Volume Contraction of Isolated Pea Chloroplasts Promoted by Bivalent Cations

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1. Chloroplasts isolated from pea seedlings were incubated in sucrose-tris medium reinforced with salts of calcium, magnesium, manganese or iron, at concentrations up to 10 mM. 2. Measurements of chloroplast-pellet volume and water content showed that the bivalent cations brought about a contraction in chloroplast volume and a loss of chloroplast water. This was further substantiated by density-gradient centrifugations. 3. Measurements of the light-scattering and apparent fluorescence of chloroplast suspensions confirmed this conclusion and eliminated the possibility of contraction being caused by centrifugal forces. 4. The uptake of $^{45}\text{Ca}^{2+}$ was measured and shown to be competitive with diluent Ca^{2+} , Mg^{2+} or Mn^{2+} ions, indicating a mechanism of low specificity. 5. The chloroplast contraction was insensitive to light but could be made sensitive by the addition of ferric EDTA. This light-sensitivity was inhibited by added 3-(*p*-chlorophenyl)-1,1-dimethylurea and so probably involves the Hill reaction. 6. On the basis of these observations it is suggested that the process of contraction does not consume much energy, but that in light-activated contraction a previous step occurs that is conducive to contraction and that is energy-transducing. It is postulated that this step results in a local increase in concentration of bivalent ions, which promotes contraction.

A considerable volume of research is currently directed towards an understanding of the relationship *in vivo* of the chloroplast to the surrounding 'soluble phase' of the cytoplasm. This is taking various forms, for example the kinetic approach of Bassham & Kirk (1960) and Smith, Bassham & Kirk (1961) with intact algae in which the movement of ^{14}C into photosynthesis products is studied quantitatively. Evidence of 'compartmentation' and the leakage of products from photosynthesis pools has been obtained.

A second approach is to isolate chloroplasts and determine the complement of enzymes, cofactors and substrates. This approach, applied to chloroplasts isolated by non-aqueous techniques, has provided evidence of enzymic activities (Heber, 1960) and cofactor contents (Ogren & Krogmann, 1965; Heber & Santarius, 1965; Santarius & Heber, 1965).

The non-aqueous isolation procedure must ultimately be supplanted by an aqueous isolation that preserves the native properties of the chloroplast. The organelles can then be incubated in synthetic media simulating the cytoplasm. The reports of Spencer & Unt (1965), Izawa & Good (1965) and Baldry, Bucke & Walker (1966) indicate

that when photosynthetic reactions are measured by the uptake of components from the medium the rate of reaction is less in the intact structure than that shown by fragments, thus stressing the importance *in vivo* of the limit membrane. The work of Spencer & Wildman (1964), Francki, Boardman & Wildman (1965) and Boardman, Francki & Wildman (1965) emphasizes the interdependence of the chloroplast and cytoplasm in the context of protein synthesis.

Interaction of metabolic activity with the structure of the organelle introduces an additional factor. To evaluate this requires the isolation of native chloroplasts, i.e. the use of aqueous systems, but there is still real doubt about the structural integrity of chloroplasts in these preparations. A percentage of swollen chloroplasts that are probably not in the native state always occurs and isolated chloroplasts gradually swell on storage. However, a number of treatments, mostly directed towards an accelerated electron-transport flow (and accompanying photophosphorylation), have been shown in contrast with this to cause chloroplast contraction (Izawa, Itoh & Shibata, 1963; Packer, 1963; Dilley & Vernon, 1964*a,b*, 1965; Hind & Jagendorf, 1965*a,b*).

The work now reported concerns chloroplast contraction promoted by bivalent cations at concentrations frequently encountered in metabolic experiments. No comparable effect has been reported previously, but Izawa & Good (1966) have reported a response of chloroplast volume to salts, including calcium and magnesium salts. The significance of the work probably lies more in the insight it affords into the process of contraction than in demonstrating a basic requirement of isolated chloroplasts.

MATERIALS AND METHODS

Peas (Sutton's variety Meteor) were germinated in vermiculite for 3 days at 25° in the dark, and then transferred to a cooled incubator for 19 ± 4 days' growth before use. The regime during this period was 30°-day and 20°-night temperatures, for a 14-hr.-day period under a mercury fluorescent lamp (Philips HPLR.700, 5762G/93) providing approx. 25000 lux supplemented with a 100w tungsten-filament light-source. Seedlings were harvested from these day conditions and used without any dark-adaptation period.

Leaves from an appropriate number of seedlings (usually 6-15) were homogenized in 25ml. of chilled 0.25M-sucrose in 27.5mM-tris-HCl buffer, pH 7.7 ± 0.2. An MSE top-drive homogenizer was used at maximum speed (14000 rev./min.) for a timed 10sec. Tissue debris was removed by straining the homogenate through four layers of nylon fabric (70 strands to 1 in.), and the filtrate centrifuged for 4min. at 450g in a swing-out head. Chloroplast pellets were resuspended in 1-2ml. of isolation medium, and stored in ice. Suspensions were used as soon as possible and incubations were completed within 2hr. or less of isolating the chloroplasts.

