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Biochemical and Ultrastructural Changes in *Tetrahymena pyriformis* During Starvation*

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SYNOPSIS. Certain of the ultrastructural and biochemical changes occurring during the first 25 hr of starvation in *Tetrahymena pyriformis* were studied. Ultrastructurally, numerous profiles of degenerating mitochondria were seen in the early stages of starvation. The presence of oxidizable substrate such as glucose and acetate did not prevent this degeneration. Numerous large nucleoli were formed, many of which seemed to be passing into the cytoplasm as forming autophagic vacuoles. There was a transient increase in Oil Red O-positive bodies, presumably lipid (triglycerides). The extent and duration of this increase were pronounced in the presence of acetate. The lipid droplets appeared to arise within the cisternae of the endoplasmic reticulum. Lipid reserves were apparently utilized prior to carbohydrates, as the disappearance of lipid droplets preceded glycogen utilization, both in the presence of acetate and in the absence of exogenous substrate. A considerable loss of cellular protein also occurred. In cells from inorganic medium supplemented with glucose, glycogen occupied much of the cell, leaving only islands of cell organelles. Acid phosphatase was localized, ultrastructurally, mainly in autophagic vacuoles which contained mitochondria and other cell organelles, and in association with small, double-membraned structures which seemed to be sequestering small areas of cytoplasm. Such sequestered areas also appeared within larger autophagic vacuoles. Residual bodies containing concentric whorls of myelin-like membranes surrounding a more solid core accumulated during starvation. Acid phosphatase activity decreased in amount but not in specific activity. The specific activity of cathespin doubled or tripled, but there was little change in total enzyme.

A number of interesting physiologic and biochemical findings have been reported by workers using suspensions of the ciliate *Tetrahymena pyriformis* in non-nutrient medium. Several reports suggest that endogenous metabolism occurs at the expense of intracellular lipids(21,35,42). Also, during the first 3 hr of starvation. there may be a loss of about 30% of cellular RNA(9) and 10-20% of protein(25). Metabolically, there may be a conversion of lipids to glycogen(21,42), the extent of this conversion being dependent upon the history of the culture from which the cells were taken(21,26,29). In *Paramecium* a transient increase in lipid reserves has been reported in starved cells(cf. 36), while in *Euglena gracilis* increases occur in the specific activities of 2 hydrolytic enzymes, acid phosphatase and cathepsin(4). At the ultrastructural level, starvation in this organism seems to be characterized by sequestration and subsequent digestion of portions of cytoplasm(5,32), a process that seems to occur in livers of starved rats as well (40).

In the present investigation, the ultrastructure of *T. pyriformis* was studied after various periods of starvation. The results tend to provide a structural basis for the physiologic findings of other workers concerning the source of carbon for endogenous metabolism and for the loss of cellular RNA. We have also investigated the effects of the presence of oxidizable substrates such as glucose or acetate on the nature and amount of reserve products. Our results suggest that degenerating mitochondria are an important source of metabolites for maintenance of endogenous respiration, altho a considerable breakdown of other cellular components also occurs. A preliminary report of this work has appeared(27).

MATERIALS AND METHODS

T. pyriformis (E) was grown with shaking in 2% (w/v) proteose peptone supplemented with 0.1% liver fraction, as described previously(25). The cultures were harvested after about 24 hours growth while they were in log phase. The cells were washed at least twice and resuspended in a phosphate-buffered inorganic medium(35) to a concentration of 0.5-1.0 protein/ml. Glucose and sodium acetate, when added, were present at an initial concentration of 1 mg/ml. Unless otherwise stated the cell suspensions were shaken in a water bath at 26 C. Static cell suspensions can be metabolically, and possibly ultrastructurally, quite different from shaken ones(25,26,29); therefore, our results do not necessarily apply to cells from non-shaken suspensions.

For electron microscopy, the ciliates were concentrated by centrifugation into a soft pellet and fixed for 20 minutes in 2% glutaraldehyde buffered with phosphate at pH 7.4-7.5. They were then post-fixed for 30 minutes in 1% phosphate-buffered osmium tetroxide, dehydrated, and embedded in Epon 812 according to the method of Luft(31). The blocks were polymerized overnight at 60 C, sectioned with a Porter-Blum MT-2 microtome, and examined with an RCA EMU 3 electron microscope. Sections were stained with lead citrate(34). Acid phosphatase was localized at the ultrastructural level by reacting the glutaraldehyde-fixed cells with Gomori's mixture(19) for 15 minutes at 25-27 C. Controls were incubated without substrate or with 0.01 M potassium fluoride.

Acid phosphatase was measured on frozen-thawed cells by the method of Torriani(41). Reaction mixtures contained 2 μmoles p-nitrophenyl phosphate, 20 μmoles acetate buffer, pH 4.9, and 0.1% Triton X-100, in a final volume of 0.4 ml. Homogenates were preincubated with the buffer and Triton for 5 min at 28-29 and reactions were started by addition of substrate. Reactions were run at 3 enzyme levels for 5 min and were stopped by addition of cold NaOH. Rates were linear with time and were proportional to the amount of homogenate. Acid proteinase was assayed by the method of Gianetto and de Duve(18) in acetate buffer, pH 3.6, also at 3 enzyme levels. Procedures for determination of protein and glycogen have been given(29). In order to detect lipid at the light microscope level, whole cells were fixed in formol-calcium and stained with Oil Red O according to the method of Lillie(28).

RESULTS

Mitochondria. Perhaps the most obvious change occurring in the early stages of starvation is the great increase in number of degenerating mitochondria. While these are not particularly rare in sections from growing cells, they become quite common shortly after cells are transferred to inorganic

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medium, and mitochondria in various stages of digestion were found at all stages tested (up to 25 hr). The presence of oxidizable substrate such as glucose or acetate did not seem to prevent this degeneration.

Figs. 1-6 show several aspects of mitochondrial degeneration. It appears that in the initial stages (Figs. 1-3) the mitochondria become surrounded by a double membrane, the origin of which is unknown. Many of the mitochondria at this stage appear to have inpocketings of cytoplasm (Fig. 1-2). In Fig. 3, bridges seem to connect the mitochondrial membrane with the surrounding membrane. In Fig. 4, which shows a mitochondrion in the early stages of digestion, acid phosphatase reaction product seems to be localized in the space between the outer membrane of the mitochondrion and the double membrane that surrounds the organelle. In the mitochondrion shown in Fig. 5, the digestive process is more nearly complete. The reaction product is present within an autophagic vacuole, which in this case is limited by a single membrane. Many mitochondria are seen with a myelin-like whorl on one end, but which are not sequestered from the cytoplasm (Fig. 6). These may also be in a stage of degeneration; similar mitochondria are seen in livers of rats exposed to elevated oxygen concentrations(37).

T. pyriformis mitochondria frequently contain a large, dense body (Fig. 7) which has been observed earlier(12). From 1-25% of the mitochondria of growing cells contain these bodies, but we have been unable to correlate them with changes in cellular metabolism, finding only a great variation in cells from the same culture. However, they do become numerous after 24 hr of starvation, some mitochondria having 2 or 3 of these bodies (Fig. 8). They also occur in starved cells when the inorganic medium is supplemented with acetate or glucose.

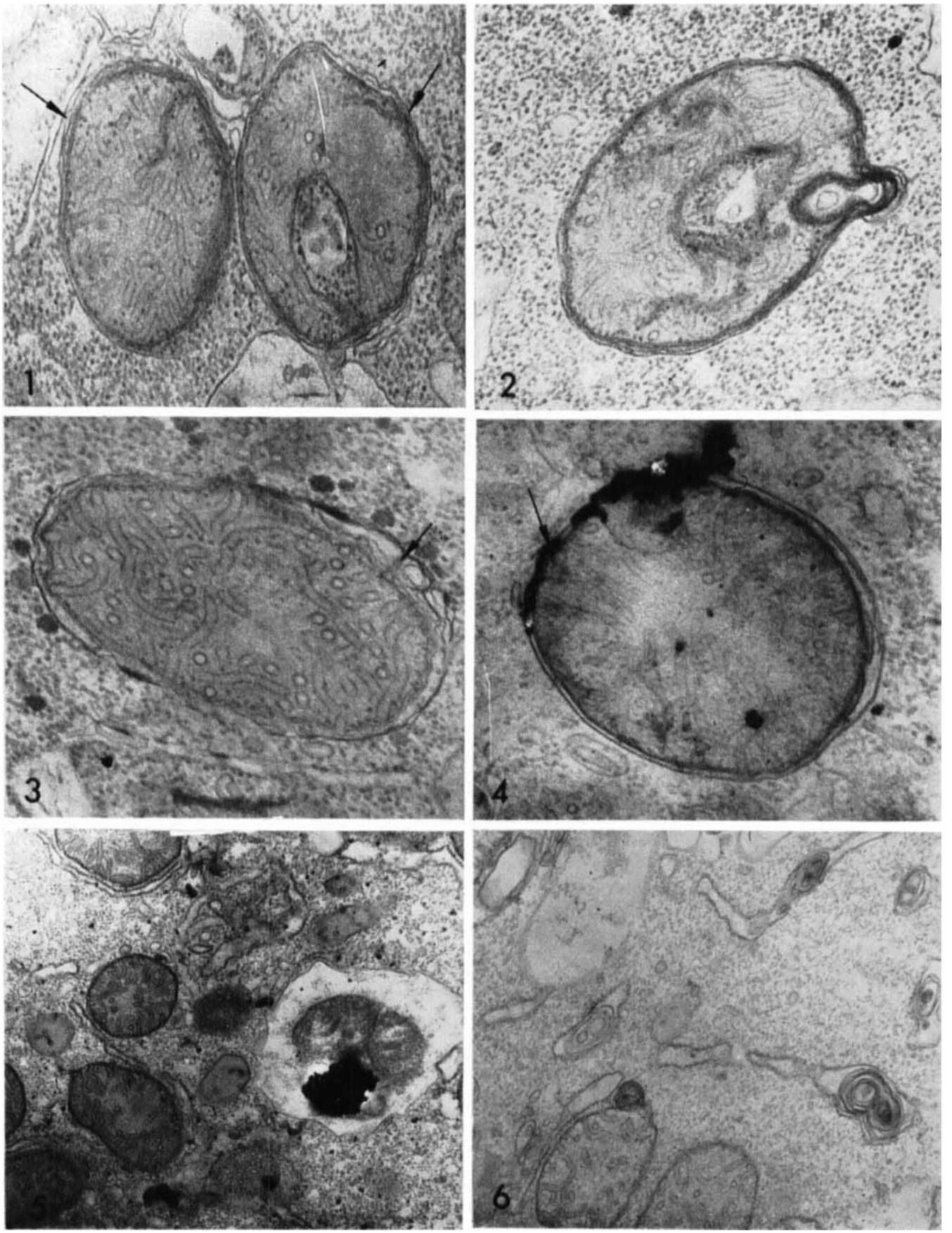
Lipids. Another interesting aspect of starved cells is the change in the content of Oil Red O-staining droplets, presumably triglycerides. A transient increase in these occurs in starved *T. pyriformis* and the increase becomes quite pronounced if the inorganic medium is supplemented with acetate. An indication of this accumulation is shown in Fig. 13. Cells were stained after various durations of starvation and scored on a 0 to 4 basis. Cells that were Oil Red O-negative, or which had only a slight positive reaction confined to the anterior tip received a "0," while cells showing an intense reaction, with droplets abundant thruout the cell, received a "4." Cells in unsupplemented inorganic medium reached a peak at about one hour, remained at this level for several hours, and then decreased sometime between 4 and 9 hours of starvation. In the presence of acetate, the peak was much higher, and occurred at about 9 hours. However, by 24 hours both groups of cells gave a negative response. The implications of these findings in terms of the endogenous respiration of the cells is discussed below.

