Absorption Microscopy of Enzymatically Treated Cell-Free Chloroplasts

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PLATES 118 TO 120

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ABSTRACT

Monochromatic light microscopy at 435 mμ shows in *Euglena gracilis*, the distribution of chlorophyll and the general orientation and geometry of chloroplasts *in vivo*. In addition it discloses, in swelling chloroplasts, a lamellar pigmented structure. Changes in this structure are observed in extruded swollen chloroplasts treated with lipolytic or proteolytic enzymes. Lipolytic enzymes produce an increase in the number of visible lamellae while proteolytic enzymes disrupt the lamellar array.

Correlation of chloroplast swelling behavior and the effects of enzymatic degradation with current electron microscope observations support the following: (1) the pigment lamellae observed *in vivo* consist of component laminae; (2) the lamellae are separated by sites of swelling; and (3) the integrity of the lamellar structure is primarily dependent upon the intact state of the protein.

The observation of an electron-dense laminate structure in ultrathin sections of chloroplasts has led to an increased interest in the distribution pattern of chlorophyll *in vivo*. Such information is difficult to obtain directly from electron micrographs since the pigments lack specific electron density over that of other cell constituents and are subject to the solvent action of dehydration agents used prior to sectioning (6).

Several investigators have utilized the spectral absorption of chlorophyll to localize the pigment *in vivo* (2, 9, 20, 25) and this property at 435 mμ has been used to demonstrate a lamellar structure in intact and swelling *Chlorella* chloroplasts (14, 15). The number of component laminae visible in swelling chloroplasts was compatible with that observed in electron micrographs of this material (1).

Euglena gracilis offers certain advantages for continuation of this study. It has large laminate chloroplasts which can be extruded without apparent damage. Furthermore, the extensive electron microscopy of its chloroplast by Wolken and coworkers (21–24) has provided a background of valuable information. Included also are statistical counts of dense lamellae, pigment concentration, and effects of environmental alteration.

The present study attempts to reconcile some of the features of the *in vivo* pigment absorption image with those observed in electron microscopy. Appropriate enzymatic degradations were induced in swelling chloroplasts in order to study the nature of the pigment arrangement.

Methods

The *Euglena gracilis var. bacillaris* was grown aerobically on a glutamic acid medium at pH 3.3 in a simple continuous culture device (8). Intermittent light (14 hours light phase, 10 hours dark phase) yielded a more homogeneous population than continuous illumination. This device produced uniform material for as long as 2 weeks after a sufficiently heavy growth was attained ($\sim 1 \times 10^3$ organisms/mm.^{3}) Cultures were harvested by low-speed centrifugation and washed twice in 0.1 M KCl to remove the acidic medium.

For chloroplast extrusion, one volume of packed cells from about 100 ml. of culture medium was mixed in a micro chamber (2.5 cm.^{3} capacity) of a Virtis homogenizer with several volumes of glass pavement marking spheres (10). The cells were ground for 30 to 75 seconds at high speed; cells and chloroplasts were

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separated from the glass spheres by aspiration with a fine hypodermic needle. No attempt was made to isolate chloroplasts from other cellular constituents. The extruded chloroplasts were capable of maintaining their structure for several days at 3°C.

The trypsin, papain, and wheat lipase used were Worthington Chemical preparations. The lipase was a crude preparation which exhibited no trypsin-like activity. The trypsin was twice crystallized and highly active. The lecithinase A and lipase were diluted in 0.1 M KCl. Trypsin was prepared in 0.02 M phosphate in 0.1 M KCl adjusted to pH 6.7. The papain was suspended in a 0.03 M cysteine solution. Dr. M. Rodbell kindly provided a purified preparation of lecithinase A which had no proteolytic activity.

Enzyme preparations and their diluents were added to freshly extruded chloroplasts to give final concentrations of 0.5 to 0.05 mg./ml. These were placed in an incubator at 37°C., except as noted. Controls were similarly treated. The digestions were carried out at pH 6.4–7.0.

The microscope optics, the photographic technique, the interference filters, and light source have been described previously (14, 15).