Under the conditions of culture, seedlings did not form starch grains until flowering commenced, and the chloroplast pellets were uniformly green. Pellets from flowering seedlings showed a banded pattern of alternating layers of chloroplasts and starch grains, and these were discarded. Microscopic inspection (Zeiss photomicroscope) of fresh chloroplast preparations showed a high proportion of strongly refractive, spindle-shaped chloroplasts that were considered to be intact (Spencer & Unt, 1965) and unswollen. After 2-4hr. storage in an ice bath such preparations showed increasing numbers of swollen chloroplasts with the characteristic rugose appearance (Nishida, 1963) until eventually these outnumbered the intact form. Other evidence of deterioration over the same time-period was forthcoming from an increase in chloroplast-pellet volume, decrease in turbidity readings and oscillations of the fluorescence measurements.

Chlorophyll was determined by the method of Arnon (1949).

Incubations. Chloroplasts respond to changes in osmotic pressure of the suspending medium by changes in volume (Nishida, 1963; Tolberg & Macey, 1965), although in my experience these adjustments do not entirely fit the equations appropriate to simple osmometers. To minimize the influence of this factor, a standard incubation medium (identical with the isolation medium) was adopted, what-

ever chloroplast property was being examined. This incubation medium (0.25M-sucrose in 27.5mM-tris-HCl buffer, pH 7.7 ± 0.2) was used in control tubes and the appropriate additions for sample tubes were dissolved in the same medium in preparing stock solutions.

A light-induced contraction of chloroplasts was first reported by Izawa *et al.* (1963), that could amount to 40-50% of the chloroplast volume, so the effect of lighting on my chloroplast preparations was investigated. Chloroplasts prepared in the dark and incubated in foil-covered test tubes were compared with suspensions prepared and incubated under laboratory lighting, and with others prepared under laboratory lighting and incubated under the illumination used for seedling growth. No effect of light on cation uptake could be detected, nor on turbidity measurements nor packed chloroplast volume. This was also true if ATP was added to 10mM final concentration, or by simulating phenazine methosulphate-activated photophosphorylation, or by changing from a sucrose to an ionic medium (with or without added ATP). It was concluded that these pea chloroplasts do not display light-sensitive volume changes and the procedure was adopted of preparing and incubating chloroplasts under laboratory lighting conditions, but of confining comparisons to within a set of sample tubes prepared from a chloroplast suspension and handled together throughout the procedure as a set.

Chloroplast-pellet properties. Packed chloroplast volume was determined in haematocrit tubes of 10ml. capacity having the capillary portion of 7.5cm. length and 0.8mm. bore. The tubes were centrifuged (in sets of up to eight at a time) at 1800g for 14min. in a swing-out head. The effect of centrifugal force on the sedimentation of blood cells in haematocrit was investigated by Jackson & Nutt (1950). They report a packed-cell volume of 26 ± 2% (of the whole blood volume) at 3500-7000g but of 29 ± 2.0% at 1800g. Clearly if this were the only consideration chloroplasts should be sedimented at a force approximately double the 1800g adopted; however, experiments in this Laboratory (M. J. Harvey & A. P. Brown, unpublished work) indicate that pea-chloroplast membranes are readily damaged during centrifugation, so the mildest successful sedimentation conditions were adopted.

Optical properties of suspensions. The fluorescence intensity of chloroplast suspensions was measured by using an instrument constructed in the laboratory. Light from a xenon lamp was passed through an Aminco monochromator (set at λ 640 m μ) and projected on to the cuvette. Fluorescence emitted at 90° to the incident light was passed through a 609 filter (transmission 20% at 680 m μ , 60% at 750 m μ) and detected by a photomultiplier.

Incident light scattered through an angle of 90° was measured in the same instrument by removal of the 609 filter. The fluorescence spectrum was determined in a similar instrumental arrangement, but substituting a second monochromator in place of the filter in the fluorescence beam.

