EFFECT OF LIGHT ON THE VOLUME AND ION RELATIONS OF CHLOROPLASTS IN DETACHED LEAVES OF *ELODEA DENSA*

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Abstract

Light-induced changes in chloroplasts of detached leaves of *E. densa* were investigated either by fixing leaves in 6% glutaraldehyde or by snap-freezing leaves and isolating chloroplasts after freeze-substitution in acetone. Individual chloroplasts were examined by electron probe analysis.

Upon illumination (450 lux green light) chloroplasts in detached leaves flattened, with a reduction in volume of about 30% within the first second. During this initial phase of chloroplast contraction the calculated osmotic potential of chloroplasts fell from about −17 bars in the dark to −21 bars after 1 s of illumination, suggesting that the rapid contraction was not the result of an osmotic mechanism of water efflux brought about by an efflux of chloride, potassium, sodium, or calcium ions from chloroplasts. During the next hour of illumination chloroplast volume was reduced by only a further 5%, but the chloroplasts lost approximately 50% of their original ion content. The calculated osmotic potential of the chloroplasts rose to approximately −11 bars.

During the first hour of illumination the average net efflux of ions (± standard error of the mean) across the chloroplast envelope was 32·6 ± 3·5 for chloride, 8·7 ± 0·8 for potassium, and 21·24 ± 1·4 p-equiv. $cm^{-2} s^{-1}$ for sodium. The estimated efflux for calcium was approximately 8 p-equiv. $cm^{-2} s^{-1}$. Typical concentrations of chloride, potassium, and sodium respectively in chloroplasts were 0·53, 0·24, and 0·31 equiv/l of chloroplast volume in dark-treated leaves and 0·32, 0·20, and 0·17 equiv/l in light-treated leaves. The chloride content of chloroplasts represents at least 20% of the total chloride content of dark-treated leaves. In the light the corresponding proportion is approximately 8%.

The effects of CO_2, ammonia, brucine, ouabain, and 3-*p*-chlorophenyl-1,1-dimethylurea on the *in vivo* ion and volume relations of *E. densa* chloroplasts are described.

I. Introduction

Although the cytoplasm is usually treated as a simple compartment in kinetic studies of ion transport, it is evident that organelles represent further compartmentation for ions in the cytoplasm (MacRobbie 1971). This may in part account for the anomalous kinetics of efflux and uptake of ions observed by Lüttge and Pallaghy (1972).

The ion and volume relations of isolated chloroplasts and mitochondria have been studied extensively. Volume changes in chloroplasts occur under a variety of

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physiological conditions (Packer and Crofts 1967; Packer *et al.* 1970; Dilley 1971; Miller and Nobel 1972). In plants studied so far chloroplasts flatten in the light and swell in the dark. Volume changes in chloroplasts can also be brought about by many other treatments. Chloroplasts shrink in CO_2-free air as compared to CO_2-containing air (Heber 1969) and they swell strongly in ammonia solution (Crofts 1966).

In these studies, however, chloroplasts were usually isolated and suspended in a variety of media containing ions, buffers, and sugars to give maximal rates of CO_2 fixation as the sole criterion. In addition, under many conditions of isolation the plastids lose their outer envelope. It is questionable therefore whether the ion relations of isolated chloroplasts, suspended in various media, are relevant to the *in vivo* situation (Larkum 1968).

Very little is yet known of the ion relations of chloroplasts *in vivo*. The first investigation of elements in chloroplasts dates back to Neish (1939), but it was not until the studies of Stocking and Ongun (1962) and Larkum (1968) that high concentrations of sodium, potassium, and chloride were demonstrated in chloroplasts isolated from leaves using non-aqueous techniques.

The investigations presented in this paper report some preliminary observations on the "ion" and volume relations of chloroplasts isolated from leaves of *Elodea densa* (Casp.) using a non-aqueous technique. The volume and ion content of individual chloroplasts could thus be determined by scanning electron microscopy and electron probe analysis (Läuchli 1972; Pallaghy 1973). In some experiments the effects of uncouplers and inhibitors of electron transport were also tested in an attempt to characterize the nature of ion transport in chloroplasts.

II. Methods and Materials

(*a*) *Plant Material*

E. densa was chosen because its chloroplasts are large and relatively uniform in size, which enables chloroplast volumes to be estimated with more precision than in leaf tissues of other species. Due to the rapid rate of cytoplasmic streaming in the leaf cells of *E. densa*, the shape of the chloroplasts can be easily observed as they tumble along in the stream.

E. densa was grown under diffused sunlight in a glasshouse in a large bucket of pond water, the ion content of which is compared with that of leaf cell sap of *E. densa* in the following tabulation:

Ion	Concn. (m-equiv/l) in:	
	Pond water	Cell sap
Chloride	0·54	28·3
Potassium	0·22	105·0
Sodium	0·56	66·5

Leaves from the fourth, fifth, and sixth sets of nodes from the apex were always selected. After leaves had been cut from the shoots they were briefly agitated in distilled water to remove the unicellular algae growing on their surface.

(*b*) *Conditions of Illumination*

E. densa is very sensitive to high-intensity white light which causes the chloroplasts to "clump" into a spherical formation in the middle of the cells. Although the chloroplasts are apparently not damaged, as clumping was alleviated when the leaves were returned to darkness, volume changes in these chloroplasts could not be detected. Green light supplied by a National (10-W) white fluorescent tube with a green Cellophane filter at an intensity of 450 lux (selenium detector, without an eye correction filter—TOSCO) was effective in preventing the clumping reaction.

