CHANGES OF THE CELL SURFACE AND OF THE DIGESTIVE APPARATUS OF *DICTYOSTELIUM DISCOIDEUM* DURING THE STARVATION PERIOD TRIGGERING AGGREGATION

CHANTAL DE CHASTELLIER and ANTOINETTE RYTER

From the Unité de Microscopie Electronique, Département de Biologie Moléculaire, Institut Pasteur, Paris, France

ABSTRACT

The effects of starvation on the cell morphology of *Dictyostelium discoideum* were studied with different cytochemical techniques, and with a morphometric method by which the surface areas of the cell membrane and of the digestive system can be determined.

During the first 2 h, the cell membrane becomes very wrinkled and many phagocytic cups and filopods are formed. These changes are in accord with the 40% increase in the cell surface area to cytoplasmic volume ratio observed, which is mainly due to a strong decrease in the cytoplasmic volume. At this time of starvation, cells are able to ingest twice as many yeast as during growth. Afterwards, while the phagocytic ability decreases, the phagocytic cups disappear, and all the cells become bristled with many thin filopods. In spite of these morphological changes, no quantitative or topological differences have been observed concerning the polysaccharide content of the plasma membrane, whether it was stained with phosphotungstic acid, silver proteinate, or ruthenium red.

During this time, the digestive vacuoles imbricate one into the other. Part of the vacuoles are degraded by this process, thus leading to an atrophy of the digestive apparatus. The digestive apparatus is progressively replaced by an autophagic system. Polysaccharide stainings and morphological observations show that the cytosegresomes seem to originate from the food vacuoles which flatten and sequester portions of cytoplasm. After 5 h of starvation, the digestive system is entirely transformed into an autophagic apparatus. The cell population appears to be homogeneous with respect to these changes. Therefore, potential precursors of prestalk and prespore cells were not observed.

KEY WORDS modifications · *Dictyostelium discoideum* · cell surface · digestive apparatus

The life cycle of the slime mold *Dictyostelium discoideum* is divided into two distinct phases: the vegetative phase, during which the organism exists as solitary ameboid cells; and the developmental phase, which is triggered by a starvation period of ~5 h, after which cells aggregate and differentiate into fruiting bodies. Many studies have been made on the biochemical and physiological events occurring during starvation and differentiation. They show that several enzyme activities decrease or increase, that new ones appear, and that the cells use their glycogen reserves and their own proteins for new synthesis (15, 16, 19, 20, 31, 34).

Several ultrastructural studies have described the characteristic events occurring during the differentiation of stalk and spore cells (8, 12, 18, 23), but, until now, the morphological and cytochemical changes appearing during starvation have been completely neglected. Yet, starvation certainly has an important effect on the digestive system and its membrane turnover. As shown in the accompanying paper (28), the surface area of the digestive apparatus is equal to that of the plasma membrane in growing cells. Despite a continual internalization of the plasma membrane due to pinocytosis or phagocytosis, the cell surface area remains constant due to its simultaneous renewal by the membranes of the digestive system. During starvation, the absence of food obviously leads to the disappearance of the digestive vacuoles, as differentiating cells do not contain any (8, 12, 23). In contrast, autophagic vacuoles, the origin of which is not well established, are particularly abundant in the prestalk cells (23), and new types of vacuoles appear in the prespore and spore cells (12, 18, 23).

We studied in detail the morphological transformations that occur during starvation to learn the fate of the digestive vacuoles, the origin of autophagic vacuoles, and to determine whether or not a distinction can already be made between two types of cells which could give rise to the prespore and prestalk cells. Different methods were used in this study. Firstly, the morphometric method (32) already applied during phagocytosis of axenically growing cells (28), was used to determine the surface area changes of the plasma membrane and of the vacuolar system at different times of starvation. Secondly, the study of the capacity to phagocytize in cells starved for different periods of time showed whether or not the alterations of the digestive apparatus during starvation affect or modify its membrane turnover. Thirdly, the cytochemical demonstration of acid phosphatase performed at the ultrastructural level gave information on the functioning of the digestive system, on the formation and digestive activity of the autophagic vacuoles, and their eventual role in the degradation of glycogen and proteins.

In addition, cytochemical techniques revealing polysaccharides were applied to detect eventual changes in the membranes of the digestive system and of the cell surface. It is certain that important modifications of the plasma membrane occur inasmuch as new antigenic determinants and cyclic adenosine monophosphate (AMP) receptors appear (3, 4, 9), and the particulate structures of the cell membrane visible on freeze-etching preparations vary in size and number (1, 11, 13, 14, 36). Furthermore, because the cells have a tendency to aggregate, the cell coat is probably implicated in this first step of differentiation. Therefore, techniques which specifically stain polysaccharides seem to be good tools for detecting some quantitative or topological modifications of the cell surface.

MATERIALS AND METHODS

Starvation Conditions

D. discoideum strain AX-2 was grown in HL-5 liquid culture medium (30) at 20°C with constant and mild shaking. Under these conditions, this strain showed a generation time of 8–10 h. Cells were grown to a concentration of 2×10^6/ml. They were harvested, washed once in 17 mM phosphate buffer, pH 6.5, and resuspended in the same buffer, keeping the same concentration of cells as initially. The cells were incubated in this starvation medium at 20°C with constant and mild shaking.

Phagocytosis

At 0 h of starvation and every hour thereafter (up to 5 h), 12–15 killed yeast (28) per cell were added to samples of myxamoebae under starvation. In each case, after 1 h of phagocytosis, the cells were fixed and prepared for electron microscopy. In the study of the kinetics of uptake, the number of ingested yeast per cell was counted with the light microscope every 20 min for 1 h of phagocytosis.

Morphometric Analysis

The surface area changes of the plasma and vacuolar membranes during starvation were studied according to the technique of Weibel et al. (32), as already used on growing cells (28). For this purpose, 100–120 micrographs of cell profiles were randomly taken. They came from samples fixed every hour between 0 and 5 h of starvation, or after phagocytosis. Micrographs were enlarged to a 14,000 final magnification, and were analyzed with a grid of 14-mm long bars. The number of intersections (I) between the bars and the different membranes were determined, as well as the number of points (P) in the cytoplasm, including the nuclear area. The surface area to cytoplasmic volume (S/V) ratio was calculated by the following formula: $S/V = (I \times 4)/(P \times l/G)$, where l = length of the bars in microns, and G = enlargement of the micrograph, here $l/G = 1$.

Electron Microscopy

The conditions of fixation, dehydration, and embedding of samples are described in the accompanying paper (28).

Scanning Electron Microscopy

Samples were harvested in axenic medium during vegetative growth, and every hour during the starvation

period (0–5 h). An aliquot of each sample was deposited on glass slides coated with Formvar. Once the cells had adhered to this support (~3-5 min), they were fixed overnight with 2.5% glutaraldehyde in 0.05 M cacodylate buffer containing 0.1 M sucrose. After several brief washings with buffer, the cells were post-fixed for 1 h with 2% osmium tetroxide in 0.05 M cacodylate buffer also containing 0.1 M sucrose. They were then washed in buffer and dehydrated in a graded series of ethanol. After being passed through absolute ethanol and Freon (Phillips Manufacturing Co., Chicago, Ill.), they were dried by the critical point method with Freon. Finally, the specimens were coated with gold, and mounted on stubs. They were observed with a CAMECA MEB 07 at 20 kV.

Staining of Polysaccharides

The silver proteinate staining procedure of Thiéry (29), slightly modified by Robertson et al. (25), and the phosphotungstic acid-chromic acid technique of Rambourg (24), modified by Roland et al. (26), were used to stain polysaccharides as described in the accompanying paper (28).

Ruthenium red (RR [22]) was also used to stain cell surface glycoproteins. Cells were fixed for 1 h in 2.5% glutaraldehyde containing 0.05% RR. They were washed overnight in cacodylate buffer devoid of RR, postfixed for 3–4 h in 2% OsO_4 containing 0.05% RR, dehydrated in acetone, and embedded in Epon. Thin sections were observed without further staining.

Cytochemistry

The cytochemical procedure used to detect acid phosphatase was the same as the one described in the accompanying paper (28). For this purpose, samples were taken at 0 min of starvation, and every hour for 5 h.

Biochemical Assays

At 0 min of starvation and every hour thereafter (up to 5 h), 5-ml samples were collected and washed once in water. The supernate of the culture and the pellet were immediately frozen and stored at −20°C. During freezing and thawing, the cells are completely broken, and this homogenate can be directly used for biochemical assays.

Acid phosphatase was measured in a medium consisting of 0.037 M acetate buffer, pH 5.0, 0.05% Triton X-100 (Rohm & Haas Co., Philadelphia, Pa.), and 0.005 M *p*-nitrophenyl phosphate. Aliquots of cell homogenate (2×10^5 cells in 1 ml of incubation medium) or culture supernate (0.1 ml/assay) were incubated at 37°C for 30 min. The reaction was linear for at least 60 min, and it was stopped by adding 5 ml of 0.1 N NaOH. The absorbency of the reaction product was measured in a spectrophotometer at 410 nm.

To assay for total cellular protein content, the thawed cells were resuspended in water to have the equivalent of 5×10^6–1×10^7 cells/ml of homogenate. The protein content was determined by the method of Lowry et al. (21), with bovine serum albumin as a standard, and expressed as milligrams of protein per 10^8 cells.

