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Nature and Development of Membrane Systems in Food Vacuoles of Cellular Slime Molds Predatory upon Bacteria

HANS R. HOHL

Department of Microbiology, University of Hawaii, Honolulu, Hawaii

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ABSTRACT

HOHL, HANS R. (University of Hawaii, Honolulu). Nature and development of membrane systems in food vacuoles of cellular slime molds predatory upon bacteria. J. Bacteriol. 90:755–765. 1965.—During the digestion of bacteria by the myxamoebae of cellular slime molds, systems of concentric lamellae begin to appear within the food vacuoles. Each constituent lamella is a unit membrane of 75 to 85 A thickness. A study of these lamellae in *Dictyostelium discoideum* and *Polysphondylium pallidum* reveals that most of them do not represent original membranes of the ingested bacteria but are formed mainly in two ways. (i) After swelling and partial digestion of the bacteria, the first membranes appear adjacent to pre-existing membranes, e.g., the membrane lining the food vacuole and the cytoplasmic membrane surrounding the bacterium. Progressive addition of lamellae leads to the formation of the systems of concentric lamellae. (ii) After digestion of the bacteria has proceeded to a high degree, the concentric lamellae are formed spontaneously from clouds of amorphous material through condensation and orientation of precursor material. The study shows that, in biological systems, unit membranes may be formed from amorphous material through template action of pre-existing membranes, and does not necessarily involve fusion of membrane-bound vesicles.

In the food vacuoles of myxamoebae of cellular slime molds growing on living bacteria, numerous systems of tightly packed membranes are frequently observed (Gezelius, 1959, 1961; Mercer and Shaffer, 1960). It is reasonable to assume that the existence of these membranes is closely related to the digestion of the bacteria. However, final proof of this had not previously been presented, since until recently the myxamoebae could not be cultivated in the absence of bacteria (Hohl and Raper, 1963*b*; Sussman, 1963).

Since the ingested bacteria contain much less membranous material than these lamellar systems, their presence in such large quantities must be accounted for. We hope that an elucidation of the mechanism of membrane formation in the food vacuoles of these organisms might throw some light on possible membrane-generating processes in the living cell in general.

This report describes the nature of the individual membranes and their configuration in the membranous systems and presents their formation from the beginning of the digestive process together with a plausible explanation of the facts observed. In addition, some other related aspects of cellular digestion in the cellular slime molds are considered.

MATERIALS AND METHODS

Dictyostelium discoideum strain NC-4(S2) and *Polysphondylium pallidum* strain FR-47 were used. The latter strain is able to grow on a soluble medium in the absence of any bacteria or bacterial components (Hohl and Raper, 1963*c*) and, therefore, was particularly useful in the present study.

Escherichia coli strain B/r was used as the food organism. The bacteria were grown on a solid medium consisting of 1% lactose, 1% peptone, and 1.5% agar. They were suspended in 0.016 M phosphate buffer at *p*H 6.25, centrifuged, resuspended, and their concentration was adjusted to 10^{10} bacteria per milliliter.

The myxamoebae were grown in 5-ml samples of the bacterial suspension in test tubes rotating at 250 rev/min at 24 to 26 C; details of the technique have been described elsewhere (Hohl and Raper, 1963*a*, *b*). The inoculum was adjusted to 10^4 spores per milliliter of bacterial suspension.

P. pallidum, strain FR-47, also was cultivated on the complex medium of Hohl and Raper (1963*c*) consisting of 4% bovine serum albumin and 2% tryptose in an inorganic salt solution.

In some experiments, bacterial suspensions were added to myxamoebae growing on the complex medium; in others, ferritin or colloidal gold solutions were incorporated in the medium or the bacterial suspensions, usually 4 hr prior to the

time of fixation. Ferritin from horse spleen (Pentex Inc., Kankakee, Ill.) was used in a final concentration of up to 25 mg/ml of medium. Colloidal gold solution (Magar Chemicals, Inc., Cornwall Landing, N.Y.) of undetermined concentration was employed in concentrations of up to 1 part of gold solution in 2 parts of medium.

Suspensions of myxamoebae were fixed by addition of an equal volume of 2% phosphate-buffered (*p*H 7.4) glutaraldehyde; the suspension was immediately centrifuged and the myxamoebae were resuspended for 2 hr in fresh fixative. After repeated washings in phosphate buffer (0.016 M, *p*H 6.25) the cells were postfixed in 1% Veronal acetate-buffered osmium tetroxide at a *p*H of 7.4 for another 2 hr. Stepwise dehydration in acetone was followed by propylene-oxide treatment and subsequent embedding in Maraglas. Polymerization took place at 60 C for at least 2 days. This method proved to be superior to all other procedures tried, including fixation in potassium permanganate; also, addition of $CaCl_2$ to the fixative did not change the quality of the pictures appreciably. Embedding in Epon-araldite or Vestopal is satisfactory, but does not quite equal that in Maraglas.

Sections were cut with a LKB ultratome or a Porter-Blum MT2 ultramicrotome and were observed in a Hitachi HU-11A electron microscope or, occasionally, in a Norelco 75 electron microscope.

RESULTS

Lamellar configurations and fine structure of individual membranes. The membrane complexes are most numerous in the food vacuoles of myxamoebae actively feeding on bacteria (Fig. 1). They occur less frequently in aggregated myxamoebae, but can be found even in the culmination stage. They also have been observed outside the cells either closely attached to the plasma membrane, or between aggregated cells, or free in the medium, as has been described by Gezelius (1961). This strongly suggests that they are expelled from the cells.

