THE STRUCTURE AND SWELLING PROPERTIES OF *NITELLA* CHLOROPLASTS

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Summary

The swelling of the chloroplasts of *Nitella cristata* A. Br. in solutions of different osmotic pressures has been examined in relation to their fine structure, isoelectric point, and permeability towards KCl.

Electron-microscope studies of ultra-thin sections have shown that the normal chloroplast consists of about 40-100 lamellae *c.* 70A apart, enclosed by an external membrane *c.* 70A in thickness. Grana do not occur. Space within the chloroplast not occupied by the lamellae is filled with a granular material resembling the cytoplasm. The chloroplast often contains a considerable number of osmiophilic globules *c.* 0·1 μ in diameter lying between the lamellae. Their staining reaction with osmic acid indicates that they are probably lipoidal. Numerous small osmiophilic bodies *c.* 100A or less in diameter are associated with the lamellae. Starch grains, when present, occur between the lamellae and appear to be enclosed within by a membrane.

During swelling in hypotonic solutions, chloroplasts change in shape from disc to sphere to cylinder. Swelling results from the formation of vacuoles between the external membrane and the rest of the chloroplast (blebs), by increase in interlamellar spacing and by the formation of vacuoles between the lamellae. The interlamellar vacuoles form by the lamellae rounding up and coalescing to enclose the granular interlamellar material.

Quantitative results for the volume:osmotic pressure relations, the volume:pH relations, the surface charge, and the apparent free space for KCl show that swelling is an osmotic process. The osmotic gradients arise from: (*a*) a Donnan system associated with the interlamellar material; and (*b*) from diffusible solutes present in the interlamellar solution. The high values for the apparent free space and the volume:pH relations indicate that the chloroplast membrane is differentially permeable, not semi-permeable.

The degree of swelling of chloroplasts in solutions is controlled by the osmotic pressure and pH of the solution, the differential permeability of the chloroplast membrane and lamellae, and the cohesive forces maintaining the structure of the membrane and lamellae.

By analogy with the chloroplast, it is suggested that the mechanism controlling the water relations of mitochondria, which also possess lamellar structures bounded by a thin membrane, may be similar to that of the chloroplasts.

I. INTRODUCTION

Some of the physical properties of the chloroplast, in particular the swelling properties, have not been satisfactorily explained. Several attempts have been

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made to interpret this property of the plastid in terms of structure. Priestley and Irving (1907) considered that either the peripheral layer of the chloroplast is semi-permeable or that the stroma imbibes water. They tended to favour the latter view because of the difficulty of explaining transfer of metabolites if the former theory were true. Zirkle (1926) denied the existence of a chloroplast membrane and from studies on *Elodea canadensis* Michx. concluded that the expansion of the plastid was due to the uptake of water by a central vacuole probably containing sugar and protein in solution. Hubert (1935) also observed such vacuoles but could not demonstrate their expansion in dilute glycerol.

Knudson (1936) observed the development of bubble-like protrusions (blebs) from the surface of chloroplasts in distilled water and claimed to have caused such blebs to contract in hypertonic sugar solutions. This membrane concept has been supported by Weiler (1936), Granick (1938, 1949), and Strugger (1951). However, Weier (1938), in reviewing the evidence for an outer membrane, points out that while some sort of surface membrane probably exists in the normal chloroplast, it is possible that a membrane with semi-permeable properties forms only when the isolated chloroplast comes in contact with an aqueous phase.

The use of the electron microscope so far has failed to solve the problem although confirming the earlier theory (reviewed by Frey-Wyssling 1948) of a lamellar structure in the chloroplast (see Steinmann 1952*a*; Cohen and Bowler 1953; Wolken and Schwertz 1953). Electron micrographs of unsectioned chloroplasts have been interpreted as showing an outer membrane (Algera *et al.* 1947; Granick and Porter 1947; Frey-Wyssling and Mühlethaler 1949; Thomas, Bustraan, and Paris 1952). Steinmann (1952*b*), however, considers that the existence of a well-defined membrane has not yet been shown by direct methods and questions the validity of the assumption that blebs represent portion of the membrane which originally surrounded the chloroplast. Examinations of sectioned plastids in the electron microscope have generally failed to affirm the existence of an outer membrane (Steinmann 1952*a*, 1952*b*; Cohen and Bowler 1953; Leyon 1953), although Wolken and Palade (1952, 1953), have shown indications of a surface membrane in sections of *Euglena* sp. chloroplasts.

In view of the inconclusive results obtained by direct observations, the present authors felt that a combination of a quantitative study of swelling properties with light and electron microscopy might yield valuable information. The chloroplasts used for experiments described here were obtained from *Nitella cristata*. This alga proved to be very suitable owing to the ease with which chloroplasts could be isolated from it in an undamaged condition. In the present paper an attempt has been made to relate the swelling properties of the chloroplast to its general structure, surface charge and permeability.

The present studies have resulted in the demonstration of lamellar structure, the absence of grana, and the presence of an external membrane in *Nitella* chloroplasts. They have shown that swelling results either from the formation of vacuoles between the lamellae or from the growth of vacuoles between the

external membrane and the rest of the plastid, or from a combination of these. In both cases the movement of water is controlled by the external membrane and possibly also by the lamellae which may have differentially permeable properties. The external membrane at least is shown to be differentially permeable. Such a membrane would permit the movement of metabolites between the chloroplasts and cytoplasm. The osmotic gradients responsible for swelling arise from the osmotic pressure of solutes in the interlamellar solution and from immobile (Donnan) ions associated with the interlamellar colloids.

II. Material and Experimental Methods

Samples of two varieties of *Nitella cristata* A. Br. were collected from ponds and cultured in their native mud plus tap water in glass troughs. Chloroplasts were isolated usually by excision of one end of an internodal cell (length 2-5 cm) followed by gentle scraping of the cell wall to expel the cell contents. The chloroplasts, which are found packed around the periphery of the cell next to the wall in regular rows, are extruded in apparently undamaged condition.

Ultra-thin sections of *Nitella* cells were prepared for electron microscope examination as follows. Intact cells or swollen isolated chloroplasts were fixed in 2 per cent. OsO_4, veronal-acetate buffered to pH 7·5 (Palade 1952*a*), and made up to *c.* 7 atm osmotic pressure with NaCl, for periods up to 48 hr. This was followed by washing, dehydration in alcohol, and embedding in *n*-butyl methacrylate, the general procedures being similar to those of Newman, Borysko, and Swerdlow (1949). Up to 15 per cent. methyl methacrylate was used because of the toughness of the cell walls. Sections between 200 and 300 Å thick were obtained using the technique and microtome of the type described by Hodge, Huxley, and Spiro (1954), and were examined without removal of plastic in a RCA model EMU electron microscope fitted with an externally centerable objective aperture (Farrant and Hodge 1950). The magnification of the instrument was calibrated by the interferometric method of Farrant and Hodge (1948).

In the experiments on swelling in hypotonic media, the chloroplasts from several cells were stored together in the extracted cell sap. Then samples were taken and placed in a relatively large volume of KCl or glucose of known osmotic pressure p. The mean volume of at least 50 chloroplasts was calculated from measurements under a high-power microscope ($\times 600$) using a calibrated eyepiece micrometer.

When larger quantities of chloroplasts were required in the investigation of the penetration of KCl, up to 5 g of clean sorted internodal cells were placed in a "Blendor" with 100 ml of glucose of the required osmotic pressure and blended for 5-10 sec. The homogenate was strained through muslin to remove cell wall fragments, and centrifuged at low speed to deposit starch grains. A further spin for 2 min at 150 g produced a residue which was resuspended in fresh glucose, recentrifuged, and the supernatant decanted. This treatment resulted in a substantially pure sample of chloroplasts.

III. Results

(*a*) *Description of the Swelling Process*

(i) *The Normal Chloroplast.—In vivo,* under the light microscope or the phase contrast microscope, the chloroplasts appear as pale green, disc-shaped bodies, of 5-10 μ dia. by 1-2 μ thick. Starch grains are readily visible. In surface view most chloroplasts appear green but not optically homogeneous. Frequently, many had a finely spotted appearance, the spots being circular, of about 0·3 μ dia. and darker in colour than the rest of the chloroplast. The spots were similar in size to the grana of some other chloroplasts (Strugger 1951). Plate 1, Figure 1, shows chloroplasts viewed from above in the living cell, compared with those isolated in 0·5M glucose (Plate 1, Fig. 2). The significance of the spotted appearance will be discussed later in relation to the chloroplast fine structure. In side view (Plate 1, Fig. 3), they appeared approximately elliptical when isolated.