To assay for total cellular carbohydrate content, the homogenate contained the equivalent of $1–2 \times 10^7$ cells/ml. Samples were then assayed for hexose by the anthrone method of Hassid and Abraham (17), with glucose as a standard, and expressed as milligrams of glucose equivalents per 10^8 cells.

RESULTS

Ultrastructural Study of Starved Cells

During the 5 h of starvation, cells acquire several characteristic features which have been served in whole cells with the scanning electron microscope and in thin sections. During growth, whole cells present an irregular shape with some filopods (27), and sometimes they present a phagocytic cup (Fig. 1*a*). The membrane remains smooth on the processes, but becomes a little ruffled on the rest of the cell surface. In contrast, when cells are starved for 1 h, the membrane becomes very wrinkled (Fig. 1*b*, [27]), and this appearance persists until aggregation. In thin sections, the membrane is very indented (Fig. 2*b*, *d*) as compared to the membrane of growing or 0-min starved cells (Fig. 2*a*, *c*). After 1 or 2 h of starvation, the majority of the cells observed in scanning electron microscopy possess several large phagocytic cups, but few filopods (Fig. 1*b*). In thin sections, the phagocytic cups appear as very thin, long processes. These cells look very "aggressive" and sometimes encircle other cells. Some succeed in phagocytizing a whole cell or part of a cell, as can be judged by the partially digested aspect of the endocytized cells observed in thin sections 1 or 2 h later. However, in the "well-fed' cultures, cannibalism is an exception.

After 2 h of starvation, phagocytic cups progressively disappear and are replaced by a high number of thin, long filopods (27, [manuscript in preparation]).

With regard to the digestive apparatus of starved cells, the food vacuoles which are initially big and contain a material of variable density (Fig. 2*a*) lose their content and imbricate, one into the other (Fig. 3*a*, *b*). This phenomenon is sometimes observed in growing cells, but becomes much more frequent at the beginning of starvation. Many vacuoles contain vesicles constituted by a double membrane (Fig. 3*b*) which correspond either to cross sections of these imbricated

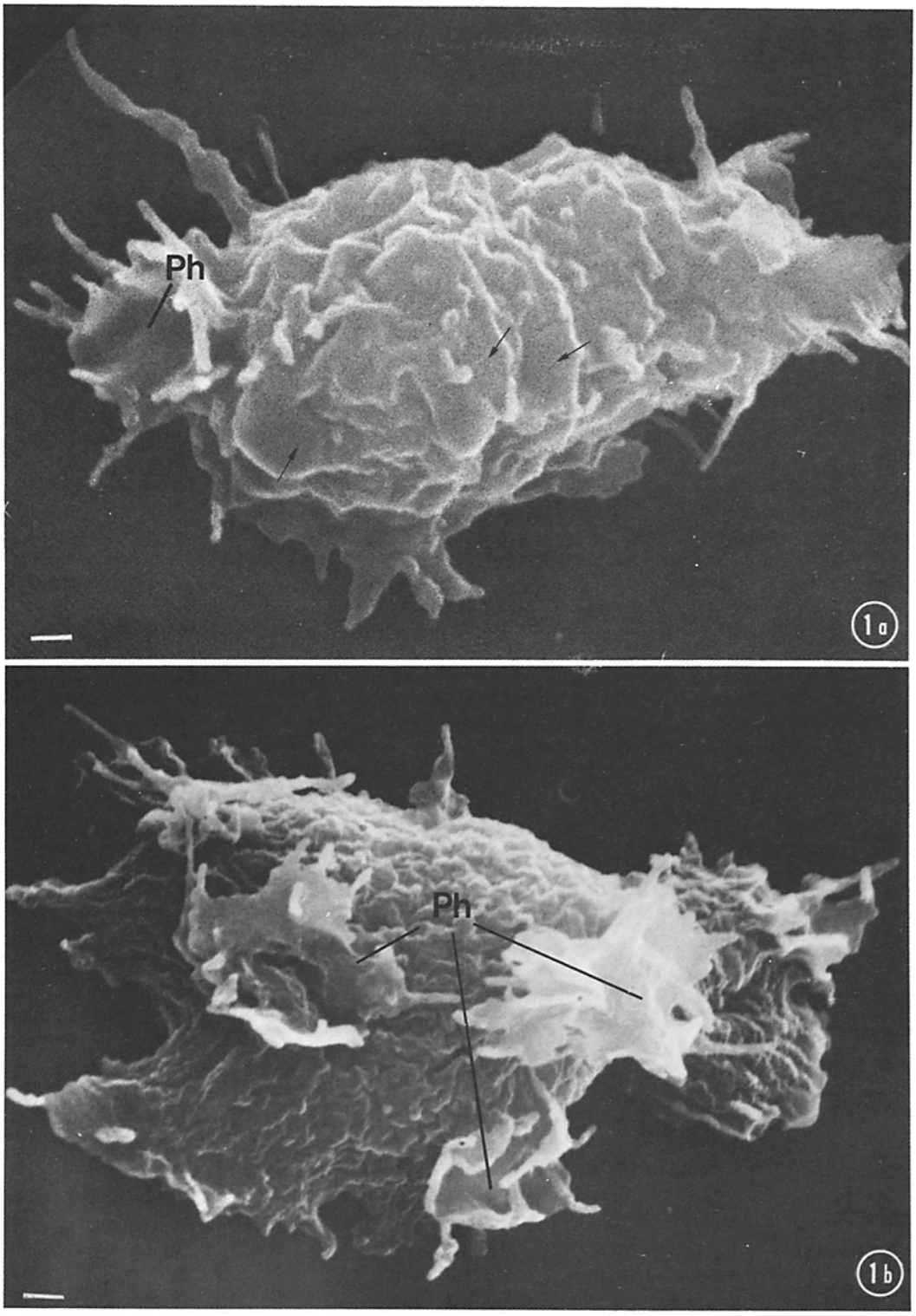


FIGURE 1 General views of whole cells observed with the scanning electron microscope. (*a*) Vegetative phase cell grown in axenic culture medium. The membrane forms many folds, some filopods, and a phagocytic cup (*Ph*). The membrane is smooth between the folds (arrows). × 6,000. Bar, 1 μm. (*b*) Cell starved for 2 h showing a very wrinkled membrane, some filopods, and several phagocytic cups (*Ph*). × 6,000. Bar, 1 μm.

