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THE SPHEROSOMES AND THE RESERVE FAT IN PLANT CELLS[1]

HELEN P. SOROKIN

The Radcliffe Institute, Cambridge, Massachusetts

ABSTRACT

The deposition of reserve fat is studied in plants with a high, medium, or low lipid content, and is contrasted with the spherosomes of the same cells. In storage tissue with a high lipid content the reserve triglycerides have the form of comparatively large globules which are quite distinct from the spherosomes. In plants with a medium lipid content the reserve fat appears in the form of granules, globules, or oil-plasm, and as a homogeneous, interstitial deposition between the protein bodies. Plants with a low lipid content contain a very large number of spherosomes and only very few small sudanophil globules. The spherosomes and the reserve fat represent distinctly separate entities.

THIS INVESTIGATION continues the study of the spherosomes and lipids which was originally carried out on the epidermal and guard cells of *Campanula persicifolia* L. (Sorokin and Sorokin, 1966) and extends it to the vegetative and storage tissues of several plants. It was found in the earlier paper that the spherosomes contain phospholipids, are of uniform diameters, and have a limiting unit membrane at the boundary of each spherosome, which in the living cells prevents their coalescence. The well-known figures of diplosomes represent a temporary association of two spherosomes which breaks up easily. In contrast to the spherosomes, the reserve oil droplets contain neutral fat, have a wide range of diameters, do not have a limiting unit membrane, and easily coalesce into larger aggregates. Nevertheless, the spherosomes and the oil droplets are frequently confused in the literature, particularly in electron micrographs (Yatsu, 1965; Engleman, 1966; Horner and Arnott, 1966), and the regular reserve lipids of the oil-bearing seed are often referred to as spherosomes. Because of this misinterpretation of the spherosomes, and also because of the difficulty in obtaining electron micrographs of triglycerides, which are highly soluble in organic solvents, a comparison has been made at the light microscope level of the living and fixed cells with various amounts of lipids in parallel with the spherosomes of such cells. Accordingly, plant organs with a high, medium, or low lipid content in the tissues have been selected from a table by Butt and Beevers (1966) and the mode of deposition of fat in such cells has been studied.

MATERIALS AND METHODS—The fruit pericarp of the avocado (*Persea americana* Mill.) and the coconut endosperm (*Cocos nucifera* L.) have been selected to represent cells with a high lipid content (about 65–72%, dry weight); sunflower seeds (*Helianthus annuus* L.) and rapeseed (*Brassica napus* L.), cells with a medium lipid content (about 22–36–49%); and the fruit mesocarp of the Hubbard squash (*Cucurbita pepo* L., percentage of fat unknown), those with a very low reserve lipid content.

The endosperm of the coconut and the pericarp and mesocarp of the avocado and the squash were sectioned directly by freehand. The ungerminated seeds were soaked for 3 hr in distilled water to allow for partial hydration before sectioning. The smaller seeds were held firm between slit pieces of pith during sectioning. The sections were either floated on distilled water containing added sucrose as a 9% solution, or were fixed for 6 to 10 hr in 10% neutral formalin with 0.02% $CaCl_2$. The thick freehand sections (50–100 μ) permitted observations in vivo of the large intact parenchyma cells, both under phase-contrast and bright-field microscopy. In the living cells the spherosomes were identified and distinguished from mitochondria by their refractivity, typical motion (Sorokin, 1958), and negative reaction to Janus green B (Sorokin, 1955). In the fixed material, after the extraction of the neutral fats, the spherosomes were distinguished from the mitochondria by their stronger affinity for a basic rosanilin dye, crystal violet (Coleman and Bell Co.), dissolved in 95 or 100% ethanol (Sorokin and Sorokin, 1966). Staining with lipid dyes was carried out on both fixed and unfixed preparations. The neutral lipids were demonstrated by staining with a 0.5% solution of Oil Red O in 60% triethyl phosphate (Gomori, 1952; Bourgeois and Hubbard, 1965).

To demonstrate the total lipids, Nile blue A was used both as 1% (I), and as 0.02% (II) solutions in distilled water (Jensen, 1962). The hydrolyzed solutions of Nile blue contain blue salts and red-colored oxazone, which is present only at higher concentrations of Nile blue. In

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Home address: 8 Cliff Street, Winchester, Mass. 01890.

my experiments with the sample of Nile blue A (National Aniline Company) the neutral lipids became rose-colored (rather than red), if the staining was carried out rapidly; otherwise the preparations became obfuscated. Thus a rose color after Nile blue (I) indicates the presence of triglycerides (Lison, 1960), while blue may indicate fatty acids, or else is of no significance because other parts of the cell might also take the dye. To check the effects of fixation, staining, and embedding on the form of the oil deposition, the cells were photographed both unstained and stained, and also before and after extraction of neutral fat by absolute alcohol.

