INTRACELLULAR CENTRIFUGAL SEPARATION OF ORGANELLES IN *PHYCOMYCES*

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ABSTRACT

Live sporangiophores of *Phycomyces blakesleeanus* were centrifuged at 35,000 rpm. The cell contents sedimented into distinct layers, and each layer was studied with an electron microscope and with cytochemical methods. The following layers were found (their volumes and their densities are shown in Fig. 3): 1. polyphosphates; 2. polyphosphates and protein crystals; 3. glycogen; 4. yellow layer with ferritin; 5. ribosomes; 6. protein crystals; 7. mitochondria; 8. mitochondria and fibrils; 9. nuclei; 10. endoplasmic reticulum; 11. vesicles, membranes, and reticulum; 12. vacuole; 13. lipoproteins, membranes; 14. fat droplet. The densities of the various layers were determined by the injection of droplets of inert oils of known density into the sporangiosphores before centrifugation. Sedimented cell organelles could be isolated. Centrifuged nuclei of a lycopene-producing mutant were injected into the intact sporangiophore of an albino host where they induced color formation. The ensuing spores, when plated, gave a mixture of white and colored colonies. It was concluded that cell organelles, sedimented by centrifugation of living sporangiophores, remain alive and can be used for biochemical studies. Microspectrophotometric examination of the layers indicated the presence of cytochromes and flavines in the mitochondria and of cytochromes in the nuclei. No pigments corresponding to the action spectrum for the light growth response were found.

INTRODUCTION

Much of the progress of molecular biology is based on studies of cell organelles isolated from homogenized cell preparations. No matter how "gentle" the disruption of the cell, one cannot expect that isolated particles are physiologically completely equivalent to organelles in the living cell. Although the isolated particles continue to perform many vital functions in vitro, their rates of activities are often only a fraction of those of the organelles in undisturbed cells. Also, it is possible that some of the activities are changed qualitatively by isolation. The damage done in isolating particles may be irreversible, and there is no proof that these particles can function normally if reintroduced into a living cell.

It has been often shown that one could sediment cell organelles inside living cells by high centrifugal forces and that the cells continued to function normally after the organelles were redistributed in the cytoplasm (Beams and King, 1939; Zalokar, 1959). One can, therefore, expect that cell organelles, displaced by the centrifugation of living cells, retain all their vital capacities, although their functions may be temporarily modified because the organelles are separated from each other. If one could isolate and recombine different cell organelles without actually killing the cell, one might learn something of their interactions and of their dependence on the normal structural organization of the cell. Studies of such organelles could also

reinforce or modify our knowledge obtained from conventional methods of molecular biology.

Sporangiophores of *Phycomyces blakesleeanus* are particularly well suited for the centrifugal separation of cell constituents inside the living cell. They are large coenocytic cells of cylindrical shape, tapered at the end, and enclosed by a relatively rigid cell wall, which, when properly mounted, can function as a minute centrifuge tube. In this paper, we present experiments which show that cell organelles can be separated by centrifugation within living sporangiophores and that they remain functional. We will also characterize the organelles morphologically and biochemically and measure their total amounts and their densities. Since *Phycomyces* is widely used in the research on phototropism,[1] our particular concern was to identify particles which may contain the photoreceptor pigments.

MATERIALS AND METHODS

Wild type of *Phycomyces blakesleeanus* was used. The fungus was grown on potato dextrose agar medium (Zankel et al., 1967) at 25°C and sporangiophores (spphs) were harvested when 1–2 cm long. Cultures were illuminated from above to assure straight growth of the spphs. Stage I spphs (Castle, 1942) were plucked from the mycelium and centrifuged in an inverted position. Since the cell wall is not strong enough to withstand high centrifugal forces, the spph had to be supported within the centrifuge tube by a liquid the density of which was similar to that of the spph. At the centrifugal end, a denser liquid functioned as a cushion to prevent the spph from being squashed against the bottom of the tube (Fig. 1 *A*). High centrifugal forces were obtained with a Spinco Model A centrifuge and swinging bucket rotor SW 39. Sporangiophores were mounted in capillary tubes, 1 mm × 4 cm, containing 40% Ficoll (Pharmacia Inc., Uppsala, Sweden) at the bottom (first 10 mm) and filled with 10% Ficoll. The capillary tube was placed in a 1.5-mm-wide axial well of a plexiglas insert for regular Spinco centrifuge tubes. Heavy fluorochemical 3M Company Kel-F3 (Minnesota Mining and Manufacturing Co., Minneapolis) was introduced into the well for supporting the capillary and preventing breakage at high speeds.

The cell contents were stratified best when the centrifugation speed was increased slowly and kept at 25,000 rpm for 10 min and then raised to 35,000 rpm for 20 min. Smaller centrifuge forces were not sufficient to separate all the particles, and a longer centrifugation time or higher forces did not yield significant improvement. The centrifuge temperature was 5°C. At the end of the centrifugation, the centrifuge was braked to a stop; the capillary tubes were removed from the containers and inspected under a dissecting microscope. For better observation, the capillaries were broken at an appropriate level and the specimens were removed and mounted in water on microscope slides. Coverslips, supported with spacers, prevented crushing the specimen. Centrifuged layers were sufficiently stable to remain in place during a short microscopic observation even when the spph was in a horizontal position.

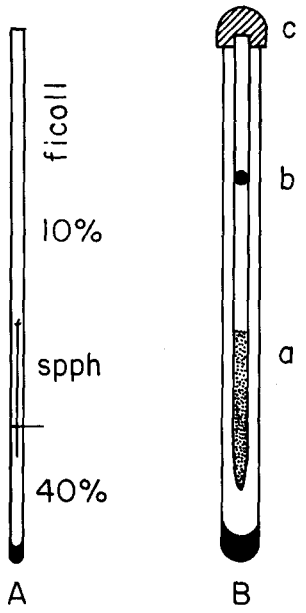

FIGURE 1 *A*, Spph mounted in a capillary for centrifugation (× 1.2). *B*, Centrifuged spph (× 6) (*a*), with injected oil droplet (*b*), mounted in a small capillary, and sealed with wax (*c*).

Cytological and cytochemical studies of spphs are rendered difficult by the low permeability of the cell wall. Added chemicals or fixatives penetrate slowly and unevenly into the cell, causing displacements and contraction of the cytoplasm. Centrifuged layers can be disorganized easily by fixation. The growing tip of the spph is more permeable, and chemicals may reach reaction sites in the cytoplasm only in its vicinity, thus falsely indicating localized reactions. For cytological staining and some cytochemical reactions, spphs were fixed with formol-acetic acid-alcohol, or acetic acid-alcohol alone. For electron microscopy, glutaraldehyde (5% in 0.1 M phosphate buffer at pH 6.8) gave good results. As good or better results could be ob-

[1] K. Bergman, P. V. Burke, E. Cerdá-Olmedo, C. N. David, M. Delbrück, D. S. Dennison, K. Foster, E. W. Goodell, M. Heisenberg, G. Meissner, W. Shropshire, and M. Zalokar, 1969. *Bact. Rev.* Data to be published.

tained with 5% glutaraldehyde and 5% acrolein in 0.1 M cacodylate buffer at pH 7.0 (F. A. Burr, personal communication). The fixed specimens were posttreated with 1% OsO_4, dehydrated in alcohol, transferred to propylene oxide, and embedded in Vestopal. The transfer from propylene oxide to Vestopal was made by dissolving Vestopal (with initiator and activator added) in propylene oxide and evaporating the solvent slowly in a dry atmosphere. Blocks were properly oriented, and thin sections were made with an LKB microtome. The sections were stained with uranyl acetate and lead citrate and were studied with a Phillips (EM 200) electron microscope, operated at 60 kv.