At the ultrastructural level, cells in the early stages of starvation, especially in the presence of acetate, have numerous lipid droplets which appear to arise within the cisternae of the endoplasmic reticulum (ER). Figs. 9-11 show several profiles of what are apparently forming lipid droplets. In Fig. 9 the membrane of the ER seems to surround the droplet. In Fig. 10 the membrane associated with the droplet continues distally as rough ER, terminating in a region of the cytoplasm which seems to be in a stage of degeneration. In Fig. 11 membranous material is present in the vicinity of what is presumably a forming lipid droplet. It is possible that the droplet may grow by coalescence with this membranous material, which could be derived from degenerating mitochondria, several of which are present in the area. Alternatively, this material may arise from a blebbing of the ER. Fig. 12 shows a great deal of such material in the cisternae of the ER, and some of this can be seen to be arising from the wall.

Glycogen. During the first several hours after transfer of cells to inorganic medium there is an increase in intracellular glycogen stores (Fig. 13). [The extent of this deposition is much greater in cells taken from statistically-grown cultures or older cultures(21,26,29,42).] In the absence of nutrients, utilization of this material begins at about 8 hr of starvation. In the presence of acetate or glucose, glycogen deposition may continue for 24 hr. In the latter case, glycogen values of more than 100% of cell protein can be obtained.

The ultrastructure of cells shaken with glucose is quite striking, as glycogen seems to occupy most of the cytoplasm, leaving only small islands of organelles. Figs. 14-17 show a series of cells incubated with glucose for 0-8 hr. Initially, individual glycogen granules are scattered about the cytoplasm (Fig. 14). Within 1.5 hr, polysaccharide is accumulating in various areas (Fig. 15), and by 3 hr (Fig. 16) is present in very large islands. Fig. 17 is from a cell incubated with glucose for 8 hr. Cells at this time contain (chemically) more glycogen than those incubated for 3 hr, but apparently due to the vast quantity, it either is retained only poorly upon fixation, or does not take the stain well. However, it can be seen that polysaccharide occupies much of the cell. (We have examined numerous cells from several similar experiments, and have almost always found this appearance in cells incubated with glucose for 8 to 25 hr, and often in cells incubated for 3 hr.)

Macronucleus. During the early stages of starvation numerous large nucleoli form in the macronucleus. These apparently result from the fusion of smaller normal-sized nucleoli. This may be occurring in Fig. 9 where several of the smaller nucleoli seem to be fusing. The distribution of the large nucleoli is shown in Fig. 16. These consist of an outer cortex of ribosome-like particles surrounding an amorphous region of material which probably is RNA and protein (Fig. 18). In some profiles there is a central less-dense region of fibrous material which may be DNA. Cameron and Guile (7) observed similar nucleoli in *Tetrahymena* during late logarithmic and stationary growth. During starvation nuclear material seems to move into the cytoplasm by means of blebbing. Evidence for this process is shown in Figs. 19-22. In Fig. 19 a small bleb containing nucleolar material and nuclear matrix is pushing out into the cytoplasm. A clear bleb is shown in Fig. 20 and just beyond it is a vacuole containing amorphous dense material suggesting that it pinched off from the macronucleus although it could be simply a nuclear outpocketing. In Fig. 21 two vacuoles, with a con-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Figs. 1-6. Aspects of mitochondrial degeneration. Fig. 1. A double membrane (arrows) surrounds each mitochondrion. The section was taken from a cell shaken 1½ hr in inorganic medium supplemented with glucose. × 60,000.

Fig. 2. A mitochondrion resembling those in Fig. 1. Also note the centrally located area of cytoplasm and the bleb consisting of several membranes. The section was taken from a cell shaken 1½ hr in inorganic medium plus sodium acetate. × 60,000.

Fig. 3. In this micrograph membranous connections may be seen between the outer membrane of the mitochondrion and those which engulf it. This section is from a cell starved for 8 hr. × 62,000.

Fig. 4. This section was incubated to show acid phosphatase activity. Reaction product is present in the space between the outer mitochondrial membrane and the surrounding membrane (arrow). This cell was starved for 2 hr. × 51,000.

Fig. 5. This section, from a cell starved 1 hr and incubated for acid phosphatase activity, shows a later stage in the digestion of a mitochondrion. The vacuole, containing the mitochondrion with reaction product, is surrounded by a single membrane. × 23,000.

Fig. 6. This section is from a cell starved 3 hr under static conditions. Note the myelin-like whorl present at the periphery of one of the mitochondria. Several whorls of membranes are also present in the cisternae of the endoplasmic reticulum. × 36,000.

nection between them, lie near the nuclear membrane. The smaller proximal one contains material similar to that of the nuclear matrix, while the other contains nucleolar-like material which might be in a stage of degeneration. Fig. 22 is from a cell starved for one hour (treated for the presence of acid phosphatase). An extremely long, finger-like protrusion, containing nucleolar material, is shown. It terminates in a swollen region which seems to be in the process of pinching off from the macronucleus. The insert (Fig. 22), which is from an adjacent section, shows that the terminal portion of this protrusion is connected to what might be termed an autophagic vacuole. Thus it appears that macronuclear contents may be disposed of by digestion in a manner similar to the degradation of mitochondria and other cytoplasmic materials. This visual loss of nucleolar material is of interest since Cline and Conner(9) showed chemically that 30% of the cellular RNA is lost in the first 3 hours of starvation in *T. pyriformis*.

Autophagic Vacuoles. In addition to the sequestration of mitochondria and macronuclear material into what appear to be autophagic vacuoles, other cytoplasmic particulates seem also to be sequestered into vacuoles when the ciliates are starved. The cell shown in Fig. 23 has been starved for only 0.5 hr and already an area of "focal degradation"(40) is seen. Three sequestered areas of cytoplasm are observed, one of which contains 2 bodies composed of concentric myelin-like membranes. Several of these structures lie free in the cytoplasm. One of the sequestered areas seems to be in the process of being enveloped itself. After 4 hr of starvation, autophagic vacuoles, containing membranes and other unidentifiable material, appear as shown in Fig. 24. In later stages of starvation (24 hr) in cells with large glycogen reserves, double-membraned vacuoles containing this polysaccharide are occasionally seen (Fig. 25). The fate of these vacuoles and of its contents is unknown.

After 24 hours starvation, numerous residual bodies appear consisting of concentric whorls of myelin-like material and a solid dense core (Figs. 8, 26). Fig. 26 also shows what are apparently earlier stages in the formation of these, since cytoplasmic material is still present in one of them. Also, some very dense, myeloid material is shown in 2 digestive vacuoles. Such material has been previously described by Swift and Hruban(40). Fig. 27 shows several residual bodies at the periphery of a large vacuole. Another such vacuole, located at the other end of the cell, was also surrounded by these bodies. The residual bodies are presumably autophagic vacuoles in which only the more slowly, or the non-digestible materials remain. The possibility that such membranous material may be only slowly digested has been discussed by de Duve(10).

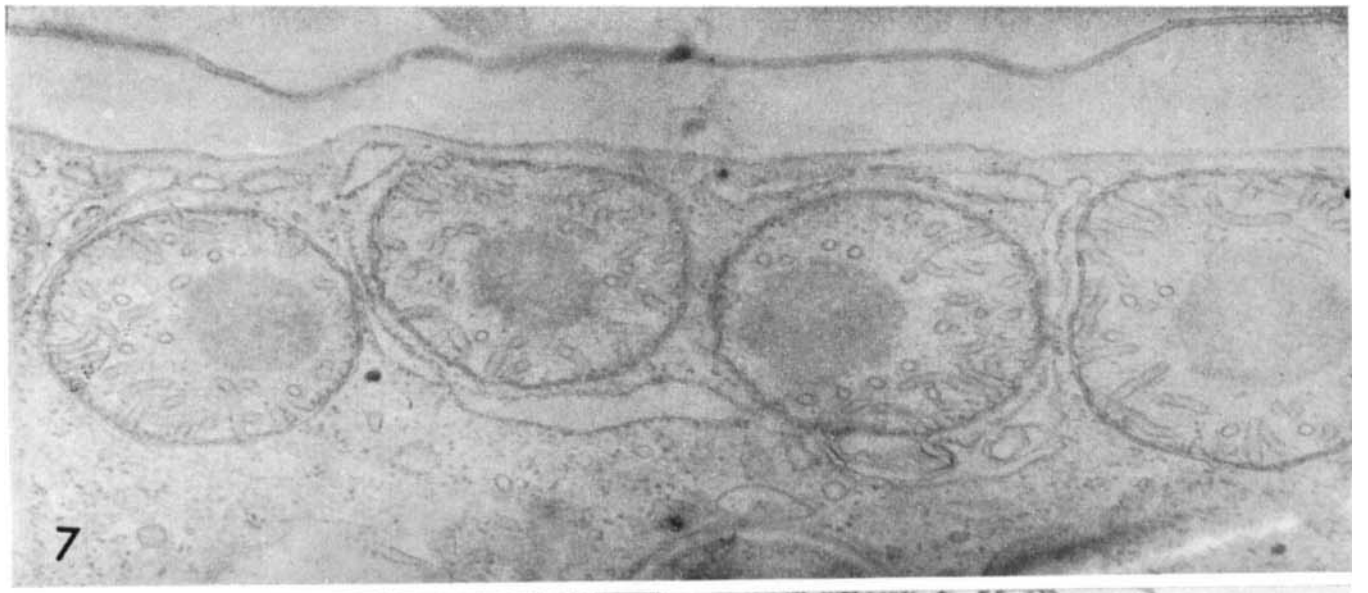
In these earlier studies(12) autophagic vacuoles containing mitochondria, as well as other cytoplasmic material, gave a positive reaction when tested for acid phosphatase, indicating that such vacuoles contain the enzyme. Just how the enzyme enters the vacuole or how the vacuole is formed remain unknown. In the present study, acid phosphatase (in so far as this test is reliable) occurs in the cisternae of some regions of the ER, in the vacuole membranes, and in the vacuoles themselves. In Fig. 28 reaction product appears in a vacuole which seems to arise from the end of the rough ER, suggesting that acid phosphatase, synthesized at the ribosomes, makes its way into the cisternae of the ER and move to the vacuole. Evidence for the sequestration of cytoplasmic materials is shown in 2 serial sections (Figs. 29, 30) which show what appears to be a forming vacuole. In Fig. 29 the vacuole is open on one side, whereas the next section (Fig. 30) shows it completely closed. Acid phosphatase reaction product appears between the double membrane.

