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Isolation of Spherosomes (Oleosomes) from Onion, Cabbage, and Cottonseed Tissues

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L. Y. YATSU, T. J. JACKS, AND T. P. HENSARLING
Southern Regional Research Laboratory,[1] *New Orleans, Louisiana 70119*

ABSTRACT

Subcellular particles, identical in appearance to spherosomes observed *in situ*, were isolated from onions (*Allium cepa* L.) and cabbages (*Brassica capitata* L.). They were minute spherules about 1 micron in diameter, filled with an evenly stained osmiophilic matrix and delimited by a single, fine-line membrane 20 to 30 Å thick.

"Spherosomes" isolated from onions and cabbages were compared with "oil-droplets" isolated from cottonseeds. Morphologically, they were similar, even at ultrastructural levels. Their chemical compositions were also similar: both types of particles containing primarily lipid with very little phospholipid or protein. Neither type of particle possessed acid phosphatase activity.

These results indicate that oil-droplets of oleaginous tissues correspond to spherosomes of nonoily tissues. Therefore, both types of particles should be referred to by the same name. Since these particles are rich in lipids, it is suggested that the name "spherosome" be abandoned in favor of "oleosome," which is also entitled to priority.

Spherosomes are intracellular particles which have been familiar to botanical cytologists for more than half a century; yet, surprisingly, they are still the object of much controversy (4, 5, 7, 8, 10, 13, 16, 19, 23). The term "spherosome" was introduced into the literature in 1922 when Dangeard (2) distinguished between spherical (spherosomes) and rod-shaped (mitosomes) "microsomes of the spherome." Earlier, Dangeard (1) had described the spherome as consisting of "microsomes" which he reported as highly refringent spherules of an oily appearance that blacken, more or less, with osmic acid. From the very outset, there was controversy concerning spherosomes: one faction (1) which looked on spherosomes as organelles, and the other (6) which considered spherosomes merely as products of cellular metabolism (lipids).

One of the most recent eruptions of this controversy was the objection voiced over the identification of "oil-droplets" from oleaginous tissues with "spherosomes" of nonoily tissues (19). Oil-droplets were purported to be droplets of oil that were free in the cytoplasm without delimiting unit-membranes, whereas spherosomes were alleged to be composed of phospholipids and proteins which were bounded by unit-membranes. Many investigators (17, 22) agreed with this distinction between oil-droplets and spherosomes—both at the light and electron microscope levels. Yet, some continued to identify oil-droplets with spherosomes (10, 23).

Clearly, this problem requires investigation. Since spherosomes do not possess distinct morphological features as seen in other organelles such as mitochondria, chloroplasts, or dictyosomes, it was felt that a morphological and chemical characterization was necessary. In this communication we describe, for the first time, the isolation of spherosomes from nonoily tissues and compare their morphology and chemical composition with oil-droplets isolated from cottonseeds.

MATERIALS AND METHODS

Light-microscopic Procedures. Microscopic examinations of fresh tissues were conducted with a Zeiss Ultraphot II light microscope using brightfield, darkfield, and phase contrast. The effects of osmium tetroxide on tissues were observed directly as the fixative contacted the tissues under the cover slips.

Electron-microscopic Procedures. Electron microscopic examinations were conducted on tissues: (*a*) fixed overnight at room temperature in 2% osmium tetroxide dissolved in 0.1 M phosphate buffer, pH 7.2, (*b*) fixed for 1 hr in 2% aqueous potassium permanganate at room temperature, and (*c*) doubly fixed with aldehyde and osmic acid fixatives. The tissue was first fixed overnight in an aldehyde fixative which consisted of 2.8% glutaraldehyde and half-saturated picric acid adjusted to pH 7.3 with 50 mM sodium cacodylate. The tissue was thoroughly rinsed in 50 mM sodium cacodylate, pH 7.3, and postfixed overnight in 1% OsO_4 dissolved in 50 mM sodium cacodylate, pH 7.3.

The tissues were serially dehydrated in a graded series of aqueous acetone and embedded in Maraglas. Sections were cut on a Servall MT-1 Porter-Blum ultramicrotome with a diamond knife, stained with uranyl acetate and lead citrate and examined with a Phillips EM 200 or EM 300 electron microscope.

Isolation of Spherosomes. Basically, spherosomes were isolated from tissue homogenates by differential centrifugation. Fifty pounds of onion bulbs (*Allium cepa* L.) were sliced into small pieces and blended in a Waring Blendor with approximately an equal volume of 0.5 M NaCl containing 50 mM tris-HCl buffer, pH 7.2. The mixture was blended for 1 min in an ice bath. The homogenate was squeezed through eight layers of cheesecloth, and the effluent was passed through a Sharples Type T-41-248RY-34 continuous-flow centrifuge at 20,000*g* to remove dense particles. The supernatant was then passed through a De Laval Gyro test unit separator to collect particles less dense than the grinding medium. No "cream" was collected from the separator; however, a light layer of creamy substance adhered to the cones inside the separator. This mate-

[1] One of the laboratories of the Southern Marketing and Nutrition Research Division, Agricultural Research Service, United States Department of Agriculture.

