ON STARVATION-INDUCED AUTOPHAGY IN TETRAHYMENA

by

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Starvation-induced autophagy in *Tetrahymena* was studied over a 48-hour period during which the cells decreased in size to a quarter of their original volume. The autophagosomes were divided into 4 types: I) Clearly identifiable cytoplasmic content delimited by 2 membranes. II) Clearly identifiable content delimited by one membrane. III) Content of remnants permitting identification of origin. And, IV) Completely disintegrated content of unknown origin. All types were present in 30-minute starved cells and Type I organelles were seen throughout starvation, thus indicating that autophagy occurred continuously. The number of autophagosomes per unit cytoplasmic area remained constant but with a decrease of the first 3 types and an accumulation of Type IV. Acid phosphatase was found in some Type II organelles and in most Types III and IV; these organelles are autolysosomes but Type I is not. Disintegration of the inner autophagosomal membrane (passage from Type I to Type II) thus occurs in the absence of lysosomal enzymes but could be ascribed to an assumed acidification of the organelles. In addition to autophagosomes, profiles were observed of double membrane systems, isolation membranes, enveloping cell constituents as an indication of autophagy in progress. These isolation membranes seemed to derive from Golgi complexes as tubular, or sac-like structures; moreover, such structures were seen in association with microtubules which indicates a possible transport mechanism to the site of autophagy. An analysis of the origin of the autophagosomal contents, revealed that cytoplasm was enclosed throughout starvation, whereas enclosure of mitochondria, peroxisomes, and membrane whorls occurred in a time-dependent manner. Hence in this ciliate, autophagy occurs in a well-defined and controlled manner.

1. INTRODUCTION

Autophagy is the mechanism by which portions of the cell's own cytoplasm is sequestered by membrane during formation of an autophagic vacuole, or autophagosome. The ultimate fate of the sequestered material is digestion after addition of hydrolytic enzymes to the autophagosome which then becomes an autolysosome.

Autophagosomes are identified initially by the cytoplasmic organellar origin of their content and later, during the early stage of digestion, by recognizable remnants of such structures; as digestion proceeds, these criteria cannot be used. However, in a ciliate like *Tetrahymena*, later digestive stages may still be recognized as auto-

phagosomes by their vacuole size, about 1 μm in diameter, being several fold smaller than normal digestive (food) vacuoles (25, 26, 27).

Autophagy may be seen in tissue cells during removal of wornout cell organelles and during pathological conditions and drug treatment (1, 6, 7, 8, 14, 15, 30, 31, 32); it may also occur during cell remodelling in metamorphosis (20, 21) and during starvation (2, 7, 24). In *Tetrahymena* autophagosomes are not seen in cells from rapidly proliferating cultures but they appear whenever growth is inhibited (27, 28). The highest frequency of autophagy occurs when cells from rapidly proliferating cultures are exposed abruptly to starvation (27).

During starvation, autophagy seems to be the mechanism which enables *Tetrahymena* to maintain its basic metabolism with the sparse reserve material characteristic of cells in exponentially multiplying cultures. Apart from the contents of food vacuoles formed prior to starvation, the cells must rely on endogenous resources for necessary metabolites; these resources are the cell's own constituents and autophagosomes may be found 20 minutes after transfer to the starvation medium (27). Previous semi-quantitative data (26) have revealed that the number of autophagosomes increases during a 3-hour starvation period of *Tetrahymena* and that this controlled autophagy ceases when exogenous nutrients are supplied.

In this reinvestigation of starvation-induced autophagy, *Tetrahymena* was subjected to prolonged starvation. The purpose has been to obtain more information on the mode of autophagy and to determine the stage at which the autophagosome becomes an autolysosome.

2. MATERIAL AND METHODS

Tetrahymena pyriformis GL-8 was grown axenically at 28 °C in 2% proteose peptone (Difco) enriched with 0.1% yeast extract (Difco) and inorganic salts (33). The 100 ml cultures were aerated and shaken; the cells were used in the exponentially multiplying growth phase ($67,000 \text{ cells} \times \text{ml}^{-1}$). For induction of starvation, the cells were transferred to an inorganic salt medium (11, 33) after 3 centrifugal washings, maintaining the initial cell density; the culture

was then replaced at 28 °C for 48 hours under the conditions described above. The cell density was determined in a particle counter, Coulter Counter model ZB, after fixation of the cells in 1% glutaraldehyde in inorganic salt medium and following dilution with 0.45% NaCl.