RESULTS—*Avocado*—The parenchyma cells from the pericarp of the avocado fruit store oil in the form of sizable globules ca. 5–8 μ in diam. In undamaged cells the globules are fairly constant in size and do not range down to the size of the spherosomes. The latter measure 0.8 μ in diam, exhibit typical motion in the cells with a smaller amount of stored oil, and are conspicuously black under phase contrast, while the oil globules are medium refractive and appear light; the number of globules is seen to be as high as 70–100 per cell (Fig. 1). However, a slight change in the environmental conditions, such as a pressure on the cover glass, a change in the *p*H of the mounting medium, and particularly the application of solvent for the Oil Red O dye (70% ethanol, or 60% triethyl phosphate), causes the globules to coalesce into aggregates of various size which often occupy the greater part of the cell. The fixation in formol-calcium does not make the lipids insoluble in organic solvents, but it somewhat reduces the coalescence of the globules. Thus after fixation in formol-calcium and staining with Oil Red O dissolved in triethyl phosphate, the globules of neutral lipid stain red selectively (Bourgeois and Hubbard, 1965) and range in size from 8.5 μ to 27.5 μ (Fig. 2), as compared with the range of 5–8 μ seen in unfixed and unstained material. In the cells stained with Oil Red O, the spherosomes remain colorless (Fig. 2, arrows).

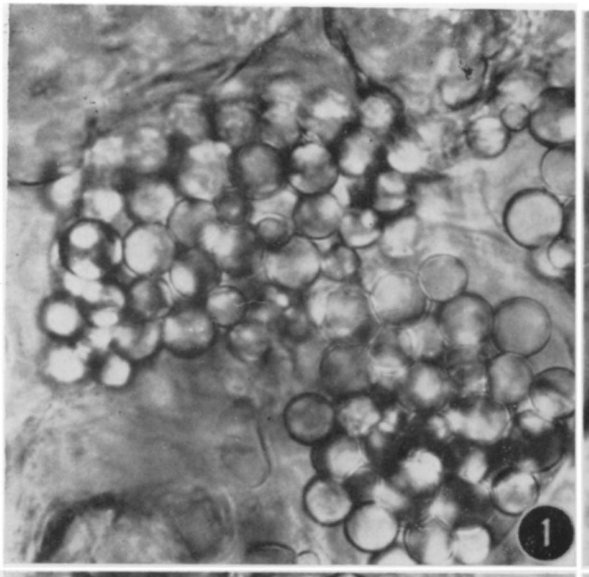
After staining with Nile blue (I), the sudanophil globules coalesce into larger bodies and become rose-colored, while one or rarely two brilliant blue spheres 3–5 μ in diam appear in every cell (Fig. 3, bb). These bright spheres do not take Oil Red O and can be seen as highly refractive colorless bodies in preparations stained with that dye. Both the bright spheres and the sudanophil globules dissolve in 100% ethanol without leaving any residue (Fig. 4). Since the unsaturated fatty acids are predominant components of the glycerides of the pericarp in the avocado (ca. 92%; Butt and Beevers, 1966), it is very likely that they are located in the sudanophil globules, while the bright-blue globules seen after Nile blue staining may represent the location of the free fatty acids which are found usually in small quantities. After the extraction of the lipids that are soluble in 100% ethanol or acetone, small spheres and granules remain visible in the cytoplasm (Fig. 4). With crystal violet these stain a deep purple or lavender, corresponding to the spherosomes and mitochondria, respectively (Sorokin and Sorokin, 1966).

Coconut—The fat content in the endosperm cells of the coconut varies. Certain cells contain a large quantity of fat, and it becomes impossible to identify any other components, whereas other cells with less fat produce a very good picture of phase contrast in which the spherosomes and the mitochondria are easily recognizable. However, the characteristic motion of spherosomes by which they are identified is usually absent, probably because of the dormancy of the seed.

The fat appears in the form of globules which range in size from the limit of visibility to very large bodies of 100 μ by 150 μ, which may occupy the whole width of the cell (Fig. 5). Both unfixed and fixed material stain well with Nile blue (I); the oil globules become bright rose-colored, and a small sphere, present in almost every cell, stains amethyst blue (Fig. 5, ab). In all probability this smaller globule corresponds to a similar body in the avocado cell.

After staining with Oil Red O, the oil globules of the same cell might show a difference in the amount of dye dissolved, appearing either bright red or paler red (Fig. 6). The significance of this is not clear; perhaps it results from the difference in the solubility of the dye in triglycerides containing different fatty acids. According to the table in Butt and Beevers (1966), coconut fat contains predominantly saturated acids (94%), with the lauric acid as the major component (51%), followed by myristic acid (18.5%), and palmitic acid (7.5%), while some other saturated acids plus a small amount of unsaturated acids make up the rest. All the neutral fat of the coconut endosperm is readily dissolved in 100% ethanol without leaving any residue in the cells. When stained afterwards with crystal violet dissolved in 100% ethanol, the spherosomes become visible and stain purple, while the mitochondria appear as lavender bodies, similar in color to the organelles in the avocado pericarp cells. Thus in the endosperm of coconut as well as in the pericarp of avocado, the spherosomes and the reserve fat represent distinctly separate entities.