Once bits of cytoplasm and cytoplasmic particulates are sequestered into vacuoles, these may coalesce into large autophagic vacuoles. In Fig. 31 an autophagic vacuole, containing partially degraded mitochondria, may have fused with a double-membraned vacuole. Both vacuoles give a positive test for acid phosphatase. In the same micrograph another vacuole, containing unidentifiable material, also gives a positive reaction. It is assumed that the ultimate fate of these autophagic vacuoles is complete degradation and that the end-products will be utilized by the starving cell. Fig. 32 shows several double-membraned structures which appear to be pinching off small bits of cytoplasm. Reaction product is associated with these structures.

Hydrolytic Enzymes. The activities of acid phosphatase (toward p-nitrophenylphosphate) and acid proteinase (toward hemoglobin) were determined in cells starved for various periods. The results of one of these experiments are shown in Table 1. The specific activity of acid phosphatase

TABLE 1. *Amounts of hydrolytic activities in starved cells. Specific activity of proteinase is given as mg albumin equivalents released/hr/mg protein. That of acid phosphatase is given as μmoles p-nitrophenol released/hr/mg protein.*

Hours of starvation	mg pro./ml suspension	Proteinase		Acid phosphatase	
		Sp. act.	Rel. amt.	Sp. act.	Rel. amt.
0	0.527	1.3	1.0	24.2	1.0
8	0.364	2.6	1.4	20.1	0.57
25	0.172	3.9	0.97	22.2	0.30



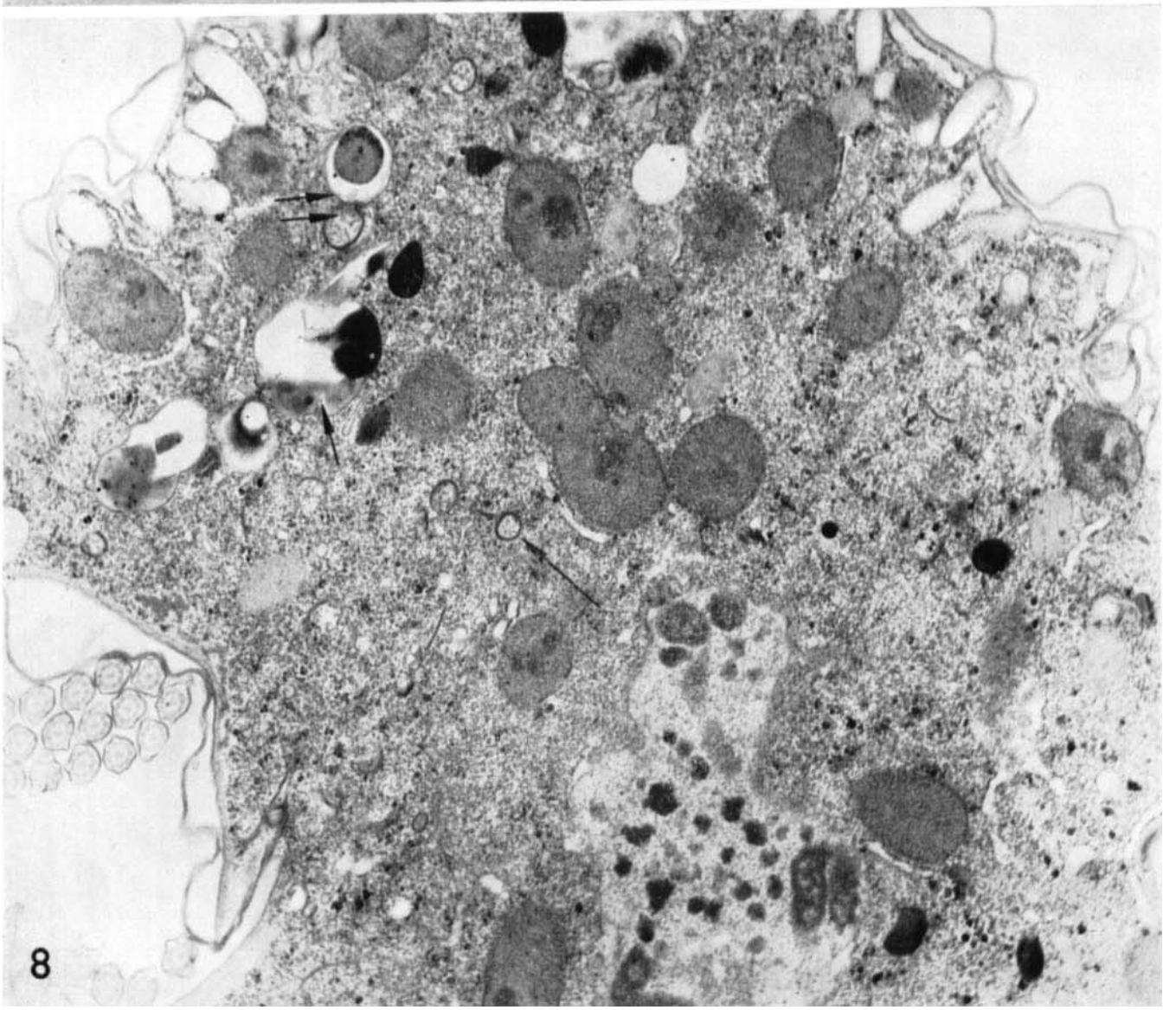


Fig. 7. This micrograph, from a cell starved 3 hr with acetate, shows intramitochondrial masses in the peripherially located mitochondria. × 47,000.

Fig. 8. Some mitochondria from cells starved 25 hr contain multiple intramitochondrial masses as seen in this micrograph. Note the very dense-staining bodies in the cytoplasm and in one of the autophagic vacuoles (short arrow). Also note the small areas of cytoplasm enclosed by a double-membraned vesicle (long arrow). One of these (double arrows) may be fusing with a vacuole which contains an unidentified organelle. × 20,000.

Figs. 9-11. These micrographs show lipid droplets which may be in stages of formation. The sections were taken from cells shaken in inorganic medium, supplemented with acetate, for either 1 hr (Figs. 10-11) or 1½ hr (Fig. 9). Since a marked increase in the number of Oil Red O-staining particles occurs during this period, it is assumed that these are stages in the formation of lipid reserves.

Fig. 9. This micrograph shows the relationship between endoplasmic reticulum and lipid droplets (L). The droplet appears to lie in the cisterna of the endoplasmic reticulum. Note the aggregation of nucleoli (N). These apparently will fuse, since in cells starved for several hours, large nucleoli are prominent (see Fig. 18). × 36,000.

Fig. 10. In this micrograph the lipid droplet (L) appears to be connected, via the endoplasmic reticulum, to an area of the cell that is undergoing degradation. An unidentified particle (X), containing a few tubules, is seen in this section. It may be a primary lysosome or a peroxisome. × 31,000.

Fig. 11. The cisternae of the endoplasmic reticulum in this micrograph contain membranous material which might contribute to the formation of the developing lipid droplet (L). Several degenerating mitochondria lie in the area (arrows). × 33,000.

Fig. 12. This micrograph is from a cell starved 4 hr. An abundance of membranous material lies in the cisternae of the endoplasmic reticulum. Some of this material may arise by blebbing from the wall of the endoplasmic reticulum (arrows). × 47,000.

usually decreased only slightly during the first 24 hr of starvation, but this represents a substantial net decrease in the amount of enzyme, since considerable cell protein is lost during this period. In contrast, the specific activity of proteinase doubled or tripled during this period, altho this represents little change in the total amount of enzyme per ml of cell suspension. It does appear, however, that there is no net loss of this enzyme during the first 25 hr of starvation despite a loss of about ⅔ of the cellular protein.

Cell Protein. Our findings suggest that about 65% of the cell protein is lost during the first 25 hr of starvation (Table 1). Values of 63%, 67% and 65% were obtained in 3 experiments. The presence of glucose in 2 of these experiments reduced the loss to 32% and 34%, respectively; indications are that acetate has a similar sparing action. Intracellular proteins therefore presumably contribute to, or at least are potentially available for maintenance of endogenous metabolism. The meaning is difficult to evaluate, however, because of the presence of broken cells in starved suspensions.

DISCUSSION

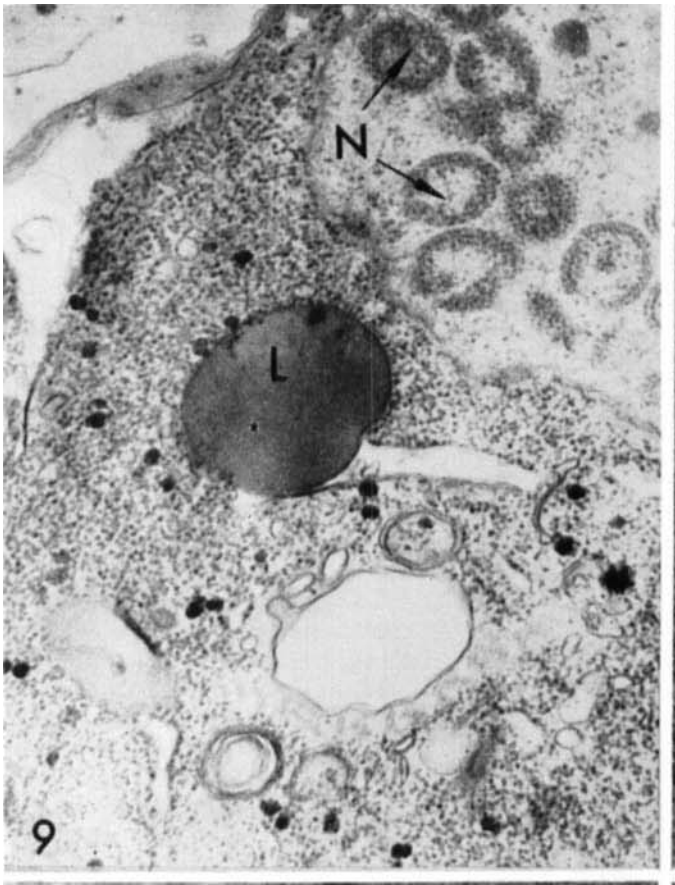
Malkoff and Buetow(32), in studies on *Euglena,* suggested that during starvation the cell uses its "most expendable organelles" for a carbon and energy source. Under our culture conditions, *T. pyriformis* has a very high rate of growth (generation time of about 4 hours) and a very high rate of respiration (about 5 μl/min/10^6 cells) (28). Much of the machinery for protein synthesis, and many of the mitochondria, could therefore be considered expendable in starved cells. Ciliates from log growth phase cultures grown under our conditions have low carbohydrate and lipid reserves. That the machinery for protein synthesis in *T. pyriformis* may be reduced in amount after a step-down transfer was shown by Cline and Conner(9), who found that about 30% of the cell RNA is lost in the first 3 hr after transfer of cells to non-nutrient medium. It was also found (Levy, unpublished data) that immediately upon transfer to inorganic medium, the respiratory rate decreases by 50-60%, and this value gradually falls for several hours. As starvation progresses, the respiratory rate can be elevated by acetate, but the final rate also drops progressively. The degeneration of mitochondria that occurs in the first few hr of starvation could, at least in part, account for this decrease in respiratory capacity.

