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Molecular size limit for movement in the symplast of the *Elodea* leaf

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Abstract. A range of water-soluble fluorescent dyes and dye conjugates have been injected into cells in *Elodea canadensis* Michx. leaves. All compounds are unable to cross the plasmalemma between living cells and the external solution, are not degraded to other fluorescent compounds by tissue homogenates, and do not affect cytoplasmic streaming. Despite being unable to cross the plasmalemma, molecules up to 874 dalton pass from cell to cell, smaller molecules showing greater mobility. The conjugate of fluorescein isothiocyanate and leucyl-diglutamylleucine (874 dalton) appears to be close to the limit for movement: in only three out of 17 injections was any movement visible; this movement was only to adjacent cells and was close to the limit of detection. Dye molecules of 1678 dalton and larger did not pass from cell to cell. From the relationship between the size of the dye molecules, measured using molecular models, and their intercellular mobility, the equivalent pore diameter of the *Elodea* leaf plasmodesmata has been estimated to lie within the range 3.0–5.0 nm.

Key words: *Elodea* – Intercellular communication – Plasmodesma – Symplast.

Introduction

Compounds as varied as inorganic ions, sugars, amino acids and auxin can move from cell to cell in leaves of the water plant *Vallisneria*, without leakage to an external solution (Arisz and Wiersema 1966; Arisz 1969). Bostrom and Walker (1975) demonstrated the intercellular transport of chloride in the charophyte *Chara corallina*. A number of dyes have also been shown to move between cells, for example fluorescein in *Vicia faba* and procion brown and procion yellow in *Elodea* (for a review see Goodwin 1976). These compounds probably move in the plant symplast – that is in cells via the cytoplasm, and between cells via plasmodesmata, at all times being within the plasmalemma. There is evidence that virus particles can move in the symplast (for a review see Gibbs 1976). However, if virus particles can pass normal plasmodesmata, then most soluble enzymes, and possibly most cytoplasmic ribonucleoprotein complexes could move between cells. All the evidence indicates that this is not the case (see review by Carr 1976). The studies described in this report were carried out in an attempt to define the molecular exclusion limit of the *Elodea* leaf symplast using a graded series of fluorescent dye-peptide conjugates as probes.

Abbreviations: LRB = lissamine rhodamine B; F = fluorescein isothiocyanate isomer I; $(Glu)_2$ = glutamylglutamic acid; $(Gly)_6$ = hexaglycine; LGGL = leucyldiglutamylleucine

Material and methods

Plant material. Elodea canadensis Michx. was obtained from local ponds in Edinburgh. Plants were held in pond water at approx. 20° C, 8 h daylength. They showed continuing growth for at least a month after collection. Generally, young leaves of about half the final leaf length were taken from growing shoot tips. Older and younger leaves gave similar results, but older leaves often had dead cells, or adhering organisms, and the small size of younger leaves made them difficult to handle.

Dyes and dye conjugates. Injected lissamine rhodamine B (LRB; Gurr, BDH Chemicals, Poole, UK or Polysciences, Warrington, Penn., USA) caused injected cells to cease cytoplasmic streaming. On the assumption that this might be because of some contaminant, the LRB was purified as follows. A mixture of LRB and one tenth its weight of acid-washed activated charcoal was prepared, dissolved in water, and then filtered (Milli-

pore, Bedford, Mass., USA; 0.1 μm pore size). The purified LRB did not inhibit cytoplasmic streaming. 6-Carboxyfluorescein (Eastman Chemicals, Rochester, N.Y., USA) was dissolved in three equivalents of 0.3 M KOH, diluted, and used without further purification. Fluorescein isothiocyanate isomer I (F), F-dextran, glutamic acid, glutamylglutamic acid [$(Glu)_2$], hexaglycine [$(Gly)_6$], insulin A chain and microperoxidase were obtained from Sigma Chemicals, St. Louis, Mo., USA. Leucyldiglutamyl-leucine (LGGL) and the pentamer (prolyl-prolylglycine)$_5$ were obtained from Vega Fox Biochemicals, Tucson, Ariz., USA.