(c) *Pretreatment of Leaves*

Detached leaves were placed in distilled water in small vials. In some vials CO_2-free air (C.I.G., Australia) was constantly bubbled through the distilled water, whereas other leaves were exposed to the normal level of CO_2 in air. After an initial pretreatment period of 2 hr in darkness the vials were illuminated or left in the dark as required.

The uncouplers chosen for investigation were ammonia and brucine, which cause severe swelling and shrinkage respectively of isolated chloroplasts suspended in aqueous solutions (Crofts 1966, 1967; Good *et al.* 1966). CMU (3-*p*-chlorophenyl-1,1-dimethylurea), like DCMU [3-(3,4-dichlorophenyl)-1,1-dimethylurea], is an inhibitor of electron transport in photosystem II. Ouabain, an effective and specific inhibitor of Na–K coupled fluxes in animal cells, was also tested. Solutions of these inhibitors and uncouplers were made up at the following concentrations in distilled water: ammonia, 10^{-2}M (pH 10·0); brucine, 10^{-3}M (pH 7·1); CMU, 5×10^{-6}M (pH 6·6); ouabain, 10^{-3}M (pH 6·8). These solutions did not contain added ions. The pH of the distilled water used in the control experiments was 6·5. Temperature was controlled at 25 ± 1°C.

(d) *Volume Measurement of Chloroplasts in Glutaraldehyde-fixed Leaves*

At the end of the various treatments described above, leaves were immersed into a 6% solution of glutaraldehyde in distilled water and allowed to fix overnight. Chemical fixation after short bursts of light (1 or 2 s) is difficult to achieve; the usual procedure was to illuminate the leaf tissue and then fix it quickly in darkness. Time intervals of 5 s or longer presented no problems, for in this time the whole procedure of illumination and fixation could be carried out.

After 12 hr of fixation the leaves were washed three times with distilled water at hourly intervals. The fourth wash was overnight so that there would be sufficient time for any residual glutaraldehyde to be eluted out. A final wash was given on the following day and the tissue was placed in a relatively large volume of distilled water. The detached leaves of *E. densa* were mounted in distilled water onto a glass slide and viewed using light microscopy. Five photographs were taken at random of leaves for each of the various treatments, which provided photographs of between 50 and 150 chloroplasts in focus for each treatment.

(e) *Volume Measurement of Chloroplasts isolated from Freeze-substituted Leaves*

Leaves were snap-frozen directly into dry acetone cooled to −78°C in an acetone–dry ice slush bath. This appeared to be a more adequate method for our purposes than using liquid Freon (Pallaghy 1973). For freeze-substitution, further amounts of pre-cooled activated molecular sieve (Union Carbide-Linde 3A) were added to the dry acetone in the sealed test tubes.

Since dry acetone is thought to extract ice from tissue in about 3–5 days (Pallaghy 1973), the specimens were allowed to freeze-substitute for 5 days at temperatures below −70°C. When freeze-substitution was completed, the sealed test tubes were allowed to come to room temperature over a period of 3 hr in a dry-atmosphere glove-box continuously flushed with a stream of air previously dried by passage through silica gel towers. The freeze-substituted tissue was then homogenized in the dry atmosphere in a small volume of dry acetone in the presence of glass beads (30 s at 4000 rev/min). There was no significant rise in temperature during homogenization.

A small sample of the supernatant solution was placed onto a Mylar-covered aluminium stub in the glove-box and the acetone allowed to evaporate. The purpose of the carbon-coated Mylar was to eliminate the interfering counts from aluminium during electron probe analysis. Samples were usually observed uncoated in the scanning electron microscope, although for photography some samples were coated with a thin film comprising 80% Pt–20% Pd.

(f) *Electron Probe Analysis*

Electron probe analysis was performed using a scanning electron microscope (JEOLCO-JSMU3) fitted with an energy-dispersive X-ray analyser (EDAX—Nuclear Diodes). The accelerating voltage of 20 keV was sufficient for the beam, used in spot mode, to excite the whole of the volume of an intact chloroplast, as the count rates did not alter when chloroplasts were analysed by scanning over their surface. In later experiments, however, it was found that spot analysis underestimated the ion content of very swollen chloroplasts by about 10–20% (e.g. for chloroplasts isolated from leaves immersed in ammonia). The beam current was 1·20 nA, providing a specimen current of about

0·86 nA. Chloroplasts were analysed for a period of 400 s and were usually viewed using a magnification factor of 3000 during analysis, since the use of a higher magnification for prolonged periods caused noticeable swelling of chloroplasts. Investigations since this work was completed have shown that a beam current of only 0·6 nA does not cause chloroplasts to swell.

