

# MORPHOMETRIC AND CYTOCHEMICAL STUDIES OF *DICTYOSTELIUM DISCOIDEUM* IN VEGETATIVE PHASE

## Digestive System and Membrane Turnover

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### ABSTRACT

The morphometric analysis of growing cells shows that the membranes of the digestive apparatus have a surface area equal to the cell surface area. After yeast phagocytosis, the surface area of the membrane surrounding the ingested yeast is equal to 40% of the surface area of the cell membrane. In spite of this internalization, the cell surface remains constant. Its renewal is insured by the translocation of the membrane of the digestive system, the surface area that concomitantly decreases by 40%. This means that the influx of plasma membrane is continually compensated for by the same outflow of internal membranes. During this turnover, the characteristic polysaccharide stainability (two different stains were used) of the plasma membrane is maintained after internalization, at the level of the digestive system, despite the presence of hydrolases in the digestive vacuoles. The cytochemical demonstration of acid phosphatase shows that this enzyme penetrates into phagosomes by fusion between phagosomes and vacuoles of various sizes. The debris of digested yeast are released into the culture medium after 2 h. This process of defecation is accompanied by the appearance of new pinocytotic vacuoles, which indicates that the uptake of axenic medium has resumed. A model of membrane turnover is proposed to explain these observations.

**KEY WORDS** phagocytosis · digestive apparatus · amoeba · membrane flow

*Dictyostelium discoideum*, like all other amoebae, exclusively feeds by pinocytosis and phagocytosis. This process of feeding leads to a rapid internalization of the plasma membrane. According to measurements or estimations made on different amoebae, internalization of the plasma membrane during pinocytosis or phagocytosis is extremely variable according to the species (5, 18, 48, 55). It can reach 0.5–10 times the cell surface area per hour. It is obvious that such a fast internalization necessitates a rapid renewal of the plasma membrane.

An attempt to elucidate this question in *Acanthamoeba castellanii* was done (Bowers and Ryter, unpublished results). The preliminary results obtained with the morphometric method of Weibel et al. (54) seem to show that the plasma membrane is replenished by the membrane of the digestive vacuoles, and that a constant equilibrium exists between cell surface area and internal surface area.

This problem will be studied in *D. discoideum* in order to learn whether this kind of turnover exists during vegetative growth, and how it evolves during the starvation period preceding cellular aggregation and differentiation (9). This

study will be applied to axenically growing cells and to cells phagocytizing yeast by measuring the plasma membrane and vacuole membranes.

Parallel to this approach, cytochemical techniques revealing acid phosphatase or polysaccharides will be applied to the same cells in order to have a better understanding of the behavior and fate of the digestive vacuoles and their recycling.

## MATERIALS AND METHODS

### *Culture Medium*

*D. discoideum* AX-2 was grown in the axenic medium HL-5 (14) at 20°C under mild agitation. In the experiments on phagocytosis, 10–20 killed yeast per cell (*Saccharomyces cerevisiae* treated with acetone and dried) were added to cultures containing about 10<sup>6</sup> cells/ml, and samples were taken 0–7 h later.

### *Fixation and Embedding*

Several fixation conditions were assayed for purely ultrastructural study and for cytochemistry of acid phosphatase. The best conditions found were those described below. The cell suspension in growth medium was prefixed at room temperature by adding an equal volume of 2.5% glutaraldehyde in 0.05 M cacodylate buffer at pH 6.8 containing 0.2 M sucrose. The presence of 0.2 M sucrose in the fixative protects the vacuolar system of the cell and prevents the rupture of vacuolar membranes as was also observed in *A. castellanii* (44). After 15–20 min, the cells were spun down and resuspended in the same fixative. For ordinary morphology and polysaccharide staining, the cells were fixed for 1 h at room temperature before being washed overnight at 4°C in the same buffer containing 0.2 M sucrose. A longer fixation renders the cytoplasm very dense, in which case the ultrastructure is almost completely masked.

After being washed, the cells were centrifuged, postfixed for 1 h in 1% osmium tetroxide in cacodylate buffer 0.05 M, pH 6.8, dehydrated in acetone, and embedded in Epon. Thin sections were contrasted with uranyl acetate and lead citrate.

### *Staining of Polysaccharides*

Two different techniques were used to stain polysaccharides. In both cases, cells were fixed for 1 h in glutaraldehyde, washed overnight in cacodylate buffer, postfixed for 1 h in osmium tetroxide, and embedded in Epon (unless otherwise indicated). Thin sections (silver to pale gold interference colors) were cut on a Sorvall ultramicrotome (Ivan Sorvall, Inc., Norwalk, Conn.) with a diamond knife. They were gathered in plastic rings with a 3-mm diameter central hole, and floated on the different reagents.

SILVER PROTEINATE PROCEDURE (51): The silver proteinate stain of Thiéry was initially used. However, the stain, as discussed in Results, was modified

because control treatments showed a nonspecific staining of certain cell structures. The following procedure (4) was adopted: ~10 vol hydrogen peroxide (HP), 25 min; bidistilled water, 10 s (three times) and 10 min (three times); 1% periodic acid (PA) in bidistilled water, 25 min; bidistilled water, 10 s (three times) and 10 min (three times); 0.2% thiocarbonylhydrazide (TCH) in 20% acetic acid, 24 h; 10% acetic acid, 10 s (three times) and 20 min (three times); 5% acetic acid, 2 min; 2% acetic acid, 2 min; bidistilled water, 10 s (three times) and 20 min (three times); 1% silver proteinate (SP) in bidistilled water, 25 min in the dark; and bidistilled water, 10 s (twice) and 10 min (twice). All these reactions were carried out at room temperature.

The tests described below were done to check the specificity of the silver staining procedure: (a) TCH + SP. Free aldehyde groups coming from glutaraldehyde or tissue-bound osmium present in the cells before PA oxidation could nonspecifically react with TCH and SP. Therefore, this control was done after a double fixation with glutaraldehyde and osmium tetroxide, and after a simple fixation with osmium tetroxide. (b) HP + TCH + SP. At this concentration, HP completely removes osmium from thin sections of fixed cells and does not oxidize glycols to aldehydes. If nonspecific staining is due to osmium tetroxide bound to cell structures, this reaction will be negative; if it is due to free aldehydes present in the cell, staining will persist. (c) SP only. This reveals cell structures having a nonspecific affinity for silver. (d) PA + SP. This reaction checks for groups able to reduce SP after PA oxidation.

PHOSPHOTUNGSTIC ACID PROCEDURE (40, 41) MODIFIED BY ROLAND (43): Thin sections were treated at room temperature as follows: 1% PA in bidistilled water, 30 min; bidistilled water, 5 min (twice) and 10 min (three times); 1% phosphotungstic acid in 10% chromic acid (PTA-CrA), 10–20 min; bidistilled water, 5 min (three times) and 10 min (twice).

It is necessary to use PA before PTA-CrA to deosmicate the thin sections, otherwise the reaction becomes nonspecific. As a control, sections were only treated with PA.

### *Cytochemical Demonstration of Acid Phosphatase*

As opposed to what was observed in ordinary morphological studies, a good preservation of the ultrastructure and a satisfying cytochemical demonstration of acid phosphatase were obtained only after an overnight fixation at 4°C. The cells were then washed twice for 30 min at 4°C with 0.05 M cacodylate buffer containing 0.2 M sucrose. They were then resuspended in the cytochemical medium, frozen in liquid nitrogen for 3 min, thawed, and immediately incubated at 23°C for 10 or 20 min. If the cells are not frozen, the demonstration of acid phosphatase remains negative. A shorter fixation does not improve the results at all. These difficulties are presumably due to the great impermeability of the plasma mem-

brane. The same problem of membrane impermeability had been met in *A. castellanii* (44), but was overcome by working with rather dense cultures. With *Dictyostelium*, we did not notice any effect of the age of the culture on membrane permeability.

The cytochemical medium contained 10 ml of 0.05 M acetate buffer, pH 5.0; 1 ml of 1.2%  $\text{Pb}(\text{NO}_3)_2$ , and 1 ml of 3%  $\beta$ -glycerophosphate. Other substrates such as cytosine-5'-monophosphate or *p*-nitrophenyl phosphate were also used in some experiments, but the reaction was more faint. Controls were incubated in the presence of  $10^{-2}$  M NaF or in the absence of substrate. After incubation, the cells were washed once in acetate buffer and twice in cacodylate buffer with sucrose before being resuspended in agar and fixed with  $\text{OsO}_4$ .

