BBA 4046

SHRINKAGE OF WHOLE CHLOROPLASTS UPON ILLUMINATION

MICHIO ITOH, SEIKICHI IZAWA AND KAZUO SHIBATA

Tokugawa Institute for Biological Research and Tokyo Institute of Technology, Tokyo (Japan)*

(Received December 28th, 1962)

SUMMARY

The effect of light on the size and shape of whole spinach chloroplasts were studied by observing the changes of the volume distribution, the packed volume, the light-scattering cross-sectional area and the axial ratio measured by electron microscopy. Upon illumination of a chloroplast suspension, the volume at the distribution maximum decreased to 51–78 % of the volume before illumination, and this volume change was reversed when the light was turned off. This phenomenon of shrinkage was confirmed also by the observations of the packed volume. On the other hand, the light-scattering cross-sectional area increased in the light and decreased in the dark. These changes of the area were interpreted as due to the changes of the axial ratios of chloroplasts, which were confirmed by electron-microscopic observations. These results are presented in this paper together with the effects of ATP and the inhibitors of photophosphorylation and the Hill reaction.

INTRODUCTION

In a previous study[1], the sizes of chloroplasts, and grana were measured by two recently developed techniques; the determination of the average sizes by the "flattening effect" of DUYSENS[2,3] as measured by the opal-glass spectroscopic method[4-6], and the electric measurement of the volume distribution with the Coulter counter[7]. The process of disintegration of whole spinach chloroplasts to grana with dodecylbenzene sulfonate could be followed accurately and quickly by use of either of these new techniques. Increasing attention has been given to the structural changes of biological particles such as the light-scattering changes upon shrinkage and swelling of mitochondria coupled with phosphorylation, which are reviewed comprehensively by PACKER[8] and LEHNINGER[9]. The success in these circumstances in measuring chloroplast sizes accurately and precisely stimulated the authors to examine whether or not illumination of chloroplasts causes any change of their sizes or shapes. The following different approaches were made to detect the changes: (i) the volume–distribution curves were measured with the Coulter counter; (ii) the light-scattering cross-sectional areas were determined by measuring the rectilinear attenuation of light through chloroplast suspensions; (iii) the packed volumes were estimated with a haemotocrit after fixation of chloroplasts in the dark or in the light and (iv) the axial ratios and structures were observed by electron microscopy. These different

* Mailing address.

measures of size and shape changed remarkably and reversibly upon illumination of chloroplasts, and the results are described in this paper together with the effects of inhibitors on this phenomenon. After the present experiments were completed and when this manuscript was in preparation, PACKER[10] reported a great change by illumination of the 90° scattering of light by chloroplast fragments, and correlated the change with photophosphorylation. The results obtained in this study are discussed referring to his data.

EXPERIMENTAL

Preparation of samples. Chloroplasts were squeezed out of leaves of spinach (*Spinacia oleracea*) through cotton cloth into 0.04 M phosphate buffer (pH 7.2). The green juice was centrifuged in a refrigerated centrifuge at 110 × *g* for 12 min. The sediment was suspended in the same and ice-cooled phosphate buffer, and filtered again through the cloth to remove cell debris. The filtrate containing whole chloroplasts was incubated in the dark at 0° for 2 h, during which the chloroplasts isolated in the room light were transformed into their dark states. After the incubation, the suspension was diluted appropriately with 0.04 M phosphate buffer (pH 7.2) and was subjected to various measurements.

Volume distribution. The volume–distribution curves of chloroplasts as well as the total number of chloroplasts per ml of suspension were measured with the Coulter counter model A (see ref. 7) with an orifice of 100 μ in diameter. The principle of this instrument and its applicabilities to various biological particles were reviewed in the previous paper[1]. The measurements were made in a dark room, since weak room light affects the results. To observe the effect of illumination, the sample chloroplast suspension of 50 ml in a beaker set in the instrument was illuminated through 5-cm water layer with an incandescent lamp. The intensity of light at the surface of the beaker was approx. 6000 lux. The same illuminating condition was adopted in the measurements by other techniques described below. The temperature of the suspensions ranged from 15–19°, but was fairly constant within $\pm 0.5°$ during a series of measurements to observe the effect of light. In this paper, the total number of chloroplasts per ml of suspension is designated as N, and the volume distribution is expressed by the number, n, of chloroplasts *versus* log. V, in which V is the volume of the chloroplast in μ^3. In most of the experiments to observe the effect of light, the change of the number, N^*, of the chloroplasts having a volume greater than the volume at the distribution maximum in the dark was followed with time. This is because it takes approx. 10 min to measure the distribution curve and, during the measurement, the volume changes appreciably in the intermediate stage of shrinkage or swelling.

