The Spherosomes of *Campanula persicifolia* L.

A Light and Electron Microscope Study

By

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With 19 Figures

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Although interest in the occurrence, behavior, and structure of spherosomes in plant cells has increased recently, they remain an enigma. A decade ago they were subjected to fairly intensive investigation by light, phase contrast, and fluorescence microscopy (Perner 1952, 1953, Perner and Pfefferkorn 1953, Drawert 1953, Sorokin 1955a, 1955b, 1956, Sorokin and Sorokin 1956, Gutz 1956), which established some of the fundamental properties of spherosomes. In most cells of higher plants, these spherical cytoplasmic bodies are conspicuous for their abundance and rapid motion. They measure some 0.7–0.9 μ in diameter. In phase microscopy of living cells, the spherosome attracts attention by appearing alternately as a dark body surrounded by a halo, and as a light body surrounded by a dark rim. The history of previous observations on these bodies, and of the evolution of the terminology, has been reviewed by Perner (1953, 1958), Wildman and Cohen (1955), Steffen (11955), Dangeard (1956), Bautz (1956), and Milovidov (1964). Additional information on spherosomes has been obtained by cinematography (Honda, Hongladarom, and Wildman 1964, Mahlberg and Venketedwaran 1963), by phase and anoptral contrast (Url 1964a, Holcomb, Hildebrandt and Evert 1965), and ultraviolet light microscopy (Url 1964b). Nevertheless, little is known of the functions of spherosomes. One of the purposes of this study has been to acquire new information on the physical and chemical properties of spherosomes, on their distribution and behavior in living cells, and on the changes that occur in them when the cell dies, either naturally, or after chemical fixation. Because the spherosomes have often been considered to be lipoid inclusions, special attention has been given to their affinities for various stains.

While spherosomes are easily seen in living cells studied by light microscopy, they have not been identified with certainty in electron micrographs. Electron microscopists have not agreed which of the cytoplasmic bodies represents the spherosome (Hohl 1960, Drawert and Mix 1962, 1963, Peveling 1962, Frey-Wyssling et al. 1963). A second aim of this study has been to provide a sound basis for identification of spherosomes in electron microscopy through selection of material that contains an unusual abundance of spherosomes and through observation of spherosomes during all stages of preparation for electron microscopy.

Materials and Methods

The epidermis from the lower side of the rosette leaves of *Campanula persicifolia* L. was the principal material used in this study. It is well suited to the study of living cells because small pieces can be stripped off the leaf and examined under the microscope without damaging the cells unduly. The same material is particularly well suited to the study of spherosomes, since unusually large numbers of them are present in guard cells of the stomata. The observations made in epidermal cells were compared with others made in cells of the spongy parenchyma of *Campanula* and in the epidermis of the inner side of the scale leaf of *Allium cepa* L., a classical material for the study of spherosomes.

For the observations on living cells, the epidermal tissue was stripped off the leaf with forceps and floated on either tap water (pH 7.2) or on distilled water containing added sucrose as 5% or 9% solutions (pH 5.7). Under these conditions the cells usually survived several days. Survival was judged by the presence of spherosomal motion (Sorokin 1958) and by the accumulation of neutral red in the vacuoles of the guard cells. The *Campanula* material usually recovered rapidly from the trauma of explantation; but (as Url [1964a] has noted) the preparations of *Allium* usually required several hours before the mitochondria were restored from a rounded to their customary elongate shape, and motion of the cytoplasm was resumed.

One epidermal cell from a slide preparation was observed under the oil immersion lens for a 75-hr. period to determine the distribution and translocations of the spherosomes within. Other cells were studied for shorter periods. The tissue was mounted in water containing 0.1% ribonuclease, and the simple medium was changed at intervals. The substage illuminator was operated only during the observations.

Supravital staining was carried out on the plant tissues using neutral red as means for increasing contrast between vacuoles and cytoplasm (Sorokin 1955b) and indophenol blue (Perner 1952) and Janus green B (Sorokin 1955a) as redox dyes. In addition, succinic dehydrogenase was demonstrated within cells at pH 7.5 using nitro-blue tetrazolium (nitro-BT) (Nachlas et al. 1957) as a 0.002% solution dissolved in 0.05 M potassium succinate. As in the case of neotetrazolium (Sorokin and Sorokin 1956), use of a dilute solution of nitro-BT permits long-term survival of the cells. Finally, 0.005% aqueous solutions of rhoda-

mine B (Strugger 1938) and Nile blue A (Bailey and Zirkle 1931) were used supravitally as lipid stains and supplemented those given below.

Staining with lipid dyes was attempted on both fixed and unfixed preparations. The best results were obtained on tissues fixed in 10% neutral formalin containing 0.02% $CaCl_2$. Nevertheless, as far as the spherosomes were concerned, the fixation was only partially successful. In the same specimen spherosomes might be well preserved in some guard cells and coalesced into larger spheroids in others. During dehydration more coalescence occurred when tissues reached 70% ethanol, and, if dehydrated to 95% or 100% ethanol, only residual bodies remained. The following lipid dyes were used: 0.5% Oil Red O dissolved in 60% triethyl phosphate, Sudans III and IV dissolved in 70% ethanol or propylene glycol (Jensen 1962), and Sudan black B in 70% ethanol. As a control for the latter, preparations were extracted with acetone prior to staining. Reactions for phospholipid were carried out using Baker's dichromate-acid hematein method, as modified by Bourgeois and Hubbard (1965), as well as with the modifications of La Cour, Chayen, and Gahan (1958). The latter was applied after fixation in Lewitsky's fluid. Since the spherosomes were poorly preserved after Lewitsky's fluid, only the procedure of Bourgeois and Hubbard gave useful results.

For the light microscopy of other fixed preparations, and for the electron microscopy of the tissues, the following schedule was used: Strips of epidermis were fixed at pH 7 in phosphate-buffered 1% osmium tetroxide (Millonig 1961) and at pH 7.3 in barbiturate-buffered 3% glutaraldehyde at room temperature, followed by osmication at 4° C. After fixation the tissues were dehydrated rapidly through cold graded ethanol and, when in 100% ethanol, were allowed to warm to room temperature. Subsequently, the tissues were placed in propylene oxide, infiltrated in a 50:50 mixture of epon and propylene oxide, and embedded in epon. At each stage of this procedure a small piece of tissue was removed to a slide and examined by bright field and phase contrast microscopy. Additionally, samples taken from 100% ethanol and the mixture of propylene oxide and epon were rinsed in absolute ethanol and stained with crystal violet in absolute alcohol. They were then hydrated and mounted in glycerine jelly. In this manner, preservation of spherosomes was ascertained through fixation, dehydration, and embedding. Other sections were cut from the epon blocks and reacted by the periodic acid-Schiff (PAS) method. Identification of the bodies could be made in electron microscopy, based on their shape and number in cells as seen by light microscopy. Electron microscopy was carried out on thin sections of the embedded material, stained with lead (Karnovsky 1961), and examined in RCA electron microscopes, EMU 3F and 3G.

