Isolation and Characterization of Spherosomes from Aleurone Layers of Wheat[1]

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Abstract

Spherosomes were isolated from aleurone layers of wheat (*Triticum aestivum* L.) by combined differential and density gradient centrifugation. The isolated spherosomes were heterogeneous in size and density with some sedimenting as floating lipid ($d<1.0$) while others sedimented with mitochondria ($d>1.18$). Analyses of acid phosphatase using β-glycerophosphate as substrate revealed only trace levels of the enzyme associated with spherosomes. Our findings show that spherosomes of wheat seeds are not lysosomal equivalents. Rather, spherosomes appear as cellular repositories of lipids and proteins which may provide a precursor pool for membrane biogenesis during germination.

Spherosomes have been defined as organelles bounded by a single membrane, rich in triglycerides, which have been considered lysosomal equivalents on the basis of the histochemical localization of acid phosphatase (38). Originally described by light microscopists as small spherical bodies refractile in dark-field illumination, spherosomes have long puzzled cytologists (7). There was controversy concerning spherosomes since their discovery. Some (3) regarded spherosomes as membrane-bounded organelles. Others (11) considered spherosomes to be products of cellular metabolism, i.e., lipid bodies or oil droplets that were free in the cytoplasm and lacked delimiting membranes. With the advent of the electron microscope, the opinion that spherosomes and lipid bodies were distinct entities (29) gained prominence. However, recent studies using glutaraldehyde (followed by osmium tetroxide) fixation have equated spherosomes with what were formerly termed lipid bodies. In these studies, single-line 'membranes", 25-40 Å in thickness, were demonstrated at the spherosome periphery (22, 30, 41, 42).

The lipidic nature of spherosomes was first deduced from lipophilic staining and extraction procedures (4, 11, 44) and their relative abundance in the storage tissues of oil seeds. Cytochemical methods have further revealed that spherosomes react freely with phospholipid indicators (32) as well as with specific reagents for proteins (14, 28, 42). Sorokin and Sorokin (32) were unable to demonstrate the presence of carbohydrate by the periodic acid-Schiff (PAS) reaction.

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The suggested equivalence of spherosomes and lysosomes is based primarily on electron miscroscope investigations of Matile (19, 20), Sorokin and Sorokin (33) and Wilson (39). In these studies, electron-dense deposits of lead phosphate were reported to accumulate within spherosomes after incubation by the Gomori (10) procedure for acid phosphatase. Other reports suggest that spherosomes have a more anabolic role either as lipid synthesizing sites (7, 32), as sites of storage of reserve lipids (13) or proteins (22), or as precursors of other structural entities within the cell (23, 24, 43).

With a view toward testing the application of the lysosome concept to spherosomes, we have isolated and purified spherosomes from wheat aleurone layers. Analyses of the isolated fractions show that the structure commonly identified in the electron microscope as an equivalent of the spherosome is not a type of lysosome. Rather, the results are consistent with a more anabolic role for this cell component.

Materials and Methods

Plant material.—Seeds of wheat (*Triticum aestivum* L., cv. Arthur, 1971 harvest) were obtained from the Illinois Seed Supply Service, Peoria, Illinois. Seeds were degermed by dissecting the embryo end (ca. ¼ of the grain containing the embryo) with a razor blade. Aleurone layers were prepared by grinding the degermed seeds with a heavy-duty metal mortar and pestle and removing excess starch with a 25 mesh sieve. The material was then imbibed in distilled water (25 ml for every 5 g of layers) for 12 hrs at 25° on a reciprocating shaker operating at about 2 cycles per sec.

Cell fractionation.—Imbibed aleurone layers in lots corresponding to 5 g (dry weight) were homogenized in 15 ml of medium for 3 min with a Polytron 20ST homogenizer (Kinematica, Lucerne, Switzerland) operated at 7-8,000 rpm. The homogenization medium consisted of 0.1 M sodium phosphate (pH 7.4), 1% dextran and 0.5 M sucrose (25) except in experiments where acid phosphatase was measured, where 0.1 M Tris-HCl replaced the phosphate buffer. The resulting homogenate was filtered through 4 double layers of cheese cloth and centrifuged at 1,000 x g (2,500 rpm, Sorvall HB-4 rotor) for 10 min to remove cell walls, nuclei and debris. This centrifugation was followed by a second differential centrifugation at 6,000 X g (6,000 rpm, Sorvall HB-4 rotor) for 15 min to yield a crude spherosome fraction (Fig. 1).

