Mitochondria and Spherosomes in the Living Epidermal Cell
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Source: *American Journal of Botany*, Vol. 42, No. 3 (Mar., 1955), pp. 225-231

MITOCHONDRIA AND SPHEROSOMES IN THE LIVING EPIDERMAL CELL[1]

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RECENT DEVELOPMENTS in the field of enzymology, as well as renewed interest in plasma inheritance have redirected the attention of investigators to the particulate components of the cytoplasm. Whereas research on mitochondria of the animal cell has made considerable prograss both in the field of biochemistry (for summary see De Robertis et al., 1954) and in that of the fine structure of these bodies (Palade, 1952, 1953), the same cannot be said for cytological studies on living plant mitochondria (see Millerd and Bonner, 1953), excepting quite recent developments (Perner, 1952a, b, c, 1953, 1954; Perner and Pfefferkorn, 1953; Drawert, 1953, 1954; Ritchie and Hazeltine, 1953). This lag is partly the result of reluctance by some to accept the living-material technique as a working basis, and of the hope for future development of new cytological methods (Newcomer, 1951). Biochemical approaches to plant mitochondrial research have meanwhile moved forward (see summaries by Laties, 1953; Millerd and Bonner, 1953; Goddard and Stafford, 1954); but, not being able to obtain more information about the morphology of these intracellular bodies, such workers adapted information on the properties of animal cell bodies, by analogy, to the plant cells. Though the mitochondria of plants and animals are most likely similar structures, the present author stresses the fundamental differences between the cells of vertebrates and higher plants: whereas the animal cell contains a large amount of cytoplasm with considerable quantities of mitochondria and submicroscopic particulates (Lazarow, 1943), the adult plant cell as a rule has but a thin layer of parietally-distributed cytoplasm with comparatively few mitochondria, plastids, and spherosomes. In addition, the plant cells have very large vacuoles containing heavy colloidal substances which have specific gravities less than that of the cytoplasm, but greater than that of the oil droplets in the cells (Zirkle, 1937). All this complicates the identification of various cytoplasmic particles in the cellular fractions after centrifugation (Stafford, 1951). Laties (1953) has accepted an abstract concept of mitochondria, which name is used interchangeably with the term, particles. Goddard and Stafford (1954) have proposed a new definition of mitochondria in terms of their specific enzymatic activities and a wide size range of 0.1μ to 6.0μ in diameter.

The immediate task before the cytologist, therefore, is to find means for identifying various cytoplasmic bodies accurately, estimating their quantity and distribution within the living cell, determining some of their properties, such as their motions within the cytoplasm, relative indices of refraction, fluorescence, and reaction to vital staining. The combination of light and phase microscopy with vital staining permits one to observe, identify, and photograph the various cell components in living condition. In the plant cells examined three particulate components of the cytoplasm could be distinguished by these methods: the mitochondria, the plastids, and the spherosomes.

MATERIALS AND METHODS.—Small pieces of epidermis with living cells exhibiting cyclosis from the perianth of 26 varieties of tulips and from the inner side of the sheath of jonquil were stained with Janus green B (Hoechst) in 1:100,000 dilution with tap water, or in double-distilled water to which ten per cent sucrose was added (for details of technique see Sorokin, 1938, 1941). Observations were made under a light microscope, and selected cells were photographed under phase contrast.

