

Internalisation of fluorescein isothiocyanate and fluorescein isothiocyanate-dextran by suspension-cultured plant cells

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Summary

The uptake of pure non-conjugated fluorescein isothiocyanate (FITC) and of the membrane-impermeant probe FITC-dextran into suspension-cultured carrot cells and protoplasts has been investigated. Commercial samples of a 70K ($K=10^3 M_r$) FITC-dextran were shown to contain contaminant FITC and/or its degradation products, which were rapidly internalised into the vacuolar system of both cells and protoplasts. However, purified samples of the 70K FITC-dextran were taken up into the vacuoles of cells but not protoplasts after a 1 h incubation period. This apparent difference in the ability of cells and protoplasts to internalise FITC-dextran was confirmed using samples of both commercial and purified 20K FITC-dextran as putative endocytotic probes. Both confocal and conventional fluorescence microscopy of FITC-treated cells have shown that FITC was internalised into similar intra-

cellular compartments as was observed in cells treated with three-times purified 70K FITC-dextran. Thus, FITC was a useful fluorophore for rapidly labelling both the putative endocytotic compartments and the pleiomorphic vacuolar system of carrot cells. Kinetic studies indicated that FITC entered the cell by diffusion in the form of the neutral molecule. We have shown that treatment of cells or protoplasts with the drug Probenecid reversibly inhibited the uptake of FITC from the cytoplasm into the vacuole. In addition, the uptake of FITC into isolated vacuoles was enhanced in the presence of Mg-ATP.

Key words: fluorescein isothiocyanate, FITC-dextran, endocytosis, suspension-cultured plant cells, fluorescence microscopy, vacuoles.

Introduction

Fluorescein isothiocyanate (FITC) is a fluorophore that is extensively used in cell biological studies. It retains the complex pH-dependent fluorescence spectra of fluorescein (Martin and Lindqvist, 1975) and therefore may exist in different ionic states in aqueous solutions of various pH values. The active isothiocyanate group reacts with unprotonated primary amines (Rinderknecht, 1962) and consequently can be covalently linked to the membranes of specific organelles and macromolecules.

In animal cells, FITC has been a useful probe in studies investigating the lateral diffusion of molecules at the cell surface (Edidin *et al.* 1976), the conformational state and activities of the plasma membrane (Na^+, K^+)-ATPase (Sen *et al.* 1981), the pH of intracellular compartments (Tycko *et al.* 1983; Geisow, 1984), cell-to-cell interaction (Segal and Stephany, 1984) and fluid-phase endocytosis (Berlin and Oliver, 1980; van Deurs *et al.* 1984). In plant cells, the applications of FITC as a fluorescent tracer have been limited to studies of cell-to-cell communication (Tucker, 1982; Goodwin, 1983; Terry and Robards, 1987) and the surface dynamics of protoplasts (Furtula *et al.* 1987; Walko *et al.* 1987).

FITC-conjugated dextrans (FDs) have been frequently used as membrane-impermeant markers for fluid-phase endocytosis in animal systems (Buckmaster *et al.* 1987;

Ferris *et al.* 1987; Lake *et al.* 1987; Swanson, 1989) and also in lower organisms including yeast (Makarow, 1985; Riezman *et al.* 1986; Makarow and Nevalainen, 1987), the amoeboid state of *Dictyostelium discoideum* (Thilo and Vogel, 1980) and the mollusc *Patella vulgaris* (Kühtreiber *et al.* 1987). The majority of studies on endocytosis in plant tissues have been carried out at the ultrastructural level using electron-opaque markers to investigate the coated-vesicle mediated pathway (see review, Coleman *et al.* 1988; Robinson and Depta, 1988; Robinson and Hillmer, 1990). Evidence for fluid-phase endocytosis in plants has come from experiments using the small fluorescent dye Lucifer Yellow CH ($M_r \sim 500$), which is negatively charged at physiological pH and hence membrane-impermeant (Oparka and Prior, 1988; Oparka *et al.* 1988; Hillmer *et al.* 1989; Wright and Oparka, 1989; Cole, 1989). However, FDs are convenient macromolecules with which to study fluid-phase endocytosis, since their molecular weights can be varied by altering the chain length of the polymer. This property is of particular importance for studies on plant cells where the size of the molecule available for endocytosis will be determined by the porosity of the cell wall.