### Morphometric Analysis

The surface area of the plasma membrane and of internal membranes was measured by the technique of Weibel et al. (54). For this purpose, wide, thin sections containing more than 100 cell profiles were deposited on a coated Formvar copper ring with a 1-mm diameter central hole. After staining with uranyl acetate and lead citrate, 100–120 micrographs of cell profiles were randomly taken, regardless of their size. This means that these micrographs contain sections of different cells cut at any level. The micrographs were enlarged to a final magnification of 14,000, and were analyzed with a 14-mm grid of bars. The following formula surface/volume (S/V) was used:  $S/V = (I \times 4)/(P \times l/G)$ :  $I$  = number of intersections between bars and membranes (plasma membrane, vacuole membranes or phagosome membranes),  $P$  = number of cytoplasmic points including nuclear area, but not the inside of vacuoles and phagosomes,  $l$  = length of the bars in microns (14,000); and  $G$  = magnification of micrographs ( $\times 14,000$ ). Here  $l/G = 1$ .

## RESULTS

### *Axenically Growing Cells*

**MORPHOLOGICAL OBSERVATIONS:** In thin sections, *D. discoideum* presents the characteristic appearance of amoebae, an irregular shape and a highly developed vacuolar system (Fig. 1a) (16, 19, 24, 32). When the cells are grown in an axenic medium, the vacuoles contain spongy material of variable electron density (Fig. 1a) (32). When the cells are fed with bacteria, the vacuoles contain bacteria at different degrees of digestion (16, 23), and many of them contain acid phosphatase (17). Obviously, these vacuoles constitute the digestive apparatus of the cell. The rough endoplasmic reticulum (RER) is poorly developed and the Golgi apparatus appears to be constituted

more by a complex system of vesicles than by stacks of cisternae (16, [Fig. 8a]). The cytoplasm is extremely rich in granules 18–19 nm in diameter which correspond to glycogen, as we shall see later. The granules are randomly distributed in the cytoplasm, except along the membrane of phagocytic cups which are surrounded by a network of filaments probably corresponding to actin (Fig. 3b). The contractile vacuole is always electron transparent, and depending upon its state of contraction, appears as a large vacuole bulging at the cell surface, or as many small vacuoles gathered along the plasma membrane (Fig. 3a, [32]). An extracellular filamentous material is always found between the cells, and it looks more abundant in old cultures.

**STAINING OF POLYSACCHARIDES:** The silver proteinate stain of Thiéry (51) (PA+TCH+SP) was used in the beginning of the studies. But control treatments showed a nonspecific staining of most of the membranous structures.

If the staining is really specific, a control treatment with TCH+SP should be negative. In our experiment, after a double fixation with glutaraldehyde and osmium tetroxide, all the membranes remained contrasted (Fig. 2c). This nonspecific reaction could be due either to free aldehyde groups of the glutaraldehyde or to osmium tetroxide bound to these structures. The first hypothesis could be eliminated, inasmuch as the nonspecific staining was also observed on cells fixed only with osmium tetroxide. This nonspecific staining due to osmium was suppressed by treating the thin sections with HP (42) before TCH+SP (at the concentration used, HP removes all the osmium bound to cell structures without oxidizing 1,2-glycols to aldehydes), in which case none of the cell structures are contrasted (Fig. 2e). This technique gives better results not only because the polysaccharides seem to be more specifically stained, but also because the silver granulation is finer at the sites of reaction. Furthermore, control treatments with SP alone and with PA+SP are completely negative. This demonstrates that no cell structures have a nonspecific affinity for silver or can reduce SP after only PA oxidation.

The 18- to 19-nm granules distributed all over the cytoplasm, often in small clusters, are highly contrasted (Figs. 1b, 2a,b). They most probably correspond to glycogen particles because cells of *D. discoideum* contain large amounts of glycogen in our growth conditions (9, 20). Different cell

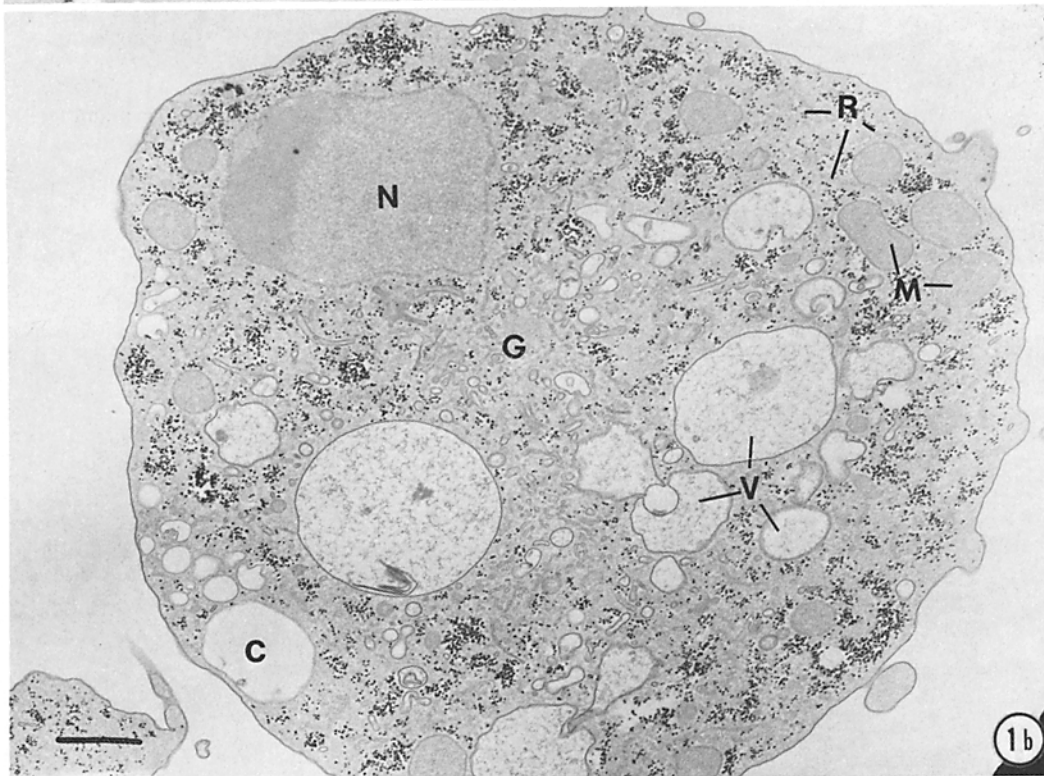
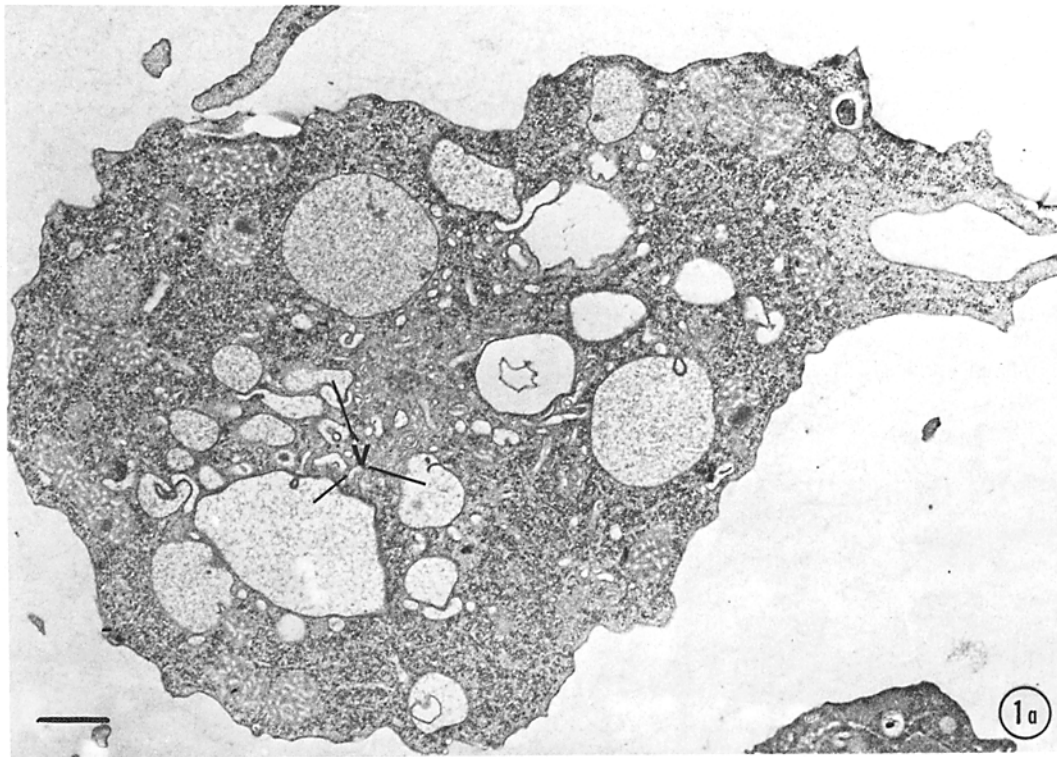
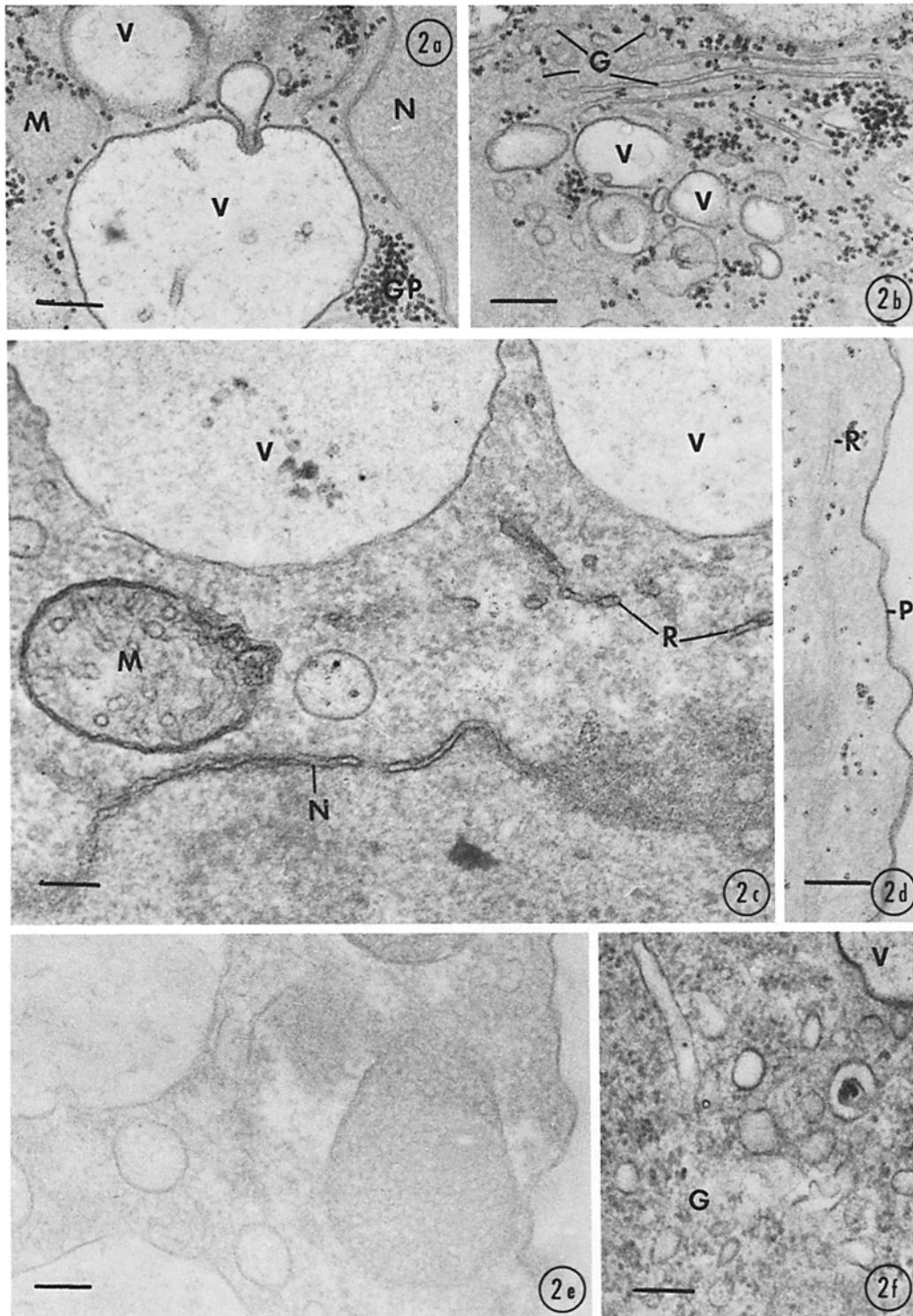