For electron microscopy, duplicate (or triplicate) samples were removed prior to starvation and at intervals during the 48-hour starvation. The cells were fixed for 30 minutes by pouring the sample into an equal volume of cold 5% glutaraldehyde in 0.05 M-cacodylate buffer containing 2% glucose. At each time point, one cell sample was washed in buffer, postfixed for 1 hour in 1% osmium tetroxide in the same buffer, rinsed in distilled water, contrasted for 1 hour in 1% aqueous uranyl acetate, rinsed in distilled water, and dehydrated in a graded series of ethanols and finally in propylene oxide before embedding in epon. The other cell samples were processed for acid phosphatase, thus after the fixation in glutaraldehyde, the cells were washed thoroughly in cold cacodylate buffer and stored in this buffer overnight in the cold. The Gomori medium (pH 5.5) was prepared according to LOCKE and SYKES (21) and the cells were incubated in freshly filtered test medium for 1 hour at 32 °C; the third samples were incubated in the presence of NaF which inhibited the reaction completely. After the enzyme reaction, the cells were washed thoroughly, postfixed in osmium tetroxide, and contrasted with uranyl as described above. The embedded material was cut on an LKB ultramicrotome and the sections were contrasted with zinc-uranyl acetate (38) for 20 minutes and with lead acetate (37) for 3 minutes. The material was then examined in a Zeiss EM9 electron microscope at primary magnifications between 1,800 and 20,000 times.

For the semi-quantitative estimates of the number of autophagosomes, mitochondria, and peroxisomes, micrographs of an 18,000 times magnification were used. The number of profiles of these organelles was counted in about 20 cells per sample and the cytoplasmic area was measured using a planimeter.

For estimation of the cell volume in the various samples, the length and width of 30 fixed cells were measured in each case in a calibrated Reichert light microscope using a 63x objective.

Figure 1. Relative size of *Tetrahymena* prior to starvation (A) and after a 1-hour (B), 3-hour (C), 24-hour (D), and 48-hour (E) starvation. Note the unchanged size of the oral structure (oa), the contractile vacuole (cv), and the nucleus (n) after stage B. The nucleolar changes in response to starvation are indicated. Mean values of 30 measured cells per sample.

3. RESULTS

3.1. General changes in starved cells

Cells from an exponentially multiplying *Tetrahymena* culture were followed during a 48-hour starvation in an inorganic salt medium (11). Duplicate cell samples were removed before transfer to the starvation medium and after 0.5, 1, 3, 24 and 48 hours in the medium; one of the 2 samples was processed cytochemically for acid phosphatase.

After transfer to the starvation medium, cells most advanced in the cell cycle will divide without increase in cell volume; in the present study a 20% increase in cell number was observed. The average cell volume decreased to 80%, 60%, 35%, and 24% of the original cell volume after 1, 3, 24, and 48 hours of starvation, respectively (mean values of 30 cells per sample); these values have been corrected for reduction of the original cell volume due to cell division. The relative size of *Tetrahymena* during starvation is shown in Figure 1; the oral structure, the contractile vacuole, and the nucleus (after division) remain unchanged in size. The large reduction in cell volume means that the nucleus which occupies 3-4% in growing cells, takes up about 15% of the volume of 48-hour starved cells. Food vacuoles formed prior to starvation decrease rapidly in size; starved cells contain few larger (debris) vacuoles but several small vacuoles (autophagosomes).

In addition to inducing autophagy (see following sections), starvation causes other fine struc-

tural changes in the cells. Mitochondria and peroxisomes change into their "dense" type (26, 27, 28), the nucleoli form aggregates, and glycogen particles, lipid droplets, and dense granules appear (27). Furthermore, laminated membranous material is seen below the cell surface in alveolar sacs, in the cytoplasm near mitochondria, and within rough endoplasmic reticulum cisternae. Transfer to the starvation medium involves centrifugal washings of the cells which causes extrusion of their mucocysts, thus new mucocysts are formed early during starvation.

Cells starved for 48 hours are capable of recovery when supplied with nutrient medium.

3.2. Autophagosomes and definition of types

All starved cells contained autophagosomes but these organelles were not seen in cells from the exponentially multiplying culture. As mentioned in the Introduction (section 1), autophagosomes are recognized by their contents of cytoplasmic origin and on their small size; they often appear in groups (Figures 2, 3). The organelles may be delimited by one or two membranes; typically, the space between the two membranes, or between the contents and the limiting membrane, is electron translucent.