Recent work using FDs to investigate fluid-phase endocytosis in walled cells of yeast have led to controversial findings. Makarow (1985) and Makarow and Nevalainen (1987) have reported that FD-70S is taken up by endocytosis in yeast cells and concentrated in their vacuoles. In

contrast, Preston and his coworkers (1987) found no evidence for endocytosis of FD-70S but reported that fluorescent contaminants of low molecular weight (e.g. FITC compounds) were present in commercial preparations of FDs. These small molecules were internalised via a non-endocytotic pathway and sequestered in the vacuoles.

In this paper we report upon an investigation into the uptake of both fluorescein isothiocyanate and fluorescent dextrans by carrot cells grown in suspension culture and by protoplasts from the same cell system. We confirm that the very rapid internalisation of fluorescence observed when cells or protoplasts are incubated with commercial FDs is a result of the presence of free FITC in the commercially supplied FDs. We indicate a difference in the uptake of purified FDs by cells and protoplasts and, in addition, show that both non-conjugated FITC and purified samples of FDs label similar intracellular compartments in suspension-cultured carrot cells. The kinetics of uptake of free FITC into the vacuoles of carrot cells are also discussed.

Materials and methods

Materials

Cell suspension cultures of carrot (*Daucus carota* L.) were grown in Murashige & Skoog medium (M & S; Flow Laboratories), supplemented with 2.5% sucrose, 0.1 mg dm⁻³ 2,4-dichlorophenoxyacetic acid, 0.1 mg dm⁻³ t-zeatin and 5% (v/v) coconut milk. Cells (10 ml) were subcultured every 10–14 days into 100 ml of sterile culture medium and maintained as a fine suspension in a New Brunswick G25 incubator shaker at 25°C, 140 revs min⁻¹ with a light intensity of 120 lumen/sq.ft for a 12 h day. Protoplasts were isolated from cells three to five days after subculturing as described by Coleman *et al.* (1987).

In every case, the viability of cells and protoplasts prior to experimentation was greater than 80%. Viability measurements were determined by the percentage of control cells showing cytoplasmic fluorescence following treatment with 0.01% (v/v) fluorescein diacetate (FDA) as described by Widholm (1972). FDA was taken from a concentrated stock solution: 10 mg ml⁻¹ 100% acetone.

Preparation of vacuoles

Vacuoles were isolated from carrot protoplasts by a modified osmotic-lysis method (van der Valk *et al.* 1987): protoplasts were resuspended in 3 ml of M & S medium (pH 5.0) containing 0.6 M sorbitol and then mixed with 10 volumes of lysis medium (90 mM K₂HPO₄, 1 mM dithiothreitol (DTT), 5 mM EDTA, pH 8.0) with gentle agitation for 5 min. After addition of 0.33 volume of a solution of sterile-filtered 0.6 M sorbitol, 5.0 mM EDTA (pH 7.5) and 20% (w/v) Ficoll the mixture was stirred and filtered through one layer of Miracloth (Henry Simon Ltd, mesh size 25 µm). The filtrate was centrifuged at 1300 g for 15 min and the vacuoles collected by suction from the surface of the Ficoll cushion. A 0.2–0.6 ml sample of vacuole suspension was diluted to 8 ml with a solution of 0.15 M sorbitol, 5 mM EDTA (pH 7.5), 250 µM DTT, 5% (w/v) Ficoll and 90 mM K₂HPO₄, the suspension was recentrifuged and purified vacuoles collected from the surface.

Purification of FITC-dextran

The 70K (K=10³ M_r) FD (FD-70S, Sigma Ltd) and 20K FD (FD-20S, Sigma Ltd) were purified by gel-column chromatography essentially as described by Preston *et al.* (1987). Two ml of FD (50 mg ml⁻¹ in 20 mM sodium phosphate buffer, pH 8.0) were microcentrifuged at 8100 g for 1 min to remove insoluble impurities and the supernatant was loaded onto a prewashed 1.5 cm×24 cm Sephadex G-25-300 (Sigma Ltd) column. FD was allowed to settle on the column for 30 min before resuming elution. On elution (flow rate=60 µl min⁻¹) FD was collected as

the first fluorescent band off the column and subsequently concentrated 10-fold with Centricon-30 Amicon filters. Finally, 400–800 µl of FD was freeze-dried overnight at -40°C. This process was repeated twice and samples of once-, twice- and three-times-purified FD were stored desiccated at 0–4°C. All procedures were carried out under low light conditions.