While spherosomes were fairly well preserved by glutaraldehyde- and osmium-containing solutions, they were less successfully fixed by 2% or 5% solutions of potassium permanganate (Mollenhauer 1959). Brown-colored precipitates, possibly of Mn_3O_4 (Rauen 1956), formed in and about the cells and obscured the spherosomes. After washing away the

surface precipitate the spherosomes were recognized, but they usually coalesced into large droplets during dehydration. Those that survived could not be stained clearly with crystal violet, owing to obfuscation by precipitates within the cytoplasm.

Results

The results of this study on spherosomes are presented in three sections. The first deals with spherosomes in living cells; the second, with staining reactions of spherosomes; and the third, with their electron microscopy.

Table 1.
Numbers of Spherosomes in Outer and Inner Layers of an Epidermal Cell of Campanula

Hr after explantation	Outer cytoplasm (apical)	Inner cytoplasm (basal)
0	—	—
24	—	—
26	—	—
34	30	—[1]
37	30	50
39	45	35
41	67	35
43	79	65
44	58	68
46	49	55
48	40	65
50	49	69
52	46	67
54	46	67
58	46	67
60	31	37
65	33	42
68	27	35
70	20	27
75	0	0

[1] Nineteen observations were made between 0 and 24 hr. Because of the rapid motion of spherosomes, they could not be counted. At 26 hr, 3 min of illumination was required to restore spherosomal motion to normal; at 34 hr, 15 min of illumination. Between 43—48 hr, only slow motion was observed after illumination; after 48 hr, no motion.

Spherosomes in Living Cells

The behavior of spherosomes in living epidermal cells has been decribed before and will only summarized. Spherosomes in such cells are conspicuous for their abundance, refractility, and motion. Their refractility is enhanced under the phase contrast microscope. Under bright field the rim of a spherosome may appear red in unstained preparations. This optical phenomenon is not exhibited by the other cytoplasmic particulates. Spherosomes move sometimes with and sometimes against the cytoplasmic stream.

They rotate, rather than oscillate (Brownian motion), which they do only in damaged cells. Motion is rapid in epidermal cells but slow in guard and spongy parenchyma cells. Overtaking one spherosome, another may pass it or join it, to form a diplosome, which subsequently separates (Sorokin 1958). This type of motion is characteristic of spherosomes and has been recorded in a short film. Nevertheless, it is not certain whether or not this motion is independent from that of the cytoplasm.

In the cells studied periodically for many hours (Table 1) spherosomal motion is rapid during the first 24 hr. Thereafter it becomes slow or stops and requires illumination for increasing periods (3 to 15 minutes) to restore it to normal. When the motion stops, the number of spherosomes can be counted. The outermost cytoplasmic layer of the epidermal cell contains spherosomes, a few chloroplasts, and almost no mitochondria. The innermost layer contains spherosomes and mitochondria, identified in other preparations by the Janus green reaction (Sorokin 1938, 1941). The mitochondria appear to lie nearer the central vacuole and the spherosomes, nearer the cell wall, much as Dangeard (1947) has observed. Between these peripheral layers the cytoplasm forms a reticulum of transvacuolar strands, which contains the nucleus, a few spherosomes, and mitochondria. In time the spherosomes tend to accumulate in the innermost layer. After 60 hr. of examination they decrease in number due to coalescence into larger bodies (diameter 1.5 μ). Still later, as the cell is dying, the spherosomes undergo autolysis. Similarly, if a living cell is killed with absolute ethanol or I_2 in KI the spherosomes coalesce into droplets of variable size. Thus, while spherosomes, unlike lipid droplets, do not coalesce while the cell is alive (Steffen 1955, Honda et al. 1964, Milovidov 1964), the may do so at its death.

Plate 1.

Figs. 1–6 illustrate guard and epidermal cells of the leaf of *Campanula persicifolia* L.

Fig. 1. Two guard cells and parts of the surrounding epidermal cells stained supravitally with neutral red. Large numbers of spherosomes (sphero) are visible in the outer, or uppermost, layer of the guard cells. Smaller numbers are visible in the epidermal cells. ×1,210.

Fig. 2. Guard cells stained with Oil Red O in 60% triethyl phosphate for 30 min. Formol-calcium fixation. Spherosomes are colorless; a few lipid droplets (dark spots) stain bright red. ×1,210.

Fig. 3. After staining with Sudan IV in propylene glycol for 24 hr, spheroidal aggregates of lipid are formed within guard cells. These stain orange-red. ×1,210.

Fig. 4. Living material stained with 0.25% Nile blue A in water. Vacuoles contract and accumulate color, while spherosomes do not become colored. They continue to move about in the cytoplasm. ×1,260.

Fig. 5. A strip of epidermis stained with Sudan black B for 4 min. Formol-calcium fixation. Spherosomes stain blue-black ;mitochondria and plastids stain gray ×1,000.

Fig. 6. Innermost layer of epidermal cells reacted for succinic dehydrogenase. Enzymic activity is indicated by dark staining. It is present in mitochondria (mito) and portions of the chloroplasts (chloro), which have become vesiculated. The spherosomes (sphero) are unreactive. Nitro-blue tetrazolium, pH 7.5. ×1,210.