Sunflower—Sunflower seed is exalbuminous, and the main reserve materials are stored in the cotyledons. The lipids constitute 22–36% of the dry weight and appear in different cells of the cotyledon in varying quantities and diverse forms. The fatty acid composition of the sunflower triglycerides is equal to 85.9% of the unsaturated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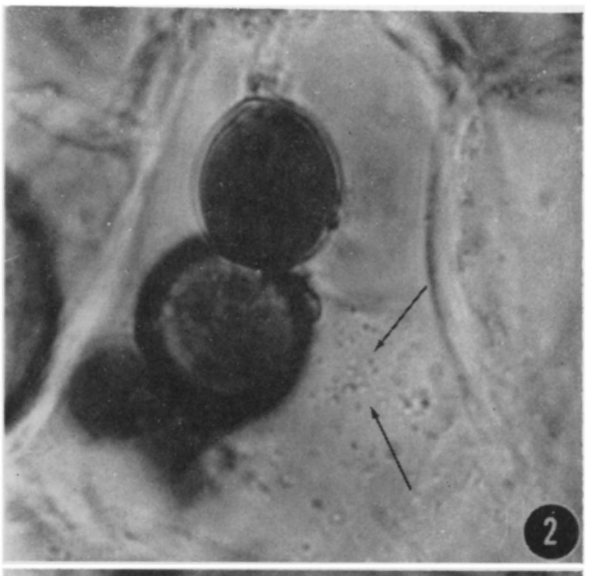
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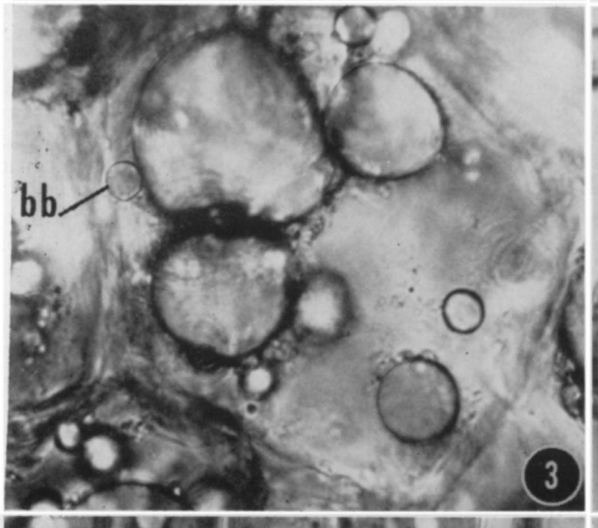
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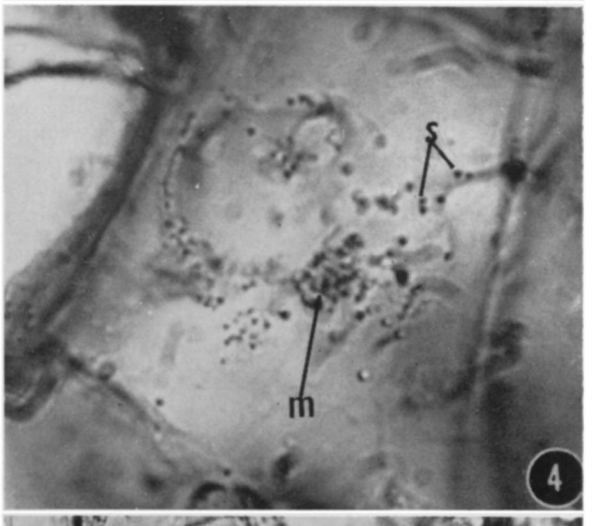
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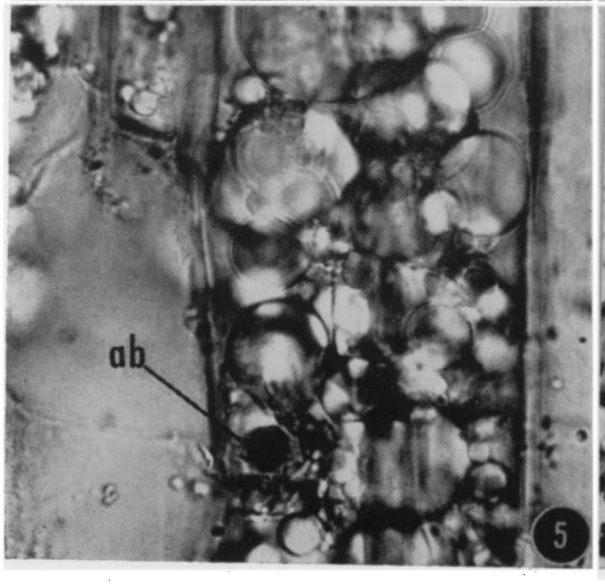
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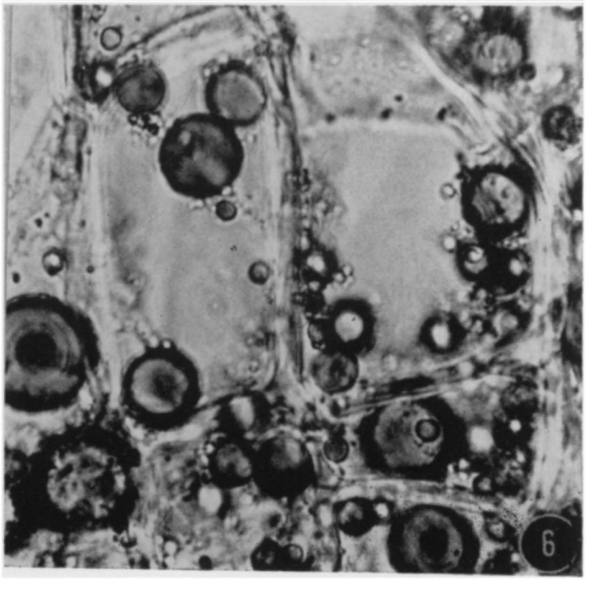
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and 14.1% of the saturated acids (Butt and Beevers, 1966). When the tissue is fixed in formol-calcium and stained with Oil Red O (Fig. 7, 8, 10, 11), the epidermal cells show red-colored granules and aggregates of fat. In the palisade parenchyma cells oil appears in two forms: (a) as a granular and globular deposit and (b) as a homogeneous network found in the interstices between the protein grains (aleurone) (Fig. 8, 10, 11). There is also a noticeable concentration of oil-plasm at the polar ends of the cells which form the palisade parenchyma tissue. Since there are several rows of palisade cells, the accumulated oil at the ends of the cells forms wide red-colored bands throughout the tissue (Fig. 7, arrows). The fat found in the interstices also liquefies easily and concentrates at the polar ends of the cells, as is seen in the central cell of Fig. 8. In the subepidermal and parenchyma cells from the abaxial side of the cotyledon the interstitial fat (Fig. 10, if) is found only in the cells with distinct aleurone bodies, whereas other cells of the same tissue contain red-colored granules of neutral fat (Fig. 10, gf) in addition to some other colorless inclusions. The distribution of red-colored oil in the transverse sections indicates that the interstitial network is three-dimensional (Fig. 11, arrows) and that it occurs in the cells with characteristic aleurone bodies. Such a distribution of fat may be observed in seeds which have undergone a period of dormancy, since in the ripe seeds collected from the head of the sunflower the oil is quite liquid and coalesces into a very large mass which forms large globules and may also line the cell wall and collect in the acute cell ends (Fig. 9).