Light-scattering cross-sectional area. The light-scattering cross-sectional areas of chloroplasts were determined with a Cary recording spectrophotometer model 14M. The rectilinear attenuance is a measure of the attenuation of light through a particle suspension pertaining to the parallel transmitted light, and was defined as ${}_pE_p = \log. I_0/I_p$, where I_0 and I_p are the intensities of the parallel incident and transmitted light beams, respectively. Two long and narrow slits were set, one between a 10-cm cuvette and the monochromator and the other between the cuvette and the photomultiplier to eliminate almost all of the light scattered by chloroplasts in suspension. As illustrated previously[6], the following relationship is obeyed for the rectilinear attenuation of light:

$$ {}_pE_p = 0.434\, dNS^* = 0.434\, dNQS \qquad (1)$$

where d and S^* are the thickness of the cuvette and the light-scattering cross-sectional area of the chloroplast, respectively. The ratio, Q, of S^* to the geometrical projected area, S, has been called the efficiency factor. In general, Q is a complicated function of the wavelength of the measuring light, the size and shape of the particles and refractive indices of the particles and the suspending medium, especially when the particle radius is comparable to the wavelength. VAN DE HULST[11] has made a comprehensive survey of the theories and numerical results in this field. It was predicted by these theories that, when the radius is comparable to the wavelength of the measuring light and when the refractive index is not widely different from that of the suspending medium, the value of Q is roughly 2 and may vary somewhat around this value. Previous observations[6] of Q for *Chlorella* and yeast cells showed that the values are fairly constant on the level of the theoretical value in the visible region, and the variations due to the selective scattering[12,13] are at most 20 %. If the value of Q is assumed to be exactly equal to 2.0, one can calculate the value of s from the observed values of ${}_pE_p$ and N. To minimize the effect of selective scattering, the observations were made at the wavelength, 750 mμ, where chloroplast pigments show no perceptible light absorption. The Beer's law was obeyed below ${}_pE_p = 0.55$ at this wavelength with a 10-cm cuvette, so that dilute suspensions of ${}_pE_p = 0.35$–0.55 were used for the measurements of S^* or S. Roughly speaking, S^* measures the projected area of the particle, since the light which has hit the particles is almost totally eliminated in the measurement. Therefore, the value of S^* should increase either when the particle swells or when it is elongated.

Electron micrographs. Chloroplasts in suspension were preincubated in the dark or in the light for 1 h, and were fixed with 1.25 % OsO_4 at pH 7.2 for 2 h under the same light conditions. After dehydration, the specimens were embedded in a mixture of styrene and *n*-butyl methacrylate (4:6) by the method of KUSHIDA[14] Thin sections were cut on a Porter–Blum ultramicrotome (Ivan Sorvall, Inc.), and the micrographs were taken with a JEM-T6 electron microscope (Japan Electron Optics Co.).

Packed volume. Chloroplasts fixed as above were sedimented in a haematocrit at 2700 · *g* for 40 min. The packed volume thus measured was divided by the number of chloroplasts in the suspension to estimate the average packed volume, V_p, per chloroplast.

RESULTS

The effect of illumination

The volume–distribution curve of the chloroplasts (Sample No. 1 in Table I) incubated in the dark for 2 h is shown by Curve A in Fig. 1, which indicates a sharp distribution peak at log. $V_{max} = 1.95$ or $V_{max} = 89\ \mu^3$. This sample, when illuminated with white light for 30 min, underwent a marked change of distribution. As seen from Curve B in the same figure, the distribution peak was shifted by illumination to log. $V_{max} = 1.65$ or $V_{max} = 45\ \mu^3$. The relative change of the volume depends considerably on the sample of chloroplasts. The measurements of several different preparations of chloroplasts showed the shrinkage to 51–78 % of the volume in the dark. The pH optimum for the volume change was found to be 7.0–7.6 in phosphate buffers of 0.04 M. All of the experiments in the present study were, therefore, performed at pH 7.2.