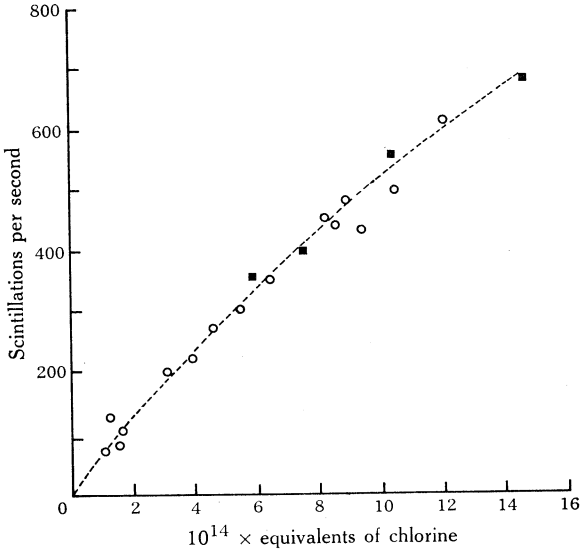


Fig. 1.—Calibration curve for chlorine obtained by spot analysis of small, flat, rectangular crystals of KCl (○) and NaCl (■). A beam current of approximately 1·2 nA was used at an accelerating voltage of 20 keV. Crystal sizes were measured by scanning electron microscopy.

The characteristic X-ray emission peaks for the elements sodium, magnesium, chlorine, potassium, and calcium occur in the range 0–4 keV (Russ 1971). For purposes of semiquantitative analysis the areas under each peak were estimated between the limits bordered by the "half-peak" heights. Figure 1 shows a calibration curve for chlorine determined by analysing small crystals of known potassium, chlorine, and sodium contents as described by Humble and Raschke (1971). On the basis of using peak areas, the count rate for the same number of equivalents of each of the elements chlorine, potassium, and sodium was in the proportion 1 : 0·86 : 0·058 respectively — i.e. the method of microprobe analysis is least sensitive for sodium.* The depth of a typical crystal used for calibration was 0·2 μm. Only flat rectangular crystals were analysed, since the count rates of X-ray emission were the same for crystals analysed in either spot or scanning mode, as found for the analysis of most chloroplasts. Cubic crystals were not used as the count rates obtained by scanning the electron beam over their surface were 20–30% higher than those obtained by spot analysis.

Evaluation of the data has since shown that the results presented in this paper could also have been obtained by using peak heights instead of the usually recommended procedure of measuring areas under the peaks as adopted by us in this investigation. At 20 keV the peak heights, above background, were equal for potassium and chlorine when analysing KCl crystals. The corresponding calibration curve was almost linear.

III. Results

(*a*) *Volume Change in Chloroplasts as determined by Glutaraldehyde Fixation of Detached Leaves*

(i) *Effect of Illumination*

The long axis of the chloroplasts remained almost constant for at least the first 4 hr of illumination (Figs. 2*a* and 2*b*). The short axis responded to illumination.

* Under these conditions the limit of detection for sodium is approximately 2 fmoles.

There is a short- and a long-term component in the contraction of chloroplasts in leaves exposed to light. The initial decrease in the length of the minor axis is large

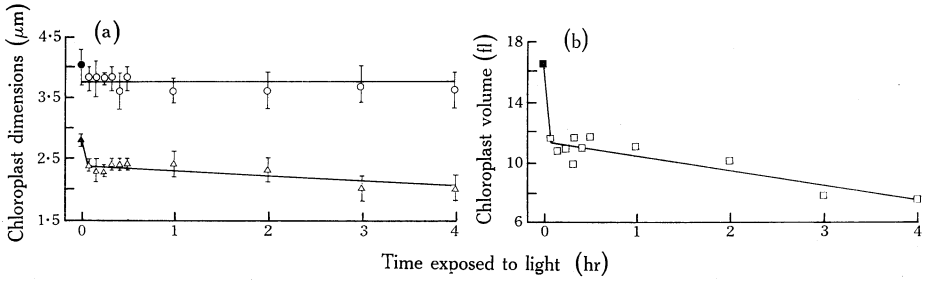


Fig. 2.—(*a*) Time course of dimensional changes in chloroplasts of *E. densa* during 4 hr of illumination. Standard errors shown are for 95% confidence limits.
● Mean of major axes in the dark. ○ Mean of major axes for various intervals of illumination.
▲ Mean of minor axes in the dark. △ Mean of minor axes for various intervals of illumination.
(*b*) Estimated time course of volume change in chloroplasts of *E. densa* during 4 hr of illumination.
■ Mean of the volumes in the dark. □ Mean of the volumes for various intervals of illumination.

and is completed within the first second of illumination (Fig. 3). This is then followed by a much slower shrinkage of the minor axis. Accordingly, the estimated volume of chloroplasts decreases slowly over the next 4 hr of illumination. Figures 6 and 7 show typical chloroplasts in light- and dark-treated leaves of *E. densa*.