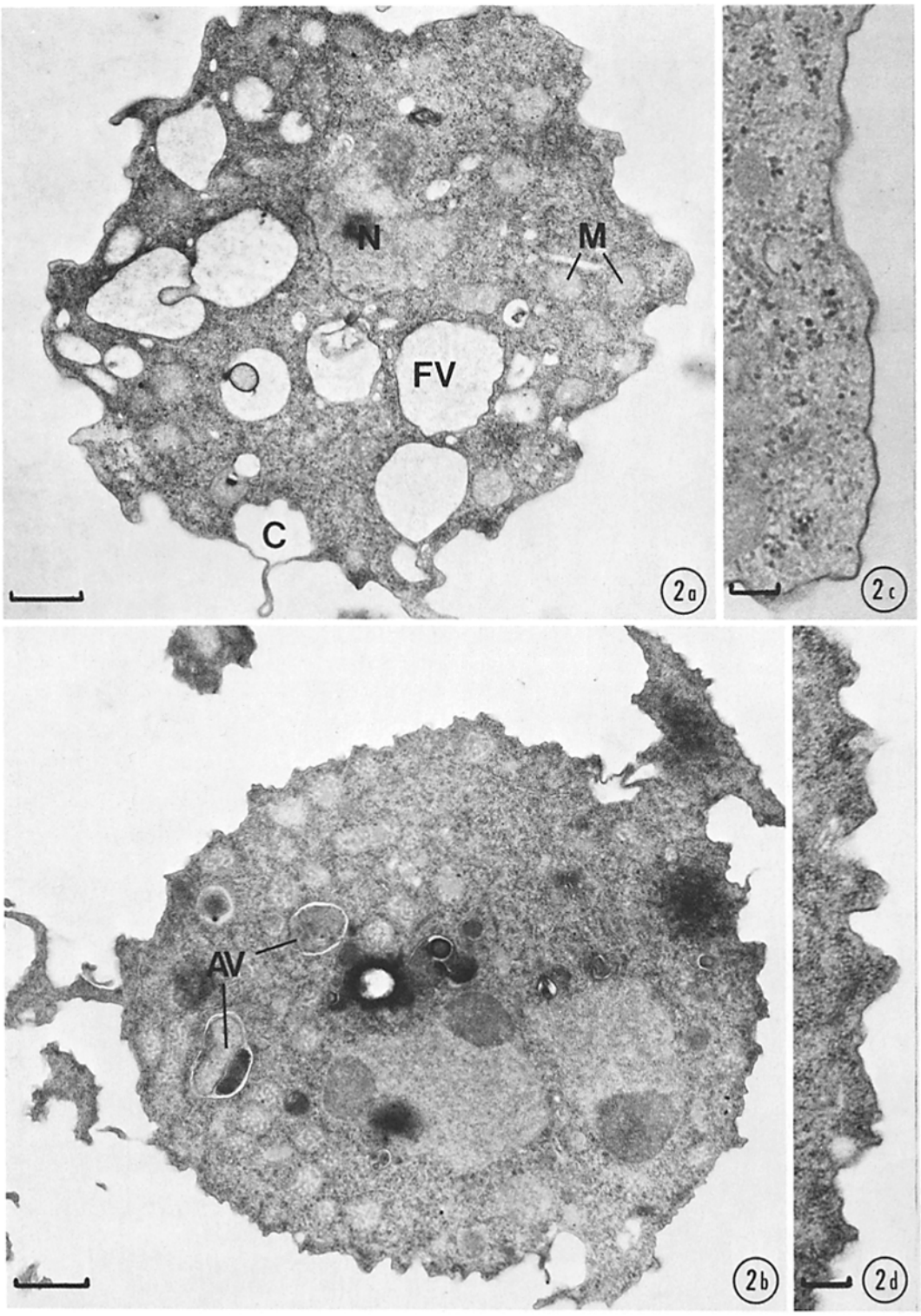


FIGURE 2 (*a*) General aspect of a cell after 0 min of starvation. There are many food vacuoles (*FV*) of various sizes. (*N*) nucleus, (*C*) contractile vacuole, (*M*) mitochondrion. × 11,200. Bar, 1 μm. (*b*) General aspect of a cell after 5 h of starvation. There are very few, if any, small food vacuoles. In contrast, cells contain many autophagic vacuoles (*AV*). × 11,200. Bar, 1 μm. (*c, d*) Details of the plasma membrane. (*c*) At 0 min of starvation, the membrane is quite smooth, (*d*) after 5 h, it is very indented. × 28,000. Bar, 0.25 μm.

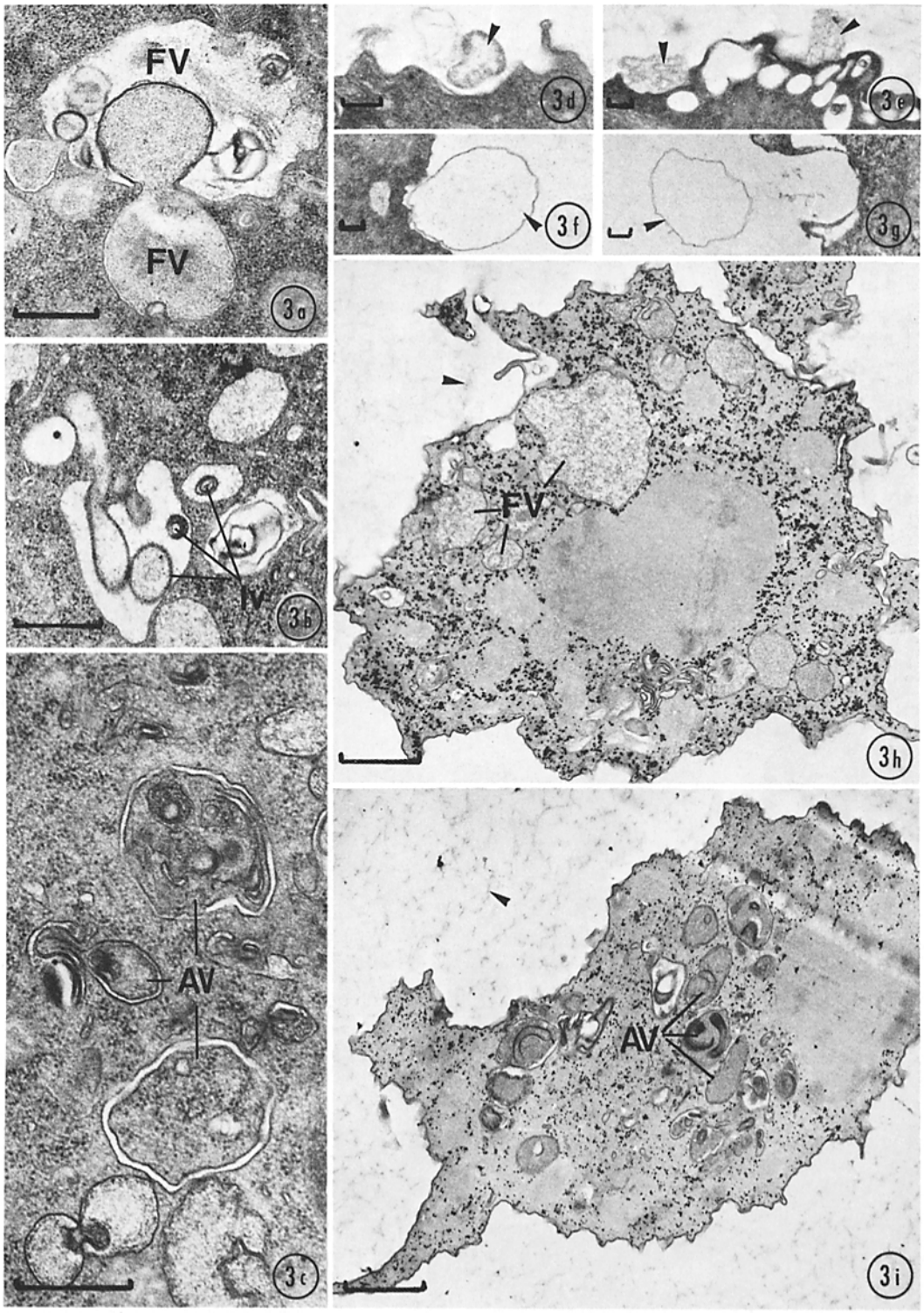


FIGURE 3 (*a,b*) Imbrication of food vacuoles (*FV*) resulting in their fragmentation and in the appearance of small intravacuolar vesicles (*Iv*) limited by a double membrane. These vesicles correspond to cross sections of imbricated vacuoles, or to pinched-off vacuoles. × 26,000. Bar, 0.5 μm. (*c*) Detail of autophagic vacuoles (*AV*). The cytoplasm is surrounded by a double membrane before being digested. × 35,000. Bar, 0.5 μm. (*d,e*) Spongy material, and (*f,g*) membranes frequently observed outside the cell along incurved portions of the plasma membrane (arrows), and suggesting a defecation process. (*d*) × 24,000, (*e,f,g*) × 14,000. Bar, 0.25 μm. (*h,i*) Staining of polysaccharides with the SP. (*h*) Cell at 0 min of starvation, (*i*) cell after 5 h of starvation. (*FV*) food vacuoles, (*AV*) autophagic vacuoles containing no glycogen, arrows indicate extracellular fibrillar material. × 12,000. Bar, 1 μm.

vacuoles or to pinched-off vacuoles (Fig. 4). The amount of these intravacúolar membranes increases during the first 2 h, and decreases afterwards.

After 2 h of starvation, vacuoles of a new type appear and its number increases with time. These vacuoles contain a dense material, and many of them are limited by a double membrane (Fig. 3*c*), but the inner membrane presents different degrees of integrity (arrows on Fig. 5*f*, 6*b*, *d*). They probably correspond to autophagic vacuoles formed by the engulfment of a cytoplasmic area in a kind of flattened sac (7 [Fig. 5*a*, *c*]). Some of them contain glycogen particles, as seen after polysaccharide staining, but never mitochondria. Changes in the number of the two types of vacuoles which occur during the 5 h of starvation are represented in Fig. 7. A continuous decrease in the amount of food vacuoles is observed during the starvation period. After 5 h, cells contain only 7% of their initial number of food vacuoles. In contrast, the number of autophagic vacuoles steadily increases during the first 4 h. In spite of a certain decrease during the last hour, the autophagic vacuoles represent 84% of the total number of vacuoles at the end of the starvation period. These observations apply to the whole cell population.

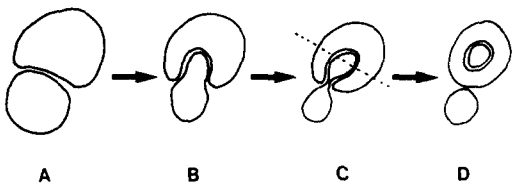


FIGURE 4 Imbrication of food vacuoles, one into the other. In thin sections, some vacuoles contain vesicles limited by a double membrane which correspond either to a cross section of imbricated vacuoles (*C*) or to pinched-off vacuoles (*D*).

During the first hours of starvation, ~10–15% of the cells exhibit a deposit of spongy material or circular membranes outside the cell close to the plasma membrane (Fig. 3*d*, *e*, *f*, *g*), which strongly suggests a defecation of the vacuolar material. Afterwards, this phenomenon decreases. A fibrillar material is also found in the extracellular space which increases in quantity during starvation. As it is stained by phosphotungstic acid-chromic acid (PTA) and silver proteinate (SP) (arrows on Fig. 3*h*, *i*, 6*a*), it could correspond to the extrusion of polysaccharides observed by Yamada et al. (35).