Because the cells of a sunflower cotyledon show a great affinity for Nile blue A (I), the staining has to be very short to avoid obfuscation. In material fixed in formol-calcium, the oil-plasm stains rose-lavender, and the smaller and larger globules become rose-colored. However, the staining is less satisfactory as compared with Oil Red O, because the storage proteins stain deep blue and obscure the lipid reaction.

After dissolving the oil with absolute ethanol and staining the preparation with crystal violet, the aleurone bodies become purple and are clearly outlined by the transparent spaces around them which were previously occupied by the interstitial oil. It is difficult to say whether the interstices contain still another substance than oil, as is suggested by Bagley et al. (1963), who cite that a fraction isolated from peanuts by Dieckert et al. (1962) has the appearance of a network and contains 7% nitrogen.

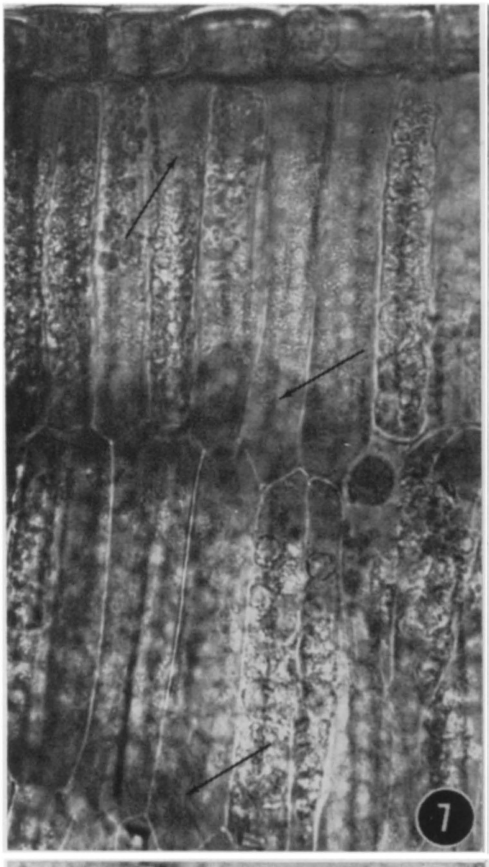
The iodine in the potassium iodide test for starch is always negative, but occasionally a few Maltese-cross granules are seen under polarized light in the cotyledon of the young seed. Under a phase contrast, dark spherical bodies of the size typical for spherosomes are seen, but because of the lack of motion a positive identification is lacking. The liquid oil of sunflower cells easily traverses the cell wall and is often found in the intercellular spaces of a young shoot (Fig. 12).

Rapeseed—The quantity of oil stored in the seeds of *Brassica campestris* varies from 22 to 49% of the dry weight. The percentage of unsaturated acids in these triglycerides is very high (ca. 95%) with the erucic acid, characteristic of the *Cruciferae* family, occupying first place (55%; Butt and Beevers, 1966). Although the precise figures for the fat content are not given for *Brassica napus*, it is assumed that they closely resemble those for *Brassica campestris*.

The transverse section through the axis of the embryo shows the oil-bearing cells scattered in a net-like pattern throughout the ground parenchyma (Fig. 13). The cells with a large quantity of oil alternate with those storing other reserve substances, and the whole central region is surrounded by a band of cells containing aleurone grains. When stained with Oil Red O, the fat appears in the form of granules, globules, and the homogeneous accumulation in the cells on both sides of the aleurone layer (Fig. 14, gf). The interstices between the aleurone grains (Fig. 14, if) also take the dye and acquire a red color of a somewhat lighter tint, as compared with the dark red of the cells containing oil predominantly. The difference in color may indicate a different composition of the oil found in these locations, since the interstices stain blue after Nile blue (I) (Fig. 16, arrows), but not rose, which is typical for neutral fats.

In the cotyledon fixed in formol-calcium and stained with Oil Red O, the oil is deposited in two forms: (a) as a bright red, thick, homogeneous lining of the inner surface of the cells, which appears light in the photomicrographs (Fig.