RESULTS

Intact Chloroplasts:

In the absorption image of an intact cell, the chloroplasts are clearly flattened and wafer-shaped and are usually oriented with their broad surface parallel to the cell membrane.[1] Under our growth conditions there are 5 to 7 chloroplasts per organism. Fig. 1 *a* shows an edge view, while the flat view is presented in Figs. 2 *a* and *b*. The latter often shows the pyrenoid as a central area of decreased pigment absorption as in *Chlorella* (14, 15). (This structure appears dense in numerous electron micrographs 1, 23.) The edge of the chloroplast becomes irregular after long exposure to light (Fig. 2 *a*).

Expanded Chloroplasts:

Organisms maintained anaerobically in 0.1 M KCl gradually increase in size and their chloroplasts expand. The time required for swelling varied from 2 to 24 hours. (It was our impression that the most motile cells were the ones most resistant to swelling.) However, when chloroplasts are extruded into 0.1 M KCl they expand immediately, suggesting that their flattened form is dependent on the cytoplasmic environment. Expanded chloroplasts appear to consist of numerous (10 to 20) fine bands arranged in one of two general patterns depending upon the aspect presented to view (Figs. 3 *a* and *b*). In one of these (designated by *P* in the figure), the bands (approximately 10) appear parallel. In the other (designated by *R*), the separated bands (approximately 20) appear radially arranged around a prominently unpigmented pyrenoid. Several intermediate aspects are shown in the extruded chloroplasts in Figs. 4 *a*, *b*, and *c*. The lamellate nature of these bands may be demonstrated in the radial aspect by their continuity at various focal planes. A typical example is presented in Figs. 5 *a*, *b*, and *c*.

The transition from the flattened form to the expanded form is difficult to follow in the same chloroplast. Not only are the organisms initially motile, but the individual chloroplasts change their location during swelling. Figs. 1 *a*, *b*, and *c* represent an attempt to follow swelling in the same cell. The expansion of chloroplasts upon extrusion occurs, of course, in the homogenizer and cannot be observed. Fig. 6 is a simplified diagrammatic representation of the intact and expanded chloroplast from the same aspect derived from the various aspects in the photographs above. It shows how the radial expansion of originally parallel lamellae appears to double their number.

Enzymatically Treated Chloroplasts:

Lipase and lecithinase A do not profoundly alter the pigment lamellae, but appear to increase their number. Fig. 7 *c* shows about 30 lamellae and Fig. 7 *a* suggests that even more are present, but not resolvable. Other treated chloroplasts are seen in Figs. 7 *b*, *d*, *e* to *h*. Even when enzyme action is prolonged for 24 hours, no further dissolution of the chloroplasts occurs. A noticeable increase in chloroplast size is also observed.

Proteolytic enzymes disrupt the pigmented chloroplast structures. Often lamellae seem to break and rejoin and pigment globules form at the interstices (Fig. 8). After prolonged digestion, only globules of pigment are left in an amorphous matrix. Figs. 9 *a* and *b* represent transitional stages in papain digestion, while Figs. 9 *c* and *d* show examples of chloroplasts digested with trypsin.

In order to follow sequential changes in chloroplasts, digestion was attempted at room temperature (24 ± 2°C.). Though fragments were attacked

[1] For a comparison of the absorption image to a conventional light microscopy image, the reader is referred to earlier papers from our laboratory (8, 14, 15).

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under these conditions (Figs. 8 *a*, *b*, and *c*) intact chloroplasts were quite resistant. At 30°C., some digestion was apparent, but rapid and characteristic digestion took place only at 37°C.

DISCUSSION

The swelling behavior we have described is comparable to that observed in extruded *Nitella* chloroplasts by Mercer *et al.* (12). They employed both electron microscopy and conventional light microscopy and concluded that a swelling aqueous phase separates the dense lamellae. A similar separation of pigment lamellae in our study suggests that both opaque and pigmented lamellate systems are closely associated or are identical.

Wolken and Schwertz (24), Thomas *et al.* (18), Goedheer (5), and Olson and Engel (15), have given values for the space occupied by each chlorophyll molecule in various laminate chloroplasts. These values depend on the interpretation of the number of pigment layers associated with the dense lamellae in electron micrographs, and this remains a point of uncertainty. Wolken and Schwertz (24) have reported that there are 21 ± 3 major-dense lamellae in the *Euglena* chloroplast, while we regularly see about ten pigmented lamellae in the edge view of the expanded chloroplast. This would indicate that the approximately ten lamellae we resolve on swelling are at least double structures. The action of the lipolytic enzymes in increasing the number of visible pigmented structures supports this interpretation. If a lipid interphase does exist between compounded pigment laminae, then digestion of this junction would tend to make the components resolvable by increasing their separation. This interpretation agrees very well with Wolken's model (22). When lipids are removed with organic solvents the dense lamellae of the chloroplast also remain intact (3, 7, 11, 17).

