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Light-Induced Chloroplast Shrinkage in vivo Detectable after Rapid Isolation of Chloroplasts from Pisum sativum[1]

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Abstract. **A light-induced shrinkage of chloroplasts *in vivo* could be detected with chloroplasts isolated within 2 minutes of harvesting pea plants. As determined both by packed volume and Coulter counter, the mean volume of chloroplasts from plants in the dark was 39 μ^3, whereas it was 31 μ^3 for chloroplasts from plants in the light. Upon illumination of the plants, the half-time for the chloroplast shrinkage *in vivo* was about 3 minutes, and the half-time for the reversal in the dark was about 5 minutes. A plant growth temperature of 20° was optimal for the volume change. The chloroplast shrinkage was half-maximal for a light intensity of 400 lux incident on the plants and was light-saturated near 2000 lux. The light-absorbing pigment responsible for the volume change was chlorophyll. This light-induced shrinkage resulted in a flattening and slight indenting of the chloroplasts. This chloroplast flattening upon illumination of the plants may accompany an increase in the photosynthetic efficiency of chloroplasts.**

Light-induced variations in chloroplast shape *in vivo* are well known. In 1942 Bunning (1) described diurnal changes in the conformation of chloroplasts in *Nymphaea, Nicotiana,* and *Phaseolus.* Busch (2) reported in 1953 that chloroplasts in *Selaginella serpens* were flat during the day, while at night they became spherical. The flat chloroplasts were in the higher energy state. Vanden Driessche (15) found that chloroplasts in *Acetabularia mediterranea* in the middle of the light period were elongated and could evolve the most oxygen, while in the middle of the dark period they became spherical and less efficient for oxygen evolution. A light-induced flattening of chloroplasts in *Mnium undulatum* has been described by Zurzycki (16) in red light, but in blue light the conformational change was different. The pigment responsible for these light-induced chloroplast shape changes *in vivo* is not known. Kushida et al. (6) in 1964 presented electron microscopic evidence indicating that the chloroplasts in spinach leaves also flattened upon illumination. On the other hand, Packer, Barnard, and Deamer (12) observed very little light-induced change of spinach chloroplasts *in vivo* unless they first infiltrated the leaves with 1 of various solutions. Hilgenheger and Menke (4) using both light and electron microscopy have estimated that the light-induced decrease in chloroplast thickness is 15 to 20 % for chloroplasts in *Nitella flexilis.* In summary, chloroplasts in many species flatten in the light. However, primarily because of the difficulty and descriptive nature of the experiments, this conformational change of chloroplasts in the plant cell has not been well characterized.

Recently, Nobel (10) described a method for gently isolating chloroplasts from *Pisum sativum* within 2 minutes of harvesting the plants. Chloroplasts isolated from plants which were in the light appeared to be about 15 % smaller than those isolated from plants which were in the dark (11), suggesting that the light-induced shrinkage *in vivo* can be observed after the chloroplasts have been rapidly isolated. Using packed volume, Coulter counter, and absorbance techniques, this light-induced chloroplast shrinkage occurring *in vivo* has been quantified, and its kinetics, temperature sensitivity, and dependence on light quality and quantity have been studied.

Materials and Methods

Pisum sativum "Laxton's Superb" was grown in moist vermiculite for 14 days (10). A light intensity of 2000 lux was provided for 12 hours each day by daylight fluorescent tubes, and the plants were grown at 20 ± 1°. Where indicated, the plant growth conditions were varied on the fourteenth day. For chloroplast isolation, 10 g of leaves and stems were quickly cut with scissors into a bag made of 2 layers of nylon cloth. The bag was transferred to a chilled mortar containing 10 ml of 0.2 M sucrose buffered with 0.02 M N-tris(hydroxymethyl)methyl-2-aminoethane sulfonate (TES)-NaOH (pH 7.9). After grinding for

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10 seconds, the material was squeezed through the nylon bag (which retained nearly all whole cells and large debris). The resulting homogenate was centrifuged for 60 seconds at 1000 × *g* at 2°, the supernatant fluid was decanted, and the pellet was resuspended by gently placing the tube on a vortex mixer. The chloroplast isolation was performed under subdued illumination and took only 120 seconds (10).