Radioisotope experiments. Chloroplasts containing 50-100 μ g. of chlorophyll were incubated in 2ml. of the sucrose-tris medium, and $^{45}\text{CaCl}_2$ (The Radiochemical Centre, Amersham, Bucks.) was added at 33.5m μ Ci in 1 μ mole of carrier CaCl_2 to each incubation. Where appropriate, CaCl_2 , MgSO_4 or MnSO_4 was added to 2.5mM or 0.125mM final concentration; K_2HPO_4 was neutralized to

2.5 mm, and potassium oxalate to 0.25 mm. After incubation the chloroplasts were collected by membrane filtration of the suspension under mild vacuum with Millipore HAWP membranes of 0.45 μ pore size supported on a sintered-glass disk. Retention of $^{45}\text{Ca}^{2+}$ by the membrane material was substantially lowered by presoaking membranes in 5% (w/v) AlCl_3 soln. and blotting off the surplus liquid immediately before use. The chloroplasts and membrane were washed, by filtration, with 2 ml. of sucrose-tris medium and allowed to dry overnight at room temperature before being counted. The membranes were transferred to supporting planchets and counted directly with a Nuclear-Chicago gas-flow counter model 183A, and a gas mixture of argon + propane (95:5). Samples of $^{45}\text{CaCl}_2$ diluted for the purpose with sucrose-tris medium and counted on membranes as though chloroplast samples gave a count of 27.7% of that calculated from the data provided by The Radiochemical Centre. Experimental samples counted repeatedly gave values agreeing in the main to $\pm 5\%$ of the mean count.

Membrane blanks were prepared by filtering a portion of incubation medium, then washing with 2 ml. of sucrose-tris medium and following the counting procedure as though it were a chloroplast sample. The count obtained was subtracted from the sample values to correct for the $^{45}\text{Ca}^{2+}$ retention by the membrane. (This procedure also eliminates the background contribution to the experimental sample count.) Membrane blanks were prepared on every occasion of incubation and, for every change in composition of the incubation medium, the blanks gave counts about 50 counts/min. for sucrose-tris media reinforced with Ca^{2+} , Mg^{2+} or Mn^{2+} , but these rose three- to four-fold in the presence of 2.5 mm-phosphate and seven- to eight-fold in the presence of 0.25 mm-oxalate. In the highest uptakes observed, approx. 10% of the $^{45}\text{Ca}^{2+}$ supplied was removed from the incubation medium, but in most incubations the uptake was less than half this.

RESULTS

Chloroplast-pellet properties. Early experiments in which various isolation and incubation media were being tested showed that the volume of chloroplast pellets responded to composition of the medium. It was quickly established that bivalent cations were significant effectors, and contractions as large as 55% of the initial volume could be obtained by adding, e.g. calcium chloride (to 10 mm final concentration), to chloroplasts in a sucrose-tris medium. Chloroplast-pellet volumes ($\mu\text{l.}/\text{mg.}$ of chlorophyll) obtained in the haematocrit experiment of Table 1 show that a final concentration of 1 mm-magnesium sulphate caused a 37% decrease in volume, whereas neither potassium chloride nor ferric chloride caused contraction. Ferrous sulphate was almost half as effective as magnesium sulphate at the equivalent concentration, though manganese sulphate and calcium chloride produced results quantitatively similar to magnesium sulphate. These results are supported by pellet-water determinations also shown in Table 1, where values for Ca^{2+} , Mg^{2+} and Mn^{2+} are identical, and values for Fe^{2+} fall midway between this and those of potassium chloride and control tubes.

The effect of ion concentration is more fully explored for Ca^{2+} , Mg^{2+} and Mn^{2+} in Fig. 1, in which the chloroplast-pellet volume in the three experiments is plotted against $\log [\text{M}^{2+}]$ (where M^{2+} indicates the effector bivalent cation) over the range 5–0.25 mm. A straight-line relationship is

Table 1. *Chloroplast-pellet volume and water content*

In the pellet-volume determinations chloroplasts in 4 ml. of the sucrose-tris medium, made to a final concentration of 1 mm with respect to the salts indicated, were centrifuged in haematocrit tubes at 1800g for 15 min. In the pellet water-content determinations the final concentration of the salt in sucrose-tris medium was 5 mm and the pellets were prepared at 1800g for 10 min.

Added salt	Pellet vol. ($\mu\text{l.}/\text{mg.}$ of chlorophyll)	Pellet fresh wt. (mg.)	Pellet dry wt. (mg.)	Water/dry wt. ratio
Nil (sucrose-tris control)	86.4	27.46 31.12	7.20 8.30	2.81 2.77
KCl	83.1	32.25 24.52	8.10 6.65	2.98 2.69
MgSO_4	54.6	24.88 23.55	7.95 6.90	2.13 2.42
FeSO_4	73.4	29.66 29.37	8.15 8.40	2.64 2.50
MnSO_4	—	24.90 22.68	7.15 7.40	2.48 2.07
CaCl_2	—	25.60 23.85	7.75 7.55	2.31 2.16
FeCl_3	105.2	—	—	—

Fig. 1. Effect of Ca^{2+} , Mg^{2+} and Mn^{2+} concentration on chloroplast-pellet volume ($\mu\text{l.}/\text{mg.}$ of chlorophyll). Chloroplasts equivalent to approx. $150\mu\text{g.}$ of chlorophyll were incubated in 4 ml. of sucrose-tris medium in haematocrit tubes, and sedimented at $1800g$ for 15 min. CaCl_2 (O), MgSO_4 (Δ) or MnSO_4 ($\square$) was added to incubations as indicated.

indicated, and the slope of the plots is regarded as identical in the three cases. The experimental values at a given cation concentration would then be expected to coincide for the three elements, but to demonstrate this the sedimentation conditions must be made identical, e.g. by the use of a centrifuge head capable of accommodating in one spin the requisite number of tubes.