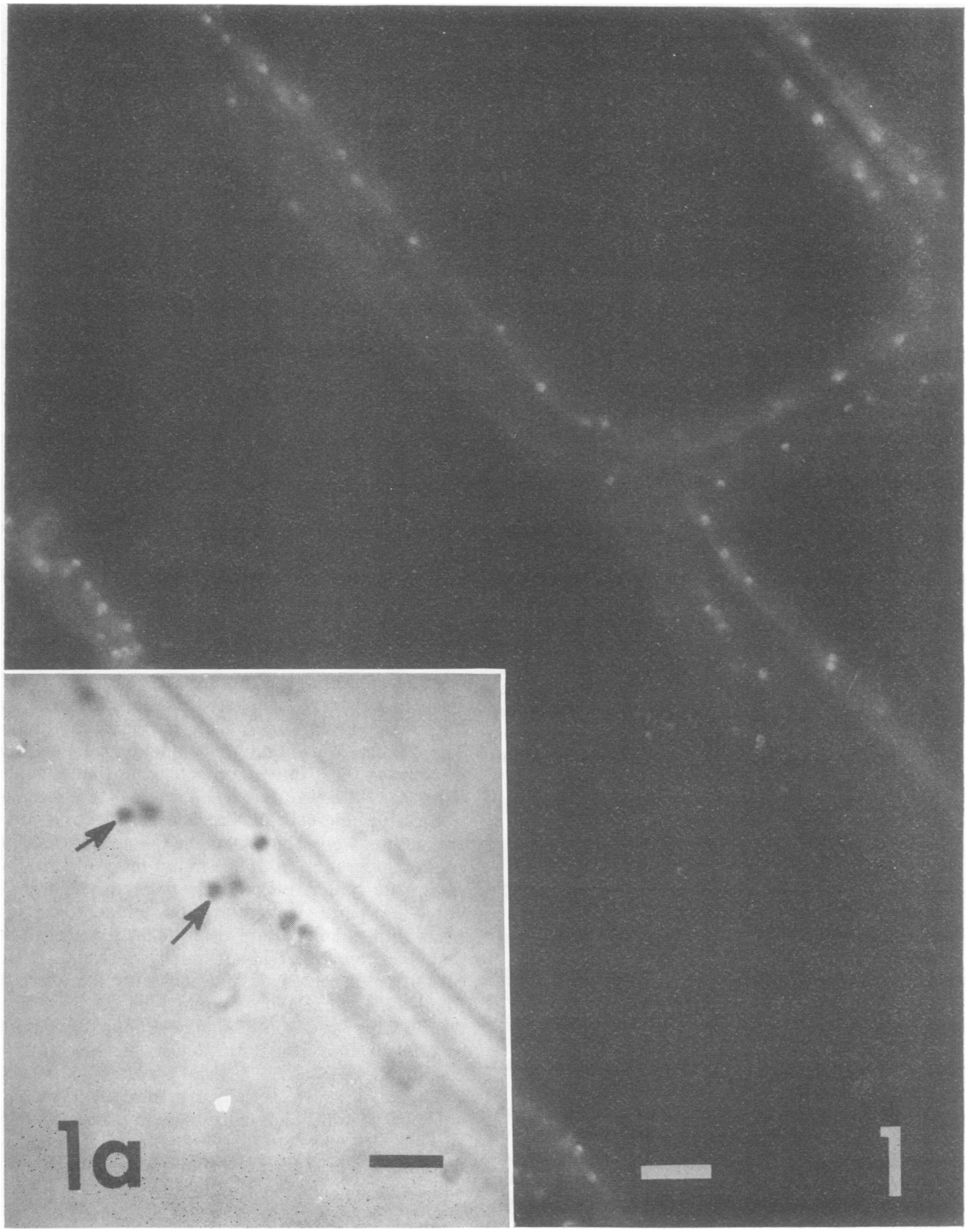

FIG. 1. Dark field light micrograph of an onion epidermal cell showing the highly refractile spherosomes in the thin layer of cytoplasm next to the cell wall. Because the highly refringent spherosomes are so bright, many of the organelles which are not in focus are visible as white dots. The apparent size is deceptive for various reasons, *e.g.*, diffraction, refraction, etc. Bar indicates 10 μ. a: Brightfield photomicrograph of a portion of an onion epidermal cell showing highly osmiophilic spherosomes (see arrows). The darkening of the spherosomes by osmium tetroxide was observed while the object was under the microscope (see text). Bar indicates 5 μ. Fixed in 1% osmium tetroxide in 0.1 M phosphate buffer, pH 7.2.

rial was rinsed off the cones with buffer and centrifuged at 25,000*g* for 20 min in the swinging bucket rotor of a Servall RC2B centrifuge. A light, creamy layer came to the top of the centrifuge tube but was present in such sparse quantity that no analytical procedures were practicable. A sample was taken from the top of the centrifuge tube with a platinum loop and examined with a light microscope.

Fifty pounds of cabbages (*Brassica capitata* L.) were sliced and treated as above, but the procedures were carried out in distilled water instead of buffered saline. Again, cream was not obtained from the separator, but the rinses from the separator cones yielded about 1 ml of fatty material. Samples were taken for light and electron microscopic studies and for biochemical analyses.

Studies of Isolated Spherosomes. Unfixed, isolated spherosomes exhibited Brownian motion under the light microscope. To eliminate this for photographic purposes, a droplet of spherosomal preparation was allowed to dry on a cover slip briefly and then observed in contact with a fresh drop of water. Many spherosomes then adhered to the cover slip enabling photomicrographs to be taken.