Crude spherosomes from a total of 40 g dry weight of aleurone layers were resuspended in 10 ml of homogenization medium and applied to a sucrose gradient consisting of 4 ml of 1.5 M sucrose, 4 ml of 1.4 M sucrose, 3 ml of 1.2 M sucrose, 3 ml of 1.0 M sucrose and 3 ml of 0.8 M sucrose. The tube was filled to a final volume of 35 ml by layering water over the resuspended spherosomes. The gradients were centrifuged 60 min at 90,000 X g (25,000 rpm, Spinco SW-27 rotor). The spherosomes from the interface between the water phase and the spherosome-free supernatant were diluted with cold distilled water and pelleted by centrifugation at 50,000 X g (20,000 rpm, Spinco SW-27 rotor) for 30 min. The fractions from the gradient were pelleted in like manner

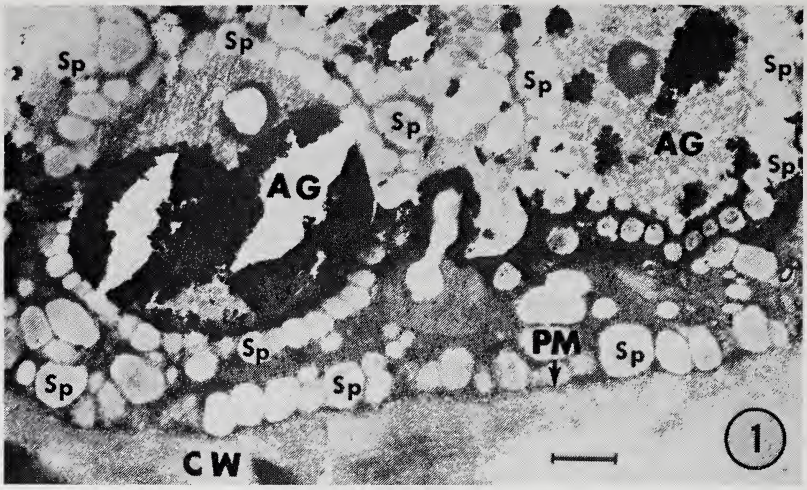

FIGURE 1. *Electron micrograph of a portion of an aleurone cell of the wheat grain. Numerous "spherosomes" (Sp) surround aleurone grains (AG) and are aligned in the cortical cytoplasm adjacent to the plasma mmebrane (PM). CW = cell wall. Scale bar = 1 μ.*

following a 1:1 dilution with 0.1 M Tris, pH 7.4, containing 1% dextran.

The spherosome-free supernatant of the 6,000 rpm centrifugation step was further fractionated by centrifugation at 16,000 X g (10,000 rpm, Sorvall HB-4) for 20 min to remove mitochondria, followed by centrifugation at 100,000 X g (40,000 rpm, Spinco 50.1 rotor) for 1.5 to 2 hrs to yield the microsome fraction. The supernatant from the last centrifugation was retained as the microsome-free supernatant.

Determination of acid phosphatase.—Biochemical assays of acid phosphatase were performed according to Holcomb *et al.* (12) using β-glycerophosphate as the substrate. Protein was determined by the procedure of Lowry *et al.* (17) using bovine serum albumin as the standard.

Electron microscopy.—For electron microscopy, tissue and pellets of cell fractions were fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, for at least 2 hr followed by post fixation in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer, pH 7.2, for 2 hr (35). The cytochemical assay for acid phosphatase was the Gomori (10) procedure adapted for electron microscopy. Samples were rinsed with buffer, dehydrated through an acetone series and embedded in Luft's (18) or Spurr's (31) Epon. Thin sections were examined and photographed with a Philips EM 200. Magnifications are approximate.

Results

Aleurone layers of wheat are characterized by numerous cytoplasmic particles comparable to those termed spherosomes by Paleg and Hyde (26), Jones (15), Yatsu and Jacks (41) and others (7-9). As illustrated

in Fig. 1, the spherosomes of wheat are approximately spheroidal in profile with a uniform-staining matrix of medium to low electron density. The spherosomes show characteristic associations with the plasma membrane at the cell surface, and surround the aleurone grains giving a halo effect to these cell components.