These findings also shed some light on the nature of the carbon source for the endogenous metabolism of *T. pyriformis.* Previous work suggested that intracellular lipids are metabolized when this organism is placed in non-nutrient medium. Warnock and Van Eys(42) reported a large deposition of glycogen in the first few hours of starvation, which presumably took place at the expense of intracellular lipids, since there was no decrease in cell protein during this period. Hogg and Wagner(21) also found that glycogen increased in washed cell suspensions, the cells having an RQ of 0.63. Ryley(35) reported an RQ of 0.85 for washed cells. The degenerating mitochondria might therefore be a source of the lipids which appear to be serving as a carbon source for the endogenous metabolism of *T. pyriformis.* Proteins derived from degenerating mitochondria and ribosomes might also be utilized during starvation, since a loss of about 65% occurs during the 1st 25 hr in non-nutrient medium.

Lipids. The studies on Oil Red O droplets also indicates that endogenous lipids are being preferentially utilized over carbohydrates. In washed cells lacking a carbon source, these increased for a short time, but had virtually disappeared after 9 hr. If the medium is supplemented with acetate, there is a much greater increase in the number of particles, but they have been utilized by 25 hr, a time at which glycogen reserves are still high.

The transient increase in lipid reserves seems to be characteristic of cells in which growth has stopped. Increases in triglycerides have been shown in tissue culture cells in the stationary growth phase, or after treatment with an amino acid analogue(23), while in *T. pyriformis,* increases occur in early stationary phase(1,16,17). In *Paramecium,* lipid reserves have been reported to increase for a short time during starvation(cf. 36). Similar changes have also been observed in liver cells under conditions of nutritional deficiency or after exposure to hepatotoxic agents(15,24). Following exposure of rats to a carcinogen, small vesicles of the ER became detached and contained small lipid droplets, which increased with time and dosage(15). The role of the ER in fatty acid esterification has recently been examined by Stein and Stein(39), who found that labelled precursors appeared 1st in the ER (both rough and smooth) and then in lipid droplets. The participation of the ER in glyceride synthesis has been studied biochemically by Brindley and Hubacher(6).

Our results suggest that the triglyceride droplets arise within the lumen of the ER. Profiles such as those shown in Figs. 8-10 are quite numerous in cells starved for short periods in the presence of acetate. It is not known whether the carbon source for these lipids is exogenous acetate mitochondrial breakdown products, or if they result from a coalescence of the membranous-like material which seems to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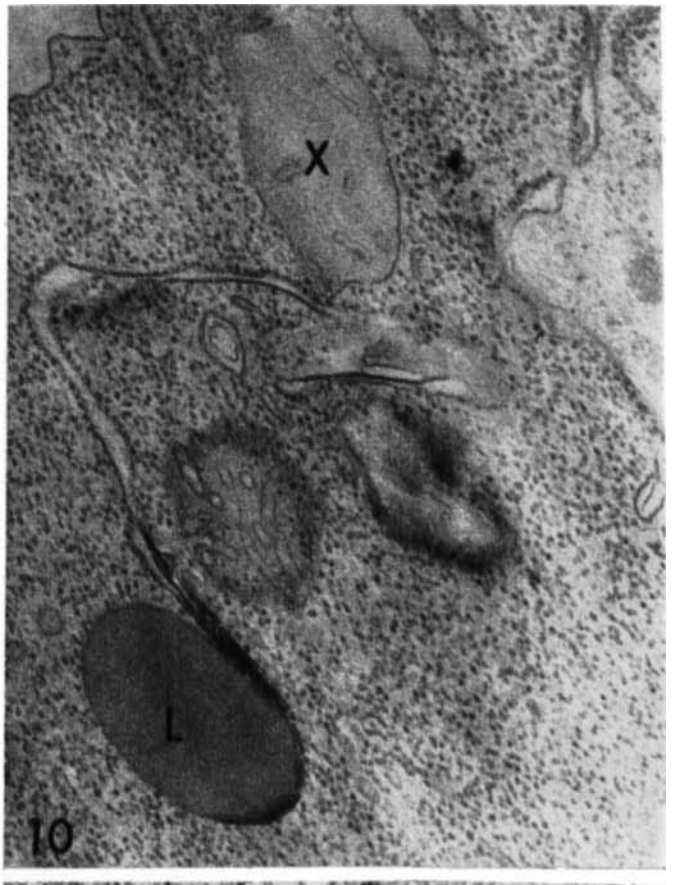
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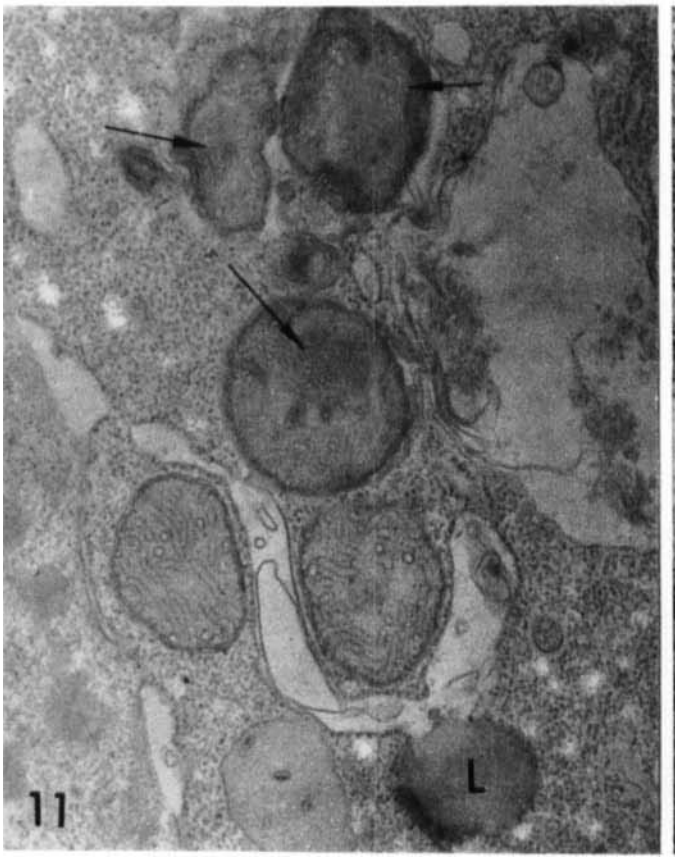
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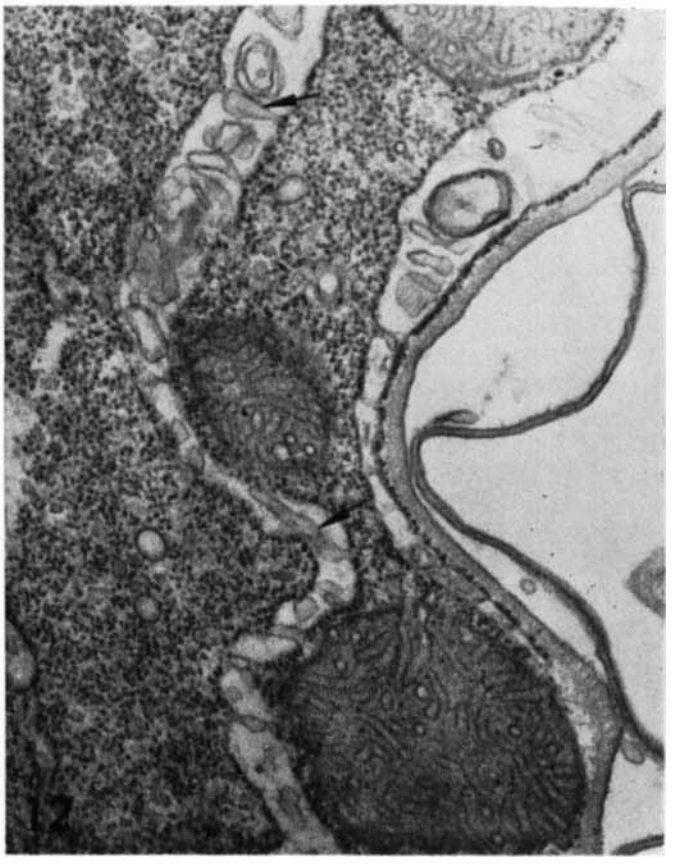
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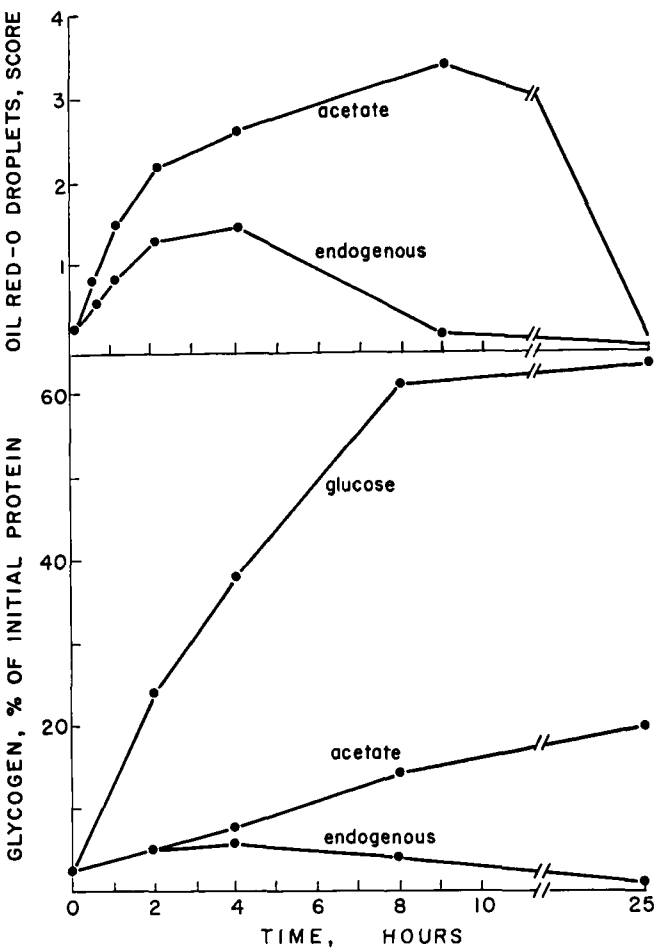


Fig. 13. Changes in lipid and carbohydrate reserves during starvation.