FIGURE 1 (a) Axenically growing cell of *D. discoideum* showing the highly developed digestive system consisting of many vacuoles (V).  $\times 10,000$ . Bar,  $1 \mu\text{m}$ . (b) Growing cell after the staining of polysaccharides with silver proteinate. Glycogen is heavily stained. The plasma membrane and the membranes of all digestive vacuoles (V) are well stained. The membranes of the Golgi vesicles located in the middle of the cell are also lightly stained (G) as well as the nuclear membrane (N), but the RER and the membranes of mitochondria (M) are negative or very faintly stained. The contractile vacuoles (C) are completely negative.  $\times 12,000$ . Bar,  $1 \mu\text{m}$ .



**FIGURE 2** (a), (b), and (d) Staining of polysaccharides with silver proteinate. (a) Detail of glycogen particles (GP), digestive vacuoles (V), the nuclear envelope (N), and a mitochondrion (M); (b) the Golgi region (G) and a few digestive vacuoles (V); (d) the plasma membrane (P). The RER (R) remains negative.  $\times 48,000$ . Bar,  $0.2 \mu\text{m}$ . (c) Staining of polysaccharides with silver proteinate. Control treatment with TCHSP only. A nonspecific staining is visible on the membranes of mitochondria (M), the nuclear membrane (N), and RER (R) which are even more contrasted than the membranes of digestive vacuoles (V).  $\times 44,000$ . Bar,  $0.2 \mu\text{m}$ . (e) Staining of polysaccharides with silver proteinate. Control treatment with  $\text{H}_2\text{O}_2 + \text{TCH} + \text{SP}$ . All the membranes are negative.  $\times 40,000$ . Bar,  $0.2 \mu\text{m}$ . (f) Region of vesicles stained with PTA-CrA which may correspond to the Golgi region. V = vacuole.  $\times 40,000$ . Bar,  $0.2 \mu\text{m}$ .

membranes are also contrasted by this technique, but with a variable intensity. The staining is most intense on the cell surface (Figs. 1*b*, 2*d*) and on all food vacuoles (Figs. 1*b*, 2*a*) appearing as a thin layer of tiny granules on the membranes. The Golgi apparatus is a little less contrasted (Fig. 2*b*). No differences have been observed between the cisternae and the vesicles. The nuclear membrane is generally less stained than the Golgi complex (Figs. 1*b*, 2*a*), whereas the endoplasmic reticulum (Fig. 2*d*) and the membranes of the contractile vacuole (Fig. 1*b*) and mitochondria are faintly or negatively stained (Figs. 1*b*, 2*a*).

With PTA-CrA (40-42), many membranes of *Dictyostelium* are heavily stained. The cell-surface and food vacuole membranes are the most contrasted (Fig. 3*a*). As in *Acanthamoeba* (4), the staining appears on both sides of the membrane (Fig. 7*c*). The cytoplasmic side of the plasma membrane is usually thinner than the exterior one (Table I). This asymmetric staining is also observed on the food vacuole membranes. The Golgi complex is less contrasted, the cisternae and vesicles appearing to be equally stained (Fig. 2*f*). The nuclear, mitochondrial, and contractile vacuole membranes, or the endoplasmic reticulum do not react with PTA-CrA (Fig. 3*a*). The spongy material found in food vacuoles is also stained with PTA (Fig. 3*a*) and silver proteinate (Fig. 1*b*).

**ACID PHOSPHATASE DEMONSTRATION:** After the cytochemical treatment, a lead precipitate is found inside many vacuoles of variable size, as found in many protozoa (13, 17, 21, 35, 44, [Fig. 3*B*]). As observed in *Acanthamoeba* (44), some vacuoles, in particular the contractile vacuole, remain free of lead deposit regardless of the duration of the incubation in the cytochemical medium. The positive or negative response of vacuoles is apparently not related to the electron density of their spongy material. The cells of controls, incubated with NaF or without substrate, are always totally devoid of lead deposit.

