Mitochondria and Plastids in Living Cells of Allium Cepa
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MITOCHONDRIA AND PLASTIDS IN LIVING CELLS OF ALLIUM CEPA[1]

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THIS INVESTIGATION presents evidence which demonstrates that it is possible to differentiate mitochondria from morphologically similar forms of plastids. In addition, it records the results of a study not only of the vital staining of mitochondria but also of the effects of various liquid media upon the survival of living tissue cells and upon the form and behavior of mitochondria and of plastids.

MATERIAL AND METHODS.—The epidermal tissue of the adult scale leaves of *Allium Cepa* L. provides unusually favorable material for microscopic examination and may be removed without seriously injuring the living cells. Small sheets of the epidermis were peeled from the inner surfaces of the scale leaves and were floated, with the cuticular side uppermost, upon various liquids. The behavior and the survival of the living cells were tested upon tap water, distilled water, mineral oil, solutions of sucrose varying from 2.5 to 30 per cent, and a series of Clark's standard M/20 buffers.[2] The liquids upon which the tissue cells survived the longest were subsequently utilized in vital staining with Janus green B. A pre-war sample of Hoechst's dye was used in a concentration of one drop of a 1 per cent aqueous solution of the dye to 50 cc. of the liquid medium. With the exception of the stock solution of the Janus green B, all other solutions were freshly prepared at the beginning of each experiment. The cytological criteria outlined by Bailey and Zirkle (1931) were employed in determining the condition of living tissue. All critical examinations were made upon cells which exhibited normal cyclosis.

SURVIVAL OF TISSUE CELLS.—During the months of November, December, January, and February, tap water from certain specific localities provides one of the most favorable media for the survival of tissue cells. Bailey and Zirkle (1931) found that cambial initials exhibit normal cyclosis for from 1200–1700 hours when immersed in tap water from Forest Hills, Boston, but subsequently discovered that such was not the case in dealing with tap water from other sources—e.g., Cambridge, Mass. My experience, in dealing with the epidermal tissue of onion, has been that tap water from Forest Hills, Boston, and from Winchester, Mass., is particularly favorable; whereas that from Cambridge, Mass., is quite unsatisfactory. Epidermal cells survive for a period of approximately 18 days in Winchester tap water.[3]

Few epidermal cells survive for more than 12 days in distilled water. In mineral oil and in solutions of sucrose varying in concentration from 2.5 to 30 per cent, the cells exhibit active protoplasmic streaming after 150 hours and may at times survive for 14 days or more. The lethal effects of Clark's standard M/20 buffers are obvious, and the epidermal cells of the onion seldom remain alive for any considerable length of time. The longest survival was 48 hours in the pH 7.8 and pH 8.2 borates. In the pH 5.4 phthalate, the cells survived for 30 hours; in the pH 5.8 phthalate, for 24 hours; in the pH 5.8 phosphate, for 26 hours; and in the pH 6.2 phosphate, for 20 hours. The more acid phthalates and the more alkaline phosphates and borates are extremely toxic.

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[2] The following series of buffers were used: pH 3.0, 4.0, 4.6, 5.0, 5.4, 5.8 (phthalates); pH 5.8, 6.2, 6.6, 7.0, 7.4, 7.8 (phosphates); pH 7.8, 8.2, 8.6, 9.0 (borates).

[3] The chemical analysis of this water for January 1937, as given by the State Department of Health, is as follows: chlorides, 4.4; nitrates, 0.05; iron, 0.15; in parts per million. Hardness, 20. The pH at 6°C. was 7.8.

VITAL STAINING WITH JANUS GREEN B. — Laguesse (1912) observed in the living cells of various vertebrates and invertebrates an elective affinity of mitochondria toward Janus green B, but indicated that the staining is erratic. E. V. Cowdry (1914, 1916) emphasized that Hoechst's Janus green B — i.e., diethylsafraninazodimethylanilin chloride—stains mitochondria specifically and that the contradictory results obtained with other samples of Janus green may be attributed to admixtures of other dyes. The specificity of Hoechst's dye depends upon the diethylsafranin group. The differential staining of animal mitochondria by Hoechst's Janus green B has been confirmed by a number of zoologists, and the use of this dye has become a standard procedure in intra vitam staining (Ludford, 1933).

N. H. Cowdry (1917) is credited with having first successfully applied this test to plant mitochondria. He found that they react to it precisely as do animal mitochondria except that the staining is much slower, owing to difficulties in penetration of the dye into plant cells. According to Guilliermond (1923), plant mitochondria stain with Janus green B while the cells are alive, but he concluded that the mitochondria undergo rapid degeneration shortly after staining and that the dye has pronounced lethal effects upon the cells. In the mycelium of *Endomyces magnesii* only, the mitochondria remained intact for several hours after staining. In accordance with his theory that the "active" and "ordinary" chondriosomes have identical physical and chemical properties, Guilliermond emphasized that leucoplasts also stain with Janus green B, but that they do so more slowly.