In Fig. 1 the plots have been extended to the values obtained in the absence of added M^{2+} , and they correspond to $[\text{M}^{2+}] = 51 \pm 10\mu\text{M}$. This could perhaps represent either the endogenous (cytoplasmic) concentration of effector ions, or the concentration experienced during isolation if this is higher, but other interpretations are also possible.

Volume changes of the pellets may not reflect similar volume changes in individual chloroplasts, and although microscopic inspection supported this interpretation the possibility of artifacts or deterioration arising during histological preparation could not be eliminated. Chloroplasts contracted by exposure to bivalent cations should, however, sediment more readily and indications of this were apparent in haematocrit experiments. To substantiate the point chloroplasts were centrifuged in a sucrose-density gradient reinforced in sample tubes with Ca^{2+} , Mg^{2+} or Mn^{2+} at 2.5mM final concentration. Linear gradients of 5 ml. of sucrose (in 7 mM-tris-hydrochloric acid, $\text{pH } 7.7 \pm 0.2$) were prepared in tubes 5 cm. $\times$ 1.25 cm. diam. Gradients having sp.gr. 1.082–1.221, prepared on a bed of 0.25 ml. of sucrose of sp.gr. 1.310, were found satisfactory and chloroplasts in 0.25 ml. of isolation medium (0.25 M-sucrose-tris medium) were added

Fig. 2. Effect of suspension concentration and MgSO_4 at 5 mM concentration on incident light scattered through 90° by chloroplast suspensions in sucrose-tris medium. The suspension concentration was estimated by the extinction at $650\text{m}\mu$. Wavelength of incident light was $565\text{m}\mu$. O, Suspensions in sucrose-tris medium; Δ , suspensions in 5 mM- MgSO_4 in sucrose-tris medium.

to the top of the gradients. After 15 min. at $900g$ tubes containing Ca^{2+} , Mg^{2+} or Mn^{2+} showed that the chloroplasts were predominantly concentrated at the bottom of the gradients (sp.gr. 1.204–1.221), whereas in control tubes they were spread fairly evenly throughout the lower half of the gradient (sp.gr. 1.150–1.221). This result supports the view that individual chloroplasts were modified by exposure to bivalent cations, but does not eliminate the possibility that chloroplast water was squeezed out by centrifugal force, and to examine this point certain optical properties of chloroplast suspensions were considered.

Optical properties of chloroplast suspensions. At wavelengths of low chlorophyll absorption the apparent absorption (turbidity) of suspensions is due to chloroplast scattering of incident light. The loss of endogenous water by chloroplast contraction would cause an increase in the particle refraction and so increase the apparent absorption (Price, Fonnesu & Davies, 1956). Spectrophotometer (Unicam SP.500) readings at 565 and $950\text{m}\mu$ were consistent with this interpretation, but because of the relative positioning of the cuvette and photo-receptor in this instrument the effect of bivalent ions was small.

A greater sensitivity can be obtained by measuring directly the incident light scattered through 90° , and in the experiment of Fig. 2 this was determined for a series of chloroplast suspensions before and after the addition of magnesium sulphate to 5 mM final concentration. The effect of the magnesium sulphate was clearly to increase the amount of light scattered, consistent with the argument outlined above and supporting the assumption of the previous experiments that

Fig. 3. Effect of suspension concentration and MgSO_4 at 5mM concentration on fluorescence of chloroplast suspensions in sucrose-tris medium. The suspension concentration was estimated by the extinction at 650m μ . Wavelength of 'exciting' light was 640m μ . O, Suspensions in sucrose-tris medium; Δ , suspensions in 5mM- MgSO_4 in sucrose-tris medium.

chloroplast volume was modified directly by the bivalent cations, accompanied by an expulsion of water.

The fluorescence of chloroplast suspensions offers a more critical test of the point. In Fig. 3 the fluorescence of the same chloroplast suspensions is shown, before and after the addition of magnesium sulphate to 5mM final concentration. Again the fluorescence was increased in the presence of Mg^{2+} ions. Straight lines have been fitted to the experimental points and on extrapolation to zero suspension concentration these coincide. This is taken as evidence that no fundamental change in the fluorescence characteristics of chloroplasts has been effected by Mg^{2+} ions but that an increase in refraction has increased the scatter component of the total apparent fluorescence. This was tested by determining the fluorescence spectrum of a chloroplast suspension (6.7mg. of chlorophyll/ml.) with a double-monochromator instrument. On the addition of magnesium sulphate to 2.5mM final concentration the peak intensity was doubled but the wavelength of fluorescence maximum remained unchanged at λ 680m μ , and the band width at half maximum intensity remained unaltered at λ 665–697m μ . These observations indicate that a substantial portion of the measured fluorescence intensity is due to refractive scatter by the chloroplasts, but that Mg^{2+} has not affected the fundamental characteristics of chloroplast fluorescence to any measurable extent.