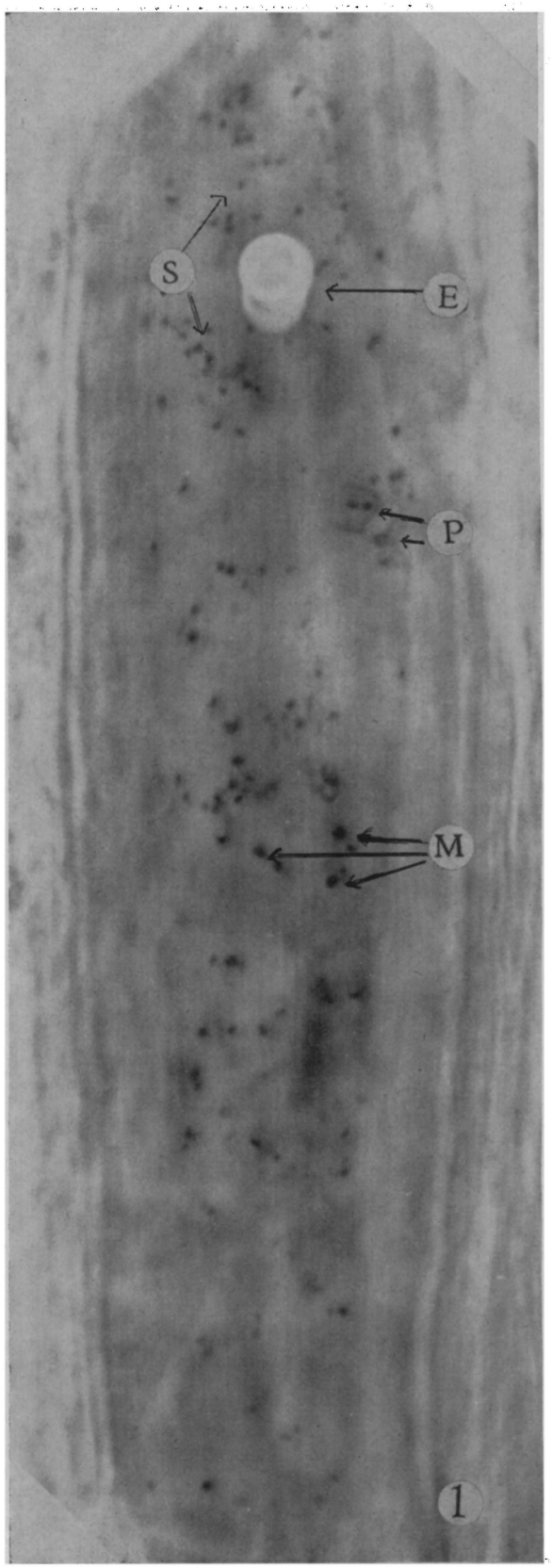
Zeiss apochromatic 2mm., N. A. 1.40, oil immersion objective (90×) and 15× compensating oculars were employed in the observations; and a Leitz phase system, including a Heine condenser, Pv. Apo. 40/0.70 mm. lens, and dark contrast were used in the photomicrography. Negatives had an original magnification of 540× and were enlarged to make final prints.

MITOCHONDRIA.—The term mitochondria used in this study corresponds to the term, chondriosomes, of the European and Latin countries (Drawert, 1953; De Robertis et al., 1954). The definition which follows applies only to the mitochondria in living cells of higher plants, as dead cells do not reduce Janus green B and hence, show no differential staining (Lazarow and Cooperstein, 1953). By mitochondria are meant the nearly spherical, the granular, and the cylindrical components of plant cytoplasm which can be observed and photographed in living cells (fig. 1–5, M) and stain an opaque medium blue with Janus green B. Under anaerobic conditions the color disappears, to reappear again at higher oxygen tensions, when material containing decolorized mitochondria is floated again upon pure sucrose solution. It is this reversible decolorization only which is considered specific for the identification of mitochondria. Under phase contrast the mitochondria appear dark gray, the intensity being increased if the material is previously stained with Janus green B.

In tulip, the mitochondria are always nearly spherical, but in most of the dicotyledons and in many monocotyledons both nearly-spherical and cylindrical forms of various lengths occur simultaneously (jonquil, onion). In different varieties of tulip the diameters of the nearly-spherical forms

[1] Received for publication July 20, 1954.