The process of the volume change can be followed sensitively and rapidly by

measuring the number, N^*, of the chloroplasts having a volume greater than the value of V_{max} in the dark. For example, this number, N_0^*, for Sample No. 1 before illumination decreases to N_t^* after illumination, as illustrated in Fig. 1. The time course of the change of N^* upon illumination is shown by Curve A in Fig. 2, which was observed for the same sample of No. 1. The value of N^* dropped rapidly in the first 5 min of illumination, and then gradually to approach a lower and constant level. Repeated experiments showed that the time required for obtaining a constant reading of N^* varies from 15–50 min, depending upon the activity of the sample. This phenomenon of shrinkage is almost completely reversible. When the light was turned off, the value of N^* increased gradually, but the rate of reversion was so slow that it usually took roughly 2 h to obtain the original reading of N^* before illumination. The shrinkage and swelling were repeatedly observable at least several times.

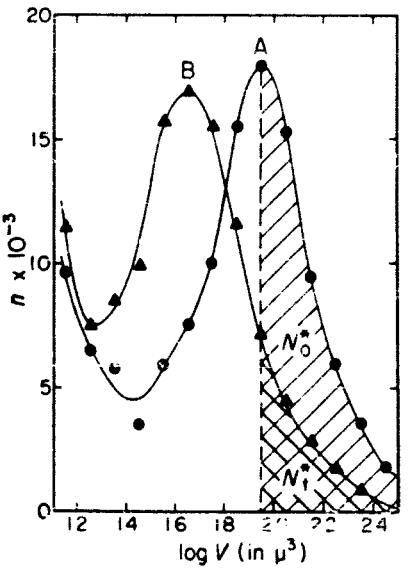

Fig. 1. The volume distribution curves of whole chloroplasts in the dark (Curve A) and in the light (Curve B).

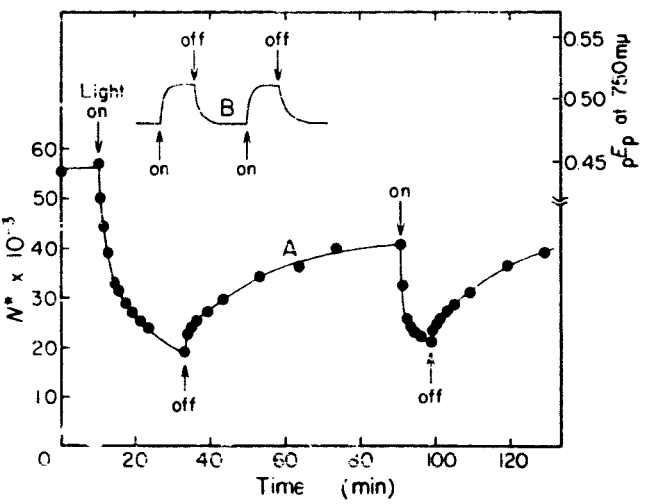

Fig. 2. The time course of the shrinkage of chloroplasts in the light and the swelling in the dark as measured in terms of N^* (Curve A) and ${}_pE_p$ at 750 mμ (Curve B).

The chloroplast suspension of No. 2 was divided into two parts. One part was illuminated for 1 h and fixed with OsO_4 also in the light, and the other part was treated similarly but in the dark. The average packed volumes, V_p, per chloroplast of these fixed samples showed also a considerable change due to illumination as listed in Table I. This fact agrees qualitatively with the result obtained with the Coulter counter. The value of V_p is however, appreciably greater than the value of V_{max} for both samples in the dark and in the light. This is, probably, due to a certain space left between chloroplasts in their packed states. From the volume–distribution curves of these samples before and after the fixation with OsO_4, the shrinkage of chloroplasts due to the fixation was estimated to be less than 10%, and the volume distributions of the fixed samples were unaffected by illumination.