Isolated spherosomes resemble those in the intact cell (compare Figs. 2 and 3 with Fig. 1) and retain their association with sheets of membrane (Fig. 3) which may represent fragments of either the membrane of the aleurone grains or of the plasma membrane (Fig. 1).

When centrifuged on sucrose gradients, the crude spherosome fraction separates into four discrete bands, collecting at the different interfaces of the gradient. Of these, two fractions were estimated from electron micrographs to be in excess of 90% spherosome-derived and were designated light and heavy spherosomes on the basis of their position in the gradient. The light and heavy spherosomes banded at sucrose densities of about 1.0 and 1.18, respectively. Except for a greater heterogeneity in size, the light spherosomes (Fig. 5), which had sedimentation characteristics of lipid bodies, resembled, morphologically, the heavier spherosomes (Fig. 6), which had sedimentation characteristics of protein-rich mitochondrial membranes.

The 1,000 X g pellet (Fig. 8) and the pellet formed during gradient centrifugation (not illustrated) contained the bulk of the starch grains, wall fragments, nuclear fragments and debris. The fraction sedimenting between 6,000 and 16,000 X g was enriched in mitochondria (Fig. 9) although a few spherosomes were also present. The predominantly mitochondrial nature of this fraction was verified by measurements of succinic-INT-reductase activity (27). The 16,000 to 100,000 X g (microsomal) fraction contained the bulk of the endoplasmic reticulum and other membrane vesicles and was essentially spherosome-free (Fig. 10).

The analysis of the acid phosphatase activity in the various fractions is summarized in Table 1. The bulk of the acid phosphatase is found in the spherosome-free supernatant (16,000 X g supernatant) and the microsome-free supernatant (100,000 X g supernatant). The supernatants were the only fractions to show relative enrichment when

TABLE 1. *Distribution of acid phosphatase activity among fractions of wheat aleurone layers.*

Fraction	Specific Activity[1]	Relative Enrichment[2]
Total homogenate	1.92	1.0
Debris	0.56	0.3
Light spherosomes	0.12	0.06
Heavy spherosomes	0.60	0.3
Spherosome-free supernatant	2.26	1.3
Mitochondria	0.44	0.2
Microsomes	0.80	0.4
Microsome-free supernatant	3.52	1.8

[1] Units of specific activity are μmoles inorganic phosphate released from β-glycerophosphate/hr/mg protein.

[2] Ratio of specific activity to that of the total homogenate.

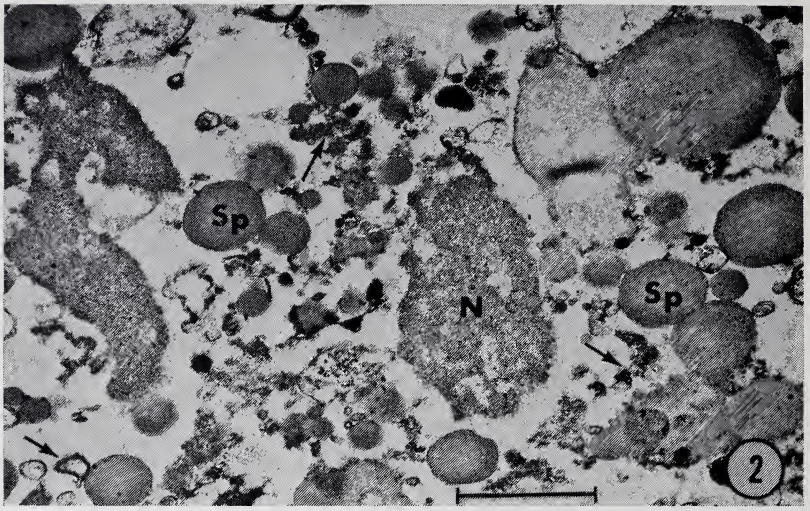

FIGURE 2. *Electron micrograph showing a portion of the crude spherosome fraction sedimenting between 1,000 and 6,000 x g. In addition to numerous spherosomes (Sp), nuclear (N) and other fragments (arrows) are present. Scale bar = 1 μ.*