Electron microscopic examination of isolated spherosomes was conducted as before (11). Electron microscopic histochemical studies were carried out as described in a previous communication (24).

Materials for chemical analyses were dried to constant weights over P_2O_5 *in vacuo* at room temperature. The dried samples (from 100–200 mg) were extracted of total lipids according to the method of Martin and Morton (14), except that the residues were collected in small, fritted glass funnels. Solvents were removed by evaporation, and the residues and total lipids were determined gravimetrically after drying to constant weight *in vacuo*.

Lipid-phosphorus was determined by procedures described previously (12); nitrogen was determined by the method of Minari and Zilversmit (15); acid phosphatase (acid phosphomonoesterase, E.C. 3.1.3.2) activity was assayed according to Salomon *et al.* (18).

RESULTS

Light Microscopy. Spherosomes, in the classical sense, were readily observable in fresh, nonoily plant tissue, *e.g.*, epidermal cells of onion bulb scales. They were minute spherules about 1 μ in diameter, highly refractile, and prone to Brownian motion. We confirmed, under direct observation in the light microscope, the fact that spherosomes stain brown to black with osmic acid. The highly refractile spherules (spherosomes) were the most intensely stained (osmiophilic) subcellular particles in the cell; the hyaloplasm and other organelles were very faintly stained (Fig. 1).

Electron Microscopy. The quality of ultrastructural preservation achieved varied with the fixative employed. Osmium tetroxide, even when buffered, produced the poorest images of the fixatives used. Spherosomes were odd shaped, perhaps best described as "star shaped" (Fig. 2), and the preservation of cytoplasm was generally poor.

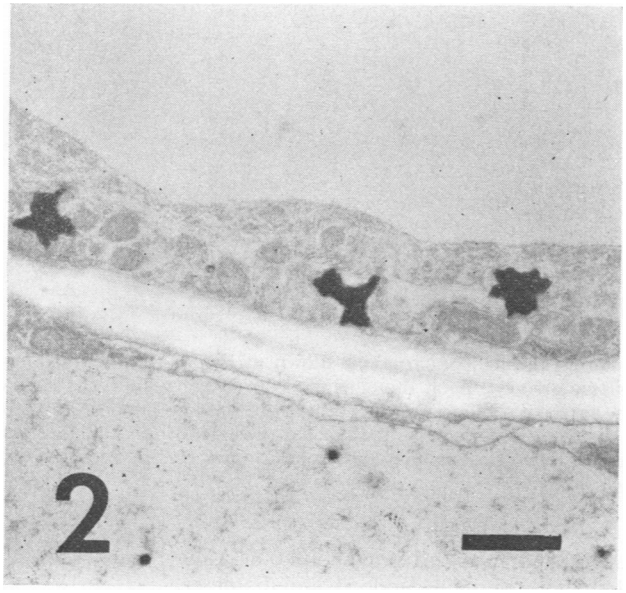

FIG. 2. Electron micrograph of a portion of an onion epidermal cell fixed in 1% osmium tetroxide in 0.1 M phosphate buffer, pH 7.2. Note the "star shaped" spherosomes which are produced by osmium tetroxide fixation alone. Bar indicates 2 μ.

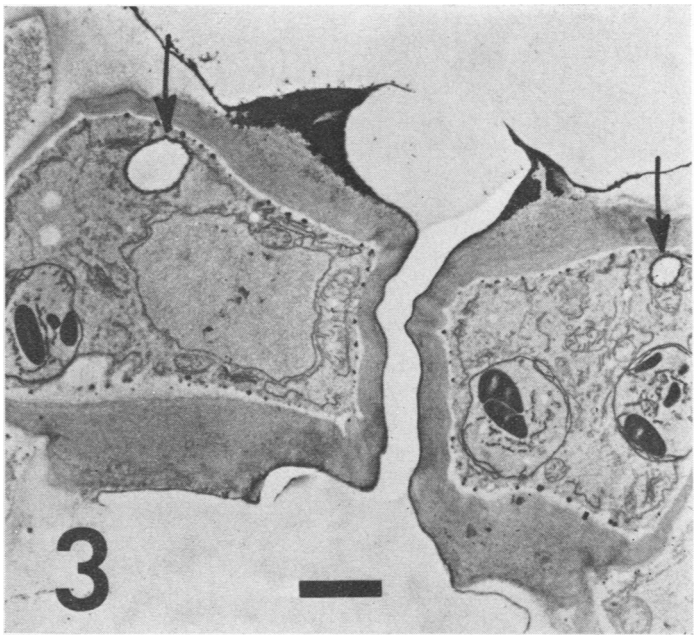

FIG. 3. Electron micrograph of a guard cell from cabbage leaf tissue fixed in 2% aqueous potassium permanganate. Arrows point to electron transparent spherosomes (note dark edges). Bar indicates 2 μ.

After permanganate fixation, spherosomes appeared as electron-transparent areas bounded by dark, electron-dense edges (Fig. 3). Permanganate-fixed spherosomes tended to be round in cross section, but they often assumed highly irregular outlines. Double fixation produced the best fixation.

The only entities found in the plant cells at the electron microscope level corresponding to the osmiophilic spherosomes observed in the light microscope were the osmiophilic spherules that are commonly referred to by electron microscopists as "lipid bodies" (Fig. 4). They were the same size, same shape, occurred in the same frequency, and were the most intensely osmiophilic bodies in the cytoplasm. The hyaloplasm and other organelles, although stained, were much less electron-dense.