Upper graph, changes in the relative amount of Oil Red O-positive bodies. Cells were scored on a 0-4 basis; at least 130 from each stage were counted.

Lower graph, changes in glycogen content during starvation. Results are the average of 2 experiments. After 25 hr, the amount of protein decreased by 65, 41, and 34%, respectively, in the suspension shaken with no addition, with acetate, and with glucose. Thus, after 25 hr, in cells shaken with glucose, glycogen was equal to 111% of cell protein.

be abundant in the ER during starvation. This material seems to arise both from degenerating mitochondria and from the membrane of the ER. It should also be mentioned that the previous history of the culture will influence the metabolism of acetate in washed suspensions. Cells taken from static or from old cultures have an active glyoxylate cycle and much of the acetate carbon is converted to glycogen (21, 26, 29).

Nuclear changes. The large nucleoli, such as those that appear in the first few hours of starvation, are apparently a general response to non-growing conditions. In *T. pyriformis* they have been reported in stationary phase cells (7), in cells subjected to cyclic(8,14) or continuous heat shock(26), and, in the present study, in starvation. The nucleoli in stationary phase cells have been described as having an outer cortex of ribosome-like particles(7), and a similar appearance has been shown here. The apparent loss of these nucleoli to the cytoplasm is of interest in that Cline and Conner(9) reported a loss of 30% of the cell RNA in the early stages of starvation. The present study suggests that much of this loss might be from nuclear RNA. The nucleoli seem to be in a type of autophagic vacuole once they have been lost from the nucleus. However, mitochondrial degeneration, alterations of the ER, and general cytoplasmic sequestration could also contribute to the loss.

Autophagic Vacuoles. In studies of rat livers, Swift and Hruban(40) pointed out that starvation stimulates the sequestration process. There was no evidence that preformed cell components were involved in this process. Brandes, et al.(5) suggested involvement of Golgi-type vacuoles and multivesicular bodies in the sequestration of mitochondria and other cytoplasmic structures in starved *Euglena.* It was suggested that these vacuoles might surround a mitochondrion and then fuse, acid phosphatase being included in the forming vacuole. In the ciliate *Campanella* it has been suggested that acid phosphatase is transferred directly from the ER, where it seems to be formed, into digestive vacuoles(20). It has been suggested by Elliott and Clemmons(13) that hydrolytic enzymes may reach autophagic

Figs. 14-17. These micrographs show the accumulation of glycogen in cells starved in the presence of glucose.

Fig. 14. This section from a cell just after removal from the culture medium shows a few isolated glycogen particles (arrows). × 12,200.

Fig. 15. After 1.5 hr starvation the glycogen particles have increased in number, and in some places have begun to aggregate (arrows). × 11,200.

Fig. 16. After 3 hr starvation with added glucose the glycogen is present in large fields, some of which contain what apparently are regions of cytoplasm. Note the very large, bizarre nucleoli (arrow) in the macronucleus. × 8,300.

Fig. 17. Cells that have starved 8 hr with added glucose contain, when analyzed chemically, more glycogen than those starved 3 hr. This micrograph is from such a cell. Apparently the glycogen is lost during preparation of the cells for electron microscopy. Note that glycogen (light areas) occupies most of the cell, leaving only islands of organelles. Several autophagic vacuoles containing very dense material can be seen (arrows). × 11,500.

Figs. 18-23. These micrographs show nucleolar changes during starvation.

Fig. 18. Section is from a cell starved 3 hr. The large nucleolus has a cortex composed of a double row of ribosome-like particles (R). The inner amorphous material is presumably ribonucleoprotein. The central core contains fibrous material which may be DNA. × 64,000.

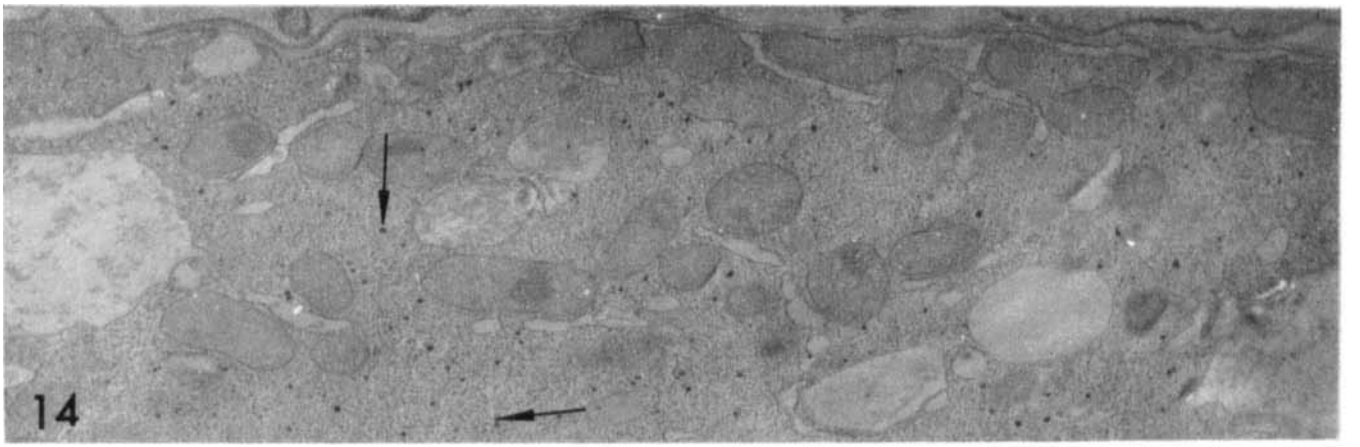
Fig. 19-21. These micrographs are a series from the same area of one cell.

Fig. 19. A portion of the macronucleus containing nucleolar material that is protruding into the cytoplasm. × 24,000.

Fig. 20. Part of the macronuclear material is present in a bleb while another portion is separated off as a vacuole which contains ribonucleoprotein (arrow). × 28,400.

Fig. 21. This section is closely adjacent to that in Fig. 20. In this case the connection between the bleb and the vacuole containing the nucleolar material can be observed. × 28,500.

Fig. 22. Low power micrograph of a cell starved 1 hr showing passage of macronuclear material to the cytoplasm. Note the nucleoli lying in the long finger-like protrusion from the macronucleus. Some of its contents has pinched off into a vacuole. The insert, from a closely adjacent section, shows the connection between the vacuole and the protrusion (arrow). (Cell stained to reveal acid phosphatase.) × 21,000. Insert × 16,300.



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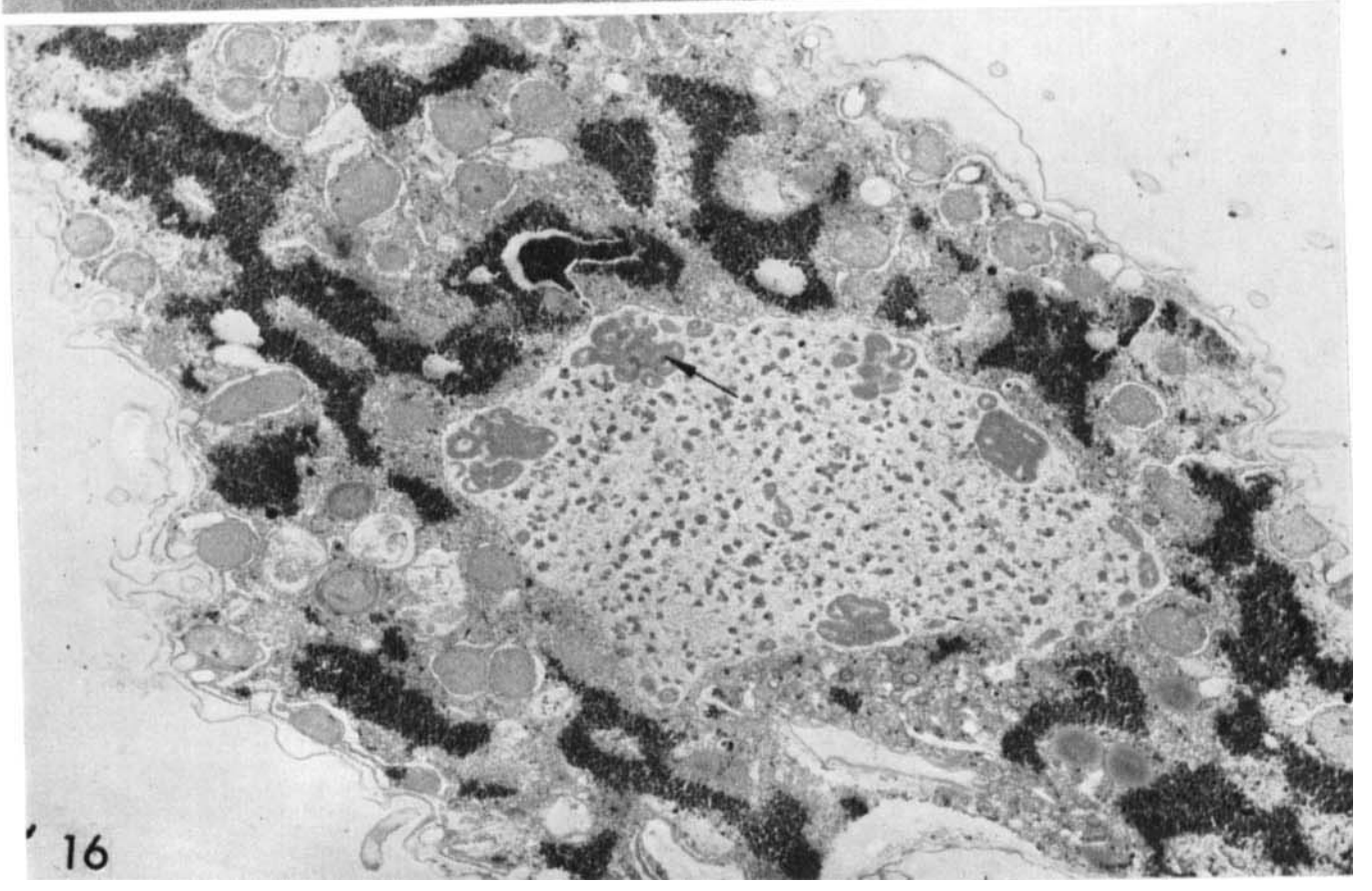