The great variety of arrangements of lamellae found can be satisfactorily grouped into a few types. Yet, although this grouping accounts for many observations, it is only an approximation and does not necessarily include all possible arrangements.

The most prominent type consists of a set of closely packed, more or less spherical lamellae whose outside diameter approximates the size of the food bacteria (Fig. 1 and 2). The number of membranes per set varies considerably, but is often about a dozen. These sets of lamellae have been described by Mercer and Shaffer (1960) and Gezelius (1961), but these authors did not make a decision as to whether the lamellae are concentric or form tight spirals. From the observations presented here, it is clear that they consist of concentric lamellae (Fig. 2, 3). Spirals have been seen occasionally, but under different circumstances: if a myxamoeba engulfs another myxamoeba, as happens not infrequently (Huffman and Olive, 1964), the plasmalemma might break and roll up to a certain degree (as in Fig. 4). In most cases, however, these tightly packed lamellae represent sets of concentric membranes comparable to the layers of an onion. The sets may or may not harbor a core of amorphous material, probably remnants of the bacteria.

Often these sets of concentric lamellae occur not singly, but in clusters, which themselves might be surrounded by concentric lamellae (Fig. 5–7). Usually there is one cluster to one food vacuole. In several cases, even more complicated forms have been observed: lamellae of two sets can cross each other, resulting in a honeycomb type of network which is restricted to a relatively small area (Fig. 5–8). The structures resemble the well-known interference pattern developing on a water surface between neighboring sets of standing waves.

Although the membranes are closely and fairly evenly packed, each individual membrane is still clearly separated from the other by a space of approximately 200 A. In a few instances, however, they have been observed as stacks without any space between membranes, thus giving an appearance similar to myelin sheaths (Fig. 9). It is noteworthy that in this figure the membranes are not closed, but have open ends. Open ends are not rare (Fig. 10), and in some instances may be due to breakage during fixation or embedding. In other instances, however, the membranes are joined at their open ends to a mass of amorphous material and give the appearance of incomplete spherical membranes (Fig. 11).

The individual membrane of the various complexes mentioned is 75 to 85 A thick and shows the familiar triple-layered structure of a unit membrane (Fig. 12). The two dark layers enclosing the lighter middle layer are of equal thickness, thus giving the membrane a symmetrical appearance. This is in contrast with the membrane lining the food vauole, which is strongly asymmetrical, the dark layer on the inside of the food vacuole being much more electron-dense than is its counterpart bordering the cytoplasm. It can thus be assumed that the unit membranes making up the complexes have a component of polar lipids and are most probably lipoproteins. The dark layer may exhibit a somewhat beaded appearance (Fig. 12) similar to that described by