Lissamine rhodamine B was converted to the sulphonyl chloride, and then used to prepare LRB-insulin A chain following Nairn (1976). The conjugate was separated from the dye on a Sephadex G15 (Pharmacia, Uppsala, Sweden) column (9 mm diameter, 300 mm long), eluting with 0.1 M NH_4HCO_3. The coloured fraction eluting at the front was freeze-dried and stored at 5° C. The F-peptide conjugates were prepared following Simpson (1978). To test for purity the conjugates were run on thin-layer chromatographs (Kieselgel 60; Merck, Darmstadt, FRG; developing solvent *n*-butanol:acetic acid:pyridine water, 150:30:100:120, by vol.). All gave a single fluorescent spot. The composition of all conjugates except F-microperoxidase and LRB-insulin A chain was confirmed by a combination of measuring the F content by adsorption at $A_{492.5}$, and amino-acid analysis.

Electrophysiological techniques. Detached leaves were held rigidly in a horizontal position against a layer of clear silicone elastomer (Sylgard 184; Dow Corning, Midland, Mich., USA) by a fine stainless-steel mesh. The leaves were covered by a thin layer of bathing buffer (Spanswick 1972) containing 0.1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.1 mM KCl, 0.5 mM NaCl, 0.05 mM Na_2SO_4 and 1.0 mM 3-(N-morpholino)propanesulphonic acid (pH 7.0). A 3 M KCl salt bridge leading to a silver-silver chloride half cell was inserted into the buffer.

Microelectrodes with a resistance of 15 MΩ (3 M KCl) were pulled from borosilicate glass capillaries 1.2 mm outside diameter (Clark Electromedical, Pangbourne, U.K.) using a locally constructed microelectrode puller. The microelectrode was held in a Carl Zeiss (Jena, GDR) micromanipulator, and connected through a BB-1 breakaway box to a M 701R Microprobe system (WP Instruments, New Haven, Conn., USA). The output from the Microprobe system was monitored on a 5103 dual-beam storage oscilloscope (Tektronix, Beaverton, Ore., USA). Current was injected via the breakaway box using a locally constructed voltage generator.

Microscopy. The mounted leaf was illuminated from above using an Intralux (Volpi, Urdorf, Switzerland) fiber-optics light, and was observed with an Olympus (Olympus Optical, Tokyo, Japan) binocular microscope, 80-fold magnification. After injection the leaf was removed, placed on a slide in bathing buffer, and observed under an Orthoplan (Leitz, Wetzlar, FRG) microscope using incident-light fluorescence. Photographs were taken on Ektachrome 400 daylight film (Eastman Kodak, Rochester, N.Y., USA), rated at 1600 ASA. Where LRB and F conjugates were injected together, they could readily be separated by their differing fluorescence characteristics: fluorescein conjugates show peak excitation at 495 nm and emission at 525 nm, whereas LRB conjugates show peak excitation at 575 nm, and emission at 590 nm. The LRB fluorescence could be removed at the fluorescein-conjugate settings of excitation and barrier filters (KP 490 excitation filter, TK 510 dichromatic beam splitter, K 515 suppression filter; Leitz) by the use of a Wratten 45 filter (Kodak, London, UK). Fluorescein-conjugate fluorescence was negligible at the LRB settings (K 580+2 mm BG 36 excitation filters, TK 580 beam splitter, K 580 suppression filter; Leitz).

Assessment of ability of conjugates to cross the plasmalemma. The leaves were soaked for 1 h in 1.0 mM solutions of each dye or conjugate, rinsed, and examined for fluorescence. In separate trials, leaves containing dyes which had shown cell-to-cell movement were returned to bathing buffer for 2 h, and then re-examined to see if dye had been lost.