[2] It is a pleasure to express my appreciation to Professors I. W. Bailey and R. H. Wetmore for loan of microscopic equipment and for their interest in my work. I am gratefully acknowledging the cooperation of Dr. C. R. Partanen, whose skill made the photomicrographs possible.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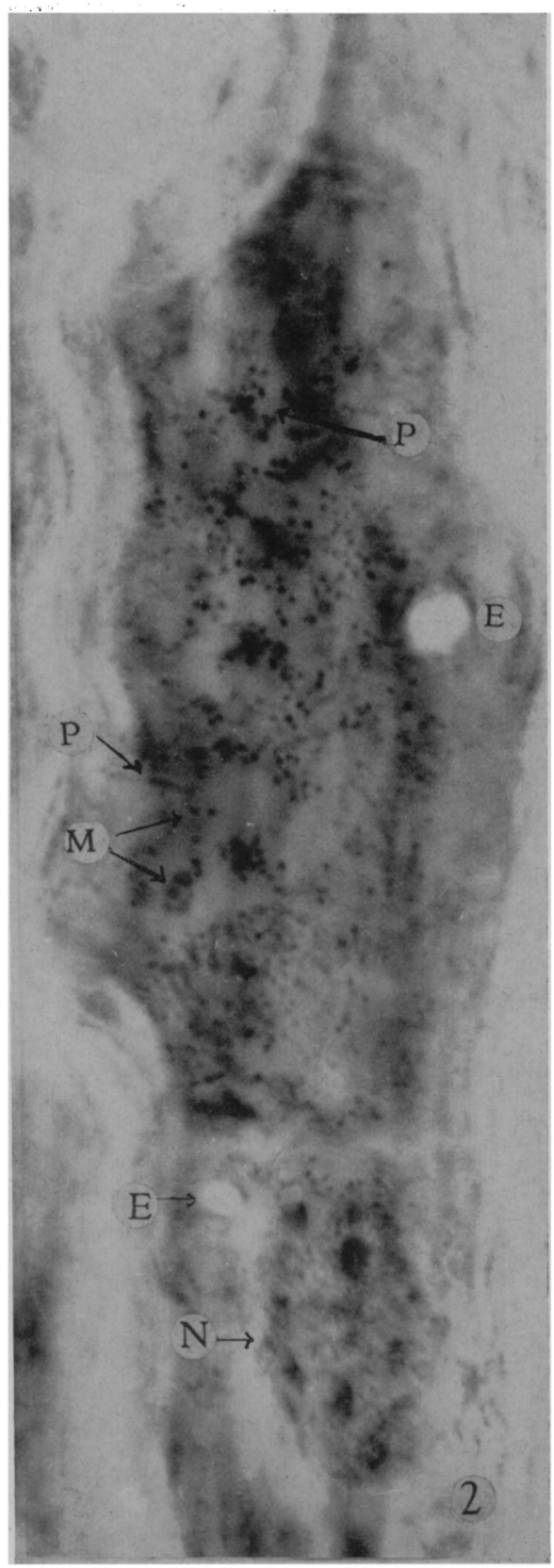
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vary from 0.4μ to 1.0μ (fig. 1, 2, M). The largest diameter recorded for the spherical forms of plant mitochondria reached 1.2μ. In animal tissues the mean diameter of the mitochondrion is 1μ or under, and only in the sarcosomes of the flight muscles of insects at the time of emergence are extreme values of 3μ found (Ishimoto, 1950). The size variation for mitochondrial diameters, 0.1μ to 6.0μ, given by Goddard and Stafford (1954) in their definition of this particulate is, therefore, unrealistic. In jonquil the girth of the cylindrical mitochondria varies from 0.8μ to 1.0μ and the length from 1.0μ to 6.0μ (fig. 3–5, M), longer forms being seen occasionally. In onion, Perner and Pfefferkorn (1953) give the following percentages of differently-shaped mitochondria in the cell: spherical, size range 0.6μ–1.2μ, 30 per cent; diplosomes, length 2.0μ–4.0μ, 10 per cent; rod-shaped, length 2.0μ–4.0μ, 50 per cent; and thread-like forms, 10 per cent. Blunt ends are typical for cylindrical forms (fig. 4, M.).