Radioisotope experiments. The experiments above suggest the possibility that bivalent cations were taken up by chloroplasts during the volume contractions observed, so experiments were devised to

measure the cation uptake. Tests with both $^{45}\text{Ca}^{2+}$ and $^{54}\text{Mn}^{2+}$ were encouraging, and on the grounds of instrumentation and counting efficiency $^{45}\text{Ca}^{2+}$ was selected for further study.

The experimental evidence above strongly implied the existence of a process that could not distinguish between Ca^{2+} , Mg^{2+} and Mn^{2+} . If this also applied to the uptake of bivalent cations then the incubation of chloroplasts with $^{45}\text{Ca}^{2+}$ diluted by non-radioactive Mg^{2+} or Mn^{2+} ions should result in chloroplast radioactivity comparable with incubations in which $^{45}\text{Ca}^{2+}$ was diluted with Ca^{2+} at the equivalent concentration. If $^{45}\text{Ca}^{2+}$ were taken up but discrimination did occur, then the chloroplast radioactivity would be lowered in the incubations containing diluent Ca^{2+} ions, to the extent that $^{45}\text{Ca}^{2+}$ was diluted with non-radioactive Ca^{2+} . The experimental technique of collecting the radioactive organelles by membrane filtration does not permit as high a level of accuracy as is normally experienced in radioisotope experiments, possibly because of the damage caused to the organelles, but it does provide an answer to the simplified choice presented here. The results of an experiment of this type are included in Table 2, where in the top line although the radioactivities as counts/min. are not identical for the three diluent ions that for Ca^{2+} falls between the other two, whereas the value would be one-fifth of this if a discriminating process occurred. The radioactivities appearing in the second and third lines of Table 2 also support this conclusion, but, because of the presence of carrier ion, no conclusion can be drawn in this context from the lower half of the Table.

Investigations of Ca^{2+} uptake by vesicle preparations of skeletal muscle by Hasselbach (1964) showed that accumulation of the cation could be enhanced by the addition of oxalate ions to the incubation medium. Sedimentation of the insoluble calcium oxalate within the vesicles then provided a driving force to prolong the accumulation until the medium was virtually exhausted. Chappell, Cohn & Greville (1963) have similarly used the ion pair Mn^{2+} and PO_4^{3-} in mitochondrial investigations. In the experiment of Table 2 the incubations already discussed were repeated with the addition of potassium phosphate at 2.5mM final concentration, or potassium oxalate at 0.25mM, to the incubation medium. However, to avoid precipitation occurring in the medium it was also necessary to repeat this at lower concentrations of added bivalent cation, and to membrane filter the incubation media before adding to it the chloroplast sample. With these cautionary points in mind, and also the errors arising from radioisotope retention by the membranes (see the Materials and Methods section), it is doubtful how much reliance should be placed on the absolute

Table 2. *Chloroplast uptake of $^{45}\text{Ca}^{2+}$ and calculated total uptake of bivalent cation*

Chloroplasts containing 87 μg . of chlorophyll were incubated in 2 ml. of the sucrose-tris medium containing 33.5 μM of $^{45}\text{CaCl}_2$, in 1 μM of carrier CaCl_2 . Where appropriate 5.0 or 0.25 μM of CaCl_2 , MgSO_4 or MnSO_4 were added and K_2HPO_4 (neutralized) to 2.5 mM, and potassium oxalate to 0.25 mM. Chloroplasts were collected by membrane filtration, washed with sucrose-tris medium and counted. A correction for membrane retention of $^{45}\text{Ca}^{2+}$ has been applied as indicated in the Materials and Methods section.

Incubation medium	$^{45}\text{Ca}^{2+}$ retained (counts/min.)				Mean cation uptake (μg . ions/mg. of chlorophyll)
	Ca^{2+}	Mg^{2+}	Mn^{2+}	Mean	
Added cation (2.5 mM)					
Sucrose-tris	127	216	84	142	1.27
Sucrose-tris + phosphate (2.5 mM)	235	210	—	223	2.01
Sucrose-tris + oxalate (0.25 mM)	—	210	276	243	2.17
Added cation (0.125 mM)	Ca^{2+}	Mg^{2+}	Mn^{2+}	Mean	
Sucrose-tris	164	190	154	169	1.31
Sucrose-tris + phosphate (2.5 mM)	211	290	233	245	1.88
Sucrose-tris + oxalate (0.25 mM)	320	386	316	341	2.64

values obtained for ion uptake by the chloroplasts, but in relative terms the addition of either phosphate or oxalate resulted in an approximate doubling of the radioactivity retained. Quantitatively this is a smaller increase than might have been expected had the deposition of ion pairs occurred in the chloroplasts, and no evidence of selective uptake of any one of these bivalent cations emerges from these incubations. It appears instead that the chloroplast has a limited capacity for bivalent cation uptake that has been extended in the presence of the bi- and ter-valent anions.