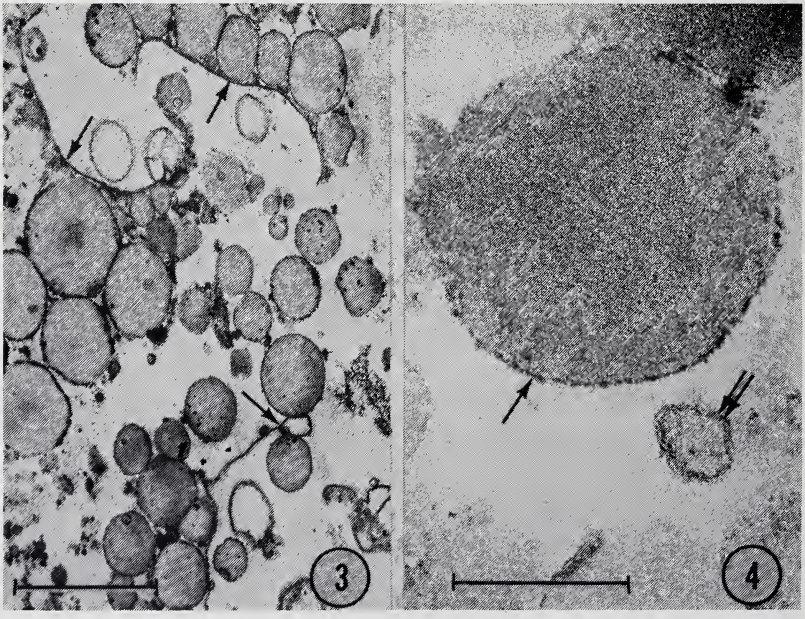

FIGURE 3. *Isolated spherosomes resemble those of the intact cell. Here extensive associations with sheets of membrane, derived probably from either membranes of aleurone grains or plasma membranes (arrows), are retained. Scale bar = 1 μ.*

FIGURE 4. *Isolated spherosome at higher magnification to show the absence of a unit-type membrane with a clear dark-light-dark pattern (single arrow). A typical unit-type appearance is shown by the adjacent membrane fragment (double arrow) probably derived from plasma membrane or membrane of an aleurone grain. Scale bar = 0.2 μ.*