The value of rectilinear attenuance at 750 mμ of a chloroplast suspension responds

also to illumination. The value increased in the light and this change was reversed when the light was turned off (Curve B in Fig. 2). The direction of this change is opposite to that of the volume change. As briefly introduced in the experimental part, the attenuance value should increase either on the swelling of chloroplasts or on the deformation accompanied by an increase of the axial ratios. Since it is certain from the changes of both V_{max} and V_p that chloroplasts shrink on illumination, the increase of the attenuance value in the light is interpreted as due to a considerable change of the axial ratios, which would compensate and overcome the drop of the attenuance due to the shrinkage. From the observed values of ${}_pE_p$ and N, the geometrical and projected areas (S in Table I) in the dark and in the light were calculated to be 29 and 31 μ^2, respectively, assuming that the value of Q is equal to 2.0. Another different feature of this attenuance change is that the change on illumination as well as the reversion in the dark is completed within a period of 10 min, which is considerably shorter than the time required for the shrinkage observed in terms of N^*. This suggests that the deformation reflected in the attenuance change is completed before the volume change proceeds to completion.

TABLE I

THE SIZE AND SHAPE PARAMETERS OF CHLOROPLASTS IN THE DARK AND IN THE LIGHT

V_{max}, the volume at the distribution maximum; V_p, the packed volume per chloroplast; ${}_pE_p$, the rectilinear attenuance; S, the geometrical and projected area calculated from the light-scattering cross-sectional area; and R, the average of the axial ratios of chloroplast sections as measured by electron microscopy.

Sample No.	*Parameter*	*Dark*	*Light*
1	$V_{max}(\mu^3)$	89	45
2	$V_{max}(\mu^3)$	89	93
	$V_p(\mu^3)$	104	90
	${}_pE_p$	0.390	0.415
	$S(\mu^2)$	29	31
3	$V_{max}(\mu^3)$	56	30
	R	1.96	2.34

The electron micrographs of the chloroplasts fixed in the dark and in the light showed a significant difference in shape (Fig. 3). The ellipsoidal chloroplasts in the dark are flattened by illumination, and some of the sections of illuminated chloroplasts look like a long spindle or the crescent. The axial ratios of one hundred such sections of the chloroplasts fixed in the dark or in the light were averaged. As listed in the line of R in Table I, the change due to illumination is appreciable and is more than the errors, ± 0.05, due to random samplings. It has to be noted here that the average of the ratios of the thin sections cut at random is by no means equal to and should be smaller than the ratio between the longest and the shortest axes of the chloroplast. Therefore, the change of the average ratio from 1.96 to 2.34 is an indication of a much greater change of the true axial ratio. This difference in shape would result from or result in a change in the internal structures. Closer comparison between the pictures of the chloroplasts fixed in the light and those fixed in complete darkness seems to indicate a structural change of grana. The osmiophilic layers appear

