Plant Physiol. (1966) 41, 1686–1694

Reversible Swelling and Contraction of Isolated Spinach Chloroplasts

Tasani Hongladarom[1] and Shigeru I. Honda[2]

Department of Botanical Sciences, University of California, Los Angeles, California 90024

Received September 12, 1966.

Summary. By use of a micro technique for producing extracts of spinach mesophyll cells, chloroplasts were isolated in a state wherein they displayed microscopically visible, reversible osmotic properties. Swollen spherical chloroplasts treated with hypertonic sucrose or mannitol media, but not NaCl, could be shrunken to a state resembling their disk appearance in living cells. Reversible osmotic behavior was more easily demonstrated when the chloroplasts were initially isolated from cells in a relatively low osmolar concentration in contrast to using 0.25 M sucrose or more concentrated media. Individual chloroplasts could be swollen and contracted repeatedly through as many as 4 cycles. The relationship between the capacity for osmotic behavior and chloroplast appearance in cell extracts is discussed.

In the living higher plant cell, chloroplasts consist of a granular, chlorophyllous, stationary lamellar component surrounded and, presumably, interpenetrated by a pleomorphic mobile phase containing stroma (4, 6, 21, 22). In chloroplasts viewed in situ by electron microscopy (14), the stationary lamellar component together with the mobile phase is enclosed by a structural membrane. Whether or not this limiting membrane has properties of differential permeability has not been clearly resolved despite evidence that chloroplasts are osmotically-sensitive structures. A view that chloroplasts are grana-less — i.e., they appear bright, refractile, contracted spheres without grana — may result from chloroplast swelling and contraction behavior. Swelling behavior of isolated chloroplasts changing from contracted homogenous spheres to unfolded granular discs has been documented by cine microphotography (5). This latter transformation is an extreme one resulting in the water-broken type of granular chloroplast (12). Here, we describe a less violent transformation involving reversible osmotic behavior as recorded earlier on cine film (7).

Studies with isolated chloroplasts using measurements of light-scattering, packed pellet volume, and volume distribution curves of chloroplast suspensions (10, 17, 19) and observations of chloroplasts by electron microscopy (10, 13) have indicated that chloroplasts can undergo reversible swelling and contraction under the influence of light and darkness. It is interesting, therefore, that several attempts failed to obtain osmotic reversal of osmotically-induced swelling of isolated chloroplasts as obtained for mitochondria (cf. 1). Chloroplasts were reported to swell readily to volumes consistent with the Boyle-van't Hoff law (15, 17). However, since an osmotic reversal of swelling could not be achieved, the latter chloroplasts may be described more closely as osmotically-sensitive structures rather than osmometers — osmotic sensitivity indicating an irreversible response engendered by an osmotic stimulation.

Wherever osmotic reversal of chloroplast swelling was not successful (15, 17, 21), chloroplasts were initially isolated in sugar media greater than 0.5 osmolar or the cells were relatively harshly treated by blending. Itoh (9) and Hongladarom, Honda and Wildman (7) used a relatively low concentration of osmoticum during the initial isolation of chloroplasts and were able to demonstrate osmotic contraction. However, in the report of Itoh (9) anomalous effects were recorded since in the dark chloroplasts after osmotic contraction did not conform to the Boyle-van't Hoff law. Indeed, the chloroplasts became swollen in concentrated sucrose medium. The anomalous behavior, which corresponds to non-osmotic swelling of chloroplasts (3) even in concentrated hypertonic sugar media (2), presumably can be prevented by use of a suitable medium containing protein, e.g. bovine serum albumin, which protects against non-osmotic swelling (3). Non-osmotic swelling occurs in protein-less media whether or not the osmotic environment is changed.

Inorganic salts, ordered in lyotropic series, contract chloroplasts (16, 18). However, the contraction may be ascribed to effects on fixed charges in the chloroplast (gel) structure — i.e., to Donnan effects not dependent on the presence of a differ-

[1] Present address: Department of Agriculture, Bangkhen Experiment Station, Bangkok, Thailand.