Originally, 2 types of autophagosomes (cytolysosomes) were defined in *Tetrahymena* according to whether the cytoplasmic origin of their contents could be identified (type 1) or not (type 2) (25). This classification has been extended in the present study by subdivision of the previous type 1 organelles into those with a clearly identifiable content surrounded by two membranes, Type I (Figure 4), or by one membrane, Type II (Figure 5), and those with enclosed remnants that permit identification of their origin, Type III (Figure 6); the previous type 2 organelles with disintegrated content of unknown origin, are now designated, Type IV (Figure 7). In addition to these 4 types, consideration is also paid to profiles of a double membrane system partially enveloping an organelle (Figure 2) or portion of cytoplasm; such profiles are believed to represent "autophagosomes in formation".

The cytoplasmic origin of the contents of autophagosomes varies. Typically, cytoplasm

Figure 2. Part of a 30-minute starved cell showing autophagosomes (a) and an autophagosome in formation (arrow); note the dense reaction product on the enveloping membrane of the latter structure. Mitochondria (m) and peroxisomes (p) are of the "dense" type. Lipid droplet (l). Light spots in cytoplasm are glycogen islands. Gomori tests. (Bar = 0.5 μ m).

Figure 3. Part of 3-hour starved cell showing autophagosomes (a) of varying electron density. Mitochondria (m) and peroxisomes (p) of the "dense" type. Light spots in cytoplasm are glycogen islands. (Bar = 0.5 μ m).

Figure 4. Autophagosome (Type I) with two limiting membranes enclosing cytoplasm. Note small vesicles (arrow). Sample: 30-minute starvation. X 60,000. (Bar = 0.5 μ m).

Figure 5. Autophagosome (a) delimited by one membrane (Type II) enclosing cytoplasm. The organelle shows negative reaction for acid phosphatase but reaction product is seen in the small vacuole (arrow). Sample: 30-minute starvation. Gomori test. X 60,000.

Figure 6. Autophagosome (Type III) containing remnants of a mitochondrion (rm) and reaction product for acid phosphatase (arrow). Sample: 30-minute starvation. Gomori test. X 60,000.

Figure 7. Two autophagosomes (1, 2) with a fluffy and more electron dense content of unidentifiable cytoplasmic origin. Both organelles show positive reaction for acid phosphatase. Sample: 30-minute starvation. Gomori test. X 60,000.

with ribosomes (Figures 4, 5, 11, 12, 14, 15, 18), mitochondria (Figures 6, 8), or peroxisomes (Figure 9) were enclosed; however, also laminated membranous material (Figure 10) or electron dense granules (Figure 13) were seen enclosed, especially after prolonged starvation; Golgi complexes or cilia components were not observed enclosed. Some autophagosomes were composite in nature (Figure 11) and in cases where the content was a mixture of digested and undigested material, the organelle was assumed to have arisen by fusion of autophagosomes of different age (Figure 12). In no instance was nuclear material (nucleoli or chromatin) seen enclosed in autophagosomes. After prolonged

starvation, the complexity of the autophagosomal contents increased, thus negative images of apparently dissolved crystals could be seen (Figures 13, 16, 18). In addition to autophagosomes, the cells contained debris vacuoles, about 3 μ m in diameter; these became electron dense and complex in structure late during starvation.

Autophagosomes were classified in the various cell samples according to the above-mentioned types and the number of profiles indicating autophagy in progress was noted. The percentage distribution of the organelles is shown in Table I. All types were found in cells starved for 30 minutes (Figures 4-7). Furthermore, early during starvation a high frequency is

Table I.
Distribution of types of autophagosomes during starvation (%)

Time	In formation	Type I	Type II	Type III	Type IV	Total positive
0.5 hour	7.3 (0)	18.7 (0)	19.5 (25%)	8.9 (100%)	45.5 (56%)	65.5%
1 hour	13.9 (0)	22.2 (0)	11.1 (28%)	6.9 (100%)	45.8 (59%)	50.0%
3 hours	8.8 (0)	7.8 (0)	11.8 (33%)	4.9 (62%)	66.7 (62%)	51.8%
24 hours	2.7 (0)	3.6 (0)	4.5 (0)	4.5 (100%)	84.8 (89%)	81.0%
48 hours	0	7.6 (0)	2.2 (0)	9.8 (80%)	80.4 (85%)	82.0%

Analysis of 100 autophagosomes per sample. Figures in parentheses indicate the percentage of each type giving a positive reaction for acid phosphatase.