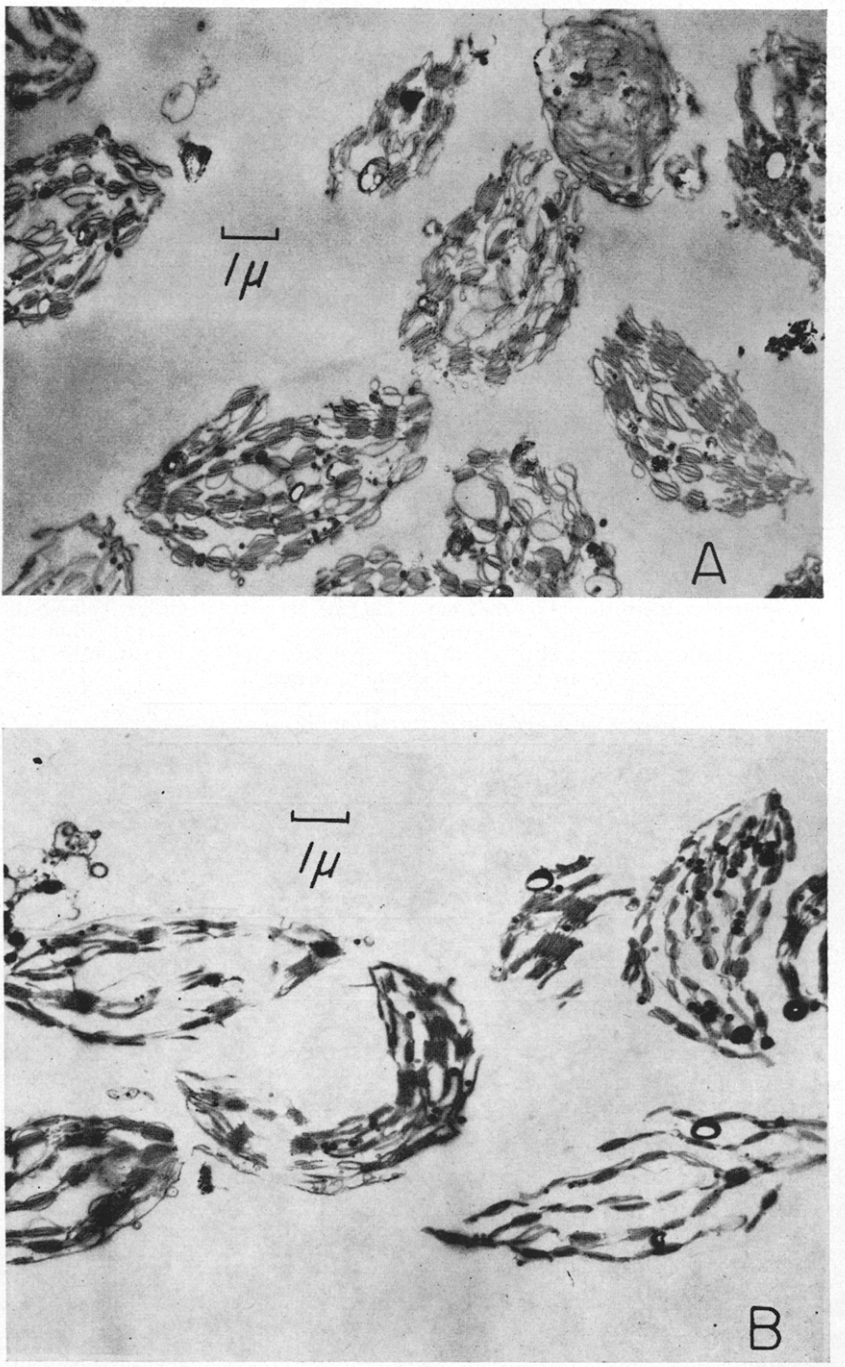

Fig. 3. Electron micrographs of the thin sections of the chloroplasts fixed with 1.25% OsO_4 in 0.04 M phosphate buffer (pH 7.2), after 1 h of preincubation in the dark (Picture A), and those fixed similarly after 1 h of illumination (Picture B).

to be more closely situated with each other in the light than in the dark. This suggests a slight expansion of each lamella after a sufficient time of incubation in the dark. The results obtainable in the more detailed observations will be reported in future.

The effect of reagents

The effect of ATP on the sizes of chloroplasts was studied both in the dark and in the light by measuring the change of N^*. As shown by Curve A in Fig. 4, the value of N^* dropped instantaneously upon addition of 2 mM ATP in the dark, and no further change occurred after the addition. However, when this sample with ATP was illuminated, the value decreased further in the same manner as observed without ATP. Curve B in the same figure shows the result obtained with 3 mM ATP added to the chloroplasts in the light. Here again, the drop of N^* due to the addition occurred instantaneously. The slow decrease of N^* after addition of ATP in the light is, probably, a residual change by light, which might occur without ATP. An increase of ATP concentration to more than those used in the above experiments did not increase the drop of N^*. It is evident from these results that the addition of ATP lowers the volume both in the dark and in the light. This effect is different from the effect of light in that the drop of N^* with ATP occurs instantaneously while the drop due to illumination proceeds with an observable rate.

Ammonium ion is known to be an uncoupler of photosynthetic phosphorylation. Chloroplasts in suspension were incubated with 3.3 mM NH_4Cl for 15 min in the dark, and the effect of illumination on this sample was examined. Curves A and B in Fig. 5 show the results obtained for the samples with and without the uncoupler, respectively, and indicate that NH_4Cl of the above concentration inhibits the shrinkage completely.