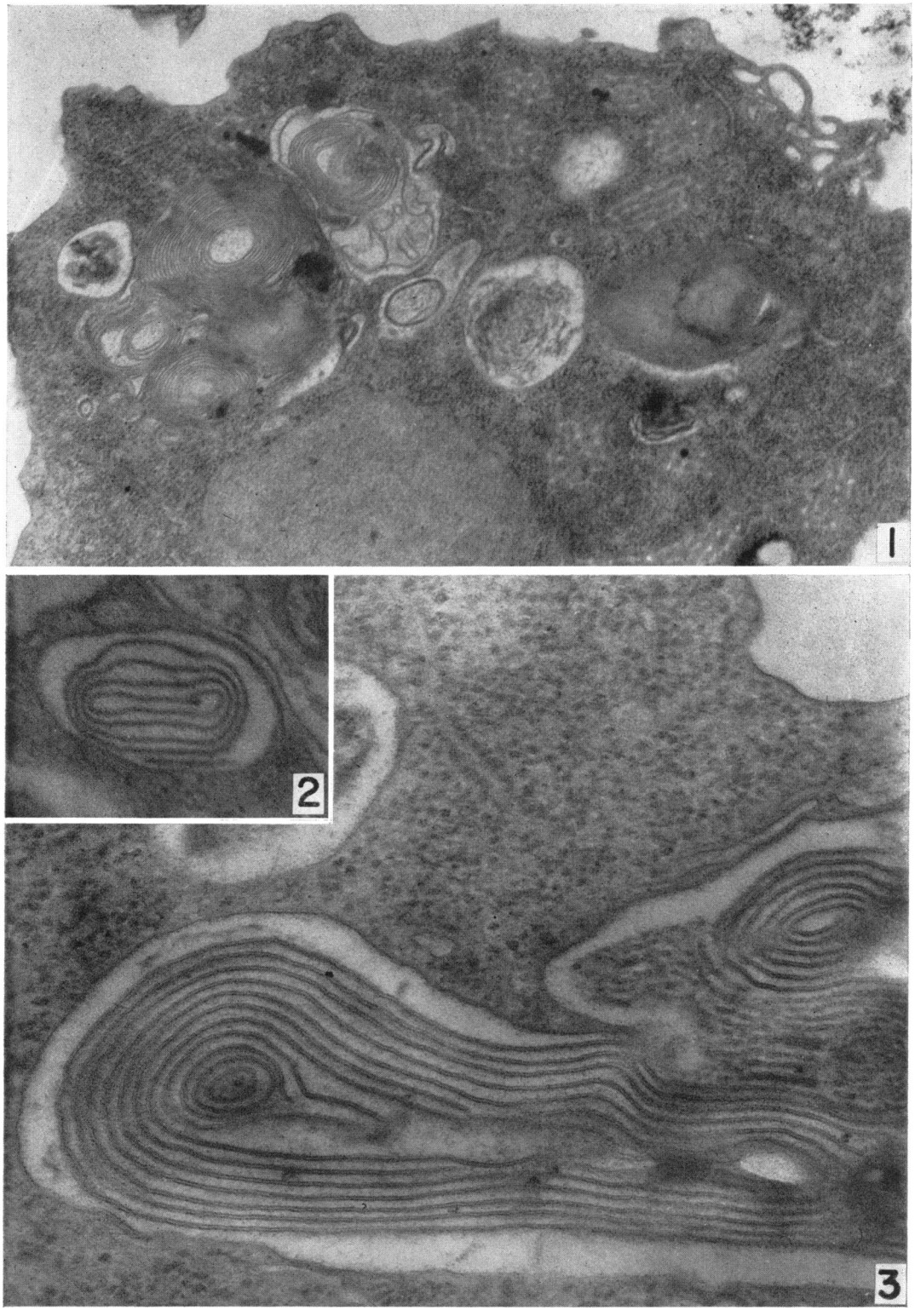
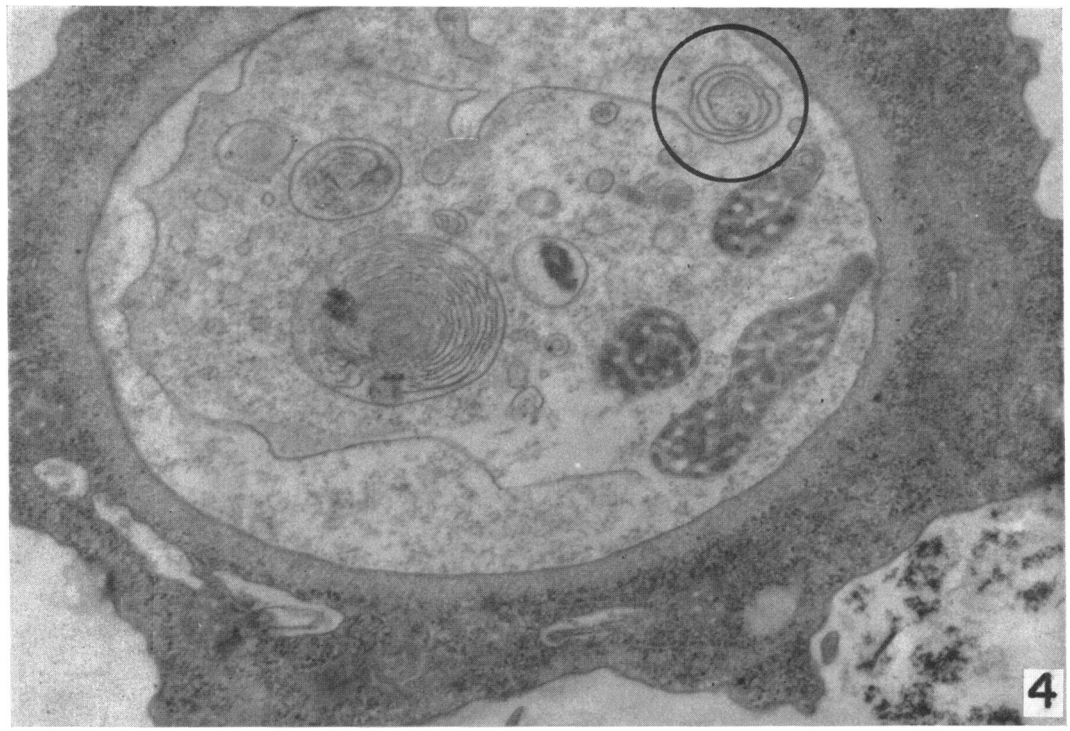


FIG. 1–3. *Sets of concentric membranes in the food vacuoles of myxamoebae of Dictyostelium discoideum. Fig. 1, × 30,400; Fig. 2, × 69,000; Fig. 3, × 99,700.*