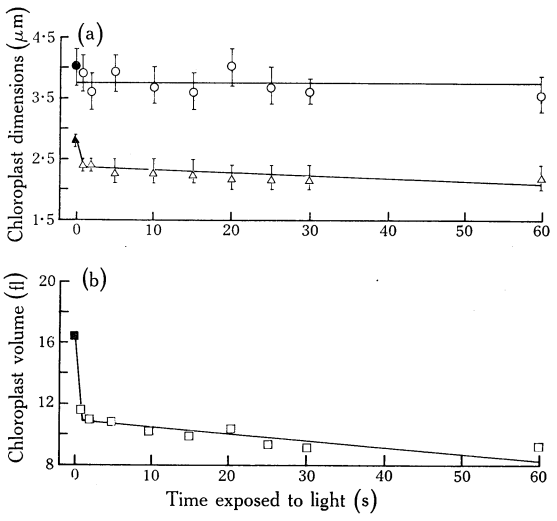
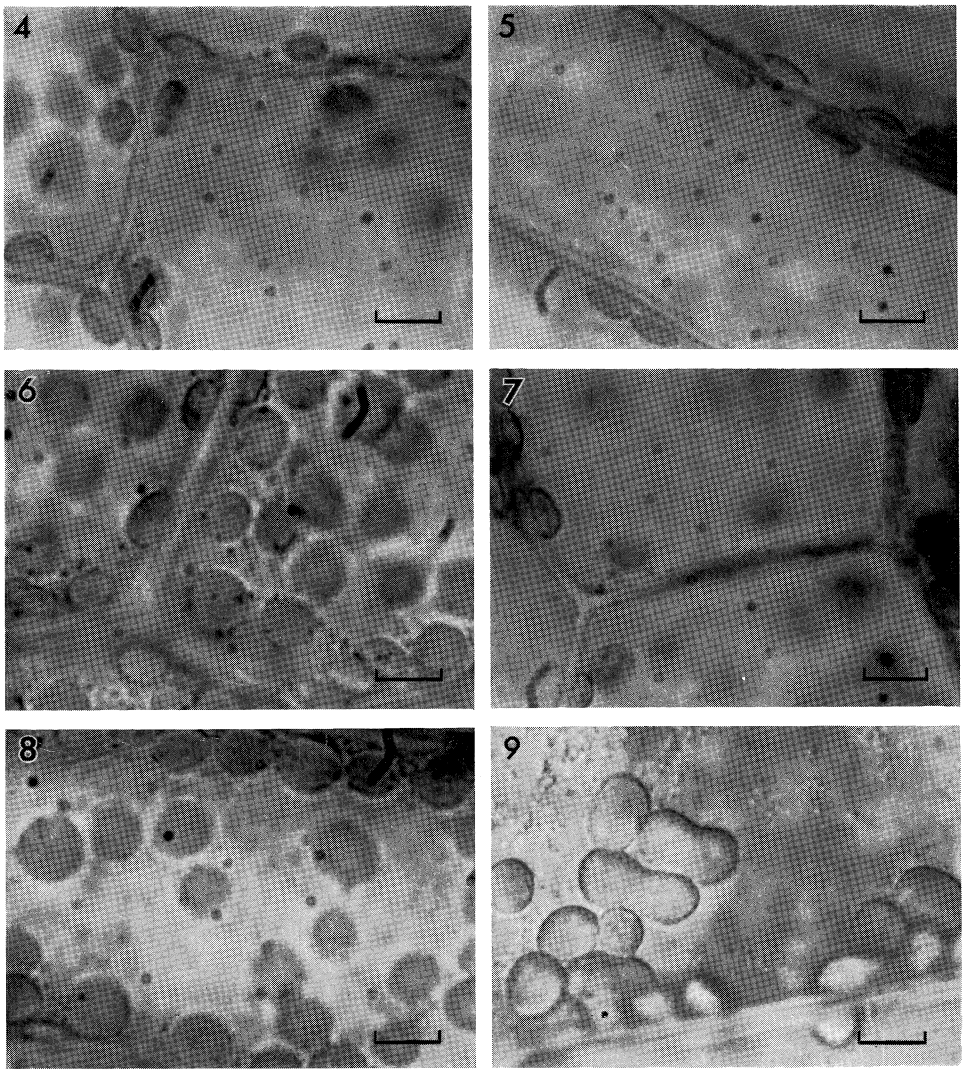


Fig. 3.—(*a*) Separate series of experiments showing the time course of dimensional changes in chloroplasts of *E. densa* during 1 min of illumination. Standard errors shown are for 95% confidence limits.
● Mean of major axes in the dark.
○ Mean of major axes for various intervals of illumination.
▲ Mean of minor axes in the dark.
△ Mean of minor axes for various intervals of illumination.
(*b*) Estimated time course of volume changes in chloroplasts of *E. densa* during 1 min of illumination.
■ Mean of volumes in the dark.
□ Mean of volumes for various intervals of illumination.

(ii) *Effect of CO_2 Concentration and Ammonia*

Light microscope evidence shown here (Figs. 4–7) has confirmed the previously demonstrated results of Heber (1969) who found that CO_2 enhances the swelling