Opinions vary concerning the extent to which Janus green B is injurious to living cells. E. V. Cowdry (1914) observed stained mitochondria in neurophile leucocytes during amoeboid movement and phagocytosis. Becker (1933) described the formation of the phragmoplast and the phenomena of cytokinesis in cells of *Tradescantia* that were stained with Janus green B. Lewis and Lewis (1924) concluded that the dye produces conspicuous abnormalities in animal cells after 15 minutes. Parat (1928), although considering Janus green B as one of the best reagents for the staining of mitochondria, considered the staining reaction to be post-vital.

If the epidermal cells of the onion are immersed in tap water containing Janus green B in a ratio of approximately 1 to 100,000 or in a 12 per cent aqueous solution of sucrose containing a similar ratio of the dye, the staining of mitochondria is extremely erratic. If sheets of epidermal tissue are floated with the cuticular surface down upon such solutions of the dye, the tissue becomes colored along the margins only. The cuticle prevents the dye from penetrating into the cells, and the mitochondria remain unstained except in cells at the periphery of the piece. On the contrary, if the epidermis is floated with the cuticular surface uppermost, the mitochondria of the living cells stain after 45–60 minutes and remain colored as long as the cells survive—i.e., for about 30 hours. It is essential, however, to transfer the sheets of tissue from time to time to a freshly prepared solution, since the dye is likely to precipitate upon standing, and the solutions become colorless. After 12 hours, or sometimes even sooner, the contents of the vacuoles and the cell walls take up the dye and tend to obscure the staining of the mitochondria.

The mitochondria may be uniformly colored a bright blue-green (fig. 1, 4, 6), or they may exhibit deeply stained granules in an uncolored matrix (fig. 2). The latter type of staining occurs commonly in cells which do not survive so long as those in which the mitochondria are uniformly colored. A study of living and of fixed material indicates that mitochondria normally are microscopically homogeneous structures and that a differential staining of granules and matrix is due to an abnormal segregation of two components of the mitochondria.

When living epidermal cells, containing colored mitochondria, are mounted under a cover glass in a drop of dye solution and are examined under an oil-immersion lens, the color suddenly disappears after a varying interval of time from all the mitochondria, and the cells assume the appearance of a freshly prepared, unstained mount. If the destained cells are floated again upon the dye solution, the mitochondria restain, and the process of staining and destaining may be repeated a number of times. This capacity for staining and destaining is characteristic of normal uninjured mitochondria and may be utilized in distinguishing mitochondria from other stainable bodies, such as the granular precipitates that occur at times within or upon the surface of vacuoles. The behavior of the epidermal cells of onion suggests that the staining and destaining of mitochondria may be correlated with variations in the available supply of oxygen.

The staining of mitochondria with Janus green B in distilled water is erratic and uncertain, and it varies markedly in different buffers of Clark's standard series. There is no staining in buffers more acid than pH 4.6 or more alkaline than pH 8.2. In the case of the phthalate series, the mitochondria stain well for 30 hours at pH 5.4 and for a somewhat shorter period of time at pH 5.8. In the phosphate series, the mitochondria exhibit an excellent coloration for 26 hours at pH 5.8 (fig. 6) and for decreasing periods of time at pH 6.2, pH 6.6, and pH 7.0. There is little, if any, staining at pH 7.4 and pH 7.8. In the borate series, the mitochondria stain well at pH 7.8 and fairly well at pH 8.2. It is evident that mitochondria are capable of staining with Janus green B over a comparatively wide range of hydrogen-ion concentrations and that their staining behavior is largely dependent upon the toxicity of specific buffers.

In none of the living epidermal cells that I have examined have I encountered evidence of the vital staining of plastids—regardless of variations in their size and form—with Janus green B (fig. 1, 3, 5, 6). Staining of the plastids occurs in obviously dying or dead cells only. A truly vital staining with Janus green B, therefore, may be utilized as a means of dif-

eter is relatively constant, and the ends are blunt except in the case of rods which have just passed through a diplosome stage of transverse division. Conspicuous changes in the girth and form of mitochondria, such as are recorded by Emberger (1927) and others, do not occur in normal living cells of the onion but are characteristic of plastids. Under normal conditions the mitochondria were neither observed to shorten and to become thicker nor to elongate and become thinner. There was no evidence of branching of mitochondria in the cells of onion nor of the formation of networks.