Table II.
Cytoplasmic origin of content in autophagosomes in starved *Tetrahymena*

Enclosed material	0.5 hour	1 hour	3 hours	24 hours	48 hours
General cytoplasm	75%	68%	72%	64%	63%
Mitochondria	25%	25%	19%	–	6%
Peroxisomes	– ^{*)}	7%	6%	7%	–
Membrane whorls	–	–	3%	29%	13%
Mucocysts	–	–	–	–	6%
Dense granules	–	–	–	–	12%

Analysis of autophagosomes (types I, II, III) from Table I. ^{*)} None observed.

seen of Type I and of autophagosomes in formation but although the frequency decreases with time, Type I was observed in all samples. The findings indicate that autophagy occurs as a controlled process throughout the 48-hour starvation simultaneously with the decreasing cell size.

An analysis of the nature of the autophagosomal content is shown in Table II. As may be

deduced from the table, the majority of autophagosomes contained cytoplasm throughout the starvation period, whereas mitochondria were enclosed primarily at an early stage, peroxisomes after 1 hour, membranous material after 3 hours, and mucocysts and dense granules only during late starvation. These observations show that different organelles become enclosed at different times during starvation.

Figure 8. Autophagosome (Type II) containing a mitochondrion (m) and disintegrated cytoplasm. Sample: 1-hour starvation. X 60,000. (Bar = 0.5 μ m).

Figure 9. Autophagosome (Type II) containing a peroxisome (p) shows negative reaction for acid phosphatase. Positive reaction for the enzyme is seen in small vesicles (arrows). Sample: 3-hour starvation. Gomori test. X 60,000.

Figure 10. Autophagosome (Type II) enclosing laminated membranous material (lm). Note microtubule (arrow). Sample: 3-hour starvation. X 60,000.

Figure 11. Autophagosome containing a mixture of remnants of cytoplasm (ribosomes) (arrow), electron dense material (dense granule?), and unidentifiable, less dense material. Sample: 24-hour starvation. X 60,000.

Figure 12. Fusion (arrow) between 2 autophagosomes of different age: Type II (top) and Type IV (bottom). Positive reaction for acid phosphatase was seen in different plane of section. Lipid droplet (l). Sample: 1-hour starvation. Gomori test. X 60,000.

Figure 13. Composite autophagosome (Type IV) containing negative image of dissolved crystal (arrow), spheres of electron dense material (dense granules?) and unidentifiable granulated material. Negative reaction for acid phosphatase. Sample: 24-hour starvation. Gomori test. X 60,000.

3.3. Acid phosphatase testing (autolysosomes)

In cell samples tested for acid phosphatase activity, the relationship between the type of autophagosome and the enzyme reaction could be determined. The reaction product for the enzyme was either finely granulated or localized in small aggregates which means that organelles may appear negative in one section but show a positive reaction in a different section.

A general positive reaction for acid phosphatase was not observed in Type I autophagosomes, whereas some Type II organelles, the majority of Type III, and most of Type IV organelles gave a positive reaction for the enzyme (Figures 12-17). The percentage of each type of autophagosome giving a positive reaction for the enzyme, is indicated in parentheses in Table I. The total number of autophagosomes showing a positive reaction for the enzyme is somewhat higher in the 30-minute sample than in the 1-hour and 3-hour samples but it is highest during prolonged starvation (Table I). The analysis indicates that only autophagosomes of Types II, III, and IV may be considered autolysosomes.

Transfer of hydrolytic enzymes to autophagosomes presumably occurs by fusion with lysosomes. As reported previously (27), *Tetrahymena* contains numerous small vesicles (about 0.2 μm in diameter); they often appear with a laminated content and show a positive reaction for acid phosphatase (Figures 14, 16) but no direct fusion of these vesicles with autophagosomes was observed. A possible mode by which an initial acquisition of hydrolytic enzymes may occur was indicated in early stages of autophagosomes enclosing general cytoplasm; these autophagosomes contained small vesicles (Figure 4) giving a positive reaction for acid phosphatase (Figures 14, 18). Liberation of the enzyme from these vesicles to the general content of the autophagosome would, however, require a breakdown of their limiting membrane. Such small vesicles were also seen accumulated in regions of apparently early stages of autophagy (Figure 19). Another mode by which autophagosomes may acquire hydrolytic enzymes is by fusion with an autolysosome as indicated by fusion of autophagosomes of different age (Figure 12), previously mentioned in section 3.2.