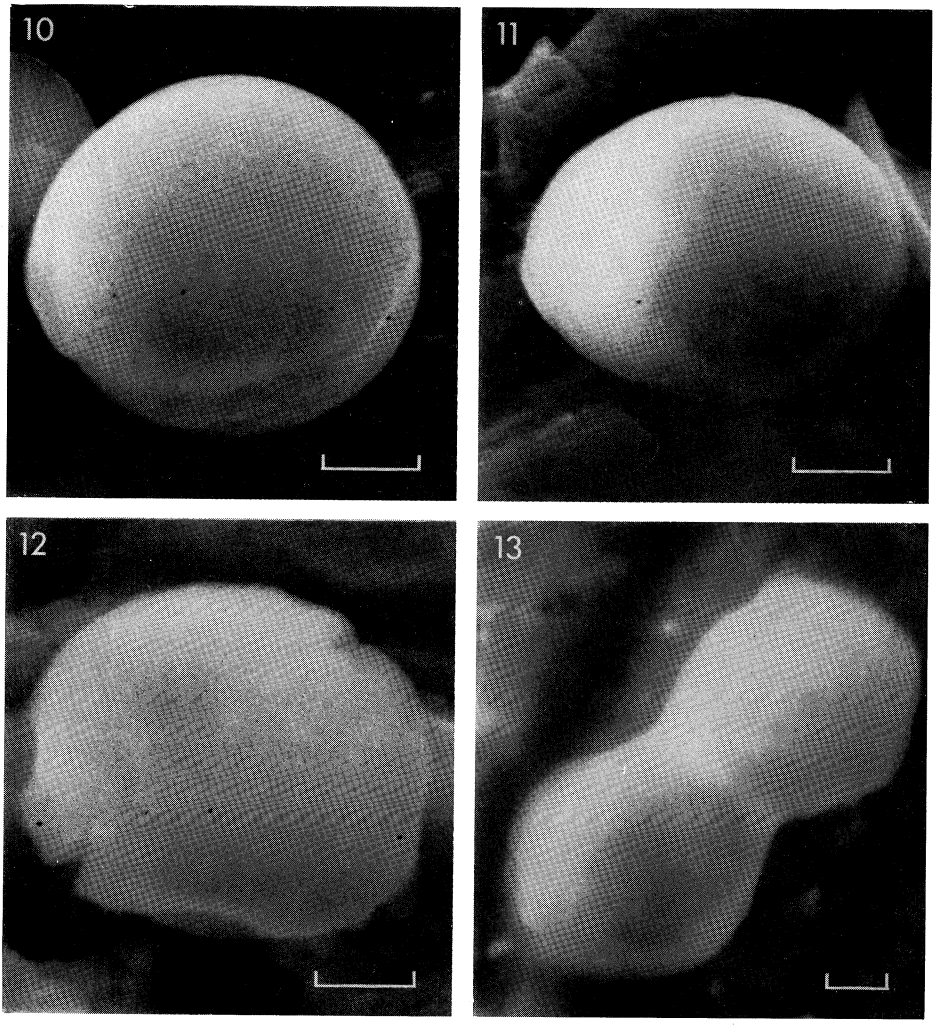
of chloroplasts. There is a distinct flattening of chloroplasts in CO_2-free air as compared to CO_2-containing air.



Figs. 4–9.—Response of chloroplasts in detached leaves of *E. densa* immersed in distilled water using a variety of treatments. After 2 hr of the appropriate treatment leaves were fixed in glutaraldehyde and photographed directly without staining. Bars indicate 5 μm. *4*, Leaves in darkness aerated with CO_2-free air. *5*, Leaves in light aerated with CO_2-free air. *6*, Leaves in darkness in normal air. *7*, Leaves in light in normal air. *8*, Leaves immersed in 10^{-2}M ammonia solution in the dark. *9*, Dumb-bell shaped chloroplasts in leaves viewed by Nomarski interference phase-contrast microscopy.

Chloroplasts were extremely swollen in the light in leaves which had been immersed in ammonia solution (10^{-2}M, pH 10) compared with the distilled water

controls. In the dark, however, there was no significant difference between the control and ammonia-treated samples (Figs. 6 and 8).



Figs. 10–13.—Isolated freeze-substituted chloroplasts of *E. densa* (coated with 80% Pt–20% Pd). Bars indicate 1 μm. Note the distinct ridge around the equator of isolated chloroplasts. *10*, Chloroplast from dark-treated leaves. *11*, Chloroplast from light-treated leaves. *12*, Damaged chloroplast. *13*, Dumb-bell shaped chloroplast(s) — these (dividing?) chloroplasts showed a most extraordinary compartmentation of elements between the two halves of the dumb-bell (see Section IV*a*).

(*b*) *Estimation of Volumes of Freeze-substituted Chloroplasts*

Many of the isolated, freeze-substituted chloroplasts appeared to be intact (Figs. 10 and 11), although damaged chloroplasts were frequently observed (Fig. 12). Damaged chloroplasts were not analysed. The dumb-bell shaped chloroplasts,

frequently observed in dark-pretreated leaves (Fig. 9), and probably representing the dividing stage of this organelle, were also preserved by freeze-substitution (Fig. 13).

Chloroplast dimensions were measured by scanning electron microscopy with the sample stage tilted at angles of 0 and 45° to the incident electron beam. The average dimensions of 10 chloroplasts isolated non-aqueously from dark-pretreated leaves of *E. densa* were as follows [volumes are expressed as femtolitres (fl)]:

Angle of tilt	Major axis (μm)	Minor axis (μm)	Estimated volume (fl)
0°	4·2	2·9	18·5
45°	4·1	2·9	18·0

Since the measured lengths of the chloroplast axes did not change upon tilting the specimen stage by 45°, the chloroplasts could be assumed to be ellipsoids.

TABLE 1

COMPARISON OF DIMENSIONS OF CHLOROPLASTS AS OBSERVED BY LIGHT MICROSCOPY (GLUTARALDEHYDE-FIXED) AND SCANNING ELECTRON MICROSCOPY (FREEZE-SUBSTITUTED)

Detached leaves of *E. densa* were immersed in distilled water exposed to air. Volumes were estimated on the assumption that chloroplasts are ellipsoids

Illumination	Treatment	Major axis (μm)	Minor axis (μm)	Estimated volume (fl)
Dark	Glutaraldehyde-fixed	4·0	2·8	16·3
	Freeze-substituted	4·2	2·9	18·5
Light	Glutaraldehyde-fixed	3·7	2·3	10·8
	Freeze-substituted	3·9	2·5	12·9

Table 1 compares a population sample of chloroplasts of *E. densa* in the light with one in the dark, as well as with the corresponding changes found in glutaraldehyde-fixed material. Irrespective of the nature of pretreatment, chloroplast volumes were always larger in freeze-substituted than in the corresponding glutaraldehyde-fixed material. It seems that glutaraldehyde fixation causes additional shrinkage of chloroplasts, unrelated to any stimuli, as was also observed previously by Diers and Schieren (1972). It is clear, however, that the reduction in volume upon illumination is mainly due to a shortening of the minor axis of the chloroplasts (i.e. a flattening), while the major axis remains fairly constant in length. Further results on volume changes are included in the next section.