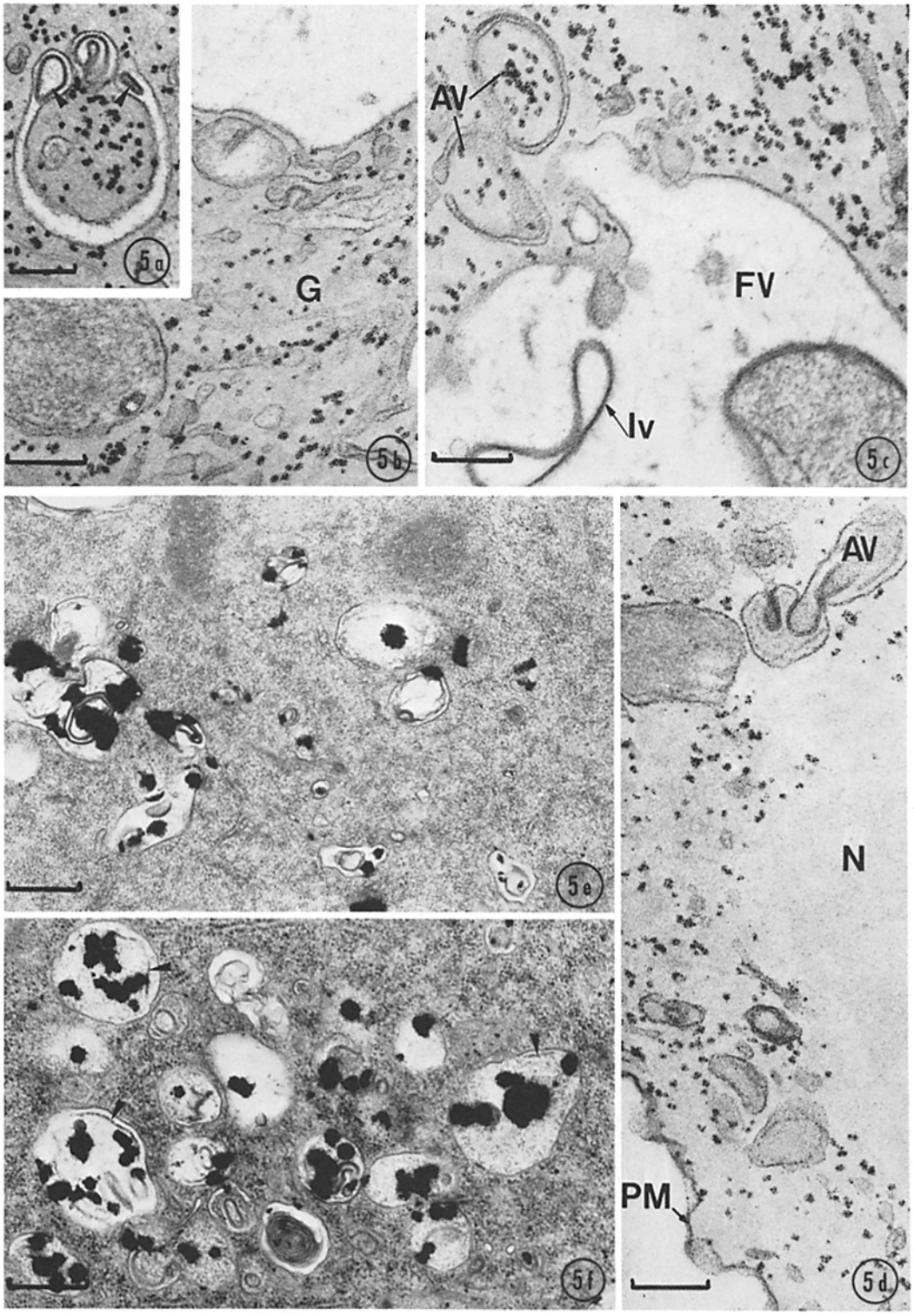
As in growing cells, the contractile vacuole appears either as many small vacuoles or as a single, round or flattened vacuole. It is always electron transparent and situated next to the plasma membrane (Fig. 2*a*, 6*a*). The amount and the appearance of the mitochondria and the endoplasmic reticulum does not change, but after a few hours, the cisternae of the Golgi complex are no longer detectable.

Staining of Polysaccharides

The SP procedure stains glycogen, mucopolysaccharides, and glycoproteins, whereas PTA-CrA reacts mainly with glycoproteins. RR was also employed as a stain. It does not penetrate into the cell, and it stains only the cell-surface carbohydrates.

As during vegetative growth, the small granules distributed in the cytoplasm and corresponding to glycogen are highly contrasted with the SP staining procedure. Their number clearly decreases during

FIGURE 5 (*a,b,c,d*) Staining of polysaccharides with SP. (*a*) Detail of an autophagic vacuole in formation. An area of cytoplasm containing many glycogen particles (dark spots) can be seen partly enclosed in a kind of flattened sac which contains intravacuolar vesicles (arrows). Both membranes surrounding this area are positively stained. × 40,000. Bar, 0.25 μm. (*b*) Detail of a starved cell showing the SP-positive reaction of many vesicles which may correspond to the Golgi region (*G*). × 48,000. Bar, 0.25 μm. (*c*) Detail of a starved cell showing autophagic vacuoles in formation (*AV*), the two membranes of which are well stained. The membrane of the food vacuoles (*FV*) is contrasted as well as the intravacuolar membranes (*Iv*). × 48,000. Bar, 0.25 μm. (*d*) Detail of a starved cell showing the contrasted plasma membrane (*PM*). The nuclear (*N*) membrane is not stained. The cytoplasm enclosed in the autophagic vacuoles (*AV*) does not contain any glycogen particles. × 48,000. Bar, 0.25 μm. (*e,f*) Demonstration of acid phosphatase in starved cells. The positive vacuoles have a variable size, and some contain intravacuolar membranes. The inner membrane of the autophagic vacuoles is more or less degraded (arrows). (*e*) × 22,000; (*f*) × 24,000. Bar, 0.5 μm.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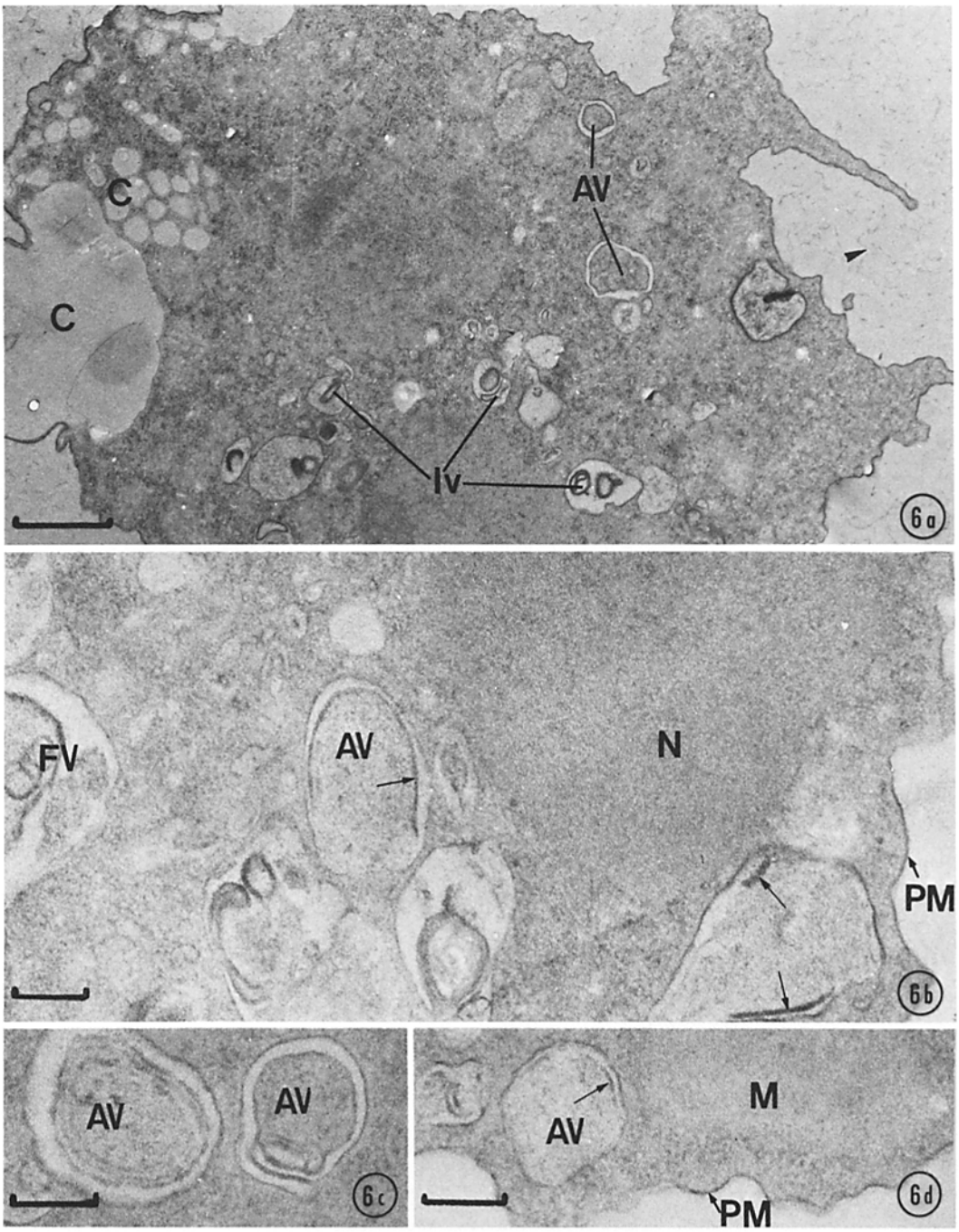

FIGURE 6 (*a,b,c,d*) Staining of cells with PTA-CrA during starvation. (*a*) General view of a cell starved for 5 h. The cell contains intravacuolar vesicles (*Iv*) and some autophagic vacuoles (*AV*). Arrow indicates extracellular fibrillar material. (*C*) contractile vacuole. × 13,500. Bar, 1 μm. (*b*) Detail of a starved cell showing a positive reaction on the plasma membrane (*PM*) and the membranes of the food vacuoles (*FV*) and autophagic vacuoles (*AV*). The inner membrane of the *AV* is more or less degraded (arrows). The nuclear (*N*) membrane does not react. × 40,000. Bar, 0.25 μm. (*c*) Detail of a starved cell showing the contrasted membranes of the autophagic vacuoles (*AV*). × 48,000. Bar, 0.25 μm. (*d*) Detail of a starved cell showing the positively stained membranes of an autophagic vacuole (*AV*), the inner membrane of which is partially digested (arrow). The plasma membrane (*PM*) is also stained, but the mitochondrion (*M*) remains negative. × 48,000. Bar, 0.25 μm.