[2] Present address: Department of Biology, Wright State Campus, The Ohio State University, Dayton, Ohio.

entially permeable membrane — since contraction also was induced in water-broken chloroplasts (18) which correspond to naked lamellar systems or chloroplasts stripped of their outer limiting membranes and stroma (12).

A technique is more completely described here (3, 7) for isolating spinach chloroplasts in a condition which permits microscopic demonstration of the osmotic reversal of chloroplast swelling. Since initial chloroplast osmotic swelling probably conforms to Boyle-van't Hoff law (15, 17), our efforts were concentrated on defining conditions necessary for the demonstration of the reversible swelling characteristic of true osmometers.

Materials and Methods

Plant Material and Extraction Medium. Commercial spinach (*Spinacia oleracea* L.) was used as the source of chloroplasts. The extraction medium developed for maximum preservation of organelles in homogenates (3) was used for some experiments. This medium contained 2.5 g Ficoll/100 ml medium, 5 g Dextran-40/100 ml medium, 10 mg bovine serum albumin/100 ml medium, 0.025 M Tris-HCl buffer, pH 7.8, and 0.25 M sucrose as the osmotic agent. This medium is called 0.25 M SFDP. The medium used for isolating chloroplasts in the slightly swollen condition was composed of the same ingredients except the concentration of sucrose was 0.025 M, and will be called 0.025 M SFDP. For contracting swollen chloroplasts, the medium contained 0.5 M sucrose and will be called 0.5 M SFDP. Ficoll, Dextran-40, and bovine serum albumin are not essential for the demonstration of the phenomenon to be described, but they maintain the chloroplasts in a condition where the phenomenon is easier to recognize.

Method for Extraction of Chloroplasts. The lower epidermis of a fully expanded spinach leaf was stripped off, exposing about 1 cm^2 of mesophyll cells. A few drops of the extracting medium were placed on the exposed cells. With a special knife, made of a razor blade chip inserted at 1 end of a wooden applicator stick, the mesophyll region under the medium was cut about 100 times. The cell-free extract was then collected by a capillary pipette and transferred to a slide. A coverslip was laid down and held in place with wax at 2 opposing edges. The chloroplasts on the microscope slide were ready for microscopic observation within about 3 minutes after the stripping of the epidermis.

The concentration of the medium on the slide was changed by placing a drop of solution on 1 edge of the coverslip and sucking the liquid under the coverslip by means of a piece of filter paper on the opposite edge. Thus, the chloroplasts could be kept under continuous observation while the medium was being changed.

If the spinach leaf cells were extracted by grinding in media with mortar and pestle only 20 % of the chloroplasts, compared with 60 to 80 % by the micro method, were capable of reversible swelling. Further concentration by centrifugation and resuspension of the chloroplasts decreased the yield of osmometer-like chloroplasts to 1 %.

Microscopy. Observations were made with a Zeiss phase contrast microscope equipped with a V Z condenser, Neofluar objective lenses, Komplan ocular lenses and basic body II. A Zeiss electronic flash illuminator was used to prevent excessive subject and camera movement during exposure of 35 mm Kodak Plus-X and Panatomic-X film.

Results

Appearance of Chloroplasts Isolated in 0.25 M SFDP Hypertonic Medium. As shown previously (3) most of the chloroplasts (60–80 %) isolated from spinach mesophyll cells in 0.25 M SFDP have a slightly contracted appearance, although closely resembling the appearance of chloroplasts in living cells. The mobile phase, from which extend conspicuous protuberances, can be seen surrounding the grana. When these irregular disk-like chloroplasts were caused to swell osmotically by decrease of sucrose concentration in SFDP medium, recontraction of spherical swollen chloroplasts was only rarely observed upon exposure to 0.5 M SFDP. It appeared that swelling had proceeded to the extent of disrupting the mobile phase (on most of the chloroplasts). This situation was obtained also when buffered sucrose (0.25 M or 0.50 M) instead of SFDP media was used.