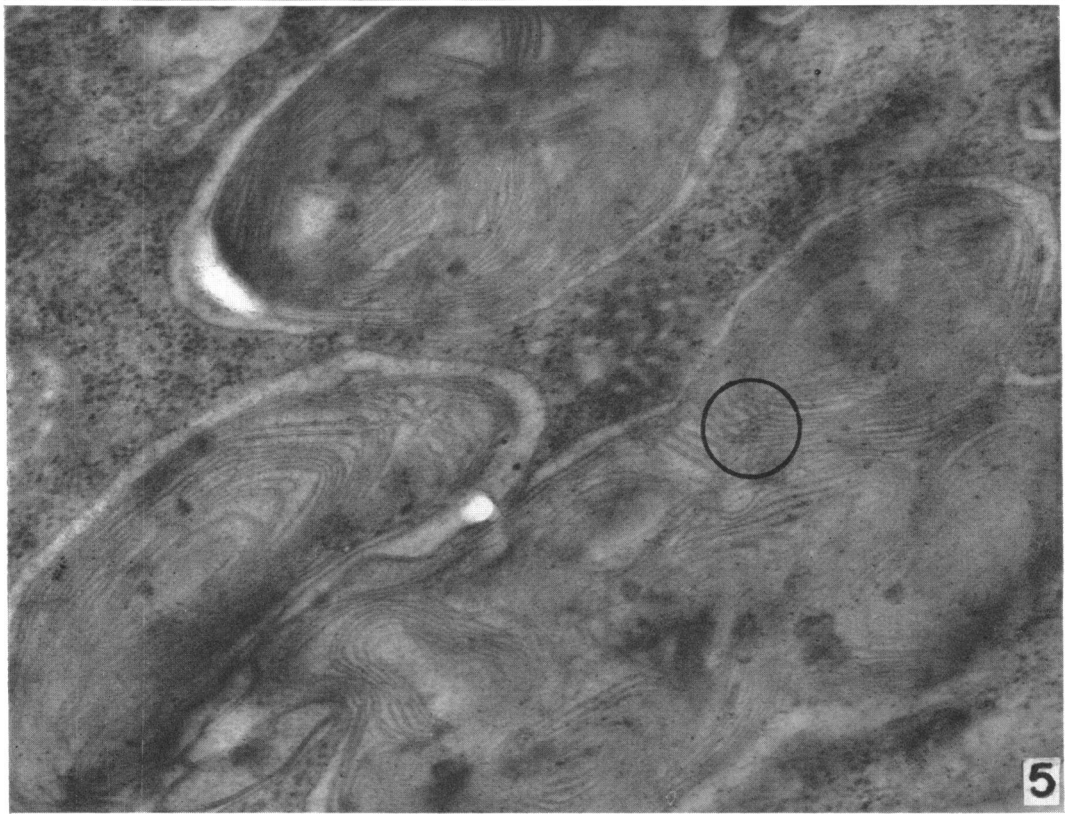


FIG. 4. *Food vacuole of a myxamoeba of Dictyostelium discoideum containing remnants of another amoeba. Encircled area shows rolled-up membrane.* × *28,200.*

FIG. 5. *Complex membrane systems of Dictyostelium discoideum with honeycomb-pattern encircled.* × *63,000.*

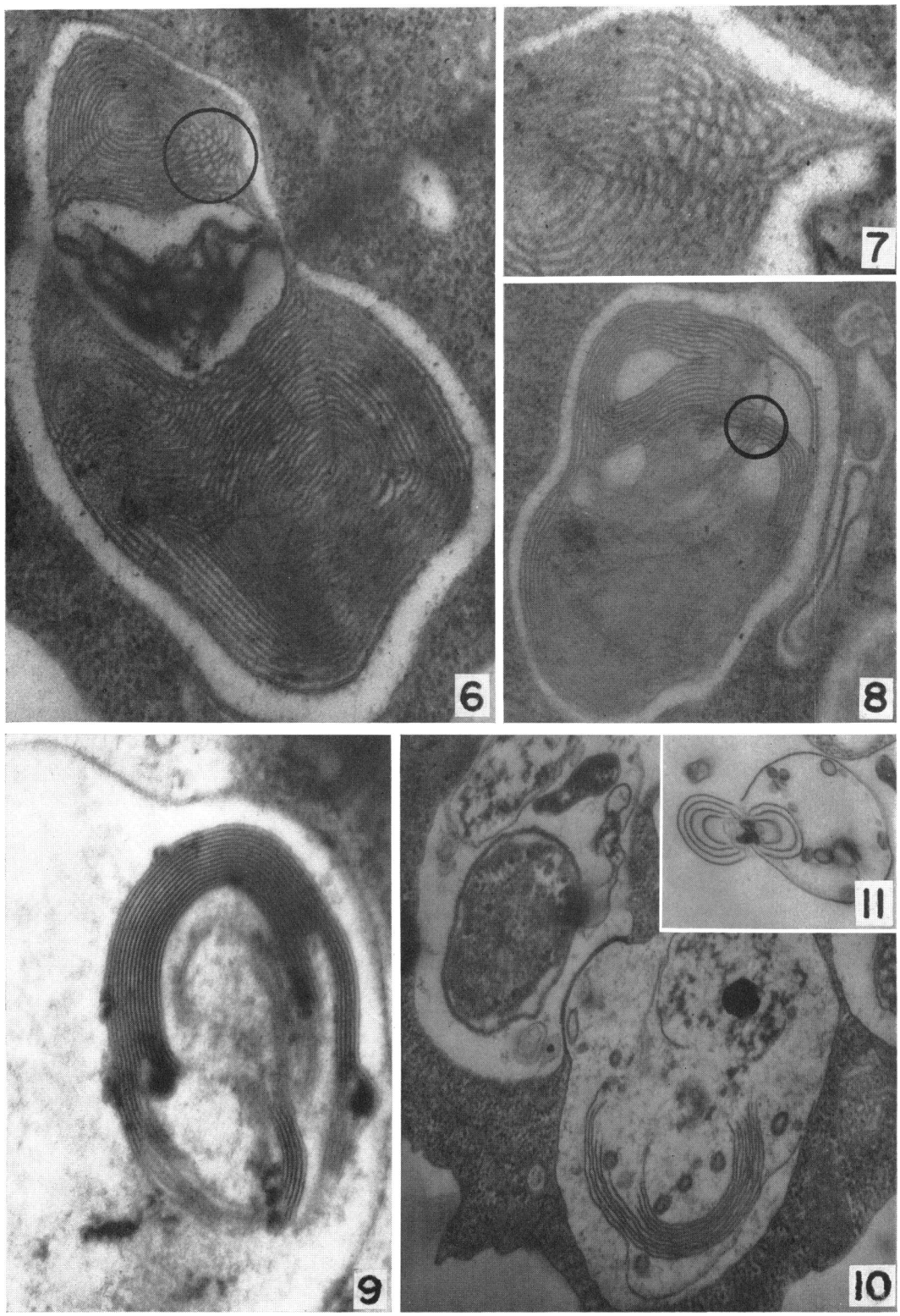


FIG. 6. *Complex membrane systems of Dictyostelium discoideum with homeycomb-pattern encircled.* × *51,000.*

