DIFFERENTIATION OF FOOD VACUOLAR MEMBRANES
DURING ENDOCYTOSIS IN TETRAHYMENA

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ABSTRACT
The origin and differentiation of Tetrahymena pyriformis food vacuolar membranes has been studied by freeze-fracture electron microscopy. By measuring the temperature needed to induce the onset of lipid phase separation (as inferred by the appearance of particle-free regions in replicas) and calculating the changes in average intramembrane particle distribution, a distinct modification of the vacuolar membrane could be observed from the time of its formation from disk-shaped vesicles to its maturation before egestion of its indigestible contents. Whereas the nascent vacuolar membrane first showed signs of phase separation at 9°C, this temperature rose to 14°C in the completed vacuole and then, after lysosomal fusion, eventually declined to 12°C. The average membrane particle density on the PF face increased from 761 ± 219 to 1,625 ± 350 per μm² during membrane differentiation. Like other membranes of the cell, the vacuolar membrane underwent adaptive changes in its physical properties in cells maintained for several hours at low temperature. This exposure to low temperature caused an equal effect in vacuoles formed before, during, or after the temperature shift-down. Normal changes in the properties of the vacuolar membrane may have some bearing on its programmed sequence of fusion reactions.

KEY WORDS food vacuoles • freeze-fracture electron microscopy • membrane fluidity • Tetrahymena pyriformis

Increasing attention is being directed to the role of membranes in a variety of cell functions. Since the realization that lipid bilayer fluidity can function in a regulatory manner with respect to membrane-mediated activities (10), the analysis of membrane physical properties and structural composition has taken on new meaning.

Many correlations between membrane structural properties and function can be observed by using the model system, Tetrahymena pyriformis (17). The functionally distinct membrane systems of this cell appear to differ from one another, sometimes markedly, in their lipid composition (17) and susceptibility to bilayer phase separation (7). Furthermore, evidence is accumulating that Tetrahymena cells can modify their intracellular lipid composition so as to maintain the fluidity of each organelar membrane within its particular optimal range despite environmental perturbations.

In this communication, we report on a somewhat different phenomenon—the programmed change in membrane properties with time in a specific membrane. It has been pointed out in a number of cases, e.g., the Golgi apparatus (20), that membrane alterations and modifications in enzyme activity accompany cytodifferentiation. The phagocytic vacuole is another example of programmed change in functional activity during the course of its formation and maturation (14).
We have previously isolated newly formed food vacuoles and shown that their membrane lipid pattern differs from that of other organelles (19). In view of the recent indications that physical alterations caused by lipid changes can be a determining factor in membrane fusion and related dynamic actions (13), there is good reason to ask whether those fusion events which occur at specific stages of the endocytosis process are also influenced by membrane changes.

At the present time, there is no satisfactory procedure for isolating purified food vacuoles at a number of discrete stages of maturity. We therefore began our study with an in vivo examination of membrane physical properties as inferred from freeze-fracture electron microscopy. The technique employed involved chilling several aliquots of cells, each to a different test temperature, before fixation with glutaraldehyde (7, 9). The resulting patterns of intramembrane protein particle distribution on the freeze-fracture replicas could be used to determine the temperature at which each membrane underwent initial phase separation of its constituent lipids (7). More specifically, the onset of phase separation was signaled by the sudden appearance of particle-free regions on the membrane fracture face. The data provided below show significant differences in this property and in several other characteristics of the vacuolar membrane during endocytosis.

MATERIALS AND METHODS

Tetrahymena pyriformis, strain NT-1 (4), was grown in 200 ml of enriched proteose peptone (16) on a rotary shaker at 39.5°C, until the cells reached mid-logarithmic phase.

1 ml of India ink solution (two drops of Pelikan drawing ink [Günter Wagner, Hannover, Germany] per ml H2O) was added to 24 ml of culture and incubated for 2 min at these temperatures. Cells were then treated with 5.7 ml of a polystyrene latex bead emulsion (Dow Latex 586 beads [Dow Corning Corp., Midland, Mich.], 0.23 μm diam, suspended to 50% by weight in inorganic medium [9] and dialyzed) and incubated for a further 30-min period. After the incubation periods, 25-ml aliquots of cell culture with India ink and/or latex beads were each chilled to a preselected temperature, e.g., 17°C, 14°C, 12°C, 10°C, 9°C, 5°C, and 0°C, over a 2-min period and maintained for 2 min at these temperatures. Cells were then fixed by adding ½ vol of precooled 4% glutaraldehyde in 0.2 M sodium phosphate buffer, pH 7.2, as described previously (7).