Proteolytic enzymes disrupt the pigment lamellae, suggesting that the pigment is present as a pigment-protein complex.[2] However, some reservations are necessary. It cannot be discerned whether the actual site of the digested protein is within the pigmented lamellae. Digestion of an interlamellar protein which provides a surface for the spreading of chlorophyll monolayers may be as important as disruption of a chlorophyll-protein layer in which an actual chlorophyll-to-protein bond exists.

The finding by Trurnit and Colmano (19) that chlorophyll monolayers at water/air and water/oil interfaces exhibit a shift of the red absorption maximum to longer wavelengths relegates to a lesser importance the argument for a chlorophyll-protein complex on a purely spectral basis. The spectral shift resulting from heating leaves, as observed by Godnev and Akulovich (4), may be due to a breakdown of chlorophyll monolayers without specific protein attachment. Certainly, disruption of the light-absorbing lamellae by proteases is no proof that they are essentially protein.

A physiological corollary to these enzymatic effects may be found in the work of Spikes (16), who has reported that proteolytic enzymes inhibit the Hill reaction in isolated chloroplasts while lipase and lecithinase leave it unaltered. This occurs at 15°C., considerably lower than the temperature of our studies. Important functional alterations may thus precede the more drastic structural changes.

[2] Mercer *et al.* (12 reported that extruded *Nitella* chloroplasts appeared normal in the light microscope after incubation at 37°C. in 1 per cent trypsin. Repeating this work with absorption microscopy, we found that swelling *Nitella* chloroplasts (in 0.1 M KCl) are disrupted by trypsin. This disagreement may be due to differences in experimental conditions and reagents as well as the method of detecting alterations. It may be that the intact membrane offers considerable protection against enzymatic digestion unless altered by the swelling process. On the other hand, Thomas *et al.* (17) found destructive changes with pepsin digestion on the dense layers of the spinach chloroplast. Pepsin treatment necessitates a considerable hydrogen ion concentration, and this alone may produce marked alterations (8). Zirkle (25) and Metzner (13), have studied proteolytic digestion of grana-bearing chloroplasts, but the conditions of their experiments, as well as their results, are not comparable to our own.

BIBLIOGRAPHY

1. Albertsson, P. A., and Leyon, H., The structure of chloroplasts. V. *Chlorella pyrenoidosa* Pringsheim studied by means of electron microscopy, *Exp. Cell Research*, 1954, **7**, 288.
2. Doutreligne, J., Note sur la structure des chloroplasts, *Proc. k. Akad. Wetensch.*, Amsterdam, 1935, **38**, 886.
3. Frey-Wyssling, A., Morphological aspects of the lipoproteins in chloroplasts, *Discussions Faraday Soc.*, 1949, **6**, 130.
4. Godnev, T. N., and Akulovich, N. K., The distri-

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bution of chlorophyll molecules in the pigment layer of chloroplast granules, *Proc. Acad. Sc., U.S.S.R., Biochem. Sect.*, A.I.B.S. translation, 1958, **120**, 136.