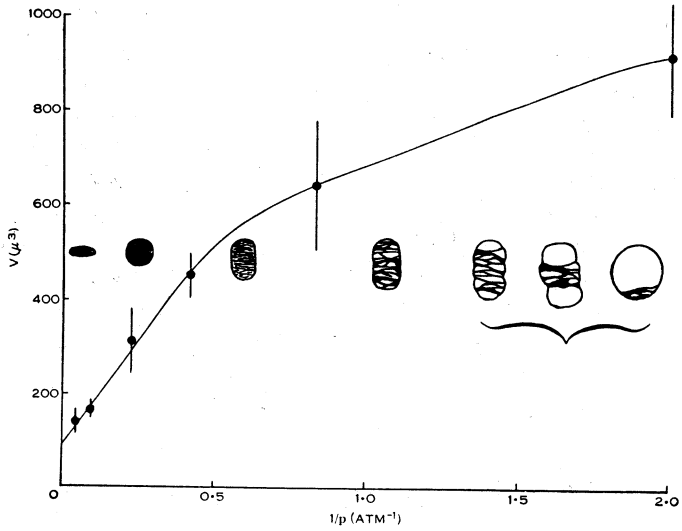

Fig. 1.—Mean volume V in μ^3 of a large number of *Nitella* chloroplasts plotted against the reciprocal of the osmotic pressure of the KCl solution ($1/p$) in reciprocal atmospheres. Each point represents the mean and standard deviation of independent samples taken from cell sap. The diagrams represent the mean appearance of the chloroplasts at the osmotic pressure below each drawing.

(ii) *Swelling in Hypotonic Solutions.*—As it was not possible to follow the swelling process in a single chloroplast, the morphological changes associated with swelling have been reconstructed from the mean changes observed in many chloroplasts. Although there was variation in the degree of morphological change which occurred, particularly in the very dilute solutions, swelling followed a definite pattern.

As illustrated in Figure 1, a chloroplast changes from disc to sphere to cylinder, and the latter continues to expand, ultimately forming large vacuoles or blebs, though a single spherical bleb is often observed. Most of the change in volume results from an expansion along the initial minor axis of the chloroplast. Over the concentration range 0·05-0·1M approximately, the chloroplast increased in volume, becoming spherical. During the disc to sphere transformation the general appearance of the chloroplast remained constant. The sphere-cylinder change between 0·1M and distilled water was associated with gross structural changes. As the cylindrical form developed, the striations became more obvious as alternating dark green and light bands. With further swelling the dark green striations appeared as a coarse reticulum with colourless vacuole-like regions between the meshes. The strands of the meshwork ranged from *c.* 2 μ to the limits of resolution of the microscope in width. At this degree of swelling a distinct membrane could be seen enclosing the reticulum.

The increase in volume resulted mainly from the increase in the vacuole regions rather than from an increase in the volume of the reticulum. Frequently blebs of up to 20 μ dia. developed from the surface of the chloroplast. Where one or two large blebs were formed from a plastid they were most commonly developed next to the hemispherical ends of the plastids. Examples of the vacuoles and blebs are given in Plate 2, Figures 4, 5, and 6.

Attempts to reverse the swelling process were not successful. This is contrary to the results of Knudson (1936) who succeeded in causing blebs to contract in hypertonic solutions. No structures identifiable as grana were observed during swelling.

Further information concerning structural changes associated with swelling was obtained from electron-microscope studies of normal chloroplasts *in situ* and of isolated chloroplasts showing different degrees of swelling.

(*b*) *The Fine Structure of* Nitella *Chloroplasts*

The general morphological features of *Nitella* are shown in Plate 3, Figure 7, which is of a section cut transversely to the major axis of a cell kept in the dark for 10 days to minimize the formation of starch grains. Starch grains, which do not stain well with OsO_4, usually swell during the embedding procedure and cause distortion. Starch-free cells were therefore usually selected for examination.

The first feature of interest in Plate 3, Figure 7, is the cell wall which exhibits a number of striations, most clearly defined in the region adjacent to the cytoplasm. These resemble the growth rings seen in cotton fibres (see references in Hock 1942). The disc-shaped chloroplasts are closely applied to the cell wall and frequently overlap to some extent. The lamellar structure of the chloroplasts is evident, as is the chloroplast limiting membrane. In places the membrane is separated from the lamellae, the intervening space being filled with some osmiophilic material (see also Plate 4, Figs. 9 and 10). In the bifurcations between the groups of lamellae are located starch grains or starch vacuoles and a number of spherical densely staining bodies of fairly uniform size (0·1-0·2 μ). Similar bodies are shown in the micrograph of a chloroplast

of *Aspidistra* published by Finean, Sjöstrand, and Steinmann (1953). The spherical shape and osmiophilia suggest that they may be oil globules. The tonoplast, separating the central vacuole and cytoplasm, appears as a thin (*c.* 70 Å) membrane of varying width. This appearance is characteristic of thin membranes which are oriented at angles less than 90° to the plane of the section. The tonoplast is better seen in Plate 4, Figure 10.

The lamellar structure of the chloroplast is shown more clearly in Plates 4-6. The lamellae are seen as thin dark lines about 40 Å thick, separated by less dense zones about 30 Å thick. The lamellae extend continuously across the entire width of the chloroplast. Stacking is more regular towards the edges of the chloroplast, where up to 40 lamellae may be seen in almost perfect array. In the more central regions of the chloroplast, groups of lamellae are separated by spaces of variable width, in the larger of which are located the starch grains or vacuoles and the dense spherical bodies described above. The existence of these spaces between groups of lamellae and their shape may offer an explanation of the inhomogeneous spotted appearance of the chloroplasts when viewed under the light microscope. Since several of the dense-staining, spherical bodies appear within each chloroplast in thin sections, it is clear that the total number of spaces must be large. Further, if the refractive index of the material filling these spaces differs appreciably from that of the lipoprotein lamellae, the spaces will show up in surface view as inhomogeneities under the light microscope.

The periodic spacing observed in the well-ordered regions of lamellae is somewhat variable, ranging from less than 70 Å to about 120 Å. Some of this variation is probably intrinsic, i.e. due to variation in the perfection of packing in the intact chloroplast and to the thickness of the interlamellar zones, but some is undoubtedly due to variation in the angle of sectioning. It therefore seems likely that the true spacing, consistent with that to be expected for a single lipoprotein layer structure, in *Nitella* is in the vicinity of 70 Å, a value very much less than has been reported for chloroplasts of other plants (Cohen and Bowler 1953; Finean, Sjöstrand, and Steinmann 1953; Wolken and Schwertz 1953). The regular structure of *Nitella* chloroplasts suggests that this alga would be suited to X-ray diffraction work, especially at small angles.

The presence of grana in the chloroplasts of higher plants has been confirmed by electron microscopy (Steinmann 1952*b*; Cohen and Bowler 1953; Finean, Sjöstrand, and Steinmann 1953). The grana are sharply defined regions of well-ordered lamellae with a periodic spacing of about 70 Å. Such differentiation is absent from the chloroplasts of *Nitella.* Since the lamellar spacing is of the same order as that observed in the grana of higher plants, the *Nitella* chloroplast might be regarded as a single giant granum.

Plates 3-6 show clearly the limiting membrane surrounding the chloroplast. This membrane appears to be about 70 Å thick, although such measurements on a single membrane are necessarily inaccurate. In Plate 4, Figure 8, the membranes of two adjacent chloroplasts can be seen closely applied to each other.

The larger spaces within the chloroplast not occupied by the lamellae are filled with a relatively homogeneous granular material. In appearance it re-

sembles the cytoplasm and might appropriately be termed chloroplasm. In addition, numerous small very dense granules of irregular shape (mostly of less than 100 Å dia.) are present, associated mainly with the lamellae, and probably represent small localized deposits of osmium (Plates 4-8). The association of these granules with the lamellae is well seen in the swollen plastids (Plate 8, Fig. 16). It is possible that they result from the reduction of OsO_4 by some reducing system within the lamellae. Numerous reducing systems, e.g. ascorbic acid, reduced coenzymes, and carotenoids, are associated with the chloroplast (Weier and Stocking 1952). It is of interest that similar dense granules were observed by Sjöstrand (1953) within the outer segments of retinal rods, which have a lipoprotein layer structure somewhat similar to that of chloroplasts.

Plate 6, Figure 13, shows a number of morphological features of interest, in addition to those already described. On the right are three cytoplasmic inclusion bodies which are probably mitochondria. They possess thin limiting membranes and exhibit internal structures resembling the cristae of animal mitochondria described by Palade (1952*b*). In the upper right are two cytoplasmic "vacuoles." The membranes of these "vacuoles" are closely applied to the tonoplast membranes, resulting in a double membrane effect. The cytoplasm has a reticular appearance and many lamellar structures are present, often in close apposition. These are identified as elements of the endoplasmic reticulum (Palade and Porter 1952). The lamellar elements (or flattened canalicular elements) are also evident in Plate 4, Figures 9 and 10, and Plate 5, Figure 11.

It has been suggested (Zirkle 1926) that starch forms in specialized starch vacuoles within the chloroplast. The results obtained here are not inconsistent with this hypothesis. Plate 5, Figure 12, shows a region of low density surrounded by what appears to be a thin closed membrane located in the space between two bands of ordered lamellae. Since some starch appeared to be present within the enclosed region (not clearly seen in the illustration because of contrast limitations), this structure is tentatively identified as a starch vacuole.

The cytoplasm of *Nitella* is clearly limited on one side by the tonoplast membrane, but a cell membrane between the cytoplasm and the cell wall has not yet been observed in the sections. This may be due to its thinness and close apposition to the cell wall or there may be no membrane in this position. The chloroplast membrane is also difficult to observe on the cell wall side.