To study the physical alterations of the membranes of food vacuoles during temperature acclimation, cells fed both ink and latex as described above were cooled from 39.5°C to 15°C over a 30-min period, with the cooling commencing as soon as the latex suspension had been added to the culture. After a 4-h period from the beginning of the temperature shift, cells were chilled to 12°C, 10°C, 9°C, 5°C, 3°C, and 0°C and fixed at these temperatures as mentioned above.

To free glutaraldehyde-fixed cells from an excess of latex beads, the fixed cells were washed with 0.05 M sodium phosphate buffer, pH 7.2, by repeating the centrifugation and resuspension, and the cells were suspended finally in 4 ml of the buffer. The suspension was layered on a density gradient containing 4 ml of 1 M sucrose and 4 ml of 0.25 M sucrose and centrifuged at medium speed in a clinical centrifuge for 10 min to separate the cells from aggregations of latex beads and India ink particles. The cells were obtained as a clean pellet at the bottom while the ink particles were in the 1-M sucrose layer and the latex beads were at the interface between 0.25- and 1-M sucrose layers.

Freeze-fracture electron microscopy was carried out basically according to the method described previously (7): cells were washed and the pellets were exposed to increasing concentrations of glycerol, terminating with a 24- to 48-h incubation with 30% glycerol. The cells were pelleted in a clinical centrifuge and samples were frozen in liquid Freon 22 and transferred to liquid nitrogen before fracturing in a Balzers apparatus (BH, 360M, Balzers, Fürstenstum, Liechtenstein) and shadowed with platinum and carbon at −100°C. Replicas were examined with a Hitachi HS-8 electron microscope at 50 kV.

Intramembrane particle density was measured by counting the particles within an area 0.2 μm × 0.2 μm (9) on three to five regions each of 7-10 cells for each final value.

The effect of temperature upon the rate of vacuole formation was determined by shifting a 7-ml aliquot of logarithmic-phase cells from their growth temperature to the test temperature over a 10-min period. Samples were fixed with formaldehyde for microscopy examination at 5, 10, and 20 min after adding five drops of diluted ink (two drops of ink/ml H2O).

For studying the effects of ingested ergosterol, 4 mg of 3× recrystallized ergosterol (Nutritional Biochemicals, Cleveland, Ohio) was sonicated in 10 ml of inorganic medium to produce small fragments. The suspension was warmed to 39.5°C and mixed with 65 ml of logarithmic-phase cells.

RESULTS

To examine the physical properties of vacuolar membranes at various times during the endocytotic process, it was desirable to have a method for distinguishing newly formed vacuoles from vacuoles at later stages of maturity. Very recently formed vacuoles were identified by exposing cells to diluted India ink suspensions for brief intervals. Vacuole formation was easily monitored by phase
microscopy, and cells could be fixed for microscopy before completion of the first vacuole or at any later time as desired. The resistance of the ingested 0.04-μm india ink particles to the chromic acid employed for cleaning the freeze-fracture replicas often caused them to adhere to the replicas in the vicinity of cleaved vacuoles and to register as electron-dense bodies in the resulting micrographs (see Fig. 3). Thus, ink-containing vacuoles could generally be identified unequivocally.

In many experiments, a dense suspension of 0.23-μm diam polystyrene latex spheres was added to cultures 5 min after addition of ink. All vacuoles formed subsequently contained a predominance of the latex spheres, which were also clearly identifiable both by their occasional retention on the acid-cleaned replica and by the characteristic depressions in the vacuolar membranes surrounding them (Fig. 1). To avoid a high level of nonspecific binding of extracellular beads to the replica surface during chromic acid digestion, it was essential to wash the fixed cells free of latex. In freeze-fracture replicas of these cells, the older vacuoles could be identified as being those containing india ink and no latex spheres.