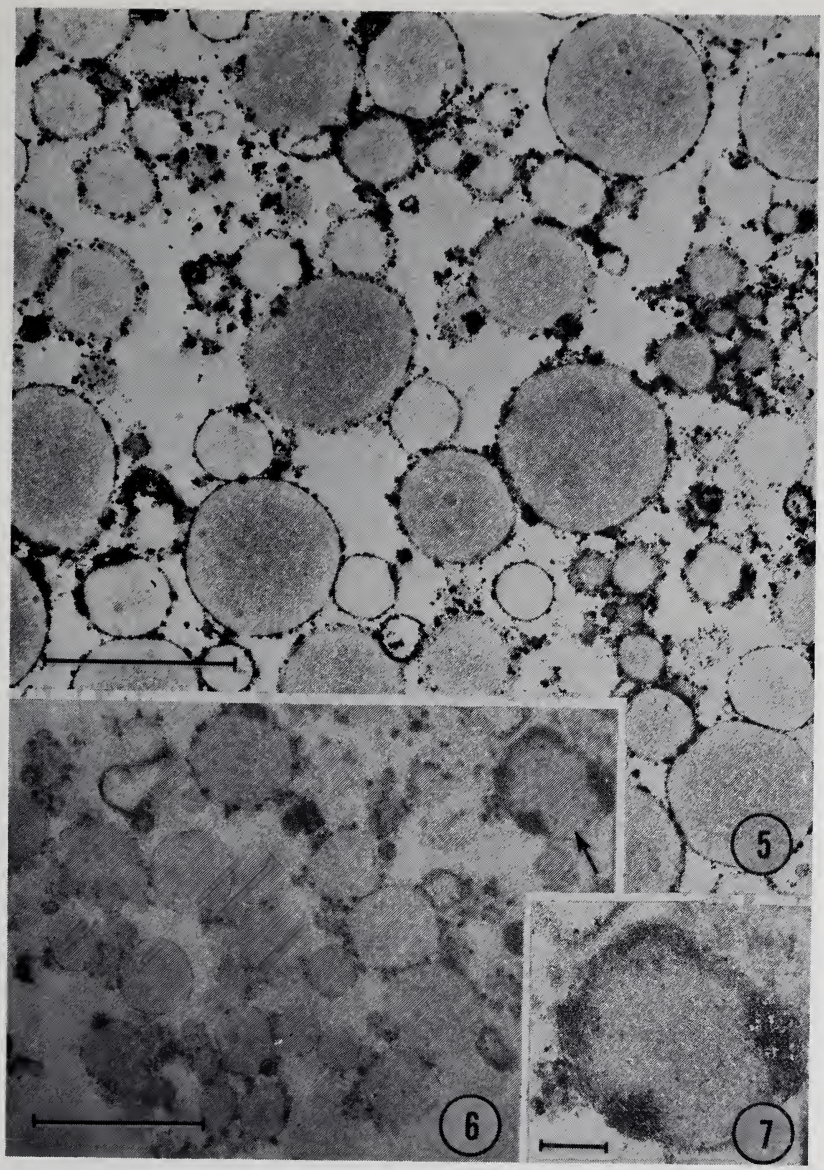

FIGURE 5. *Purified fraction of "light" spherosomes collected from the water-homogenate interface of the sucrose gradient. This fraction was extremely heterogeneous with a 5-fold range in spherosome diameter. Scale bar = 1 μ.*

FIGURE 6. *As in Figure 5 except from the 1.2 M/1.4 M sucrose interface of the sucrose gradient. This fraction is designated as "heavy" spherosomes. Spherosome diameters are less variable and correspond to the smaller spherosomes of the "light" fraction (Fig. 5). Except for the absence of very large spherosomes, this fraction cannot be distinguished morphologically from the "light" spherosomes. Compare with figure 5. Scale bar = 0.5 μ.*

FIGURE 7. *Isolated "heavy" spherosome indicated by arrow in Figure 6 at higher magnification. "Myelin" forms were seen within the interior of some but not all "heavy" spherosomes. Scale bar = 0.1 μ.*

compared with the total homogenate (1.3-fold in the spherosome-free supernatant and 1.8-fold in the microsome-free supernatant). Neither the light nor the heavy spherosomes showed any enrichment with respect to acid phosphatase activity. On a protein basis, the specific enzymatic activities of the spherosome fractions were less than that of the microsomal fraction and approximately equal to that of the mitchondria and debris fractions.

To further verify the low levels of acid phosphatase in the isolated spherosomes and to attempt to explain the residual activity associated with them, fractions were analyzed by enzyme cytochemistry. Electron-dense deposits were present, but principally on membrane fragments associated with the spherosomes. The peripheries and interiors of the spherosomes were relatively free of specific reaction product (Fig. 11). Non-specific electron-dense deposits were present at the spherosome periphery in control preparations (Fig. 118) including those which had not been incubated by the Gomori procedure (see Figs. 4-7). Based on these findings and the results of the *in vitro* acid phosphatase assays of Table 1, we conclude that spherosomes of aleurone cells of mature wheat grains do not contain acid phosphatase.

Discussion

Matile (20, 21), Sorokin and Sorokin (33), Wilson *et al.* (39) and others (1, 31) base their conclusion that spherosomes are lysosomes on the cytochemical demonstration of acid phosphatase in these organelles. Their findings contrast those of Yatsu and Jacks (42) and those reported here, both of which were based on analyses of isolated fractions. The basis for the positive cytochemical results of others is not certain. However, our findings indicate: 1) a propensity for spherosomes to precipitate electron-dense deposits in the region of the spherosome boundary (Figs. 4-7) and 2) a concentration of acid phosphatase activity in adhering membrane fragments (Fig. 11). The membrane fragments are most probably derived from aleurone grains, which are known to contain acid phosphatase (19, 39). The acid phosphatase activity associated with these membrane fragments could well explain the residual acid phosphatase activity found in the fractions since the isolated spherosomes do retain their association with these membrane fragments (Fig. 3).

A second reason for lack of homology between spherosomes and animal lysosomes is the membrane. With lysosomes, the limiting membrane is a unit type with a clear dark-light-dark pattern in osmium-fixed preparations. With plant spherosomes, a clear dark-light-dark pattern is not indicated (Fig. 4); the existence of a unit type membrane around plant spherosomes remains to be demonstrated.