To take this aspect of the investigation further, chloroplasts in sucrose-tris medium were exposed to $^{45}\text{Ca}^{2+}$ and collected by membrane filtration with glass-fibre (Whatman GF/C) membranes. The membranes were washed, dried and counted as in previous experiments, and then returned to the filter. The lipid and chlorophyll components of the chloroplasts were removed by a chloroform-methanol wash (2:1, v/v; 5 ml.), and the colourless samples were then dried and re-counted. The count was not appreciably lowered by this extraction (e.g. 610 counts/min./sample before extraction, 596 counts/min./sample after extraction). Similar extractions of wet chloroplast filtrates with acetone (to destroy membrane properties as well as extracting lipid and chlorophyll) produced a similar result. This is taken as evidence that the $^{45}\text{Ca}^{2+}$ is associated with the structural (protein) component of the chloroplast rather than lipid or the endogenous aqueous phase.

DISCUSSION

The experiments showed that these chloroplasts responded vigorously to the bivalent ions Ca^{2+} , Mg^{2+} , Mn^{2+} and Fe^{2+} by a contraction in volume,

accompanied by the expulsion of chloroplast water, but two basically different interpretations of this can be envisaged. These cations could modify the chloroplast structure so that water is forced out of the organelle under hydrostatic pressure exerted by the structure, or else modify the metabolism to lower the water content (e.g. by lowering the osmotic pressure) so that water diffuses out and the structural components contract. Both of these effects could occur in the special case of an allosteric effect of cations on transport mechanisms in the membranes.

Data from Table 2 and Fig. 1 have been combined to estimate the final concentration of Ca^{2+} in the chloroplast after exposure to $^{45}\text{Ca}^{2+}$, and the values are presented in Table 3. The values obtained show clearly that concentration of the bivalent cation in the chloroplast phase occurs, and that the bivalent cation is present in greater than catalytic amounts and must be regarded as a reactant. When this is taken together with the low specificity, and in particular the positive response to Fe^{2+} , the probability of a direct cation effect on enzymes of metabolism must be small, but an effect similar to mitochondrial behaviour is not eliminated.

Mitochondria supplied with a suitable fuel to promote a flow of electrons will perform osmotic work; this phenomenon has been demonstrated with the uptake of Ca^{2+} , Mg^{2+} and Mn^{2+} (Lehninger, 1964) and so it too has a low specificity similar to, but not identical with, that of the pea chloroplasts. However, there are two important points of difference: (a) The chloroplast system did not respond to anions that result in ion pairs of low solubility. (b) The chloroplast system is indifferent to illumination. Electron flow in the chloroplast is light-driven, so a chloroplast requirement for light is the parallel to mitochondrial requirement for a fuel

Table 3. *Chloroplast uptake of Ca^{2+} (calculated from the radioisotope experiment of Table 2) related to chloroplast-pellet volumes (calculated from Fig. 1)*

(1) Concn. of Ca^{2+} in incubation medium (mM)	(2) Uptake of Ca^{2+} ($\mu\text{g. ions/mg. of}$ chlorophyll)	(3) Pellet vol. ($\mu\text{l./mg. of}$ chlorophyll)	(4) Ratio (2)/(3) ($\mu\text{g. ions of Ca}^{2+}/$ $\mu\text{l. of pellet}$)	(5) Conversion factor for obtaining (4) from (1)
0.625	1.25	73	0.0171	$27.4 \times$
3.0	1.13	50	0.0226	$7.5 \times$

in performing osmotic work. A form of reversed electron flow in non-illuminated chloroplasts could be envisaged driven by the breakdown of photosynthesis products, but if this existed (and there is no positive evidence of it) some response to illumination would still be expected. Several attempts to demonstrate a light-effect on $^{45}\text{Ca}^{2+}$ uptake and chloroplast-pellet volume have all failed (see the Materials and Methods section and below).

In view of these important differences from the mitochondrion and the evidence of $^{45}\text{Ca}^{2+}$ association with structural components of the chloroplast it is proposed as a working hypothesis that the bivalent ions taken up by the chloroplast are absorbed on to sites on the structural protein. The accessibility of the sites, or the spatial arrangement of them, is regarded as the feature that permits only certain cations to bring about the volume contraction. The contraction itself is regarded as being the result of a closer association of protein moieties.