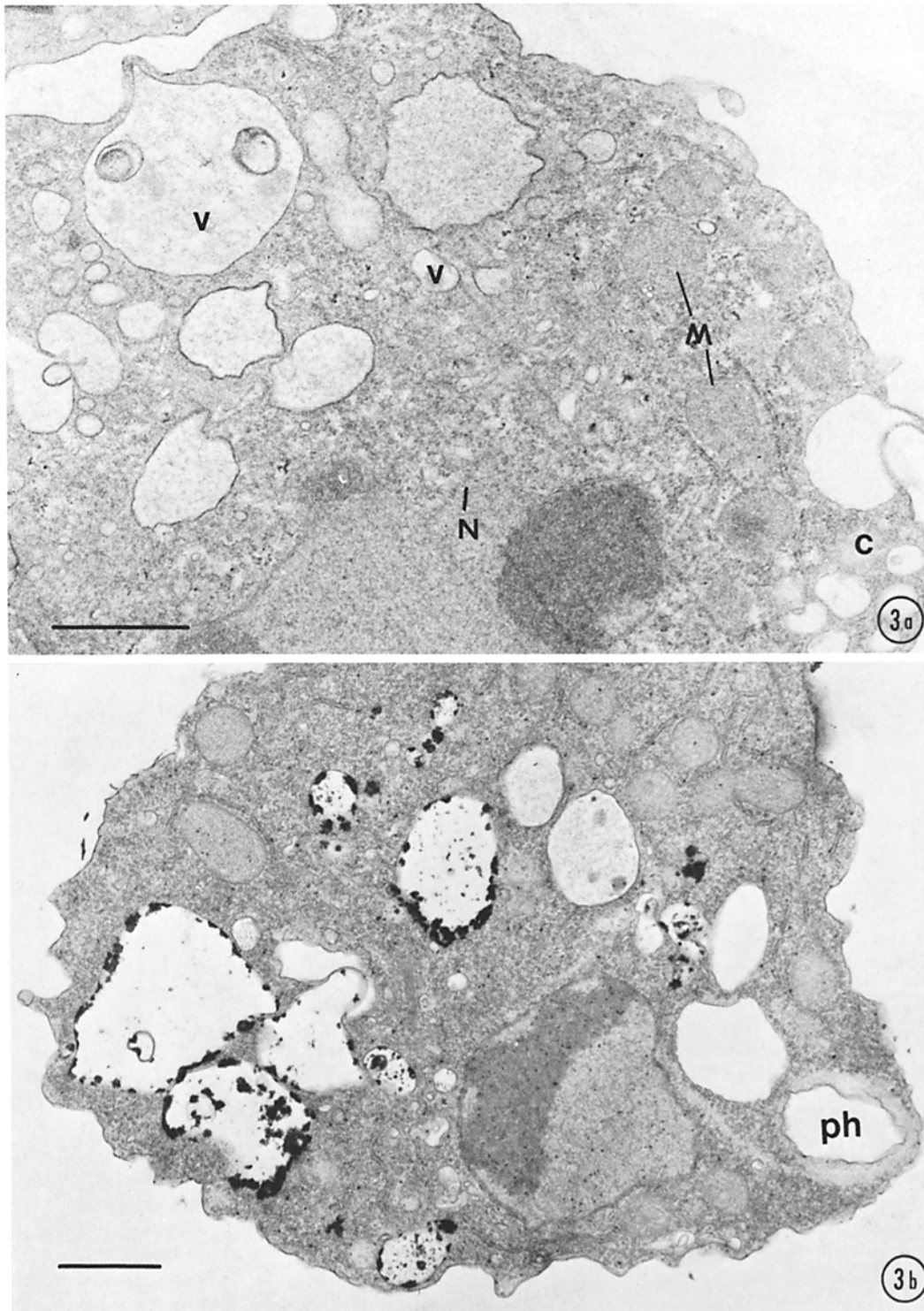
The RER and the nuclear membrane always remain negative, but the nucleoplasm is frequently studded with a small lead precipitate, even after a short cytochemical treatment (10 min) (Fig. 3*b*). Although this reaction is not observed in the cells incubated with NaF or without substrate, it cannot be considered as a specific staining without additional proof (12). Contrary to what was observed in other amoebae (21, 44, 56), no reaction product could be found in the cisternae of the Golgi apparatus, but some vesicles of the Golgi region

are labeled (Fig. 8*a*). However, it is not possible to decide whether or not these vesicles belong to the Golgi apparatus.

### Phagocytosis

**MORPHOLOGICAL OBSERVATIONS:** Cultures of axenically growing cells received 10-12 yeast/cell, and samples were taken between 0 and 7 h. Because of their rather large size and refringency, the number of ingested yeast can be easily counted with the light microscope. As shown in Fig. 4, the uptake reaches its maximum after 90 min of phagocytosis. The number of yeast/cell slightly varies from one culture to another (3.5-5), and especially from one batch of culture medium to another, but the kinetics of uptake is always the same. Afterwards, the number of ingested yeast slowly decreases. This decrease was initially attributed to the loss of refringency of yeast during their digestion as observed in *Acanthamoeba* (44), but the electron microscope study reveals a rather different phenomenon. The cells taken during the first 90 min of phagocytosis and observed in thin sections contain an increasing number of ingested yeast (Fig. 5). In parallel to this increase, the number of digestive vacuoles decreases. This disappearance of vacuoles is particularly visible in the cells which have taken up five to six yeast (Fig. 6*a*). During the 1st h, ingested yeast have a dense cytoplasm and are tightly enclosed in the membrane surrounding them. Later, many phagosome membranes present hemispherical deformations of various sizes (Figs. 6*a,b*). A spongy material, similar to that in digestive vacuoles, is located in these regions, between the deformed membrane and the yeast. Such pictures very strongly suggest that fusion has occurred between the phagosome membrane and the membrane of vacuoles. This phenomenon seems especially frequent between 30 and 120 min.

90 min after the beginning of phagocytosis, some ingested yeast have lost their spherical or ovoid shape and are completely distorted (Fig. 6*b*). Their cell wall still looks intact in spite of its deformation, but the cytoplasm seems partially digested. Unlike what was observed in *Acanthamoeba* (44), this aspect represents the most advanced state of digestion, and phagosomes containing only yeast debris were never found, even in the cells taken 6-7 h after the addition of yeast. Moreover, their number does not increase with time, but yeast presenting the same aspect start to appear in the culture medium at 2 h (Figs. 6*d,g*).



**FIGURE 3** (a) Growing cell stained with PTA-CrA. This technique lightly contrasts the cytoplasmic background but more heavily contrasts the membranes of all food vacuoles (V) and the cytoplasmic membrane. In contrast, the membranes of mitochondria (M), of the nucleus (N), of contractile vacuoles (C), and the RER remain negative.  $\times 20,000$ . Bar,  $1 \mu\text{m}$ . (b) Demonstration of acid phosphatase in a growing cell. The lead deposit is located in many digestive vacuoles but not in cross sections of phagocytosis cups (Ph) recognizable by the actin network surrounding the membrane.  $\times 15,000$ . Bar,  $1 \mu\text{m}$ .

TABLE I  
 Thickness of the Two Faces of the Different Membranes after Staining the Polysaccharides\*

Membrane	Hours of Phagocytosis			
	Normal growth (no phagocytosis) 0 h	1 h 30 min	4 h	6 h
	<i>nm ± SEM</i>			
Plasma membrane				
cytoplasmic face	5.21 ± 0.20	5.47 ± 0.15	5.06 ± 0.14	5.16 ± 0.16
outer face	5.36 ± 0.14	5.67 ± 0.14	5.40 ± 0.25	5.16 ± 0.11
Phagosomes				
cytoplasmic face	—	5.21 ± 0.14	4.79 ± 0.12	5.06 ± 0.12
inner face	—	5.75 ± 0.21	5.54 ± 0.16	6.19 ± 0.15
Food vacuoles				
cytoplasmic face	5.16 ± 0.20	5.32 ± 0.21	4.76 ± 0.15	4.71 ± 0.16
inner face	5.41 ± 0.15	5.32 ± 0.21	5.17 ± 0.15	5.29 ± 0.15
Dense food vacuoles				
cytoplasmic face	—	5.96 ± 0.20‡	5.47 ± 0.24	4.60 ± 0.19
inner face	—	7.66 ± 0.39‡	7.26 ± 0.51	9.25 ± 0.36

\* Staining technique used by Rambourg (40).

‡ 2 h instead of 1 h 30 min.