Metabolism of conjugates. Ten *Elodea* shoot tips were ground with 1 ml of 1.0 mM 3(N-morpholino)propanesulphonic acid buffer, pH 6.5. Fifty μl of the homogenate was mixed with 50 μl of a solution containing 10 nmol of the conjugate, and left for 3 h at 20° C. The solutions were then spotted on thin-layer chromatograms as described earlier, together with samples of the conjugates which had not been incubated with homogenate. After development and drying, the plates were examined under UV light.

Injection of chemicals. The fluorescent compounds were dissolved in water to a concentration of 0.5 mM. Approximately 5 μl of the solution was added to the tip of the microelectrode, and the remainder of the electrode filled with 0.1 M KCl. Such electrodes typically had a resistance of approx. 100 MΩ. Injections were made from above into cells near the centre of the leaf, three to six cells from the midvein. Membrane potentials recorded with them varied greatly, from −250 to −50 mV. The most satisfactory indication of cell entry was a sharp fall in the noise level. The F-dextran and LRB-insulin A chains were injected by pressure, using 1,000 kPa for 15 min. All other compounds were injected by iontophoresis, generally at 25 V (square wave, 500 ms s^{-1}) for 15 s, with the microelectrode left in the cell for a further 15 min. Movement was usually scored in the fluorescence microscope within 20 min of the beginning of dye injection.

As judged by visual observation during injection, the initial injection of LRB could be into either the vacuole or the cytoplasm, vacuolar injection being the more common. The F-conjugates could not be seen under the binocular microscope during injection. However, by the time the tissue was examined under the fluorescence microscope all dyes had come to occupy both compartments in the injected cell. On rare occasions only the vacuole filled, and then the dye did not move from cell to cell. Such injections were not included when compiling the data.

A sufficiently heavy injection of any of the dyes would bring about an equilibrium in dye content between the injected cell and one or more adjacent cells, so that they all showed equally heavy fluorescence. Such 'movement' may have been the consequence of damage to the plasmodesmata rather than to cell rupture, as there was no obvious leakage of dye to the external solution. This type of intercellular dye movement was discounted when compiling the results.

Results

Movement through the plasmalemma. In the soak trials, although dead or damaged cells often became fluorescent (Fig. 1 a, b), none of the chemicals tested penetrated healthy, streaming cells from the external solution. In the tests for loss from injected cells, loss sometimes occurred from the original injected cell, but fluorescence persisted in

Table 1. Intercellular movement of fluorescent probes in *Elodea canadensis* Michx.

Molecule	Size (daltons)	Charge[a]	Number of trials where movement[b]	Extent of movement[c]	Mobile control molecule[d]
6-Carboxyfluorescein	376	−2	9/9	+++++	
Lissamine rhodamine B	559	−2	16/16	+++	
F-glutamic acid	536	−3	9/9	+++++	
F-glutamylglutamic acid	665	−4	9/9	+++++	
F-hexaglycine	749	−2	7/9	++	
F-leucyldiglutamylleucine	874	−4	3/17 (9)	+	LRB
F-(prolyl-prolyl-glycine)$_5$	1,678	−2	0/12 (7)	−	LRB
F-microperoxidase	2,268	?	0/10 (1)	−	LRB
LRB-insulin A chain	4,158	?	0/5 (4)	−	Fluorescein
F-dextran	19,400	?	0/5 (2)	−	LRB

[a] Negative charges only, at pH 7.0

[b] The ratio of the number of trials where intercellular movement occurred to the total number of trials. In brackets: number of trials where the test and the control molecules were coinjected

[c] This is the maximum movement seen in any trial:
+++++ = 10 or more cells in each direction
+++ = three to four cells in each direction
++ = two to three cells in each direction
+ = Fluorescence in adjacent neighbours just above detection threshold
− = No movement

[d] Control molecule injected with the test molecule, to establish whether or not intercellular movement could occur

the cells to which the dye had moved. The only exception was fluorescein, which showed high mobility, but was lost from cells near the injected cell within about 1 h of injection, presumably by leakage through the plasmalemma. Accordingly fluorescein was not used in the main study.