FIG. 7. *Detail of Fig. 6.* × *102,500.*

FIG. 8. *Membrane crossing other membranes (in circle).* × *43,500.*

FIG. 9. *Myelinoid structure in food vacuole of Polysphondylium pallidum.* × *103,100.*

FIG. 10. *Parallel set of membranes with open ends, Polysphondylium pallidum.* × *49,600.*

FIG. 11. *Set of lamellae with open ends connected to amorphous material, Dictyostelium discoideum.* × *29,000.*

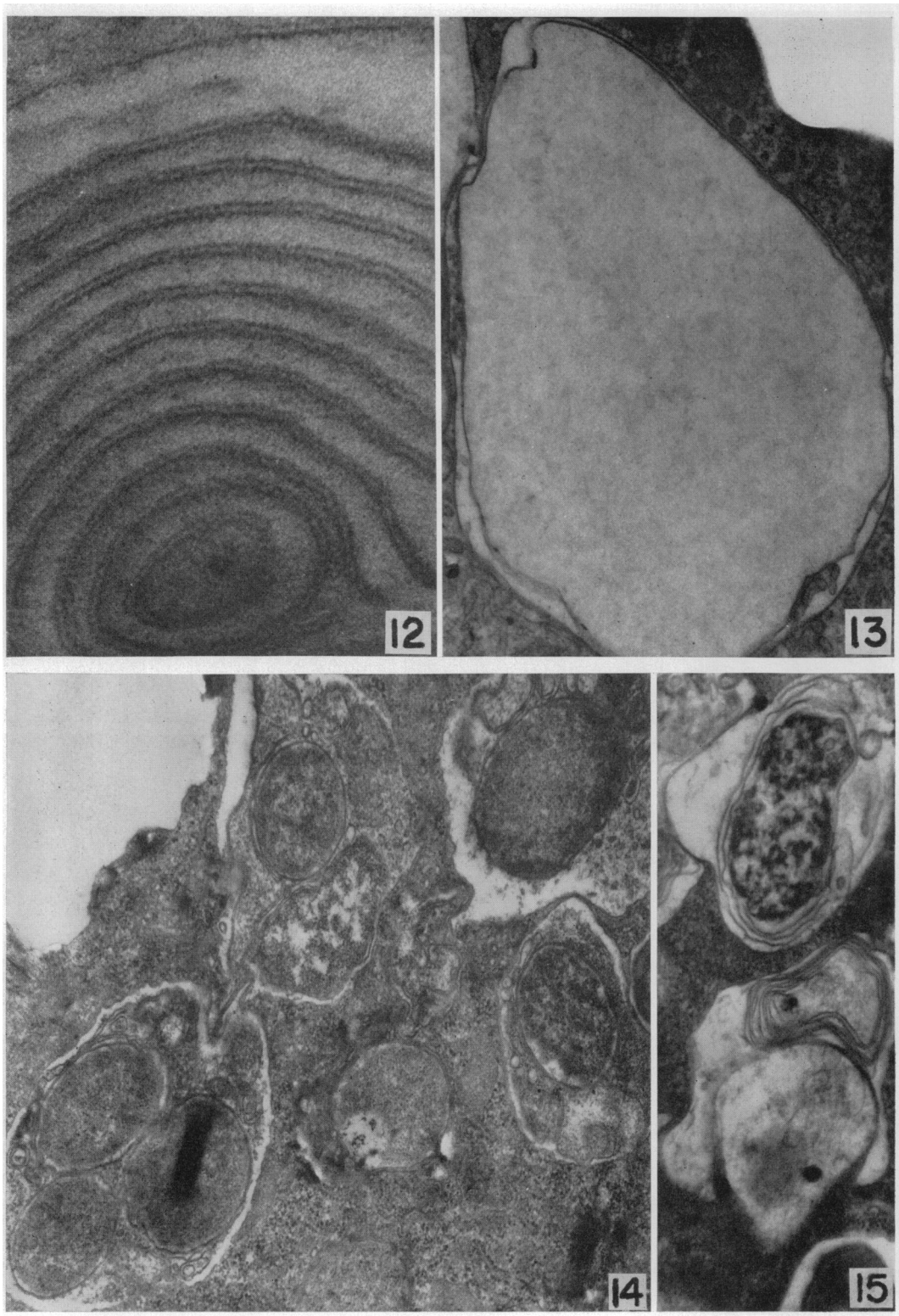


FIG. 12. *Part of Fig. 3 at higher magnification.* × *290,000.*

FIG. 13. *Food vacuole of Polysphondylium pallidum lined with an additional membrane.* × *32,800.*

FIG. 14. *Part of myxamoeba of Polysphondylium pallidum with food vacuoles containing one or more bacteria.* × *46,400.*

FIG. 15. *Food vacuole of Polysphondylium pallidum containing half-digested bacterium surrounded by several membranes.* × *46,400.*

Slautterback (1965) for mitochondrial membranes of heart muscle cells.

Development of lamellar systems. To study the initial formation of the lamellae, myxamoebae of *P. pallidum* were cultivated on the soluble medium. When the cell density reached 10^6 to 2×10^6 myxamoebae per milliliter, a heavy suspension of *E. coli* was added. At different time intervals thereafter, samples of myxamoebae were prepared for electron microscopy.

In myxamoebae grown on the medium alone, very few membranes were found in the food vacuoles. These had a tendency to lie close to the vacuolar membrane. Since cannibalism is not infrequent in these organisms (Huffman and Olive, 1964), the occurrence of these lamellae is not surprising.