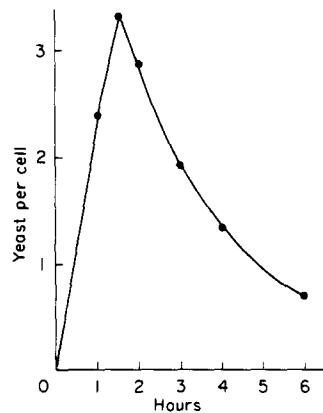


FIGURE 4 Number of yeast per cell counted with the light microscope with respect to the number of hours after the addition of yeast. (Experiment I).

The number of these extracellular digested yeast increases regularly during the next hours. As shown in Fig. 5, their appearance in the culture medium coincides with the decrease of intracellular phagosomes (intact and partially digested yeast) observed in thin sections as well as in the light microscope. It is clear that the decrease of ingested yeast found by light microscopy is not due to their loss of refringency, but to their release into the culture medium.

The progressive disappearance of phagosomes, between 2 and 7 h, is accompanied by the reappearance of new pinocytotic vacuoles, indicating that the uptake of the axenic medium has re-

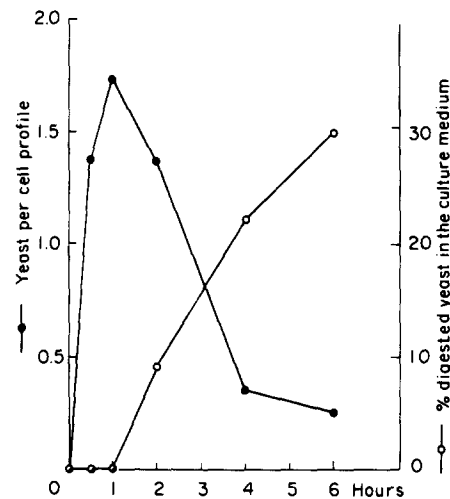
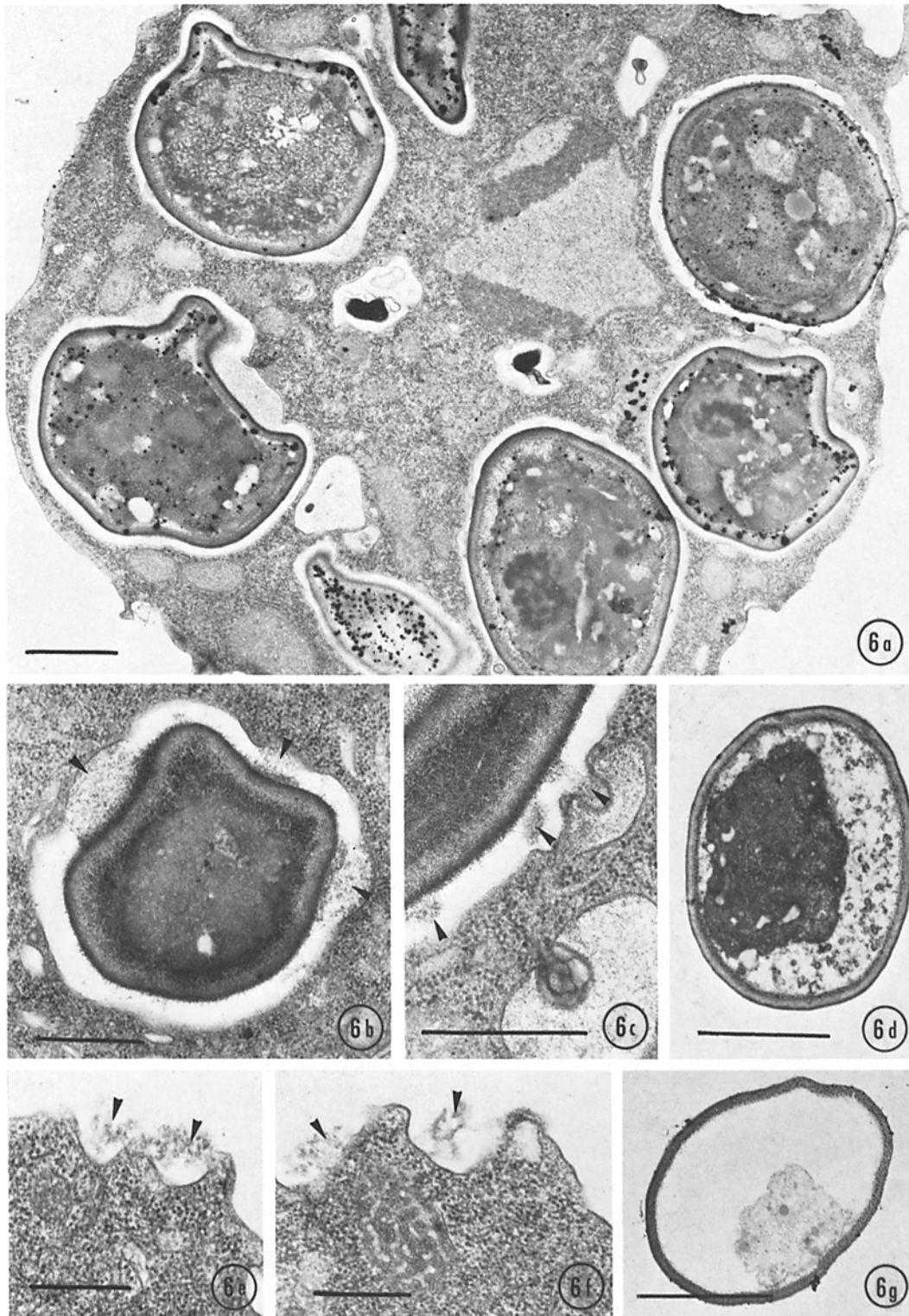


FIGURE 5 Number of ingested yeast per cell profile counted on thin sections (●), and percentage of partially digested yeast found in the culture medium with respect to all extracellular yeast (○). As the cells had received 10 yeast each at time 0, and had phagocytized 3.3 after 90 min, about 6 yeast/cell remained in the culture medium. These yeast sedimented with *Dictyostelium* cells during centrifugation and therefore were found among the cells in thin sections. The partially digested yeast start to appear when the number of ingested yeast starts to decrease. (Experiment II).

sumed. The number of vacuoles progressively increases, and reaches the usual number of vacuoles found in axenically growing cells after 7 h. Because the cells continue to grow as these events





**FIGURE 6** (a) Demonstration of acid phosphatase in a cell having ingested many yeast. All phagosomes contain lead precipitate. Most of the digestive vacuoles have disappeared.  $\times 14,000$ . Bar,  $1 \mu\text{m}$ . (b) and (c) Phagosomes showing several patches of spongy material located between the yeast and the phagosome membrane (arrows). In these regions, the membrane bulges around the patch of material. These pictures suggest that fusion has occurred between the phagosome and vacuoles.  $\times 17,000$ ,  $25,000$ . Bar,  $1 \mu\text{m}$ . (d) and (g) Partially digested yeast found in the culture medium after 2 h of phagocytosis.  $\times 20,000$ . Bar,  $1 \mu\text{m}$ . (e) and (f) Spongy material (arrows) frequently observed outside the cells along the plasma membrane suggesting a defecation process.  $\times 33,000$ . Bar,  $0.5 \mu\text{m}$ .

occur, one can conclude that during the yeast defecation period, the cells feed preferentially on axenic medium instead of phagocytizing the numerous intact yeast still present in the culture medium (6–7 yeast/cell). In contrast, cells starved in phosphate buffer continuously ingest and release yeast (under study).

**STAINING OF POLYSACCHARIDES DURING PHAGOCYTOSIS:** After a silver proteinate stain, the membranes of the organelles found in normally growing cells react as previously described. The only new element, the phagosome membrane, reacts at the beginning of phagocytosis as strongly as the cell-surface and food vacuole membranes react (Fig. 7a). The membranes of the vacuoles which have apparently fused with phagosome are stained with the same intensity as the phagosome membrane (Fig. 7a), but their content is not contrasted. Also, as the phagosomes age, the reactivity on their membranes seem to stay constant, even in the ultimate state of digestion.

With the PTA-CrA stain, the cell-surface and food vacuole membranes have the same appearance as in normally growing cells, and the phagosome membrane is as intensively stained (Figs. 7b,d). The membrane resulting from the fusion between phagosomes and vacuoles presents a normal thickness, and the material which is discharged is slightly stained (Fig. 7b).