Microscopy

FITC. Carrot cell suspensions (3- to 5-day-old) were diluted 1:1 (w/v) with 0.1 mg ml⁻¹ FITC (FITC stock solution: 5 mg FITC ml⁻¹ 100% ethanol) in M & S culture medium (pH 5.6–6.5) to give a final concentration of 4×10⁶ cells ml⁻¹ and incubated at 25°C for 30–60 min with constant agitation. Protoplasts were incubated with 0.1 mg ml⁻¹ FITC in M & S medium (pH 5.0) containing 0.4 M sorbitol as described above. Control cells and protoplasts were also incubated in the appropriate medium containing 1% ethanol only, as above. Following treatment, samples of cells and protoplasts were washed and mounted for fluorescence microscopy.

Fluorescent dextrans. Samples of 3- to 9-day-old cell suspensions (5×10⁶ cells ml⁻¹) were treated with an equal volume of FD (25 mg ml⁻¹ in M & S culture medium, pH 5.6) and incubated as described above. Protoplast suspensions (4.8×10⁶ protoplasts ml⁻¹) were also incubated with FD in M & S medium containing 0.4 M sorbitol as above. In addition, cells and protoplasts were treated with non-fluoresceinated dextran polymers as control specimens for the observation of autofluorescence.

Vacuoles. Prior to the final centrifugation step, 500 µl samples of vacuole suspension were mixed with 500 µl of 0.1 mg ml⁻¹ FITC±10 mM Mg-ATP in isolation medium for 30 min at 25°C and agitated gently. Vacuoles were then diluted to 8 ml with isolation medium and reconcentrated by centrifugation on a Ficoll cushion prior to microscopy.

Inhibitors. Pre-washed 3- to 5-day-old cells (1.0 ml samples) were diluted 5–10 times with M & S medium containing 50 mM Mes (pH 6.3) and 5 mM Probenecid (Sigma Ltd) and preincubated for 3 or 17 h at room temperature. One-ml samples were removed, the cells pelleted and incubated with an equal volume of Mes-buffered medium containing Probenecid±FITC (0.1 mg ml⁻¹) for 5–30 min at 25°C. Finally, treated cells were washed in buffered medium±Probenecid and samples were observed with the fluorescence microscope.

Specimens were observed with a Zeiss Axiophot epifluorescence microscope fitted with an FITC filter set and micrographs were recorded on Ilford HP5 film. Confocal microscopy was carried out with a BIORAD Lasersharp MRC 500 laser scanning confocal microscope. Stereo-reconstructions were made with the stereo-projection facility in the microscope control programme.

Fluorimetry

Cells were pre-rinsed with M & S culture medium containing 50 mM Mes (pH 6.5) at 0–4°C or 25°C or 20 mM Hepes (pH 7.2) at 25°C. At the appropriate temperature and pH, 7.5 ml of buffered M & S culture medium containing 0.1 mg ml⁻¹ FITC was mixed with 7.5 ml of cell suspension (2.85–3.28 (×10⁶) cells ml⁻¹). One-ml samples were taken at regular intervals between 0 and 40 min, added to 4 ml of appropriate buffered medium and filtered with pre-wetted 25 mm diameter Whatman cellulose nitrate filters (0.45 µm pore size) under low suction using a Hoefer FH224 filtration unit. Cells were immediately resuspended in 5 ml of buffered medium on the filters and then refiltered. This step was repeated twice, after which the filter and cells were submerged directly in 2 ml of 5% SDS for 10 min. After SDS treatment, 1-ml samples were pelleted at 15 000 g for 1 min and 500 µl of the supernatant was made up to 3 ml in distilled water for fluorimetric analysis.

Quantities of FITC and FITC derivatives were determined by the fluorescence measured at excitation (400–450 nm) and emission (500–550 nm) wavelength bands, with the appropriate filters in a Fluorimet FM-200 fluorimeter connected to a potentiometric recorder.