Fig. 1–6.—Fig. 1–4. Pericarp of avocado.—Fig. 1. Reserve oil in form of globules 5–8 μ in diam; 9% sucrose, in vivo with green filter VG 9, × 1000.—Fig. 2. Oil globules coalesce into large spheroids and stain deep red. Spherosomes colorless, visible in cytoplasm (arrows); For/Ca, Oil Red O, × 1600.—Fig. 3. Oil globules vary in size and stain rose; one sphere is bright blue (bb); For/Ca, Nile blue (I), × 1000.—Fig. 4. A cell treated with For/Ca, Oil Red O, 100% ethanol, and crystal violet. All reserve fat is dissolved without leaving any residue; spherosomes (s), and mitochondria (m) stain purple and lavender, respectively, × 1600.—Fig. 5, 6. Endosperm of coconut.—Fig. 5. Neutral fat stains rose; one globule stains amethyst (ab); For/Ca, Nile blue (I), × 1000.—Fig. 6. Red globules differ in size and in intensity of color; For/Ca, Oil Red O, × 1000.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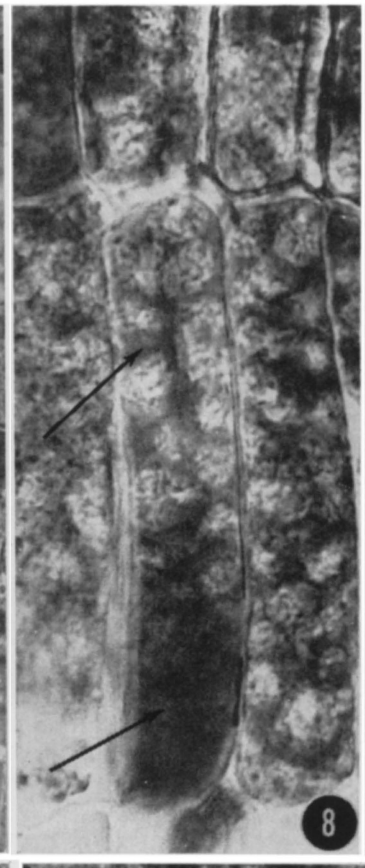
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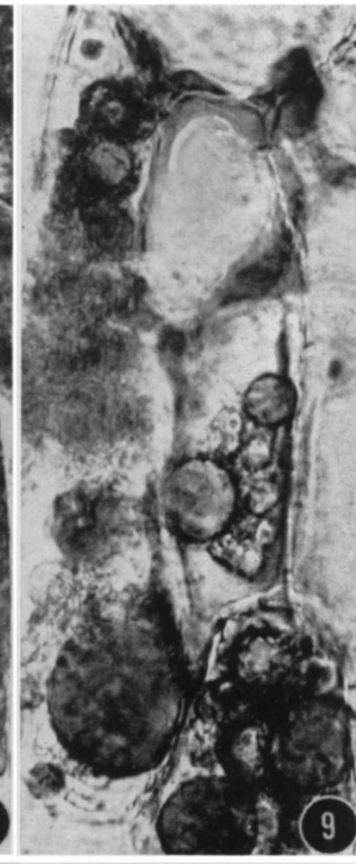
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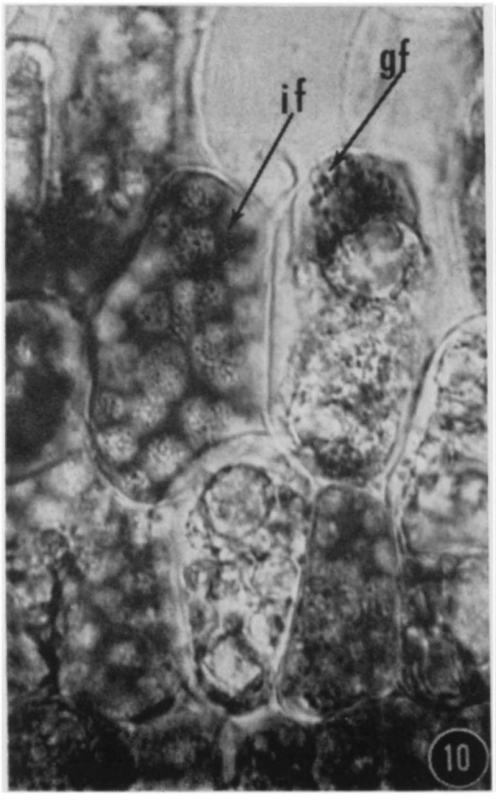
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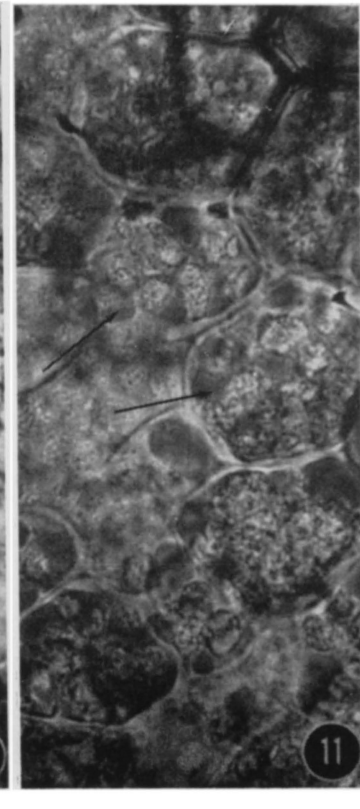
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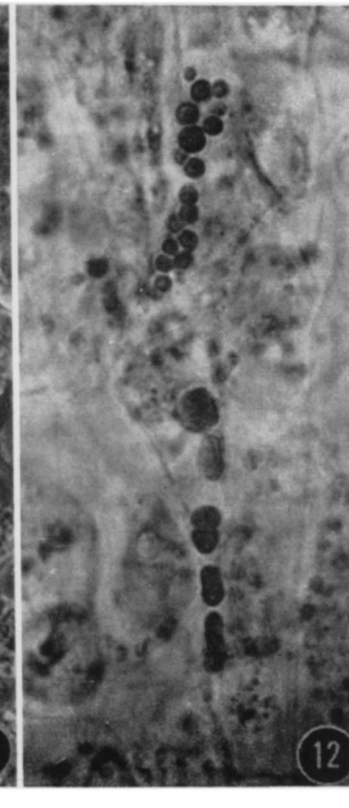
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15, br); and (b), as numerous dark red-stained granules which fill the cytoplasm of the cell and photograph black (Fig. 15, dr).