Various substances can be injected into spphs after centrifugation. The normal spph loses its turgor and collapses as soon as an opening is made in its wall, unless special precautions are taken. For protection during manipulation, the spph is introduced, tip first, into a capillary filled with 20% Ficoll. This capillary is closed at one end and has an inside diameter of about 200 μ (Fig. 1 *B*). All operations are performed under water. The protruding part of the spph is cut with a sharp razor. A micro-injection needle, about 10 μ in diameter, is introduced through the opening into the vacuole, and an appropriate amount of material is injected. The spph is then pushed farther into the capillary, so that the protruding end is about 200 μ long. The capillary is then taken out of the water, placed in a vertical position, and the open end is sealed with a droplet of molten beeswax. The wax seals the spph and keeps it attached to the rim of the capillary. This capillary is then introduced into a 1-mm × 4-cm tube filled with paraffin oil and is centrifuged. Spphs prepared in this way can withstand centrifugal forces up to 25,000 rpm. For most work, the spph was first centrifuged at 35,000 rpm for 20 min, then injected according to this procedure, and centrifuged again at 25,000 rpm for 20 min.

RESULTS

Description of the Layers

When a stage I spph is centrifuged for 10 min at 25,000 rpm and for 20 min at 35,000 rpm, its contents sediment in several well defined layers (Fig. 2). Typical results for a 1-cm spph grown under optimal conditions are presented in Fig. 3. Starting from the centrifugal end, the following layers were observed:

1. Polyphosphates (Fig. 4). The heaviest material settles to the bottom in the form of large droplets, confluent at the tip into a colorless mass, not revealing any structure. In the electron microscope, one sees that the droplets are enclosed by a simple membrane, taking up stain avidly. At least two types of droplets are present: one is more homogeneous, with stained material aggregating in the center; the other is coarsely reticulated. The interstitial material is composed of barely percepti-

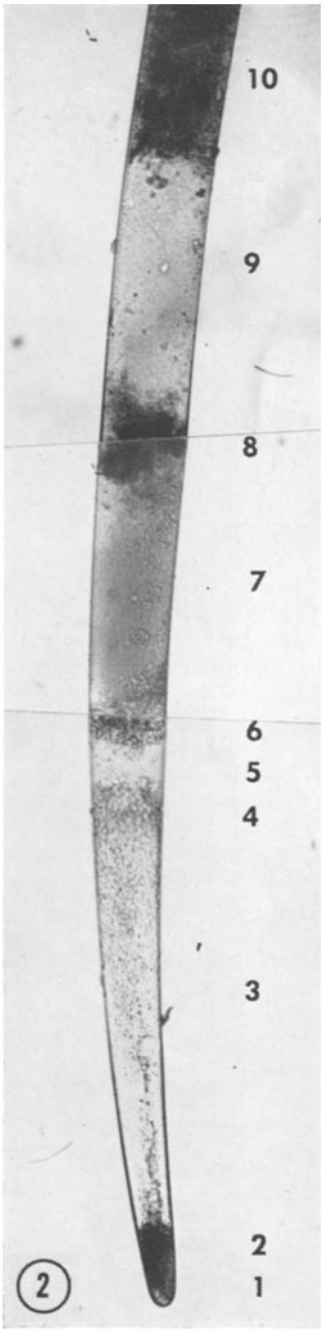

FIGURE 2 Photograph of a centrifuged spph. Numbers corresponding to the ones in the text identify each layer.

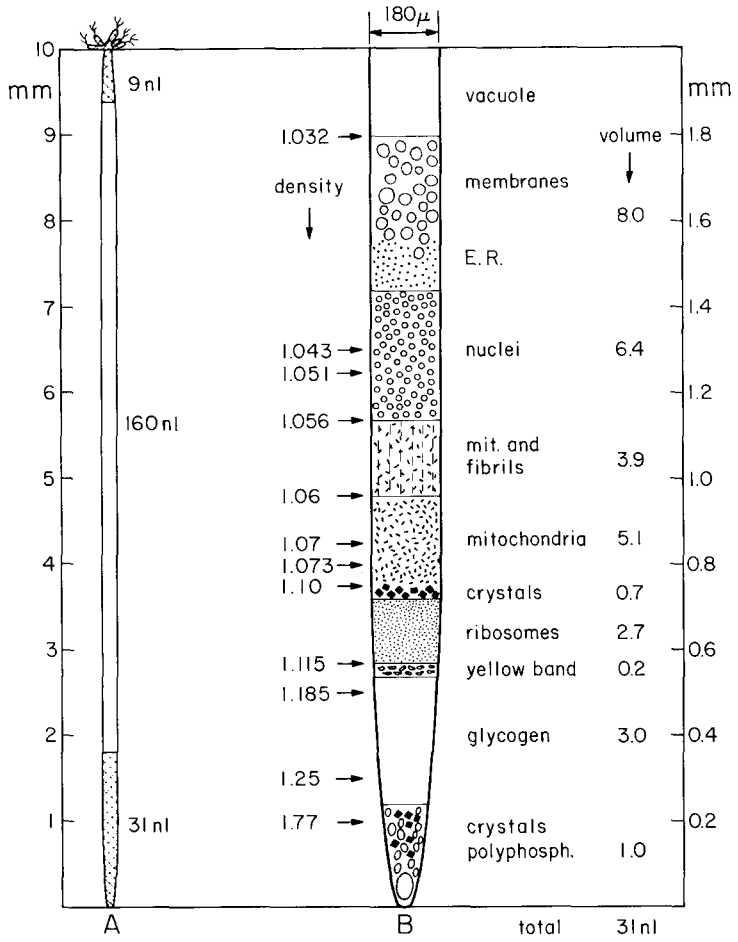

FIGURE 3 Drawing of a centrifuged spph with layers and their volume given at the right and specific gravity determined by oil drops at the left. The arrows indicate the position that oil drops took after centrifugation.

ble fibrils. Cytochemical tests indicate that this layer contains polyphosphates. The layer stains heterochromatically with toluidine blue, turns black after reaction with lead acetate and hydrogen sulfide, and gives a faintly positive molybdate reaction for phosphates. It could not be determined which of the three types of sedimented materials correspond to polyphosphates. The droplets give only very weak reactions for carbohydrates by periodic acid–Schiff reaction and for proteins (basophilic cytological stains).

2. Polyphosphates and crystals (Fig. 5). The first layer changes gradually into the dark second layer. The droplets described above become smaller and are intermixed with rhombohedral protein crystals. In electron micrographs, these crystals display a regular lattice pattern (Fig. 6). The crystals were first described by Thornton and Thimann (1964).

3. Glycogen (Figs. 7 and 8). This layer appears transparent and structureless in a microscope, and has a bluish cast in reflected light. It stains reddish-brown with iodine and gives a positive periodic acid–Schiff reaction, both characteristic for fungal glycogen. Electron micrographs show tightly packed granules about 800 A in diameter (Fig. 7). It is very difficult to make thin sections of the glycogen layer, since glycogen swells and prevents the sections from spreading properly. In most sections, the glycogen must have been dissolved, leaving the corresponding empty spaces unstained with lead citrate (Fig. 8).