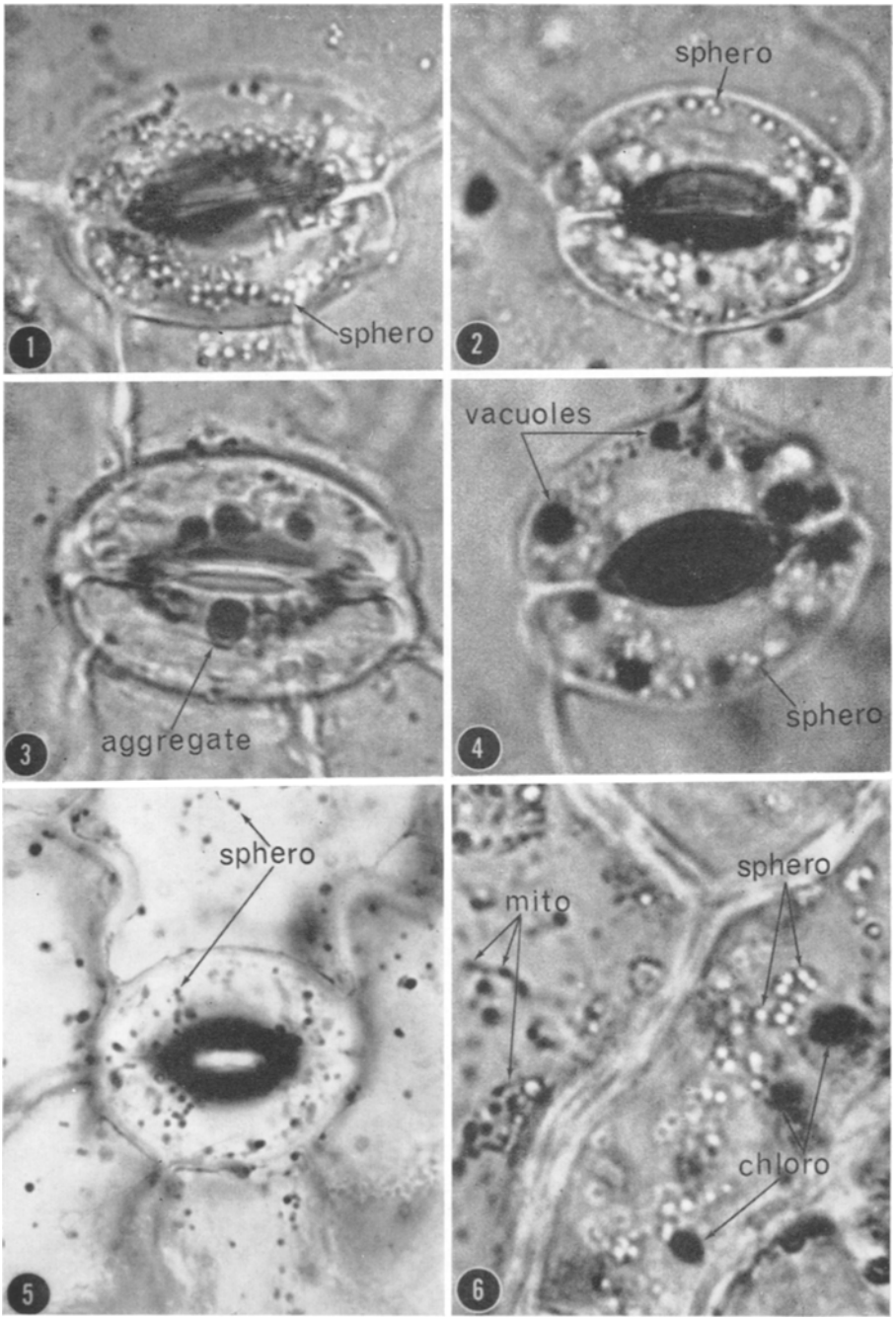
sphero
1
sphero
2
aggregate
3
vacuoles
sphero
4
sphero
5
sphero
mito
chloro
6

Plate 1.

In guard cells spherosomes are some 10–15 times more densely packed than in the larger epidermal cells. They occur in both outer and inner layers of the cytoplasm as is demonstrated in fixed and stained preparations (Figs. 7, 8). In living guard cells the spherosomes move little, but they occasionally exhibit the bumping and turning motions of epidermal spherosomes. After supravital staining, neutral red accumulates in the vacuoles of guard cells and facilitates observation of the spherosomes (Fig. 1). Although the presence of large numbers of such bodies in guard cells has not been specifically mentioned in the literature, Mix (1959) has pictured numerous fluorescent spherosomes in the chloroplast-free guard cells of *Tradescantia.* In cells of the spongy parenchyma, which contain large, fully developed chloroplasts, spherosomes are few in number; but they exhibit all the characteristics typical of spherosomes in guard cells.

Staining Reactions

All authors are not agreed on the staining properties of spherosomes. Our results are described below and are summarized in Table 2.

Lipid dyes. In general, spherosomes in either fixed or unfixed preparations are not stained by methods for simple fats or fatty acids but are stained by methods for phospholipids. This statement may be qualified as follows:

1. In fixed or unfixed preparations of *Campanula,* Sudans III and IV and Oil Red O do not stain intact spherosomes (Fig. 2). The spherosomes nonetheless are recognizable in well fixed strips of epidermis because they retain the characteristic shape and distribution of spherosomes in living cells (compare Figs. 1, 2). In fixed tissues, however, a small number of other cytoplasmic bodies are colored by the lipid dyes (Fig. 2).

Plate 2.

Figs. 7, 8, and 10 represent guard cells of *Campanula* fixed in Millonig's buffered osmium tetroxide, extracted with ethanol and acetone, and stained with crystal violet in 100% ethanol. ×1,000.

Fig. 7. At the outer surface many dark stained spherosomes are visible in the cytoplasm. Preparation mounted in glycerine jelly.

Fig. 8. Guard cells as they appear from the inner surface of the epidermis. Purple colored spherosomes (sphero) occur singly or in pairs. They are also visible in the parietal cytoplasm of neighboring epidermal cells. Chloroplasts (chloro) stain pale lavender. Preparation mounted in glycerine jelly.

Fig. 9. *Campanula* guard cells fixed in formol-calcium and stained with crystal violet in 100% ethanol. The spherosomes are not recognizable. ×1,000.

Fig. 10. Spherosomes stained with crystal violet lose color if the tissue is cleared in xylene and mounted in balsam.

Fig. 11. After osmication, dehydration, and infiltration with epon in propylene oxide, spherosomes stain well with crystal violet. They appear reddish-purple with clear centers. ×1,000.

Fig. 12. Epidermis of the inner side of the scale leaf of *Allium cepa* L. prepared as in Figs. 7 and 8. The spherosomes (sphero) stain purple. Spherical and rod-shaped mitochondria (mito) and the proplastids (pro) stain lavender. The nucleus stains lavender-brown. ×1,000.