Engulfment of bacteria occurs singly or in groups (Fig. 14). The bacteria are either tightly enclosed by the membrane of the food vacuole or are more or less free within a rounded food vacuole. As the first sign of digestion, the bacteria swell up (Fig. 10 and 14). Lysis of the cells is accompanied by a gradual loss of electron-dense material, such as ribosomes. Plasma membrane and cell wall are much more resistant than the cytoplasmic contents and do not seem to be degraded appreciably. During this time of cell degradation, the membranous systems begin to form. Judging from a large number of pictures, the assembling of the membrane material occurs mainly in three ways: (i) formation adjacent to the membrane of food vacuoles; (ii) formation adjacent to bacterial envelopes; or (iii) spontaneous formation from amorphous pools of precursor material.

(i) Whereas in myxamoebae feeding on the soluble medium the membrane of the food vacuole is clearly a single asymmetric unit membrane, upon the addition of bacteria it seems often to consist of two or even more membranes (Fig. 13, 16, and 17). Gezelius (1959) mentioned that the food vacuoles are sometimes lined with a double membrane, sometimes with a single unit membrane. All our observations indicate that these additional membranes are formed on the membrane lining the food vacuole during the digestion of bacteria. The use of ferritin or colloidal gold as electron-dense markers provides some additional evidence: the marker, added to the bacterial suspension upon which the myxamoebae feed, is found preferentially towards the center of the lamellar sets (Fig. 18), indicating that at least some of the lamellae originate from the periphery of the vacuole. The possibility that these membranes originate from repeated shedding of the membrane of the food vacuole can almost certainly be excluded, since the additional membranes are symmetric, in contract with the membrane of the food vacuole, and the same mechanism should work when the myxamoebae grow on the soluble medium. (However, this is not the case, since, under those conditions, membranes are found only rarely.)

(ii) Membranes also appear adjacent to the membranes surrounding the bacterium. Since the cell wall of *E. coli* appears in the electron microscope as a triple-layered structure similar to a unit membrane, it cannot be decided whether the lamellae form initially inside the plasma membrane or on the outside of the cell wall. However, the fact that the sets of concentric lamellae are normally the size of a bacterium indicates that most of the lamellae are produced inside the plasmalemma. Figure 15 shows an early stage of membrane formation. As lysis of the cells proceeds, progressively more lamellae are formed, eventually replacing the whole bacterial cytoplasm. It seems as if each lamella formed mediates the formation of the next lamella.

(iii) Several observations indicate an additional mechanism of membrane formation. First, areas within the food vacuoles give the appearance of intermediate stages in membrane formation. The whole area consists of a mosaic pattern of small pieces of lamellae intermingled with amorphous material. The short pieces of lamellae are arranged in a way already suggesting the outline of the future lamellae (Fig. 3, 19, and 21). The point here is that a whole area of precursor material seems to be transformed spontaneously and simultaneously into a set of lamellae. This would be in contrast with the previous two cases, where the lamellae are formed progressively or stepwise.

The second observation, pointing in the same direction, refers to the honeycomb pattern described earlier. If, on a water surface, standing waves from two different sources meet, an interference pattern develops that resembles the arrangement observed in Fig. 5, 6, and 7. This would indicate, in our case, that the impulse for lamella formation has started at two points and that the pool of precursor material is under the influence of the two sources while the membranes are being formed. In other words, the molecules in the interference area arrange themselves according to the pattern of forces created by the two sources, the resulting membranes thus being their visible expression. A mechanism assuming a stepwise formation of lamellae could hardly account for a phenomenon of this type.

Figure 23 summarizes the development of the membrane complexes as suggested by our observations. The diagram does not include the possibility of growth of these membranes by intus-