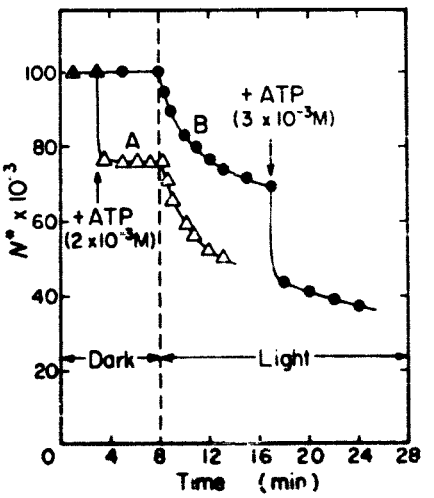

Fig. 4. The effect of ATP on the shrinkage of chloroplasts in the dark and in the light. ATP was added before illumination in the measurement of Curve A and during illumination in the measurement of Curve B.

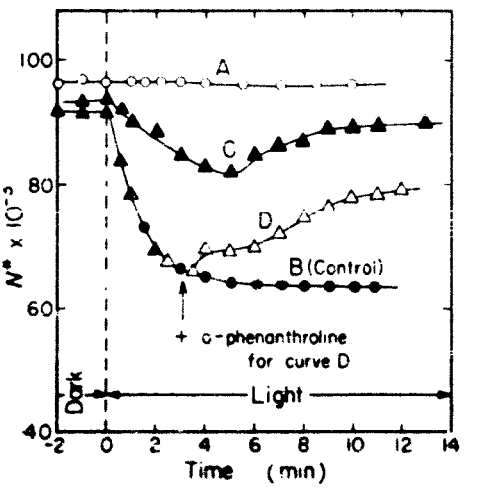

Fig. 5. Effect of NH_4Cl and *o*-phenanthroline on the shrinkage of chloroplasts; Curve A, chloroplasts preincubated with 3.3 mM NH_4Cl in the dark for 15 min; Curve B, the control without inhibitor; Curve C, preincubated with 2 mM *o*-phenanthroline for 15 min, and Curve D, 2 mM *o*-phenanthroline added after 3 min of illumination.

The rectilinear attenuance of the same sample with NH_4Cl was also unaffected by illumination. At this concentration, NH_4Cl did not inhibit the Hill reaction at all as measured with 2,6-dichlorophenolindophenol as the electron acceptor, or rather slightly stimulated the reaction as observed by GOOD[15].

A similar experiment was conducted with 2 mM *o*-phenanthroline. The treatment with this reagent for 15 min inhibited completely the shrinkage of the chloroplasts with a low activity. However, the inhibition was incomplete for a different sample with a high activity of shrinkage. An example of the latter case is shown by Curve C in Fig. 5, which indicates a drop of N^* once lowered in the earlier stage of illumination goes up to the value close to the level before illumination. The same concentration of *o*-phenanthroline was added to the same sample, but after 3 min of illumination (an arrow in Fig. 5). The inhibition in this case started almost immediately after the addition of the reagent. Hydroxylamine is less effective as the inhibitor of the Hill reaction than *o*-phenanthroline. The same sample of chloroplasts as used for the measurement of Curve B was treated with 2 mM hydroxylamine for 15 min. The rate of the decrease of N^* by light was reduced by this treatment to 75 % of the control without the inhibitor, the rate being greater than that observed with the same molar concentration of *o*-phenanthroline. In this case again, the value of N^* rose gradually after a certain time of illumination. By contrast, the treatment with 2 mM KCN showed no effect, and the value of N^* dropped with the same rate as that observed for the control. In a previous study[1], it was demonstrated that chloroplasts are swollen to the maximal extent by treatment with 0.2 mM dodecylbenzene sulfonate for 5 min and the Hill activity is completely lost on the swelling. The volume distribution of the chloroplasts treated with the same concentration of dodecylbenzene sulfonate was unaffected by illumination. The data obtained with these inhibitors indicate a close parallelism between the shrinkage and photophosphorylation.

DISCUSSION

It was established from the measurements of both V_{max} and V_p that chloroplasts shrink reversibly upon illumination. On the other hand, the rectilinear attenuance increased also reversibly in the light. This phenomenon was interpreted as due to a deformation of chloroplasts accompanied by an increase of the axial ratios, which was confirmed by electron-microscopic observations. As discussed above, the change of the average of the axial ratios of thin sections from 1.96 to 2.34 would be an indication of a much greater change of the true axial ratios. According to the most recent observations by PACKER[16], the 90° scattering of light by chloroplast fragments increased in the light and decreased in the dark. In our measurements of the rectilinear attenuance, the light scattered by chloroplasts was almost totally eliminated, so that the increase of ${}_pE_p$ by illumination indicates an increase of the total scattering which accords with the result obtained by PACKER. This agreement suggests that chloroplast fragments also undergo a considerable shrinkage and deformation on illumination, although he did not discuss the scattering changes in terms of size and shape.