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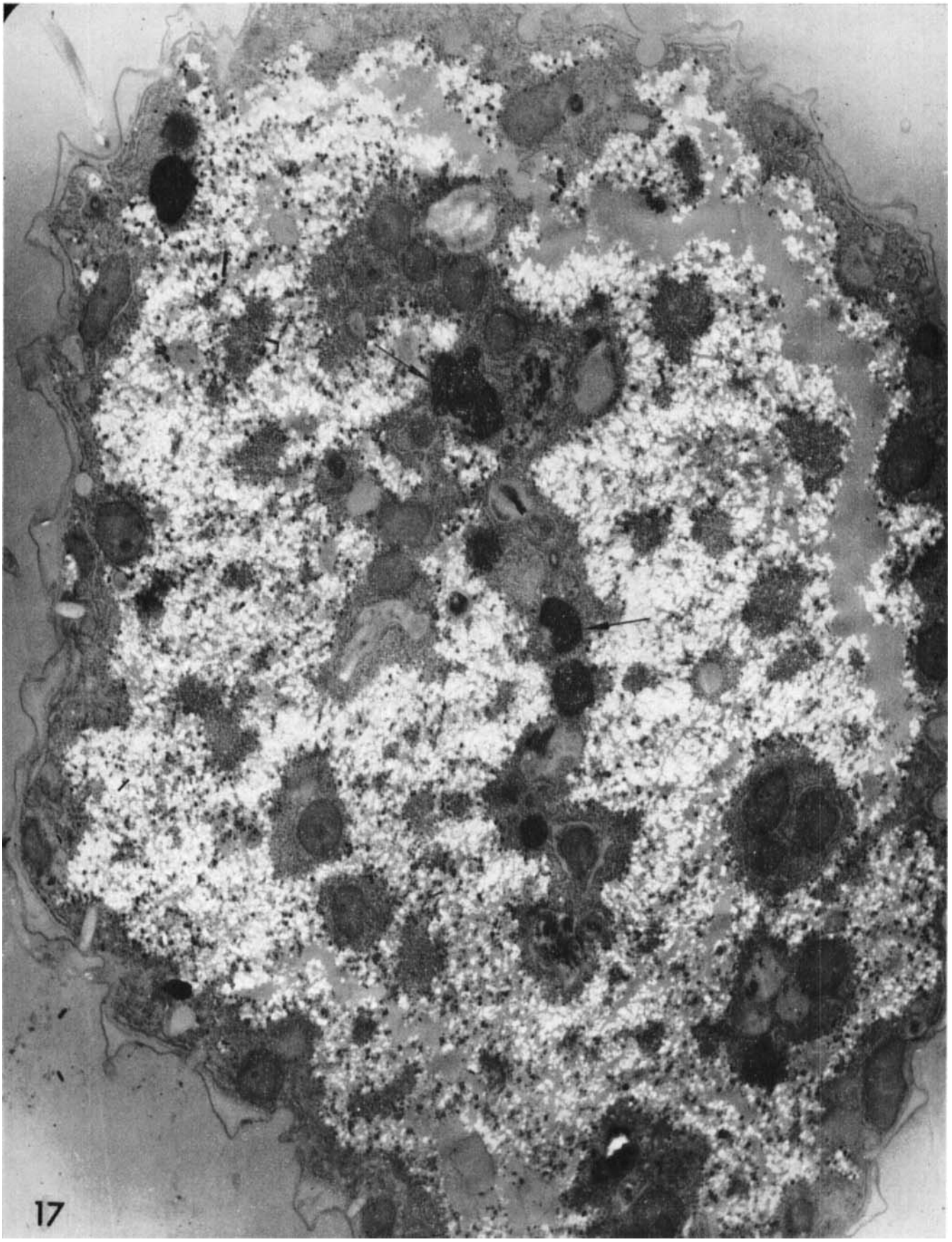
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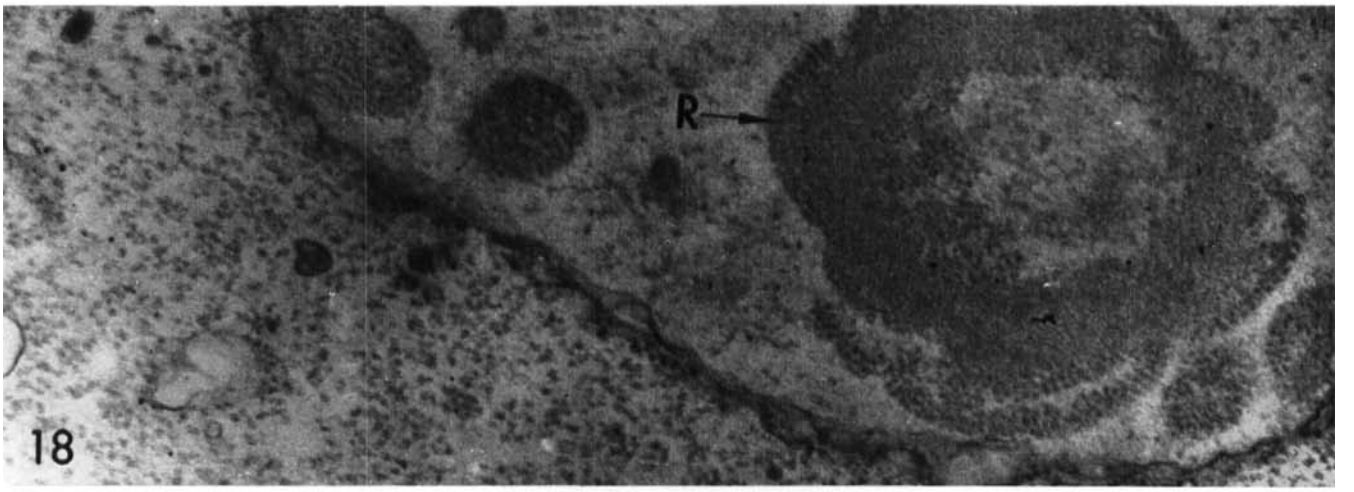
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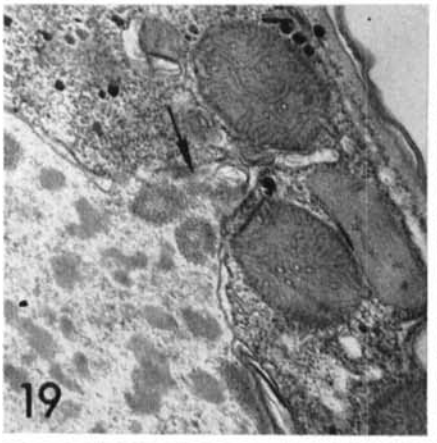
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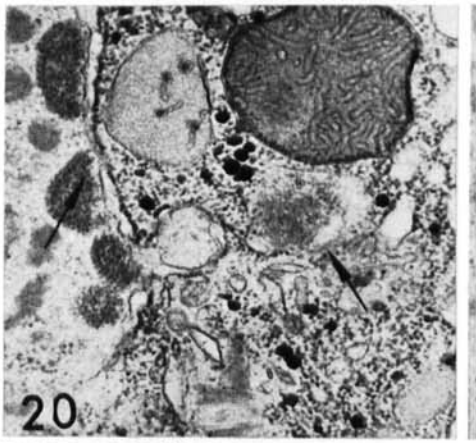
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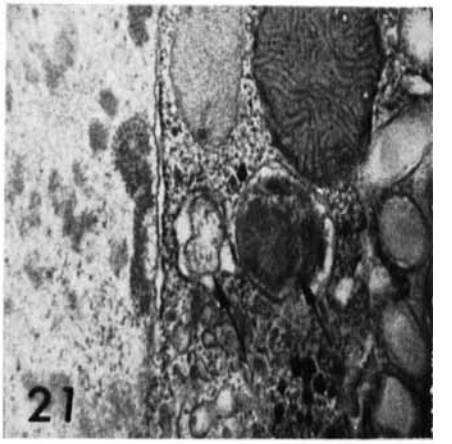
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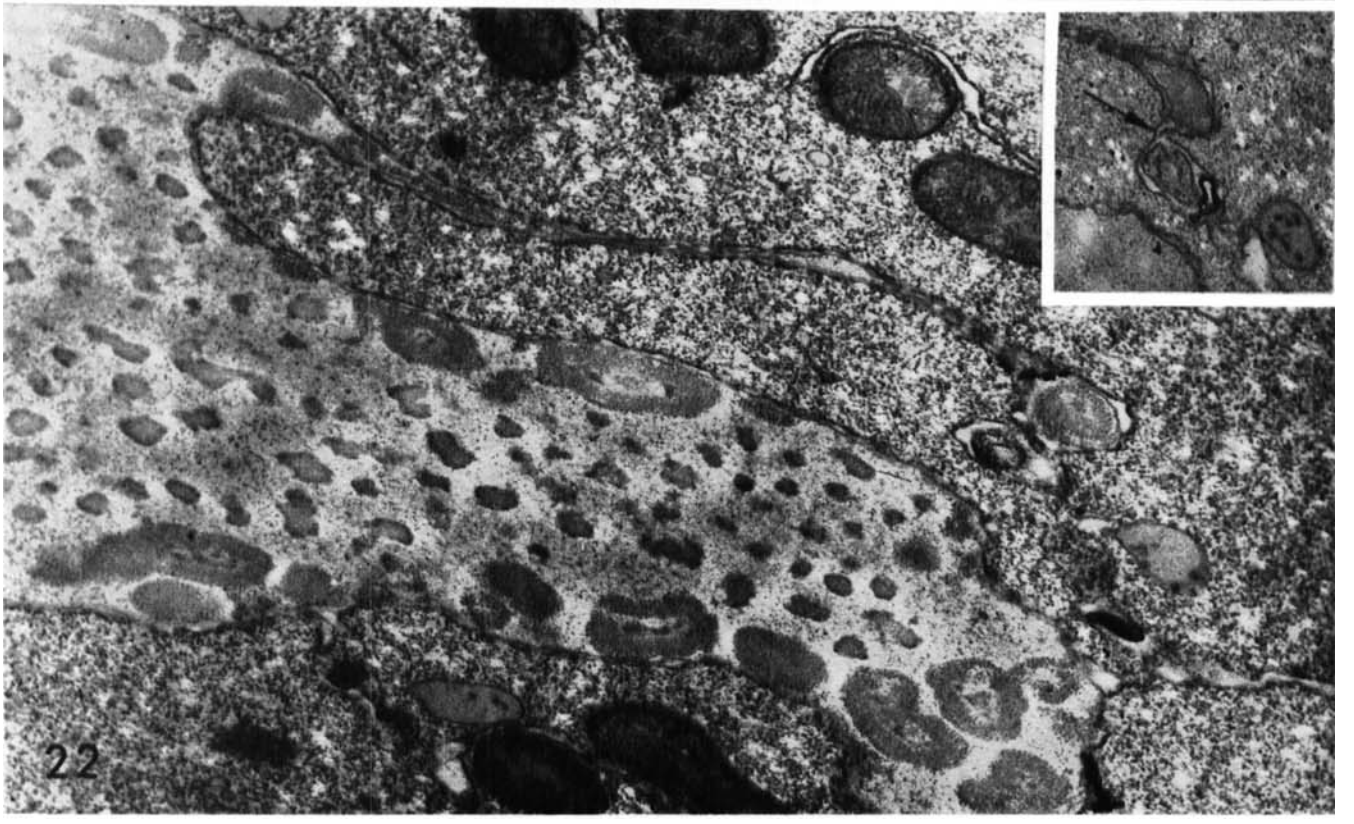
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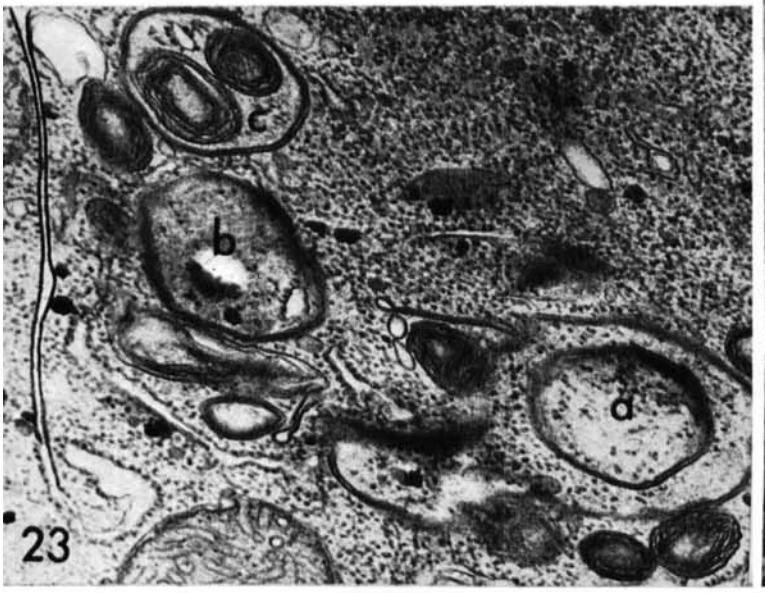