If spherosomes are not lysosomal equivalents, another function must be assigned to them. Three facts must be considered for any new hypothesis: 1) Spherosomes are widely distributed among plants and fungi but are not known as such for animal cells. 2) Spherosomes tend to be prominent in seeds (2, 15, 42) and embryos, and to a lesser extent in active cells, i.e. meristematic (22, 43) or recently stimulated secretory

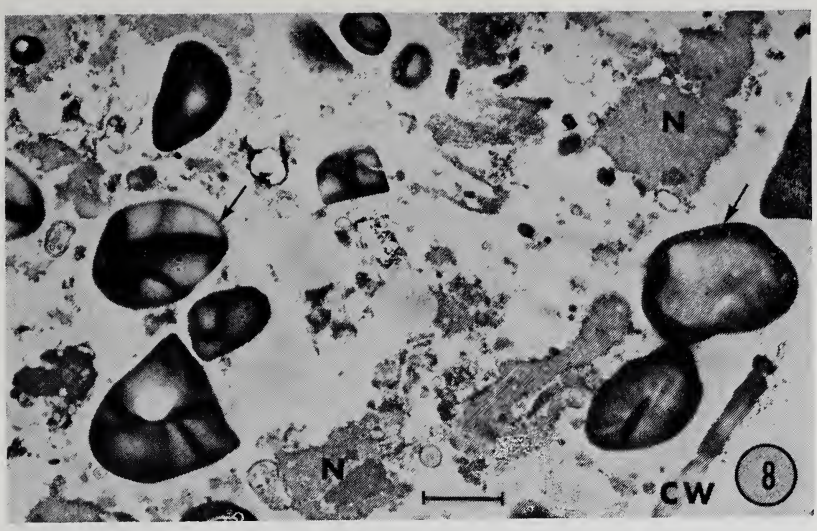

FIGURE 8. *Portion of the 1,000 x g "debris" fraction showing a predominance of starch grains (arrows), nuclear fragments (N) and cell wall fragments (CW). Spherosomes are absent. Scale bar = 1 μ.*

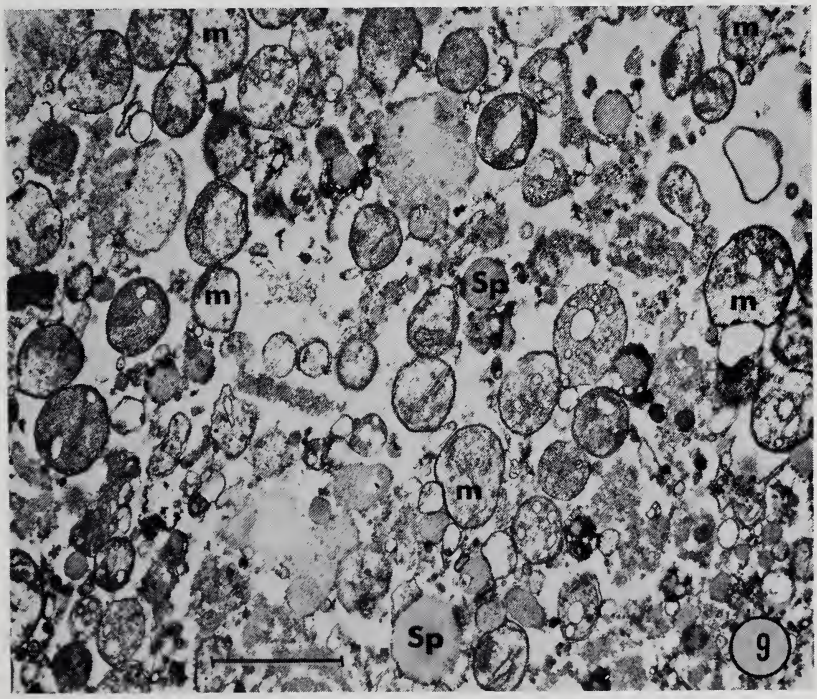

FIGURE 9. *Portion of the mitochondrion-rich fraction obtained by differential centrifugagation of the spherosome-free supernatant at 16,000 x g for 20 min. Mitochondria (M) appear swollen but resemble those of the imbibed seeds. A few spherosomes (Sp) are present in this fraction. Scale bar = 1 μ.*