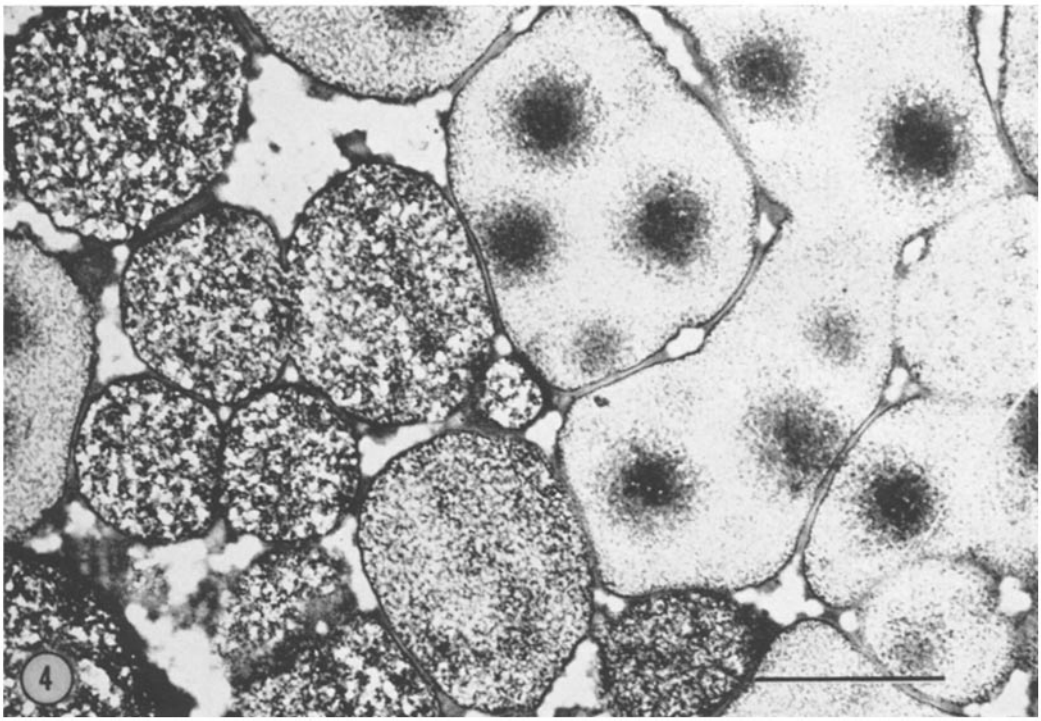

FIGURE 4 Polyphosphate layer. Two types of droplets, one with coarsely reticulate structure, the other with spotty concentration of stained material. Both aspects are probably artifacts of fixation. Interstitial material is not very distinct in this micrograph. × 25,000.
(The bar indicates 1 μ, except in Figs. 6, 10–12, and 17 where it is 0.1 μ.)

4. Yellow layer (Figs. 9 and 10). An intensely yellow narrow band separates the glycogen from the ribosomes. Under the microscope it appears to be composed of irregular granules, many of which are at the limit of visibility. At high resolution one sees a mixture of glycogen and ribosomes, some small vesicles, and a large quantity of ferritin-containing particles. On these particles, ferritin molecules occur in two-dimensional arrays as on the surface of lipid droplets (David and Easterbrook, personal communication.). However, these arrays are irregularly folded, as if they were attached to crumpled patches of membranes. No membranes could be resolved in the electron microscope. The particles were probably stripped from the surface of the fat droplets and centrifuged to a layer of appropriate density. Small vesicles mixed with this layer are probably the autophagic vesicles described by Thornton (1968).

5. Ribosomes (Fig. 11). This is a very well defined brownish, translucent layer. Electron microscopy reveals tightly packed ribosomes, completely devoid of membranes. Polysomes, if any, cannot be discerned because of the packing. A few ferritin-bearing vesicles are scattered throughout the layer. The layer takes up basic stains (acridine orange, toluidine blue) which indicate the presence of nucleic acids.

6. Crystals (Fig. 12). This layer contains mainly rhombohedral crystals, similar to the ones in layer 2, embedded in a ribosomal matrix. The crystals are of various shapes and sizes and correspond to the crystals found in the vacuole of normal spphs. Their crystal lattice pattern is less conspicuous than that of the crystals at the centrifugal end. The crystals give a positive reaction for proteins (acidic cytological stains) and a negative one for carbohydrates. Mixed with the crystals are dense, often kidney-shaped bodies, corresponding to those that Thornton (1968) found in the cytoplasm and inside autophagic vesicles.

7. Mitochondria (Fig. 13). The layer is brownish and finely granular. Electron micrographs show loosely packed, typical mitochondria, pre-

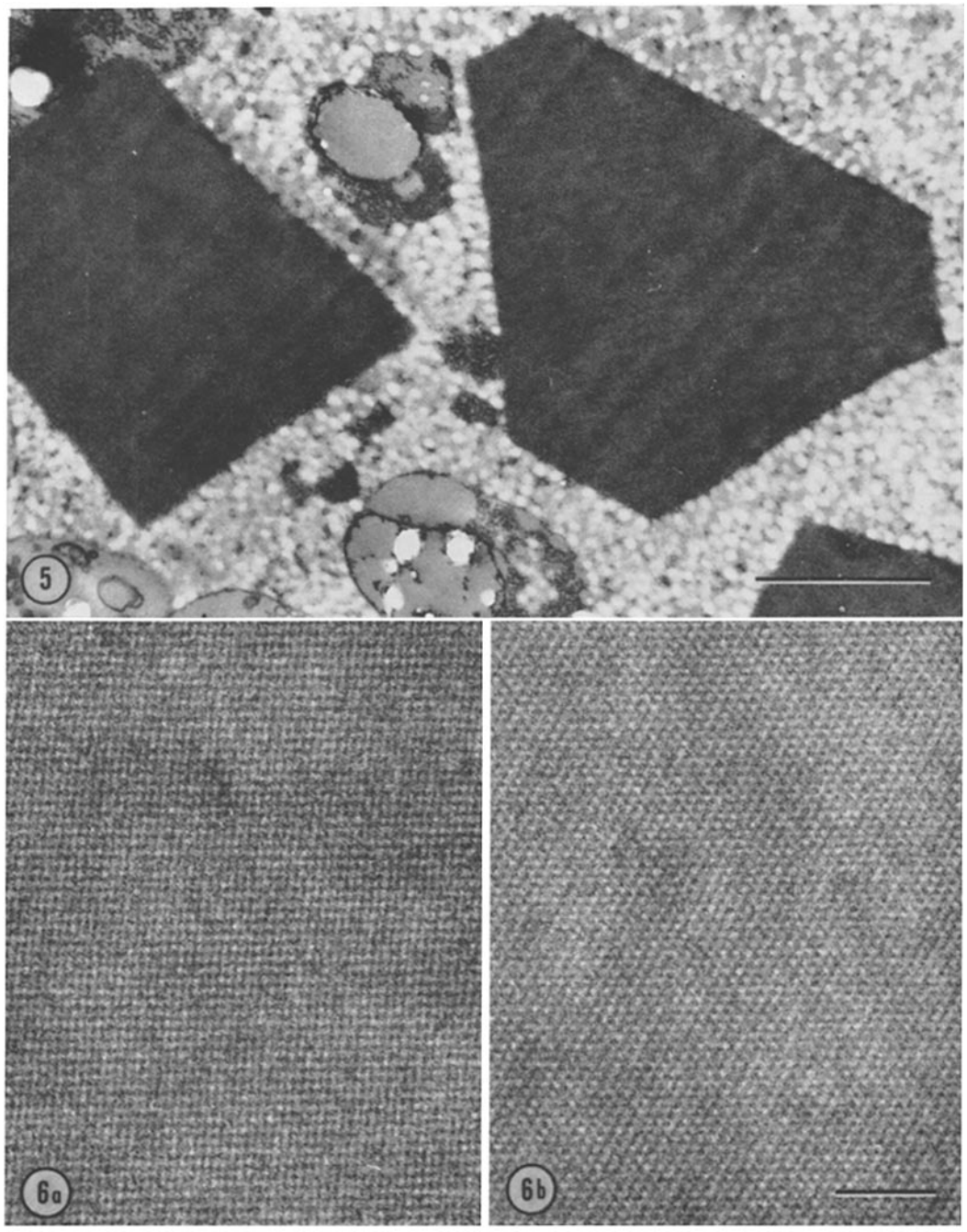

FIGURE 5 Polyphosphate droplets and crystals with background of glycogen. The two crystals were sectioned at different angles. × 25,000.