After 2 h of phagocytosis, when defecation starts to occur, a new type of vacuole is found presenting a highly contrasted membrane in which the asymmetry of the staining existing in other vacuoles is considerably enhanced (Fig. 7e). The number of these vacuoles increases with time, so that after 6 h there are more of this kind than of the less stained food vacuoles.

The thickness of both faces of the membrane was measured and is reported in Table I. During normal growth, the cytoplasmic face of the cell-surface and ordinary food vacuole membranes is usually the thinnest, and its thickness does not vary in the course of phagocytosis. But the membranes of yeast-containing phagosomes seem to slightly change with time. The thinner cytoplasmic face keeps its original width, but the side facing the yeast cell increases about 10% after 4 h when phagosomes age. As for the highly contrasted vacuoles appearing after 2 h, the cytoplasmic face of the membrane conserves its original thickness, but the inner one thickens about 30% between 4 and 6 h.

**ACID PHOSPHATASE DEMONSTRATION:**

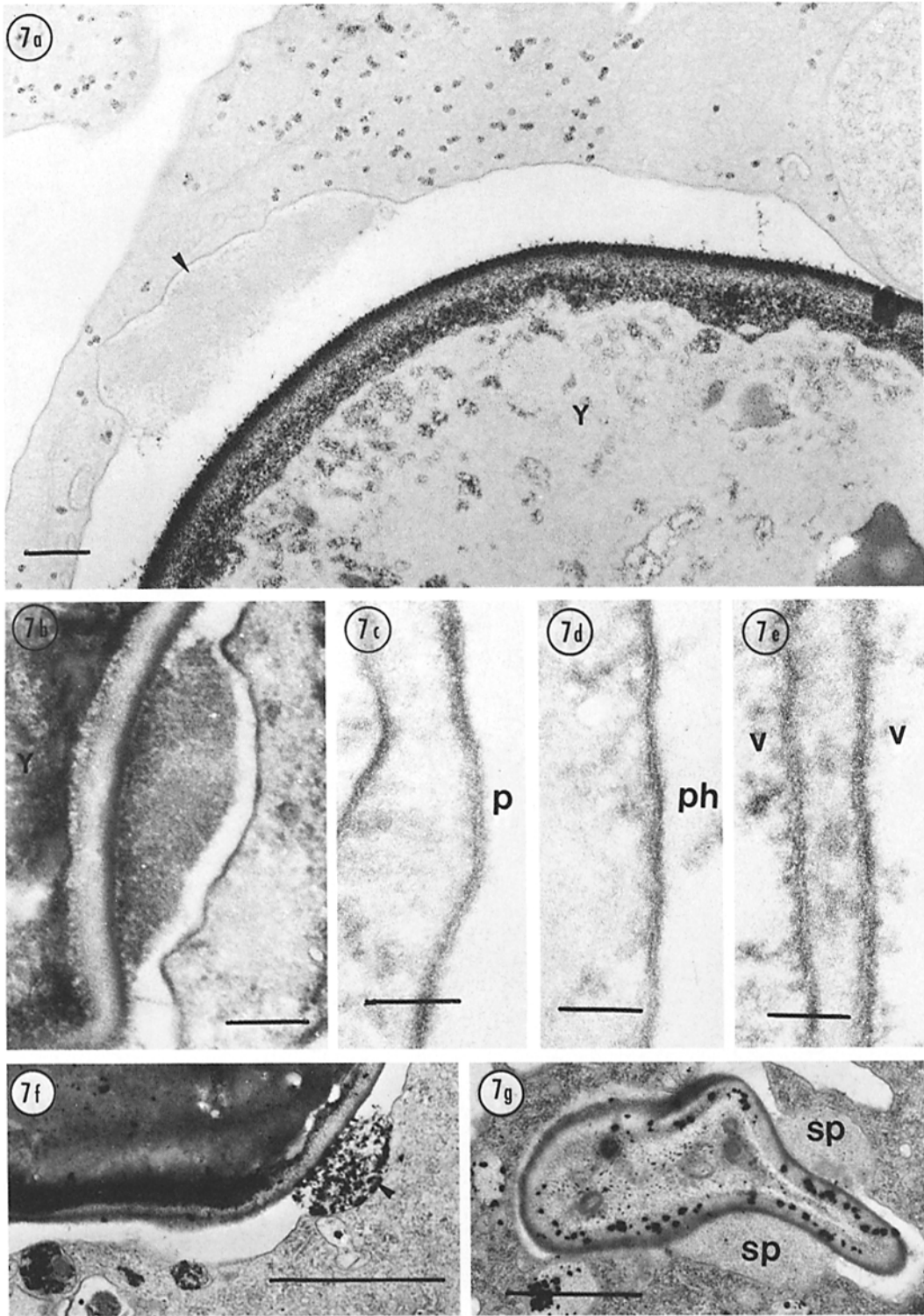
Cytochemical studies show that during the first 30 min of phagocytosis most of the phagosomes are still negative, but are surrounded by many small, positive vacuoles (Fig. 8b). After 1 or 2 h, these positive vacuoles are less numerous, but most of the phagosomes are positively stained (Fig. 6a, 8c). As we have already shown, many phagosomes at this stage seem to have fused with small vacuoles. The dense material coming from these vacuoles is sometimes covered with lead precipitate (Fig. 7f), but in most cases it is negative in spite of the positive reaction inside the yeast cell (Figs. 7g, 8c). The lack of reaction of this content after fusion could be due either to the diffusion of acid phosphatase into the yeast cell, or to the fact that only part of these vacuoles contain acid phosphatase. The partially digested yeast cells are generally acid phosphatase positive (Fig. 7g). However, some of them are negative in spite of the near presence of positive vacuoles (Fig. 8d).

**MORPHOMETRIC STUDIES DURING PHAGOCYTOSIS:** As described in detail in Materials and Methods, measurements of the cell-surface, vacuole, and phagosome membranes were performed by the method of Weibel et al. (54). This method was applied to samples taken at 0, 30, and 60 min after the addition of yeast (same experiment as in Fig. 5). At 60 min, the uptake measured in the light microscope was 3.6 yeast/cell.

The magnification at 14,000 chosen for this analysis allows one to take into account the vesicles of ~70 nm in diameter (1  $\mu$ m on the micrographs) or more. This means that all the food vacuoles are measured, possibly with the exception of the Golgi vesicles, which are generally difficult to distinguish at this magnification. As for the pseudopods or filopodia formed by the plasma membrane, their size is sufficient to allow the measurement of their surface without significant errors.

Table II summarizes the results of this analysis. We can see that the mean number of cytoplasmic points (P cytoplasm + nucleus) per cell profile is similar in the three samples. Because it can be assumed that the cytoplasmic volume does not change during the 1st h of phagocytosis, the 100 cell profiles analyzed in these three samples can be compared. In contrast, the total number of points (cytoplasm + nucleus + vacuoles + phagosomes) increases by 30% during yeast ingestion. This indicates that the total cell volume increases during phagocytosis.

The number of intersections ( $I$ ) per cell profile



with the plasma membrane remains constant. It decreases by half with the vacuole membranes, and it increases from 0 to 6 with the phagosome membranes.

The average membrane surface area to cytoplasmic volume (S/V) ratios give more precise data on the evolution of the membrane surface area (Fig. 9). It appears that the S/V ratio of the vacuole membranes at 0 min is equal to that of the plasma membrane. Therefore, the surface area of the digestive system is equal to that of the cell surface area. During phagocytosis, the S/V ratio of the plasma membrane remains absolutely stable in spite of the fact that yeast ingestion leads to the internalization of ~40% of the plasma membrane after 1 h. The total surface area of the digestive system (vacuoles + phagosomes) also remains stable. This paradoxical situation can be explained by the decrease of the S/V ratio of the vacuole membranes which also corresponds to ~40%. Therefore, it can be concluded that during phagocytosis a strict equilibrium is maintained between the cell surface area and the internal surface area.

#### DISCUSSION

Polysaccharide stainings show that the plasma membrane of *D. discoideum* does not possess a thick cell coat as do larger amoebae (2, 28, 38), but contains two thin layers of carbohydrates, revealed with PTA, which are located on each side. Such a symmetric location of polysaccharide has not yet been reported in mammalian cells and seems to have been observed only in *A. castellanii* (4). The extensive chemical study of the membrane of this amoeba showed that this staining mainly corresponds to a complex polysaccharide associated with phosphorus and fatty acid (termed

lipophosphoglycan) which is intimately integrated in the membrane (31).