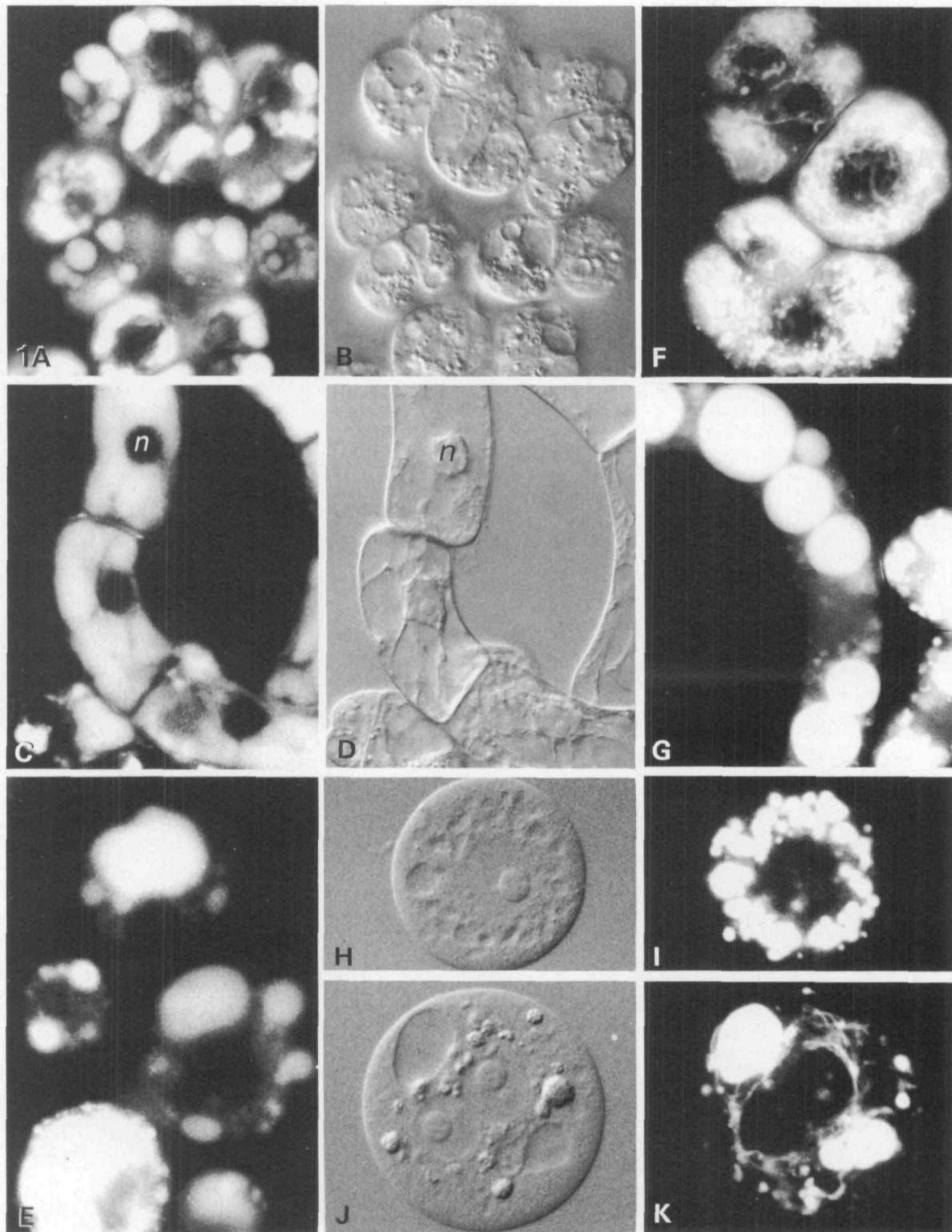


Fig. 1. Uptake of commercially available FD-70S and FITC by carrot cells and protoplasts. A,B. Micrographs show the accumulation of fluorescence in vacuoles of isodiametric carrot cells (8-day-old) after incubation with FD-70S (12.5 mg ml^{-1}) for 30 min. $\times 660$. C,D. Elongate cells (7-day-old) also appear to accumulate FD-70S (12.5 mg ml^{-1} , 60 min) in their vacuolar system. Note the total exclusion of probe from the nucleus (n) and cytoplasmic strands. $\times 900$. E. Uptake of FD-70S (12.5 mg ml^{-1}) into vacuoles of carrot cell protoplasts after 30 min. $\times 1150$. F. Fluorescence micrograph showing uptake of FITC (0.025 mg ml^{-1}) into tubular compartments of isodiametric cells (4-day-old) after a 5-min incubation. $\times 440$. G. Fluorescence micrograph showing uptake of FITC (0.05 mg ml^{-1}) into large vacuoles of elongate cells after 30 min. $\times 720$. H–K. Uptake of FITC (0.05 mg ml^{-1} , 30 min) into small vacuoles (see I) and tubular compartments (see K) of protoplasts. $\times 920$.