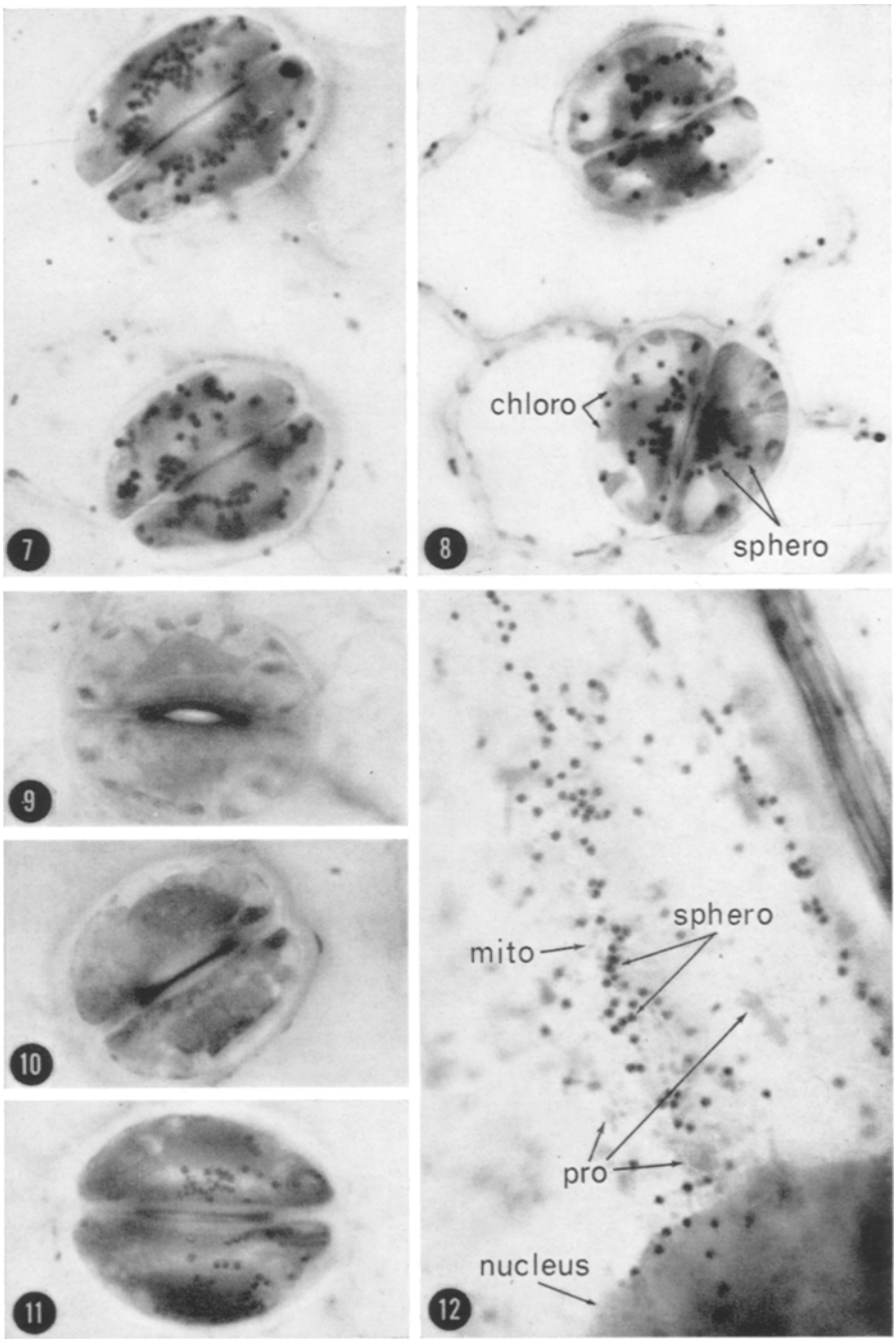

Plate 2.

After poor fixation, spherosomes are not recognizable. Instead, a few spheroids appear in the cytoplasm. When first seen they are larger than spherosomes. In time they increase in size and number. The spheroids are stainable with the Sudans (Fig. 3) and are interpreted to be "unmasked" lipids, derived partly from the coalescence of damaged spherosomes, and partly from other cytoplasmic constituents. Lipid staining in *Allium* epidermis is similar to that in *Campanula,* and we can confirm Walek-Czernecka's observation (1963) that the number and size of sudanophil particles increase in onion cells after prolonged staining.

2. Aqueous solutions of rhodamine B and Nile blue A in supravital preparations stain spherosomes slightly or not at all. After rhodamine B, for example, a faint red color seen on one side of a spherosome cannot with certainty be ascribed to the dye, because of the optical effect that reddens the rim of an unstained spherosome. In extensive experiments with rhodamine, Strugger (1938, 1949) showed that only chondriosomes (mitochondria) and leucoplasts stain electively when the dye is used supravitally, but that spherosomes stain weakly after fixation. Perner (1953) reported strong staining of mitochondria and weak staining of spherosomes. In dilute solutions of Nile blue A, *Campanula* and *Allium* cells survive for several hours. The vacuoles become stained but the spherosomes do not become colored. When stomata of living guard cells are open, the darkly stained vacuoles are contracted and permit one to see unstained spherosomes in the cytoplasm (Fig. 4). Nile blue, a mixture of components, stains both by partitioning itself between the solvent and a lipid (Schneider and Zimmermann 1922) and by acting as a basic dye (Bailey and Zirkle 1931). It also exhibits a golden yellow fluorescence, and with fluorescence microscopy, but not light microscopy, has been found to stain spherosomes (Drawert 1953, Drawert and Gutz 1953, Kuttig 1957, Mix 1959, Holcomb et al. 1965).

3. After fixation in formol-calcium, well preserved spherosomes readily are stained with Sudan black B (Fig. 5). Some spherosomes stain uniformly, while others stain only on one side and appear crescent-shaped. If neutral lipids are extracted with acetone prior to staining spherosomes can be stained subsequently; but they are difficult to recognize in such preparations. Staining with Sudan black in the absence of staining with Sudans III and IV provides better evidence than the acetone control that spherosomes contain phospolipid. Mitochondria and other phospholipid-containing cytoplasmic components also become stained with Sudan black, but they appear grayer than the spherosomes. Application of Baker's dichromate-acid hematein in general confirms the presence of phospholipids in spherosomes. In guard cells, however, the spherosomes become somewhat distorted during chromation and cannot be distinguished clearly from the numerous mitochondria present, which also stain.

Periodic acid-Schiff reaction. Thick (1 μ) epon sections of *Campanula* epidermis were reacted for carbohydrates by the PAS reaction. While starch grains in the chloroplasts stain brilliantly, and the cell walls stain moderately, spherosomes fail to stain at all.