From the results of isolating chloroplasts in a series of SFDP media differing only in sucrose concentration, the isotonicity of the chloroplasts was determined to be near 0.08 osmolar (by freezing point depression). Since most isolation media are at least 0.25 M in sugar, it follows that chloroplasts usually are subjected to hypertonic shock during the initial stages of isolation from cells. Partial loss of mobile phase may occur in the initial stages of hypertonic contraction during isolation, e.g. by segmentation of protuberances, and may account for the subsequent inability of the chloroplasts isolated in 0.25 M SFDP to recontract following osmotically-induced swelling.

The Appearance of Chloroplasts Isolated in 0.025 M SFDP Medium and Contraction of the Swollen Chloroplasts. When 0.025 M SFDP was used for isolation, the majority (80 %) of the isolated chloroplasts appeared slightly swollen and more or less spherical in form as shown in figure 1A. However, the grana could be seen in most of the swollen chloroplasts. In these chloroplasts the grana-containing stationary components were still compact and unswollen within the mobile plane which had swollen into the shape of a balloon. Since the

swollen chloroplasts were spherical, the photomicrograph of figure 1A through a single phase of focus does not reveal this situation as clearly as direct observation with the microscope where the spherical structures can be explored through all planes of focus.

When a drop of the 0.5 M SFDP medium was drawn under the coverslip, the majority of the swollen chloroplasts shown in figure 1A, gradually contracted and changed to the configurations shown in figure 1B, the arrows and numbers in figure 1A and B indicating identical chloroplasts.

Similar contractions were observed when sucrose was replaced by 0.5 M mannitol in the hypertonic SFDP medium. However, 0.35 M NaCl (Arnon's medium) could not replace sucrose or mannitol as osmoticum. On the other hand, chloroplasts swollen in 0.35 M NaCl (NaCl ineffective as osmoticum) could be subsequently contracted by mannitol or sucrose media. Therefore, the chloroplast system was not damaged by swelling in NaCl. The chloroplast osmometer system clearly is controlled by a differentially permeable membrane as opposed to an ideal semi-permeable membrane. When the spinach chloroplasts were isolated and manipulated in buffered sucrose instead of SFDP media, the results were the same except that fewer plastids (60–80 %) could be reversibly shrunken.

A few of the swollen chloroplasts in 0.025 M SFDP did not contract but maintained their swollen forms or even continued to swell further in the contracting medium. However, the majority (80 %) of the chloroplasts regained a flattened disk appearance wherein the mobile phase was inconspicuous and the grana conspicuous. The simultaneous contraction of many swollen chloroplasts to the condition shown in figure 1B, was highly reproducible each time chloroplasts were extracted from a fresh sample of spinach leaf tissue with 0.025 M SFDP medium. The phenomenon could also be readily demonstrated with mesophyll chloroplasts isolated from *Nicotiana tabacum* and *N. glutinosa* leaves. It was also possible to record the phenomenon by cine photomicrography (7). The chloroplasts in figure 1B which were contracted, i.e. were capable of osmotic behavior, became swollen spheres again upon addition of 0.025 M SFDP. It should be recalled that the swelling of chloroplasts has always been easy to demonstrate. The problem is to demonstrate cycles of contraction and swelling, the property of reversible behavior of osmometers.

Reversible Swelling and Contraction Through Several Cycles. Of the many swollen chloroplasts that have the capacity to contract, some are able to complete more than 1 cycle of swelling and contraction. Figure 2 shows 1 example of a chloroplast that was induced by different concentrations of sucrose to undergo 4 complete cycles of swelling and contraction.