With use of the above criteria, 39.5°C-grown cells containing vacuoles were chilled to one of several test temperatures and fixed with glutaraldehyde. Examination of many replicas, as described below, established that membranes of the endocytosis cycle each had its characteristic temperature at which lipid phase separation was first evident.

Forming food vacuoles showed no particle-free domains until chilled to 9°C (Table I and Fig. 2). However, 2-10 min after the vacuole had been completed and released, particle-free areas appeared at a higher temperature (14°C, Table I and Fig. 3). Vacuoles which had remained in the cell for 30 min and were ready to discharge their indigestible contents via the anal pore (14) exhibited particle-free areas at a slightly lower temperature.

**Figure 1** The PF face of a mature food vacuole showing a polystyrene latex particle (dark sphere) and the unusual depressions in the membrane (arrows) which provide a further indication that this vacuole contained latex particles. The resistance of the polystyrene (and the india ink) to the chromic acid digestion frequently caused them to adhere to the replicas, predominantly near the vacuoles that contained them. Although the latex particles are thought to be bound only to the lower (tissue) side of the replica, this cannot be confirmed from the micrographs. The small, raised areas are thought to represent either the late stages of lysosome fusion (see later discussion) or fusion points with vesicles discharging digestion products. Circled arrow indicates direction of shadowing. × 56,000.

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Table I

The Appearance of Particle-Free Domains within Various Tetrahymena Membranes Fixed at Different Temperatures*

| Fixing temperature (°C) | 17 | 14 | 12 | 10 | 9  | 5  | 0  |
|--------------------------|----|----|----|----|----|----|----|
| Forming food vacuole     | -  | -  | -  | -  | +  | +  | +  |
| Disk-shaped vesicles     | -  | -  | -  | -  | +  | +  | +  |
| Newly released vacuoles  | -  | +  | +  | +  | +  | +  | +  |
| Mature vacuoles          | -  | +  | +  | +  | +  | +  | +  |
| Endoplasmic reticulum    | +  | +  | +  | +  | +  | +  | +  |
| Alveolar membrane PDI§   | 80 | 90 | 90 | 90 | 90 | 95 | 100|

* - = No change from random particle distribution, + = presence of particle-free areas.
† Membrane faces often showed indications of vesicle fusion.
§ PDI = particle density index (9), measuring % of maximum particle aggregation.

In addition to the change in physical properties, the vacuolar membrane underwent a significant modification in its protein distribution during maturation. The most striking change we noted was a sudden increase in protein particle density on the PF face of vacuolar membranes during a period of 1-4 min after their release from the formation site at the base of the oral apparatus (compare Figs. 2 and 3). Whereas the PF face of membranes surrounding forming vacuoles contained 761 ± 219 particles/µm², the same face of completed vacuolar membranes had 1,625 ± 350 particles/µm². The increase in density occurred before the discernible interaction of the vacuoles with lysosomes (see below) and then did not change significantly during subsequent maturation. Although we examined replicas from cells fixed at their growth temperature as well as lower temperatures, we were unable to detect an equal distribution of particles on the EF and PF faces of forming vacuolar membranes as reported by Batz and Wunderlich (3). Our cells contained 146 ± 85 particles/µm² on the EF face of forming vacuolar membranes and approximately the same density in mature vacuoles.

The disk-shaped vesicles first identified by Allen (1) as being probable vacuolar membrane precursors in Paramecium were much in evidence near the oral apparatus (Fig. 4). The close physical similarity between the two organelles is suggested by the fact that the disk-shaped vesicles and the adjacent forming food vacuolar membranes had similar particle densities and both underwent phase separation at 9°C, a temperature lower than that for any other membrane of the cell. The endoplasmic reticulum, elements of which were usually seen in the vicinity, contained large domains free of protein particles at all of the temperatures used in Table I and has been shown elsewhere (7) to undergo phase separation at 24°C. The outer alveolar membrane, which was found previously (9) to be very prone to low temperature-induced lateral membrane particle movement, sustained a very pronounced phase separation at these temperatures, as evidenced by the high Particle Density Index (PDI), a measure of the percentage of maximum particle aggregation. The plasma membrane was shown in the earlier report to undergo phase separation at 12°C.