Results

When carrot suspension-cultured cells and protoplasts were incubated with FD-70S for 30 min followed by washing as described in Materials and methods, intense fluorescence was detected within the vacuoles (Fig. 1A–E). This vacuolar compartmentation of fluorescence appeared to be similar to that described previously for the fluid-phase endocytosis of Lucifer Yellow CH in plant tissues (Oparka *et al.* 1988). In order to test whether the fluorescence observed in the vacuoles of FD-70S-treated cells and protoplasts was a result of low molecular weight impurities present in FD-70S we investigated the uptake of three-times-purified FD-70S (FD-70S3). Results (Fig. 2A,B) show that after a 60-min incubation period with FD-70S3 only very low levels of fluorescence were detected in vacuoles of cells and no fluorescence was observed in protoplasts. With both commercial samples and highly purified FD-20S, fluorescence was detected in vacuoles of cells after a 30-min incubation (Fig. 2E,F). However, in both cases no fluorescence was observed in protoplasts after similar incubation periods.

By fluorescence and differential interference contrast

(DIC) microscopy it was observed that after incubation for 30 min with pure non-conjugated FITC, fluorescence was exclusively localised in the vacuoles of both cells and protoplasts. This phenomenon was particularly apparent in the large vacuoles of elongate cells (Fig. 1G). Intense fluorescence was also observed within vesicles or small vacuoles located in the cytoplasm of both cells and protoplasts (Fig. 1F–K). Further to this, fluorescence was detected within an elaborate network of tubules that appeared to ramify throughout the cortical cytoplasm (Fig. 1F,J,K). These fluorescent tubular compartments were often visible after only 5 min of incubation with FITC (Fig. 1F) and the fluorescence in these tubules appeared to diminish with time. The tubules showed considerable saltatory movement, connecting with small mobile and highly fluorescent vacuoles, which would then fuse to form larger vacuoles. Fluorescence was excluded from the nucleus and remaining cytoplasm of both FD- and FITC-treated cells and protoplasts (Fig. 1A–D and H–K). Confocal laser scanning microscopy in the epifluorescence mode confirmed the accumulation of FITC fluorescence inside the vacuolar system of carrot cell suspension cultures (Fig. 3A–I). The three-dimensional organisation of the