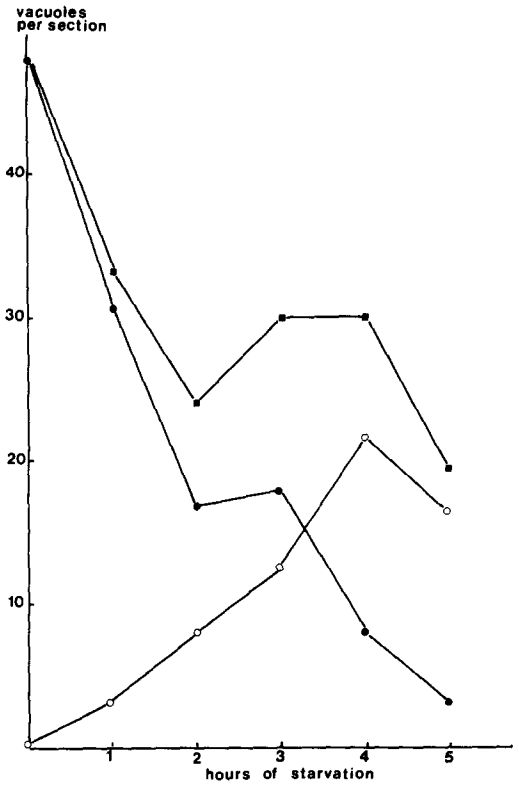

FIGURE 7 Number of vacuoles per cell profile during starvation, counted on 100–120 thin sections per sample. ●, food vacuoles; ○, autophagic vacuoles; ■, food + autophagic vacuoles.

the 5-h starvation period (Fig. 3*h*, *i*). This is in accord with the changes in glycogen content observed during the same period of time as shown by biochemical determinations of carbohydrate content in the cells (Fig. 8).

As in growing cells, the intensity of the staining with SP and with PTA-CrA varies with the different types of membranes. The plasma membrane has strong contrast and remains well stained during starvation (Fig. 3*h*, 5*d*, 6*b*, *d*). With PTA-CrA, both faces are always visible and their thickness is not modified. With RR, no differences in the thickness of the external layer of the plasma membrane have been observed during the starvation period, even when cells begin to aggregate.

The membrane of all digestive vacuoles react in the same manner as the plasma membrane with SP and PTA-CrA (Fig. 5*c*, 6*a*). The two limiting membranes of autophagic vacuoles show strong contrast, thus facilitating their observation (Fig. 5*a*, *c*, 6*b*, *c*, *d*). For two reasons, the kind of sac which encloses the cytoplasm seems to be issued from flattened food vacuoles. Firstly, it sometimes contains double-membraned vesicles (Fig. 5*a*) similar to those found in food vacuoles, and secondly, the staining with SP or PTA-CrA is similar on the membranes of food and autophagic vacuoles. The cytoplasm trapped in the vacuole contains many glycogen particles during the formation of the vacuole (Fig. 5*a*, *c*), which later become invisible, probably because they are degraded (Fig. 3*i*, 5*d*). The inner membrane is probably also digested because many autophagic vacuoles contain only some fragments (Fig. 6*b*, *d*) which can be particularly well seen during a cytochemical demonstration of acid phosphatase (Fig. 9*d*) (see following paragraph). During the 5-h starvation period, no qualitative or quantitative changes have been observed in the polysaccharide staining of the membranous organelles. The nuclear membrane is faintly stained, or not stained at all (Fig. 2*g*, *h*, 3*d*, 5*a*, *b*). The mitochondria, endoplasmic reticulum, and contractile vacuole are not contrasted (Fig. 3*i*, 6*a*). As previously mentioned, cisternae of the Golgi complex are no longer visible, but small vesicles gathered in certain areas of the cytoplasm are lightly stained with SP (Fig. 5*b*), and could correspond to the Golgi apparatus.

Acid Phosphatase Demonstration

The cytochemical demonstration of acid phosphatase shows that in the cells starved for any

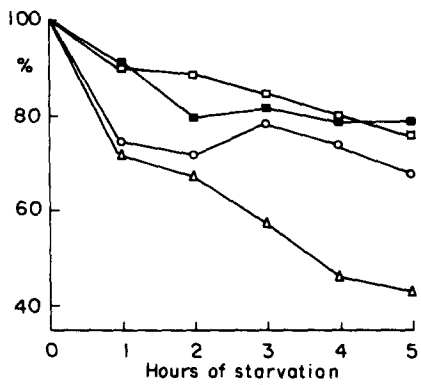

FIGURE 8 Changes in different cellular components during starvation. The results, expressed in percentages, were normalized to 100 by taking as a reference the values found at 0 min of starvation. △, anthrone-positive material (essentially glycogen); □, proteins; ■, acid phosphatase; ○, cytoplasmic volume measured with the morphometric method and expressed in number of "points" per cell profile.

period of time, most of the vacuoles contain lead deposit (Fig. 9*a*). The size of these vacuoles is quite variable (Fig. 5*e, f*). The vacuoles which contain intravacuolar membranes are generally all positive (Fig. 5*e, f*). When cytosegresomes begin to form, the flattened sacs are sometimes labeled with a very tiny lead precipitate located between the two membranes (Fig. 9*a*), but it is difficult to know whether such a weak labeling corresponds to a specific reaction. The more advanced stages of autophagic vacuoles are generally heavily labeled (Fig. 9*b, c*), but some of them contain only a fine lead precipitate (Fig. 6*d*). However, the intensity of the reaction does not seem to be related to the more or less advanced state of digestion of their content. The controls in the presence of NaF or without substrate remain completely negative.

Pictures illustrating the fusion of vacuoles with "lysosomes," as observed after yeast ingestion, were never found with cytosegresomes. Therefore, the way in which cytosegresomes acquire their acid phosphatase remains unknown. Moreover, the site of synthesis of this enzyme and its transportation remain as mysterious as in growing cells, as no recognizable Golgi apparatus could be found after 2 h of starvation, and because the rough endoplasmic reticulum (RER) and nuclear membrane are always devoid of lead precipitate (Fig. 9*a*).

Changes in Carbohydrate, Protein, and Acid Phosphatase Content of Cells during Starvation

The data obtained are in agreement with those of Hames and Ashworth (15), but give more precise details on the changes in carbohydrate and protein contents of the cells which occur during the 5-h starvation period preceding aggregation.

The total cellular carbohydrate content decreases considerably (Fig. 8), because 57.5% of the anthrone-positive material is lost after 5 h. This important loss is very rapid during the 1st h (−28.4%), and later it slows down. This is probably essentially due to myxamoebal glycogen degradation, because at least 90% of the anthrone-positive material of amoebae, grown in the presence of glucose, can be accounted for by their glycogen content (2, 15).

In addition, the total cellular protein content decreases by 24.4% during this 5-h starvation period (Fig. 8). This loss, probably mainly due to excretion of protein in the medium (15), is very rapid during the 1st h (−10.2% which represents nearly 50% of the total decrease). It is then much slower and regular for the next 4 h. The biochemical determination of the intracellular acid phosphatase content, made during starvation (Fig. 8), shows a 20% decrease in the first 2 h, after which time the level of this enzyme seems to stay constant. As shown in Fig. 10, this decrease is accompanied by the release of this enzyme into the starvation medium.

Morphometric Study of Starved Cells

This method was applied under the same conditions as described in the accompanying paper (28). The analysis was done on 100–120 micrographs of cell profiles randomly taken for each sample fixed every hour between 0 and 5 h of starvation. The chosen × 14,000 enlargement was sufficient to take into account the small villosities of the plasma membrane and the small digestive vacuoles.

One difficulty of this morphometric study concerns the interpretation of the results, because, as it appears in Table I, the cytoplasmic volume (given by the number of cytoplasmic points per cell profile) does not remain constant during starvation. The mean number of cytoplasmic points decreases by 33% during the 5 h of starvation, the main decrease occurring during the 1st h (−25%). This is in accord with the important loss of proteins and carbohydrates which is also essentially observed during this period.

In addition, as previously described, the loss of these cell components is accompanied by a change of the cellular shape which becomes very irregular and indented. The comparison of the numbers of points and intersections per cell profile between the different samples is more difficult, as these values may slightly vary with the shape of the cells. Consequently, the values given in Table I must be interpreted with caution. They only give a rough estimate of the changes that occur during starvation. However, they indicate that the plasma membrane and the membranes of the digestive system also decrease mainly during the first 2 h, with a larger decrease for the vacuole membranes.