Thin sections of swollen chloroplasts show clearly the nature of the structural changes taking place during the disc-sphere-cylinder transformation. The initial stage (disc-sphere) involves mainly an increase in the average spacing of the lamellae. However, wide variations exist, and the general pattern is often complicated by the formation of large blebs and vacuolar regions between the bands of lamellae. Plate 7, Figure 14, illustrates a chloroplast which is at a stage of swelling similar to that shown optically in Plate 2, Figure 4. The lamellar organization is still quite well preserved, but the chloroplast membrane has formed two large blebs at the ends. The membrane is cut obliquely and therefore appears as a thin ribbon. The interlamellar spacing here is about 500 Å in the plane of the section. As swelling progresses the lamellae coalesce to

form small spherical vacuoles. Plate 7, Figure 15, shows a cylinder stage in which vacuolation has occurred, although stacks of lamellae still remain.

In greatly swollen chloroplasts the structure is completely vacuolated (Plate 8, Fig. 16). As is evident in this section the membrane of the chloroplast is usually still intact. The interior of the chloroplast is now filled entirely with spherical vacuoles, each enclosed by a thin membrane formed by fusion of lamellae. A starch grain enclosed in a membrane can also be seen. The very small dense granules described earlier are located on the lamellae (Plate 8, Fig. 16) and the vacuoles contain a reticular material. This presumably represents the remains of the chloroplasm or interlamellar material, and its appearance is consistent with denatured precipitated protein. The transformation of the lipoprotein lamellae to enclose spherical vacuoles indicates the extreme lability of these structures, and suggests the necessity for caution in the interpretation of lipoprotein systems which have been exposed to hypotonic solutions.

Samples of swollen chloroplasts were also examined in the electron microscope using the conventional method of drying a suspension on a collodion-covered specimen screen, washing several times with distilled water, and shadow-casting with uranium. Plate 8, Figure 17, is a typical example of such a preparation. The appearance of this micrograph is entirely consistent with that of Plate 8, Figure 16. The outer limiting membrane is clearly defined, as are a number of flattened vacuoles in varying size. The outer limiting membrane, presumably of double thickness as expected if a spherical bleb is flattened onto a surface, is about 150 Å thick as measured by the length of a shadow. This value is in good agreement with the single membrane thickness (*c.* 70 Å) determined from thin sections. The vacuoles, trapped between the two layers of the chloroplast membrane, throw sharp shadows of a length similar to that of the chloroplast membrane. The thickness of the single vacuole membranes measured in this way is also about 70 Å.

(i) *The Nature of the Swelling Process.*—The results obtained with the light microscope and the electron microscope show that the overall volume changes which occur in a chloroplast in solutions of various tonicity are determined by volume changes in the interlamellar regions and in the vacuoles which form within the chloroplast.

Information on the nature of the processes controlling these volume changes was obtained by studying the water relations of chloroplasts under various experimental conditions.

(*c*) *The Water Relations of Isolated Chloroplasts as a Function of Osmotic Pressure*

Nitella chloroplasts, extracted and kept in cell sap of approximately 12 atm osmotic pressure, were placed in KCl solutions of various osmotic pressures and the mean volume measured. This was done with an eyepiece micrometer of which one small division was approximately 2 μ in the object. If the chloroplasts were platelets, they were treated as oblate spheroids and the major and

minor axes of a number were measured. Measurement of diameter sufficed when the shape was spherical and the much elongated or sausage-shaped chloroplasts in very dilute media were treated as cylinders.

It was found that the mean volume was inversely proportional to the osmotic pressure p of the medium over a large range of p. Figure 1 shows volume plotted against $1/p$ for KCl solutions 0·5-0·01M ($p = 22 - 0{\cdot}5$ atm). Each point is the mean and standard deviation for five experiments in each of which 50 chloroplasts were measured. Figure 1 shows that between KCl concentrations of 0·5 and 0·05M, the experimental relation between the volume V and p satisfies the Boyle-van't Hoff law:

$$p\ (V - b) = \text{const.}, \quad .. \quad .. \quad .. \quad (1)$$

where b is a "non-osmotic volume" corresponding to the volume at infinite p. This is approximately 90 μ^3 in the variety used in the experiments of Figure 1. Swelling in glucose followed the same general pattern.

Reasons for the non-linearity of the $V : 1/p$ graph in Figure 1 for $c_0 < 0{\cdot}05$M, where a halving of the osmotic pressure does not produce a doubling in volume, are discussed in Section IV.

Thus the chloroplast contains either a system comparable to the protoplast (Levitt, Scarth, and Gibbs 1936), i.e. an aqueous (vacuolar) phase surrounded by a semi-permeable membrane, or a gel-like structure, or both. In the former, swelling is due to osmotic pressure differences between the media and a solution of sugars and possibly salts, maintained by a semi-permeability of the separating membrane. In the second system osmotic pressure differences are due to the unequal distribution of ions as a result of the Donnan effect of fixed charges on protein molecules in the gel system. Experiments are to be described which bear on this question.

(*d*) *Electrophoresis of Isolated Chloroplasts*

The electrophoretic mobility of *Nitella* chloroplasts, isolated as described in Section II, in glucose + buffer solution was measured as a function of pH in a cell of special design.*

The results are shown in Figure 2 in which electrophoretic mobility in μ $\text{sec}^{-1}/\text{V cm}^{-1}$ in buffers of ionic strength 0·01 is plotted against pH of the buffer solution. It is seen that migration is towards the cathode (positive charge on the chloroplasts) at pH values less than 4·2 and towards the anode at pH values more alkaline than this. The isoelectric point of the surface of these bodies is thus at pH 4·2 in sodium acetate-acetic acid buffer. This is similar to the isoelectric point of the "chlorophyll-protein complex" measured by Fishman and Moyer (1942). The mobility values have been corrected for the viscosity of the glucose solutions. Table 1 gives charge density σ for a number of pH values, calculated according to Abramson and Moyer (1936). The charge density at near neutral reaction is similar to that calculated by these authors

* A fuller account of the electrophoresis of chloroplasts and other cell components will be published later.

for various blood cells, but the isoelectric point is much higher. Red cells are isoelectric at pH *c.* 1·7 (Furchgott and Ponder 1941).

The electrophoretic behaviour of the isolated chloroplasts is thus consistent with the presence of a protein or lipoprotein component at the surface. It is unlikely to be a phospholipid as these commonly have an isoelectric point at pH *c.* 3 (Frey-Wyssling 1948). The possibility that the results are due to the adsorption of a cytoplasmic protein retained after isolation cannot be dismissed, although repeated washing, centrifugation, and resuspension in fresh buffered glucose did not alter the results.

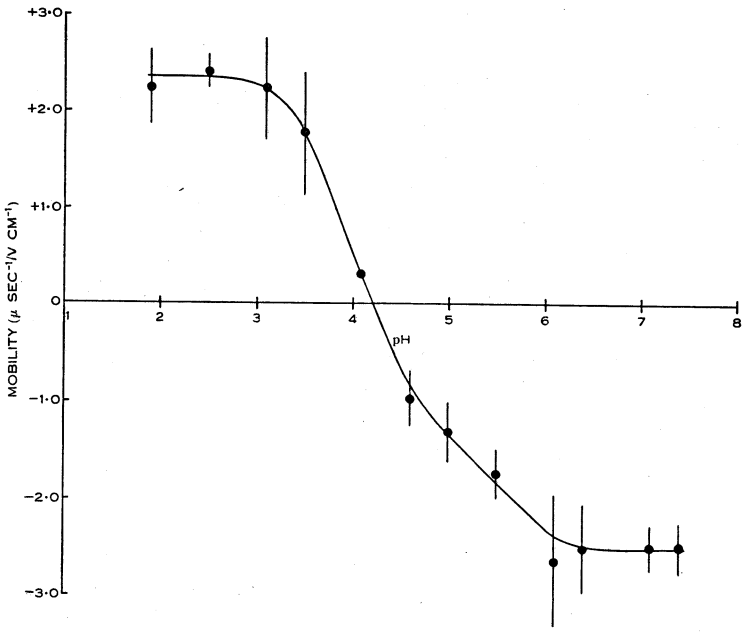

Fig. 2.—Mean mobility v in μ sec^{-1}/V cm^{-1} plotted against pH of buffer solutions for electrophoresis of *Nitella* chloroplasts. Each point is the mean and standard deviation of a number of measurements.

The mobilities and isoelectric point were also unaltered after treatment with 1 per cent. solution of crystalline trypsin, buffered to pH 8 for 2 hr at 37°C. The chloroplasts appeared normal under the light microscope after such treatment. Thus the component of the chloroplast responsible for the electrophoretic behaviour described above appears to be resistant to the proteolytic enzyme trypsin, or else this component is continuous from the surface inwards, the trypsin merely removing some of the external molecules. However, prolonged trypsin treatment left the chloroplast unaltered except for a disorganization attributable to the alkalinity of the medium. The experiment will be repeated with pepsin and lipase.

The possible existence of a three-dimensional system of fixed charges (Donnan system) will now be considered in regard to the water relations of the chloroplast as a function of pH.