For packed volume studies, the centrifugation step of the chloroplast isolation procedure was carried out in 50 ml polyethylene tubes which were pre-weighed to 0.05 mg. Immediately after isolating the chloroplasts, the resuspended pellet was centrifuged in the dark for 3 minutes at 10,000 × *g* at 0°, the supernatant fluid was decanted, the walls of the tube were carefully wiped dry, and the weight of the tube plus pellet was determined. The isolation medium had a density of 1.03 g/ml, and this number was used to convert pellet weight to volume. Results for the packed volume of chloroplasts are expressed in μl/mg chlorophyll. To measure chlorophyll, 1.00 ml of 80 % acetone was added to the tube, the pellet was thoroughly resuspended using a vortex mixer, an additional 19.00 ml of 80 % acetone were added, the tube was recentrifuged at 1000 × *g* for 5 minutes, and the absorbance of the supernatant fluid was determined at 645 and 663 mμ with a Cary model 14 spectrophotometer. The chlorophyll concentration was calculated using the absorption coefficients of Mackinney (7).

The supernatant fluid remaining in the pellet (the so-called "dead space") was determined using ^{14}C-dextran (molecular weight of 60,000-90,000; obtained from New England Nuclear). Immediately after isolating the chloroplasts, 0.20 ml of the resuspended pellet was added to 1.00 ml of the isolation medium containing various amounts of ^{14}C-dextran, and the tubes were centrifuged as for the packed volume studies. Approximately 4 mg aliquots of the resulting pellet and of the supernatant fluid were placed on pre-weighed planchets, and the planchets re-weighed to 0.01 mg. The samples were then thinly and uniformly spread on the planchets and dried for 15 hours at 60°. Radioactivity was measured using a gas flow Geiger-Muller system. To determine what fraction of the pellet is penetrated by the supernatant fluid, the results are expressed as cpm/mg pellet divided by cpm/mg supernatant fluid.

A Coulter counter Model B with a 100 μ orifice was employed using maximum instrumental gain and a low aperture current. Volume distributions were obtained for chloroplasts suspended in the isolation medium [0.2 M sucrose, 0.02 M TES-NaOH (pH 7.9)]. Measurements were made at 20°. The chloroplast concentration was approximately 25,000/ml. The entire volume distribution curve was obtained within 4 minutes of isolating the chloroplasts. Control distributions (without chloroplasts) were subtracted to correct for noise. The Coulter counter was calibrated with ragweed pollen (19 μ in diam) and erythrocytes (91 μ^3 under the conditions employed). The mean chloroplast volume in μ^3 was calculated from the volume distribution curve.

Results are expressed as the average ± standard error of the mean.

Results

Daily changes in chloroplast volume are shown in figure 1. Each point is the packed volume of chloroplasts isolated from pea plants maintained under the indicated illumination. For chloroplasts

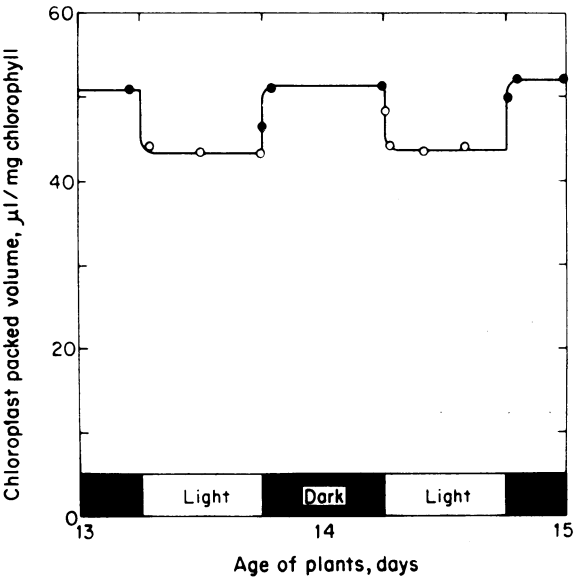

FIG. 1. Daily changes in chloroplast volume. Chloroplasts were isolated at the times indicated from pea plants harvested during the dark (●) or light (○) on the thirteenth and fourteenth days.