The sudden alteration of vacuolar membrane properties at low temperature might be expected to reduce the rate of vacuole formation. Fig. 5 portrays the effect of low temperature upon vacuole formation by 39.5°C-grown cells and by 26°C- and 15°C-grown cells as well. We have shown that cells grown at the latter two temperatures manifest the same hierarchy of intracellular membrane differences in phase-transition points as do 39.5°C cells, but with correspondingly lower absolute values (7). In all cases, vacuole formation continued until only a few degrees above the initial point of recently formed vacuolar membrane phase separation (which was 5°C-8°C in 26°C-grown cells and below 0°C in 15°C-grown cells). Although the temperatures blocking endocytosis were in each instance well below the phase-separation point of all other membranes except the plasma membrane (7), it should be pointed out that the cells displayed no obvious abnormalities of appearance or motility under these conditions.

Cells fixed at various temperatures during the first 2 min after the addition of india ink contained ink vacuolar membranes having relatively smooth, uninterrupted contours. However, vacuoles which
FIGURE 2. The PF face of a food vacuolar membrane forming in a cell fed india ink. Immediately before fixation, this cell was chilled from 39.5° to 9°C, thereby causing the formation of particle-free domains (arrows). The ridges emanating from the right side represent the cytopharyngeal terminal of the oral rib (11). × 56,000.

FIGURE 3. The PF face of the membrane surrounding a completed vacuole in a cell chilled to 5°C for fixation 2 min after feeding ink. The particle-free domains, such as are seen here, first became visible at 14°C, but at a lower frequency. The dark areas represent india ink particles, whose adherence to the replica permitted the vacuole’s time of formation to be determined. × 56,000.
A concentration of disk-shaped vesicles (arrows) adjacent to a forming food vacuole in a cell fed india ink for 2 min. Before fixation, the cell was chilled from 39.5°C to 0°C, inducing pronounced phase separation (double arrows) in the PF face of the disk-shaped vesicle membrane as well as the PF face of the vacuolar membrane (PF). Numerous ink particles underlie the cross-fractured vacuolar contents, and traces of the ink are also visible in the fracture plane. × 56,000.

The effect of temperature on the rate of vacuole formation from india ink by cells grown at 39.5°C, 26°C, and 15°C. Cells were chilled to the temperatures indicated on the abscissa and held at those temperatures in the presence of ink for 20 min before being fixed with formalin for counting vacuoles. Each point represents the average of 8–20 cells. Generally similar curves were obtained using cells counted after 5 or 10 min.

had been in the cell for 2–5 min exhibited a quite different pattern under certain fixation conditions. When fixed at 12°C or below (see Table I), these vacuoles contained variable numbers of depressions, fracture rims, and other indications that fusion with smaller vesicles (presumably lysosomes) was occurring (Fig. 6).

The interaction of the small vesicles with these chilled cells took several forms. Fig. 6 presents what we postulate to be a time sequence of early events at the fusion site. The pattern of protein particle distribution is reminiscent of that exhibited by mucocysts fusing with the plasma membrane (15). Our failure to discern substantial numbers of fusion events in cells fixed at higher temperatures indicates that lysosomal fusion is normally a very rapid process.

Although vesicles ~0.3–0.5 μm in diam could often be seen just outside the vacuoles during the lysosome-fusion stage, the fracture faces of these vesicles were never broad enough to permit an evaluation of possible phase separation at various temperatures.

We interpret the freeze-fracture data to indicate a change in the physical properties of the food vacuolar membrane from the time of its formation through its merger with lysosomes and final maturation. Although it is not presently possible to correlate the microscope properties of membranes with their physical-chemical properties in a quanti-
tative way, the evidence is in agreement with a change in membrane fluidity of significant proportions (7, 22). By what mechanism could the inferred fluidity alterations be achieved? One possibility is through changes in the membrane lipid composition. Much evidence has been accumulated to establish that *Tetrahymena* can alter its membrane fluidity very rapidly by producing specific lipid species on the endoplasmic reticulum and transferring them to other membranes (6, 7, 9). We modified one of these earlier experiments to test the ability of food vacuoles to respond to a fluidity-modifying stimulus.