(*e*) *The Water Relations of Isolated Chloroplasts as a Function of pH*

When samples of chloroplasts were placed in 0·1M KCl buffered with McIlvaine's (citric acid + disodium hydrogen phosphate) buffer, the mean volume was found to be a function of pH as shown in Figure 3. At a pH in the vicinity of 4 the volume is less than at more acid or alkaline reactions. This effect is similar to that obtained with gelatin (Loeb 1921-22) and supports the alternative mentioned previously that swelling may result from osmotic pressure differences caused by a Donnan distribution of ions. If this is so, the swelling would be expected to be a minimum at the isoelectric point where the net concentration of immobile ions is zero. With the chloroplast a minimum of swelling was found at pH 4, which is close to the isoelectric point of the external surface material and that of the fragmented chloroplast. It seems reasonable to suppose from this evidence also that a material isoelectric at pH *c.* 4 (protein or lipoprotein) extends throughout the chloroplast. It should be noted that the mean volumes in Figure 3 should not be compared with those of Figure 1 since a different variety of *Nitella* was used in these experiments. For example, the "non-osmotic volume" *b* of this variety is 50 μ^3 and therefore the volume at pH 4 is much greater than the "non-osmotic volume."

If the swelling process in the chloroplast is similar to that found in the mature plant cell, then the size of the osmotic gradient between the interior and exterior of the chloroplast will be a major factor in controlling the degree of swelling in solutions. The size of the gradient will be influenced by the permeability of the chloroplast and the chloroplast membrane. Data bearing on this point were obtained from measurements of the apparent free space of the chloroplast.

TABLE 1

SURFACE CHARGE DENSITY σ AS A FUNCTION OF pH IN *NITELLA* CHLOROPLASTS

pH	3·1	3·5	4·1	4·6	5·5	6·25
σ (e.s.u./cm^2)	2411	1841	317	−957	−1795	−2455

(*f*) *The Apparent Free Space of Isolated Chloroplasts*

The apparent free space of the chloroplasts or the percentage of the plastids which appears to reach equality of concentration with the medium by diffusion was calculated as described in Hope and Stevens (1952), from measurements of conductivity of the medium. Approximately 80-90 per cent. by weight of the chloroplast pellet is apparent free space and this value does not seem dependent on the KCl concentration or osmotic pressure of the medium.

Chloroplasts, from a second variety of *Nitella cristata* because of its greater abundance, were isolated as described in Section II in glucose. The final result was a pellet of chloroplasts 0·05-0·1 g in weight which was mopped of excess water although some remained packed with the chloroplasts. This excess water results in an over-estimation of the apparent free space due to dilution of the medium, and may account for 30 per cent. of the change in conductivity

when the chloroplasts are spheres but less when they are oblate spheroids ($p > 4$ atm). Exchange adsorption was not expected to change the conductivity appreciably. 5 ml of KCl or KCl + glucose were added and readings of conductivity made of samples of the supernatant after centrifugation for 2 min at 150 *g*. After correction for water held in the pellet, the apparent free space is approximately that given in Table 2.

As the equilibration between chloroplast and medium is completed before the first possible reading (at 3 min) it is not possible to estimate the diffusivity of KCl in the chloroplast material except that it is much greater than 2×10^{-9} cm^2 sec^{-1} (cf. $1{\cdot}7 \times 10^{-5}$ cm^2 sec^{-1} for 0·1M KCl in H_2O at 20°C). This

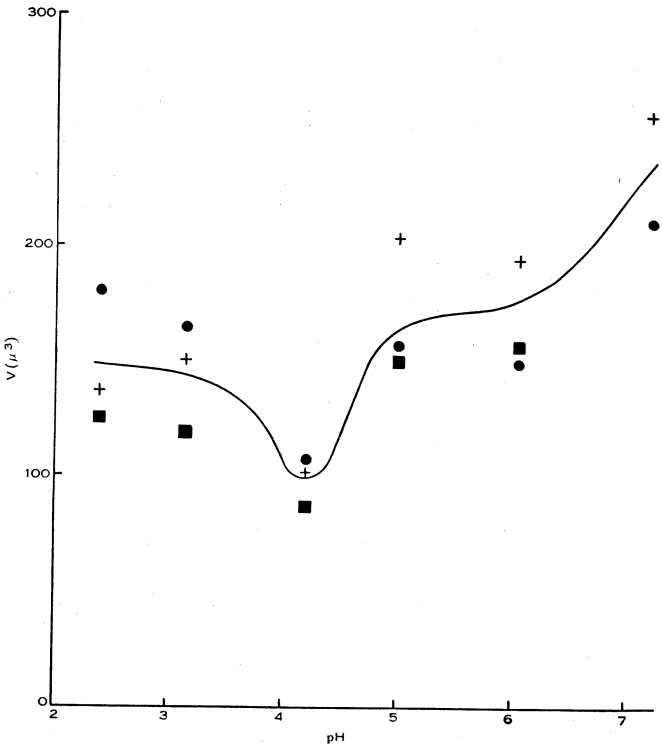

Fig. 3.—Mean volume V in μ^3 of isolated *Nitella* chloroplasts, plotted against pH of buffer solutions.

value is calculated from Fick's equation (see Davson 1943) by assuming diffusion into a sphere of radius 5 μ and $c_i/c_0 = 0{\cdot}99$ at $t = 180$ sec.

Summarizing, these results show that most of the chloroplast volume can be penetrated by KCl in a short time and that the chloroplast membrane is permeable to KCl.

IV. Discussion

The results show that over a wide range of hypotonic solutions the mean volume of the chloroplast of *Nitella* is proportional to the reciprocal of the

osmotic pressure of the solutions. The relationship $V \propto 1/p$ is usually attributed to the swelling of an aqueous phase enclosed by a semi-permeable membrane (Höber 1945). On the other hand, the swelling of a gel system because of the Donnan distribution of ions may also satisfy this relationship (Ponder 1946). Compliance with the Boyle-van't Hoff law does not distinguish between the two types of systems. The only conclusion justified is that osmotic forces, caused either by the presence of a semi-permeable membrane or by a gel system or both, control the swelling of the chloroplasts.

The importance of the Donnan system is suggested by the relationship between swelling and pH, and also by the presence of the coagulated interlamellar material in the vacuoles. Further, as swelling shows a minimum at approx. pH 4, which is also close to the isoelectric point of the chloroplast surface and "chlorophyll-protein complex," proteins or lipoproteins could be thought to form the non-diffusible ions of the Donnan system. As chloroplasts contain *c.* 40-50 per cent. protein on a dry weight basis (Menke 1938; Frey-Wyssling 1948), there is sufficient protein present to yield a large number of fixed charged groups. Additional support for a Donnan system is shown by the apparent free

TABLE 2

APPARENT FREE SPACE OF *NITELLA* CHLOROPLASTS IN KCl AND KCl + GLUCOSE

KCl (mol/l)	Glucose (mol/l)	Osmotic Pressure (atm)	Apparent Free Space (%)
0·05	0·32	10	84
0·05	—	2·5	70, 66, 71
0·10	—	4·6	87, 80

space data for KCl. If the permeability for the ions of the buffer system used in the isoelectric point experiments is similar to that for KCl, the pH effect most probably operates on substances within the structure of the chloroplast.

At least two structural arrangements within the chloroplast could result in a Donnan system. Either the non-diffusible ions are prevented from diffusion by a membrane, or they are fixed in a three-dimensional structure, or both. Although the electron micrographs show that swelling results from an increase in the volume of the interlamellar regions, they do not demonstrate the relative distribution of the polyvalent ions between the lamellae and the interlamellar regions. The size of most vacuoles would exclude Donnan forces arising from fixed ions in the lamellae, except during the initial stages of the discsphere transformation. Donnan forces resulting from ions attached at a surface would not exert an influence on water molecules beyond *c.* 10 Å from the surface. The vacuoles contain a reticular material which is probably derived from the interlamellar substance. This material, which appears to disperse throughout the vacuole during swelling, may provide free polyvalent ions. As the lamellae and the external chloroplast membrane appear to be impermeable to

this substance, a Donnan system would exist; in this case, the water associated with the polyvalent ions would be dispersed throughout the vacuoles.

The quantitative results on swelling do not permit a distinction to be drawn between these possibilities. An analysis of the fine structure of the chloroplasts favours the view that the interlamellar material rather than the lamellae is the site of the Donnan ions. If the structure of the lamellae is similar to that of the lipoprotein layers in the myelin sheath (Finean 1953) the protein component of the lamellae may not be able to contribute fixed ions because it is screened from the aqueous phase by the lipid component. If this is so, the protein of the interlamellar material would be the dominating factor. Wolken and Schwertz (1953) have postulated that the lipid and protein are arranged in alternate layers, with the hydrophilic porphyrin heads of the chlorophyll molecules in the interface between the lipid and the aqueous protein layer. However, by analogy with the probable structure of myelin (see Finean 1953 for references), it seems more likely that the lamellae comprise *c.* 30 Å layers of protein, with the lipid molecules oriented with axes normal to the protein

TABLE 3

THEORETICAL OSMOTIC PRESSURE DIFFERENCES δp BETWEEN IONIZED GEL (NON-MOBILE ION CONCENTRATIONS A) AND MEDIUM, AND ACROSS A SEMI-PERMEABLE MEMBRANE, FOR EXTERNAL CONCENTRATIONS c_0

c_0 (mol/l):	0·5	0·25	0·1	0·05	0·025	0·01
δp (atm) for:						
$A = 0{\cdot}02$M	0·0048	0·0072	0·024	0·048	0·092	0·199
$A = 0{\cdot}10$M	0·12	0·24	0·86	1·00	1·48	1·97
Semi-permeable membrane	0	11·0	17·1	19·1	20·2	22·0

layers on one or both sides. Aqueous phases would exist between the hydrophilic surfaces of such compound layers. The phytol chains of chlorophyll could penetrate the lipid layers, the hydrophilic porphyrin heads remaining in the aqueous phases. The important distinction between these two views is that in the scheme of Wolken and Schwertz (1953), all of the protein of the chloroplast is in the aqueous phase, thus contributing Donnan ions, while the latter view suggests that the protein of the lamellae may not contribute to the Donnan effect nor to the apparent free space for KCl. Rather the protein of the aqueous phase, i.e. the interlamellar material, would fulfil this function.