PACKER observed a close parallelism between the scattering changes and photophosphorylation. A shrinkage by addition of ATP has been observed for mitochondria[5,9] and erythrocytes[10] after aging these particles. A similar effect of ATP was observed in our experiments on chloroplasts. However, the addition was effective

both in the dark and in the light, and the change on the addition occurs instantaneously while the change by illumination proceeds with an observable rate. This may or may not be accounted for by a simple mechanism that ATP is the substance directly responsible for the shrinkage. Apart from the mechanism involved, the shrinkage seems to have a close connection with the photophosphorylation by chloroplasts, since it was inhibited by ammonium ion, *o*-phenanthroline and hydroxylamine but not by cyanide.

A parallelism exists between the time required for the shrinkage of chloroplasts and the time of induction to attain a steady rate of photosynthesis by higher plants and algae. The shrinkage is nearly completed within 10 min in the light, and the induction period of photosynthesis is usually several minutes or more as summarized by RABINOWITCH[17]. The induction period is, of course, dependent on the prehistory of the sample before illumination, and a sample once illuminated requires 1 h or so to return back to the completely dark state. Approx. 2 h required for completion of the swelling of chloroplasts in the dark may be related to the time of the reversion to the dark state as measured by the induction period of photosynthesis.

ACKNOWLEDGEMENTS

The present study was supported partly by the research grant from the Rockefeller Foundation and the grant from the Kettering Foundation, which were donated to the Tokugawa Institute for Biological Research, one of the organizations where the present study was conducted. The authors wish to express here their heartfelt gratitude to these foundations, and also to Dr. H. KUSHIDA for his great contribution to the electron-microscopic observations of chloroplasts.

REFERENCES

[1] M. ITOH, S. IZAWA AND K. SHIBATA, *Biochim. Biophys. Acta*, 69 (1963) 130.
[2] L. N. M. DUYSENS, *Thesis*, Utrecht, 1952.
[3] L. N. M. DUYSENS, *Biochim. Biophys. Acta*, 19 (1956) 1.
[4] K. SHIBATA, A. A. BENSON AND M. CALVIN, *Biochim. Biophys. Acta*, 15 (1954) 461.
[5] K. SHIBATA, *J. Biochem. (Tokyo)*, 45 (1958) 599.
[6] K. SHIBATA, in D. GLICK, *Methods of Biochemical Analysis*, Vol. 7, Interscience, New York, 1959, p. 77.
[7] W. H. COULTER, *Proc. Natl. Electron. Conf.*, 12 (1956) 1034.
[8] L. PACKER, in T. W. GOODWIN AND O. LINDBERG, *Biological Structure and Function*, Vol. 2, Academic Press, New York, 1961, p. 85.
[9] A. L. LEHNINGER, in T. W. GOODWIN AND O. LINDBERG, *Biological Structure and Function*, Vol. 2, Academic Press, New York, 1961, p. 31.
[10] L. PACKER, *Biochem. Biophys. Res. Commun.*, 9 (1962) 355.
[11] H. C. VAN DE HULST, *Light Scattering by Small Particles*, John Wiley & Sons, New York, 1957, p. 172.
[12] P. H. LATIMER, *Science*, 127 (1958) 29.
[13] P. H. LATIMER, *Year book Carnegie Inst.*, 56 (1956-57) 259.
[14] H. KUSHIDA, *J. Electronmicroscopy (Tokyo)*, 10 (1961) 16.
[15] N. E. GOOD, *Biochim. Biophys. Acta*, 40 (1960) 502.
[16] M. NAKAO, T. NAKAO AND M. TATIBANA, *J. Biochem. (Tokyo)*, 47 (1960) 694
[17] E. I. RABINOWITCH, *Photosynthesis and Related Processes*, Vol. 2, Part 2, Interscience, New York, 1956, p. 1313.