Isolation of Spherosomes. Differential centrifugation of onion and cabbage homogenates, both of which contain very little fat (9), yielded a whitish, creamy fraction reminiscent of the "fat pad" obtained from oilseed homogenates. The quantity was meager—from 50 pounds of onions we collected insufficient material for chemical or ultrastructural studies. However, about a milliliter of "fat pad" (spherosomes) was obtained from 50 pounds of cabbages. This slightly greater yield from cabbages may have been due to a greater spherosomal content or better technique. In any event, enough spherosomes were collected from cabbages for both microscopic and chemical studies.

Cottonseeds (*Gossypium hirsutum* L.) contain so much lipid that recourse to methods described in this paper (use of a Sharples centrifuge and De Laval cream separator) was not necessary. Instead, a previous procedure was used (11).

In the light microscope, the isolated spherosomes (from both onions and cabbages) were identical in appearance to those observed *in situ*, even to the point of exhibiting Brownian motion. These in turn were virtually indistinguishable from oil-droplets isolated from cottonseeds (Fig. 5). All appeared as refractile spherules, ranging in diameter from approximately 0.5 μ (cabbages) to as large as 4 μ (cottonseeds).

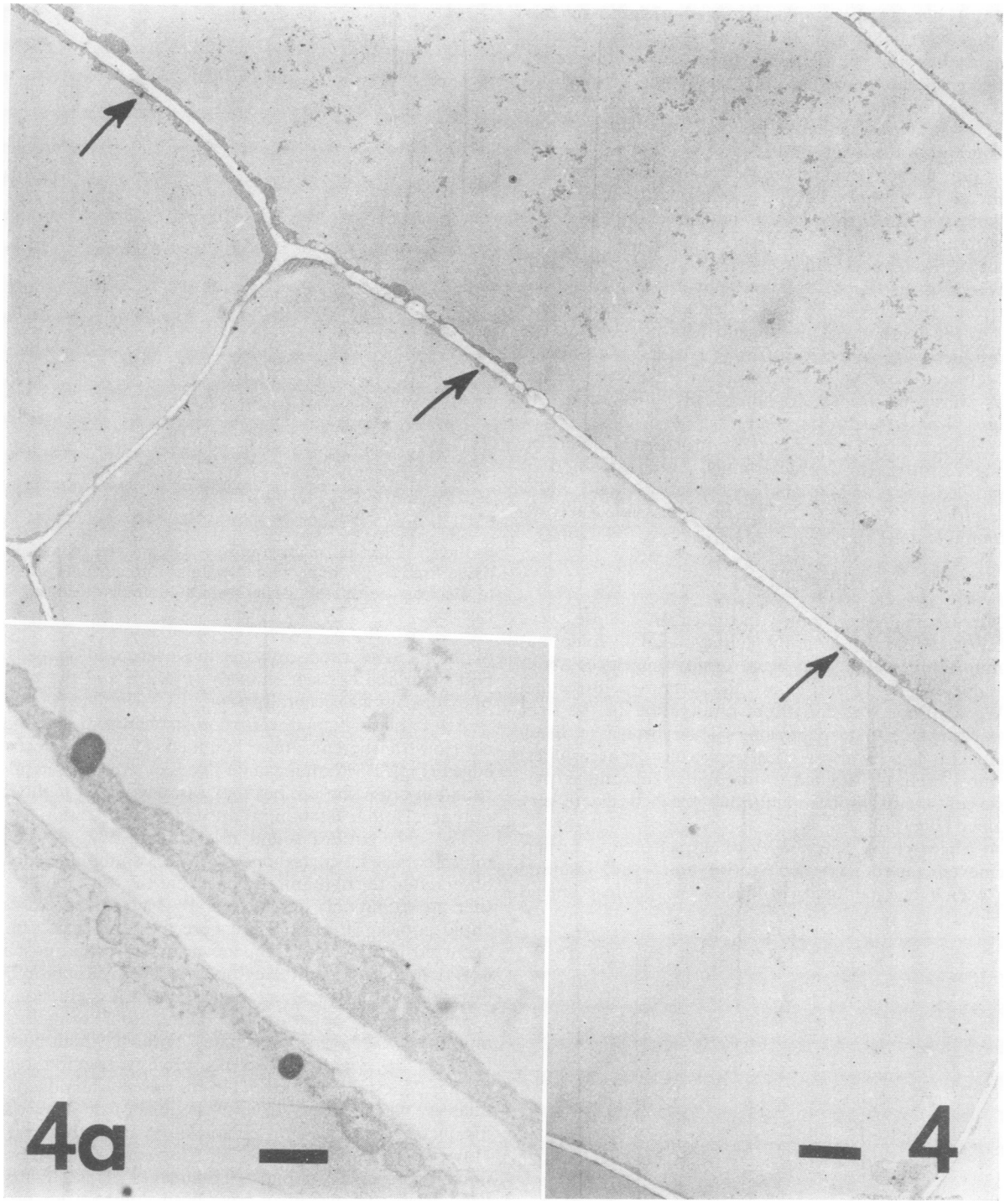

FIG. 4. A low power electron micrograph of an onion epidermal cell similar to the light micrograph shown in Figure 1. Note the intense osmiophilia of the spherosomes compared with the other cellular constituents (see arrows). Even though the electron microscope has a much greater depth of focus than the light microscope, the section is only about one-tenth of a micron in thickness, therefore fewer spherosomes are visible than in the light micrograph. Fixed in glutaraldehyde-picric acid and postfixed in osmium tetroxide. Bar indicates 10 μ. a: Higher magnification of a portion of an onion epidermal cell fixed with aldehyde/osmium with no poststaining. The spherosomes are the most intensely osmiophilic organelle in the cell. Bar indicates 2 μ.