Two types of motion could be observed in plant mitochondria. The displacement motion is connected with cyclosis of the cell. The other motion in smaller forms is agitated and in larger forms is worm- or snake-like. When mitochondria are stained with Janus green B the second type of motion is inhibited (fig. 3–5, M), although mitochondria can still be carried in the cytoplasmic stream. At the point at which a stained cylindrical form begins to lose its color, the stain becoming reduced, the motion of the mitochondrion begins to be restored; and with complete decolorization the mitochondrion slithers away from the spot at which it had remained during the time it showed the stain. This is suggestive of a connection between the second type of motion and the oxidation-reduction state of mitochondria.

The morphology and motion of mitochondria described above are typical for normal epidermal cells of a great number of higher plants (Sorokin, 1941). In the vegetative fungous mycelium the behavior of mitochondria is different (Ritchie and Hazeltine, 1953), and changes of girth and the formation of pseudopodia by such mitochondria indicate a difference in viscosity between mitochondria of the different types of plants.

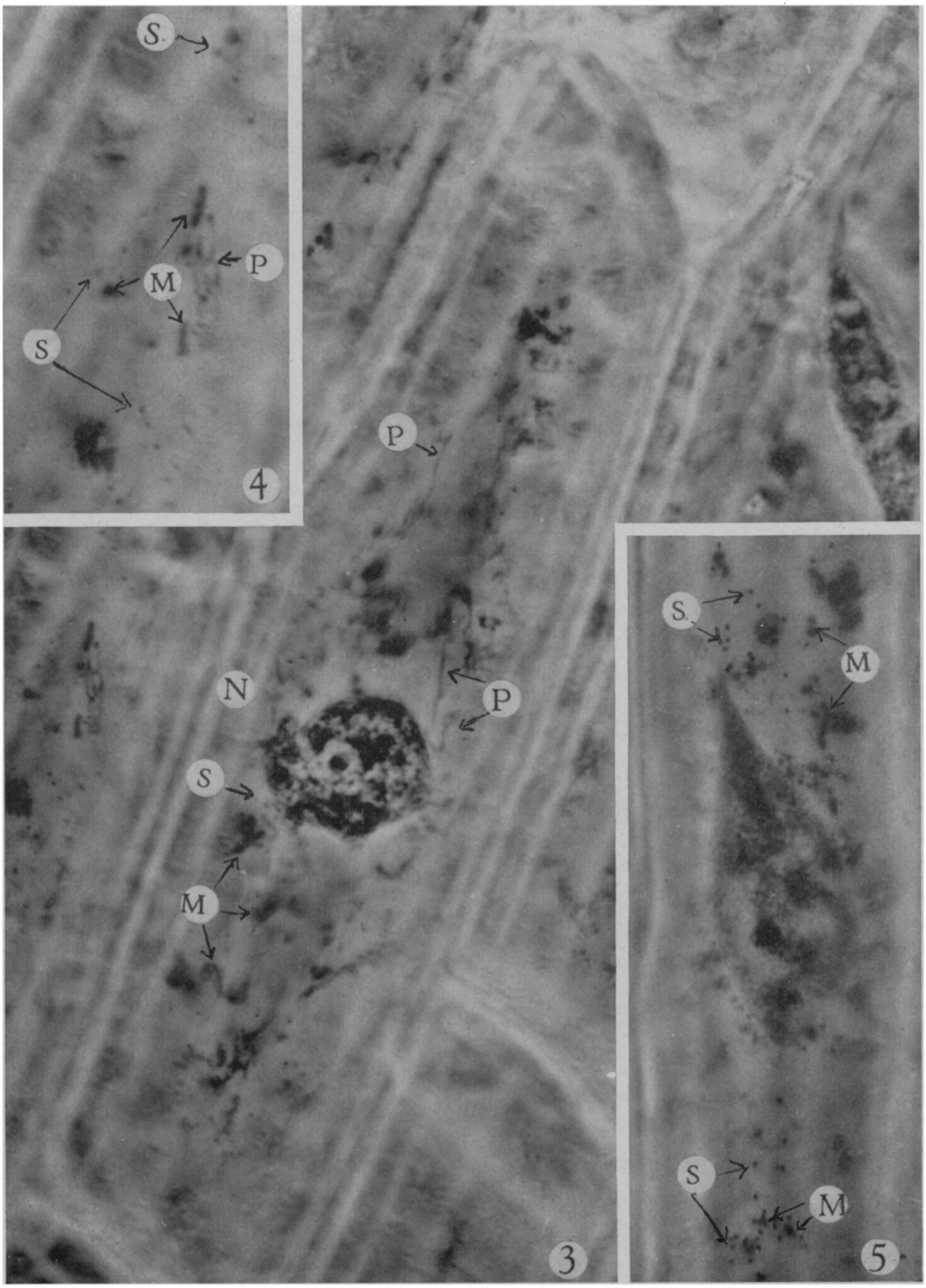
Mitochondrial distribution is not always uniform throughout the cell, and possibly depends upon light (Sorokin, 1941). In tulip, in the parietal layer of cytoplasm immediately adjacent to the striated, cutinized cell wall the mitochondria are not very numerous (fig. 1, M), or they may be entirely lacking, while they are always abundant along the opposite cell wall (fig. 2, M). The photographs here presented do not show the color distinction between mitochondria and proplastids, which can be striking, as in the cells from yellow tulips, where the blue-stained mitochondria contrast with the plastids containing xanthophylls.