Figure 14. No general positive reaction for acid phosphatase is seen in the autophagosome (Type II); note, however, localized reaction product in enclosed vesicle (arrow). Lysosome (ly) shows positive reaction for the enzyme. Sample: 3-hour starvation. Gomori test. X 60,000. (Bar = 0.5 μm).

Figure 15. Autophagosome (Type III) containing remnants of cytoplasm, shows scattered reaction product for acid phosphatase (arrows). A mitochondrion (m) with associated Golgi complex facing autophagosome. Sample: 30-minute starvation. X 60,000.

Figure 16. Autophagosome (Type IV) with heavy reaction product for acid phosphatase (arrows) between images of dissolved crystals and electron dense material. Note 2 lysosomes (ly) showing positive reaction for the enzyme. Mitochondrion (m). Sample: 24-hour starvation. Gomori test. X 60,000.

Figure 17. Composite autophagosome (Type IV) presumably derived from fusion of several autophagosomes as deduced from the large mass of electron dense (d) lipid material, negative images of dissolved crystals, and other material showing heavy reaction product for acid phosphatase (arrows). Sample: 48-hour starvation. Gomori test. X 60,000.

Figure 18. Autophagosome (Type II) containing small vesicles showing positive reaction for acid phosphatase (arrows), apart from these localized sites, the reaction is negative. Sample: 30-minute starvation. Gomori test. X 60,000.

Figure 19. Small vesicles (arrows) showing positive reaction for acid phosphatase collected in region of autophagy in progress. Configuration could explain how autophagosomes may acquire initial enzyme activity, see text for further explanation. Isolation membrane (im). Sample: 30-minute starvation. Gomori test. X 60,000.

3.4. On formation of autophagosomes

As mentioned earlier (section 3.2.), several profiles indicated autophagosomes in formation (Figure 2). The enveloping membrane system seems to consist of flattened membranous sacs, the lumen of which is electron translucent and variable in thickness (Figures 2, 20). In some instances, the ends of the enveloping system appear to "grow" towards one another (Figure 20) with a localized positive reaction for acid phosphatase at one end (Figure 2). Different profiles indicate that the enveloping system could arise from several small vesicles accumulating around the organelle or portion of cytoplasm to become sequestered (Figures 21, 30); the vesicles could also be interpreted as cross sections of tubular membrane systems which eventually could fuse sideways. Typically, the membranes of such enveloping systems were not associated with ribosomes and often the membranes were more distinct than membranes of the rough endoplasmic reticulum (Figure 28).

The cytoplasm of starved cells contained numerous tubular membrane systems, the lumen of which appear electron translucent. Such areas often included curved profiles (Figure 22) which in other instances indicated autophagy in progress (Figure 23); furthermore, a positive reaction for acid phosphatase was observed

along, or on portions of, the luminal side of these membrane profiles (Figures 22, 23). In other cases, oblong, or other unusually shaped, "vacuoles" were observed (Figure 24); these may represent chance sections through enveloping membrane systems, or isolation membranes, as those mentioned above. Moreover, the "vacuole" shown in Figure 24, has one truncate end giving a positive reaction for acid phosphatase; this might represent the advancing zone of the membrane system.

In search of evidence for the origin of such isolation membranes, it is natural to focus on the Golgi complexes. In *Tetrahymena*, these complexes are found near mitochondria where one side of the associated rough endoplasmic reticulum is differentiated with small blebs (Figures 25, 26); at the maturing side of the Golgi complexes, larger vesicles are seen. Moreover, these vesicles seem to form sac-like structures (Figure 27) not unlike the membranous profiles shown in Figure 22. A possible transport mechanism of such membranous structures to the site of autophagy was indicated by several findings of an association of these structures with thin bundles of microtubules (Figures 28, 29).

No clue was found, however, to indicate criteria determining selection of particular organelles to become sequestered.

Figure 20. Profile indicating autophagosome in formation, a mitochondrion (m) is enveloped by double membrane system (im), isolation membrane; ends of membrane system are indicated by arrows. Note the variable distance between membranes of the enveloping system and the electron translucent lumen. Sample: 3-hour starvation. X 60,000. (Bar = 0,5 μ m).

Figure 21. Mitochondrion (m) surrounded by small vesicles (tubules?) which indicates future enclosure of organelle in autophagosome. Sample: 1-hour starvation. X 60,000.