Chloroplasts prepared in 0.025 M SFDP medium invariably exhibited reversible swelling and contraction when the tonicity of the medium was varied with sucrose. In contrast, if the chloroplasts were originally isolated in a high concentration of sucrose, either in Tris buffer alone or in the SFDP medium, then caused to swell subsequently by decreasing the sucrose concentration, the majority of the swollen chloroplasts did not contract when the sucrose concentration was increased again. In this respect, the behavior of the chloroplast shown in figure 2 was exceptional.

The chloroplast was isolated in 0.25 M SFDP and had the appearance shown in figure 2A. Although not readily apparent in the photograph, the chloroplast was a flattened irregular disk, not a sphere. The mobile phase was conspicuous together with a well preserved protuberance. The grana were also readily resolved. When SFDP medium without sucrose was added beneath the coverslip, the chloroplast began to change its configuration to that shown in figure 2B and finally resulted in the spherical configuration shown in figure 2C. The spherical shape was determined by examination at various focus planes. Some of the mobile phase moved to the tip of the protuberance (fig 2B) and a portion of it accumulated in the form of a small sphere indicated by the arrow in figure 2C. At the same time, the remaining part of the mobile phase still enclosing the chlorophyll-bearing stationary component swelled into a bigger sphere as seen in figure 2C. At this point, 0.5 M SFDP medium was added, the chloroplast contracted, gradually changing from the spherical form through configurations shown in figure 2D, E and F until regaining its original disk shape shown in figure 2G. The chloroplast in figure 2G was induced to swell again by adding SFDP medium without sucrose. It assumed the spherical configuration shown in figure 2H and on addition of 0.5 M SFDP it again reverted to a configuration similar to that shown in figure 2G. Two more cycles of swelling and contraction were observed with this particular chloroplast, but after the swelling in the fifth cycle, the chloroplast failed to respond to 0.5 M SFDP. Suddenly, the small sphere from the protuberance which had decreased in size during each successive cycle then appeared half-filled. It remained so for a few minutes and then it gradually assumed an empty appearance. The sphere containing the chlorophyll-bearing component which had slightly increased in size in each successive cycle continued to swell slowly. This phenomenon was also sufficiently reproducible to be recorded by cinemicrophotography.

Discussion

When chloroplasts do not behave completely as true osmometers (15, 17, 21) it is difficult to account for their volumes conforming to the Boyle-