That mitochondria have complex structures which could be partly revealed by the light microscope was known before (Guillermond et al., 1933; Sorokin, 1938, 1941). In some cells of a tissue stained with Janus green B, the matrix of the mitochondrion becomes lightly colored, and dark-stained granules become visible about the periphery of the body. In the long cylindrical mitochondria of onion several densely colored granules become visible in a row in the almost colorless stroma (Sorokin, 1938; Drawert, 1953). The studies of Herman (1950) and Harman and Feigelson (1952), have indicated that the dense portions of the mitochondrion contain the integrated enzyme complex of the citric acid cycle, cyclophorase, and that at certain stages of the transformation of the homogeneous mitochrondrion into the complex, segregated body, the reaction is reversible. On the other hand, electron microscopic studies of both plant and animal cells (Palade, 1953) present mitochondria as organized structures consisting of a double limiting membrane, a system of internal ridges or cristae, regularly arranged and extending from the periphery towards the central matrix. At the present state of our knowledge, it is hard to reconcile the fine structure of mitochondria revealed by the electron microscope in fixed material with the reversibility of phenomena observed in living condition.

It is well known that mitochondria are labile structures which respond quickly to changes in environmental conditions. Rod-shaped mitochondria of onion were observed to resolve by division into spherical forms within ten minutes after replacement of the environmental fluid by distilled water. Subsequently, these were observed to coalesce and return to the cylindrical shape (Sorokin, 1938). However, if the cell is injured the subsequent fusion of spherical forms does not occur. Pathological changes of a different nature were observed in mitochondria of the same material by Perner (1952a) after treatment for five or six days with berberine sulfate. The normally spherical and rod-shaped forms assumed the shape of very long threads. This change occurred among cells having a respiratory rate reduced by fifty per cent. Similarly Buvat (1953) has induced the formation of extremely long chondriocontes by submerging chicory roots under water for a prolonged time.