5. Goedheer, J. C., Optical properties and *in vivo* orientation of photosynthetic pigments, Doctoral thesis, Utrecht, 1957.
6. Granick, S., Chloroplast structure and its relation to photosynthesis, *in* Research in Photosynthesis, (H. Gaffron, editor), New York, Academic Press, Inc., 1957, 459.
7. Granick, S., and Porter, K. R., The structure of the spinach chloroplast, as interpreted with the electron microscope, *Am. J. Bot.*, 1947, **34**, 545.
8. Greenblatt, C. L., and Sharpless, N. E., The effect of metabolic inhibitors on the pigments of *Euglena gracilis* in an acidic medium, *J. Protozool.*, 1959, **6**, 241.
9. Gross, J. A., Wirtschafter, S. K., Bernstein, E., and James, T. W., Monochromatic microscopy of *Euglena*, *Tr. Am. Micr. Soc.*, 1956, **75**, 480.
10. Lamanna, C., and Mallette, M. J., Use of glass beads for the mechanical rupture of microorganisms in concentrated suspensions, *J. Bact.*, 1954, **67**, 503.
11. Menke, W., Untersuchungen über den Feinbau des Protoplasmas mit dem Universal-Electronenmikroskop, *Protoplasma*, 1940, **35**, 115.
12. Mercer, J. V., Hodge, A. J., Hope, A. B., and McLean, J. D., The structure and swelling properties of *Nitella* chloroplasts, *Australian J. Biol. Sc.*, 1955, **8**, 1.
13. Metzner, H., Über den Nachweis von Nucleinsaüren in den Chloroplasten höherer Pflanzen, *Naturwissensch.*, 1952, **39**, 64.
14. Olson, R. A., and Engel, E. K., Visible absorption microscopy of pigment systems in living cells using interference filters: *Chlorella* chloroplast, *Proc. Micr. Symp.*, Chicago, McCrone Associates, 1958.
15. Olson, R. A., and Engel, E. K., Chlorophyll absorption microscopy of *in vivo,* cell free, and fragmented *Chlorella* chloroplasts, *in* The photochemical apparatus, its structure and function, *Brookhaven Symp. Biol.*, No. 11, 1958, 303.
16. Spikes, J. D., Inactivation of isolated chloroplasts by crystalline trypsin, *Plant Physiol. Suppl.*, 1957, **32**, V.
17. Thomas, J. B., Burstraan, M., and Paris, C. H., On the structure of the spinach chloroplast, *Biochim. et Biophysica Acta*, 1952, **8**, 90.
18. Thomas, J. B., Minnaert, K., and Elbers, P. D., Chlorophyll concentrations in plastids of different groups of plants, *Acta Bot. Neerl.*, 1956, **5**, 314.
19. Trurnit, H. J., and Colmano, G., Chloroplast studies. I. Absorption spectra of chlorophyll monolayers at liquid interfaces, *Biochim. et Biophysica Acta*, 1959, **31**, 434.
20. Vavra, J., The action of streptomycin on chloroplasts of the flagellate, *Euglena gracilis* Klebs, *Folia biol.*, 1957, **3**, 108.
21. Wolken, J. J., A molecular morphology of *Euglena gracilis* var. *bacillaris*, *J. Protozool.*, 1956, **3**, 211.
22. Wolken, J. J., Photoreceptor structures. Pigment monolayers and molecular weight, *J. Cell. and Comp. Physiol.*, 1956, **48**, 349.
23. Wolken, J. J., and Palade, G. E., An electron microscope study of two flagellates. Chloroplast structure and variation, *Ann. New York Acad. Sc.*, 1953, **56**, 873.
24. Wolken, J. J., and Schwertz, F. A., Chlorophyll monolayers in chloroplasts, *J. Gen. Physiol.*, 1953, **37**, 111.
25. Zirkle, C., The structure of the chloroplast in certain higher plants. I and II, *Am. J. Bot.*, 1926, **13**, 301.