Although the evidence demonstrates the importance of Donnan effects, it can be shown that the theoretical osmotic pressure differences due to the Donnan system would account for only a small fraction of the total volume changes observed.

As a first approximation consider the chloroplast, or that part concerned with swelling, as a homogeneous gel containing A mol/l of monovalent cations adsorbed to fixed negative valencies, of number n per protein molecule; i.e. the

protein concentration is A/n. Consider the simpler case where ionic equilibrium occurs without swelling, as this enables us to calculate osmotic pressure differences without the complication of having a variable A.

If the adsorbed cations are all K^+ ions and the medium is KCl, it can be shown that

$$c_i = \tfrac{1}{2}\left[(A^2 + 4c_0^2)^{\frac{1}{2}} - A\right], \qquad (2)$$

where c_i is the concentration of "extra" K^+ and Cl^- ions at equilibrium as a function of A and external concentration c_0. Assume further that n is very large (molecular weight of the protein high) so that the contribution of the protein itself to the osmotic pressure is negligible. Then the osmotic pressure difference between chloroplast and medium is given by

$$\delta p = RT\left[(A^2 + 4c_0^2)^{\frac{1}{2}} - 2c_0\right]. \qquad (3)$$

Now a value of A for calculation purposes can be arrived at as follows. The net negative charge on the external surface at pH 6-7 may mean the presence of a three-dimensional array of protein chains or lattices with similar charge distribution. In such a case at pH 6·25, σ (charge density of surface) = 2455 e.s.u./cm^2 (see Table 1), corresponding to $5{\cdot}11 \times 10^{12}$ unit charges/cm^2, and therefore to 11.55×10^{18}/cm^3. Thus $A \simeq 0{\cdot}02$ "mol"/l of such negative charges. Non-diffusible anion concentrations of this order of magnitude were calculated for the surface and interior of root cell cytoplasm (Hope 1953).

Using (**3**), values for δp for various c_0 values are calculated in Table 3 for $A = 0{\cdot}02$, 0·1M, and for a semi-permeable membrane + "vacuole" phase arbitrarily made isotonic with $c_0 = 0{\cdot}5$M KCl. Thus it is seen that the osmotic pressure differences between the chloroplast phase and the medium, when considering a Donnan system, are less by more than an order of magnitude than those present across a semi-permeable membrane. Only if A is very much greater than indicated by the surface charge density could such a mechanism account for volume : osmotic pressure relations such as in Figure 1. If $A = 0{\cdot}1$M, however, an appreciable swelling tendency would be present owing to Donnan effects.

Thus it seems likely that Donnan forces could not account for all the swelling of chloroplasts in hypotonic solutions. As swelling obeys the Boyle-van't Hoff law, it follows that the main volume changes must arise from an osmotic system consisting of solutes enclosed by a membrane. The fine structure of the chloroplast is consistent with this conclusion. The chloroplast is enclosed by a membrane and the fused lamellae form membrane-like structures. As both the lamellae and the external membrane are permeable to KCl, they are probably also permeable to other small solutes. Hence the interlamellar regions would probably be in equilibrium with the solutes of the cytoplasmic sap. In addition metabolites concerned with photosynthesis would also occur in the interlamella. When the chloroplast was transferred to an external solution water would diffuse into or from it according to the direction of the osmotic gradient between the interior and exterior. The degree of swelling which occurred would depend on the relative rates of movement of water and the solutes.

Several experimental results support this view. Conspicuous vacuoles do not form when chloroplasts are transferred to water by gradually diluting the external solution. Also the departures from the ideal $p : V$ relationships observed are to be expected since swelling would vary according to the rate of loss of solutes from the chloroplast.

The non-reversibility of swelling may indicate that the membranes become completely permeable when stretched, or it may mean that the lipoprotein system becomes disorganized when much swollen.

It is concluded that both a Donnan system and osmotic pressure due to diffusible solutes are responsible for the water relations of the chloroplast. A similar explanation could account for the water relations of mitochondria, particularly since as shown by the electron microscope data they also consist of a laminated system enclosed by a membrane.

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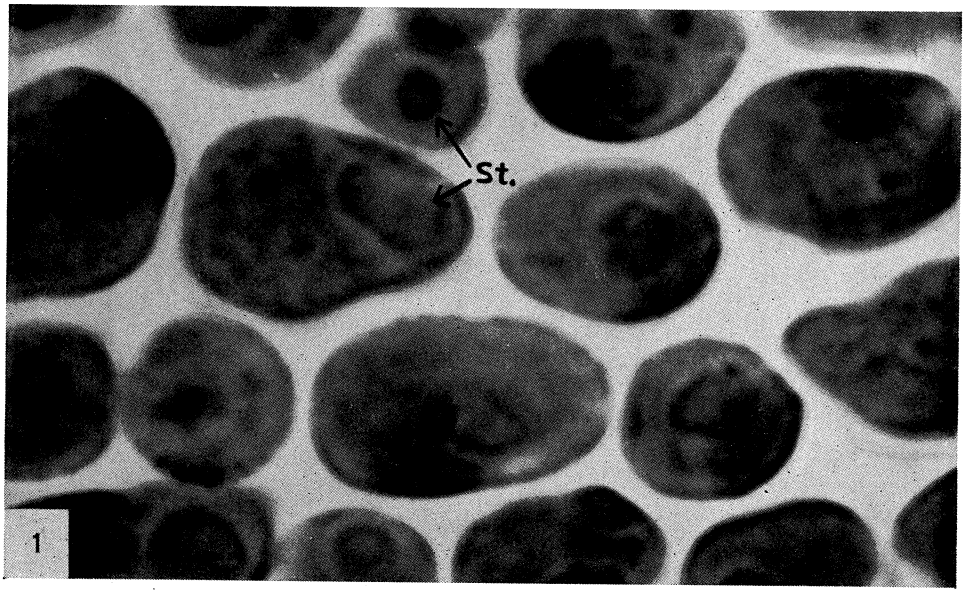