The staining of polysaccharides with both techniques applied to axenically growing cells revealed a reaction, identical to that of the plasma membranes, on the membranes of all food vacuoles. In the cells containing phagocytized yeast, the membranes of all phagosomes present this staining, regardless of the state of yeast digestion. This means that the carbohydrates revealed by these techniques are not removed by hydrolases, as is the case for the thick coat of larger amoebae (26). Therefore, it is not possible by these methods to distinguish between old food vacuoles and newly formed ones. This is not very surprising, as Ulsamer et al. (53) found no differences in the chemical composition between the plasma membrane and the membranes of phagosomes in *A. castellanii*. In the culture samples of cells fixed between 2 and 6 h after the addition of yeast, the PTA technique revealed the presence of vacuoles with a thicker membrane. This increase of thickness occurs only on the side facing the inside of vacuoles, and seems to be due to a polysaccharide material contained in these vacuoles which deposits onto the membrane. This phenomenon coincides with the beginning of yeast defecation and seems to only affect the new vacuoles which appear in the cells. Because it is not observed in normally growing cells, it is probably related to the digestion and release of yeast into the culture medium. It could correspond to partially digested yeast material expelled outside the cells which may have been re-taken.

The membranes of the Golgi apparatus are also slightly stained by both techniques. This positive reaction is in agreement with the fact that in

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FIGURE 7 (a) Detail of the phagosome membrane with a patch of spongy material (arrow) after staining with silver proteinate. The incurved part of the membrane presents the same staining as the rest of the phagosome membrane. Yeast (Y).  $\times 48,000$ . Bar,  $0.2 \mu\text{m}$ . (b) Spongy material (SP) released into the phagosome (Y) stained with PTA-CrA. It is more contrasted than the cytoplasmic background. The membrane of the phagosome presents the same intensity of staining in the incurved region as elsewhere.  $\times 54,000$ . Bar,  $0.2 \mu\text{m}$ . (c), (d), and (e) PTA staining of the plasma membrane (P) in (c), of the phagosome (Ph) membrane in (d), and of the membrane of two vacuoles (V) appearing during the defecation of yeast in (e). All these membranes are stained on both faces, but the leaflet facing the inside of vacuoles in (e) is thicker and seems covered with a polysaccharide material contained in the vacuole.  $\times 128,000$ . Bar,  $0.1 \mu\text{m}$ . (f) Demonstration of acid phosphatase at the level of the spongy material released into the phagosome (arrow).  $\times 26,000$ . Bar,  $1 \mu\text{m}$ . (g) Partially digested yeast is still acid phosphatase positive whereas the spongy material (SP) is negative.  $\times 20,600$ . Bar,  $1 \mu\text{m}$ .

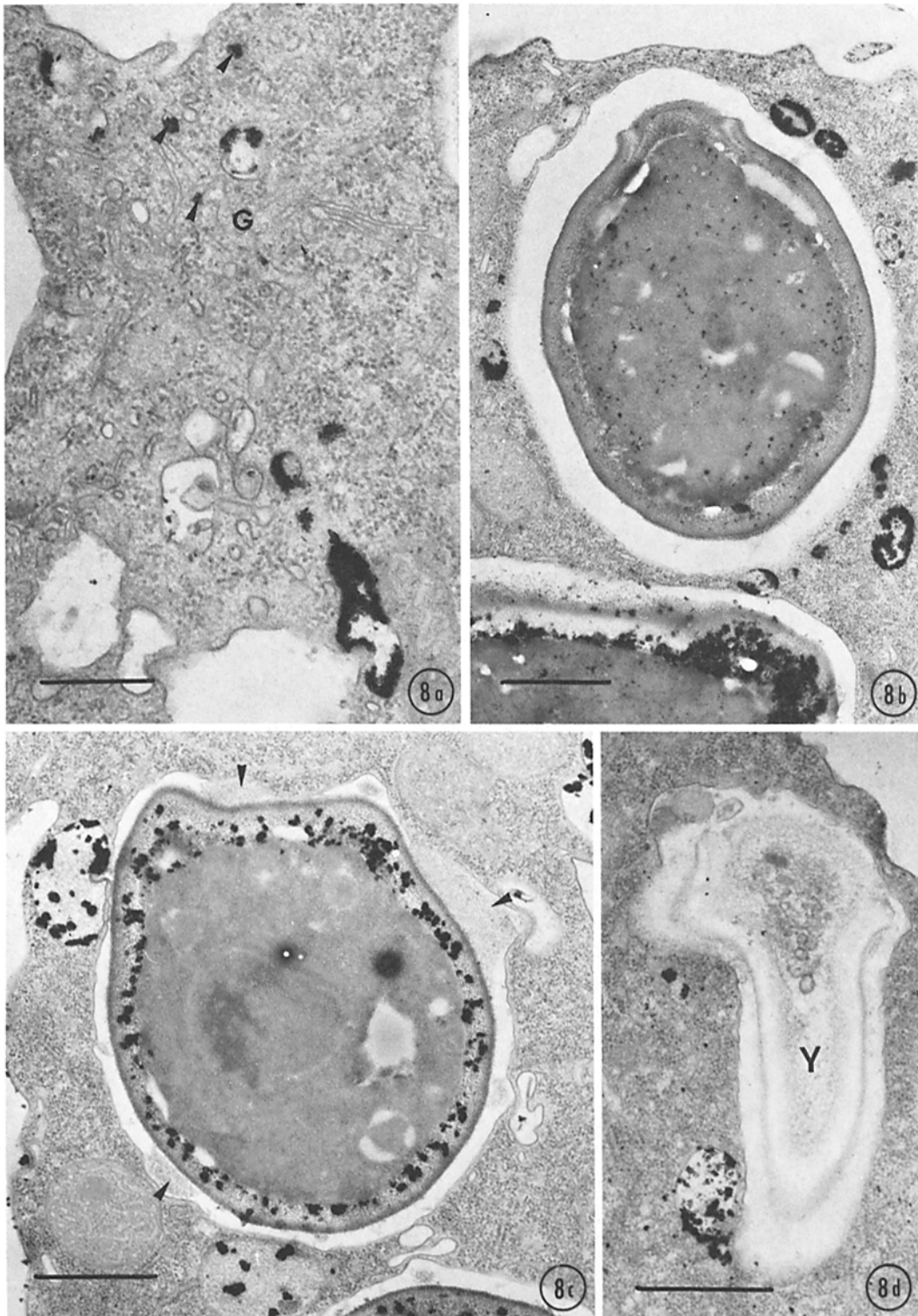


FIGURE 8 (a) Demonstration of acid phosphatase in the Golgi region (G). The Golgi cisternae are devoid of lead precipitate, but tiny positive vesicles (arrows) are often seen nearby.  $\times 34,000$ . Bar,  $0.5 \mu\text{m}$ . (b) Slightly acid phosphatase-positive phagosome surrounded by several small positive vesicles which may correspond to primary lysosomes.  $\times 16,000$ . Bar,  $1 \mu\text{m}$ . (c) Phagosome having received acid phosphatase. The spongy material visible between the phagosome membrane and the yeast (arrows) indicates that primary lysosomes or vacuoles have fused with the phagosome.  $\times 18,000$ . Bar,  $1 \mu\text{m}$ . (d) Partially digested yeast (Y). It is acid-phosphatase negative and its shape is completely distorted.  $\times 20,000$ . Bar,  $1 \mu\text{m}$ .

TABLE II  
Quantitative Data Obtained in the Morphometric Analysis of Cells during Phagocytosis of Yeast\*

	Time of sampling after yeast addition		
	0 min	30 min	60 min
No. of points			
cytoplasm + nucleus	33.0 ± 1.9	34.0 ± 1.9	30.0 ± 1.8
vacuoles	5.4 ± 0.6	3.2 ± 0.3	3.0 ± 0.4
phagosomes	—	9.8 ± 1.1	12.8 ± 1.4
total	38.4	47.0	45.8
total/cytoplasm	1.2	1.37	1.5
No. of intersections			
plasma membrane	13.7 ± 0.7	14.0 ± 0.6	12.2 ± 0.6
vacuole membrane	17.3 ± 1.6	11.9 ± 1.0	8.6 ± 1.9
phagosome membrane	—	4.9 ± 0.5	6.0 ± 0.7
vacuole + phagosome membrane	17.3	16.8	14.6
total	31.1	30.7	26.8

\* The mean values ± SEM of points and intersections were obtained from the analysis of 100–110 cell profiles in each sample.