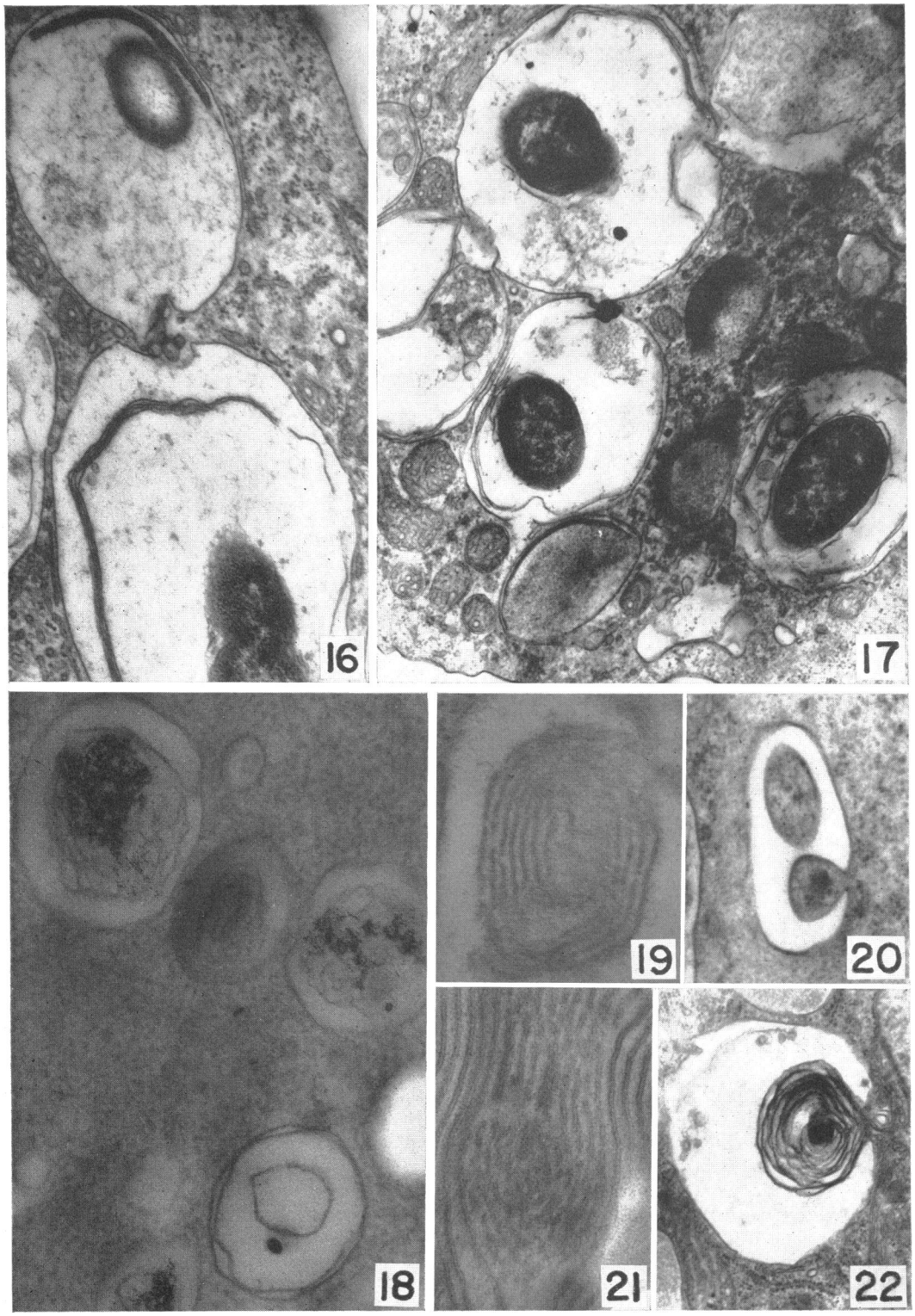


FIG. 16–17. *One to several membranes lining food vacuoles of Polysphondylium pallidum containing ingested bacteria.* × *46,400 and* × *20,800, respectively.*

FIG. 18. *Food vacuoles of Dictyostelium discoideum containing membranes and ingested ferritin.* × *58,400.*

FIG. 19. *Set of incomplete membranes in food vacuole of Dictyostelium discoideum, interpreted as intermediate stage of membrane formation.* × *72,500.*

FIG. 20. *Cytoplasmic protrusion into food vacuole of Polysphondylium pallidum.* × *43,500.*

FIG. 21. *Structures similar to those in Fig. 19.* × *116,000.*

FIG. 22. *Lamellar protrusion into food vacuole of Polysphondylium pallidum.* × *24,500.*

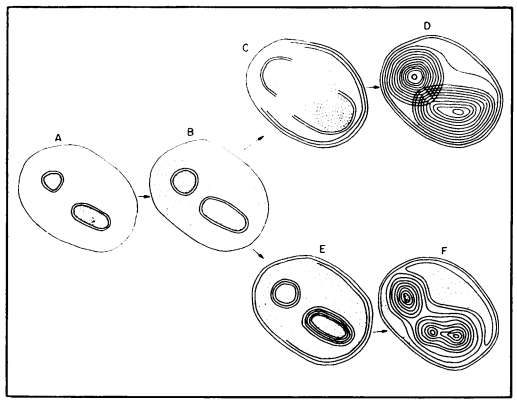


FIG. 23. *Diagram of proposed membrane formation during digestion of bacteria in food vacuoles of cellular slime molds. Each line represents a unit membrane. The sequence starts with the presence of bacteria in the food vacuole (stage A). The bacteria swell up and begin to lyse, releasing breakdown products (stage B). Subsequent membrane formation, most probably using the breakdown products of the bacteria as precursor material, proceeds in at least two ways, depicted as C–D and E–F, respectively. In C–D, many lamellae form spontaneously and simultaneously; in E–F, lamellae are formed in sequence through template action of pre-existing membranes, as described in the text.*

susception, that is, enlargement by incorporating membrane material into existing membranes. Although this might occur, we have no clear indications of it.

Other observations on the process of digestion. The process of engulfment and ingestion of food material does not seem to be extremely specific, since cannibalism is quite frequently observed (Huffman and Olive, 1964). So far, we have considered only the intake of *E. coli*. If myxamoebae are engulfed, the process of digestion is similar at the beginning. The cytoplasmic matrix with the ribosomes is degraded, whereas the membranous elements of the cell, e.g., the plasmalemma, the mitochondria, and also the concentric rings within the food vacuoles of the engulfed myxamoeba, are fairly well preserved (Fig. 4). The sets of concentric lamellae characteristic for the digestion of the bacteria have not been observed to form during digestion of myxamoebae.

The subsequent fate of the degraded, ingestible material in the food vacuole is obscure. Few morphological observations were made that might provide some clues.

Sometimes an invagination of the cytoplasm into the food vacuole has been observed. This invagination can be either plain groundplasm or may be more lamellar in nature (Fig. 20, 22). On other occasions, a zone of cytoplasm clear of ribosomes surrounds the food vacuole (Fig. 4). Strands of the endoplasmic reticulum or other membranes might parallel the membrane of the food vacuole, but this, too, does not occur frequently enough to be significant. There are no obvious groups of small vesicles around the food vacuole to suggest a process of micropinocytosis. Small breaks in the membrane of the food vacuole and in other membranes, as reported by Gezelius (1961), were observed but are considered artifacts of fixation.