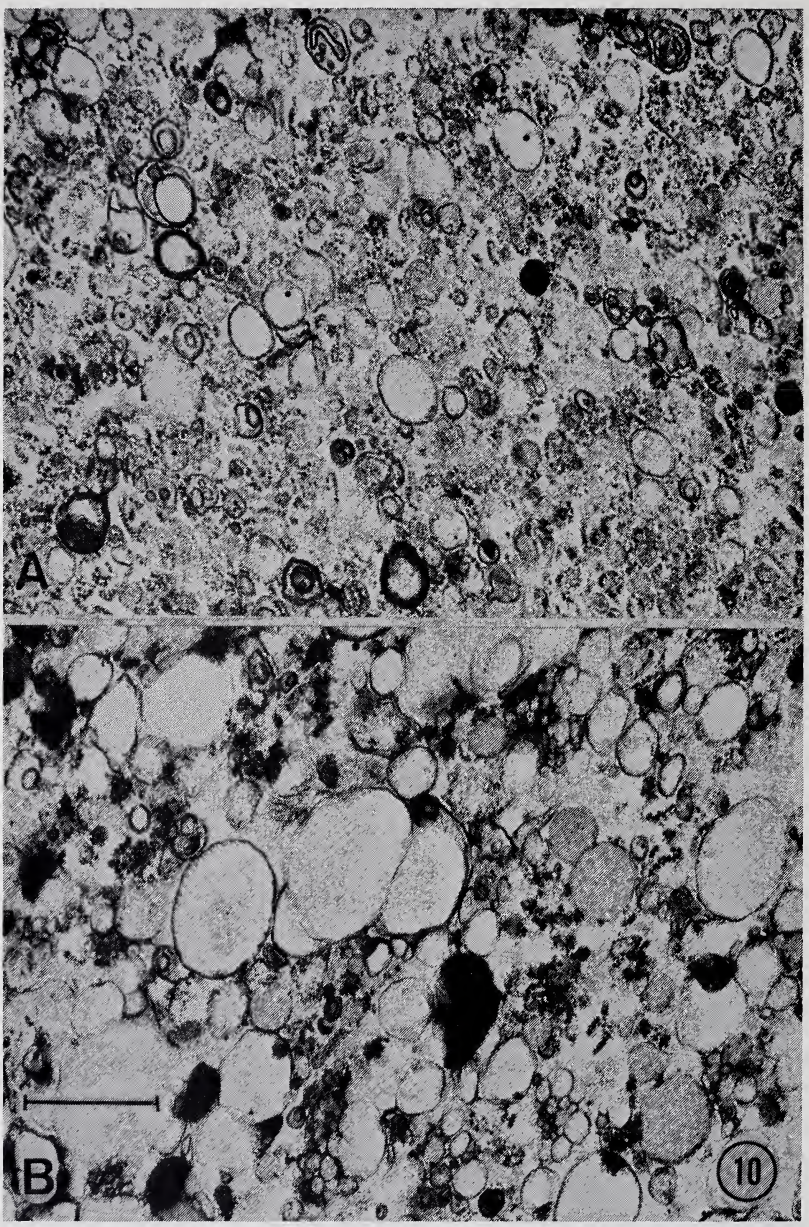

FIGURE 10. *Microsome fraction obtained by centrifugation of the post-mitochondrial supernatant for 1.5 hrs at 90,000 x g. This fraction represents a mixture of endoplasmic reticulum and other membrane fragments but contains only occasional spherosomes. A. Bottom half of pellet. B. Top half of pellet. Scale bar = 0.5 μ.*