FIGURE 6 Crystal lattices of the left (*a*) and the right (*b*) crystals shown in the previous micrograph. Lattice periods at the left are 90 and 100 A. × 147,000.

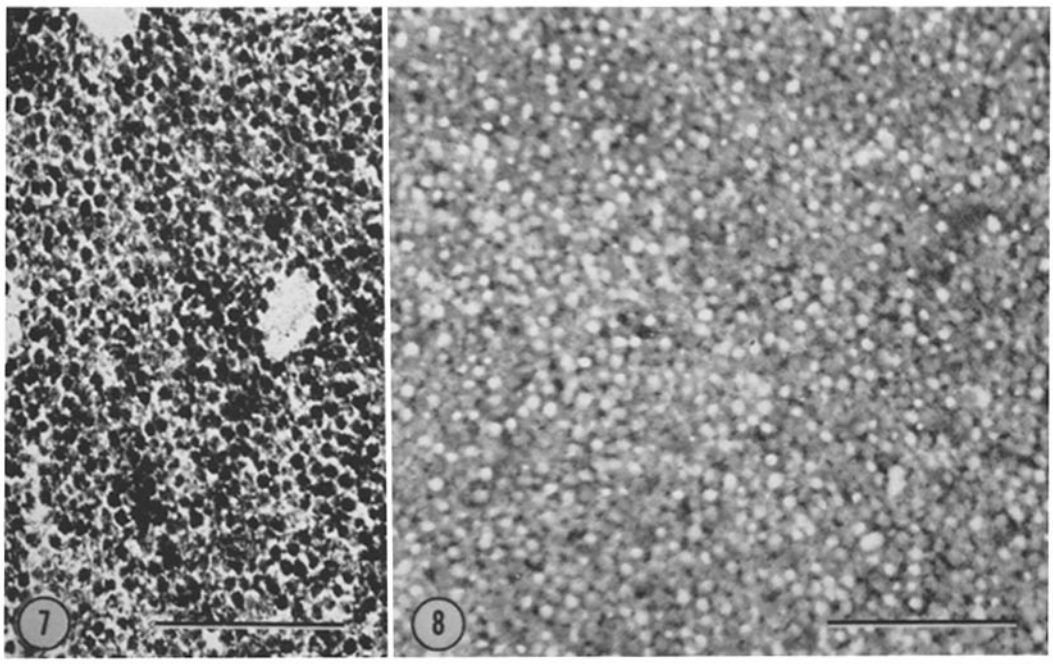

FIGURE 7 Glycogen granules stained with lead citrate. × 25,000.

FIGURE 8 Glycogen layer showing empty emplacements of glycogen granules. × 25,000.

dominantly circular or oval, with well developed cristae. The spaces between the mitochondria are filled with granules which, owing to their size and appearance, can be identified as ribosomes. Some ferritin-bearing particles are scattered between the mitochondria, but there are no other types of particles and no nuclei. The mitochondrial layer gives a positive Nadi reaction and it reduces tetrazolium salts, indicating active cytochrome oxidase and succinic dehydrogenase systems.

8. Mitochondria and fibrils. The upper portion of the mitochondrial layer is less homogeneous. It contains some opaque vesicles and an indication of fibrils parallel to the longitudinal axis. Distinct fibrils are not seen in the electron microscope, but they may have been poorly fixed. There are, however, elongated vesicles, probably corresponding to the cytoplasmic strands which support cytoplasmic streaming. Some of the irregular vesicles in this layer resemble the autophagic vesicles in the micrographs published by Thornton (1968). The upper end of the layer is a zone of transition to the next layer.

9. Nuclei (Fig. 14). This layer contains uniformly packed nuclei, approximately 2 μ in diameter. Electron micrographs show loosely packed nuclei with their nucleoli randomly oriented. One would expect the denser nucleolus to be at the centrifugal end of the nucleus, but it is possible that the nuclear layer was strongly disturbed by penetrating fixatives. Interstices between the nuclei are occupied by small vesicles of endoplasmic reticulum. Very few, if any, mitochondria were found in this layer. The layer gives a strong positive Feulgen reaction and a negative test for succinic dehydrogenase.

10. Endoplasmic reticulum (Fig. 15). On top of the nuclear layer, a layer of finely granular material often separates. It exhibits brownian motion. Electron micrographs show vesicles of the same kind as those which fill the interstices between the nuclei, and which are considered to be endoplasmic reticulum. The layer was not observed under high enough power to see the fine structure of the membranes enclosing the vesicles.

11. Membranes and vesicles (Fig. 16). This layer is composed of various vesicles and clumps of cytoplasm, often containing material stained with carotenoids, and is difficult to characterize. Electron microscopy reveals many irregular large

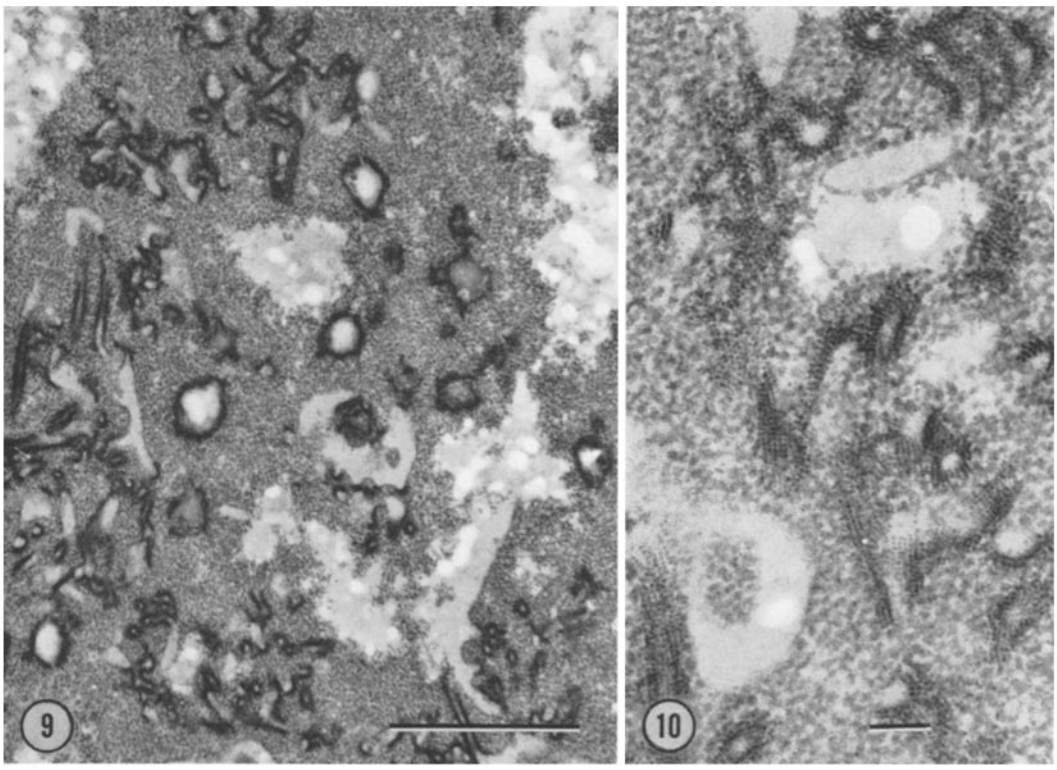

FIGURE 9 Yellow layer with electron-opaque ferritin particles, embedded in ribosomes (gray area) and glycogen (clear area). × 25,000.

FIGURE 10 Ferritin of yellow layer at high power. Ferritin molecules in rows measure 90 A. × 86,000.