The loss of dye from the original injected cell is probably a consequence of damage, most particularly of damage caused by withdrawal of the microelectrode. Such damage was seen in experiments in which two electrodes were inserted into one cell. Insertion of a second electrode generally caused little fall in membrane potential (as measured by the first electrode), but electrode withdrawal usually caused a loss of membrane potential. The loss of dye indicates that the dyes do not bind to any immobile cell component. However, the possibility of dye binding to water-soluble molecules cannot be ruled out.

Metabolism of conjugates. There was no indication of metabolism of any of the conjugates to other fluorescent compounds. The R_F's of the incubated and non-incubated conjugates were identical: 6-carboxyfluorescein 0.84; F-glutamic acid 0.74; F-glutamylglutamic acid [F(Glu)$_2$] 0.48; F-hexaglycine [F(Gly)$_6$] 0.56; F-leucyldiglutamylleucine (FLGGL) 0.81; F-(prolyl-prolyl-glycine)$_5$ 0.40; F-microperoxidase 0.88. Thus it is probable that it is the injected conjugates rather than any metabolites which move or fail to move between cells.

Intercellular movement. The results of the injection experiments are summarised in Table 1. No polarity of movement, as measured by cell number, was seen (Fig. 1c–f); the compounds moving the same number of cells towards the leaf tip or base. They also showed movement over a similar number of cells towards the midvein or leaf margin. However, the number of cells reached was greater in the transverse than in the longitudinal direction. This was particularly obvious with the more mobile compounds, where the ratio of cell numbers reached in the two dimensions was approx. 1.4. Nevertheless, since the cells are elongated in the longitudinal direction of the leaf, the distance moved was greater in this dimension.

High concentrations of LRB were visible in the binocular microscope during injection, and LRB could be seen to move from the injected cell to adjacent cells within the 15 s injection period. When the leaf was injected for 15 s, and then immediately transferred and examined under incident-light fluorescence, the extent of movement could be observed about 1 min after injection. Within this period the more mobile molecules (up to 665 dalton) had moved one or two cells in each dimension. Over 15–20 min, more extensive movement occurred in leaves left with the microelectrode inserted, than in leaves on slides. The extent of movement was not obviously affected by light or dark during the movement period.