(c) *Ion Content of Freeze-substituted Chloroplasts*

(i) *Effect of Illumination*

Table 2 shows the results of an experiment where leaves were kept in light or darkness for 1 hr. In the light the concentration of chloride and potassium in the chloroplasts was much lower than that observed in chloroplasts of dark-treated leaves. It is likely that there was also a net efflux of sodium, as this ion could not be detected in these light-treated samples.

Over a period of illumination of 1 s there was little change in ionic content of chloroplasts as compared with chloroplasts obtained from dark-treated leaves

TABLE 2

COMPOSITION OF *E. DENSA* CHLOROPLASTS IN DETACHED LEAVES EXPOSED FOR 1 HR IN EITHER DARKNESS OR LIGHT, IN DISTILLED WATER AERATED WITH NORMAL AIR

Prior to the experiment leaves were kept in darkness for 2 hr. For each treatment 10 intact chloroplasts were selected at random, measured, and analysed. Concentrations are calculated on the basis that the ions are uniformly distributed throughout the chloroplast. The dashes indicate concentrations below the limit of detection for that element

Treatment	Amount (fmoles) per chloroplast of:			Estimated volume per chloroplast (fl)	Estimated concentration in chloroplast (equiv/l):		
	Cl	K	Na		Cl	K	Na
Dark	13·1	5·3	12·9	18·5	0·71	0·29	0·70
Light	3·6	3·2	—	12·9	0·30	0·25	—

(Table 3). After 1 hr of illumination, however, there was a large net efflux of all of the three measurable ions. Thus, the long-term but not the short-term contraction

TABLE 3

COMPOSITION OF CHLOROPLASTS OF *E. DENSA* IN LEAVES EXPOSED FOR 1 HR TO DARKNESS, THEN ILLUMINATED AND SAMPLED BY SNAP-FREEZING AFTER EITHER 1 S OR 1 HR OF ILLUMINATION

Concentrations and osmotic potentials were calculated on the basis that the ions are uniformly distributed throughout the chloroplasts. Activity coefficients used in the calculation of osmotic potentials were assumed to be those of equivalent aqueous solutions. The values for calcium in experiment 2 were not included in the estimations of osmotic potential. For each treatment 10 intact chloroplasts were selected at random, measured, and analysed. The dashes indicate concentrations below the limit of detection for that element

Treatment	Amount (fmoles) per chloroplast of:				Estimated volume per chloroplast (fl)	Estimated concentration in chloroplast (equiv/l)				Osmotic potential (bars)
	Cl	K	Na	Ca		Cl	K	Na	Ca	
Experiment 1										
Dark (1 hr)	10·17	4·30	8·47	—	18·6	0·55	0·23	0·45	—	−17·5
Light (1 s)	10·16	4·00	8·33	—	14·0	0·73	0·29	0·60	—	−22·7
Light (1 hr)	3·86	3·26	—	—	13·2	0·29	0·25	0·05	—	−10·0
Experiment 2										
Dark (1 hr)	9·91	4·42	5·38	1·45	18·9	0·53	0·24	0·31	0·15	−17·0
Light (1 s)	8·58	3·70	5·21	1·10	13·3	0·65	0·28	0·39	0·17	−19·6
Light (1 hr)	4·01	2·48	2·13	0·67	12·7	0·32	0·20	0·17	0·11	−10·9

of chloroplasts appears to be correlated with a large loss of ions into the cytoplasm. Further "light–dark" results are incorporated in Table 4.

(ii) *Effect of CO_2*

Table 4 (experiment A) shows the results of an experiment for leaves treated in light, dark, CO_2-free air, and CO_2-containing air. CO_2 had an effect apparently independent of conditions of illumination, inhibiting chloroplast contraction and efflux of ions into the cytoplasm.

TABLE 4

COMPOSITION OF CHLOROPLASTS OF *E. DENSA* IN THREE SEPARATE EXPERIMENTS IN LEAVES EXPOSED IN DARKNESS FOR 2 HR FOLLOWED BY EITHER 1 HR IN DARKNESS OR 1 HR IN LIGHT

Ammonia, brucine, CMU, or ouabain were applied to the leaves at the appropriate concentrations (Section IIc) during the second hour of the dark pretreatment to allow time for permeation of these substances to the chloroplasts. $+CO_2$ represents aeration of the vials with normal air; $-CO_2$ indicates aeration with CO_2-free air. Experiments were performed several weeks apart. For each treatment 10 intact chloroplasts were selected at random, measured, and analysed. The dashes indicate concentrations below the limit of detection for that element