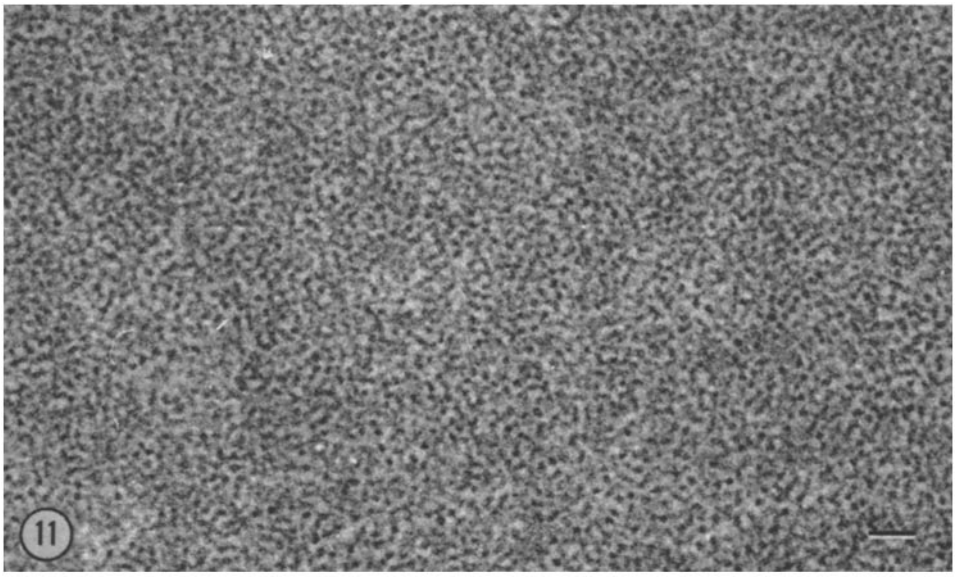

FIGURE 11 Ribosomes, showing uniformity of the layer. Approximate size of granules, 180–220 A. × 59,500.

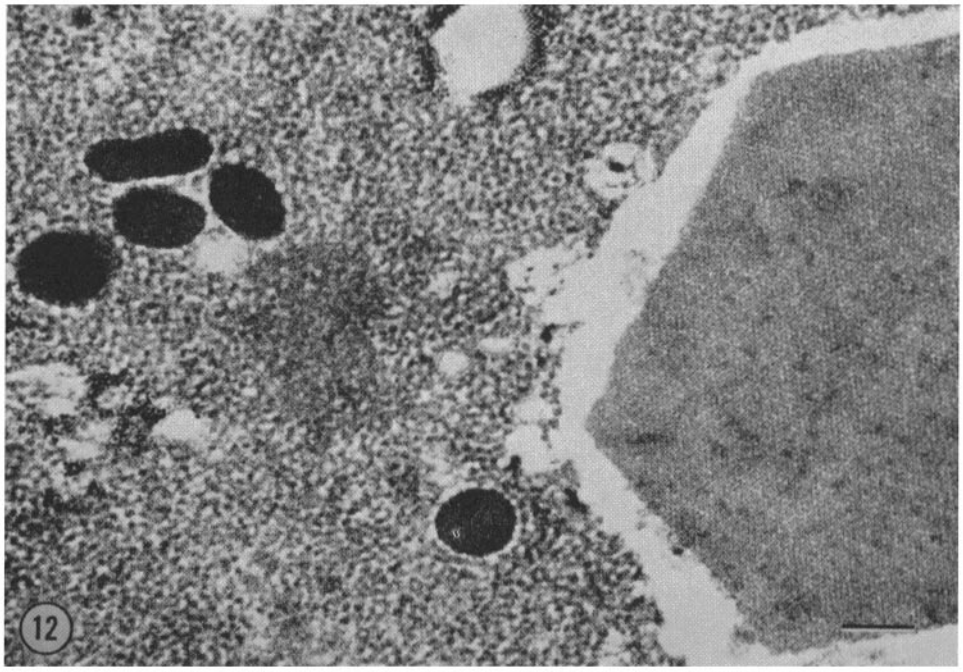

FIGURE 12 Crystals and dense bodies embedded in ribosomes; ferritin-bearing particle above. × 86,000.

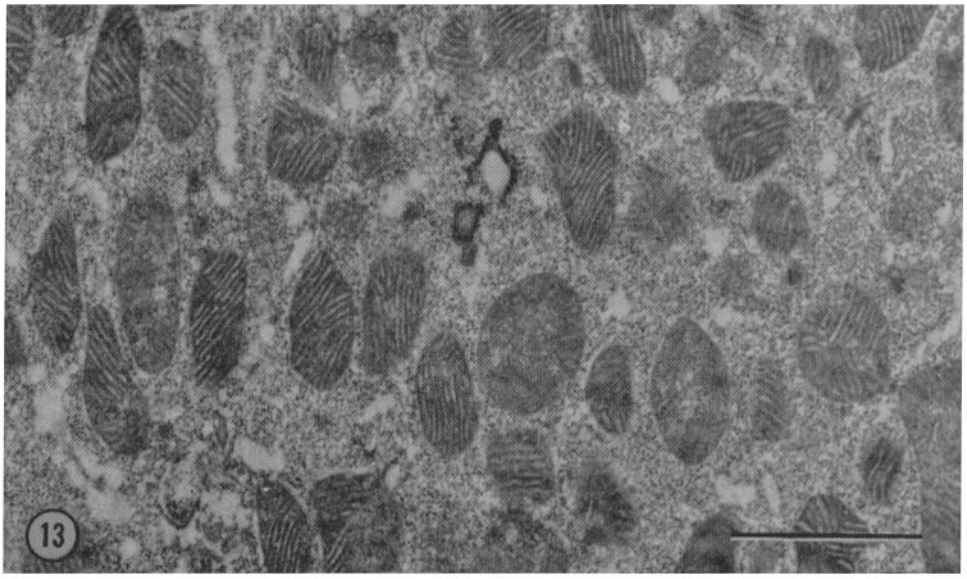

FIGURE 13 Mitochondria with ribosomes in the interstices. × 25,000.

vesicles with conspicuous unit membranes. Some of these vesicles probably belong to the vacuome, while some of them may be autophagic vesicles. Many smaller vesicles have membranes covered with granules, resembling ribosomes, and may be ergastoplasm.

12. Vacuole. The major portion of the centrifuged spphs is occupied by the vacuole, which ap-

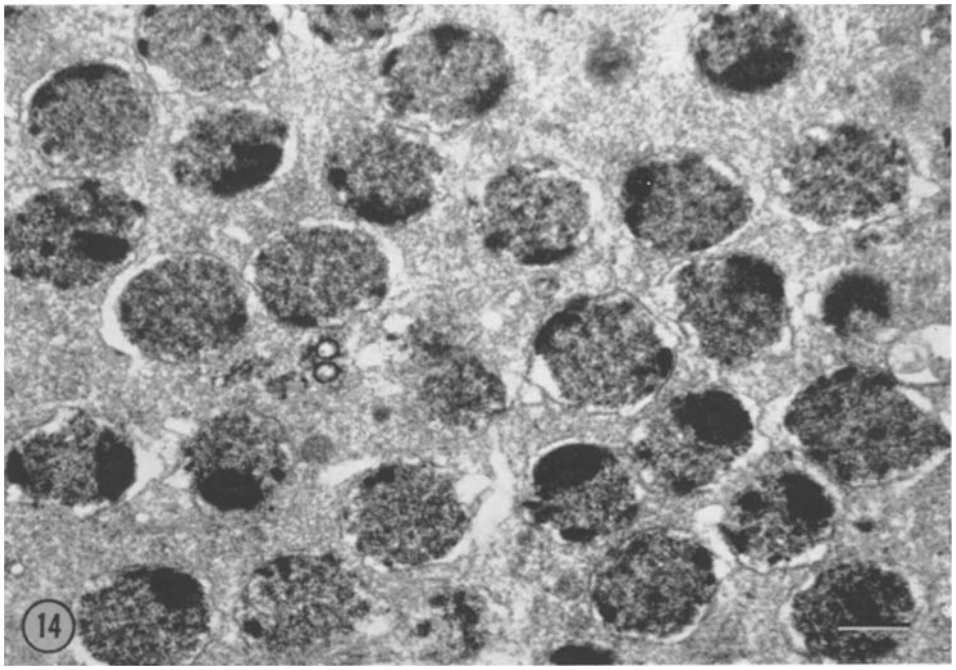

FIGURE 14 Nuclei with dark chromocenters and nucleoli. Diameter is 1.5–2 μ. × 9450.