Cells grown at 39.5°C were treated with India ink in the usual manner. After 5 min, a large excess of latex beads was added, and the cells were chilled to 15°C over a 30-min period. The culture was incubated at 15°C for 4 h, wherein aliquots were quickly chilled to appropriate temperatures and fixed with glutaraldehyde.

The quantitatively most prevalent membrane species (endoplasmic reticulum, pellicle, mitochondria, etc.) underwent a pronounced decrease in their temperatures of initial phase separation, in agreement with our published findings (7). The vacuolar membranes and membranes of disk-shaped vesicles responded in a like manner, so that no indication of particle rearrangement was seen, even at 0°C.

It is worth noting that none of the vacuolar membranes exhibited phase separation despite the fact that some were newly formed while others (those containing ink particles) had been formed and had completed their lysosomal fusion before the cell temperature was reduced to 15°C. A further point of interest is the observation that many of the food vacuoles containing latex beads displayed numerous signs of associated vesicles, even though there was no apparent phase separation. Because many of the latex vacuoles had been formed for a considerable period of time in this experiment, we cannot be certain as to whether the scars present on their membranes arose from lysosomal fusion or from micropinocytosis associated with the resorption of digested material (11). Whatever their nature, the scars were not lost when such cells were warmed briefly to 39.5°C before being chilled for fixation.
To complement the above experiment suggesting an introduction of new lipids into the vacuolar membrane from the other organelles, the possible modifying role of lipids present in the vacuolar contents was also tested. We selected ergosterol as the test substance because its incorporation has a pronounced effect on the fluidity of Tetrahymena membranes in long-term feeding experiments (12). Cells were mixed with a preparation of ergosterol which had been sonicated in inorganic medium until a large proportion of the sterol was reduced to fragments small enough to be ingested by the cells. Endocytosis of the birefringent ergosterol particles by most cells was confirmed by phase microscopy.

After allowing a period of 30 min for digestion and resorption of the ergosterol to commence, aliquots of the cells were fixed at different temperatures for electron microscopy. Surprisingly, there were no significant perturbations of the normal freeze-fracture-indicated phase-separation temperature of the vacuolar membrane. Thus, one lipid capable of significant membrane perturbation does not readily affect the membrane directly enclosing it.

**DISCUSSION**

The phagosome or food vacuole of ciliates such as Tetrahymena is an excellent example of an organelle whose enclosing membrane undergoes a continuously changing interrelationship with the rest of the cell. As recently described in great detail by Rasmussen (14), the vacuole appears to originate via the fusion of precursor vesicles, then gains its digestive capacity through a later phase of fusion with lysosomes, and finally at the end of its development appears to disintegrate into small vesicles for reuse in another round of vacuole formation. The entire process requires <1 h. The present study by electron microscopy was designed to determine whether these clearly defined transitions in specificity of interaction are correlated with modifications in membrane physical properties.

Because of the enormous technical problems to be faced in isolating vacuoles at distinct stages of maturity, we have restricted ourselves for the moment to an in vivo analysis.

The appearance of the endocytic membrane fracture faces was generally similar to that reported by Batz and Wunderlich (3). Examination of their micrographs revealed the same higher particle density in completed vacuoles that we find. For unknown reasons, we could not duplicate their finding of a nearly equal particle distribution on both the EF and PF faces of the forming vacuolar membrane. Thinking that the distribution might be temperature-dependent, we fixed some cells at their growth temperature of 39.5°C, but the particles were always found mainly on the PF face. The fact that Allen (2) has recently observed a particle distribution in the nascent vacuolar membranes of Paramecium that differs from either that reported here or that seen by Batz and Wunderlich (3) suggests that some as yet undetermined parameters may influence this property.

As a principal criterion for estimating changes in membrane properties, we employed a measurement of temperature-induced appearance of particle-free domains in the various membranes. These intramembrane particle rearrangements have been shown to arise from lipid phase separations (5, 8, 22). To some extent, then, such measurements are an estimate of membrane fluidity, although not necessarily a direct one, because phase separations can also be modulated by other factors, such as cation binding, sterol content, etc.