In the electron microscope, isolated cabbage spherosomes fixed with osmium tetroxide were identical in appearance to those observed *in situ* (Fig. 6). They were bounded by a single fine-line on the order of 20 to 30 Å thick. The matrices of the spherosomes, after osmium tetroxide fixation, were uniformly electron dense with no apparent internal structure. They ranged in size from about 0.4 μ to slightly over 2 μ in diameter, averaging under 1 μ in diameter.

Isolated cottonseed oil-droplets were essentially the same as cabbage spherosomes in appearance; however, they were about two to three times as large in diameter (Fig. 7).

Biochemistry. When cottonseed oil-droplets were dried over

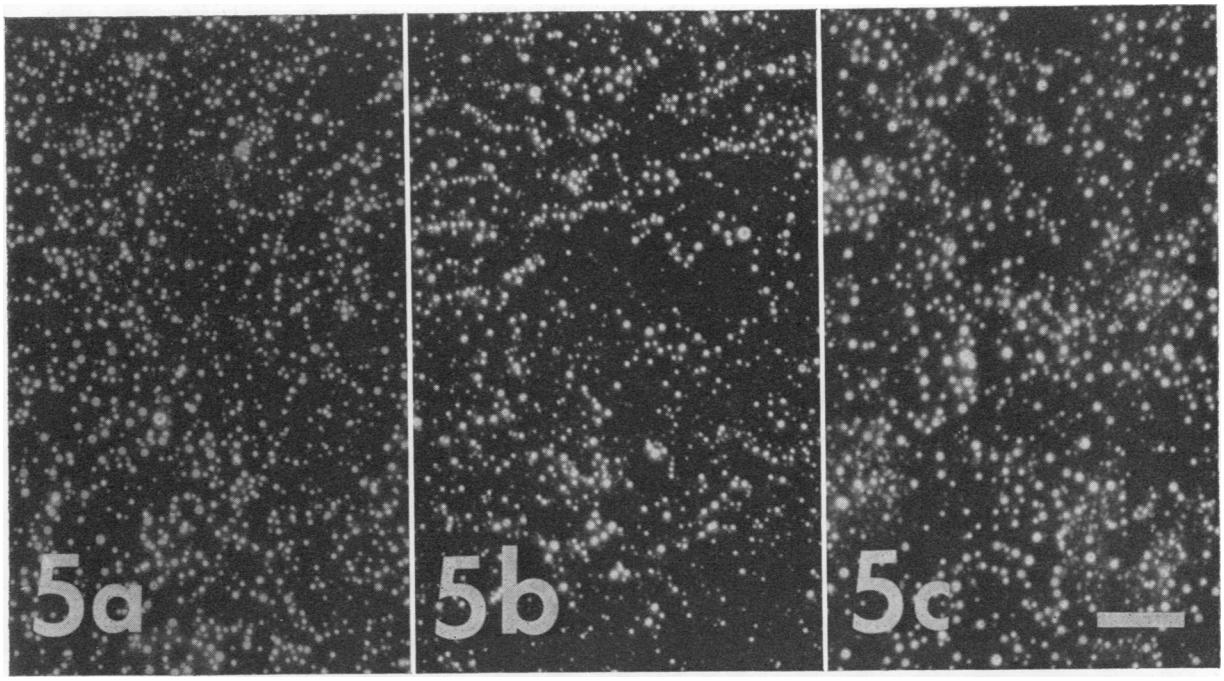

FIG. 5. Darkfield light micrographs of isolated spherosomes from; (a) onion tissue, (b) cabbage tissue, and (c) cottonseed. Bar indicates 10 μ.

P_2O_5 in a vacuum desiccator, the white, mayonnaise-like material assumed a clear, translucent, jelly-like appearance. The mass did not become a liquid but maintained a lumpy appearance, indicating a maintenance of structure. Cabbage spherosomes, on the other hand, never lost their white appearance; dried cabbage spherosomes appeared as a white, powdery mass. The bulk of cottonseed oil-droplets was lipid (98.8%). Cabbage spherosomes were also highly lipoidal, yielding 81.0% lipid (Table I).

The phosphorus content of lipid from cottonseed oil-droplets was 0.015% and cabbage spherosomal lipid 0.007%, corresponding to 0.38% and 0.16% phospholipid, respectively (Table I). Protein content of cottonseed oil-droplets and cabbage spherosomes were 0.63% and 4.0% respectively.