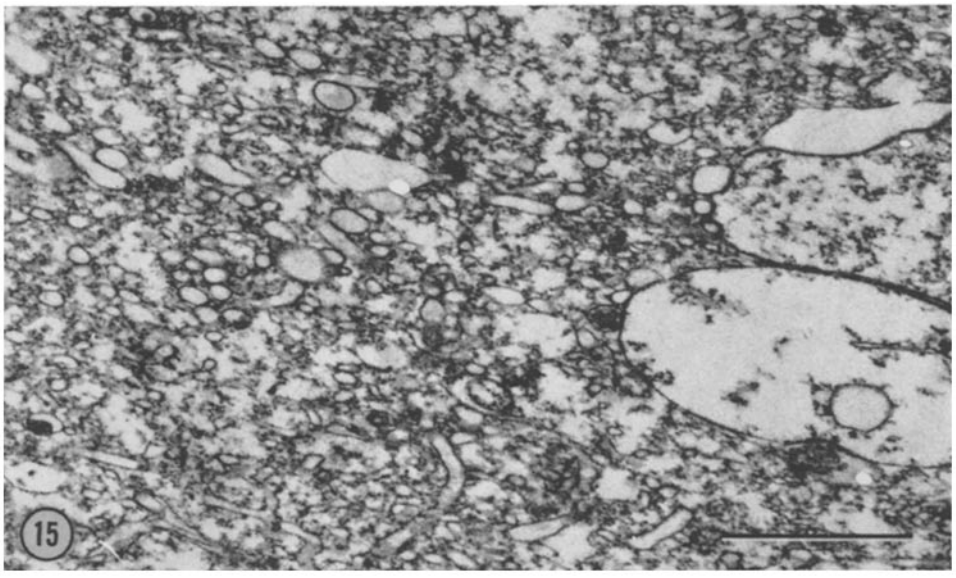

FIGURE 15 Endoplasmic reticulum composed of vesicles of various sizes, oriented slightly in the axial direction. Larger vesicles are probably autophagic vesicles. × 25,000.

pears optically empty, except for scattered granules in Brownian motion. Fixation produces a noticeable deposit of coagulated material, which was probably colloidally dispersed in the vacuole. The vacuole also contains gallic acid which can be detected with ferric chloride.

13. Lipoproteins (Fig. 17). Material which is less dense than the vacuole accumulates at the

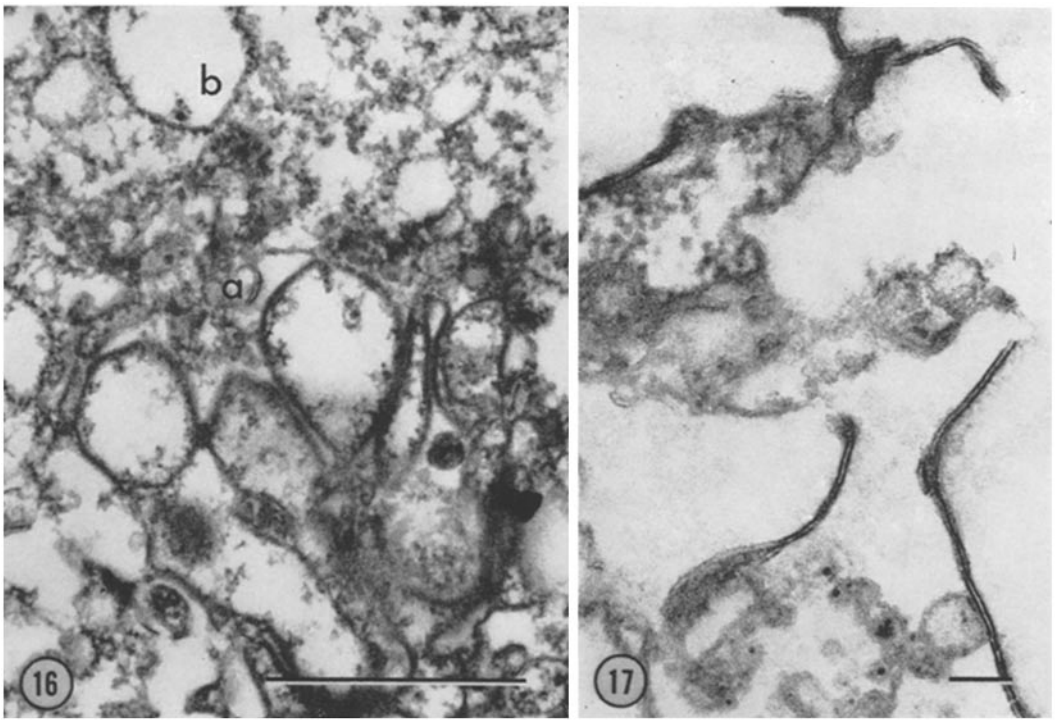

FIGURE 16 Vesicles with unit membranes (*a*) and ribosome-like particles (*b*). × 35,000.

FIGURE 17 The lipoprotein layer is composed of large irregular vesicles enclosed in unit membranes about 100 A wide. × 86,000.

centripetal end of the spph. This material can be observed best if the spph is centrifuged with its tip at the centripetal end. Below a terminal oil droplet, one finds a dark brown oblong plug containing droplets of oil stained with carotenoids. Many cross-sections of membranes are seen in electron micrographs, so that this layer can be interpreted as an accumulation of lipid-containing cell membranes. Near the centripetal end of this layer, there are many vesicles covered with ferritin granules.

14. Fat. The centripetal layer is a large drop of fat which results from the confluence of small fat droplets. In the wild type, the fat is stained bright yellow by β-carotene. In albino mutants, the layer is colorless.

Size of the Layers

The size of the layers indicates the relative proportions of the various particles in the cytoplasm, although the actual volume of the particles is less than that of the layer which they form. Table I indicates the volume of each layer in spphs of various lengths. During early growth (0–1 cm) the size of the sedimented layers increases with the size of the spph. Later, the size of the layers is nearly constant, while the vacuole volume increases continuously. The glycogen layer also continues to increase relative to the other layers, so that it is ten times larger in 2-cm-long spphs than in spphs 0.5 cm long. The volume of the ribosomal layer, on the contrary, changes little with age. Since all the material needed for growth of the aerial spph must enter through the spph foot, this indicates either that it is not accumulated indiscriminately as the cytoplasm is pushed into the spph or that the cytoplasm entering the spph varies in its composition with age.

Buoyant Density of the Layers

The density of layers can be determined by introducing oil droplets of known density into the cell before centrifugation. At the early stages of this work, phthalic acid esters were used since they did not appear to be injurious to the cytoplasm.

TABLE I

Volume of Different Layers

(Relatively small volumes of crystals and yellow band were divided between neighboring layers.)