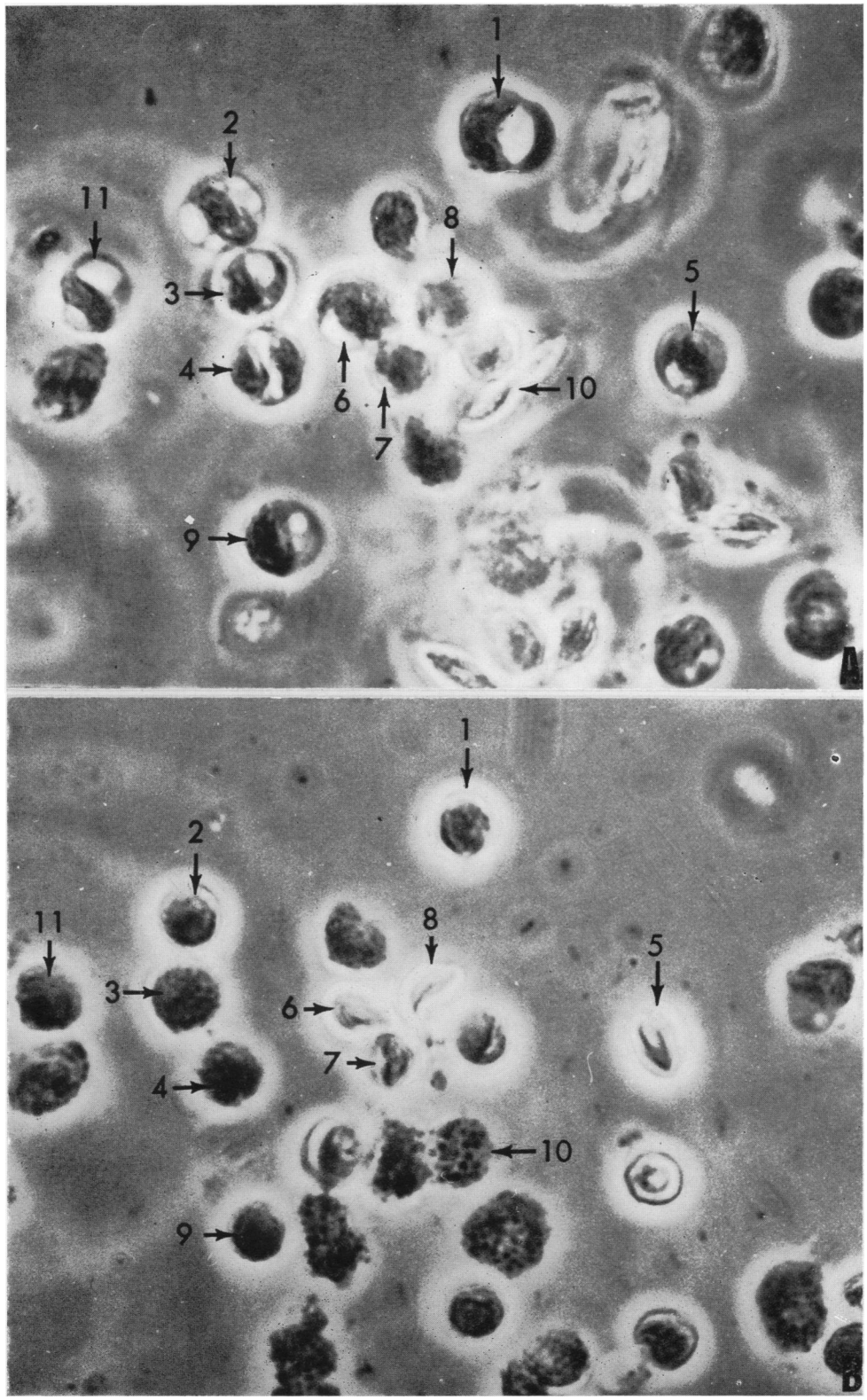

FIG. 1A. Swollen chloroplasts extracted in 0.025 M SFDP. The arrows and numbers indicate identical chloroplasts with those in figure B. Magnification 1600.

FIG. 1B. Shrinkage of the swollen chloroplasts in figure A after 0.5 M SFDP was added. Every chloroplast indicated by arrows and numbers responded to the change of osmotic environment. Magnification 1600.