Crystal violet. After fixation in osmium tetroxide, spherosomes can be stained intensely with the basic rosanilin dye, crystal violet, dissolved in 95% or 100% ethanol. In aqueous solutions, this dye has been used to stain the mitotic spindle, cilia, starch grains, chloroplasts, and nucleoproteins; but its application is rather tricky (Chamberlain 1924). Spherosomes do not stain with aqueous crystal violet and require prolonged dehydration in acetone or ethanol to stain with the alcoholic solution. Under these conditions the spherosomes are stained deep purple, while plastids and mitochondria stain pale lavender. Two paired guard cells are photographed from a piece of epidermis mounted on a slide with the cuticular side uppermost (Fig. 7) and reveal an abundance of purple colored spherosomes in the outer layer of cytoplasm. In a piece mounted in the opposite way (Fig. 8) the guard cells are examined from the side adjacent to the spongy parenchyma. Evidently the spherosomes abound in the peripheral cytoplasm and stain more intensely than any other cell component. They vary little in size and often occur as diplosomes, and triplosomes. In the same figures, smaller numbers of spherosomes can be seen in the peripheral cytoplasm of epidermal cells. In contrast, spherosomes do not stain with crystal violet after formalin fixation (Fig. 9). After osmication, the stained spherosomes retain their color if tissues are hydrated after staining and are mounted in glycerine jelly. On the other hand, when stained tissues are cleared in xylene, the spherosomes lose their color (Fig. 10). Once cleared in xylene, spherosomes cannot be stained with crystal violet, whether or not they were stained previously. After tissues are infiltrated in propylene oxide and epon, however, crystal violet stains the rims of spherosomes a dark reddish purple (Fig. 11). Spherosomes exhibit similar staining reactions in *Campanula* and in onion epidermis (Fig. 12). These results may be compared with those of Perner (1953). After first extracting lipids from epidermal preparations, he could stain spherosomes, mitochondria, and proplastids with rosaniline and the basic dye Victoria blue. Sometimes he found the spherosomes to stain more intensely than the other particulates.

Redox dyes. Spherosomes are not reactive to several redox dyes used supravitally. We estimate any staining of spherosomes present in these preparations to be both faint and non-specific.

Under aerobic conditions, spherosomes of living cells do not stain with Janus green B, which stains mitochondria. This finding is in agreement with those of Perner and Pfefferkorn (1953), Bautz (1956), and Milovidov (1964). After reduction of the main constituent of Janus green, other components of the dye can be seen (Lazarow and Cooperstein 1953). Some of these faintly color the spherosomes a green that differs in tint from stained mitochondria (Sorokin 1955 a, 1955 b). One of the leucoforms of Janus green is fluorescent (Zirkle unpublished) and causes the spherosomes to fluoresce after the mitochondria become colorless (Drawert 1953). In *Campanula* cells stained with Janus green, formation of a blue precipitate in A-type vacuoles (Bailey and Zirkle 1931, Sorokin 1955 b) may further confuse the unwary.

The G-Nadi reaction for the demonstration of indophenol (cytochrome) oxidase is absent from spherosomes. The cytochemical reaction product, indophenol blue, accumulates in spherosomes, mitochondria, oil globules and the cytoplasmic matrix. While Perner (1952) had interpreted the staining of spherosomes as due to enzymic activity, Drawert (1953) and Gutz (1956) considered that it followed diffusion of the reaction product from its site of formation in the mitochondria. Strauss (1956) indeed questioned the enzymic basis of the observed reaction. He found the same degree of coloration in spherosomes of test and control preparations containing sodium azide, which strongly inhibits indophenol oxidase.

Table 2. *Staining Reactions of Spherosomes in Campanula.*

Treatment before staining	Stain	Resultant coloring of different parts of the cell[1]					
		Sphero	Aggreg	Mito	Chloro	Vacuole	Wall
For/Ca	Sudan III	—	orange	—	—	—	—
For/Ca	Sudan IV	—	red	—	—	—	—
For/Ca	Oil red 0	—	red	—	—	—	—
In vivo	Rhodamine B	—	—	rose	rose	—	—
In vivo	Nile blue A	—	blue	—	—	blue	blue
For/Ca	Sudan black B	black	black	black	—	—	—
For/Ca	Hematein	black	—	black	black	—	—
OsO_4	PAS	—	—	—	red	—	rose
OsO_4	Crystal violet	purple	—	lilac	lilac	—	—
In vivo	Neutral red	—	—	—	—	red	—
In vivo	Janus green B	—	—	blue	—	—	—
In vivo	Indophenol blue	blue	blue	blue	—	—	—
In vivo	Nitro-BT	—	—	blue	blue	—	—
In vivo	Neotetrazolium	—	rose	red	red	—	—

[1] The following abbreviations are used: Sphero = spherosome; Aggreg = lipid aggregates; Mito = mitochondria; Chloro = chloroplasts.

After living *Campanula* epidermal cells are incubated for 24 hr with nitro-blue tetrazolium or neotetrazolium, the spherosomes remain colorless, while diformazan is formed on mitochondria and chloroplasts (Fig. 6). The diformazan of nitro-BT is blue; that of neotetrazolium is dark red. In chloroplasts the diformazans acquire a grayish cast, owing to the presence of chlorophyll. In a medium containing succinate, the tetrazoliums demonstrate the presence of succinic dehydrogenase at these locations. Like spherosomes in several species of *Narcissus* (Sorokin and Sorokin 1956), spherosomes of *Campanula* and *Allium* do not reduce tetrazolium salts. Nevertheless, they may acquire a faint rose color, particularly after neotetrazolium is used. This coloration of spherosomes is due in part to the optical effect that gives unstained spherosomes a reddish rim, and in part to the diffusion of diformazans and to their solubility in lipids of spherosomes. Formazans of different tetrazolium salts are soluble

in organic solvents, triglycerides, and phosphatides to differing degrees. The diformazan of neotetrazolium is more soluble in lipids than that of nitro-BT (Pearse 1960). Accordingly, it is not surprising that with the former reagent coloring of spherosomes is greater than with the latter.

Electron Microscopy of Spherosomes

Spherosomes frequently are included in thin sections of guard cells, owing to their unusual abundance in the cytoplasm. In this paper, identification of the spherosomes in electron micrographs of these cells is certain, because the spherosomes had been under observations in the light microscope during all of the stages of preparation for electron microscopy. Once spherosomes were identified in guard cells, they could be recognized as well in other plant cells, where they are scattered more thinly.

As seen in the electron microscope (Figs. 13–17), the spherosome has a structure that accords well with its structure and staining properties as seen in the light microscope. It is an ovoid to spherical body that varies in size within a range from 0.55 μ by 0.7 μ to 0.7 μ by 0.9 μ. It contains osmiophilic material and appears to be surrounded by a limiting membrane. Usually it is difficult to distinguish a trilaminar structure in the limiting membrane. Indeed, often it seems that there is present no more than a rim of material with heightened osmiophilia at the interface between cytoplasm and spherosome, like that sometimes observed at the margins of fat droplets, which lack bounding membranes. At times, however, the limiting membrane is visible (Figs. 16, 17). Usually the spherosome is seen to contain an electron permeable medullary zone surrounded by a cortex of osmiophilic material (Figs. 14–17). The medulla usually occupies the center of the spherosome and has a diameter about three-quarters that of the whole body. In guard cells, however, the medulla may be considerably reduced in size and may be displaced to one side (Fig. 13). After osmium fixation (Millonig 1961), no structure is seen within the medulla (Fig. 14); but after glutaraldehyde and osmium it is seen to be traversed by a reticulum of fine threads (Fig. 15), which are continuous with the osmiophilic material of the cortex and are comparable to it in electron density. In accepting the foregoing description of spherosomes in electron micrographs, one must reserve judgment concerning the heterogeneous appearance of the contents, for it may reflect nothing more than inadequate penetration of osmium during fixation.