isolated from plants which were in the dark, the packed volume averaged 51.5 ± 0.5 μl/mg chlorophyll, whereas it was 43.7 ± 0.3 μl/mg chlorophyll for chloroplasts isolated from plants in the light. This light-induced (light minus dark) change in chloroplast volume was rapid compared with the 12 hour duration of the light and dark periods. The kinetics of such changes were investigated by isolating chloroplasts from plants which were placed in the dark or light (2000 lux) for only 30 minutes (fig 2). Upon illuminating the plants, the half-time for the chloroplast shrinkage was about 3 minutes; the reversal upon putting the plants back in the dark had a half-time of about 5 minutes. Hence pea chloroplasts *in vivo* reversibly shrink when the plants are illuminated, and furthermore, this light-induced shrinkage can be detected after the chloroplasts have been rapidly isolated.

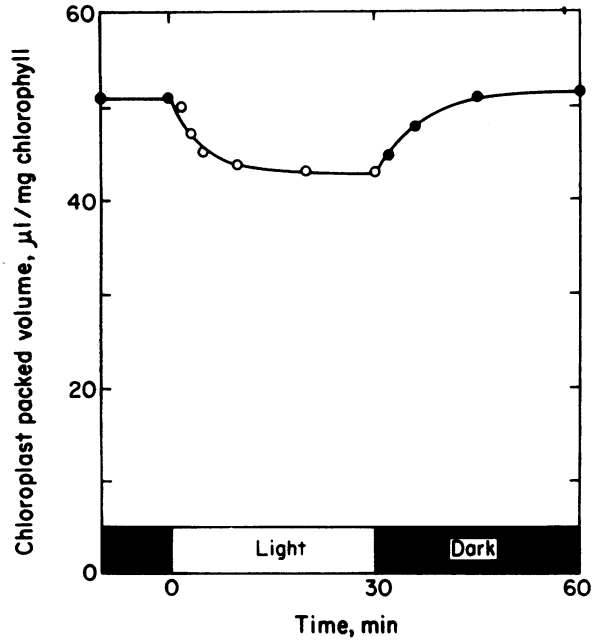

FIG. 2. Changes in chloroplast volume upon rapid cycling of the illumination condition of the plants. Where indicated, plants were illuminated with the usual growth light (2000 lux). Chloroplasts were isolated within 2 minutes of harvesting plants which were in the dark (●) or light (○).

Both the quantity and the quality of the light incident on the plants was varied. The chloroplast shrinkage increased as the light intensity was raised (fig 3). This shrinkage was half-maximal near

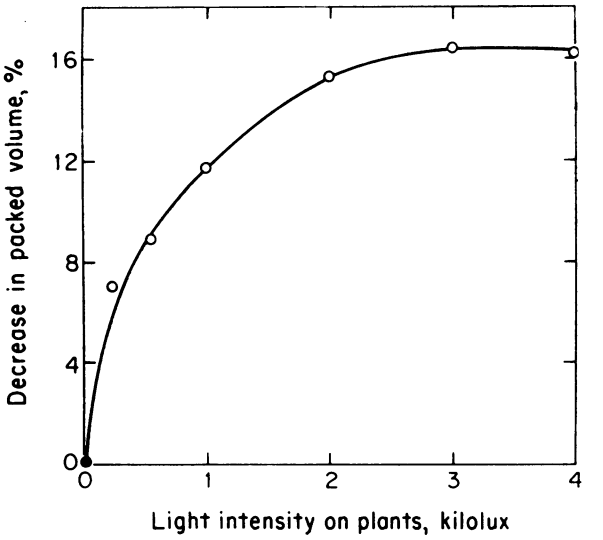

FIG. 3. Light intensity incident on the plants versus chloroplast volume after isolation. The plants were placed for 1 hour under the light intensities indicated (provided by daylight fluorescent tubes). The packed volumes (µl/mg chlorophyll) for the different light intensities were compared with the packed volume of chloroplasts from plants in the dark.