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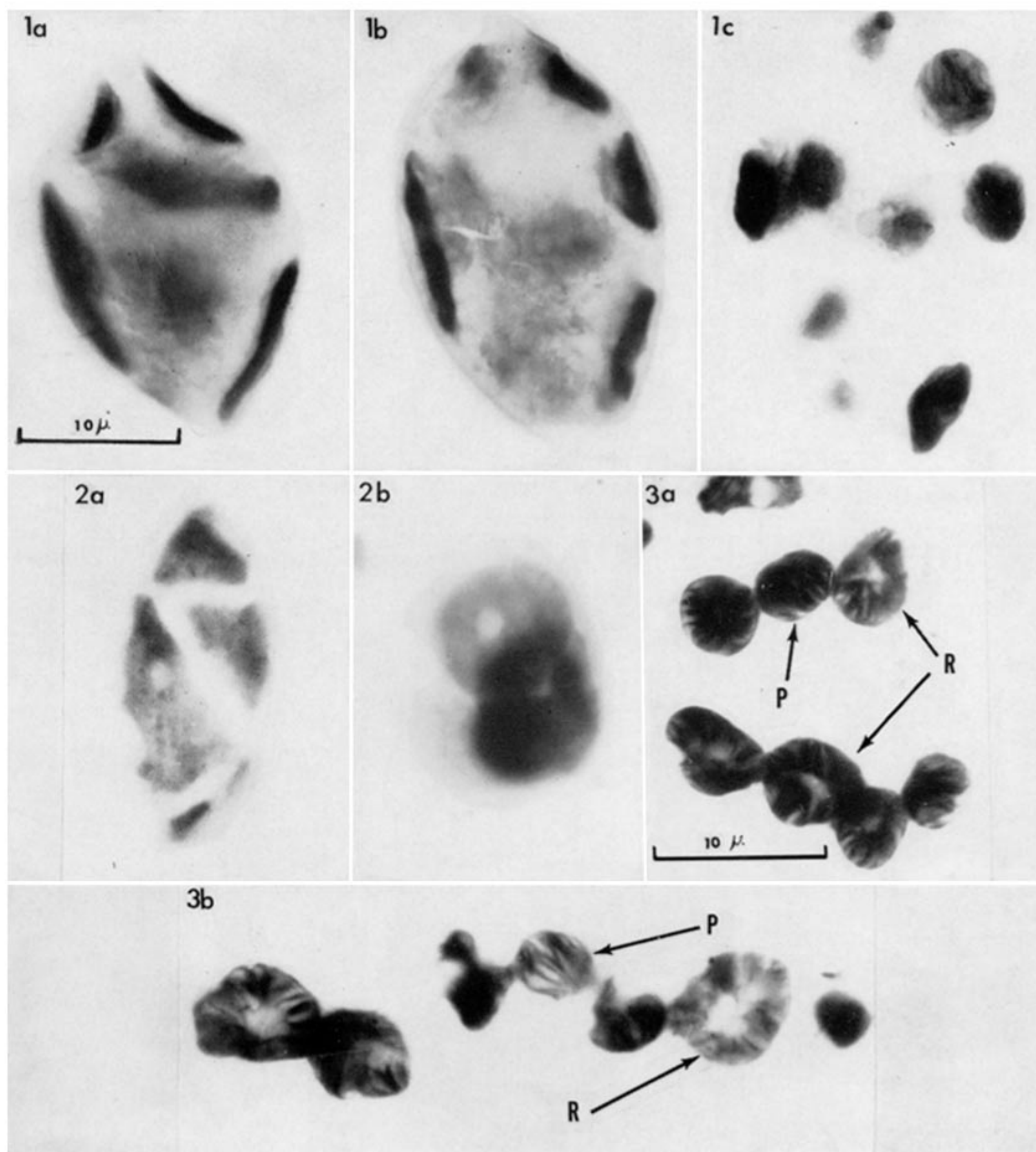
Explanation of Plates

Plate 118

Figs. 1 *a*, *b*, and *c*. The same organism in 0.1 M KCl is seen at various stages of swelling. The photographs were taken at 0 time, 7 hours, and 20 hours, respectively. Motion and changes in focal level make sequences difficult to obtain. × 2200.

Figs. 2 *a* and *b* are views of the flat surface of intact chloroplasts. The focal plane is near the outer surface of the organism. The irregular edge of a chloroplast is seen in Fig. 2 *a*, and both figures show the pyrenoid as a central region of decreased absorption. The scale in Fig. 3 *a* applies to Figs. 2 *a* and *b*. × 2400.

Figs. 3 *a* and *b* present two general aspects of chloroplasts observed in swollen organisms. That indicated by *R* shows the lamellae radially arranged around the pyrenoid, while that marked *P* shows only parallel lamellae. There are about twice as many separated lamellae appearing in the radial aspect. × 2400.



(Greenblatt *et al.*: Absorption microscopy of chloroplasts)

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PLATE 119

FIGS. 4 *a*, *b*, and *c* are examples of extruded chloroplasts in 0.1 M KCl. × 3800.

FIGS. 5 *a*, *b*, and *c* are radial views of an extruded chloroplast at three focal planes about 1 μ apart. The continuity of the bands at different focal levels indicates a true lamellate structure. × 3400.

FIG. 6 is a diagrammatic representation of a chloroplast from the same aspect before and after expansion.