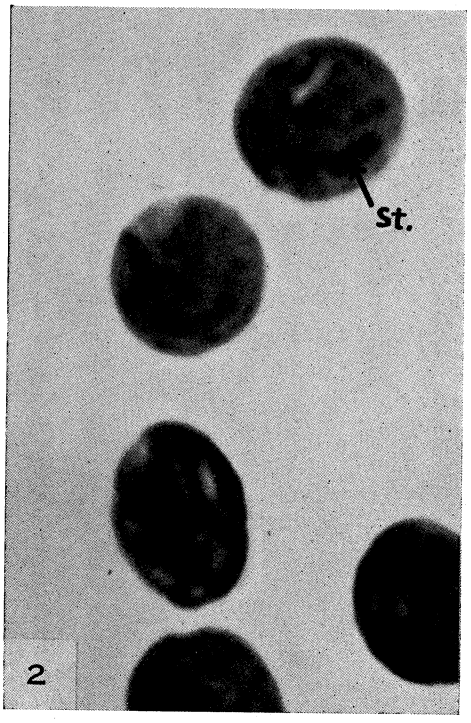

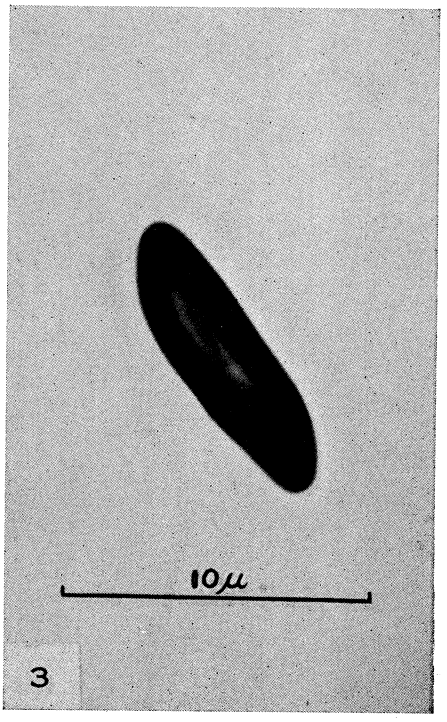

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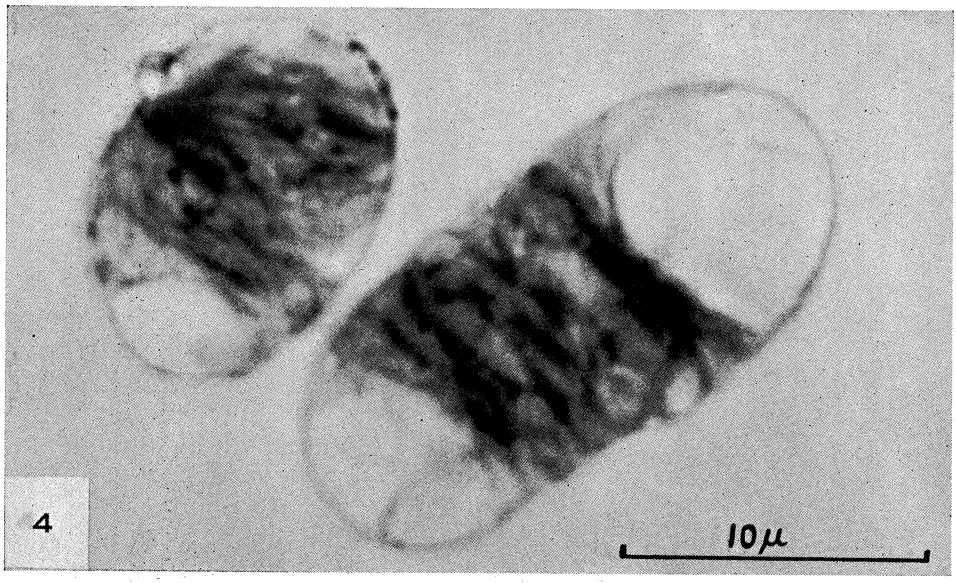

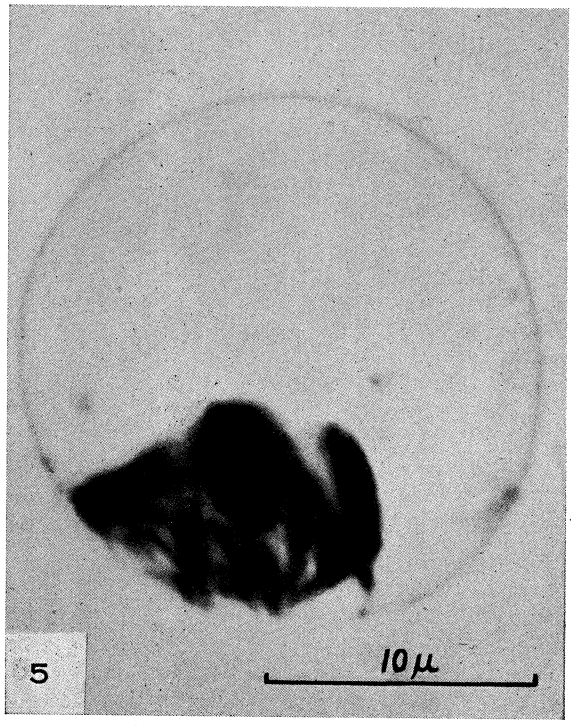

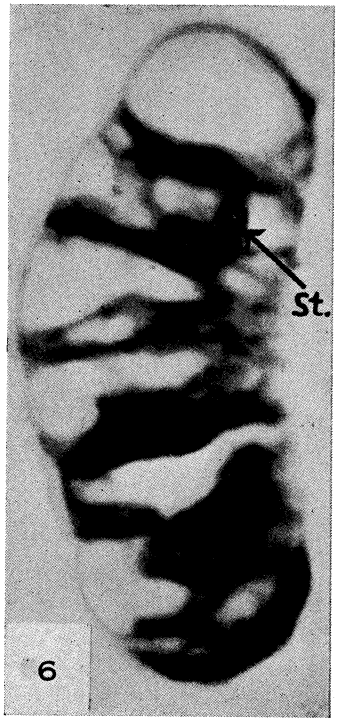

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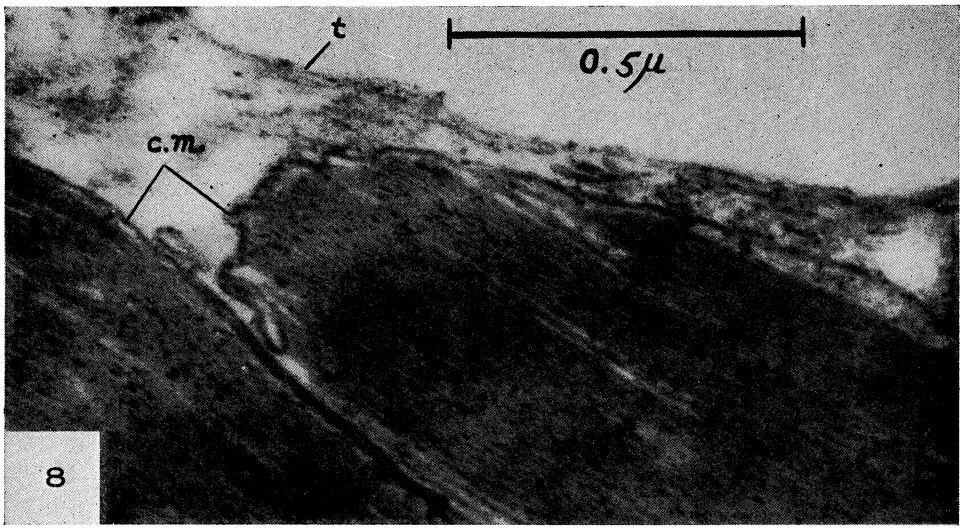