Treatment	Amount (fmoles) per chloroplast of:			Estimated volume per chloroplast	Estimated concentration in chloroplast (equiv/l)		
	Cl	K	Na	(fl)	Cl	K	Na
				Experiment A			
Dark							
$+CO_2$	9·66	3·46	7·73	18·8	0·51	0·18	0·41
$-CO_2$	5·22	2·68	6·42	15·7	0·33	0·17	0·41
Light							
$+CO_2$	1·93	1·49	—	14·2	0·14	0·11	<0·1
$-CO_2$	1·73	1·46	—	11·1	0·15	0·13	<0·1
				Experiment B			
Dark							
Distilled water	6·52	3·79	5·90	19·2	0·34	0·20	0·31
Ammonia	6·56	3·94	6·45	18·8	0·35	0·21	0·34
Brucine	6·14	3·65	5·87	19·3	0·32	0·19	0·31
Light							
Distilled water	1·68	2·02	—	14·0	0·12	0·15	<0·1
Ammonia	33·24	8·84	29·92	25·2	1·32	0·35	1·18
Brucine	1·50	1·86	—	12·5	0·12	0·15	<0·1
				Experiment C			
Dark							
Distilled water	7·61	3·36	6·98	19·0	0·40	0·18	0·37
CMU	8·13	3·68	8·06	18·5	0·44	0·21	0·44
Ouabain	7·71	3·36	7·13	18·9	0·41	0·18	0·38
Light							
Distilled water	2·28	1·63	—	13·3	0·17	0·12	<0·1
CMU	5·06	3·31	4·67	16·8	0·30	0·20	0·28
Ouabain	2·25	1·44	—	13·1	0·17	0·11	<0·1

(iii) *Effect of Amines*

Compared to the dark controls, ammonia caused swelling of chloroplasts in the light while brucine enhanced chloroplast contraction. The marked swelling

of chloroplasts in ammonia was paralleled by a very large increase in their ion content (Table 4, experiment B).

(iv) *Effect of CMU and Ouabain*

Whereas ouabain had no effect on chloroplast contraction, CMU inhibited light-induced long-term chloroplast flattening by 61% and net ion efflux by 51% (Table 4, experiment C).

IV. Discussion

(*a*) *General Observations*

Scanning electron microscopy of non-aqueously isolated chloroplasts shows that the majority of chloroplasts remain intact. Because freeze-substitution was carried out in acetone, chlorophyll was extracted from the tissue. As magnesium is probably mainly associated with chlorophyll, it was not surprising that little or no magnesium was detected in freeze-substituted chloroplasts. This result is in marked contrast with freeze-dried chloroplasts, where magnesium can usually be detected (Larkum 1968).

Freeze-drying had been previously used successfully in retaining high concentrations of sodium, potassium, and chloride in *Tolypella* chloroplasts (Larkum 1968). The range of concentrations of these ions obtained by Larkum (1968) and Larkum and Hill (1970) is similar to that found for light-pretreated freeze-substituted chloroplasts of *E. densa*. Although the concentration of ions in chloroplasts is very high, the ion content of chloroplasts represents only a small fraction of the total ion content of the leaves of *E. densa* (Table 5). For leaves kept under dark conditions,

TABLE 5

RELATIVE DISTRIBUTION OF IONS BETWEEN CHLOROPLASTS AND OTHER LEAF CELL PARTS IN *E. DENSA*

These estimates were obtained on the basis that leaf cells contained approximately 100 chloroplasts per cell. The mean concentration of ions in the leaf was as given in Section II(*a*)

Treatment	Total volume of chloroplasts (cm^3)*	Amount (mmole) of element in leaf* present in chloroplasts			Percentage in chloroplasts of total element content of leaf		
		Cl	K	Na	Cl	K	Na
Dark (1 hr)	10·3	5·67	2·3	4·6	20%	2·2%	6·9%
Light (1 hr)	7·3	2·11	1·8	2·3	7·5%	1·7%	3·5%

* Per 1000 cm^3 of fresh leaf tissue.

however, the chloride content of chloroplasts may exceed 20% of the total chloride content of the leaves. Thus the fourth component in the wash-out of chloride in leaf disks of *Limonium* (Hill 1970) is probably due to the chloroplasts acting as a fourth compartment in addition to those normally observed for sodium, i.e. the free space, cytoplasm, and vacuole.

Localization of high concentrations of chloride in chloroplasts have also been demonstrated by autoradiography using *Limonium vulgare* (Ziegler and Lüttge 1967), by silver ion precipitation in barley seedlings (van Steveninck *et al.* 1972), and by electron probe analysis of freeze-substituted epoxy-embedded leaf tissue of *Mesembryanthemum crystallinum* (Pallaghy, unpublished data). Thus, although chloroplasts of many plant cells show marked chloride accumulation, there is evidence that this is not always the case (Pallaghy 1973; Eshel and Waisel, unpublished data).

Individual chloroplasts in leaves of *E. densa* occasionally appeared to contain much more calcium than either sodium or potassium (cf. Schorer-Mörtel 1972). In a few preliminary observations it was found that whereas one of the chloroplasts making up the two chloroplasts (?) of the dumb-bell (Figs. 9 and 13) contained a very high concentration of potassium and sodium, its twin would contain a very high concentration of calcium and almost no sodium or potassium. This phenomenon deserves further attention.