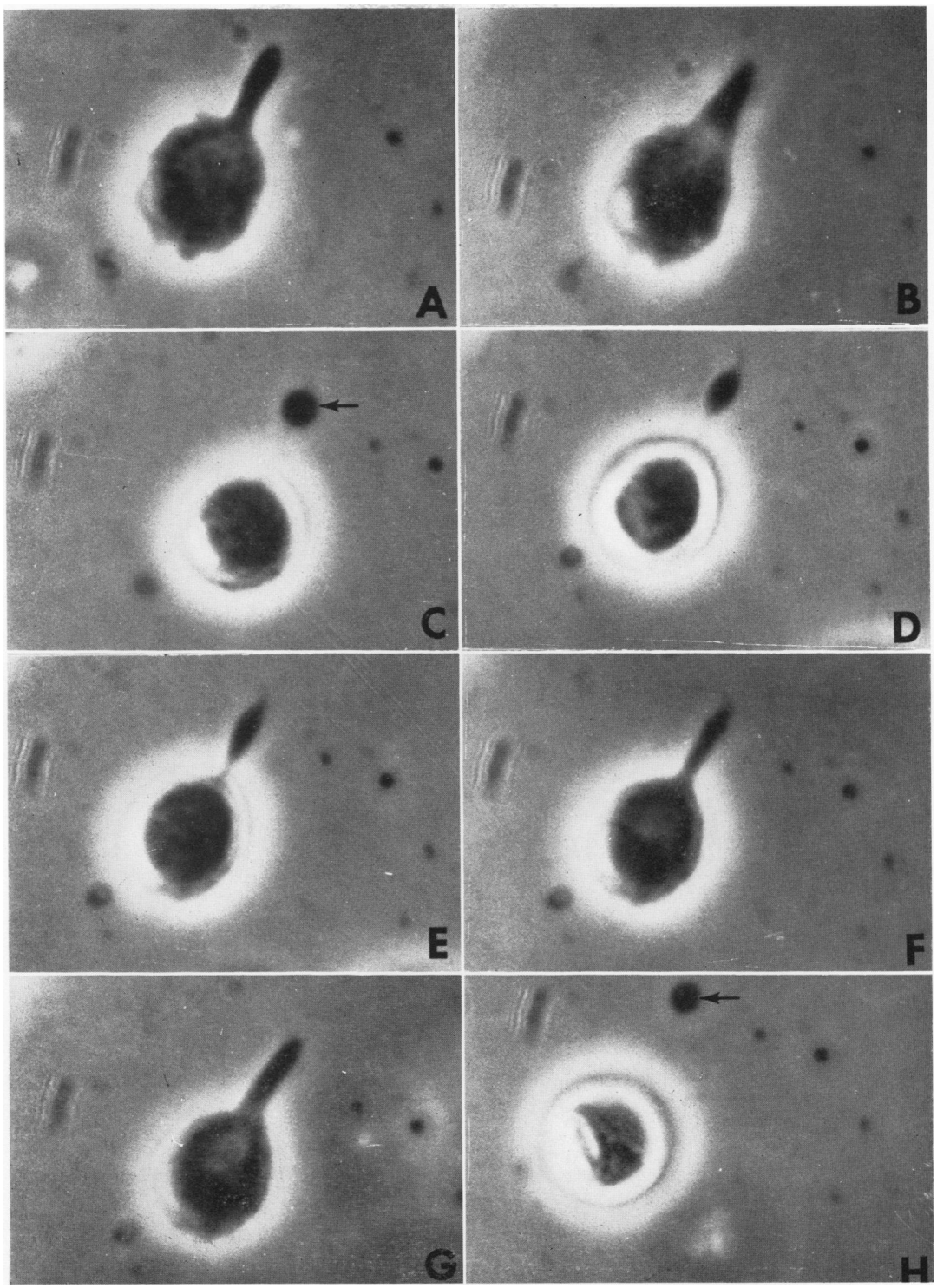

FIG. 2A to H. Reversible swelling and contraction of a chloroplast through several cycles. For explanation, see text. Magnification 3200. Estimated chloroplast volumes based on form changes from disk (A) to sphere (D) to disk (G) to sphere (H) are: (A) 53.7 $\mu^2 \times 0.5\ \mu = 26\ \mu^3$; (D) $4/3\ \pi\ (2.6\ \mu)^3 = 74\ \mu^3$; (G) $47.2\ \mu^2 \times 0.5\ \mu = 24\ \mu^3$; and (H) $4/3\ \pi\ (2.7\ \mu)^3 = 82\mu^3$. Shapes were established by examination at different focus planes.

van't Hoff law after swelling. If differentially permeable membranes were damaged, i.e. became permeable by stretching during chloroplast swelling, the usual explanation to account for failure to reverse osmotic swelling, could the chloroplasts be expected to continue to swell and attain their final volumes in conformity with the Boyle-van't Hoff law? If initially the chloroplasts were osmometers controlled by an elastic differentially permeable membrane, could the chloroplast volumes remain expanded upon the membrane subsequently becoming permeable?