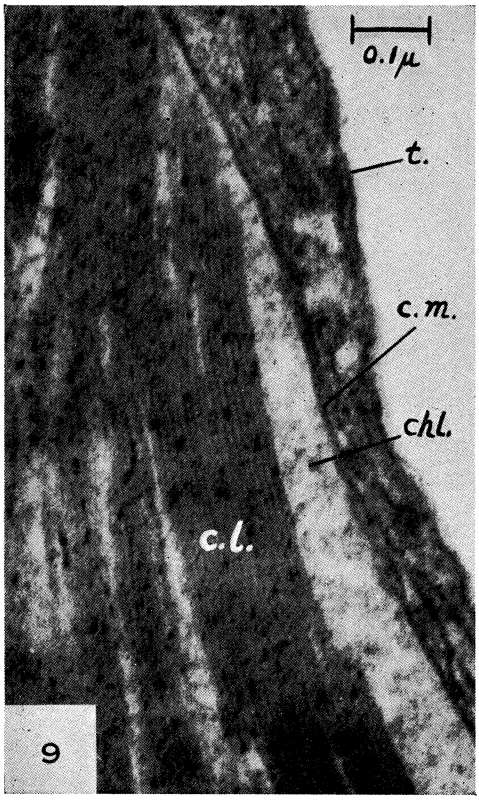

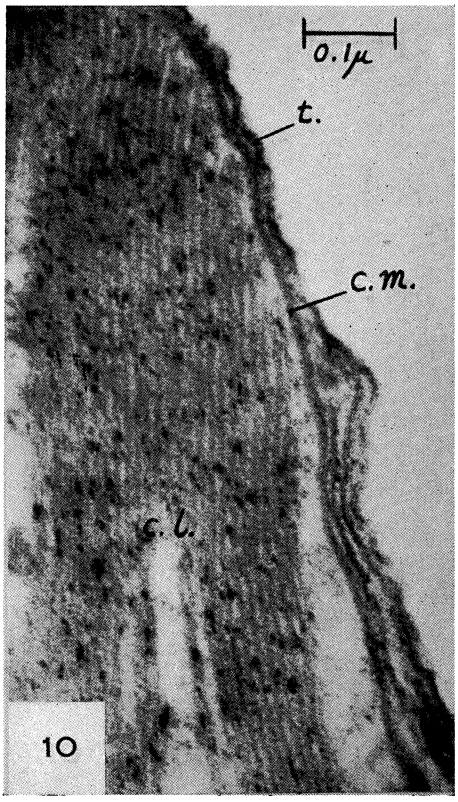

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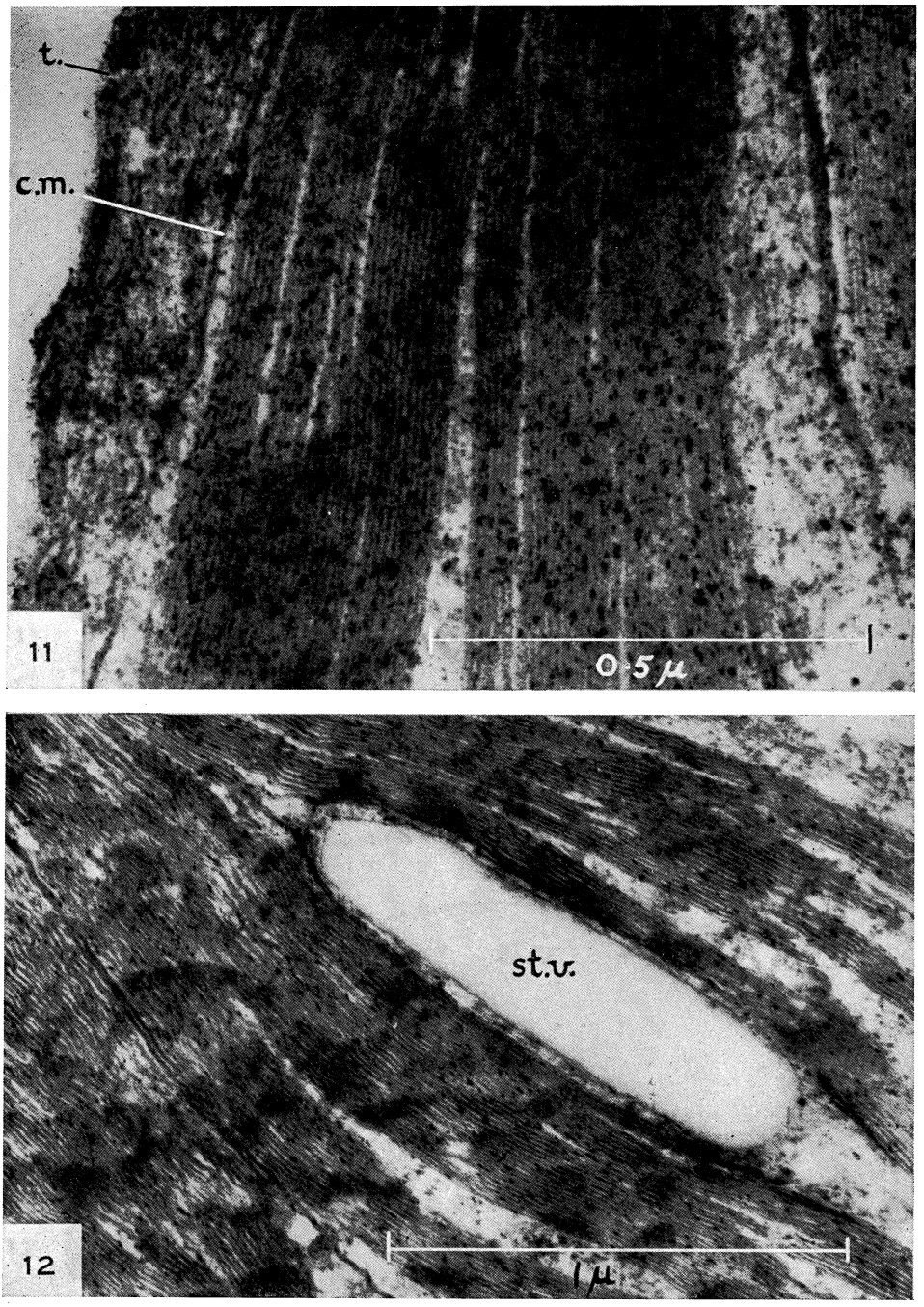

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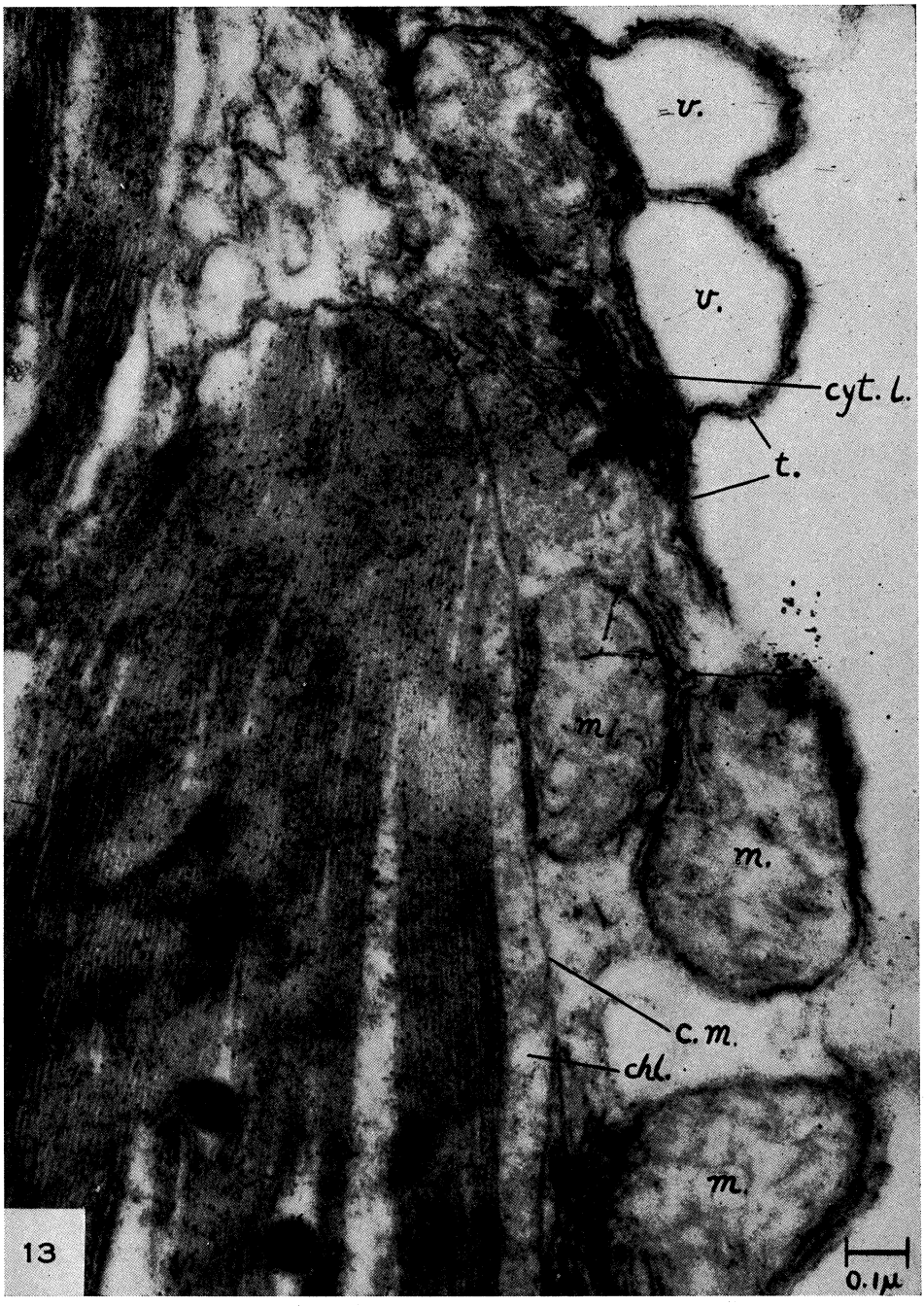

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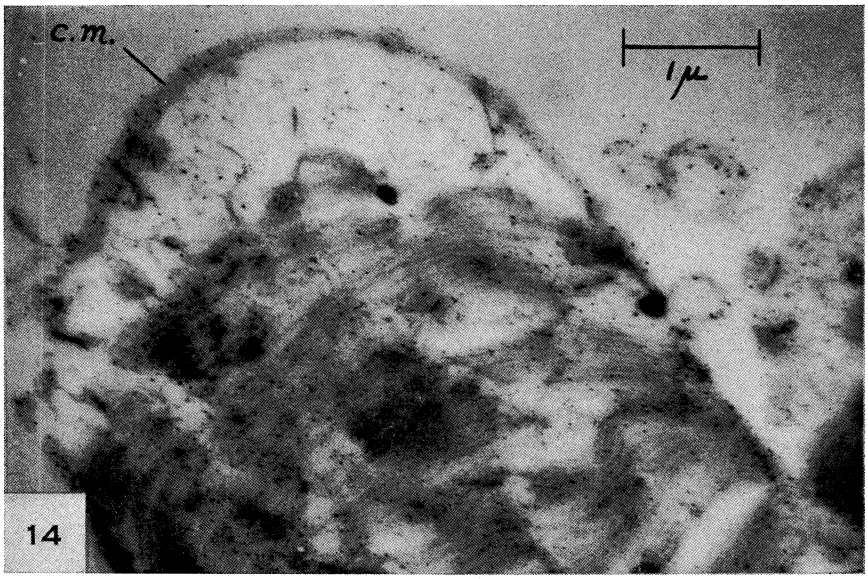