In homogenates of plant material no positive identity has so far been shown for mitochondria as they exist in the tissues and the small particles obtained in the homogenates (Goddard and Stafford, 1954). Rod-shaped mitochondria would certainly

Fig. 1–2. Living epidermal cells of tulip stained with Janus green B and photographed under Leitz phase contrast. *M*, mitochondria; *S*, spherosomes; *P*, plastids; *E*, elaioplast.—Fig. 1. Layer of cytoplasm adjacent to the cutinized cell wall. Spherosomes spherical, refractive (halo), numerous; mitochondria larger, not so numerous; plastids gray, very few; elaioplast, white. ×1620.—Fig. 2. Layer of cytoplasm adjacent to the innermost cell wall. Polymorphic plastids, numerous; spherical mitochondria, numerous; spherosomes; elaioplast, white. ×1620.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resolve into small spheres under centrifugation, but whether these spheres remain intact or dissolve needs further investigation. It is interesting that Perner (1952b) could not distinguish mitochondria in homogenates by the Janus green B criterion, as the dye was adsorbed by the protein-containing portions of the protoplast, e.g., the nucleus, leucoplasts, mitochondria, granules, and various débris. In the absence of oxygen there was no reduction of the dye. Furthermore, artifacts were formed in the lipoid-rich portion of the hyaloplasm that bear superficial resemblance to the mitochondria, and in the protein-rich portion that show similarity to the leucoplasts. In isolated granules (mitochondria, leucoplasts, and spherosomes) autolytic processes were patent and could not be forestalled by adjustment of external conditions.

Cytoplasmic particles sedimented under precise conditions from a homogenate of the mung bean revealed granules of dimensional resemblance to mitochondria (Millerd et al., 1951). These particles stained brilliantly with Janus green B and were considered to be mitochrondria. Because there is no mention of a following decolorization, and because under somewhat similar conditions Price (1952) and Stafford (1951) obtained dark blue sediments giving a greenish cast by reflected light, the present writer believes that the Janus green B reaction revealed precipitation of A-type vacuoles (Bailey, 1930), but not mitochondria. As the presence of tannin colloid complexes in the vacuoles of some bean seedlings is known (Herszlik, cit. Guillermond et al., 1933), their formation of flocculent, colored precipitates with Janus green B is probable, especially since Janus green B is known to form insoluble precipitates with a variety of biological agents (Lazarow and Cooperstein, 1953). In epidermal cells after prolonged staining copious precipitates of A-type vacuoles appeared often. A study of the formation of such precipitates is now in progress.

SPHEROSOMES.—With the development of phase-contrast microscopy interest in living material observation has increased, and with the use of the fluorescent and ultraviolet microscopes attention of the plant cytologists has been drawn to the most conspicuously visible small component of the cytoplasm, the spherosomes (microsomes) (Strugger, 1939; Perner, 1952a, 1952c, 1953; Drawert, 1952, 1953). Their existence in plant cells was known long ago and their properties and relative importance has been considered from time to time in the literature of plant cytology. Dangeard (1919), classifying cytoplasmic components, created the special subdivision of *sphérome*, in which he included the microsomes, to be distinguished from the *plastidome* and *vacuome*. Guillermond recorded numerous observations of living cells belonging to representatives of both higher and lower plants, and, with very few exceptions, has pictured his *granulations lipoïdiques* in the cells examined (Guillermond et al., 1933). In English spherosomes are called lipid or oil globules and often are not distinguished from reserve oils, lipid granules within the plastids, and the elaioplasts. The whole science of plant enzymology disregards the existence of visible spherosomes or other lipid globules in plant cells (see review by Goddard and Stafford, 1954), even though they are as numerous or in some cases more numerous than either mitochondria or proplastids. Because the term microsome may cause confusion with the submicroscopic particulates of the animal cell it is abandoned in the present study, regardless of its priority, for the term spherosome (see also Perner, 1953).