400 lux and approached saturation at 2000 lux (the light intensity used for growth of these plants). To identify the pigment system responsible for this light-induced shrinkage, various wavelength bands were selected for illuminating the plants (table I). Blue light (460 mµ) at 3×10^{14} quanta cm^{-2} sec^{-1} led to a 12 % decrease in packed volume compared with the dark control, and the same intensity of red light (665 mµ) yielded a 7 % shrinkage. For

Table I. *Wavelength Dependence of the Light-Induced Chloroplast Shrinkage* in vivo

The packed volume of chloroplasts from plants in the dark was compared with that after 1 hour illumination with 3×10^{14} quanta cm^{-2} sec^{-1} (11) at the indicated wavelengths. The wavelengths were selected by filters yielding bandwidths at half-maximum of 11 to 18 mµ (11). Each determination was performed twice.

Peak wavelength	Light-induced decrease in packed volume
mµ	%
460	12.4, 11.9
525	1.2, 1.4
590	1.3, 2.8
665	6.3, 7.7
724	1.4, 0.9

green (525 mµ), orange (590 mµ), and far-red (724 mµ) illumination at the above level, the chloroplast volume decreased only about 1 %. These observations strongly implicate chlorophyll as the pigment responsible for the light-induced volume decrease of chloroplasts *in vivo*.

To convert packed volume into chloroplast volume, the amount of supernatant fluid trapped in the pellet must be determined. In preliminary experiments using 0.2 % (w/v) ^{14}C-dextran, the pellet actually contained more radioactivity than did an equal volume of the supernatant fluid. Dextran was entering the chloroplasts and/or adsorbing onto them. Various concentrations of dextran from 0.5 to 3 % were employed and the pellet/supernatant specific radioactivity for chloroplasts from plants in the light and in the dark was determined (fig 4). Since the abscissa in figure 4 is the reciprocal of the dextran concentration, the intercept on the ordinate is the pellet/supernatant radioactivity at an infinite dextran concentration. The adsorption is negligible on a percentage basis at an infinite dextran concentration, and so this intercept can be taken as a measure of the supernatant fluid trapped in the pellet assuming that the chloroplasts are impermeable to dextran. Two interesting results are obtained: A) Dextran apparently adsorbs onto chloroplasts, since the pellet/supernatant ratio of radioactivity decreases as the dextran concentration increases, and B) the "dead space" is 28 % of the pellet for chloroplasts from plants in the dark and 33 % of the pellet for chloroplasts from plants in the light. This latter finding strongly suggests that

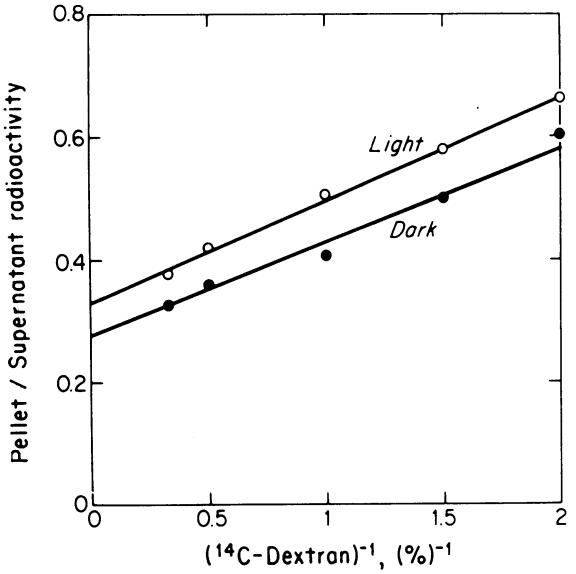

FIG. 4. "Dead space" determination for chloroplasts from plants in the light and dark. After isolation, various amounts of ^{14}C-dextran were mixed with the chloroplast suspension, and the cpm/mg of the pellet following a centrifugation at 10,000 × *g* for 3 minutes was compared with the cpm/mg of the supernatant fluid.