Figure 22. Membranous profiles of various shapes with localized positive reaction for acid phosphatase (arrows). These membrane systems are believed to be "isolation membranes". Sample: 30-minute starvation. Gomori test. X 60,000.

Figure 23. Curved membrane profile (im) indicating autophagy in progress. Note the electron translucent lumen and the localized membrane-associated reaction product for acid phosphatase, enhancing outline of membrane. Note positive reaction for the enzyme in small vesicles (arrows). Sample: 30-minute starvation. Gomori test. X 60,000.

Figure 24. Unusually shaped vacuole with truncate end showing localized reaction product for acid phosphatase (arrow), could represent section through an isolation membrane system with "advancing zone" at truncate end. Electron translucency of the structure resembles that of profiles in Figures 22 and 23. Mitochondria (m). Sample: 3-hour starvation. Gomori test. X 60,000.

Table III.**Distribution of organelles per unit cytoplasmic area ($25 \mu\text{m}^2$) during starvation**

Type of organelle	zero	0.5 hour	1 hour	3 hours	24 hours	48 hours
Autophagosomes (A)	0	1.4	1.7	2.5	3.3	2.8
Mitochondria (M)	7.0	5.7	5.6	5.3	5.1	3.5
Peroxisomes (P)	3.0	4.3	4.0	2.8	1.1	0.8
Ratio M/P	2.3	1.3	1.4	1.9	4.6	4.4
A + M + P	10.0	11.0	11.3	10.6	9.4	7.1

Counts of 20 cells per sample (cytoplasmic area: $2000\text{--}1000 \mu\text{m}^2$).

3.5. The occurrence of autophagosomes during starvation and their relationship to other cell organelles

A semi-quantitative estimate was made of the number of autophagosomes, mitochondria, and peroxisomes per cytoplasmic area in 20 cells per sample. The mean values for the various samples are shown in Table III. As may be deduced from the table, the number of autophagosomes increases during the first 3 hours after which the number remains almost constant during prolonged starvation. Since newly formed autophagosomes (Types I, II) decrease in frequency during prolonged starvation, the

finding indicates an accumulation of Type IV organelles which, as mentioned previously (section 3.2), become complex in substructure (Figures 13, 16, 17).

The number of mitochondria per unit cytoplasmic area decreases after 30 minutes to 81% of the value found in cells from the exponentially multiplying culture. However, after this period, the number of mitochondria remains almost constant up to 24 hours of starvation but decreases to 50% of the original number in 48-hour starved cells. These latter cells have little cytoplasm and their mitochondria are exclusively found in association with the cell surface.

Figures 25 and 26. Two different sections through a Tetrahymena Golgi complex (g) which is a differentiated region of mitochondria (m) associated rough endoplasmic reticulum (er). Note the initial bleb formation and later fusion into larger membrane systems (arrows) at maturing face. Weak reaction product for acid phosphatase is seen within small vesicles. Membrane differentiation within complex is evident. Peroxisome (p). Gomori test. X 60,000. (Bar = $0.5 \mu\text{m}$).

Figure 27. Section through 2 Golgi complexes (g) with maturing face towards one another; note the interjacent membranous structures (arrows). Mitochondrion (m). Sample: 1-hour starvation. X 60,000.

Figure 28. Section through membrane structures, isolation membranes (im), aligned along a thin bundle of microtubules (arrows), indicating a possible transport mechanism for the membranes. Note that the structures (im) have membranes distinctly thicker than those of the rough endoplasmic reticulum (er). Sample: 30-minute starvation. X 60,000.

Figure 29. Another example of apparent association of membrane systems (im) with microtubules (arrows). See also Figure 28. Sample: 1-hour starvation. X 60,000.

Figure 30. Membranous whorl (w) free in the cytoplasm but partially surrounded by membrane system, isolation membranes (arrows), indicating autophagy in progress. Small vesicles (v) seem to contribute to membranous whorl, they may represent excess membrane removed from various structures such as mitochondria (m). Enclosed membranous material is seen in autophagosome (a). Sample: 24-hour starvation. X 60,000.

The number of peroxisomes, on the other hand, increases initially to 43% above the value found in unstarved cells. The number remains elevated after 1 hour of starvation but returns to the original value after 3 hours (see also Figure 9); during prolonged starvation, the number of peroxisomes decreases drastically. Hence a remarkable fluctuation is seen in the ratio of mitochondria to peroxisomes, whereas the total number of these 2 organelles and the autophagosomes show little variation in cells starved for up to 24 hours (Table III).