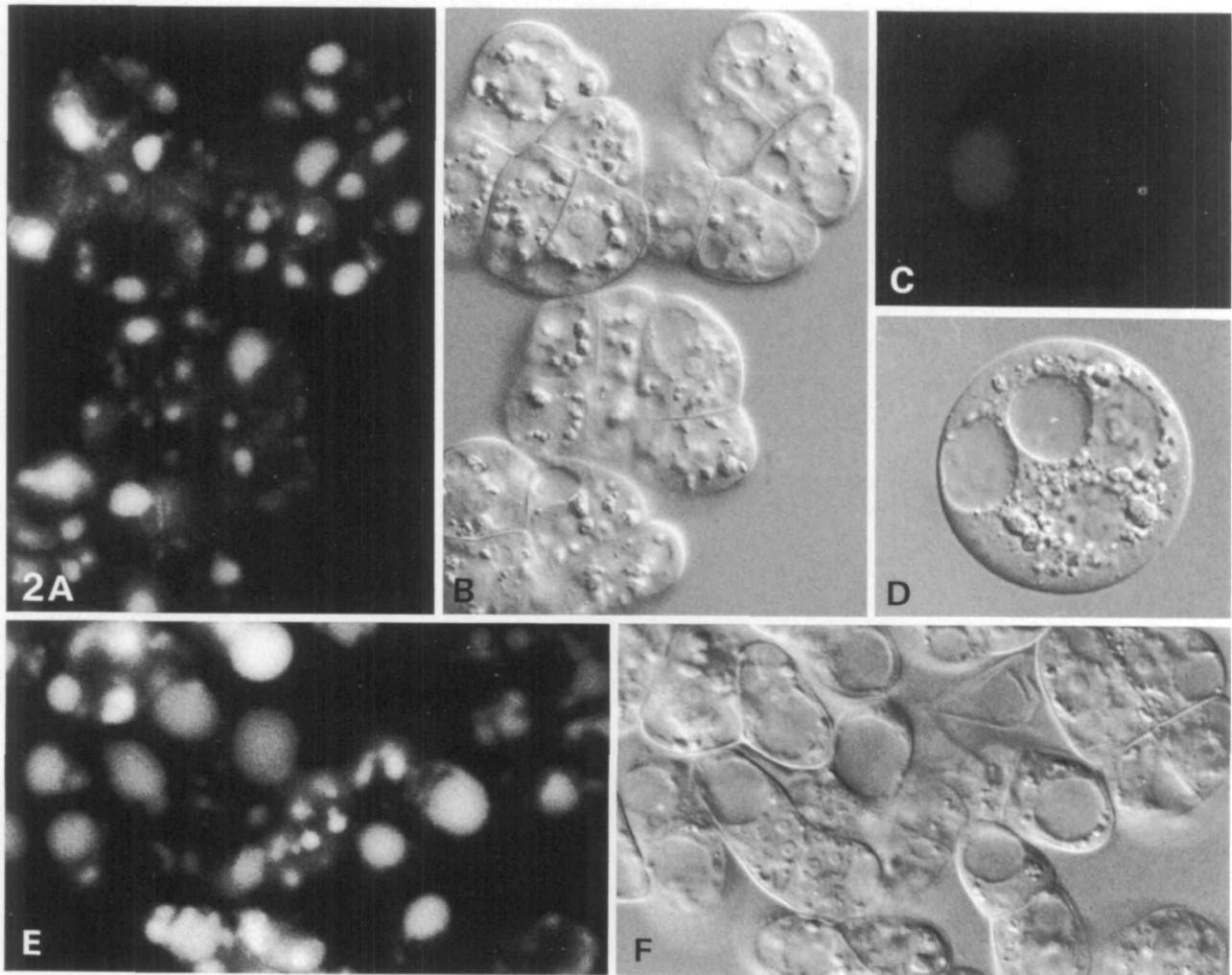


Fig. 2. Uptake of the purified FD, FD-70S3 by carrot cells and protoplasts. A,B. Uptake of FD-70S3 (12.5 mg ml^{-1}) into vacuoles of isodiametric cells (8-day-old) after a 30-min incubation. $\times 670$. C,D. Protoplasts incubated with FD-70S3 as in A,B. Note that the probe is not internalised. $\times 870$. E,F. Uptake of FD-20S3 at (12.5 mg ml^{-1}) into vacuoles of isodiametric cells after a 30-min incubation. $\times 690$.

vacuolar system can be seen in the stereo-reconstruction of the confocal images (Fig. 3I). The dynamic nature of the tubular network that often interconnected with the vacuolar system was also confirmed by this technique (not shown here). Furthermore, these results have shown that both FITC and the purified FDs label similar intracellular compartments in suspension-cultured carrot cells.

The uptake of FITC by cells was characterised by kinetic analyses. At 25°C and pH 5.6, where FITC exists as a mixture of neutral and anionic forms (Fig. 4), cells accumulated FITC in a biphasic manner (Fig. 5, open circles). A rapid initial linear phase was followed by a slower phase, which continued for more than 20 min. At 25°C and pH 7.2, where the concentration of neutral forms