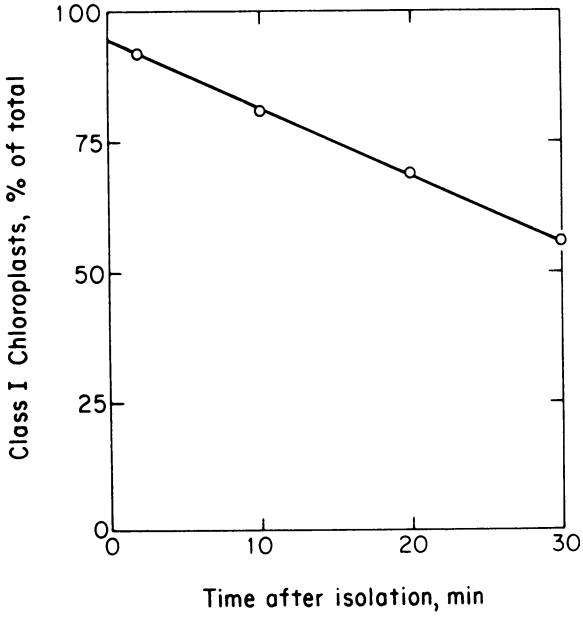

FIG. 5. Change of chloroplast appearance after isolation. The proportion of Class I (bright, refractile, indistinct grana) chloroplasts was observed with a Zeiss Phase Contrast Research Microscope. One hundred or more chloroplasts were counted within 1 minute centered at the indicated times after isolation for each of 4 preparations of chloroplasts from plants in the light (a similar curve was obtained for chloroplasts from plants in the dark).

chloroplasts from plants in the light have changed their shape so as to pack less closely than chloroplasts from plants in the dark.

To obtain the average volume per chloroplast from packed volume data, the number of chloroplasts/mg chlorophyll must be known. Using a Coulter counter, 0.93 ± 0.02 × 10^9 chloroplasts were obtained per mg chlorophyll. Chloroplasts were also observed in a Hemocytometer (Bright-Line counting chamber made by American Optical) which indicated there were 0.92 ± 0.04 × 10^9 chloroplasts/mg chlorophyll. After correcting the packed volume/mg chlorophyll (fig 1, 2, 3) for trapped supernatant fluid (*cf.* fig 4) and dividing by 0.93 × 10^9 chloroplasts/mg chlorophyll, the volume of chloroplasts isolated from plants in the light was 31.3 μ^3, whereas the volume for chloroplasts from plants in the dark was 39.7 μ^3.

Isolated chloroplasts appeared folded and more refractile than those *in vivo*. Also, many chloroplasts isolated from plants in the light were initially cup-shaped. Subsequently the cup "filled up," and the chloroplasts became more disc-like. After 30 minutes, very few cup-shaped chloroplasts remained. Chloroplasts from plants in the dark appeared slightly thicker than those from plants in the light, and very few were initially cup-shaped. The percent of chloroplasts that were Class I (regarded as intact, see below) decreased linearly with time after isolation (fig 5). Judging from the intercept on the ordinate, 95 % of the chloroplasts were Class I at the time of isolation. After 30 minutes, about 56 % of them were Class I.