Those conjugates which failed to move might

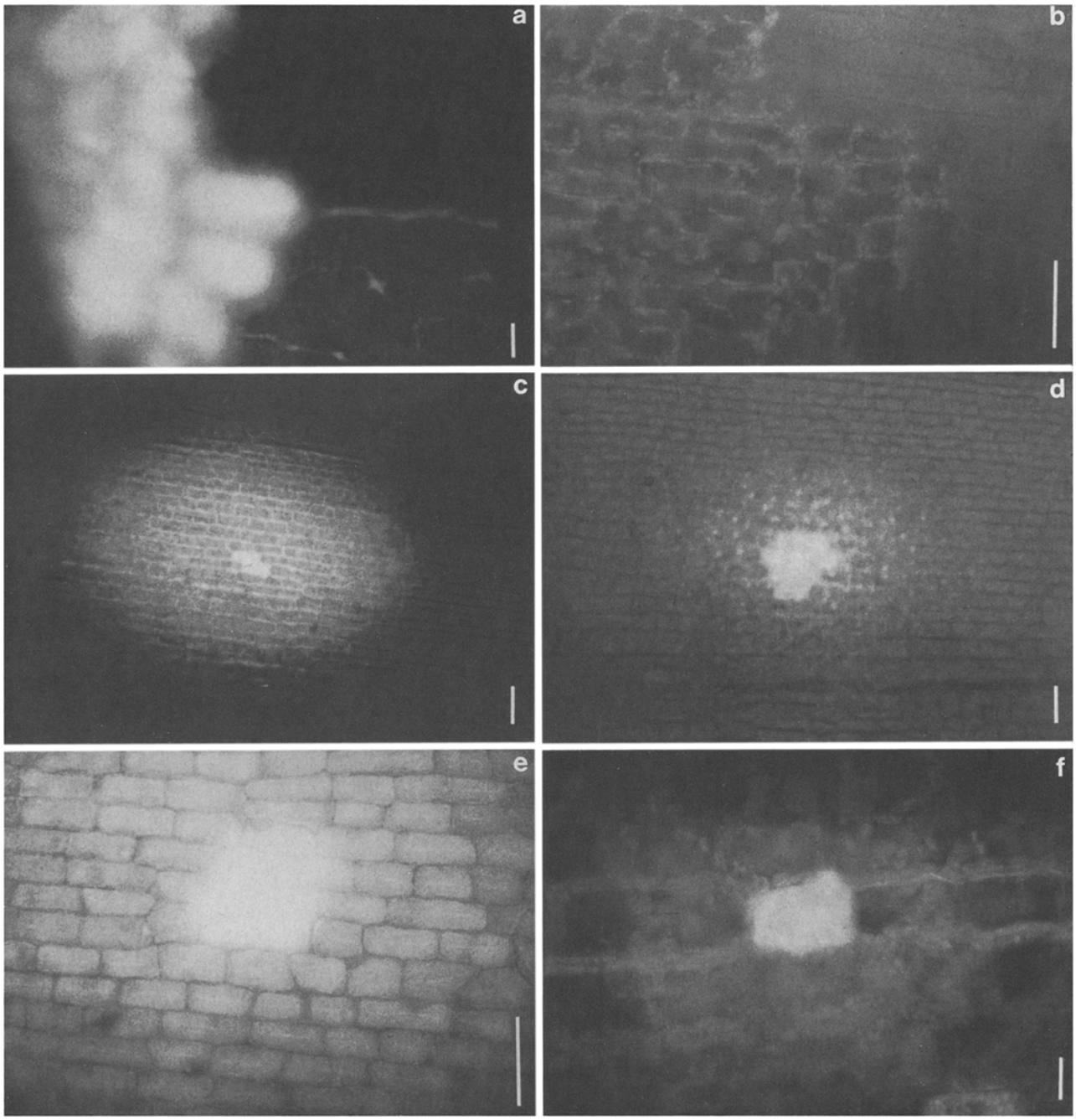

Fig. 1a–f. *Elodea* leaf soaked 1 h in **a** 6-carboxyfluorescein or **b** $F(Gly)_6$ in bathing medium. **a** ×250, **b** ×320. In **a** the apical cells of the leaf, to the left, have taken up 6-carboxyfluorescein. None has passed to the remaining leaf cells. In **b** the cut edge of the leaf is to the left, and cells have been damaged by cutting, and also by handling. These cells took up $F(Gly)_6$, much of which has been lost on rinsing. In both **a** and **b** the cells which took up the dye were not streaming, and were presumably damaged or dead. Movement of **c** 6-carboxyfluorescein (×58); **d** F-glutamic acid (×58); **e** $F(Glu)_2$ (×136); and **f** $F(Gly)_6$ (×320) following injection of the dyes into single cells. Bars = 20 μm in **a**, **f**, 40 μm in **b**, and 100 μm in **c**, **d**, **e**

have caused some type of injury response, leading to closure of the plasmodesmata, the presumed routes of movement between cells. This was tested by injecting a mobile fluorescent dye as a control with each of the non-moving conjugates, and using distinct fluorochromes so that they could be distinguished in the fluorescence microscope (Table 1; Fig. 2a–c). In each case it was found that the mobile compound retained its mobility in the presence of the immobile compound – that is to say,