4. DISCUSSION

The present observations have shown that *Tetrahymena* becomes reduced to a quarter of its original volume during a 48-hour starvation. Moreover, concomitantly with this reduction, autophagy occurs continuously as deduced from the findings of newly formed autophagosomes throughout starvation.

The autophagosomes were divided into 4 types in the present study whereas only 2 types were considered previously (25, 26). This distinction into more types was useful in the determination of the stage at which autophagosomes become autolysosomes. Thus, organelles delimited by 2 membranes and containing readily identifiable cell components (Type I) never gave a general positive reaction for acid phosphatase, whereas some of the Type II organelles with the same content but delimited by one membrane, gave a positive reaction for the enzyme, as was also found in the majority of Types III and IV with more or less disintegrated contents. Although determination of the type of the organelle thus permits its classification as an autolysosome, the more general term, autophagosome, will be used in the following discussion.

The exact turnover time of autophagosomes in *Tetrahymena* is not known. However, the passage from type I to type IV may occur in less than 30 minutes since all 4 types of the organelles were found in cells starved for this length of time, although these cells still contained food vacuoles formed before the transfer to starvation medium. The general response of *Tetrahymena* to starvation is a drastic decrease

in the rate of respiration to 30% within 1 hour and to 20% after 3 hours; this low value is kept almost constant for an additional 3 hours (12) as an expression for maintenance of a basic metabolism. In view of these observations, it might be expected that the rate of autophagy would decrease after the first hours of starvation as also indicated by the present findings of a decreasing number of newly formed autophagosomes during prolonged starvation. However, the number of autophagosomes per unit cytoplasmic area remains almost constant from 3 hours and onwards but this is due to an increasing frequency of type IV organelles. Concomitantly with this accumulation of type IV autophagosomes, their substructure becomes extremely complex, an indication that the extensive accumulation of residuals could have derived from frequent fusion of autophagosomes. Fusion of autophagosomes of different ages could represent an economical usage of hydrolytic enzymes under stress situations. During a 25-hour starvation, the specific activity of proteinase increases 3 fold although the enzyme content per cell remains constant, whereas the specific activity of acid phosphatase remains constant while the enzyme content per cell decreases to 30% (19); thus a certain reuse of the enzymes may be important. Apparently, the rate of turnover of autophagosomes may alter according to conditions, the total number of the organelles per unit cytoplasmic area is not, therefore, a satisfactory measure of the rate of autophagy, also the frequency of the different types must be known.

Another important aspect of a detailed analysis of autophagosomes is a determination of the cytoplasmic origin of their contents. Whereas general cytoplasm with ribosomes is found in the majority of the autophagosomes throughout starvation, enclosure of other cell constituents seems to follow a time-dependent pattern. Mitochondria were primarily enclosed early during starvation, as also reported elsewhere (3, 19, 26), leaving after this time an almost constant number of mitochondria per unit cytoplasmic area; the decreased rate of respiration of starved cells does not correlate directly with the removal of mitochondria but is related to their change into the "dense" type (cf. 28). Peroxisomes were

not enclosed early during starvation which is in agreement with the initial increase of their number per unit cytoplasmic area, as also briefly reported previously (3); the initial activity of gluconeogenesis in *Tetrahymena* early during starvation (18) seems to correlate with the about 40% increase in the number of peroxisomes and with their change into the "dense" type (cf. 28). However, later during starvation the peroxisomes decrease drastically in frequency, which causes great fluctuations in the ratio of mitochondria to peroxisomes during starvation. Laminated membranous material becomes enclosed in autophagosomes around 3 hours with an increased frequency later during starvation; such unenclosed membranous whorls are seen in all starved cells and they seem to derive from a diminishing of the overall membrane system, for example, the surface area of 48-hour starved cells decreases to 37%. Enclosure of mucocysts and dense granules is seen only late during starvation; Golgi complexes were not observed enclosed. In no case was enclosure of nucleoli, or other nuclear material, seen as reported by others (19); only cytoplasmic components were found in autophagosomes. Starvation of *Tetrahymena* occurs at the expense of protein (5, 19, 23), RNA (4, 16, 17), and phospholipids (13, 18) which is in agreement with the observed sequestration of cell organelles, ribosomes, and membrane material. Moreover, the sequential removal of the various cell constituents indicates that autophagy occurs in a well-defined and controlled manner; however, no evidence was found to indicate how material to be sequestered may be selected.