The phase-separation temperatures of the pertinent membranes in 39.5°C-grown cells are summarized in Fig. 7. Membranes of the food vacuoles and the apparent precursor vesicles have at all times much lower phase separation temperatures than do the adjacent endoplasmic reticulum or alveolar membranes. Of the cellular membranes examined here and previously (7), only the plasma membrane sustains particle rearrangement in the same temperature range, but the plasma membrane is clearly distinguishable from vacuolar membranes by virtue of the different size and density of its intramembrane particles.

The second finding of interest is the temporal modification in the physical stability of the vacuolar membrane, as revealed by the reproducible variations in phase-separation temperatures. As long as the vacuole was still forming at the base of the buccal cavity (stage a, Fig. 7), particle separation was observed at 9°C. The identical property of the associated disk-shaped vesicles coupled with the similarity of protein particle appearance and density in the vesicles and the forming vacuoles adds fresh evidence to the proposal by Allen (1) that these vesicles provide the bulk of the membrane needed for vacuole expansion.

Once the completed vacuoles were separated from the oral apparatus, the onset of phase separation occurred at a much higher temperature, at 14°C (stage b, Fig. 7). In a nondifferentiating
organelle, a change of this magnitude would require acclimation of the cells to a 5°C elevation in growth temperature. The pronounced change was visible in vacuoles only 2 min or less old, and as yet containing no evidence of lysosomal fusion. Thus, the alteration was clearly not the result of an admixture of lysosomal membrane lipids with preexisting vacuolar membrane lipids.

As the vacuoles continued to mature, the phase-separation temperature again dropped slightly to 12°C (stage c, Fig. 7). This was the period at which much of the now digested vacuolar contents was transferred to the cytoplasm by transmembrane diffusion and micropinocytosis (11). Shortly after this phase, undigested material was discharged through the anal pore (stage d, Fig. 7) and the remaining membrane was presumably recycled in the form of small vesicles (1).

Although freeze-fracture electron microscopy data of the type reported here clearly showed reproducible alterations in the physical properties of the vacuolar membrane, extrapolation of the findings to gain information about the membrane's fluidity at the cell's growth temperature can be only speculative at present. And even if an increase in the phase-separation temperature faithfully parallels a reduction in fluidity, the technique offers no way of detecting which of several potential fluidity-altering factors, i.e., cations, pH, or lipid changes, might have been responsible. It was to gain more information on this point that we conducted the experiment involving the incubation of cells at 15°C for 4 h.

We observed earlier that the more easily isolable *Tetrahymena* cells organelles, such as the endoplasmic reticulum and pellicles, undergo a fairly rapid decrease in freeze-fracture-inferred phase separation after being shifted from 39.5°C to 15°C (9). In these cases, the drop in phase-separation temperature was closely correlated with changes in phospholipid fatty acid composition. We therefore propose that the striking change in phase-separation temperature of the vacuolar membranes and associated structures after temperature shift may also result at least in part from lipid compositional changes. If true, this observation would add credence to the concept that single lipid molecules or small groups of molecules can exchange rather freely from one membrane to another, perhaps via some kind of exchange protein (21). There is no indication that the food vacuole merges with aggregates of membrane material as large as vesicular elements subsequent to the stage of lysosome fusion.

It therefore seems likely that the properties of vacuolar membranes are closely regulated by interaction with lipids from other regions of the cell. We have presented suggestive evidence that the proper physical state of the vacuolar membranes or their precursor vesicles may be essential for continued vacuole formation. It will be interesting to determine whether the low temperature-induced phase separation is really responsible for
the inhibition of vacuole formation shown in Fig. 5. The role of membrane fluidity in this process might be tested by studying the dramatic inhibitory effect of calcium ions on vacuole formation and the stimulatory effects of sodium or potassium ions (11). It may be more than a coincidence that divergent cations decrease and monovalent cations increase membrane fluidity in synthetic membranes (18).

This work was supported by National Institutes of Health Grant GM 20148, Robert A. Welch Foundation Grant F-350, National Science Foundation Grant OIP74-22029, and National Cancer Institute Grant TE32 CA09182.

Received for publication 16 February 1977, and in revised form 19 July 1977.

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