Diplosomes commonly represent stages in the division of mitochondria and were present in all the living cells examined. The actual process of division was frequently observed both in freshly prepared mounts and in material kept in vitro. During division (fig. 8), the threadlike connection between the halves of the diplosome is stretched and suddenly ruptured. The halves of the diplosome jerk apart as if the connecting thread broke under considerable tension. The interval of time that elapses between the formation of a diplosome and the completion of a division is extremely variable. It is possible that certain of the diplosomes may, at times, reassume a rod-shaped form without undergoing division. When the length of a rod is more than twice its diameter, the division may not be equatorial. Thus, I have observed divisions in which a spherical body is cut off from one end of a long rod-shaped mitochondrium.

It should be noted in this connection that, although various investigators have described and figured diplosomes, or so-called dumb-bells, as stages in the division of mitochondria, relatively few observations of the actual process of division in normal living cells are recorded in the botanical literature. Friedrichs (1922) worked to some extent with living material of *Elodea*, but it is not clear from his descriptions whether he observed actual divisions or based his conclusions merely upon the occurrence of supposedly transitional stages. Kassman (1926) gives an accurate description of the division of small granules, presumably mitochondria. However, certain of these granules gave a positive test for starch, which indicates that Kassman may have been concerned with small plastids rather than with mitochondria. Horning (1926) followed the process of binary fission in living mitochondria of a heretotrichan infusorian, vitally stained with a sodium salt of diethylsafranin monocarboxylic acid. His descriptions of the process of division, as also Guilliermond's (1927) based upon plant material, are in close agreement with the phenomena that I have observed in living epidermal cells of the onion.

The plastids of the epidermal tissue occur in a wide variety of forms (fig. 3), but they never grade down to the limits of microscopic visibility. The small granular forms are slightly smaller than the granular forms of mitochondria, and the short rod-shaped plastids are more slender than the mitochondria of corresponding length. There are "dumb-bell"-shaped plastids which resemble diplosomes. These "hantelförmige Gebilde" are easily confused with the diplosome forms of mitochondria and have served as one of the principal arguments for deriving plastids from mitochondria (Cunha, 1929; Loui, 1930). There are, in addition, numerous transitional forms between small and large plastids. The smaller plastids may be observed to develop into large ones, and conversely the latter may at times divide to form small plastids (compare Guilliermond et al., 1933, p. 147).

The behavior of the granular and short rod-shaped forms of mitochondria and of plastids in living epidermal cells which exhibit normal cyclosis is similar, except for the fact that mitochondria stain vitally with Janus green B, whereas the plastids do not. The larger forms of plastids appear to be less viscous and exhibit amoeboid movements and striking changes of form when kept under observation for a considerable interval of time; whereas the longer rod-shaped mitochondria are pliable but of relatively constant diameter throughout their length.

EFFECTS OF HYPOTONIC AND HYPERTONIC SOLUTIONS.—Fauré-Fremiet (1910), Lewis and Lewis (1915), Bang and Sjövall (1916), N. H. Cowdry (1917), Guilliermond (1919), Anitschkow (1923), and Kamnev (1934) are of the opinion that mitochondria swell and become vesiculate in hypotonic solutions and that they shrink and become slender in hypertonic media. Unfortunately, it is not possible to determine whether the changes observed by these investigators are due directly to osmotic phenomena or to the effects of a complex of factors in injured or dying cells. In a heterogeneous system, such as a living cell, it is extremely difficult to distinguish the effects of various factors influencing the form and the behavior of mitochondria, and specific changes may be due to injury rather than to the direct effects of variations in osmotic pressure. It is significant in this connection that Bang and Sjövall record cases of the swelling and vesiculation of mitochondria in isotonic solutions and note that such changes are concomitants of the degeneration of mitochondria.

The mitochondria of uninjured epidermal cells of onion do not swell or become vesiculate in tap water (fig. 11), distilled water, or in dilute solutions of sucrose, nor do they exhibit conspicuous contraction in saccharine solutions of 15–30 per cent. On the contrary, the mitochondria of injured or dying cells frequently swell and vesiculate when the cells are placed in hypotonic solutions, but they may do so at times in isotonic (fig. 7) or hypertonic solutions. The specific degenerative changes that the mitochondria undergo are extremely variable and are apparently dependent upon the condition of the "explanted" cells at the time when they are transferred to specific solutions.

Division or fusion of mitochondria may be induced at times in living cells that are transferred to culture solutions. I have observed these phenomena particularly in the case of distilled water. In certain cases all the rod-shaped mitochondria are resolved by divi-

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