The usually reported permanence of the expanded volume (15, 17, 21) reflects, therefore, the plastic nature of damaged chloroplasts. However, dynamically pleomorphic chloroplasts in vivo (8) and chloroplasts in vitro which show osmotic reversal of swelling (7) were observed to behave elastically. In some cases, we observed that the mobile phase stretched several hundred microns because of the rapid flow of medium over the chloroplasts but still retracted again to a length of 5 to 10 microns after the flow diminished or stopped. Therefore, stretching, in itself, should not disorganize the lipoprotein system during swelling, as suggested by Mercer, et al. (15). There is no evidence, moreover, that organelle membranes stretch and thin during organelle swelling. Rather, the relative constancy of 75 Å unit membrane dimensions (20) may indicate the addition of materials to the membrane during surface area expansion.

In hypertonic media usually employed to isolate chloroplasts, loss of mobile phase followed by self-repair (as evidenced by subsequent swelling behavior (7)) of the differentially permeable membrane may result in extreme shrinkage of the remaining mobile phase. Some loss of mobile phase can occur by mechanical breaking off of protuberances formed during shrinkage and also of those pre-existing protuberances. Shrinkage forces the stationary component to fold on itself and may account for the high frequency of folded chloroplasts in homogenates (class I chloroplasts of reference 21), whereas folded chloroplasts are never seen in living cells. Although the rapidity of the event following cell extraction prevents cine film recording, the actual folding process was observed. An excellent example of a section through a folded chloroplast is shown in an electron micrograph by Kahn and Wettstein (12, fig 4). Swelling of a folded chloroplast may extend the surface area to a point where self-repair of the differentially permeable membrane fails for lack of mobile phase. Subsequently, reconstruction of the chloroplast cannot be induced by hypertonic media.

Chloroplasts which lose enough mobile phase during cell extraction so that repair of the differentially permeable membrane cannot be effected, probably turn into the unfolded chloroplasts with sharply defined grana found with high frequency in homogenates (e.g., class II chloroplasts of ref 21). Because such chloroplasts, corresponding to the naked lamellar systems of Kahn and Wettstein (12), as well as intact plastids, can show light-dark induced swelling and contraction (cf. fig 3 of ref 10), it seems likely that the light-dark induced swelling and contraction properties belong to the stationary lamellar component and are maintained whether or not the mobile phase and differentially permeable membrane are intact.

Chloroplasts which lose none, or only a small amount of the mobile phase, and whose membranes are repaired successfully, are intermediate in appearance between the 2 extreme forms — folded, refractive chloroplasts and unfolded naked lamellar systems, described above. Chloroplasts of this type are likely to swell and contract as long as sufficient mobile phase is present to effect repair of the outer membranes.

Grana-less chloroplasts can be made to appear from granular chloroplasts by reversible osmotic behavior. The contracted, bright, refractile grana-less spherical chloroplasts and the water-broken dark, granular disc chloroplasts are extreme forms which may be produced according to osmotic environment and physical treatment.

Acknowledgments

The authors are grateful for the advice and helpful suggestions received from Drs. Albert Kahn, N. K. Boardman and S. G. Wildman. This work was supported in part by Research Grant AT-(11-1)-34 from the Division of Biology and Medicine, United States Atomic Energy Commission to S. G. Wildman and United States Public Health Service Research Grant 5-RO1 GM 10487 and Research Career Development Award 5-K3-GM-17,795 from the Institute of General Medical Sciences to S. I. Honda. T. Hongladarom is indebted to the Institute of International Education for a Smith-Mundt Fulbright Scholarship and for financial aid from the Thai Government.