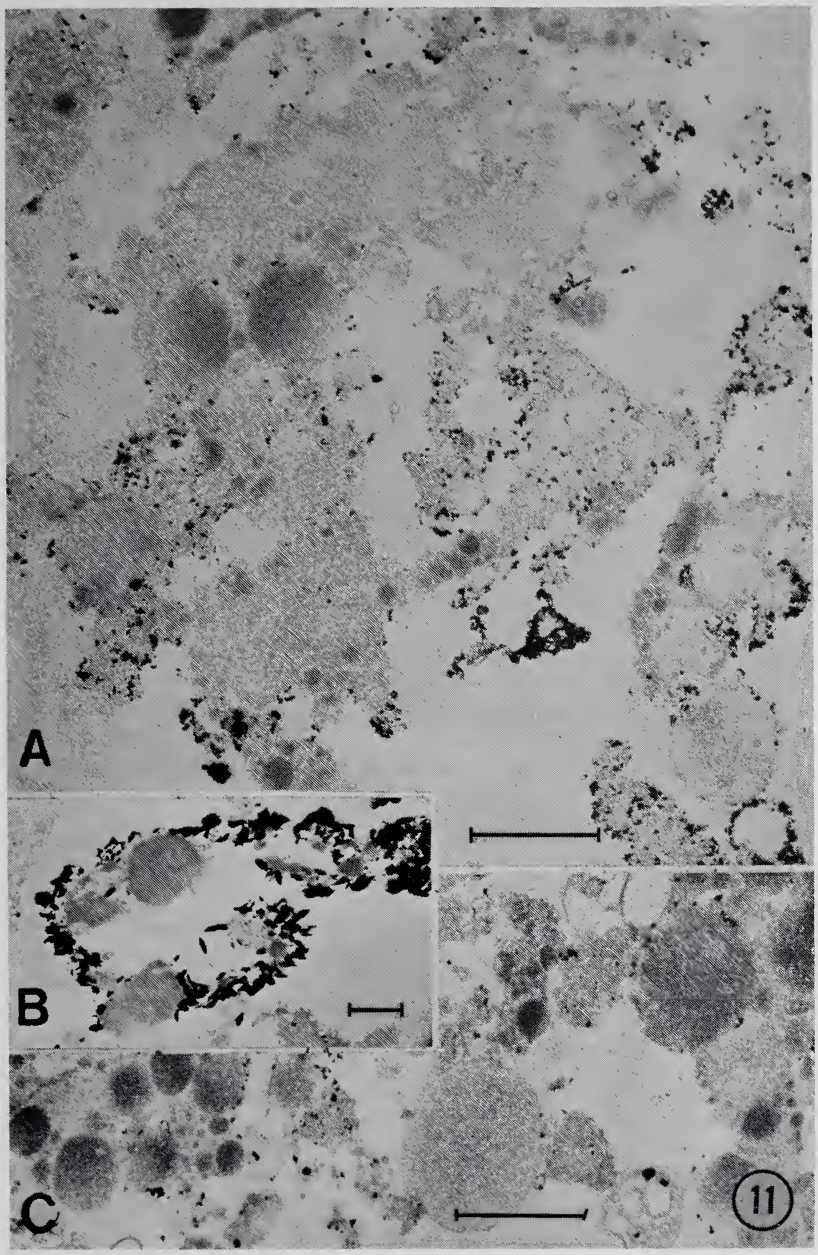

FIGURE 11. *Cytochemical demonstration of acid phosphatase activity in an isolated "heavy" spherosome fraction. The section is unstained so that membrane profiles are not heavily contrasted from the background. A. Substrate present. B. Deposits are found associated with the spherosome periphery, especially with adhering membrane fragments. C. Substrate absent. Some deposits are still present at the spherosome periphery although not as pronounced as with substarte present. The spherosome interiors are relatively free of lead deposits, verifying the non-presence of acid phosphatase. Scale bar = 0.5 μ.*

cells (15, 26, 36). 3) Spherosomes disappear as cells mature (15, 22, 24, 26). These observations are consistent with the suggestion that spherosomes are storage or transport organelles unique to plants and fungi which are consumed during growth or germination. In cereal aleurone cells, the disappearance of spherosomes is accelerated by treatment with the germination-promoting hormone, gibberellin (15, 26, 36). Concomitant with the disappearance of spherosomes is an increase in internal membranes, primarily rough endoplasmic reticulum. Significantly, this increase in endoplasmic reticulum occurs without evidence of net synthesis of phospholipids (5, 6, 16, 37).

The findings reported here suggest that spherosomes are lipoprotein bodies. In electron micrographs, "myelin" forms (Fig. 7) are sometimes observed within the spherosome interior. A role in the storage of membrane precursor materials is indicated.

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