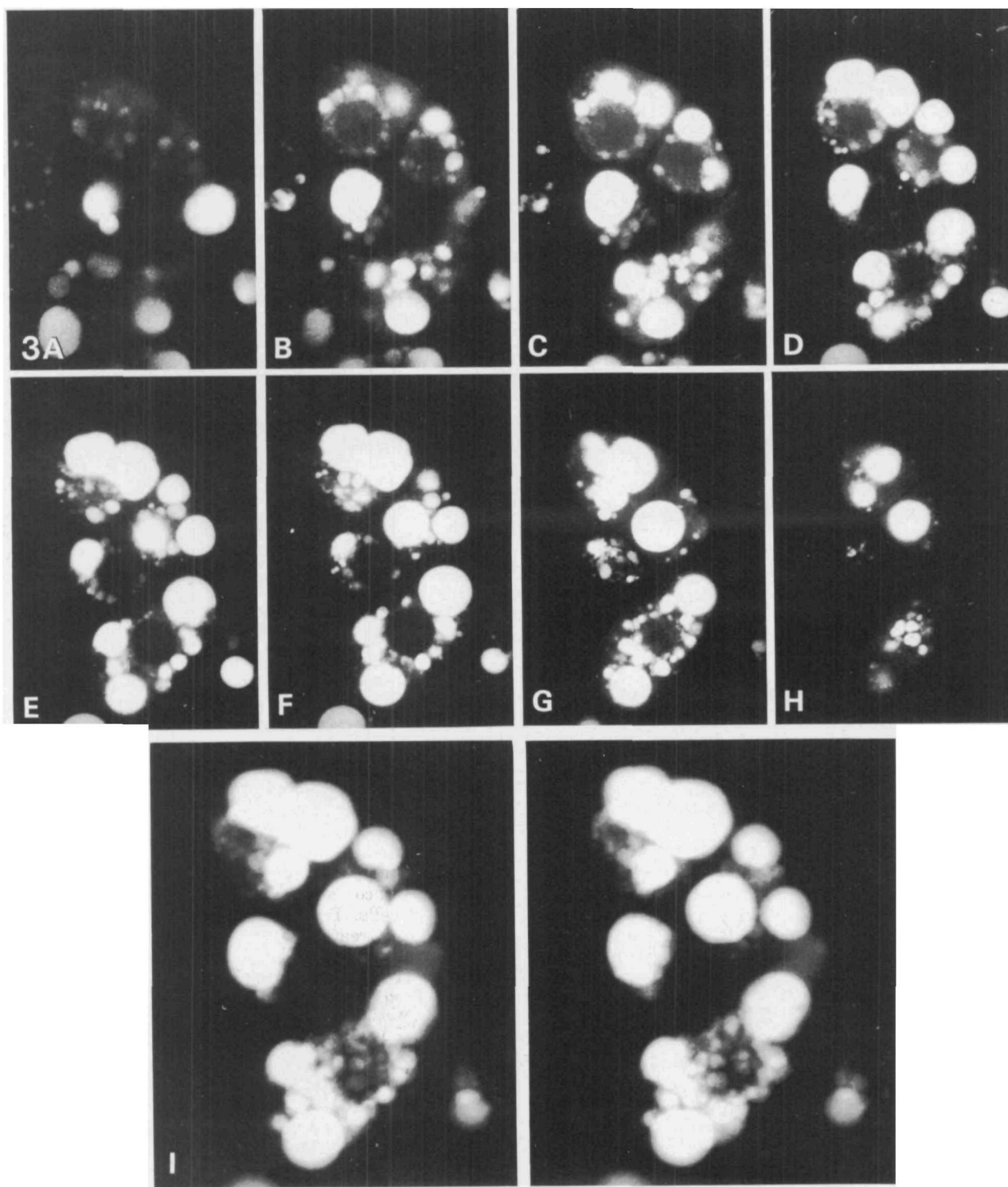


Fig. 3. Laser scanning confocal fluorescence microscopy of an FITC-treated elongate cell. A–H. Series of optical sections, of approximately 1 μ m depth of field, through cell. Alternate sections from the complete series of 16 are shown. $\times 500$. I. Stereo-reconstruction of the same cell from the series A–H.

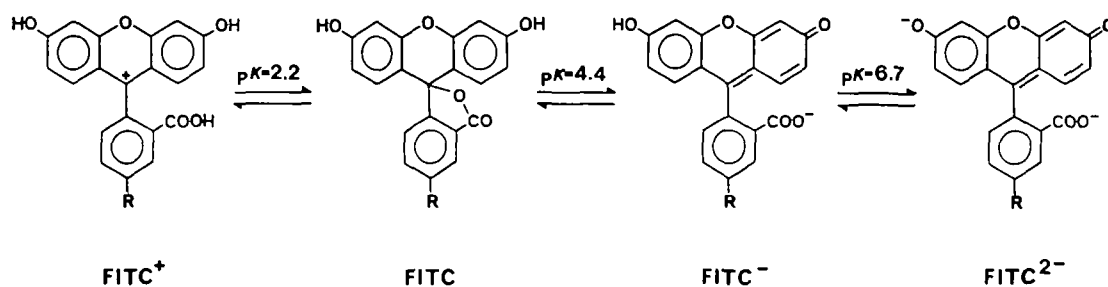


Fig. 4. Diagram showing the protonic equilibria of FITC in an aqueous solution. The pK values 2.2, 4.4 and 6.7 represent the dissociation constants for the cationic (FITC⁺), neutral (FITC) and mono-anionic (FITC⁻) forms of FITC, respectively (R shows position of isothiocyanate group attached to the fluorescein molecule).