Relation of cation-activated contraction to light-activated contraction. Light-activated contraction of chloroplasts was first reported by Itoh, Izawa & Shibata (1963), who used the techniques of chloroplast-pellet volume, light-scattering and mean particle size (assayed by means of a Coulter particle counter). Concurrent work by Packer (1963) and Hind & Jagendorf (1965a,b) substantiated the phenomenon, and Dilley (1964) and Dilley & Vernon (1964a,b, 1965) have since further investigated it. There is therefore no reason to question the reality of this phenomenon, although the work is almost entirely confined to experiments with chloroplasts or fragments of chloroplasts from spinach.

It caused concern that isolated pea chloroplasts would not respond to light in this way, although they were capable of contracting to a similar extent (approx. 50%) on exposure to bivalent cations, and demonstrated Hill reactivity and light-activated uptake of carbon dioxide or acetate. Unsuccessful attempts to stimulate a light-induced contraction including the addition of ATP and conditions of photophosphorylation are outlined in the Materials and Methods section.

During this work, however, conditions used by Petrack, Craston, Sheppy & Farron (1965) to demonstrate chloroplast light-activated hydrolysis of ATP were tested and a contraction was observed, comparable in size with cation-activated contraction. This, however, was quickly shown to be light-insensitive, and promoted in the dark by 100mM-cysteine neutralized in sucrose-tris medium, without requiring the other components of the ATP-hydrolysing incubations.

The only possible way of reconciling this effect of cysteine with the previously observed effects of bivalent cations was to assume that cysteine was reducing endogenous Fe^{3+} to Fe^{2+} , which then caused cation-activated contraction. This hypothesis was also attractive in offering a basis for comprehending light-activated contraction, since in the light the Hill reaction would cause reduction of Fe^{3+} to Fe^{2+} , thus promoting contraction that might understandably be reversed when illumination ceased.

To test this idea chloroplasts were incubated in sucrose-tris made 10mM with respect to ferric EDTA. (Neutralized ferric chloride was also tested, but was unsatisfactory because of precipitation.) After illumination the chloroplast-pellet volume was determined. The results given in Table 4 show that a light-activated contraction was indeed imparted by this treatment and that this was inhibited in incubations containing 0.125mM-3-(*p*-chlorophenyl)-1,1-dimethylurea.

The success of this demonstration may possibly be fortuitous and not related to the hypothesis on which it was based, but a closer examination of the natural light-activated contraction should help to determine the relevancy of the model.

With the volume changes of mitochondria in mind, Packer (1963) explored the relationship of chloroplast contraction to phosphorylative activity, and concluded that high-energy compounds were required for chloroplast shrinkage. Hind & Jagendorf (1965a,b) showed that this was based on a superficial resemblance. Their detailed investigation showed phosphorylative activity differed from light-scattering responses (interpreted as conformational changes) to a number of experimental variables including: (a) Addition of pyocyanine,

Table 4. *Effect of light on pellet volumes of chloroplasts incubated in 10 mM-ferric EDTA*

Chloroplasts containing 144 $\mu\text{g.}$ of chlorophyll were incubated in haematocrit tubes containing 4 ml. of sucrose-tris. Where appropriate CaCl_2 was added to 10 mM final concentration, ferric EDTA to 10 mM and 3-(*p*-chlorophenyl)-1,1-dimethylurea (CMU) to 0.125 mM. Illuminated tubes received 5 min. at a 275 w tungsten-filament spotlight, screened by a 2.5 cm. thickness of a filter solution, 10% (w/v) $\text{CuSO}_4 \cdot 5 \text{H}_2\text{O}$. The pellets were spun down at 1800 g for 15 min.

	Pellet volume ($\mu\text{l.}/\text{mg.}$ of chlorophyll)			
	Light — CMU	Light + CMU	Dark — CMU	Dark + CMU
Sucrose-tris + ferric EDTA	46.5	64.4	55.7	62.0
Sucrose-tris	73.4	—	72.7	71.6
Sucrose-tris + CaCl_2	—	—	50.3	—

which doubled the phosphorylation yield but modified only the rate of volume change. (b) Changes in pH from 6.5 to 8.5 that modified phosphorylation but not the volume change. (c) Light-flashes of 5 sec. duration were followed by light-scattering changes for a further 30 sec. in the dark whereas phosphorylation showed no overshoot. A short lag period preceded the volume change but not phosphorylation. (d) Sucrose inhibited the volume change but not phosphorylation.

These observations effectively rule out the possibility that the volume change depends on ATP generated during photosynthesis.