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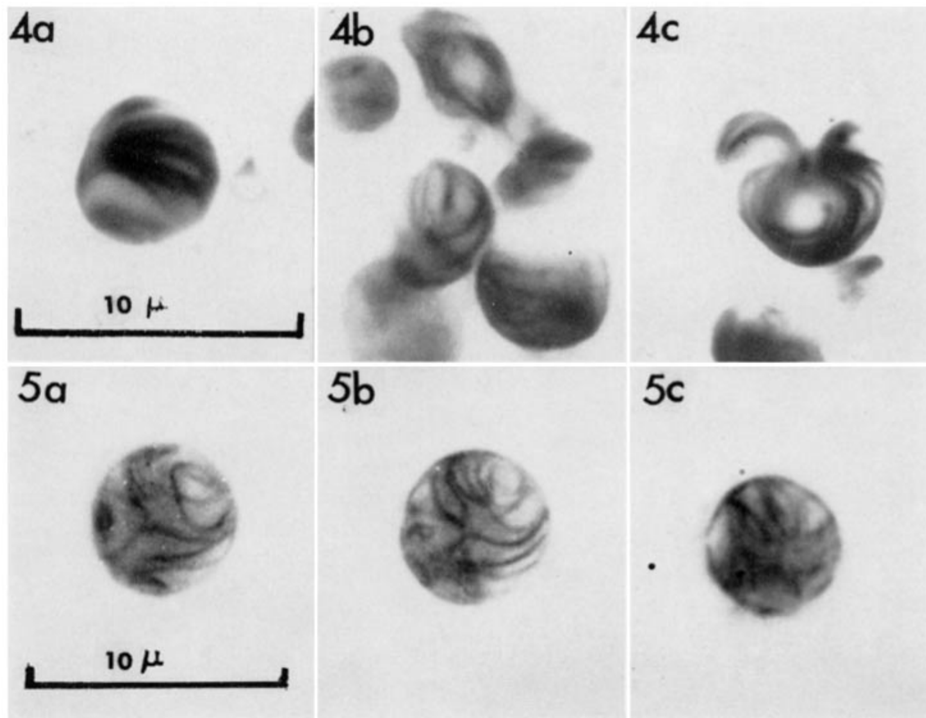

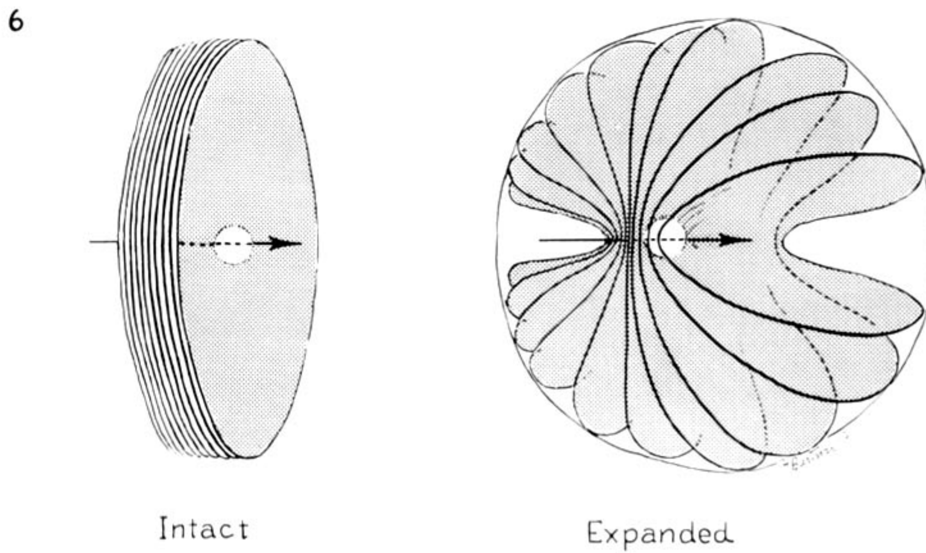

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Plate 120

Figs. 7 *a* to *f* are lipase-treated (0.5 per cent) chloroplasts. The incubation time varies from 15 minutes to 2 hours in the first five examples. Fig. 7 *f* represents a 24 hour incubation. × 3300.

Figs. 7 *g* and *h* are lecithinase A (0.5 per cent)-treated chloroplasts, which have been incubated for 30 minutes. × 3300.