Fig. 11 gives the variations of the S/V ratios for different kinds of membranes. As already pointed out, these ratios do not reflect the exact changes of the membrane surfaces, as volume is not constant during starvation. For the plasma membrane, this ratio increases by 40% during the first

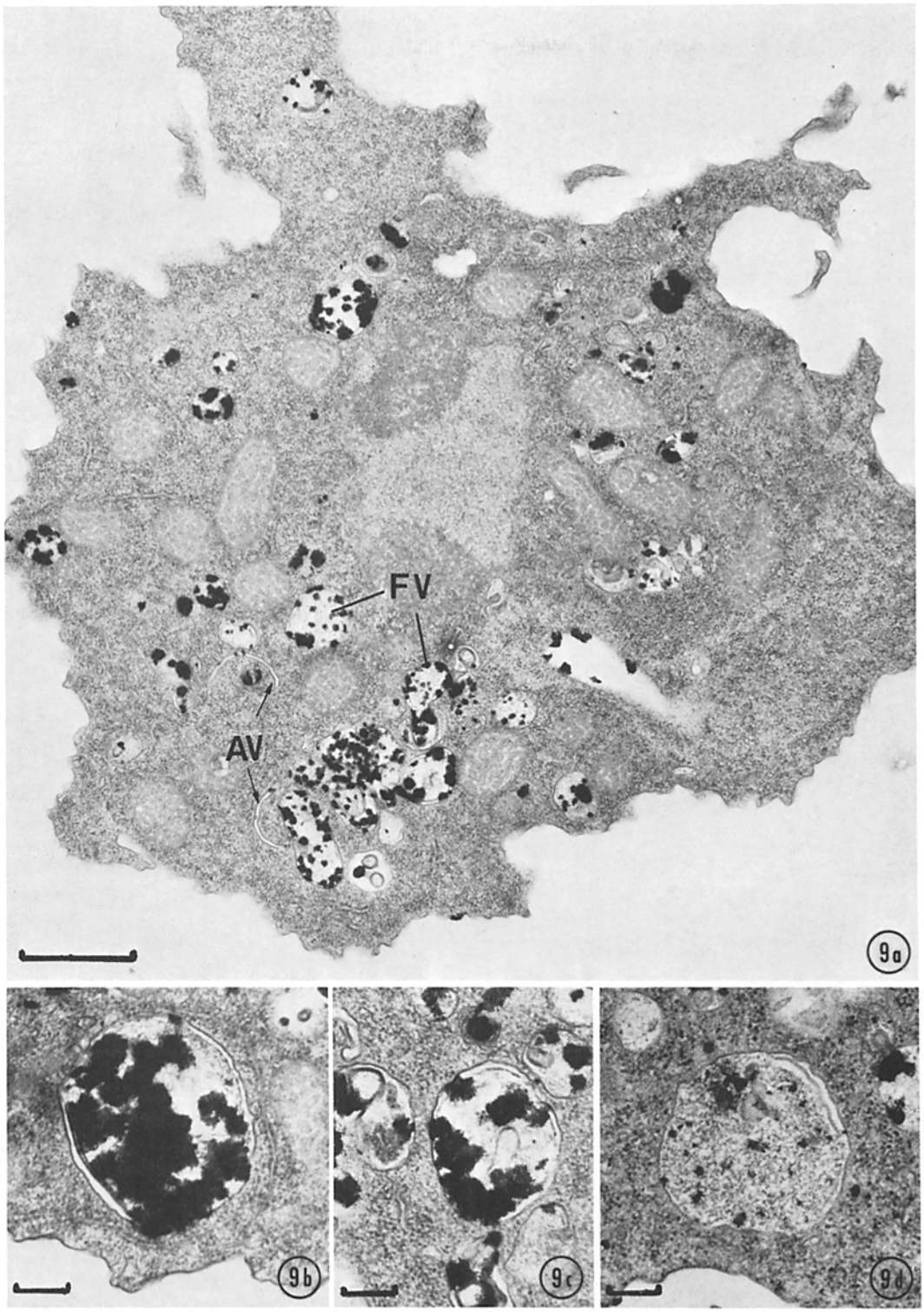

FIGURE 9 Demonstration of acid phosphatase in cells starved for 2 h. (*a*) General view of a starved cell. Most food vacuoles (*FV*) are positively stained. A tiny lead deposit can be seen on autophagic vacuoles (*AV*) in formation. × 17,500. Bar, 1 μm. (*b,c,d*) Detail of autophagic vacuoles. They are usually heavily stained (*b,c*), but some only contain a fine lead precipitate (*d*). In these three vacuoles, the cytoplasm and part of the inner membrane are partially digested. (*b*) × 31,500; (*c*) × 33,000; (*d*) × 32,000. Bar, 0.25 μm.

2 h, and then remains stable. The formation of long filopods and the indentation of the plasma membrane reflects this high ratio.

In contrast, the S/V ratio of the vacuole membranes decreases by 24% during the first 2 h, and the S/V ratio of the intravacuolar membranes increases, but the addition of these two ratios gives a stable value. This means that the intravacuolar membranes are derived from the vacuole membranes, as suggested by morphological observations, and that there is a loss of internal membranes which is probably of the same order as that of the cytoplasmic volume, inasmuch as S/V remains constant. The formation of cytosegresomes after 2 h coincides with an increase in surface area of the vacuole membranes, and an important diminution of intravacuolar membranes.

In order to obtain more precise data on the evolution of the cell surface area and on that of the internal membranes, we measured the nuclear volume, which should be more constant than the cytoplasmic volume, because DNA synthesis is

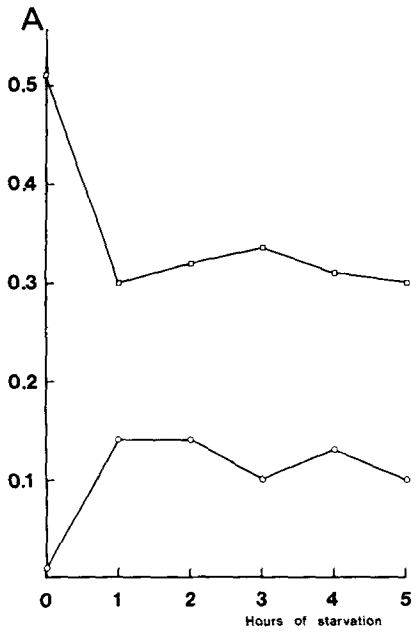

FIGURE 10 Changes in the acid phosphatase content during the starvation period. *A*, absorbency; ○, supernate; □, cells.

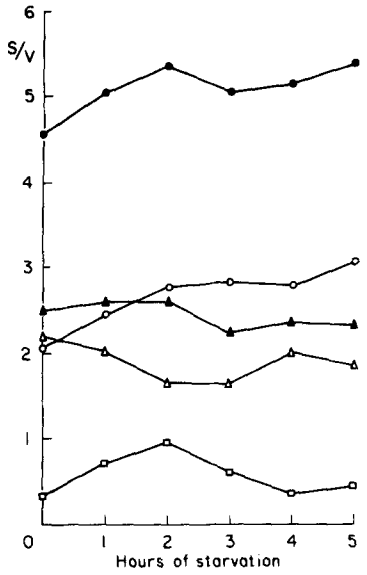

FIGURE 11 Results of the morphometric studies (32) giving the changes in the mean values of the S/V ratios of the different types of membranes during the starvation period. The SEM always represents less than 10% of the mean value. $S/V = (I \times 4)/P$ (I and P values are given in Table I). ○, plasma membrane; △, vacuole membranes; □, intravacuolar membranes; ▲, total internal membranes; ●, total membranes.

TABLE I

Results of the Morphometric Studies Made on Thin Sections

Number of points (P) or intersections (I) per cell profile	Hours of Starvation					
	0	1	2	3	4	5
Points						
Cytoplasm + nuclei	37.65 ± 1.73	28.03 ± 1.65	26.89 ± 1.72	29.60 ± 1.70	27.50 ± 1.66	25.34 ± 1.62
Vacuoles	6.75 ± 0.42	5.21 ± 0.64	3.69 ± 0.39	4.70 ± 0.50	5.42 ± 0.66	3.53 ± 0.38
Nuclei	1.85	1.65	1.70	1.82	1.75	1.80
Intersections						
Plasma membrane	17.50 ± 0.74	13.81 ± 0.72	14.76 ± 0.78	13.50 ± 0.63	12.04 ± 0.52	14.45 ± 0.63
Vacuole membranes	20.15 ± 1.15	14.58 ± 1.22	11.97 ± 0.99	13.30 ± 0.93	14.13 ± 0.99	12.70 ± 1.06
Intravacuolar membranes	2.84 ± 0.29	5.00 ± 0.45	6.88 ± 0.62	4.70 ± 0.42	2.40 ± 0.30	3.47 ± 0.44
	22.99 ± 1.32	19.58 ± 1.27	18.85 ± 1.52	18.00 ± 1.27	16.53 ± 1.19	16.09 ± 1.40
Vacuole + intravacuolar membranes						
Total	40.53 ± 1.86	33.39 ± 1.69	33.49 ± 1.91	31.50 ± 1.65	28.57 ± 1.46	30.54 ± 1.84

stopped when cells are starved (20). This is confirmed by the fact that the number of nuclear points per cell profile remains constant in the six samples (Fig. 12, Table I), and the cytoplasmic to nuclear volume ratio decreases by 30% during starvation. Therefore, the nuclear volume can be used as a reference. However, the calculation of S/V_n cannot be made by applying the formula $(I \times 4)/(P_n \times l/G)$ for each micrograph, as was done with the cytoplasmic volume, because only 40% of the micrographs contain a nucleus. Therefore, we calculated the S/V ratios by using the total number of I and P for each sample (Fig. 13*b*). In order to compare these ratios with the data obtained with the cytoplasmic points, the surface area to cytoplasmic volume ratios were also established with the total number of I and P_c per sample (Fig. 13*a*).