Layer	Size of spph		
	5 mm	10 mm	15 mm
Lipoprotein membrane	5.0	9.0	15.0
Vacuole	52.0	160.0	400.0
Membranes Endoplasmic reticulum	6.5	8.0	10.0
Nuclei	2.0	6.4	8.0
Fibrils, mitochondria Mitochondria	2.1	9.0	13.0
Crystals Ribosomes	1.5	2.8	3.4
Yellow band Glycogen	0.8	3.0	9.6
Polyphosphates, crystals Polyphosphates	0.4	1.0	0.8
Total below vacuole	13.3	30.2	44.8

Various densities were obtained by mixing diethyl phthalate and dibutyl phthalate. Later, mixtures of silicone oil and fluorochemicals were used. Dow Corning (Midland, Mich.) silicone oil 706 (density 1.10 g/cc) is miscible with 3M Company's fluorochemical Kel-F3 (density 1.96 at 5°C) and also with common paraffin oil (density 0.9). Thus it is possible to make mixtures of inert oils with densities between 0.9 and 1.96. Also, these oils can serve to establish density gradients in this range.

The oils were injected into spphs which previously had been centrifuged at 35,000 rpm for 20 min. After a second centrifugation at 25,000 rpm, the position of the oil droplets relative to the cytoplasmic layers was observed (Fig. 3, left; Fig. 18). The oil droplets indicate the average density of the layers, i.e. particles and suspension fluid, and not the density of the particles themselves.

Particle density can be determined if particles of similar size and known density are introduced. Bacteria can be injected into the cell without any apparent interaction with the cytoplasm. Densities (ρ) of several bacteria were determined by centrifugation in step density gradients made of Ficoll (Pharmacia Inc.) or Urographin (E. R. Squibb and Sons, New York) (Tamir and Gilvarg, 1966). The osmotic action of the latter is great enough to increase the densities of bacteria by dehydration.

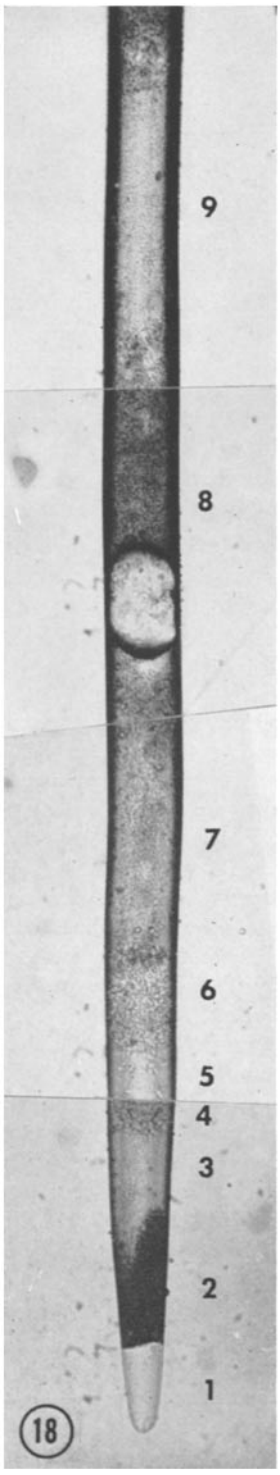

FIGURE 18 Oil droplet (density = 1.06) inside centrifuged spph settles above mitochondria. Numbers identify each layer.

Densities obtained with the former should approximate the densities that the bacteria assume in cell fluids. Bacteria can be recognized in the cell by their appearance, coloration, and by Feulgen staining.

E. coli (ρ = 1.16 in Urographin, ρ = 1.07 in Ficoll) settles below the mitochondria and above the crystals. *E. coli*, fixed with formalin and washed with distilled water (ρ = 1.22 in Urographin, ρ = 1.10 in Ficoll), settles below the ribosomes, but above the yellow zone. *Micrococcus lysodeikticus* (ρ = 1.10 in Ficoll) settles above and mixes with the ribosomes. *Serratia marcescens* (ρ = 1.14 in Ficoll) settles below the yellow layer and above the glycogen. It is easily recognized by its red color. This kind of experiment may be extended by the use of bacterial spores or other microorganisms. Viruses can provide particles of ribosomal size, and bacteriophage T4 (ρ = 1.475 in a $CsCl_2$ gradient) when injected settles in a compact layer below the ribosomes.

Search for the Photoreceptor

The action spectrum of the light growth response of the *Phycomyces* sporangiophore has been determined (Delbrück and Shropshire, 1960) and should indicate the absorption spectrum of the photoreceptor pigment. Direct measurement of the light absorbance of spphs, even in albino mutants, did not show any absorption peak corresponding to the action spectrum (Meissner and Delbrück, 1968). It was hoped that the photoreceptor might be associated with a particular cell organelle and should be sedimented in a distinct layer. This layer should then exhibit light absorption akin to the action spectrum.

The light absorption of each of the layers was measured with a Cary recording spectrophotometer, adapted for microspectrography (Zankel et al., 1967). It was possible to scan an area 10 × 50 μ and detect small differences in absorbance in the region from 400 to 550 nm. Each layer showed a steady increase in light absorption towards the blue owing to light scattering. The strength of this scattering varied from layer to layer. This made it particularly difficult to detect any small differences in light absorption due to pigments. The oil droplet had a strong absorption due to β-carotene in the wild type and due to lycopene in the rl mutant (Fig. 19). Some β-carotene was present also in other layers, so that it was necessary to use albino mutants (alb 5 or alb 10) to look for other pigments. Cytochromes were detected easily by a peak near 420 nm, which varied according to the oxidation state of cytochrome (Fig. 20). The mitochondria had a maximum at 415 nm in fresh preparations, but upon standing their maximum moved towards 422 nm. It is probable that the packed mitochondria continued to respire and thus reduced the cytochromes. A smaller but significant amount of reduced cytochrome was found in the nuclear fraction, and traces were also present in the bottom (polyphosphate) layer. The mitochondrial layer had a slight absorption shoulder around 446 nm, owing to flavins. The yellow layer had a steadily increasing absorption from the red to the blue end of the spectrum, without discernible peaks. This absorption can be attributed to the ferritin found in this layer. The membrane and lipoprotein layers scattered light too much for spectrographic study. We could not find any layer giving a spectrum corresponding to the action spectrum of the light growth response.

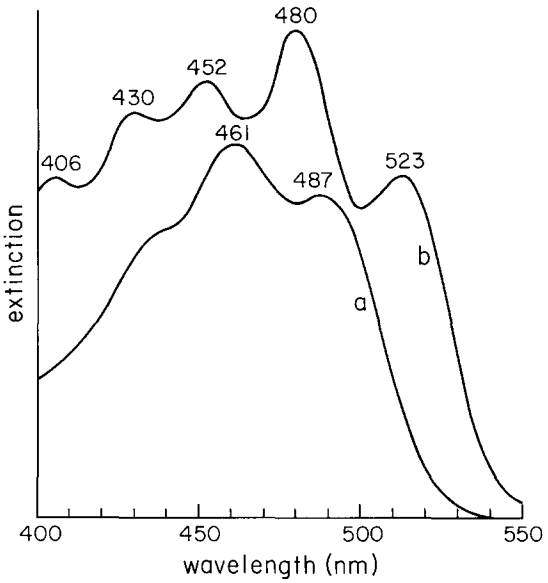

FIGURE 19 Absorption spectrum of colored fat, mainly due to β-carotene in the wild type (*a*) and to lycopene in the mutant rl (*b*).