Figs. 8 *a*, *b*, and *c* represent a chloroplast fragment being disrupted by 0.4 per cent papain. The photographs were taken at 2, 10, and 110 minutes. × 3100.

Figs. 9 *a* and *b* are other papain-treated chloroplasts after 10 minutes of incubation. × 3200.

Figs. 9 *c* and *d* are trypsin (0.5 per cent)-treated chloroplasts after 15 minutes digestion. × 3200.

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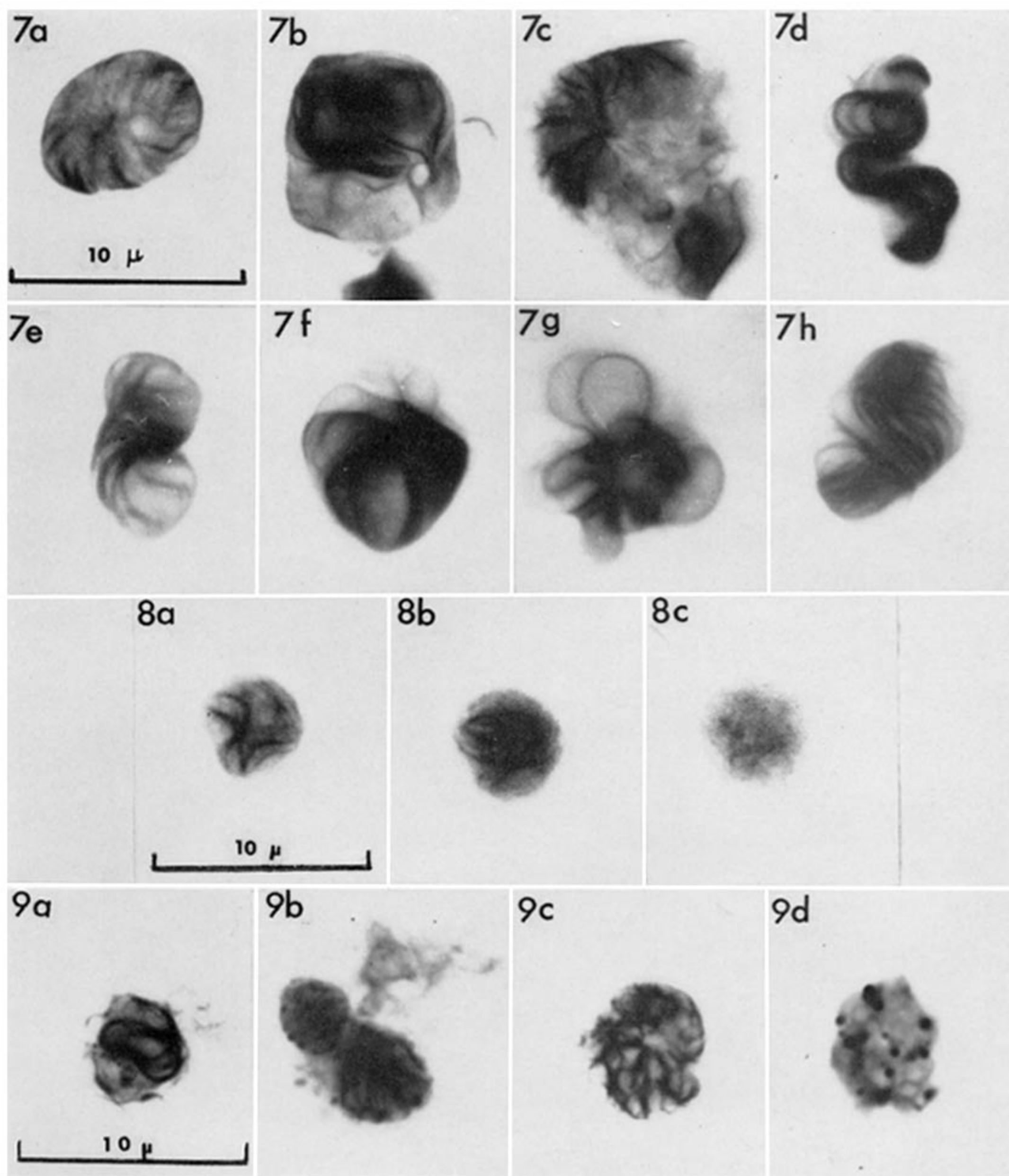

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