It appears that the S/V ratio for the plasma membrane, which is rather constant in Fig. 13*a*, has decreased by ~18% after 5 h of starvation in Fig. 13*b*. Even if these values are not precise, they indicate that the cell surface area certainly does not increase.

In Fig. 13*b*, the S/V_n ratio for the total intracellular membranes (vacuole + intravacuolar membranes) presents a stronger decrease than the ratio for the plasma membrane, which confirms the atrophy of the digestive apparatus.

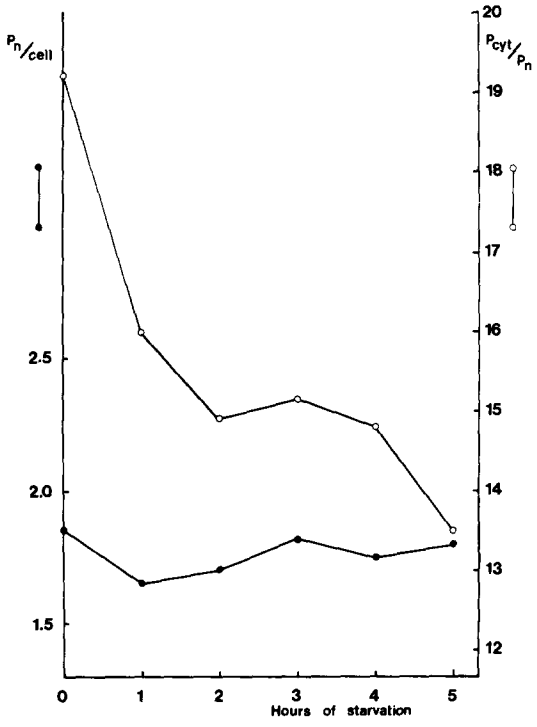

FIGURE 12 Evolution of the mean number of nuclear points (*Pn*) per cell profile ● (read values on left vertical axis) and of the ratio of cytoplasmic to nuclear points (*Pcyt/Pn*) ○ (read values on right vertical axis) during starvation.

Phagocytosis after Different Times of Starvation

ABILITY FOR PHAGOCYTOSIS: The transformations of the digestive system observed during starvation in *Dictyostelium* suggested that they could influence the ability to phagocytize, and could eventually explain the decrease of this ability observed in other amoebae (10, 33). In order to study this question, a culture of 2×10^6 cells/ml was transferred into phosphate buffer (starvation medium), and samples were taken each hour. Each sample received 15 yeast per cell, and the ingested yeast were counted with the light microscope after 20, 40, and 60 min. The results are given in Fig. 14. The uptake by cells that received yeast before their transfer was 5.5 yeast per cell after 60 min. Starvation has two different effects on yeast phagocytosis. Firstly, it doubles the final uptake in the cells starved for 30 min to 3½ h. After a longer starvation, the uptake decreases, and at the beginning of cellular aggregation, it resumes its initial value. The second effect concerns the speed of uptake which is increased considerably, and reaches its maximum in cells starved for 90 min. Afterwards, it remains constant, even in cells starved for 4½ h.

MORPHOMETRIC ANALYSIS OF CELLS AFTER PHAGOCYTOSIS: This experiment was made on the same culture as the one used for the morphometric study of starved cells. After 2 h of starvation, a sample of the culture received about 15 yeast/cell, and was fixed 1 h later.

As already described, the cells in thin sections at 2 h of starvation present a very irregular shape with many long cell processes, corresponding to an increase in the S/V ratio. After yeast ingestion, most of these projections disappear, and the cells are more spherical. As observed in growing cells having phagocytized yeast, the number of vacuoles and vesicles simultaneously decreases with the increase in the number of phagosomes. We can also point out that phagocytosis completely stops the formation of autophagic vacuoles because these vacuoles are never found after yeast ingestion.

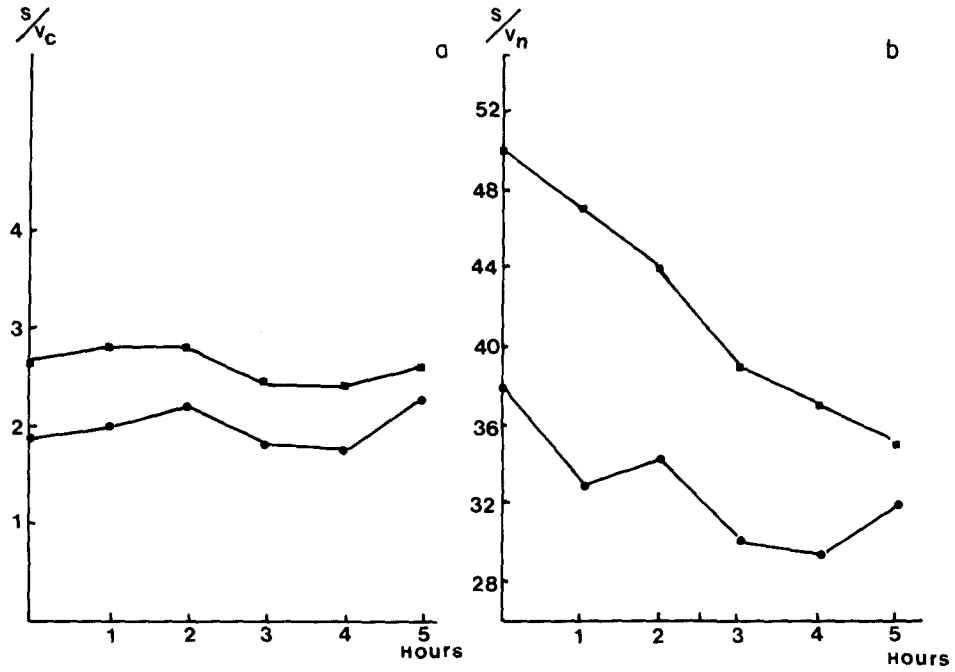

FIGURE 13 (*a*) S/V ratio for the plasma membrane (●) and the total vacuole membranes (vacuole + intravacuolar membranes) (■). These ratios were established for each sample by using the total intersections and points counted in all micrographs. (*b*) S/V_n ratios for the plasma membrane (●) and the total vacuole membrane (■) calculated as in (*a*). $S/V = (I \times 4)/P$.

As no important changes in the cytoplasmic volume were found between 2 and 3 h of starvation (Table I), the comparison of the S/V ratios before and after phagocytosis can be easily interpreted. Table II shows that the phagosome surface area, equal to 1.40, represents 50% of the initial surface area of the plasma membrane. This means that 50% of the plasma membrane has been internalized during phagocytosis. But, as opposed to what was observed during phagocytosis in normally growing cells, the internalized surface area was not totally replenished because the S/V ratio of the plasma membrane, which was 2.76, passes to 2.10 after phagocytosis. The loss of plasma membrane (0.66) corresponds to 24% of its initial value, and to about half of the internalized membrane. The decrease of vacuole membranes (0.66) is also equivalent to half of the internalized cell surface area. Therefore, the addition of the loss of plasma membrane and of vacuole membranes (0.66 + 0.66 = 1.32) is close to the surface area of phagosomes. At first sight, this seems to indicate that even if the equilibrium between plasma membrane and internal membranes is not maintained during phagocytosis in starved cells, the balance of the total membranes is conserved. However, the presence of intravacuolar membranes complicates the situation. These membranes, which are rather numerous after 2 h of starvation (S/V = 0.96), completely disappear during phagocytosis. This strong decrease is probably not entirely related to the process of endocytosis, because 0.36 of the S/V ratio is also lost between 2 and 3 h of starvation. Therefore, the real loss due to phagocytosis would correspond to 0.60 (13.4% of the S/V ratio of the total cell membranes). This suggests that such a fast endocytosis accelerates the recycling of the digestive vacuoles, and increases the release of intravacuolar membranes into the starvation medium.

DISCUSSION

Important morphological and biochemical changes have been observed during the starvation period which triggers cellular aggregation, particularly during the first 2 h. Observations of thin sections and of scanning electron microscope preparations have shown that the plasma membrane becomes wrinkled very rapidly. Also, the number of phagocytic cups increases for 2 h;

they are later replaced by many thin, long filopods. These changes are accompanied by various modifications in the composition or organization of the plasma membrane, as previously shown by different authors (1, 3, 4, 9, 11, 13, 14, 36). But the attempts to detect quantitative or topological modifications of the plasma membrane polysaccharides with specific stains did not reveal any differences between the cell surface of growing cells and that of starved cells. Both faces of the plasma membrane, which are equally stained by PTA-CrA in growing cells, conserve their original thickness, and no modifications have been observed with SP or RR.