The light-induced shrinkage of chloroplasts occurring *in vivo* was also studied with a Coulter counter (fig 6). The mean volume for chloroplasts from plants in the dark was 39.1 μ^3, whereas

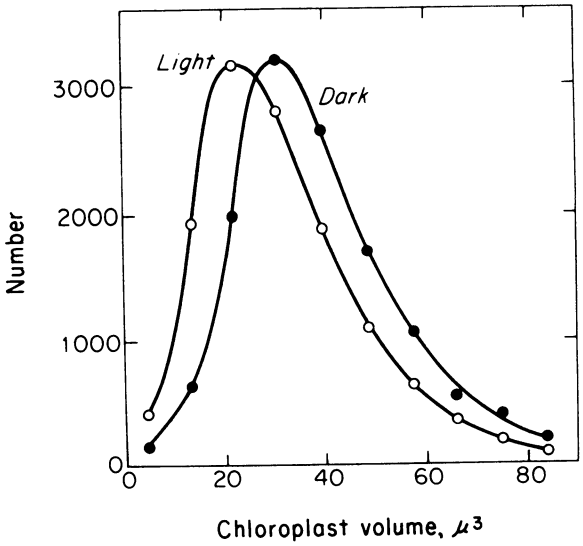

FIG. 6. Volume distribution of chloroplasts from plants in the light and dark as obtained with a Coulter counter.

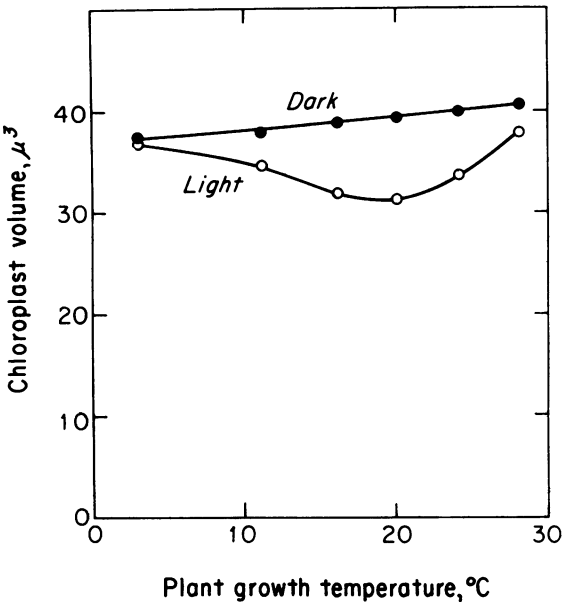

FIG. 7. Temperature dependence of the volume of chloroplasts from plants in the dark and light. The plants were placed for 2 hours in the dark or light at the temperature indicated, chloroplasts were isolated, and the mean volume was determined by calculation from the distribution curve obtained with a Coulter counter.

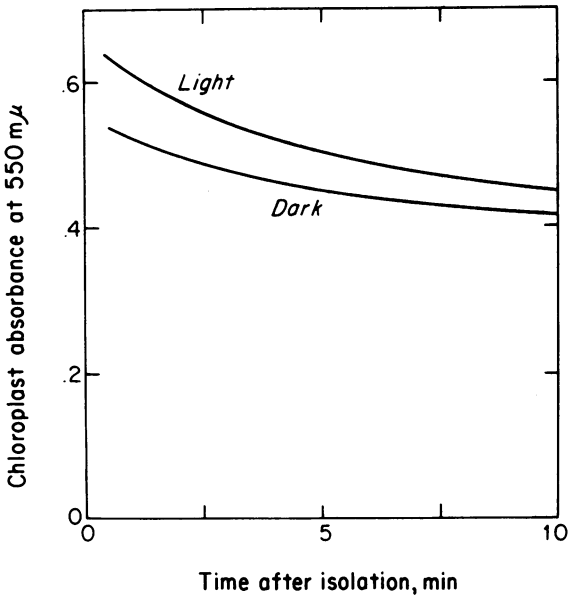

FIG. 8. Absorbance changes of isolated chloroplasts from plants in the light and dark. The absorbance of solutions containing chloroplasts comprising 5 μg chlorophyll/ml suspended in the isolation medium and maintained at 1 ± 1° was measured with a Cary Model 14 recording spectrophotometer. The cuvette was placed near the entrance slit (so-called "bad geometry") such that very little of the scattered light was collected. Measurements were made at 550 mμ which is near the minimum of the chloroplast absorption spectrum (11).

it was 31.3 μ^3 for those from plants in the light. The effect of the temperature used for growing the plants on the volume of the isolated chloroplasts was investigated (fig 7). The volume of chloroplasts from plants in the dark gradually increased as the plant growth temperature (on the fourteenth day only) was raised from 3 to 28°. The volume for chloroplasts isolated from plants in the light was minimal at 20°. Therefore, at the usual growth temperature, the light-induced chloroplast shrinkage *in vivo* was maximal.