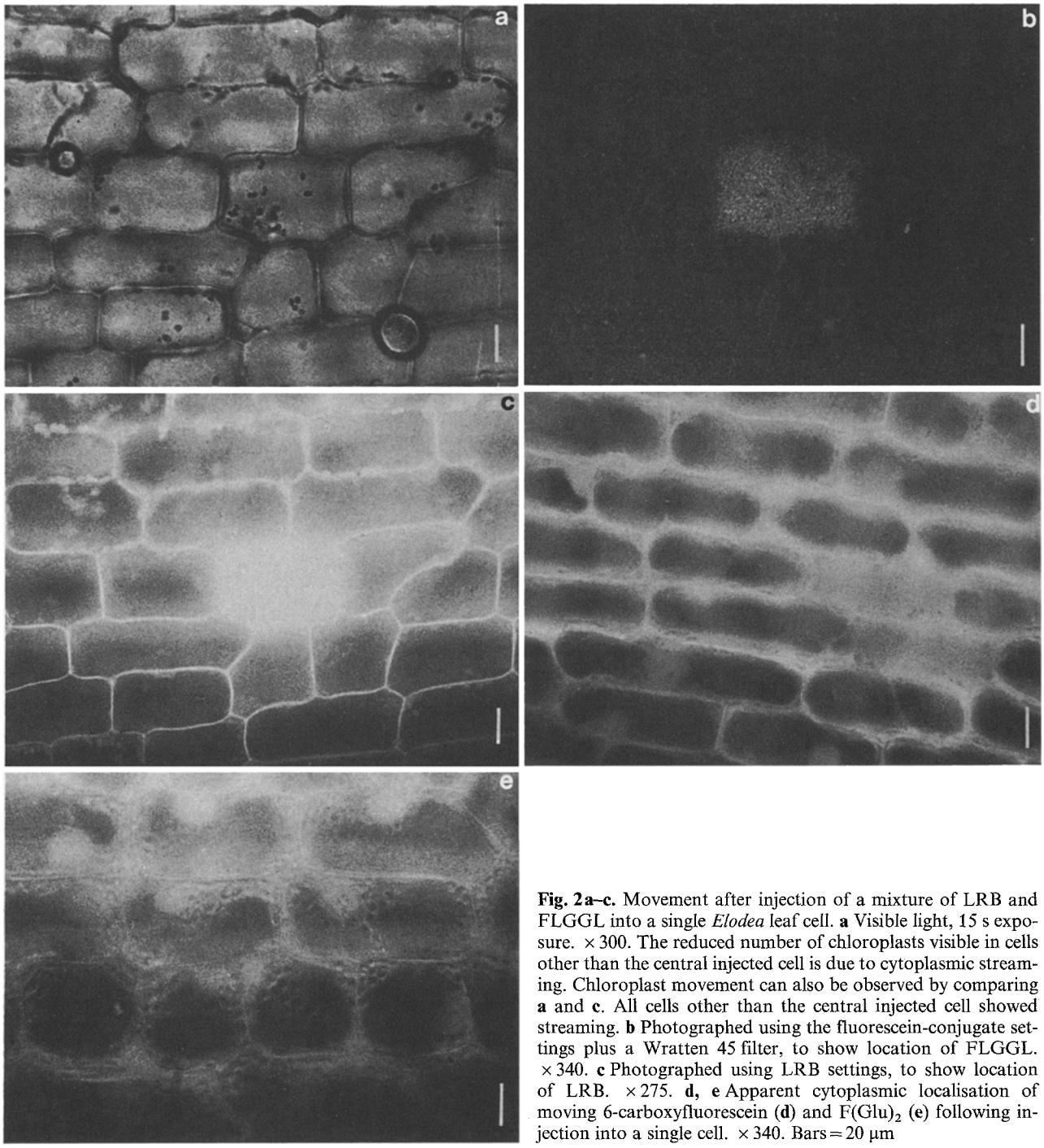

Fig. 2a–c. Movement after injection of a mixture of LRB and FLGGL into a single *Elodea* leaf cell. **a** Visible light, 15 s exposure. × 300. The reduced number of chloroplasts visible in cells other than the central injected cell is due to cytoplasmic streaming. Chloroplast movement can also be observed by comparing **a** and **c**. All cells other than the central injected cell showed streaming. **b** Photographed using the fluorescein-conjugate settings plus a Wratten 45 filter, to show location of FLGGL. × 340. **c** Photographed using LRB settings, to show location of LRB. × 275. **d, e** Apparent cytoplasmic localisation of moving 6-carboxyfluorescein (**d**) and $F(Glu)_2$ (**e**) following injection into a single cell. × 340. Bars = 20 μm

the immobile compounds do not block the intercellular channels.

