatase in the Golgi complex of human hepatic parenchymal cells infected with viral hepatitis. Cohn and Benson (8, 9) observed that when mouse mononuclear phagocytes are grown in tissue culture, they differentiate into mature macrophages. This is accomplished by an increase in both the number of lysosomes and the amount of acid phosphatase activity present in the cells. It is not known how starvation stimulates the intracellular digestive system in *O. malhamensis*.

The appearance of autophagosomes as a function of culture age has been reported in *O. danica* (37).

Recent observations in *T. pyriformis* and *E. gracilis* confirm the existence of focal autolysis in these organisms whereby cytoplasm becomes sequestered in digestive vacuoles with acid phosphatase activity (6, 13, 25).

This process was observed also in some cells of *O. malhamensis* when they were starved for 24 hr. Autophagy usually involves first the formation of autophagic vacuoles without significant acid phosphatase activity as seen in Fig. 5. However, large empty vacuoles in the same cell do have significant reaction product. Although the fusion of autophagic vacuoles with lysosomes has been shown in numerous studies, none was observed in *Ochromonas* starved for only 24 hr.

Focal autolysis indicated by degradation of cytoplasm within vacuoles containing acid phosphatase reaction product is shown in Fig. 6. It is interesting to note that while a portion of a starved cell's cytoplasm is undergoing autophagy, the cell can still phagocytize and digest bacteria in food vacuoles. The activated intracellular digestive system of starved cells can function in both autophagy and phagotrophy at the same time.

O. malhamensis is nutritionally versatile, as are *T. pyriformis* and *E. gracilis*. It may ingest particulate food and rely on autophagy to sustain itself during periods of starvation. Its ability to adapt to environmental changes is very clearly illustrated by the doubling of the specific activity of lysosomal acid hydrolases when the organism, grown osmotrophically, is starved. We have demonstrated that phagotrophy and starvation-induced autophagy in this flagellate are quite similar to these processes in ciliates and metazoan phagocytes. The sequence of events appears to be as follows: Starvation increases the synthesis of acid hydrolases. Primary lysosomes are formed in the Golgi apparatus in both normal and starved cells. There is very little cytochemically-detectable acid hydrolase activity in the vacuoles of osmotrophically-growing cells. The digestive enzymes appear in the vacuoles of starved or phagotrophic cells, probably as a result of the joining of primary lysosomes with the vacuoles. Particulate food or particles of the cells' own cytoplasm (in starvation) are engulfed in food vacuoles, which subsequently join with digestive vacuoles to complete the catabolic process. When the cells are grown in a nutritionally complete osmotrophic medium, phagocytosis causes appearance of acid hydrolases in the digestive vacuoles, whereas the total activity of the enzymes remains unchanged. An organic carbon-free medium strongly stimulates acid hydrolase activity and causes these enzymes to appear in the digestive vacuoles whether or not phagocytosis occurs.

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