In electron micrographs, as in living cells, spherosomes may occur singly (Figs. 16, 17), or in diplosomal (Fig. 14), or more numerous groupings (Fig. 15). When aligned along the cell wall, as is often the case, spherosomes may appear to contact the wall (Fig. 14). Such contact may persist in cells that have suffered some shrinkage at the time of fixation.

Discussion

Some correlations of the foregoing observations on spherosomes may now be attempted, even if their firmness is in places undermined by technical limitations.

Content. Intact spherosomes readily stain with Sudan black B, which stains phospholipids as well as neutral fat. The other Sudan dyes employed, Sudans III and IV and Oil Red O, all stain neutral fats but do not stain intact spherosomes. All four Sudan dyes are structurally related molecules that range in size from ca. MW 350–450 (Barka and Anderson 1963). Accordingly, it is unreasonable to argue that of these, only Sudan black stains the spherosomes because only it penetrates the spherosome. The alternative seems more acceptable, that staining by Sudan black indicates the presence of phospholipid in the spherosome. Lipid aggregates, formed from spherosomes and other cytoplasmic components after death of the cell, or after inadequate fixation, nonetheless are stainable by all the Sudan dyes. We interpret such stainable lipids to be result of "unmasking," or hydrolysis, of lipoprotein complexes. In this sense we agree with Perner (1952), who stated that the lipids of spherosomes are masked. Our results with crystal violet, while difficult to interpret, carry the suggestion that protein is present in spherosomes. Staining is best after unfixed lipids are removed. Finally, negative results after the PAS reaction make it seem unlikely that carbohydrate is present in spherosomes.

The apparently heterogeneous structure of the spherosomes as seen in our electron micrographs would confer on them optical properties similar to spherosomes of living cells. They would have the spherosomes' appearance of hollow spheres (Honda et al. 1964) or ring-bound bodies (Walek-Czernecka 1962, Url 1964a, 1964b). Nonetheless, the heterogeneous appearance of spherosomes may be artifactual, since similar cortical and medullary regions are at times seen in micrographs of starch grains (Figs. 13, 14) and droplets of triglyceride. Even without sure confirmation by electron microscopy, the presence of a red rim on unstained spherosomes viewed by light microscopy leads one to suspect that spherosomes possess a dense cortex. If the refractive index of the cortex is nearly that of the surrounding cytoplasm, and if the cortex absorbs green light, a red rim would appear in the microscope, because the path through the cortex is greater near the rim than near the center (Fig. 19).

Limiting membrane. In living cells, spherosomes often bump into one another without fusing, subsequently to travel together as pairs or as longer chains, and eventually to separate into units. Without a limiting

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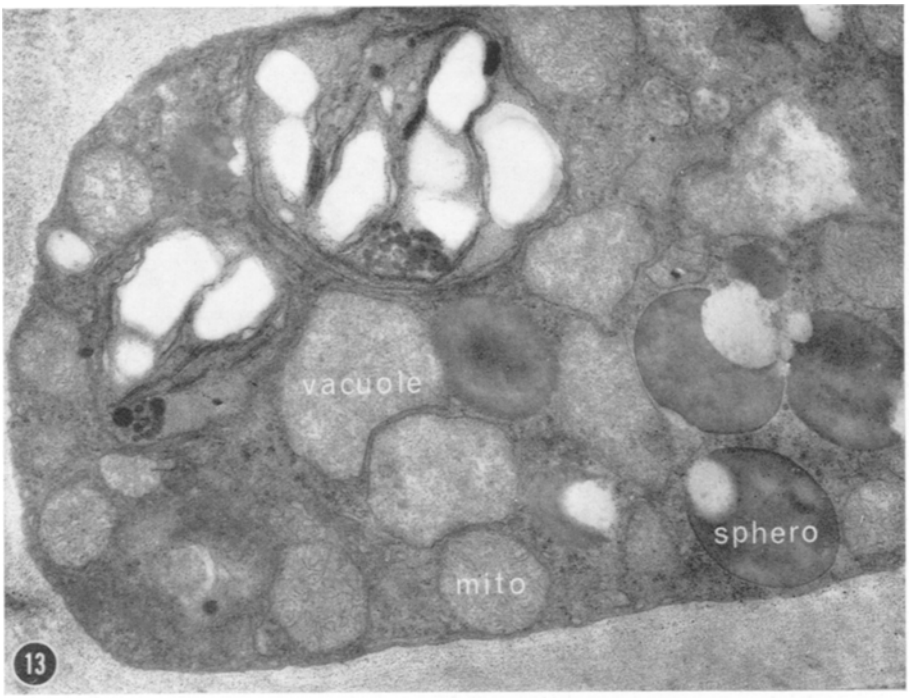
Plate 3.

Figs. 13 and 14 are electron micrographs of guard cells in *Campanula.*

Fig. 13. Low power micrograph of one end of a guard cell. The cytoplasm is rich in spherosomes (sphero), mitochondria (mito), chloroplasts, and various organelles. Numerous small vacuoles contain a finely granular precipitate. The spherosomes appear to be sectioned somewhat tangentially and display the osmiophilic cortical material. Glutaraldehyde-osmium fixation. ×18,900.

Fig. 14. A view of the mid-portion of two guard cells after fixation in Millonig's phosphate buffered osmium. Spherosomes (sphero), a chloroplast (chloro), part of the wall between the cells, and the stoma are visible. Each spherosome appears to be composed of an electron dense cortex that surrounds an electron permeable medulla. Compare with Fig. 11. ×27,900.