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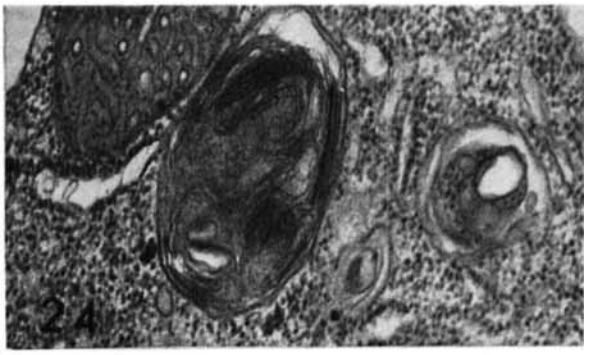

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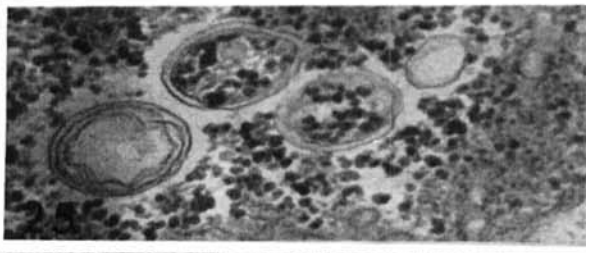

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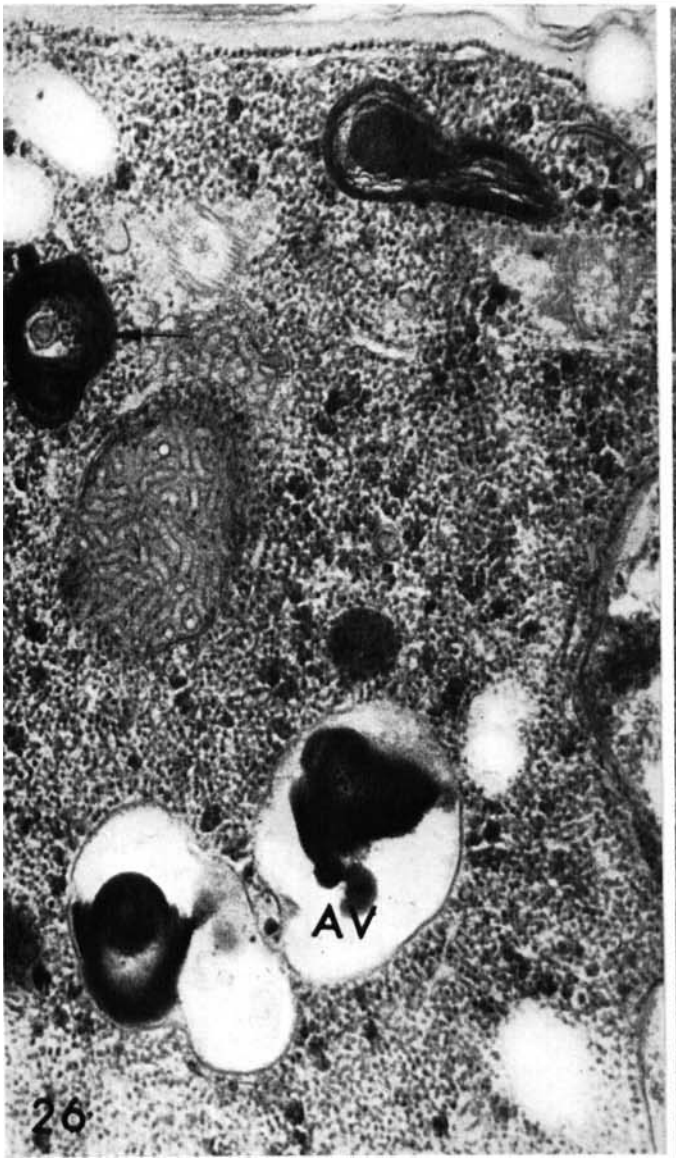

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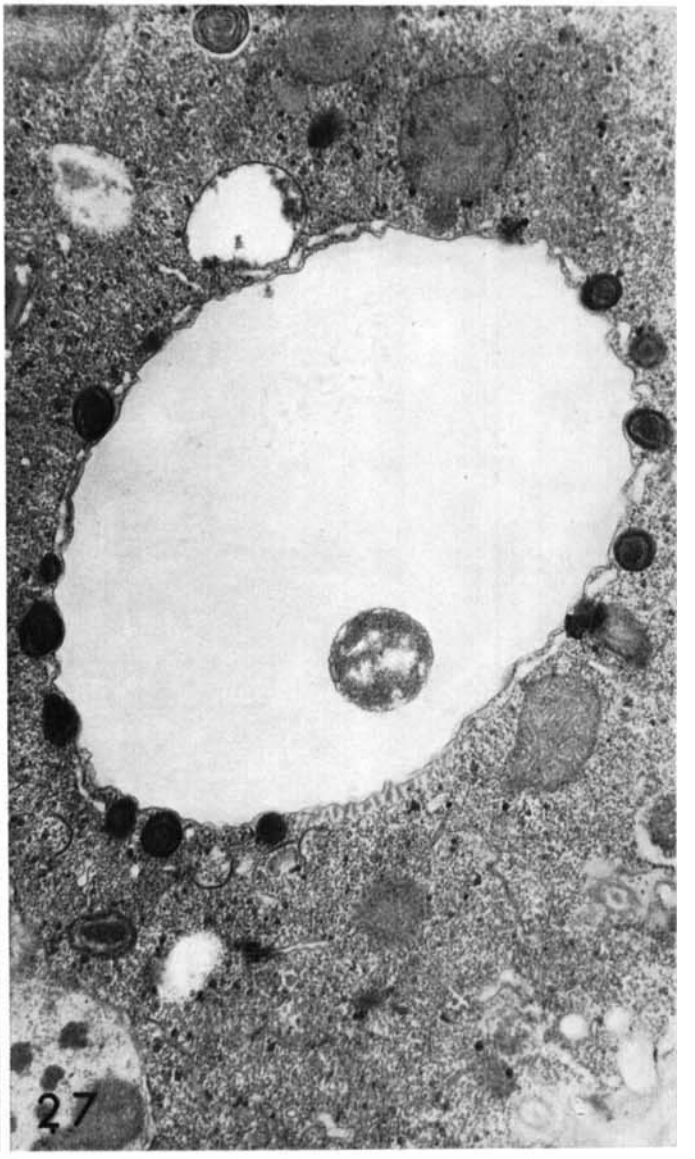