An alternative possibility is that those compounds which pass from cell to cell do so by damaging each cell in turn, and leak out of the damaged cell into the adjacent cells. There are two lines of evidence against this. Firstly, as just discussed, mixing a mobile and immobile compound did not enable the immobile compound to move. Secondly, none of the mobile compounds caused any check to cytoplasmic streaming except in the directly injected cell.

Of the various compounds injected two, 6-carboxyfluorescein and $F(Glu)_2$, appeared to be localised in the cytoplasm (Fig. 2d, e). However, this may simply be a pH effect, as fluorescein shows

little fluorescence at pH 4, with a peak at around pH 8. The cytoplasm has a pH above 7, whereas the vacuole and cell wall may have much lower pH's (Smith and Raven 1979). On the other hand, all the F conjugates would be expected to show the same response to pH, and the same apparent cytoplasmic localisation. It is possible that the tonoplast, and perhaps also the chloroplast envelope are relatively impermeable to these two dyes.

Discussion

Compounds moving between cells could use two possible pathways. They could pass the plasmalemma and then the cell wall, or they could go through the plasmodesmata. The cell walls in the leaves of this plant are fully accessible to the external solution, as indicated for example by calcofluor staining, and so compounds passing from the cytoplasm to the cell wall would be expected to leak into the solution and be lost. Furthermore, none of the compounds was able to pass the plasmalemma, as indicated by the soak test, and by the stability of injected compounds. Hence, although there is no direct evidence for it, the compounds presumably pass from cell to cell via the plasmodesmata.

It is possible that it is degradation products of the conjugates which move or that immobile dyes are bound in some way. However, no such products were obtained after incubation with *Elodea* homogenates. Furthermore, since movement is visible within 1 min, degradation or binding would need to be very rapid. The possibility of cell-damage responses causing artifacts appears to be ruled out by the results of the injection of mixtures of immobile and mobile compounds, and by the continuation of cytoplasmic streaming.

The evidence strongly indicates that the mobile conjugates are moving from cell to cell in the leaf symplast. The simplest explanation of the retarded movement of $F(Gly)_6$, and the virtual lack of movement of FLGGL is that these molecules are approaching the molecular exclusion limit of the *Elodea* leaf symplast. The same types of conjugates have been tested on a number of animal cell cultures, where the largest conjugate passing is over 1830 dalton (insect cells) or 901 dalton (mammalian cells) (Flagg-Newton et al. 1979). Movement between insect cells appears to be insensitive to charge, but in mammalian cell lines highly negative molecules such as $F(Glu)_2$ show a low permeability compared with larger, less charged molecules such as $F(Gly)_6$. In *Elodea* there is no evidence for a charge effect, $F(Glu)_2$ moving freely, but FLGGL and larger molecules showing little movement, regardless of charge. The *Elodea* leaf symplast thus combines similarities to both the mammalian junctional channels (in limiting molecular size) and to the insect junctional channels (in insensitivity to charge).

Using molecular models it can be demonstrated that the conjugates consist of a rigid T-shaped fluorescein component, 1.3 nm wide, 1.2 nm high and 0.5 nm deep, with the flexible peptide tail attached at the base. The peptide is 0.9 nm long in $F(Glu)_2$ and 1.7 nm long in FLGGL. The models do not predict the orientation of the peptide tail in the cell, but if the peptide is assumed to be in the extended β-chain configuration, the maximum adaxial length can be estimated. $F(Glu)_2$ has a maximum adaxial length of 2.1 nm, and FLGGL of 2.9 nm.