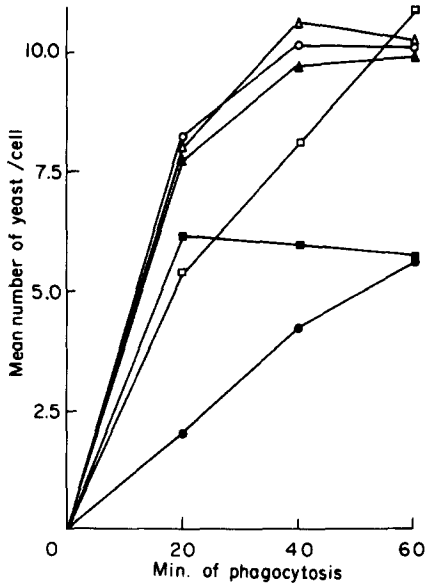

FIGURE 14 Yeast uptake in cells after different times of starvation. These values were obtained for each sample by counting the number of ingested yeast in ~100 cells with the light microscope after 20, 40, and 60 min of phagocytosis. ●, before starvation; □, 30 min starvation; △, 1 h 30 min; ○, 2 h 30 min; ▲, 3 h 30 min; ■, 4 h 30 min.

These techniques did not reveal any visible changes at the level of the membranes of the digestive system. With PTA-CrA, both faces of the vacuole membranes are stained throughout starvation, and they keep their initial thickness. This means that the membrane of the digestive vacuoles conserves the characteristics of the plasma membrane, although all or nearly all the vacuoles contain acid phosphatase regardless of their size or content.

However, a sort of atrophy of the digestive system occurs, characterized by a decrease in the size and number of the food vacuoles during the first 2 h. This decrease seems to result from the imbrication of the food vacuoles, one into the other, after which occurs the pinching off of part of one vacuole into the other, a process similar to autophagy. The presence of acid phosphatase in all or nearly all the vacuoles, and the partial digestion of some intravacuolar double-membraned vesicles suggests that part of the digestive apparatus is degraded by its own hydrolases. However, it is not the only process of elimination of intravacuolar membranes, because small extracellular vesicles are mainly observed during the first 3 h of starvation, thus suggesting a defecation process resulting from the fusion of food vacuoles with the plasma membrane. Such a fusion has never been observed in thin sections, the phenomenon probably occurring too rapidly. This process of defecation is most likely responsible for the presence of spongy material located at the surface of the cell. It would also explain the increasing amount of fibrillar material observed between the

TABLE II

Results of the Morphometric Analysis

S/V	S/V = (*I* × 4)/*P*				
	After 2 h of starvation 1	After yeast phagocytosis 2	Difference between 1 and 2 3	After 3 h of starvation 4	Difference between 1 and 4 5
Ipl/P	2.76 ± 0.20	2.10 ± 0.08	−0.66	2.82 ± 0.16	+0.06
Iv/P	1.64 ± 0.12	0.98 ± 0.10	−0.66	1.62 ± 0.10	−0.02
Iiv/P	0.96 ± 0.08	0	−0.96	0.60 ± 0.06	−0.36
Iv/P + Iiv/P	2.60 ± 0.18	0.98 ± 0.10	−1.62	2.22 ± 0.14	−0.38
Iph/P	–	1.40 ± 0.08	+1.40	–	–
Itotal/P	5.36 ± 0.20	4.48	−0.88	5.04 ± 0.20	−0.32

Ipl, intersections with the plasma membranes; *Iv*, intersections with the vacuole membranes; *Iiv*, intersections with the intravacuolar membranes; *Iph*, intersections with the phagosome membranes; *P*, cytoplasmic points.

cells in thin sections, and the release of acid phosphatase and several other hydrolases (P. Brachet, unpublished observations).

As starvation progresses, an increasing number of autophagic vacuoles are found. They begin to appear after 2 h, and are distributed throughout the cell. As previously mentioned, they are formed by a flattened sac which surrounds a cytoplasmic area, as in all cell types (7). At the end of their formation, the vacuoles are limited by a double membrane.

It has been suggested that in other cell types the endoplasmic reticulum (ER) or the Golgi complex could give rise to cytosegresomes (7). In *Dictyostelium*, the autophagic vacuoles are not found in the close proximity of the ER or the Golgi complex when they start to form. Furthermore, the polysaccharide stainings applied to starved cells indicate that their origin is probably different. As previously described, the flattened sacs and autophagic vacuoles are always heavily stained with SP and PTA-CrA. In contrast, the ER is not stained with SP, or is very faintly stained, and the Golgi complex is also much less stained than the membranes of autophagic vacuoles. It seems unlikely that the membrane polysaccharide content of these organelles could be subjected to important alterations before giving rise to flattened sacs. Moreover, the Golgi apparatus which is poorly developed in growing cells is seldom found after 2 h of starvation. In addition, we have observed that certain autophagic vacuoles in formation contain double-membraned vesicles inside the flattened sac (Fig. 5*a*). This suggests that the sacs correspond to flattened digestive vacuoles still containing intravacuolar membranes. These observations are in agreement with the strong stainability with SP or PTA-CrA of autophagic vacuoles, and we can conclude that in *Dictyostelium*, the autophagic vacuoles are formed from digestive vacuoles, as proposed by Ericsson (7).

The cytochemical demonstration of acid phosphatase showed that almost all the digestive vacuoles and cytosegresomes are positive. However, the question of the origin and transportation of acid hydrolases during starvation is still unsolved in *Dictyostelium*, as during growth, because acid phosphatase cannot be detected in the ER or the Golgi complex. In addition, primary lysosomes cannot be distinguished from all the other vacuoles present in the cell because nearly all of them, regardless of their size or content, contain this hydrolase.

It is certain that autophagic vacuoles play a role in the degradation of cell compounds. However, we can conclude that this process is not responsible for the strong decrease in proteins (20%) and carbohydrate (60%) essentially occurring during the first 2 h, because at this time, autophagic vacuoles are not yet formed. Therefore, the extent of degradation of these cellular components in cytosegresomes is very limited during starvation. The role of these vacuoles is probably more important for cell differentiation, because they become more abundant in prestalk cells than in prespore cells (23). However, even after 5 h of starvation, no differences in the amount of cytosegresomes could be found among the cell population. Therefore, at this early stage, we have not been able to distinguish between different cell types which could give rise to prestalk and prespore cells.

This study indicates that the surface areas of the plasma membrane and of the digestive system are modified. As shown by the morphometric study, the plasma membrane S/V ratio increases considerably. This is confirmed by the presence of many long filopods and by the wrinkled appearance of the plasma membrane. This increase seems to be essentially due to the loss of cytoplasmic volume, and not to a real increase of the cell surface, because the S/V ratio with the nuclear volume does not increase but, on the contrary, decreases. The decrease of the total surface area of the internal membranes (vacuoles + intravacuolar membranes) is still larger, as shown by the S/V_n ratio.

All these data clearly show an atrophy of the digestive system, but give no information on the dynamics of the process. The release of vesicles, spongy material, and hydrolases into the extracellular medium indicates that some digestive vacuoles continue to be recycled at least during the first hours. This recycling is probably accompanied by endocytosis, because pictures taken with the scanning electron microscope show the presence of many phagocytic cups per cell (Fig. 1*b*). Although we do not know whether the phagocytic cups are functional in the absence of food, we can say that horseradish peroxidase is internalized (personal observation). Moreover, experiments of yeast phagocytosis with starved cells indicate that endocytosis is not only possible, but that it is faster and greater in magnitude than during growth. We think that this acceleration and increase of uptake occurs for two reasons. Firstly, most of the digestive vacuoles, having nothing to digest when the yeast are added, are immediately available for plasma membrane renewal. This would also ex-

plain the decrease in yeast uptake after more than 2 h of starvation, inasmuch as this period also corresponds to the appearance of autophagic vacuoles which immobilize internal membranes and prevent their immediate recycling.

Secondly, the quick and high uptake is probably related to the presence of many phagocytic cups which are ready to internalize yeast, and to the high value of the S/V ratio of the plasma membrane. We have seen that this ratio has considerably decreased after phagocytosis, as opposed to what was observed in vegetative phase cells (28). This means that part of the internalized plasma membrane, which served to form phagosomes, has not been renewed. Therefore, the equilibrium between cell surface membranes and internal membranes is not maintained in starved cells.

The increased ability for phagocytosis does not seem to exist in other amoebae, except for *Amoeba proteus* (6). In the slime mold *Polysphondylium pallidum*, Githens and Karnovsky (10) found a slight increase in the speed of uptake after 1½ h, but not a higher final uptake. In *Acanthamoeba castellanii*, a small amoeba, Weisman and Moore (33) observed a 30% increase of the uptake after 1 h of starvation, and a decrease during the next hours. It would be interesting to know whether or not these different behaviors are related to differences in the evolution of the digestive system (for example, earlier formation of autophagic vacuoles), or to a different evolution of the S/V ratio of the cell surface. Although no measurements have been done in *Acanthamoeba* during starvation, morphological observations seem to indicate that the cells become more spherical before encystment, suggesting a decrease rather than an increase of the S/V ratio (5).

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