(*b*) *Effect of Illumination*

The light-induced flattening of chloroplasts in detached leaves of *E. densa* is virtually completed within the first second of illumination. Following this initial contraction chloroplasts continue to decrease in volume, but at a much slower rate. The half-time for the first phase of chloroplast flattening is much shorter than that found in previously examined tissues (Nobel 1968; Nobel *et al.* 1969; Miller and Nobel 1972), where it varied between 0·5 and 5 min and approached that found for the photo-induced changes of the transmembrane electric potential of chloroplasts in *Peperomia metallica* (Bulychev *et al.* 1972). The initial phase of the photoelectric response was attributed to the transfer of hydrogen ions, while the slow phase was thought to be due to changes in the activity of cations in chloroplasts and the cytoplasm. This suggestion, which is consistent with Mitchell's chemiosmotic hypothesis (Mitchell 1972), is also consistent with our findings.

The two separate experiments given in Table 3 suggest that the first phase of chloroplast flattening is not an osmotic response resulting from the loss of potassium, chloride, and sodium, since the absolute magnitude of the calculated osmotic potential of the chloroplasts increases. This initial chloroplast flattening may possibly be due to a conformational change of the chloroplast proteins brought about by the rapidly changing ATP and hydrogen ion concentrations (Murakami and Packer 1969; Nobel *et al.* 1969; Heber and Santarius 1970; Miller and Nobel 1972; Tzapin *et al.* 1972). Ohnishi (1964) demonstrated the presence of a contractile protein in chloroplasts.

On the other hand, it seems unlikely that the outer chloroplast envelope can withstand pressure differentials as high as the 3–5 bars developed during the first second of contraction (P. S. Nobel, personal communication). Since our estimates of concentration of ions do not include corrections for the non-osmotic spaces of chloroplasts, as applied by Larkum and Hill (1970) for example, the pressure differentials may be even higher, suggesting a substantial movement of other materials (eg. H^+) not detectable by electron probe analysis. The large volume flow of water during contraction could be accounted for if the chloroplast envelope had an electro-osmotic efficiency of about 420 moles of water per faraday of positive ion flow, which

falls within the range of values observed for some plant systems (D. S. Fensom, personal communication).

The long-term chloroplast contraction is, however, accompanied by a large net efflux of potassium, chloride, and sodium from the chloroplasts into the cytoplasm. During this phase the absolute value of the osmotic potential decreases by about 50% while the volume of the chloroplasts is reduced by only a further 5% (Table 3). One should bear in mind that the osmotic potential of the free cytoplasm will also change upon illumination. Jeschke (1971) calculated that in *Elodea* light primarily enhances the plasmalemma influx and increases the cytoplasmic content of potassium and chloride ions. This is further complicated since it appears that chloroplasts behave like a two-compartment system (Miller and Nobel 1972; van Steveninck *et al.* 1972).

During the first hour of illumination the average net efflux of ions ($\pm$ standard error of the mean for six series of experiments) was calculated from estimated values of the surface areas of chloroplasts, using the assumption that chloroplasts are prolate spheroids. The average net efflux was $32{\cdot}6 \pm 3{\cdot}5$ p-equiv. $cm^{-2}\,s^{-1}$ for chloride, $8{\cdot}7 \pm 0{\cdot}8$ for potassium, and $21{\cdot}24 \pm 1{\cdot}4$ for sodium. These fluxes are much higher than the plasmalemma fluxes usually observed for Characeae from fresh and brackish water (MacRobbie 1971). They fall within the range of values normally found for fluxes across the tonoplast (Hope 1971) and for hydrogen ion extrusion across the plasmalemma of some Characeae (MacRobbie 1971).

Chloroplast flattening was also correlated with an efflux of calcium, but the magnitude of the calcium efflux is as yet unknown (approximately 8 p-equiv. $cm^{-2}\,s^{-1}$), since the instrument was not accurately calibrated for calcium.

(*c*) *Effect of CO_2, Inhibitors, and Uncouplers*

CO_2-free air induced chloroplast shrinkage (Heber 1969) and a net efflux of ions from chloroplasts into the cytoplasm, even in darkness (Table 4, experiment A). The most noticeable effect of CO_2 is the enhancement of chloride content of chloroplasts in the dark. The effect of CO_2 on the ionic gradient in chloroplasts has not been observed previously, and may be related to the effect of CO_2 on the shuttle of ions between the guard and subsidiary cells in stomata (Pallaghy 1971).

Ouabain, an inhibitor of active cation transport in some plant cells (MacRobbie 1971), had no effect on either the volume or the ion content of chloroplasts. Ammonia and brucine are uncouplers of electron transport in photophosphorylation (Good *et al.* 1966) and, as would be expected, neither of these amines had a marked effect on chloroplasts in the dark. In the light, however, ammonia caused chloroplasts to swell enormously (cf. Crofts 1967), due mainly to an uptake of NaCl. Brucine caused a noticeable chloroplast flattening, coupled with a slight net efflux of ions as was also observed *in vitro* by Good *et al.* (1966). The opposing effects of brucine and ammonia are not understood (Good *et al.* 1966).

CMU (5 μM) inhibited the light-induced chloroplast shrinkage by 61%. In this experiment (Table 4, experiment C) CMU inhibited chloride efflux by 51%, sodium efflux by 34%, and potassium efflux almost entirely. The net efflux of chloride ($3{\cdot}1$ fmoles per chloroplast) was almost exactly balanced by that of sodium ($3{\cdot}4$ fmoles per chloroplast). This apparent differential effect of CMU on sodium and potassium is being further investigated.

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VI. References

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