The oil is readily extracted from the unfixed tissue and is frequently seen accumulated in large globules on the surface of the sections. Conversely, the large globules are easily broken into the small spherical bodies which occur in hastily made preparations. Colorless, spherosome-like bodies are frequently seen in the sections stained with Nile blue (II), but because spherosomal motion was not observed, a positive identification of spherosomes is lacking. Generally, the cells of the plants belonging to the *Cruciferae* survive poorly when explanted in a medium, and therefore cytoplasmic motion cannot be seen.

Hubbard squash—The parenchyma cells from the fresh mesocarp of the Hubbard squash survive well when mounted on the slide in 9% sucrose and they represent a favorable material for observing the spherosomes. They are distributed in the parietal layer of the cytoplasm as well as in the trabeculae traversing the vacuole. There are two types of spherosomes; the larger measure 0.8 μ in diam and the smaller 0.3 μ without any transitional size between the two types being observed. The spherosomes show very good motion during which the bumping of two spherosomes, their association into a diplosome, and their disassociation are constantly observed. After the motion subsides they can be photographed even without staining under bright-light illumination (Fig. 17) and appear as black spheres with a white halo, or, at different depth of focus, as white spheres with a black rim. After staining with Oil Red O, a few small red globules appear in the cell, indicating the presence of a small amount of neutral fat. If after the staining the same preparation is made to react with I_2 in KI, the proteins take on a yellow color, the starch becomes purple-black, and the small red globule of oil remains visible (Fig. 18, o). Because of the larger size of the cell, the photograph has been made at a lower magnification, and the spherosomes appear smaller than in Fig. 17.

DISCUSSION—Although lipids can be extracted from almost all plant tissues, only the neutral fats are quantitatively important as components of the storage tissues (Nyman, 1965; Butt and Beevers, 1966). Phospholipids, on the other hand, make up the larger proportion of the lipids of vegetative cells which do not store oil, and the content of simple lipids is usually very small in such cells. The pathways of synthesis of both the unsaturated and the saturated acids from the acetate are still undetermined, but there is evidence indicating that the isolated particles of cytoplasm participate in this process (Butt and Beevers, 1966). Thus oleic acid has been synthesized from two-carbon fragments (acetyl CoA) by particles from the avocado pericarp (Mudd and Stumpf, 1961), and from lettuce chloroplasts (Stumpf and James, 1962). More specifically, it has been suggested that fatty acids are made in the cytoplasm in association with the endoplasmic reticulum, but that the triglycerides themselves are synthesized in the mitochondria (Jensen, 1964, p. 71). When the particles of homogenized preparations are isolated by centrifugation, they usually represent a mixture of mitochondria, spherosomes, and fragments of both the plastids and the nuclei. It is possible to distinguish spherosomes from mitochondria by their greater affinity for crystal violet in the cells of avocado pericarp after extraction of the triglycerides with absolute ethanol. It is impossible, however, to say which of these two types of particulates is actually participating in the synthesis of fat and what its role is in this process.

In cells of the coleorhiza of corn seedlings and in two cruciferous oil seeds (*Brassica napus* and *Sinapsis alba*), Frey-Wyssling, Grieshaber, and Mühlethaler (1963) attempted to trace the development of spherosomes. They claimed that spherosomes originated from enlarged fragments of the endoplasmic reticulum and attributed to the spherosomes the function of producing neutral fat, which they thought was synthesized in the central part of the spherosome. This theory, based mainly on electron micrographic studies of Grieshaber (1964), is now considered by other scientists in the same institute to be in need of revision. They demonstrated the presence of several hydrolytic enzymes in isolated spherosomes, such as acid phosphatase, acid ribonuclease, acid protease, and non-specific esterase, and suggested that these cytoplasmic bodies are sites of catabolic activity in the cell. Whether or not they have anabolic functions as well remains undetermined (Matile et al., 1965; Balz, 1966).