Spherosomes have been found abundantly in the cytoplasm of all cells investigated. They move rapidly in the cytoplasmic stream and are highly refractive. Because of this they are difficult to photograph. In shape spherosomes resemble spheres, but their birefringency indicates the possibility that they have more complex structure. In tulip they range from 0.25μ to 1μ in diameter, though most seem to have half the diameter of the mitochondrion (fig. 1, S). Spherosomes of jonquil have diameters of 0.25μ to 0.5μ (fig. 3, 4, 5, S), while those of onion vary from 0.6μ to 1.0μ, according to Perner (1953). In phase contrast they appear as reflecting black bodies. Photomicrographs, which could be taken only when motion had slowed down, show dark gray or black spheres surrounded by a halo (fig. 1, 3, 4, S), probably the result of motion.

When the cell is examined through the striated cell wall (fig. 1, white lines) and is focused on the upper layer of the cytoplasm, it is evident that there the spherosomes are the principal particulates (fig. 1, S). Because the material had been stained with Janus green B beforehand, it was possible to distinguish spherosomes from mitochondria by the former's absence of color, their high fringency, rapid motion, and size. When the microscope was focused to the level of the innermost layer of the cytoplasm of the cell and photographs (fig. 2) taken through the cell wall, the uppermost layer, and the central vacuole, the above distinctions were harder to make, especially where the size and shape of the bodies were similar (as in tulip). However, under the light microscope distinction could again be made on the basis of color.

The senescence of cells is accompanied by slower cytoplasmic streaming, rendering photography more simple. In fig. 3 the focus of the camera is on the

Fig. 3–5. Living epidermal cells of jonquil stained with Janus green B and photographed under Leitz phase contrast. *M*, mitochondria; *S*, spherosomes; *P*, plastids.—Fig. 3. Mitochondria cylindrical, granular; spherosomes refractive (halo); plastids with attenuated ends. ×1485.—Fig. 4. Spherosomes arranged in curved rows; mitochondria with blunt ends; plastids polymorphic. ×2268.—Fig. 5. Motion of cytoplasm stopped. Spherosomes without halo, distinct, small; mitochondria cylindrical and granular. ×1485.

nucleus, shown suspended by cytoplasmic strands. The spherosomes (fig. 3, S) are easily recognized by their halos (see near nucleus). Figure 4 shows an enlarged detail of the cell below. Spherosomes show a regular arrangement, groups of four or more in curved lines, presumably indicating local streams within the cytoplasm (fig. 4, S). Such streaming could usually be seen in most of the material examined. Again, in fig. 5, the cell was stained over 90 minutes. Motion of the cytoplasm and spherosomes stopped. The latter are distinct, black, and do not have halos (fig. 5, S).

Within the parietal layer of the cytoplasm spherosomes are more abundant in the portion adjacent to the cell vacuole. With the exception of a few mitochondria, and also a few plastids, the spherosomes make up the whole particulate portion of the thick trabeculae which cross the vacuole. They also are found in the thick stream of cytoplasm which envelops the nucleus, together with the mitochondria and plastids associated with the nucleus' surface. Weier (1942) pictured small granules (presumably spherosomes) in the rapidly streaming colorless cytoplasm which surrounds the inner layer, tinged with carotene, inside of which the nucleus was situated. In the latter layer larger granules (presumably mitochondria), remained stationary near the nucleus. Perner (1953) shows a photograph of spherosomes in the parietal layer of the cytoplasm of living onion cells after the Nadi reaction had taken place. Indophenol blue is localized in the spherosomes only. The mitochondria and the leucoplasts remain colorless. His conclusions, based upon that reaction, are that cytochrome oxidase is located on the spherosomes, but not in the mitochondria. Fully realizing that this view is in contrast to that of all other workers, Perner suggests that different results are due to contamination of the mitochondrial centrifugation fraction with spherosomes. The possibility that the Nadi reaction is not entirely reliable has not been excluded (Goddard and Stafford, 1954). However, the whole problem evidently needs careful reinvestigation, the more so because small granules, never positively identified as mitochondria were "stained" red with triphenyl tetrazolium chloride (Ritchie and Hazeltine, 1953), an indicator of dehydrogenase activity (Pratt and Dufrenoy, 1948). From the appearance of photographs the "stained" granules look like spherosomes.