Paine et al. (1975) provide curves relating mean effective cross-sectional area for diffusion to permeating-molecule radius, for several pore sizes. This type of analysis may not apply to the annuli which appear to be the pathways in plasmodesmata, but does provide a guide for estimating the equivalent pore size of the plasmodesmata. Using 5.0 nm as an estimate of the equivalent pore diameter, and maximum adaxial length as the permeating molecule diameter, the correction factors for the mean effective cross-sectional areas are 6-carboxyfluorescein 0.32, $F(Glu)_2$ 0.12, and FLGGL 0.010. This parallels the mobilities shown in Table 1, and indicates that 5.0 nm may be taken as a crude estimate of the maximum equivalent pore diameter of the *Elodea* leaf plasmodesmata.

A minimum estimate can be made by assuming that it is the width of the molecule which is limiting, and that FLGGL folds to give an overall width appreciably greater than 6-carboxyfluorescein or $F(Glu)_2$, both of the latter being taken as 1.3 nm wide. If FLGGL is assumed to fold to one turn of a helix, with the leucine chains overlaying, then the molecular width is 1.9 nm. Using 3.0 nm as an estimate of pore diameter, the correction factors for the mean effective cross-sectional areas are 6-carboxyfluorescein and $F(Glu)_2$ 0.10; and FLGGL 0.0087. Thus 3.0 nm may be taken as a crude estimate of the minimum equivalent pore diameter.

These estimates can be compared to the dimensions of plasmodesmata. Robards (1976) gives an extensive review of plasmodesmata dimensions. In higher plants the pore has an outer diameter of 35–70 nm, and an inner diameter of approx. 25–60 nm. This pore is traversed by a central structure, the desmotubule, with an outer diameter of the order of 16–20 nm, and an inner diameter of

7–10 nm. It, in turn, is traversed by a central rod of diameter 3 nm. The two annuli in the plasmodesmata have interradial gaps of 5–20 nm: outside the desmotubule, and 2–3.5 nm: between the inner wall of the desmotubule and the central rod. *Elodea* leaf plasmodesmata fit the general pattern (Falk and Sitte 1963). The maximum estimate of equivalent pore size might be regarded as favouring the outer annulus, and the minimum estimate the inner annulus as the pathway of intercellular movement. However, Hepler (1982) and Overall et al. (1982) have provided evidence that the inner annulus is the negatively-stained inner layer of a membrane, continuous with the endoplasmic reticulum. Hence the cytoplasmic annulus is the only available channel. The cytoplasmic annulus appears to contain particles, further restricting the intercellular pathway.

If the equivalent pore size estimated for the peptide conjugates applies to other molecules moving in the leaf symplast, then protons, most ions, amino acids, sugars, nucleotides, intermediary metabolites and even adenosine triphosphate, coenzymes and peptides could move freely between cells, but not proteins, enzymes, ribonucleic acids or cell organelles. This is in agreement with general concepts of symplast function. However, the symplast must also function in such a way as to maintain each cell's genetic and metabolic individuality. Thus distinctive small molecules involved in the differentiation and-or function of specialised cells in a tissue must be isolated from the symplast, or the cells themselves isolated. Even common molecules may need to be isolated from the symplast, for example sucrose moving from the leaf to and through the phloem (Evert et al. 1978). Appropriate protective plasmodesmatal responses to cell damage and environmental stress would also be expected. Electron micrographs indicate that the neck constrictions of the plasmodesmata contain specialised structures, called spincters, which may control flow through the symplast (Evert et al. 1977; Olesen 1979). Similar structures may be seen in *Elodea* leaf plasmodesmata (Falk and Sitte 1963). The technique used in this study offers a simple but sensitive method for estimating the equivalent pore diameter of the intercellular channels, and will aid the elucidation of the properties of the plant symplast.

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