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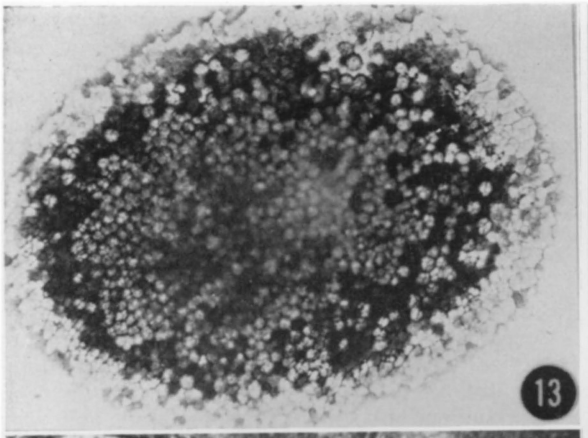
Fig. 7–12. Reserve oil distribution in sunflower; fixed in For/Ca, stained with Oil Red O.—Fig. 7. Palisade parenchyma cells from cotyledon. Oil-plasm is concentrated at the ends of the cells forming red-colored bands throughout the tissue (arrows), × 640.—Fig. 8. Interstitial oil forms three-dimensional network around the aleurone grains; it is partially liquefied and is concentrated at the bottom of the central cell (arrow), × 1000.—Fig. 9. Seed is in the process of ripening; oil is very liquid, × 1000.—Fig. 10. Abaxial side of cotyledon. Red-colored fat appears in the interstices (if) between the aleurone bodies, and as red grains (gf) in the cells which have other reserve material, × 1000.—Fig. 11. Transverse section. Interstitial lipid shows honeycomb-like, three-dimensional structure, × 1000.—Fig. 12. A young shoot. Oil traverses the cell wall and is found in the intercellular spaces, × 1000.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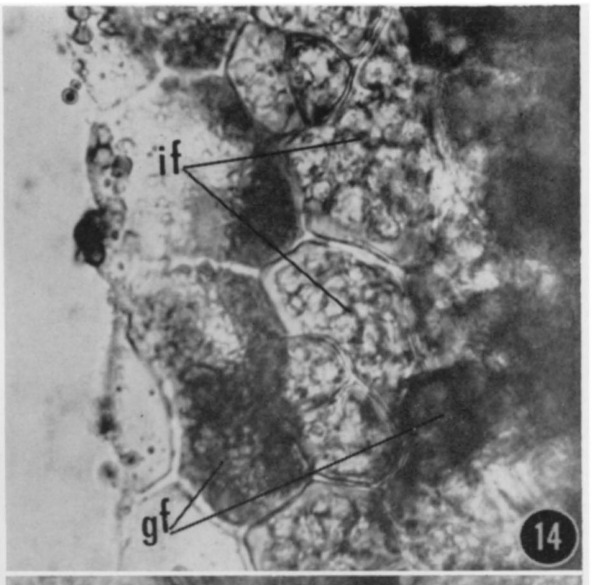
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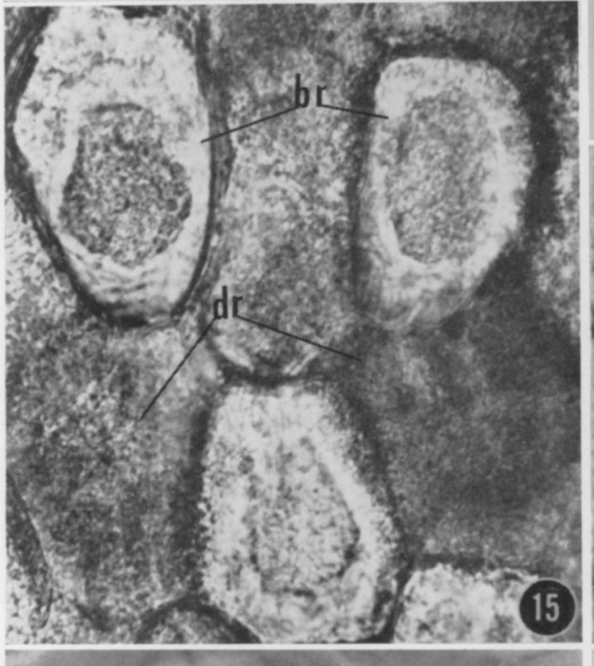
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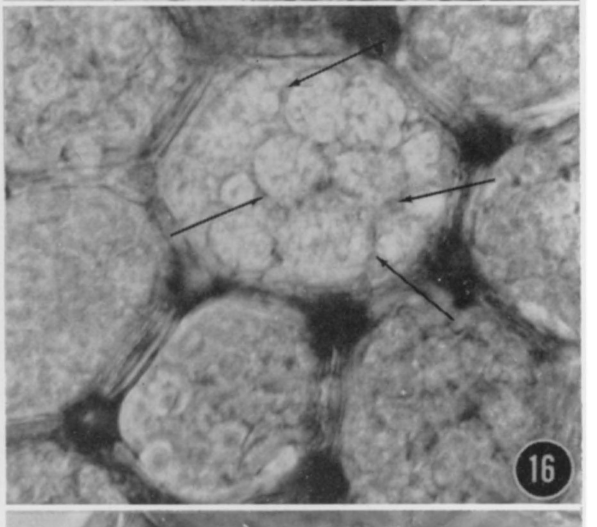
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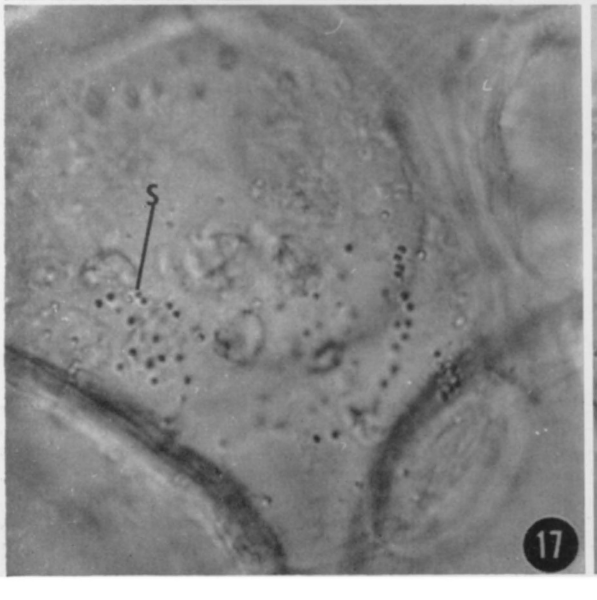
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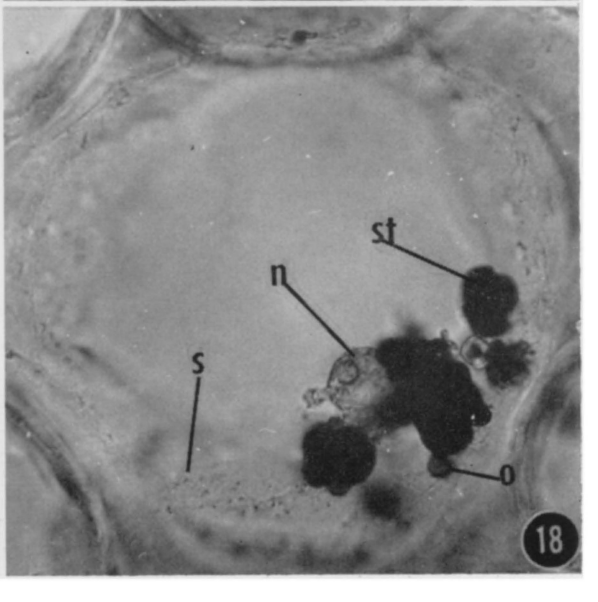
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Frey-Wyssling et al. (1963) other authors somewhat uncritically described as spherosomes certain vacuolar areas within the cytoplasm. Vacuoles (0.3–3 μ in diam) in cells of cotyledonary tissue of *Gossypium hirsutum* were described as spherosomes by Yatsu (1965) in an electron microscopic study using material fixed in potassium permanganate and dehydrated in acetone. In other micrographs of the same tissue, Engleman (1966) identified large transparent spaces in the cytoplasm as spherosomes, although in life these spaces undoubtedly contained reserve oil. In yet another study Horner and Arnott (1966) equated spherosomes with oil bodies of *Yucca* seeds.