Several profiles indicated autophagosomes in the process of formation, thus providing some evidence for the mode of autophagy in this ciliate. In advanced stages, cell organelles or general cytoplasm were partially enveloped by a flattened membrane system, the lumen of which has the electron translucency seen between content and limiting membrane of autophagosomes. Furthermore, the same electron translucency was also found in otherwise "empty" vacuoles of obscure shapes, for example oblong; these vacuoles could represent sections through the enveloping membrane system, i.e. the so-called isolation membranes described

for other cell systems (14, 20, 21). In *Tetrahymena*, localized regions on the luminal side of such membrane systems often showed positive reaction for acid phosphatase and it is tempting to speculate that these regions could represent the advancing zone of the membrane system during sequestration. Different profiles revealing organelles surrounded by small vesicles, or tubules, could conceivably represent a pre-stage of isolation membranes since a sidewise fusion would result in flattened sacs. The overall findings seem to establish that autophagy in *Tetrahymena* occurs by means of a double membrane system, or isolation membranes, enveloping the portion of cytoplasm to become sequestered and that newly formed autophagosomes are delimited by two membranes (type I). This is in agreement with the general mode of autophagy described for a variety of cells (10, 14, 20, 21).

The origin of the isolation membrane system is an intriguing question. Since autophagosomes are part of the lysosomal system, a membrane similarity with the plasma membrane would be expected, as the latter forms the limiting membrane around endocytic vacuoles (6, 7, 14, 30, 31). In the present study, tubular or sac-like structures were seen at the maturing face of Golgi complexes which are very prominent in starved cells. These Golgi-derived structures show great similarity to the membrane systems engaged in autophagy; in addition, a membrane-associated reaction for acid phosphatase was observed. Moreover, such tubular membrane structures were frequently found associated with thin bundles of microtubules, thus providing evidence for a possible directed transport mechanism of the membranes to the site of autophagy; the phenomenon could also explain the finding that autophagosomes often appear in groups in the cytoplasm of *Tetrahymena*.

The general assumption is that autophagosomes acquire hydrolytic enzymes after fusion with primary, or recycling, small lysosomes (6, 7, 14, 20, 21, 30, 31) but evidence has also been presented for the presence of acid phosphatase within isolation membranes (9, 10). In *Tetrahymena*, vesicles with laminated content have been described as lysosomes (27) and their positive reaction for acid phosphatase has been demonstrated in the present study, but in addi-

tion, the above-mentioned localized positive reaction was found in isolation membranes. The intriguing question is, however, that a general positive reaction for acid phosphatase is seen only in some of Type II autophagosomes, i.e. after disintegration of the inner membrane typical of Type I organelles. It remains obscure how this disintegration of the inner membrane is initiated, a possible cause could be a lack of supply of energy-rich compounds to that membrane once the autophagosome is formed, or it could be due to an intra-vacuolar decrease of pH which occurs in endocytic vesicles in other cells (35, 36, 39) before fusion with lysosomes (22). In fact, within 10 minutes, surface-derived coated vesicles may fuse with autophagosomes of types I and II in *Tetrahymena* (29), thus indicating a possible connection. These pinocytotic vesicles already had a changed internal environment as their initially surface-bound cationized ferritin had lost its membrane association (29); whether this change is due to a decreased pH (34) is not known, nor is the role of the vesicles in the presumed acidification of the autophagosomes known. Some indication of how *Tetrahymena* autophagosomes may acquire acid phosphatase initially, was found in cases where cytoplasm was enclosed. In these instances, small vesicles showing positive reaction for acid phosphatase, were among the contents; to provide a general positive reaction for the enzyme in the autophagosome, the limiting membrane of these vesicles should disintegrate to liberate the enzyme, which would require the same factor as that needed for disintegration of the inner autophagosomal membrane. Small vesicles containing acid phosphatase were abundant in the cytoplasm and they may represent Golgi-derived primary lysosomes; although not observed directly, such vesicles could conceivably be enclosed together with cell organelles in the small amount of associated cytoplasm. A bulk acquisition of hydrolytic enzymes could then be provided by the above-mentioned larger secondary lysosomes.

In conclusion, the present reinvestigation of starvation-induced autophagy in *Tetrahymena* has revealed further evidence for the mode of autophagy in this ciliate, although several ques-

tions remain unresolved. In particular, the study has revealed that autophagy occurs in a controlled manner involving removal of different cell organelles at different times of starvation, an indication that a selective mechanism is operating.

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