Since spherosomes have been shown to possess acid phosphatase activity (20), it was of interest to determine acid phosphatase activity in our spherosomal preparations. The oil-droplet fraction from cottonseeds contained virtually no acid phosphatase activity. Cabbage spherosomes, on the other hand, contained a slight amount of acid phosphatase activity; however, when distribution of the activity was analyzed (Table II), only 0.001% of the activity in the homogenate was associated with the spherosomal fraction, leading us to suspect contamination. This suspicion was corroborated by electron-microscopic histochemistry (Fig. 6c). Spherosomes allowed to react with Gomori's reagent for 4 hr showed a slight amount of acid phosphatase activity in the "fat pad" fraction. However, the activity was not associated with spherosomes; instead, it was associated with fine, membranous material found with the spherosomes as "contaminant." The presence of "contamination" was not surprising. Due to apprehension over the loss of our meager spherosomal preparation, this particular sample was not washed. All preparations of oilseed oil-droplets, on the other hand, were routinely washed and rewashed at least five times.

DISCUSSION

The object of this study was to eliminate the confusion concerning the cytological status of spherosomes, particularly at the electron microscope level, and to compare spherosomes of nonoily plant tissues with oil-droplets of oily plant tissues. In order to achieve these goals, spherosomes were isolated from nonoily plant tissues and studied in the cell-free state.

Identification of Spherosomes. Spherosomes are easily recognizable in the light microscope. They are highly refractile, rapidly displaced spherules about 1 μ in diameter which become black after contact with osmium tetroxide. Spherosomes stain so intensely that they stand out in stark contrast from the rest of the cytoplasm. There is no question concerning the identity between the bright, refractile particles and the black spherules obtained after osmium tetroxide staining; this can be shown under direct observation. Difficulty seems to arise at the electron microscope level.

At low magnification in the electron microscope, the only cytoplasmic inclusions that were intensely osmiophilic and spherical (corresponding to spherosomes observed in the light microscope) were the bodies commonly referred to as "lipid droplets" (compare Fig. 1 and Fig. 4). At these low magnifications, mitochondria and plastids, although stained, were not as conspicuously osmiophilic nor did not stand out as markedly from the groundplasm as did the spherosomes. The spherules not only exhibited intense osmiophilia, they were the same size and shape as spherosomes observed under light microscopy. We concluded, therefore, that the highly osmiophilic round bodies observed in the electron microscope (often referred to as "lipid bodies") and the highly refractile, osmiophilic spherical bodies (spherosomes) seen in the light microscope are one and the same.

One might wonder why confusion arose in the first place. Undoubtedly, the profiles observed in the electron microscope following osmium tetroxide or permanganate fixation were largely responsible for this problem. Spherosomes in doubly fixed (aldehyde and osmium) tissues are spherical bodies similar to the spherosomes seen in the light microscope; however, spherosomes in tissues fixed with permanganate or osmium tetroxide (fixatives employed prior to the introduction of double fixation) are not. Spherosomes in tissues fixed with permanganate are irregular-shaped with an electron-transparent matrix. The edges are generally very electron dense and membranes are not discernible. Spherosomes in tissues fixed