For a given chlorophyll concentration, the absorbance of chloroplasts from plants in the light was higher than for those from plants in the dark (fig 8). This is consistent with the light-induced shrinkage, since volume decreases are associated with absorbance increases. The light-induced absorbance difference was fairly rapidly reversed (initially about 6 % per min) once the chloroplasts were isolated. After about 10 minutes at 1°, the absorbance difference between the 2 types of chloroplasts was halved. This is only 12 minutes after harvesting the plants. To observe the light-induced shrinkage occurring *in vivo* sensitivity and reliably, the chloroplast isolation plus volume determination should take less than 10 minutes.

Discussion

The light-induced chloroplast shrinkage which occurs *in vivo* (1, 2, 4, 6, 11, 15, 16) can be detected using chloroplasts isolated within 2 minutes of harvesting the plants. Both the Coulter counter volume distribution curve and the centrifugation step involved in the packed volume determination were completed within 4 minutes of isolating the chloroplasts. From the Coulter counter data, the volume of pea chloroplasts isolated from plants in the light was 31.3 μ^3, whereas the volume for chloroplasts from plants in the dark was 39.1 μ^3. Using the packed volume data after correcting for trapped supernatant fluid and dividing by the number of chloroplasts, the mean volumes were 31.3 and 39.7 μ^3 for chloroplasts from plants in the light and dark, respectively. Hence, both techniques indicate a light-induced chloroplast shrinkage of about 20 %.

Assuming osmotic equilibrium, the chloroplast volume after isolation depends upon the osmotic pressure of the isolation medium compared with that within the chloroplasts *in vivo*. Since the same isolation medium was used throughout and the chloroplasts were smaller from plants in the light, such chloroplasts apparently contained fewer osmotically active particles than those isolated from plants in the dark. To identify this decrease in osmotically active particles within the chloroplast as a light-induced shrinkage *in vivo* presupposes that the osmotic pressure of the cytoplasm is not markedly different for plants in the light compared with the dark. Although this supposition requires

experimental justification, it seems reasonable. Furthermore, the light-induced chloroplast shrinkage *in vivo* has been indicated by both light (1, 2, 4, 15, 16) and electron (4, 6) microscopic studies.

As seen by examination of these rapidly isolated chloroplasts with a light microscope, the light-induced shrinkage *in vivo* involves a decrease in thickness (flattening) plus the indenting of 1 side of the disc. This latter indenting probably causes the chloroplasts to pack with more trapped supernatant in the pellet and hence underlies the larger "dead space" for chloroplasts from plants in the light (33 %) compared with the dark (28 %) as seen by using ^{14}C-dextran. The amount of dextran adsorbed onto the chloroplasts can also be estimated. After subtracting out the contribution of the trapped supernatant fluid to the pellet/supernatant radioactivity ratio, about 0.24 % dextran remained with the chloroplast fraction of the pellet for all dextran concentrations above 0.5 % (*cf.* fig 4). This adsorbed dextran might affect metabolic properties or influence chloroplasts morphologically. The inclusion of dextran in certain isolation media is reported (5) to help preserve chloroplast structure. This may be due to the adsorption of dextran onto the chloroplast membranes.