Microsurgery

The size of *Phycomyces* spphs allows an easy separation of the centrifuged particles (layers), by one of the following operations, all performed on spphs submerged in paraffin oil: (*1*) By cutting off the tip and exerting gentle pressure on the vacuole,

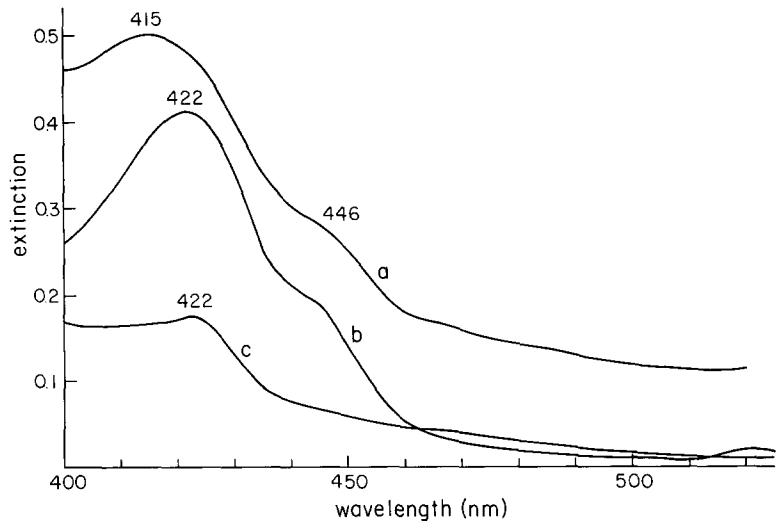

FIGURE 20 Absorption spectra of mitochondria and nuclei. *a*, Fresh preparation of mitochondria (alb 5). Note abs. peak at 15 nm and a shoulder due to flavins at 446 nm. *b*, Preparation of mitochondria after standing, with reduced cytochrome peak (alb 10). *c*, Nuclei (alb 5), same specimen as *a*.

one can extrude one layer after another. The layers behave as viscous fluids and are not packed into solid sediments. The extruded layers can be separated into droplets, with fine needles. (*2*) With a sharp razor, one can cut the spph between layers and so obtain sections containing the separate layers. Some mixing may occur at the interphases, and so this method works best only for isolation of one particular layer. (*3*) One can section the spph at the level of the vacuole and introduce a micropipette to aspirate one layer after another. This method was used to obtain nuclei for transplantation. The isolated layers should provide enough material for biochemical studies, provided the usual methods are scaled down by a few orders of magnitude. For example, one spph would yield about 3 μg of ribosomal material and 10 μg of mitochondria, free of nuclei.

A spph injured in any way does not continue to grow, but some injured spphs can regenerate a new smaller spph from the side, usually near the tip. Centrifuged spphs do not show such regeneration, and there is a question whether this indicates an irreversible damage to the cytoplasm. In other plant and animal cells, centrifuged at high speeds, normal functions are resumed after redistribution of the sedimented cell organelles. Such redistribution cannot occur in a centrifuged spph, and this may be the reason for the lack of regeneration. If the cytoplasmic fractions were not damaged, they should resume their normal functions when reintroduced into intact spphs.

Since one might expect that the nuclei would be injured first, their recovery after transplantation was studied. The viability of transplanted nuclei can be detected easily by the use of specific mutants. If the donor is a lycopene-producing mutant (rl) and the host is an albino (alb 5), transplanted nuclei should induce pigment formation in the graft. Since a transplantation of nuclei would lead to a heterokaryon, the pigmentation is characteristic of the heterokaryon, and in this case a yellow pigment is produced (Heisenberg and Cerdá-Olmedo, 1968). Spores obtained from such a heterokaryon will contain one or both types of nuclei, so that parental types will reappear in the next generation.

The centrifuged nuclear fraction of an rl spph was aspirated in a micropipette and injected into a short section of an albino spph. In about half of the cases, the spph regenerated a small secondary spph which showed a distinct yellow color in the immature sporangium. When the spores of this spph were plated, they gave white, red, and yellow colonies. Thus the centrifuged nuclei were functional and proliferated in the host to which they had been transplanted.

DISCUSSION

With respect to the order of sedimentation of cell organelles, centrifuged spphs differ from centri-

fuged cell homogenates. In cell homogenates, the cell debris and the nuclei sediment at low speeds, the mitochondria at intermediate speeds, and the ribosomes only after prolonged centrifugation at 100,000 *g*. This order of sedimentation is determined by the velocity of sedimentation, and this velocity, for spherical particles, is proportional to $a^2\Delta\rho$ (a = radius of the particle, $\Delta\rho = \rho_p - \rho_o$ = density difference of particles and fluid). Thus, the radius of the particle is the dominant feature, larger particles coming out at low speed, smaller particles at successively higher speeds. In contrast, the layering inside spphs is essentially the result of equilibrium centrifugation, the order being determined by the density of the layers formed by the particles.

The density of the layer differs from that of the particle depending on the volume-packing fraction (the fraction of the total volume occupied by the particles). For spheres of uniform size, this fraction is 0.739, independent of the size of the sphere, and the layer density is

$$\rho_{\text{layer}} = \rho_o + (\rho_p - \rho_o)\,0.739.$$

For nonspherical uniform particles, the packing fraction can be anything from unity (for cubes) to near zero (very spiny particles or inflexible tangled threads). Spheres of equal size and different density will form, under equilibrium conditions, separate layers.

For nonuniform particles, it is useful to consider the case of a mixture of large and small spheres, of equal density $\rho_p > \rho_o$. The small spheres fill interstices between the larger spheres and thus form a mixed layer which has a higher packing fraction than either of the pure layers. Thus, centrifugation would produce at the bottom a mixed layer and above it a pure layer of either larger or smaller particles, depending upon which particle was in excess. A more complex situation obtains when the particles are of different densities, but then also, mixed layers can be formed.

An immiscible fluid will form a layer by itself, whose position is determined solely by its density relative to that of the particle layer. Densities of the various layers, as found by the position of oil droplets, are given in Fig. 3. The ribosome layer has a density 1.10, and *M. lysodeikticus* (ρ = 1.10) mixes with the ribosomes, as might be expected. The density of the ribosomes must be less than 1.475—since phage T4 settles below the ribosomes —but higher than the density of the layer they form. The density of the glycogen particles must be more than that of the glycogen layer (1.25). In a cesium chloride gradient, a density of 1.66 has been measured, and the relatively low density of the glycogen layer may be due to a loose structure of glycogen particles. The density of the mitochondria must be close to 1.07, since this is the average density of the layer in which they are found and since *E. coli* (ρ = 1.07), which is similar in size to the mitochondria, sinks below them. The density of the nuclei is less than 1.07, since they float on top of the mitochondrial layer.

These estimates of particle densities are significantly different from the densities of particles obtained by centrifugation of cell homogenates in a sucrose density gradient. Thus, mitochondria obtained from *Phycomyces* spphs have a density of 1.13–1.14 in a sucrose gradient, and mitochondria from the mycelium a density of 1.17–1.19 (E. W. Goodell, personal communication). Mitochondria of *Neurospora* have a density of 1.16–1.20 in a sucrose gradient (Luck, 1963). The differences must be at least partially due to the osmotic effect of the sucrose solution, as discussed by de Duve et al. (1959). These authors corrected the density of rat liver mitochondria, measured by Thomson and Mikata (1954) as 1.22, to 1.103 for isosmotic 0.25 M sucrose. The density of isolated nuclei is generally considered to be high, but this may be owing to improper interpretation of the high sedimentation constant. In centrifuged cells, the nuclei sometimes move towards the heavy end, but often prove to be lighter than the mitochondria, as in *Phycomyces* and *Neurospora* (Zalokar, 1959).

The separation of cell organelles in a living state may open new ways for studying their properties by properly refined techniques of molecular biology. It may be valuable to obtain particles which are not contaminated with the debris of other cellular components, as so often happens in cell homogenates. Thus, the ribosome layer is practically pure, the mitochondria are free of nuclei, and nuclei are free of mitochondria. However, the separations are not perfect since the mitochondria are mixed with the ribosomes and the nuclei are mixed with the endoplasmic reticulum. Various layers may be recombined for a study of their metabolic interdependence. The organelles may be tested for genetic and other functions by their injection into appropriate hosts.

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