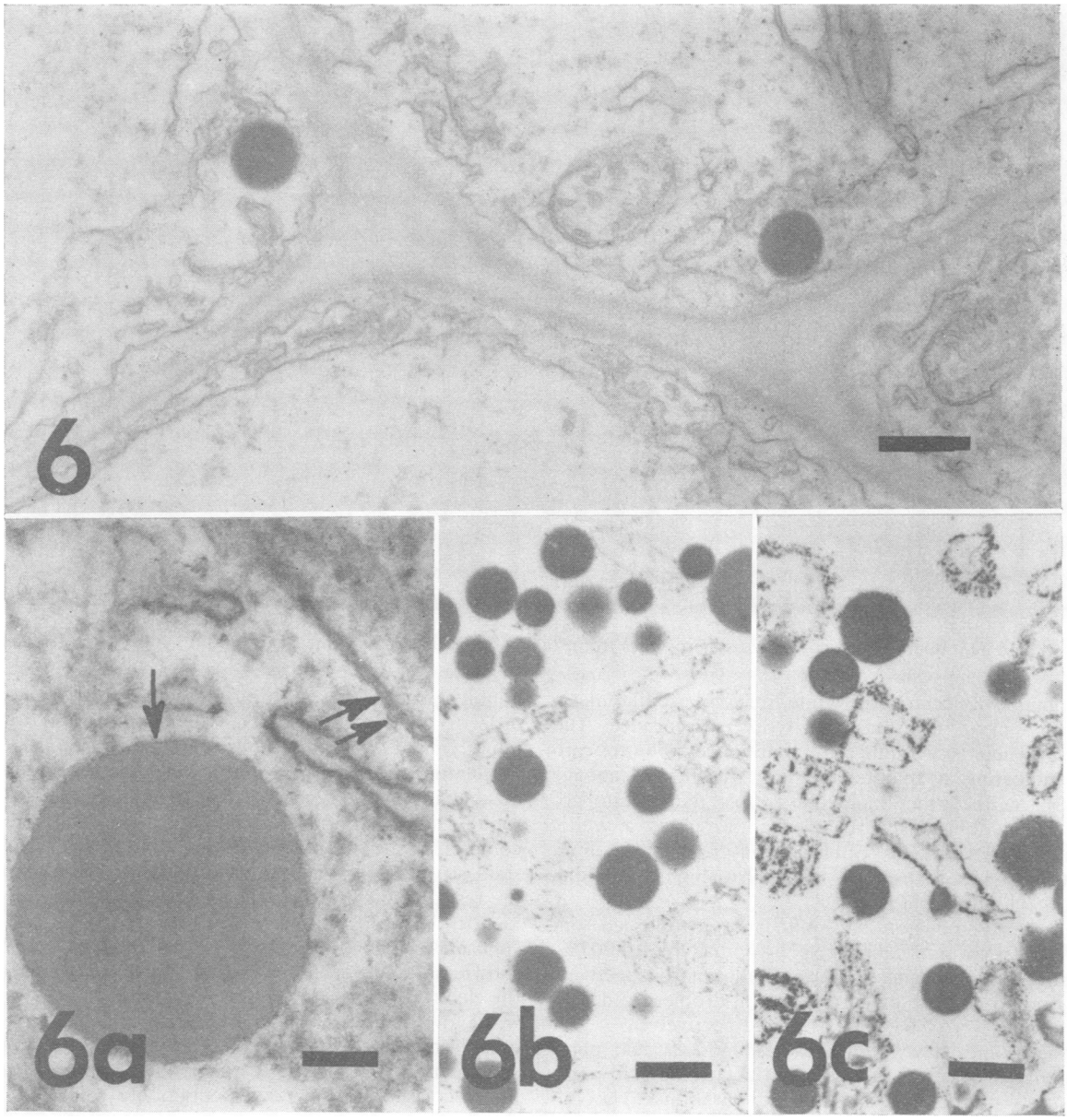

FIG. 6. Electron micrograph of a portion of a leaf cell showing the highly osmiophilic spherosomes in cabbage tissue. Fixed in glutaraldehyde, postfixed in osmium tetroxide. Bar indicates 0.5 μ. a: High magnification of a spherosome from cabbage leaf tissue fixed with glutaraldehyde, postfixed in osmium tetroxide followed by uranyl acetate and lead citrate staining. Note the "unit membrane" indicated by the double arrows and the "single membrane" bounding the spherosome indicated by the single arrow. Bar indicates 0.1 μ. b: Electron micrograph of isolated spherosomes from cabbage tissue. Note the debris between the spherosomes. Fixed with glutaraldehyde-osmium tetroxide. Bar indicates 1 μ. c: Electron microscopic histochemical study of isolated spherosomes from cabbage tissue. Note deposition of lead phosphate indicating enzymic activity associated with "debris" rather than the spherosomes. Bar indicates 1 μ.

with osmium tetroxide have very irregular and aberrant forms. Drawert and Mix (4) correctly identified spherosomes with "sternformige" bodies observed in the tissues after osmium tetroxide fixation, but apparently other workers (prior to double fixation techniques) could not, or would not, identify a star-shaped or irregular-shaped body with the neat, spherical body seen in the light microscope. In their quest for trim, round bodies (the counterpart of the neat, refractile spheres observed in the light microscope), many microscopists apparently ignored the star-shaped bodies and sought a round body. Consequently, many bodies labeled "spherosome" in the literature are not spherosomes but simply spherical organelles such as microbodies or round vesicles. Perhaps, if doubly fixed tissue had been observed first, this issue may not have arisen.

Isolation of Spherosomes. Our working hypothesis in this study was that spherosomes are lipid bodies. The known properties of spherosomes support our thesis. Firstly, lipid droplets would form spherical shapes in an aqueous milieu. Since vegetable oils have a higher refractive index than water, lipid droplets should be refractile in water. Lipid droplets would also tend to exhibit Brownian motion more readily than proteinaceous bodies because of their lower specific densities (lower inertia), and, since they are composed of highly unsaturated fatty acids, they should exhibit very high osmiophilia.

Thus, if our thesis is correct, a fraction of plant homogenate should be obtained that moves centripetally in a centrifugal field. Since low fat tissues would be expected to contain very sparse quantities of fatty spherosomes, a continuous method of centrifugation was sought to handle the large quantities of

plant material required. By utilizing a Sharples continuous centrifuge (which sediments dense particles and yields effluent continuously) together with a De Laval separator (which separates the light particles from the denser medium continuously), large volumes of material could be handled.

Although modest in quantity, a "fat pad" which was composed of fatty particles was obtained from both onion and cabbage tissues using the techniques described above. The isolated particles were identical in appearance to the spherosomes *in situ*, both at the light and electron microscope levels. They were the same size and shape, exhibited the same refractile quality, the same osmiophilia, and even the same Brownian motion.

The very low yield of spherosomes obtained from cabbages was disappointing. A rough calculation, however, shows that the yield of spherosomes is reasonably satisfactory. Assuming that 50 pounds of cabbages occupies about 1 bushel (approximately 0.35 cubic meters), half of which is air, and also assuming that the average cell is about 50 μ in diameter, one can calculate that 50 pounds of cabbages should contain about 3×10^{11} cells. If we assign a value of 0.5 μ diameter for the average spherosome, 1 ml of spherosomes would contain about 8×10^{12} spherosomes. This corresponds to about 25 spherosomes per cell, which agrees with the values Sorokin and Sorokin (20) reported for epidermal cells of *Campanula*. Therefore, although this is a crude calculation (*e.g.*, spherosomal populations vary from cell to cell, they vary in size, cell sizes vary), the yield appears to be of the correct order of magnitude.

The chemical data which were obtained show that isolated spherosomes are mainly lipid with very small quantities of phospholipid and protein. These data argue well for the correctness of our hypothesis.