Literature Cited

1. CLELAND, K. W. 1952. Permeability of isolated rat heart sarcosomes. Nature 170: 497.
2. GRANICK, S. 1938. Quantitative isolation of chloroplasts from higher plants. Am. J. Botany 25: 558–61.
3. HONDA, S. I., T. HONGLADAROM, AND G. G. LATIES. 1966. A new isolation medium for plant organelles. J. Exptl. Botany 17: 460–72.
4. HONDA, S. I., T. HONGLADAROM, AND S. G. WILDMAN. 1961. Phase microscopic observations of mitochondria and chloroplasts in living spinach mesophyll cells. Fed. Proc. 20: 146.
5. HONDA, S. I., T. HONGLADAROM, AND S. G. WILDMAN. 1962. The appearance and properties of spinach chloroplasts isolated in NaCl, sucrose, and Ficoll-dextran. Plant Physiol. 37: xli.

6. HONGLADAROM, T., S. I. HONDA, P. G. GRUN, AND S. G. WILDMAN. 1962. The fluid nature of the plastid stroma. Plant Physiol. 37: xli.
7. HONGLADAROM, T., S. I. HONDA, AND S. G. WILDMAN. 1964. Chloroplast structure and properties. Abstracts, Tenth International Botanical Congress, Edinburgh, Scotland. p 213–14.
8. HONGLADAROM, T., S. I. HONDA, AND S. G. WILDMAN. 1965. Organelles in living plant cells (revised edition) (16 mm sound color film). Extension Media Center, University of California, Berkeley, California.
9. ITOH, M. 1965. Osmotic effect on the photoshrinkage of chloroplasts. Plant Cell Physiol. 6: 221–31.
10. ITOH, M., S. IZAWA, AND K. SHIBATA. 1963a. Shrinkage of whole chloroplasts upon illumination. Biochim. Biophys. Acta 66: 319–27.
11. ITOH, M., S. IZAWA, AND K. SHIBATA. 1963b. Disintegration of chloroplasts with dodecylbenzene sulfonate as measured by flattening effect and size distribution. Biochim. Biophys. Acta 69: 130–42.
12. KAHN, A. AND D. VON WETTSTEIN. 1961. Structure of isolated spinach chloroplasts. J. Ultrastruct. Res. 5: 557–74.
13. KUSHIDA, H., M. ITOH, S. IZAWA, AND K. SHIBATA. 1964. Deformation of chloroplasts on illumination in intact spinach leaves. Biochim. Biophys. Acta 79: 201–03.
14. MERCER, F. V. 1960 The submicroscopic structure of the cell. Ann. Rev. Plant Physiol. 11: 1–23.
15. MERCER, F. V., A. J. HODGE, A. B. HOPE, AND J. D. MCLEAN. 1955. The structure and swelling properties of *Vitella* chloroplasts. Australian J. Biol. Sci. 8: 1-18.
16. NEISH, A. C. 1939. Separation of chloroplasts, a study of factors affecting their flocculation and the calculation of the chloroplast content of leaf tissue from chemical analysis. Biochem. J. 33: 293–99.
17. NISHIDA, K. 1963. Osmotic swelling of isolated chloroplasts. Plant Cell Physiol. 4: 247–56.
18. NISHIDA, K. AND K. KOSHII. 1964. On the volume-changes of isolated spinach chloroplasts caused by electrolytes and sugars. Physiol. Plantarum 17: 846–54.
19. PACKER, L. 1962. Light scattering changes correlated with photosynthetic phosphorylation in chloroplast fragments. Biochem. Biophys. Res. Commun. 9: 355–60.
20. ROBERTSON, J. D. 1958. Structural alterations in nerve fibers produced by hypotonic and hypertonic solutions. J. Biophys. Biochem. Cytol. 4: 349–64.
21. SPENCER, D. AND S. G. WILDMAN. 1962. Observations on the structure of grana-containing chloroplasts and a proposed model of chloroplast structure. Australian J. Biol. Sci. 15: 599–610.
22. WILDMAN, S. G., T. HONGLADAROM, AND S. I. HONDA. 1962. Chloroplasts and mitochondria in living plant cells: cine photomicrographic studies. Science 138: 434–36.