The selective vital staining of spherosomes, made prominently golden-yellow under the fluorescent microscope by berberine sulfate, differentiates them from mitochondria and plastids, which are unaffected by the dye (Strugger, 1939; Perner, 1952a; Drawert, 1953). According to Drawert (1952), Nile blue will accumulate in the vacuole, while the spherosomes, colorless under the light microscope, will take on a golden-yellow color under the fluorescent microscope. He reports also (1953) that spherosomes will cause Janus green B to fluoresce white-green, or yellow-green, in the preparations stained with Janus green B and at the moment when mitochondria decolorize. The reaction observed under the fluorescent microscope takes place in living cells only and is anaerobic. The present author has also observed spherosomes to acquire a greenish tint after decolorization of mitochondria. Because after the reduction of Janus green B several leucoforms of it are produced (Lazarow and Cooperstein, 1953), some of these forms apparently are adsorbed on spherosomes, and thus an explanation could be given of the fact that some investigators obtained staining with Janus green B of particles other than mitochondria.

The phase-contrast method permits the separation of different lipid bodies in the cell. Under phase contrast the elaioplast of tulip cells had a dim luster and appeared white in the photomicrographs (fig. 1, 2, E). Also, reserve fat globules could be distinguished from the spherosomes in onion cells treated with berberine sulfate (Perner, 1952a). After five to six days from the beginning of the experiment a number of newly formed oil globules appeared in the cytoplasm, which gave a positive reaction for fat but which did not show the spherosomal ability to fluoresce berberine sulfate a golden-yellow color. It is apparent, therefore, that mitochondria, spherosomes, and reserve oil globules are distinct entities.

PLASTIDS.—Under phase contrast the colorless plastids are medium gray, distinct from the shades of the cytoplasm and mitochondria. In fig. 2, P, the variety of form encountered is well illustrated. There are many plastids in view. In fig. 1, P, there are but a few. Again, plastids with typical attenuated ends and containing small vacuoles are observed in fig. 3, P.

The distinction of mitochondria from small colorless plastids on the basis of staining with Janus green B has been fully discussed before (Sorokin, 1938, 1941). Drawert (1953) confirmed the observations reported then and the reaction now serves as a reliable criterion for distinguishing these two organelles (Perner, 1952b, 1953, 1954; Perner and Pfefferkorn, 1953; Drawert, 1953, 1954). Further work on distinguishing between mitochondria and plastids from different approaches was done by Strugger (1953, 1954), Drawert (1954), and Perner (1954). In fixed and stained preparations of meristematic, postmeristematic, and epidermal cells the distinction of rod-shaped mitochondria from the amoeboid-shaped plastids was demonstrated by differences in shape, staining reactions, and by the presence of *primary grana* in the plastids.

SUMMARY

The combination of light and phase microscopy with the Janus green B stain technique permits one to observe, identify, and photograph three particulate components of the cytoplasm in living condition, the mitochondria, the spherosomes, and the

plastids. With preliminary staining mitochondria are identified by color and the reversible decolorization under the light microscope. The dye intensifies the contrast under phase optics and decreases the cytoplasmic motion, a condition essential for photomicrography. Under phase contrast mitochondria are dark gray or black, nearly spherical, granular, or cylindrical bodies with blunt ends. Spherosomes, by their refractivity and fast motion, are the most conspicuous elements of the cytoplasm, appearing as black bodies of 0.25μ–1.0μ in diameter under phase contrast. In photographs they appear surrounded by a halo due to light scattering and to motion. The elaioplast has a dull luster under phase contrast and appears white in photographs. This facilitates the segregation of lipoidal bodies within the cell. The colorless plastids are distinguished by a negative response to Janus green B, by polymorphism, and by a medium gray shade under phase contrast.

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