Comparison between "Spherosomes" and "Oil-Droplets." Aside from differences in size, isolated cabbage spherosomes were virtually identical in appearance to oil-droplets isolated from cottonseeds. In the light microscope, cottonseed oil-droplets were larger than spherosomes from onions or cabbages, but both types of bodies were spherical, refractile, and highly osmiophilic. In the electron microscope, both entities were round in cross section, filled with an evenly stained osmiophilic ground substance (oil), and bounded by the same type of single-line membrane that is distinctly different from tripartite, unit-membranes. These particles are similar to the "lipid bodies" of corn embryo cells described by Trelease (21), namely lipid droplets bounded by a single-line membrane 25 to 40 Å in thickness.

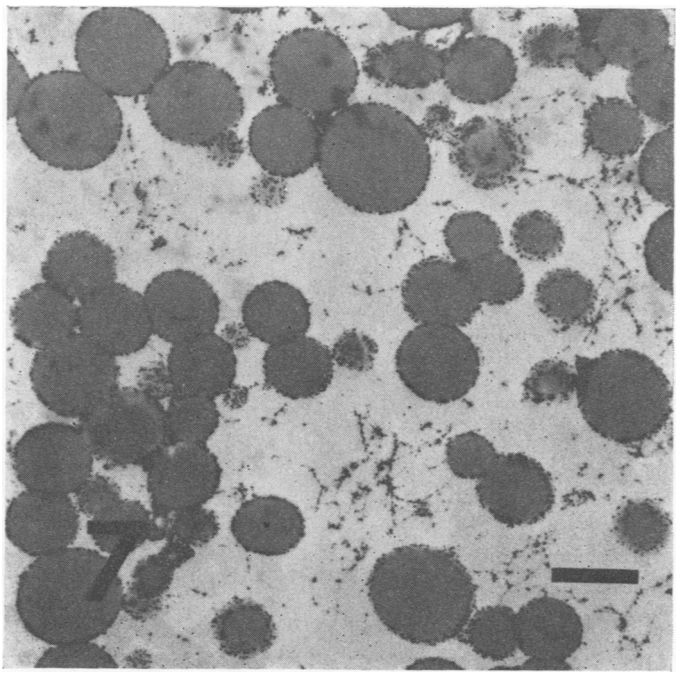

FIG. 7. Electron micrograph of isolated cottonseed "oil-droplets" (spherosomes). Fixed with glutaraldehyde-osmium tetroxide. Bar indicates 2 μ.

Table I. *Composition of Spherosomal Fractions*

Spherosomes were prepared and analyzed as described in "Materials and Methods." Values represent averages of duplicate determinations on two samples and are given as percent of the dry weight of recovered material.

Material[1]	Total Lipid	Nonlipid Residue	Recovery[2]	Phospholipid[3]	Protein[4]
	%				
Peanut[5]	99.55	0.45	100.3	0.09	0.22
Cotton[6]	98.8	1.2	100.0	0.37	0.63
Cabbage[7]	81.0	19.0	100.9	0.16	4.0

[1] Total lipid + nonlipid residue = 100%.
[2] Dry weight of sample = 100%.
[3] Phospholipid = lipid phosphorus × 24.8.
[4] Protein = nonlipid nitrogen × 6.25.
[5] Washed 10×.
[6] Washed 5×.
[7] Washed 2×.

Table II. *Distribution of Acid Phosphatase Activity in Spherosomal and Nonspherosomal Fractions*

Fractions were prepared and analyzed as described in "Materials and Methods." Values are given as percent of recovered activity.

Fraction[1]	Peanut	Cotton	Cabbage
	%		
Spherosomal	0	0	trace[4]
Nonspherosomal[2]	100	100	100
Recovery[3]	99	98	100

[1] Activities of spherosomal + nonspherosomal fractions = 100%. The original homogenate contained, per ml, 3.24 units of enzyme from peanut, 3.23 units from cottonseed and 4.48 units from cabbage. An enzyme unit is defined as the amount of enzyme which produces an increase in absorbance of 1 per 15 min.
[2] Nonspherosomal fraction is mitochondrial and supernatant liquid fractions combined.
[3] Activity in supernatant liquid after centrifuging homogenate at 1000 × g for 5 min = 100%.
[4] Trace = 0.001%.

Chemically, the composition of spherosomes from cabbages was similar to that of oil-droplets isolated from cottonseeds. Since the cabbage spherosomal preparation was not washed, a certain amount of contamination occurred; however, both types of bodies were mainly lipid with very small quantities of phospholipid and protein.

It has been reported that spherosomes possess acid phosphatase activity (20). However, we were unable to detect acid phosphatase activity in our spherosomal preparations. Since the enzymic activity (20) appeared to be a function of physiological status, this negative finding is inconclusive—the lack of acid phosphatase activity in spherosomes from the quiescent cabbage and onion tissues could be analogous to the situation

in quiescent cottonseeds. It may be of interest, however, that acid phosphatase activity was also not detected in oil-droplet preparations from 3-day germinated castor seed endosperm and 7-day germinated peanut seed cotyledons.

Our results, therefore, lead us to the conclusion that spherosomes are lipid droplets bounded by a single fine-line membrane and that they are the same as the so-called "oil-droplets" found in oleaginous plant tissues. Therefore, it is deemed incorrect to distinguish between "oil-droplets" and "spherosomes." Finally, since the name spherosome was chosen through default (17), we would like to suggest the more meaningful name "oleosome" (which has equal priority) (2) as the preferred terminology for "microsome" of the spherome.

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