Spencer and Wildman (13) have described 2 classes of chloroplasts. Class I chloroplasts under phase contrast appear bright, highly refractile, irregular in outline, and the grana are not clearly distinct. Such chloroplasts retain a limiting membrane. The decrease of the proportion of Class I chloroplasts with time after isolation observed here suggests that such chloroplasts may be representative of those *in vivo*. Since 95 % of them were Class I when isolated, this rapid isolation technique yields a high fraction of intact chloroplasts. Class II chloroplasts are less refractile, appear darker green, have distinct grana, and are not surrounded by an outer membrane, *i.e.* are naked lamellar systems. For chloroplasts isolated by the present technique, grana were visible in many of the Class I chloroplasts which were not extremely refractile. This appearance of grana in Class I chloroplasts may be because the isolation medium was chosen to be roughly iso-osmotic (10). Most chloroplast isolation procedures employ hypertonic media in which the chloroplasts shrink; this may cause them to become more refractile and the grana less visible. After the chloroplasts were isolated, their absorbance rapidly decreased. Although this may indicate swelling, more likely this reflects the decrease of Class I chloroplasts. Class I chloroplasts are more refractile and would have a higher absorbance than Class II. The loss of the chloroplast limiting membrane in the Class I to Class II transition may release the substances which cause this difference in absorbance. Thus, the rapid change in absorbance of isolated chloroplasts may simply indicate their fragility.

Spinach chloroplasts appear to be smaller than pea chloroplasts. For spinach chloroplasts in sucrose media, Tolberg and Macey (14) obtained a packed volume of 53 μl/mg chlorophyll, and Nobel (9) found 51 μl/mg chlorophyll (both corrected to the osmolarity used here). The number of spinach chloroplasts/mg chlorophyll has been found by Coulter counter (8) and hemocytometer (8, 14) to be $1.5 \pm 0.1 \times 10^9$ (*cf.* 0.93×10^9 pea chloroplasts/mg chlorophyll found above). Assuming a dead space of 30 %, the volume of spinach chloroplasts is then 24 and 25 μ^3 for the data of Nobel (9) and Tolberg and Macey (14), respectively. For spinach chloroplasts suspended in a sucrose solution of the same osmotic strength as used here, Dilley and Rothstein (3) reported a volume of only 11 μ^3 per chloroplast using a packed volume technique, but their actual calculations are not given. In any case, pea chloroplasts, which are about 39 μ^3 from plants in the dark, appear to be larger than spinach chloroplasts.

The chloroplast volume changes should also be considered from the point of view of the plant. For these peas, there were 798 ± 5 mg leaves/mg chlorophyll (8 determinations using leaves from plants in the light). Based on the packed volume studies and including the correction for dead space, chloroplasts weighed 38 mg/mg chlorophyll when isolated from plants in the dark and 30 mg/mg chlorophyll in the light. From these data, the leaf is 3.8 % chloroplasts by weight in the light and 4.8 % in the dark. This light-induced change in the ion and water content of chloroplasts may affect the ionic composition of the cytoplasm and thereby influence other reactions occurring in the plant cell.

Various experimental techniques have indicated that chloroplasts undergo a light-induced shrinkage *in vivo* which can be detected after the chloroplasts are isolated. The light responsible for this volume change appears to be absorbed by chlorophyll, since the action spectrum for the packed volume decreases obtained with the 5 wavelengths employed (table I) closely agrees with the absorption spectrum of pea chloroplasts (11). As already speculated (11, 15), such a conformational change may have important effects on chloroplast activities such as photosynthesis. In fact, chloroplasts isolated from illuminated pea plants had double the rate of endogenous photophosphorylation *in vitro* compared with those isolated from plants in the dark (11). An action spectrum indicated that light absorbed by chloroplasts *in vivo* was responsible for the increased photophosphorylation *in vitro*. Furthermore, the kinetics and the dependence on growth light intensity were similar for both the light-induced chloroplast shrinkage *in vivo* described here and also for the changes in level of endogenous photophosphorylation of rapidly isolated chloroplasts reported previously (11). The enhancement of endogenous photophosphorylation *in vitro* upon illumination of the plants from which the chloroplasts are isolated may reflect an actual increase in the photosynthetic

capability of the plants. If so, the light-induced chloroplast shrinkage *in vivo* is correlated with increased efficiency of photosynthesis.

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