DISCUSSION

Digestion of *E. coli* by the myxamoebae of *D. discoideum* and *P. pallidum* begins with a swelling of the bacterial cell, followed by gradual lysis of the cytoplasmic contents. Fragmentation of the cell does not seem to occur in *E. coli*, but has been observed during digestion of the much larger *Bacillus megaterium* by Raper (1937). Although the bacterial cell wall can break open, it seems to be most resistant to the digestive process and retains its morphological distinctiveness. These observations are substantiated by Rosen, Rosen, and Horecker (1965), who found that the polysaccharide component of the cell-wall lipopolysaccharide of *Salmonella typhimurium* is not significantly altered by *P. pallidum* and is excreted quantitatively into the culture medium.

Two fractions result from the digestive process: a digestible and an undigestible one. The subsequent fate of the digestible material is hard to trace; very few morphological peculiarities have been observed that might be related to the uptake of the digested products into the host cytoplasm. It seems most likely that the material permeates the intact membrane of the food vacuole, since optimally preserved cells show few openings in the membrane, and these are most probably artifacts. The halo deprived of ribosomes observed around the food vacuole might be due to the large amount of digested material entering the cell cytoplasm; it could also be due to accumulation of digestive enzymes. The role of the cytoplasmic intrusions into the food vacuoles might be placed in the area of resorption, but here again we know very little, since tracers such as ferritin and colloidal gold could not be followed into the cell cytoplasm.

Of the material that is not digested by the myxamoebae, the lamellar systems described in detail are by far the most conspicuous ones. Their presence in large amounts is surprising, since they seem to be lost for any nutritional purposes and are excreted into the surrounding medium. Thus, a large amount of the material taken in by the

myxamoebae is wasted, which seems to render the digestive process fairly inefficient.

These sets of concentric lamellae are not restricted to the cellular slime molds, but have been observed in the plasmodial slime molds (Schuster, 1964) and a variety of other cells. Focal degradation in mammalian cells, as described by Swift and Hruban (1964), leads to formation of somewhat similar membranous bodies. Thus, the phenomenon is widespread among biological systems.

In our system, a pool of precursor material is formed by the digestion of the bacterial cytoplasm. This step is followed by the assembling of the material into membranes. Membranous material was only rarely observed in food vacuoles of myxamoebae growing in the absence of bacteria. Thus the composition of the bacteria, particularly the lipid components, must play an important role. Apart from the bacteria, precursor material could possibly be provided by the host cell, e.g., in the form of digestive enzymes. However, no evidence for this has been obtained so far.

The membranes formed have a three-layered configuration typical for unit membranes. From this it is concluded that most probably they are built from lipoproteins. They are symmetrical in nature, the bimolecular lipid leaflet being lined on either side by a protein layer of identical thickness. Our observations suggest two main ways of membrane assembly: (i) the membranes form adjacent to pre-existing membranes, such as the membrane lining the food vacuole and the cell wall of the bacterium; and (ii) the membranes form spontaneously from a pool of precursor material. Spontaneous formation of membranous material from mixtures of lipids or mixtures of lipids and proteins have been studied intensively (Stoeckenius, 1962; Luzzati and Husson, 1962). Little attention has been given to a possible role of pre-existing structures in determining the configuration of newly forming membranes.

Our results suggest that, given a membrane and a pool of precursor material, the new membrane tends to be formed through mediation of the preformed membrane. In other words, the preformed membrane serves as a pattern or template for the precursor material, the newly formed membrane lying parallel to it and taking up the same shape. The term "template" is used here in a wide sense, such that the resulting new membrane would not have to be a copy of the old one. That this is the case in our system is obvious from the fact that the membrane lining the food vacuole is strongly asymmetric, in contrast with the symmetrical membranes forming upon it. It is reasonable to assume that the rather unspecific template action is due to preferential adsorption of the hydrophilic parts of the lipoproteins by the protein layer of the pre-existing membrane. The hydrophobic ends of the lipoproteins would thus point away from the pre-existing membrane, thereby attracting hydrophobic ends of lipoproteins from the pool, and so on. This process, repeating itself, eventually leads to the formation of the lamellar stacks. This mechanism could explain the complex patterns observed: the set of concentric lamellae reflecting the form of the bacterial envelope, and the lamellae surrounding several sets reflecting the form of the membrane lining the food vacuole.

Morré and Mollenhauer (1964) found sets of concentric lamellae in isolated Golgi apparatus from plant cells, and Mercer (1962) discussed the possibility that the Golgi body in the cells of the ovotestis of the snail might arise from sets of concentric lamellae formed from lipoid droplets. The sets of concentric lamellae would then break up, eventually leading to the well-known arrangement of stacked lamellae in a Golgi body. The relation between this example and the mechanism discussed for the formation of the membranes in the food vacuoles is obvious. The question is, does this mechanism have much wider application, and should it be considered in other cases where stacks of lamellae develop? The idea is not entirely new. Waddington (1962), including earlier suggestions by Rebhun (1956) and by Swift (1956), discussed the possibility that stacks of annulate lamellae in various kinds of cells are formed by some type of copying mechanism from the nuclear envelope, since they bear strong resemblance to it.

The idea that membranes can be formed from a pool of precursor material through template action by pre-existing membranes might, in some cases, be a fruitful alternative to the prevailing view that membranes form by fusion of vesicles lined with preformed membranes (Blondel and Turian, 1960; Humphreys, 1964; Kessel, 1963; Schuster, 1964).

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