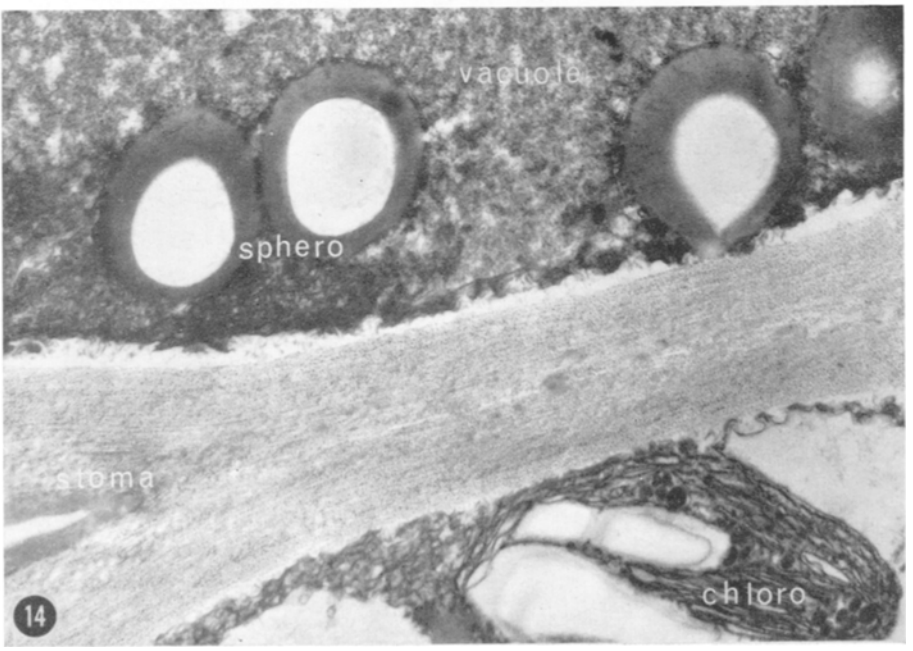


Plate 3.

membrane such bodies might be expected to fuse, were they lipid droplets, or else to mix with the cytoplasm, were they filled with water soluble materials. Consequently, a limiting membrane may be inferred to exist about a spherosome, and this is supported by evidence from electron microscopy.

Other electron microscopic studies. At times various cytoplasmic bodies have been seen in electron micrographs of plant cells and identified as spherosomes. Most frequently, identifications have been made in permanganate-fixed material, and concern the following structure: an ovoid body, irregular in outline (possibly consequent to dehydration), that contains granular material of low to medium electron density, and is surrounded by a continuous membrane 70–80 Å thick (Peveling 1962). The membrane sometimes is said to be trilaminar (Frey-Wyssling et al. 1963). Several investigators have come to essentially the same conclusions (Mühlethaler 1955, Perner 1957, Hohl 1960, Paleg and Hyde 1964). Jensen (1965 a, 1965 b), however, attempted to identify spherosomes in tissues alternately fixed in permanganate, in osmium, and in glutaraldehyde-osmium. After permanganate fixation, his presumptive spherosomes were seen to resemble those described by the authors cited, except for the presence of a small electron dense region within. After osmium or glutaraldehyde-osmium, his spherosomes seemed spherical and extremely electron dense. Drawert and Mix (1962, 1963), on the other hand, identified a different body as the spherosome. This was a stellate inclusion that resembled a lipid droplet. A third body, evidently similar to that identified by us as a spherosome, was described by Press (1957) in osmium-fixed cells from the root and shoot apices of *Cucurbita pepo*, *Hordeum vulgare*, and *Vicia faba*. Press considered it to be the equivalent of an "osmiophilic platelet," described by Bowen (1927, 1928) in osmium-impregnated plant cells as a new component of the cytoplasm. Although

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Plate 4.

Figs. 15–17 are electron micrographs of the parietal cytoplasm in epidermal cells of *Campanula*. The content of the central vacuole is precipitated coarsely. Compare with Fig. 13. Structural features of spherosomes are illustrated. Glutaraldehyde-osmium fixation.

Fig. 15. A characteristic grouping of spherosomes in the cytoplasm. Medullary regions of the spherosomes are traversed by a reticulum (ret) of fine strands. The strands are continuous with the material of the cortex and have a similar electron density. ×27,900.

Fig. 16. A view of parietal cytoplasm, showing a solitary spherosome (sphero), a Golgi zone, other largely agranular membranes, and free ribosomes. The central vacuole is at the left; the cell wall, at the right. ×49,500.

Fig. 17. Spherosome, vesicular body (vb), and mitochondrion. The spherosome appears to have a limiting membrane. The vesicular body opens into the central vacuole. ×35,100.

Fig. 18. A membrane-bound granular body in the parietal cytoplasm of a cell in the spongy parenchyma. It is comparable to a spherosome in size but not in fine structure. Glutaraldehyde-osmium fixation. ×31,500.