Dilley & Vernon (1964*a,b*) and Izawa (1965) reported a natural light-activated contraction of chloroplasts that was inhibited by Hill-reaction inhibitors, and enhanced as much as tenfold by the addition of Hill quinone reagents. At first sight this firmly implicates the Hill reaction, but it must be recalled that the nature of the coupling (obligatory or otherwise) of the Hill reaction to other redox changes (especially those of Duysens's system I) is almost unexplored so that other sequences in addition to the Hill reaction were possibly modified by the experimental treatments. Dilley & Vernon (1964*a*) suggested that a high-energy-state precursor of ATP may be involved in conformational changes.

The process of phosphorylation in chloroplasts has been resolved into two steps (Jagendorf & Hind, 1963; Neumann & Jagendorf, 1964), the first requiring light-energy to achieve a high-energy state, in the second this being dispersed with concomitant phosphorylation of ADP in a dark reaction. They suggested that the high-energy state might be a trans-membrane pH gradient, having a higher concentration of protons on the inner face of the membrane. Testing the suggestion by Dilley & Vernon (1964*a*), Hind & Jagendorf (1965*a,b*) measured the rate of formation (and dark decay) of the high-energy state, and showed considerable agreement between this and light-scattering changes, indicating chloroplast

contraction. However, in this instance, the establishment of the high-energy state is probably best regarded as a measure of photosynthetic electron transport, for the response to inhibitors distinguished the two properties measured and the formation of the high-energy state was not always associated with conformational change. The only possible conclusion is that, although a close relationship between electron transport and the volume change exists, in detail the coupling of these processes has not yet been established.

The dark decay of light-activated chloroplast contraction was shown by Hind & Jagendorf (1965*a,b*) to be a rapid process, taking a few minutes only, which could be shortened to seconds by the addition of certain redox agents. This almost certainly rules out the possibility of pea chloroplasts' storing photosynthetic energy for use in cation-activated contraction and, since this contraction occurred without an exogenous energy source, it seems reasonable to assume that the contraction itself does not consume energy appreciably.

The pea chloroplasts also responded to the presence of cysteine by contraction and cysteine was regarded above as an electron donor, i.e. an energy source. This observation can be accommodated by assuming that the redox energy was used to bring about a state conducive to contraction, e.g. a localized high concentration of endogenous effector cations. The major energy-consuming step would thus be one involving ion migration, which resulted in a contraction process requiring little energy, as in the cation-activated process.

It will be apparent that a similar scheme could accommodate many of the observations on light-activated chloroplast contraction; the light-energy would produce an electron flow, which would create the high-energy state conducive to contraction, the contraction process again being a process that requires little energy. Clearly there is now a basis for reintroducing the high-energy state

envisaged by Hind & Jagendorf that obviously must closely resemble the model pictured here. Moreover, because the contraction process is no longer equated with the energy transduction, some of the previous difficulties no longer apply. Thus Hind & Jagendorf (1965b) report that 0.3 mM-EDTA at low ion concentration reversibly eliminated the light-activation of spinach-chloroplast contraction but had a smaller effect on the high-energy state. Similarly other inhibitor treatments could selectively influence contraction more than transduction.

Two further points on the response of chloroplasts to light have contributed towards the concept of contraction described here. First, evidence exists of light-activated ion movements in illuminated incubations of spinach chloroplasts. This was first suggested by Nobel & Packer (1964, 1965) and further investigated by Dilley & Vernon (1965) and has been supported by reports (Neumann & Jagendorf, 1964; Jagendorf & Neumann, 1965) of a light-activated pH rise of the medium, and by MacRobbie's (1965) account of ion movement in illuminated *Nitella*. Secondly, the property of contraction resides in the entire lamellar system of the chloroplast, and is not confined to the limit membrane. This can be deduced from the work with chloroplast fragments of Izawa & Good (1966), and that of Hind & Jagendorf (1965a,b) with chloroplasts subjected to hypo-osmotic rupture, and it supports the evidence of electron micrographs published by Kushida, Itoh, Izawa & Shibata (1964). These show that the volume change was largely represented by decrease in the stromal volume, but in addition the granal lamellae appeared to be drawn closer together, and the diameter of the granal disk was less. These are the visible symptoms that would be expected in the case of a general contraction in volume of the structural protein elements.

According to the interpretation above the pea chloroplasts failed to contract in response to light, either because of a low concentration of endogenous cations, or because of a poor electron-transport system, or a failure to couple these two processes. The addition of ferric EDTA could have compensated for either deficiency in cation concentration or for a feeble electron transport, and so the success of the model experiment does not allow a choice between these alternatives. In future work with pea chloroplasts more critical tests will be applied to this and other aspects of the scheme outlined above.

Note added in proof. Chloroplasts isolated in 500 mM-potassium phosphate (pH 7.7) and incubated in sucrose-free phosphate solutions show light-activated contraction in haematocrit tests, in the absence of added ferric or bivalent cations. This

accords with the report of Hind & Jagendorf (1965a) that sucrose inhibits light-activated contraction.

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