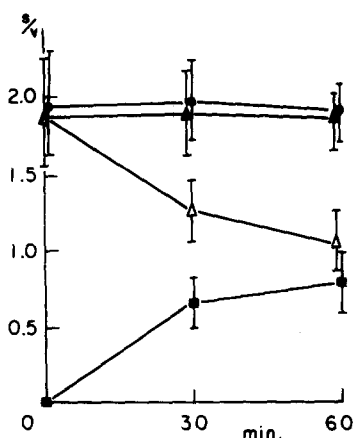


FIGURE 9 Evolution of the surface/volume ratios obtained by the morphometric analysis during phagocytosis. These ratios remain constant for the plasma membrane (●), and the membranes of the whole digestive apparatus (vacuoles + phagosomes) (▲). The ratio for vacuole membranes decreases (△), and the ratio for phagosomes increases by the same value (■).

*Amoeba proteus* and *Chaos chaos*, polysaccharides of the plasma membrane are synthesized in the Golgi cisternae and transported to the plasma membrane by small vesicles (15, 47).

The observation of light staining with silver proteinate on the nuclear membrane may be due to glycoproteins known to be present in the nuclear membranes of different mammalian cells (3, 27, 28, 34).<sup>1</sup> With PTA, the nuclear membrane is not

<sup>1</sup> Monneron, A., and J. d'Alayer. Manuscript in preparation.

stained, but as the cytoplasm is always slightly contrasted in our conditions, it may mask a light reaction on the nuclear membrane.

The cytochemical demonstration of acid phosphatase shows that this enzyme is located in most of the vacuoles of axenically growing cells, but it is not possible to distinguish food vacuoles from primary lysosomes. Moreover, the site of synthesis of this hydrolase remains hypothetical because the Golgi cisternae and the RER are devoid of lead precipitate, as opposed to what is observed in other amoebae or protozoa (13, 20, 44, 56), and in many mammalian cells (10, 37). However, because the Golgi apparatus of *Dictyostelium* is mainly constituted of small vesicles, the positive vesicles frequently found in the Golgi region could correspond to primary lysosomes.

After phagocytosis, it is clearly visualized how phagosomes receive acid phosphatase. The membranes of phagosomes fuse with several vacuoles of variable size. Because the membranes of such vacuoles are positively stained with silver proteinate and PTA, the vacuoles may correspond either to food vacuoles or to primary lysosomes formed in the Golgi apparatus. The vacuoles contain a spongy material which is lightly stained with PTA. After fusion, this material is sometimes acid phosphatase positive, but in most cases it remains devoid of lead precipitate, although the yeast itself is positive. The hydrolase probably diffuses quickly inside the yeast, whereas the spongy material can be seen for a longer period of time between the cell wall of the yeast and the phagosome membrane (Fig. 7g). This sort of material was not

observed in *A. castellanii* fed with yeast (44), and its role is obscure. Its carbohydrate nature, demonstrated with the PTA staining, suggests that it may correspond to the glycolipid matrix associated with hydrolytic enzymes found by Koenig and Jibrik (29).

Yeast digestion lasts about 1 h because ingested yeast receive hydrolases during the 1st h and start to be released 1 h later. When the yeast are expelled from the cells, their cytoplasm is often only partially digested, and their cell wall appears to stay intact. The defecation process seems to be the usual way by which the cells release the material they are unable to digest. This has been observed by Malchow et al. (33), who have shown that *D. discoideum* releases the cell wall lipopolysaccharide of gram-negative bacteria that it cannot degrade. Therefore, the early defecation of yeast seems to occur because the cells do not possess the necessary hydrolases for the digestion of the yeast cell wall. This does not appear to be the case for another slime mold (24) or for *Acanthamoeba* (44), in which the yeast are almost completely digested and remain in the cells much longer.

This kind of process probably also occurs in axenically growing cells. Yamada et al. (57) have observed an excretion of polysaccharides during vegetative growth, and we have frequently seen a spongy material, similar to the one found in many digestive vacuoles, outside the cells in depressions of the plasma membrane (Fig. 6*e,f*). This material is slightly stained with PTA, like the content of vacuoles and the extracellular filamentous substance. It may correspond to the content of digestive vacuoles released outside the cells after fusion of the vacuoles with the plasma membrane.

Before the release of undigestible material, acid phosphatase is probably inactivated or inhibited in "old" phagosomes, as it was observed in other cell types (11). In *A. castellanii* (44), the number of old phagosomes in which acid phosphatase is no longer detectable increases with time. In *Dictyostelium*, acid phosphatase-negative phagosomes are also found, but their number does not increase, probably because of the rapid expulsion of their material from the cell.

The process of defecation is accompanied by the reappearance in the cells of new food vacuoles, the number of which concomitantly increases with the decrease of old phagosomes. This phenomenon corresponds to the inverse of the process observed during yeast ingestion, characterized by the disappearance of food vacuoles and the increasing number of phagosomes.

Therefore, all these morphological observations suggest that the plasma membrane and the membrane of the digestive vacuoles are submitted to a continual turnover. The morphometric analysis made during phagocytosis entirely confirms this impression and brings more precise data on this membrane flow. Firstly, it shows that the membrane surface area of the vacuolar system of axenically growing cells is identical to that of the plasma membrane. After 1 h of phagocytosis, no changes are noted, either in the area of the plasma membrane or in the area of internal membranes (vacuoles + phagosomes). Yet, the phagosome surface area corresponding to the plasma membrane internalized during this hour represents 40% of the digestive system. However, the surface area of vacuoles has decreased, and this decrease also corresponds to 40%. This means that when yeast are ingested, the internalization of plasma membrane is compensated for by the translocation of the equivalent amount of vacuole membrane. The way in which this translocation occurs during phagocytosis has not been visualized, but it probably corresponds to a direct fusion between food vacuoles and the plasma membrane, as defecation seems to be a common process in growing cells. However, we cannot exclude the possibility of a fragmentation of some food vacuoles into smaller ones, as concluded by Holter (25) from his observations on *Amoeba proteus*.

All these results obtained with *Dictyostelium* confirm and complete previous observations made on the functioning of the digestive system and membrane turnover of other amoebae, which can be schematically represented by Fig. 10.

The equilibrium between endocytosis and exocytosis was already suspected in other amoebae (5, 18, 48, 55), and in leukocytes (45) or macrophages (46). It also seems to exist, but in an inverse way, in secretory cells (1, 39) and in nerve endings during the release of a neurotransmitter (6, 7, 22, 36, 50, 52). However, many questions remain unanswered, especially those dealing with the specificity of membrane fusions occurring between vacuoles, lysosomes, phagosomes, and plasma membrane. The study of this important problem common to the functioning of all kinds of cells is now in progress in different laboratories.

One interesting question is raised by our observations on yeast phagocytosis. During yeast defecation, why do *Dictyostelium* cells not ingest the numerous intact yeast still present in the culture medium (~6 yeast/cell), and prefer to pinocytose axenic medium, as can be judged by the progres-

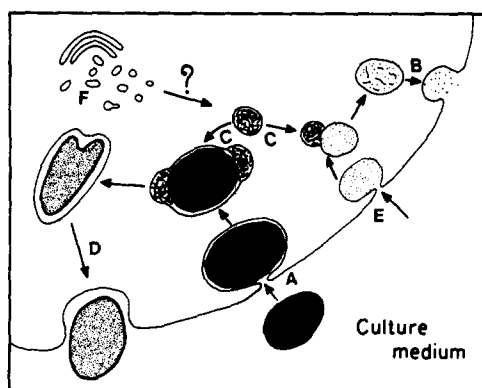


FIGURE 10 Schematic drawing representing the membrane turnover in *Dictyostelium*. During yeast phagocytosis (A), the internalized plasma membrane surrounding the yeast is replaced by the membranes of old pinosomes which fuse with the plasma membrane (B). After fusion between phagosome and lysosomes (C), the yeast is partially digested. This old phagosome fuses with the plasma membrane (D) and releases the partially digested yeast in the culture medium. The increase of plasma membrane produced during defecation is compensated for by the formation of new pinosomes (E). During growth in axenic medium, the influx of membrane produced by pinocytosis (E) is probably also compensated for by the outflow (B). It was not possible to show in *Dictyostelium* that primary lysosomes come from the Golgi apparatus (F).

sively increasing number of pinocytotic vacuoles? This behavior recalls an induction process observed in larger amoebae (8, 49). It indicates that the capability of ingesting food can be modified, and suggests the disappearance of membrane receptors. This phenomenon is under study.

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