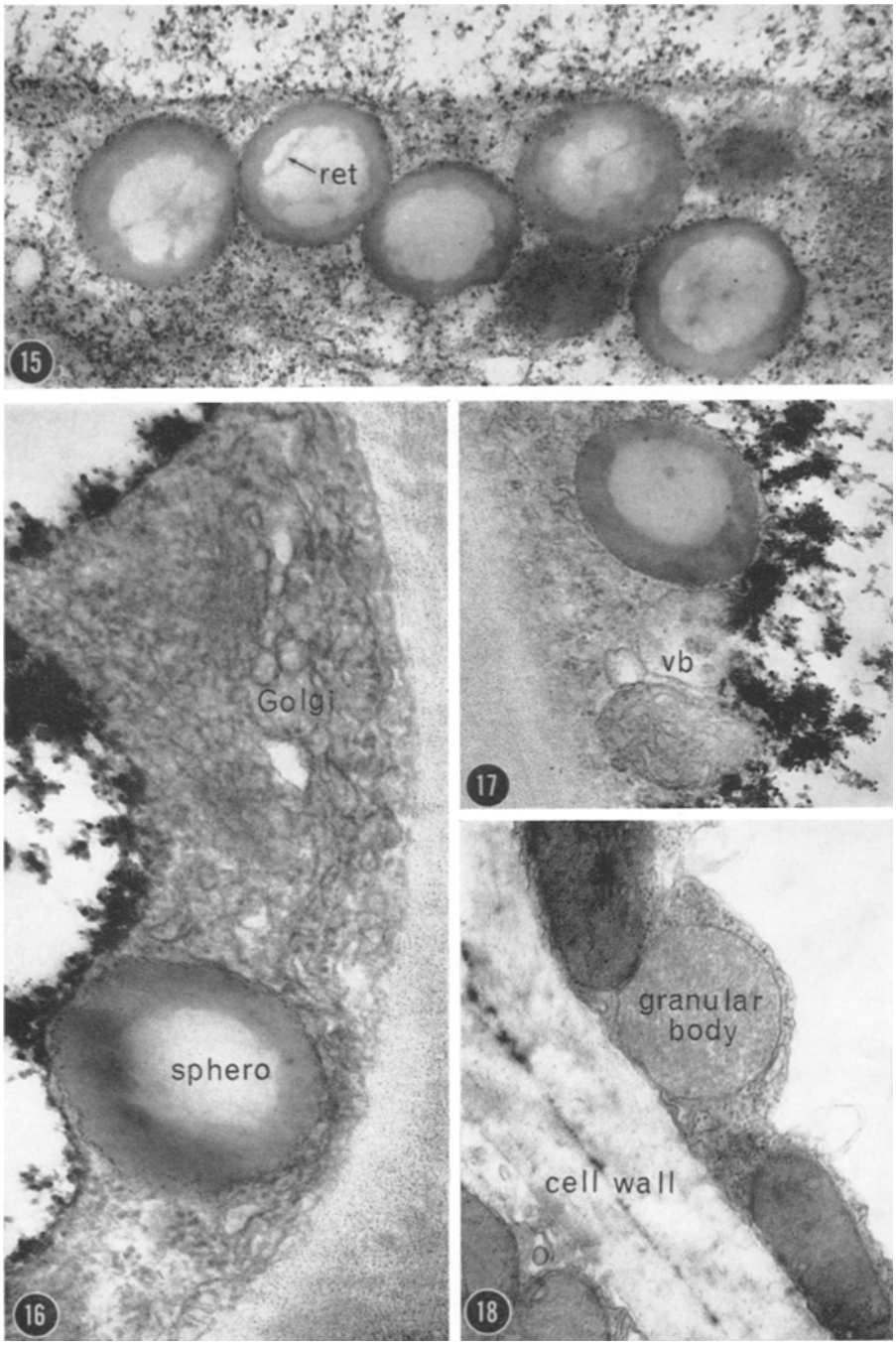

Plate 4.

Bowen considered the platelet to be homologous with the dictyosome of insect cells, Dangeard (1947) later suggested its possible identity with the microsome (spherosome).

Since we could not recognize spherosomes by light microscopy after fixation of tissue in permanganate, we did not attempt to obtain electron micrographs of such material. Consequently, we hesitate to discuss the possible identity of the bodies described as spherosomes in permanganate-fixed material with the one identified by us in osmium-fixed material. It is worth considering, however, that osmium-fixed plant cells contain bodies that differ little in appearance from those described as spherosomes in permanganate-fixed material (Fig. 18).

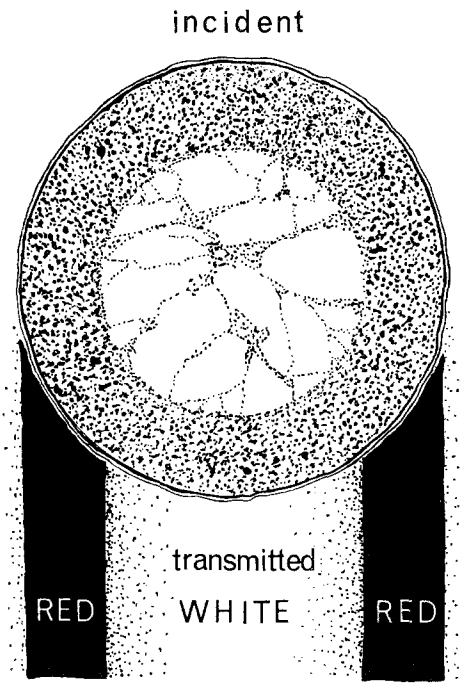

Fig. 19. Diagram of a spherosome sectioned to reveal its medulla, the surrounding cortex, and the limiting membrane. When viewed in transmitted light, the spherosome has a red rim and a white center. This may be due to the absorption of green light by the cortex, since light passing through the rim has a longer path through material of the cortex than light passing through the center.

Nature of spherosomes. Since spherosomes frequently are not identified with certainty, very little is known about their nature, origin, and function. In the coleorhiza of *Zea mays* and in the epidermis of onion, Frey-Wyssling et al. (1963) derived the spherosomes from the endoplasmic reticulum and attributed to them the function of fat production. Nonetheless, spherosomes are abundant in epidermal cells, which store very little oil; and the epidermis of onion in particular stores glycogen. That the spherosomes themselves are not droplets of storage lipid can more readily be denied: the staining reactions, the near uniformity of spherosomal diameters, and the presence of a membrane at the boundary of each spherosome all argue against the concept. The fatty reserve of pine seeds, moreover, occurs in the form of a homogeneous deposit between the aleurone grains and within the cell wall (Nyman 1965), while in the ripening fruit of *Persea* (avocado) fat deposition occurs in the absence of visible change in the spherosomes (unpublished results). These examples indicate that there is insufficient reason to associate spherosomes with storage of fat.

As a cytoplasmic constituent of plant cells, spherosomes resemble more than fat the specific granules in leucocytes of animals (Fawcett 1966). Both contain phospholipids and proteins, both are osmiophilic, and both

are limited by a membrane. Possibly spherosomes share another characteristic with the granules of heterophil and eosinophil leucocytes, since they have been reported to contain several hydrolytic enzymes associated with lysosomal functions (Walek-Czernecka 1962, 1965, Gahan 1965, Gorska-Brylass 1965, Olszewska et al. 1965). If their functions remain uncertain, spherosomes at least seem particularly to be associated with metabolically active cells, such as the aleurone tissue of barley (Hyde and Paleg 1963, Paleg and Hyde 1964), the synergids of cotton (Jensen 1965 a), and the guard cells of the leaf.

Summary

The results of a study on spherosomes in cells of the epidermis and spongy parenchyma of *Campanula persicifolia* L. are presented in three sections. The first section is concerned with observations on living material, particularly on the distribution and translocations of the spherosomes within the outer and inner layers of the cytoplasm, their "bumping" type of motion, and their coalescence into larger spheroids when the cell dies, or is killed by a number of chemical fixatives.

The second part describes staining reactions of spherosomes. Unaltered spherosomes do not stain with the lipid dyes, Sudan III and IV, Oil Red O, rhodamine B, and Nile blue A. They stain with Sudan black B and acid hematein. They are PAS-negative. Spherosomes do not react with several redox dyes used supravitally, including Janus green B, indophenol blue, neotetrazolium chloride, and nitro-blue tetrazolium. We estimate any staining of spherosomes in such preparations to be faint and non-specific. Spherosomes are also unaffected by ribonuclease. Excellent staining of spherosomes can be achieved with crystal violet in 100% ethanol after fixation in osmium tetroxide and partial extraction of lipids.

The third section describes the electron microscopy of these bodies. After observing spherosomes during all of the stages of preparation for electron microscopy, we can identify them with certainty in electron micrographs. Spherosomes occur singly, in pairs, or in larger groups. They are ovoid or spherical membrane-bound bodies that contain an osmiophilic material.

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