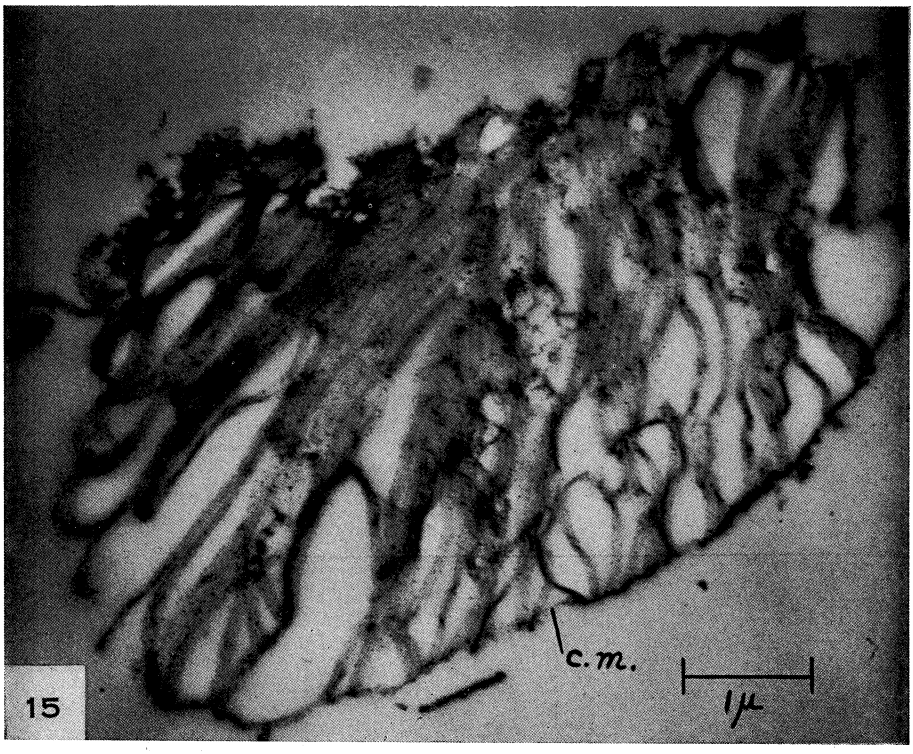

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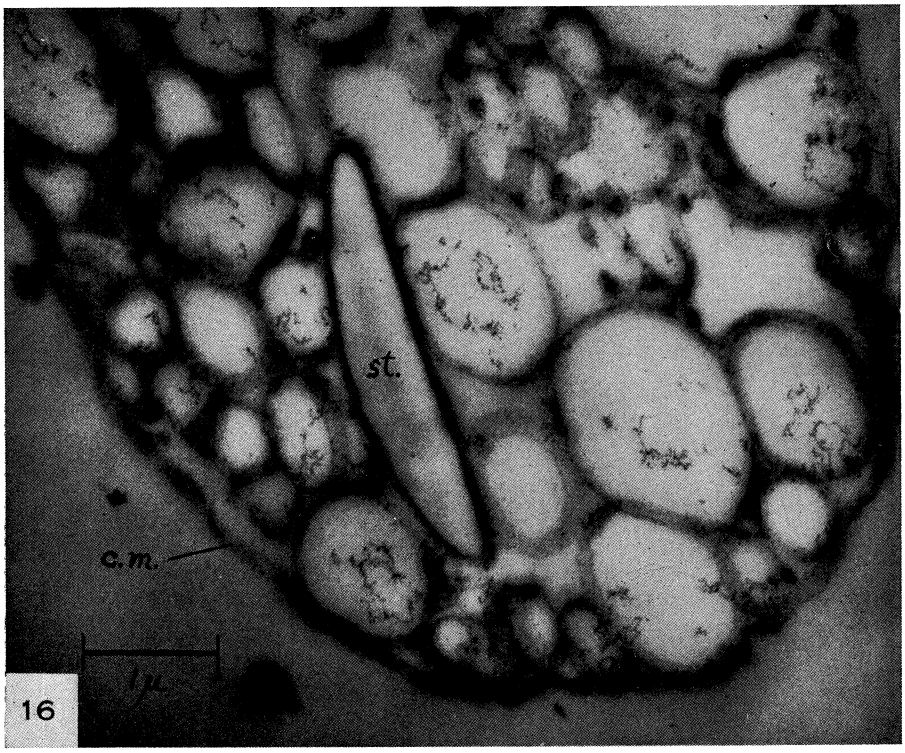

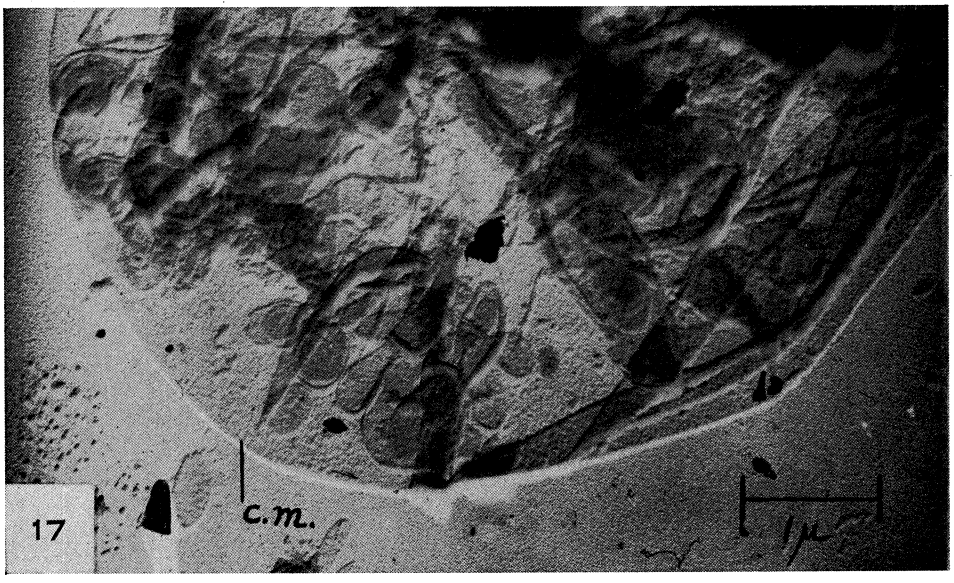

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EXPLANATION OF PLATES 1-8

chl., chloroplasm or interlamellar material; *c.l.*, chloroplast lamellae; *c.m.*, limiting membrane of chloroplast; *c.w.*, cell wall; *cyt.l.*, cytoplasmic lamellar structures; *m.*, mitochondrion; *st.*, starch granule; *st.v.*, starch vacuole; *t.*, tonoplast; *v.*, cytoplasmic vacuole.

PLATE 1

Fig. 1.—Photomicrograph of chloroplasts *in vivo*, surface view. Starch grains present. The line represents 10 μ. Taken in oil immersion. × 4100.

Fig. 2.—Isolated chloroplasts viewed from above. Isolated in 0·5M glucose. × 4100.

Fig. 3.—Side view of isolated chloroplasts. Same medium as for Figure 2. × 4100.

PLATE 2

Fig. 4.—Cylindrical form of chloroplast isolated in distilled water, showing "vacuole" formation. The dark areas appeared green and the light ones almost transparent under the light microscope. The accompanying line represents 10 μ. × 4100.

Fig. 5.—Spherical bleb formation in chloroplasts isolated in distilled water. × 3600.

Fig. 6.—Chloroplasts isolated in distilled water showing conspicuous blebs at the ends and the green coloured reticulum between. × 4100.

PLATE 3

Fig. 7.—Transverse section of osmium-fixed cell of *Nitella*, showing the cell wall and two overlapping chloroplasts lying in the thin cytoplasmic layer. Note the chloroplast membrane, general lamellae organization of the chloroplasts, the starch vacuole, and the dense staining round bodies situated between bands of lamellae. Electron micrograph, × 29,500.

PLATE 4

Fig. 8.—Enlargement of upper left portion of Figure 7 to illustrate more clearly the stacks of lamellae enclosed by a limiting membrane. Note also the tonoplast. Electron micrograph, × 94,000.

Fig. 9.—Portion of a chloroplast *in situ*, showing clearly the well-ordered lipoprotein lamellae and the interlamellar material or chloroplasm within the chloroplast membrane. Note tonoplast and lamellar structures in the cytoplasm. Electron micrograph, × 100,000.

Fig. 10.—Field similar to that of Figure 9, but showing more clearly the tonoplast and the cytoplasmic lamellar structures. Electron micrograph, × 120,000.

PLATE 5

Fig. 11.—Transverse section illustrating the orderly stacking of the lamellae in groups or bands, the intervening spaces being filled with interlamellar material or chloroplasm. Electron micrograph, × 120,000.

Fig. 12.—Transverse section of cell kept in darkness. Note the thin dense membrane of the starch vacuole. Two closely applied chloroplast membranes run diagonally across the bottom left of the field. Electron micrograph, × 63,000.

PLATE 6

Fig. 13.—General view of the cytoplasmic layer in transverse section, including parts of two overlapping chloroplasts, several mitochondria, and a number of cytoplasmic vacuoles. Note the two dense spherical bodies within the chloroplast (lower left), the cytoplasmic lamellar structures (presumably elements of the "endoplasmic reticulum"), and the tonoplast closely applied to the cytoplasmic vacuolar membranes. Electron micrograph, × 80,000.

PLATE 7

Fig. 14.—Thin section of a swollen isolated chloroplast similar to those in Plate 2, Figure 4, showing cylindrical form with end bleb formation. This bleb involves only the intact chloroplast membrane. The chloroplast lamellae are still relatively well ordered, but much more widely separated than in the intact plastid. Electron micrograph, × 18,000.

Fig. 15.—Thin section of swollen isolated chloroplast similar to that in Figure 14, but showing much vacuolization between bands of lamellae. Electron micrograph. × 17,000.

PLATE 8

Fig. 16.—Thin section of a greatly swollen isolated chloroplast. The intact chloroplast membrane encloses a number of thin-walled vacuoles formed by fusion of the individual lamellae. Vacuolization is complete at this stage of swelling. The starch granule is apparently enclosed in a thin dense-staining membrane. The vacuoles contain reticular material, presumably the remains of the chloroplasm. Electron micrograph, ×18,000.

Fig. 17.—Swollen chloroplast similar to that in Figure 16, but mounted by drying on a collodion film, followed by shadow-casting with uranium; ratio of shadow length to object height, 4 : 1. The intact chloroplast membrane is flattened on to the supporting film and can be seen to contain a number of similarly flattened vacuoles. Electron micrograph, × 18,000.