The spherosomes are fairly constant in size and usually range from 0.8 to 1.0 μ in diam. It is true that smaller spherosomes of about one-fourth to one-third the diameter of the larger forms have been described in a number of plants, including the present material, Hubbard squash; but the dimensions of each type of spherosome remain constant, and no transitional forms between them have been observed (Url, 1964). The reserve oil bodies, on the other hand, vary widely in size, ranging from the limit of visibility to very large globules and aggregates.

Although in many seeds fat is dispersed in globules and granules, it occurs also in the form of cytoplasmic emulsion. Thus Nyman (1965, 1966) observed in the seeds of *Pinus silvestris* (with a fat content of 32.3%) that the reserve oil does not occur in the shape of droplets and globules but forms a homogeneous deposition of oil-plasm between the aleurone grains and in parts of the cell wall, both in the endosperm and in the embryo tissues. In sunflower seeds reserve fat occurs not only in the interstices between the aleurone grains but also as a cytoplasmic emulsion concentrated at the polar ends of the palisade cells and as granular deposition in other cells. Similarly, rapeseed plants store neutral fat in an intracellular, three-dimensional network between protein bodies and as a granular deposit in some other cells. At the level of the light microscope, the oil stored in the interstices represents a homogeneous emulsion which may liquefy and form large drops, as was seen in sunflower seeds, and in this emulsion there are no discernible units of the size of spherosomes. Bagley et al. (1963) have mentioned presence of an intracellular honeycomb-like structure connecting the protein bodies of the seed of peanuts; however, they do not say that oil is present there. Since their preparations were made from the cells fixed in formalin–acetic acid–ethanol and embedded in paraffin, all the neutral lipids were dissolved by this technique.

Since the histochemical tests have shown that the spherosomes contain phospholipids and do not react positively on being tested for neutral fats (Sorokin and Sorokin, 1966), they cannot be considered as direct precursors of reserve oil droplets. After demonstrating the presence of several hydrolytic enzymes in the spherosomes, Walek-Czernecka (1962, 1965) came to the conclusion that they are involved in the processes in intracellular hydrolysis but not in the synthesis of fat.

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Fig. 13–18.—Fig. 13–16. Rapeseed cells.—Fig. 13. Transverse section of an embryo axis stained with Oil Red O. Cells with large amount of oil are scattered throughout the ground parenchyma, which is surrounded by an aleurone layer, × 50.—Fig. 14. A part of Fig. 13, showing cells with large amount of granular fat (gf) on both sides of the aleurone layer. The interstitial fat (if) is found between the aleurone bodies, × 1000.—Fig. 15. Cotyledon. In certain cells the fat lines the cell walls and stains bright red (br)—(light color in the photographs); dark-red (dr) granules of fat are found in other cells; For/Ca, Oil Red O, × 1000.—Fig. 16. The interstitial fat (arrows) surrounds the aleurone bodies and stains blue. Aleurone layer; For/Ca, Nile blue (I), × 1200.—Fig. 17, 18. Hubbard squash parenchyma.—Fig. 17. Living cell. Spherosomes (s) are very refractive and photograph well with a green filter VG 9, × 1000.—Fig. 18. A larger cell stained with Oil Red O, and treated with I_2 in KI; starch (st)—black-purple; nucleus (n)—yellow; spherosomes (s)—colorless; oil (o)—red, × 640.

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