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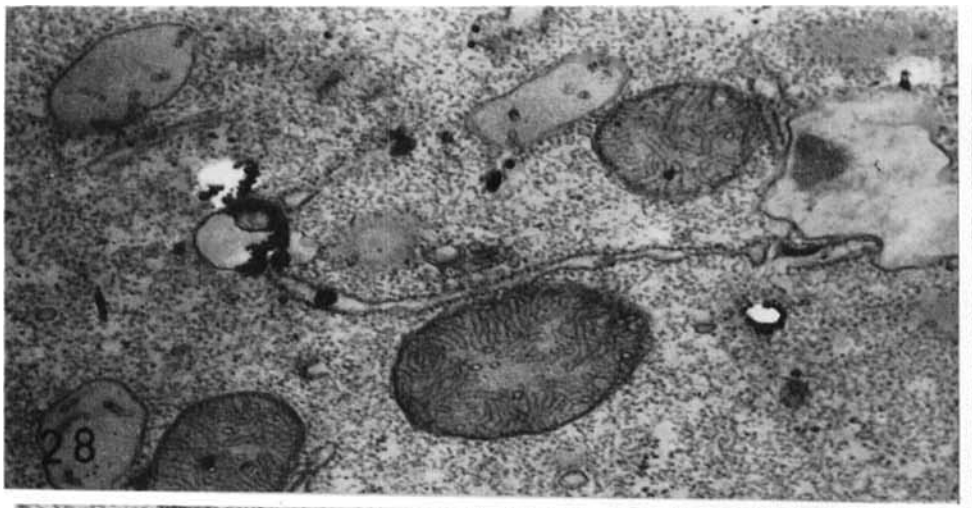
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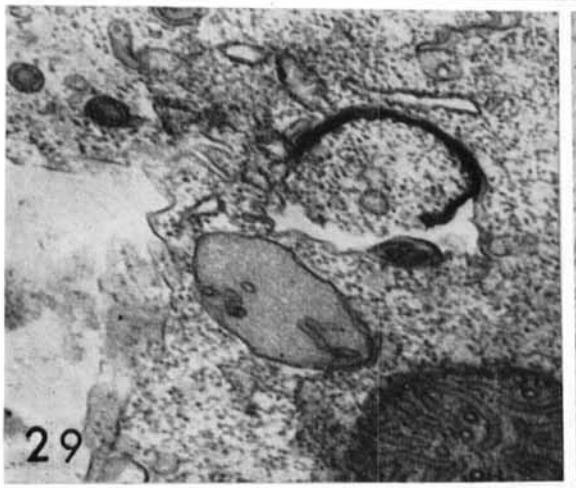
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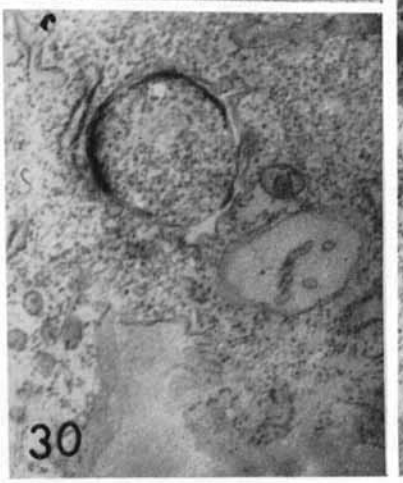
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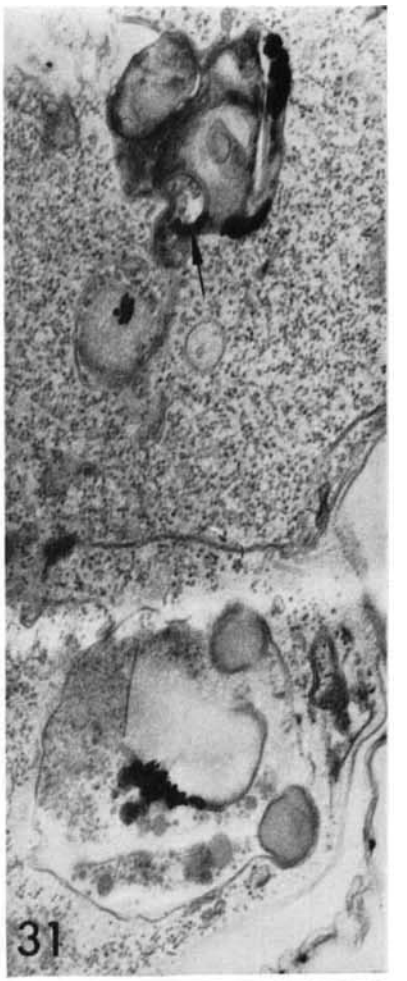
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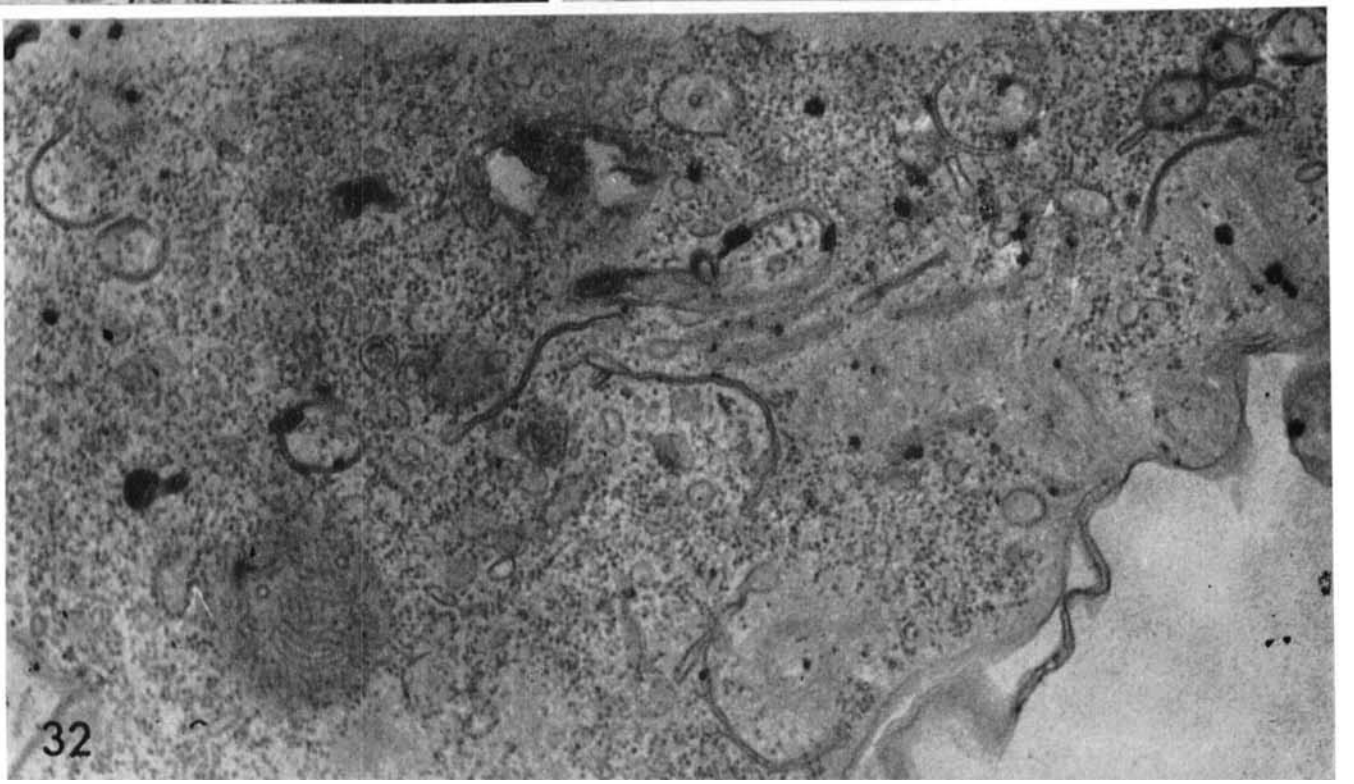
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Fig. 23. Micrograph from a cell starved ½ hr, showing several regions where cytoplasm is being sequestered into vacuoles. At (a) a double-membraned area has sealed off a bit of cytoplasm and the resulting vacuole itself appears about to be enveloped by another double membrane. A similar area is at (b). At (c), 2 bodies composed of concentric, myelin-like rings have been sequestered, together with some cytoplasm. × 41,200.

Fig. 24. Some double-membraned vacuoles seem to sequester different kinds of particulates into the same vacuole as shown in this micrograph of a cell starved 4 hr. The contents of this vacuole cannot be identified with certainty, but appear to be membranous remnants of organelles. × 50,000.

Fig. 25. Section from a cell starved 25 hr (with glucose) in which glycogen has been sequestered. The glycogen is also enclosed by double-membraned vacuoles. × 40,100.

Figs. 26-27. Cells that are starved 25 hr have numerous residual bodies in various stages of formation. One of these (Fig. 26, arrow) still contains some cytoplasmic material. Most others contain several concentric myelin-like membranes surrounding a dense core. The autophagic vacuoles (AV) contain very dense, presumably undigestible material, which seems to accumulate during starvation (see Figs. 8, 17). The residual bodies often accumulate on the membrane of large clear vacuoles as seen in Fig. 27. Fig. 26, × 45,000. Fig. 27, × 18,000.

Figs. 28-31. Micrographs from cells treated for the localization of acid phosphatase.

Fig. 28. This cell, starved for 2 hr, shows reaction product in a small vacuole that may be associated with the endoplasmic reticulum. × 31,000.

Figs. 29-30. Serial sections from a cell starved 1 hr. What appears to be reaction product is between the double membranes of the vacuole that is sequestering portions of the cytoplasm. Fig. 29 shows a portion of the forming vacuole while the adjacent section (Fig. 30) shows it closed. Fig. 29, × 39,500. Fig. 30, × 32,000.

Fig. 31. Micrograph from a cell starved 24 hr (with acetate) showing acid phosphatase in autophagic vacuoles. The upper vacuole contains 2 unidentifiable bodies (presumably mitochondria) together with one small, double-membraned vacuole (arrow) with reaction product between the membranes. The lower autophagic vacuole contains reaction product suggesting that the contents are being digested. × 33,900.

Fig. 32. Section from a cell similar to the one in Fig. 31 showing several small, double-membraned vacuoles with acid phosphatase reaction product either between or near the double membrane suggesting the association of the enzyme with the small vacuoles. × 43,000.

vacuoles by fusion with primary lysosomes in *T. pyriformis*.

The present study suggests that formation of autophagic vacuoles in *T. pyriformis* takes place in a variety of ways. In the early stages of mitochondrial degeneration, these organelles are often enclosed within a double membrane. Acid phosphatase seems to be localized between this membrane and that of the mitochondrion, at least in the initial stages of degeneration. We have no evidence concerning the origin of the surrounding membrane, nor of the acid phosphatase. The membrane may arise from the ER which is often seen in close association with the mitochondria. In the later stages of digestion the mitochondria are often seen within more or less typical autophagic vacuoles, or cytolysomes (12), which now appear to be walled off by a single membrane.

Some micrographs suggest that autophagic vacuoles may arise by sequestration of cytoplasm. This appears to be the case in Figs. 23, 29, 30, and 32. In the last 3 of these, acid phosphatase-positive membranes seem to be in the process of pinching off small areas to form small vesicles. Fig. 31 shows what might be the fusion of one of these vesicles with other degenerating organelles to form a larger autophagic vacuole. One small vesicle can be seen within an autophagic vacuole. In another ciliate, *Campanella,* acid phosphatase may arise from the rough ER, and this could be the case in *T. pyriformis,* altho we have no unequivocal evidence that it occurs. Autophagic vacuoles may also arise by nucleolar blebbing as discussed above.

The numerous residual bodies that appear after several hours of starvation seem to contain myelin-like material, which is apparently either not at all, or only slowly digested. Similar bodies have been reported to increase in number in crushed nerve(22). Myelin-like whorls seem to be quite characteristic of degenerating cells(40).

Hydrolytic Enzymes. In *Euglena,* starvation is accompanied by large increases in acid phosphatase and cathepsin (4). Ultrastructurally, the Golgi region becomes prominent and acid phosphatase-positive(5). In *Tetrahymena,* there is considerable net loss of acid phosphatase during starvation, altho the specific activity remains relatively constant. The specific activity of acid proteinase doubles or triples during the first 24 hr of starvation, but there is little or no net increase in the amount of enzyme, and the increase in specific activity can be accounted for by the loss of cell protein.

The present studies utilized, for the most part, cells from shaken, log phase (24 hr) cultures, and shaking was continued after washing. Cells from static cultures are metabolically quite different, and the present findings do not necessarily apply to such cells. In preliminary experiments, we have indeed found large differences in enzyme levels between cells from shaken and static suspensions. However, it does appear that *T. pyriformis* can rapidly rid itself of excess organelles when placed in non-nutrient medium, and that the breakdown products help the organism to maintain itself during starvation.

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