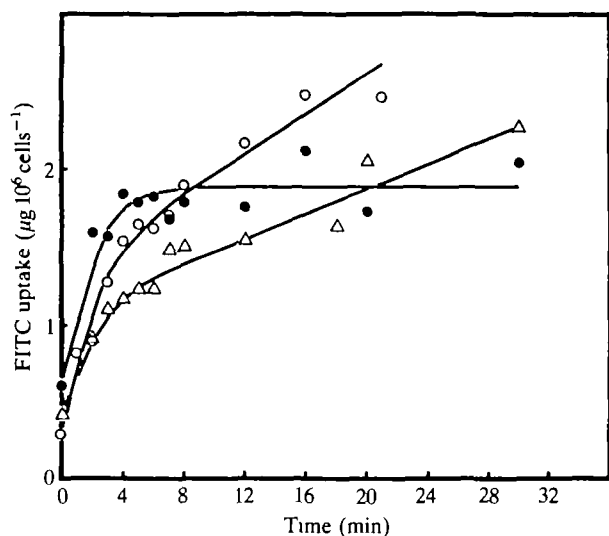


Fig. 5. Graph showing the results of kinetic studies investigating the effects of low temperature and an increase in pH upon the uptake of FITC by suspension-cultured carrot cells. Cells were incubated in Mes-buffered culture medium (pH 5.6) containing FITC (0.05 mg ml⁻¹) at 25°C (○) or at 0–4°C (●). Cells were incubated in Hepes-buffered culture medium (pH 7.2) containing FITC (0.05 mg ml⁻¹) at 25°C (△). In cells exposed to control treatments, negligible amounts of fluorescence were recorded throughout the incubation period (not shown here).

of FITC is smaller (Fig. 4), a similar pattern of uptake as described above (Fig. 5, triangles), i.e. biphasic, was observed, but the rate of uptake in both phases is considerably slower. The initial rapid uptake of FITC was unaffected by low temperature (0–4°C), but the slower phase was completely inhibited (Fig. 5, filled circles). These kinetic studies indicated that FITC entered the cells by diffusion in the form of the neutral molecule. However, it was not clear how and why FITC fluorescence was located exclusively in the vacuolar system. The possibility that FITC was selectively taken up into the vacuolar system as the anionic form, which would predominate at the alkaline pH of the cytoplasm, was investigated by testing the effects of the drug Probenecid on the uptake of FITC by suspension-cultured cells and isolated protoplasts. Probenecid has been shown to inhibit the uptake of the anion, Lucifer Yellow, into the lysosomal systems of macrophages (Steinberg *et al.* 1987).

Fig. 6A–D and G–J show that the uptake of FITC into vacuoles of cells and protoplasts was inhibited by Probenecid. FITC fluorescence was restricted to the cytoplasm of cells pretreated with Probenecid for 3 h and no FITC

fluorescence was detected in the vacuoles (Fig. 6A,B). After prolonged exposure to Probenecid (17 h) FITC appeared to accumulate in the nuclei of cells (Fig. 6C,D). When Probenecid-treated (3 h) cells were washed in Probenecid-free medium, fluorescence was no longer detected in the cytoplasm or nuclei (Fig. 6E,F) but reappeared in the vacuoles. Similar results were obtained with protoplasts (Fig. 6G–L).

The uptake of FITC into the vacuolar system was also studied, using isolated vacuoles. When isolated vacuoles were incubated with FITC (pH 7.5) for 30 min, 20% of the vacuoles fluoresced (Fig. 7A,B). However, with the addition of 10 mM Mg-ATP, the number of vacuoles that were fluorescent increased to 90% (Fig. 7C,D).

Discussion

By comparison of the uptake of FITC and of both commercial and purified samples of FDs by suspension-cultured carrot cells and isolated protoplasts we have shown that samples of commercial FD-70S (Sigma Ltd) contain low molecular weight fluorescent contaminants. Such contaminants, e.g. FITC compounds, were taken up rapidly by carrot cells and protoplasts, producing intense labelling of the vacuolar system. In our experiments small contaminant molecules were removed by gel filtration yielding purified FDs, which were tested as endocytotic substrates. In contrast, our results indicated little difference in the uptake of commercial and three-times-purified FD-20S by carrot cells. The absence of fluorescence in protoplasts following treatment with FD-20S indicated that this probe was contaminant-free.

When FD-70S3 was used as the endocytotic substrate we observed weak fluorescence in the vacuoles of cells. By comparison, when FD-20S3 was the substrate the fluorescence in the vacuoles was stronger. Over similar incubation periods, no fluorescence was detected in the vacuoles of protoplasts incubated with either FD-70S3 or FD-20S3. At least two conclusions may be drawn from these results. First, FD-70S3 and FD-20S3 are taken up by cells *via* fluid-phase endocytosis. In view of the ultrastructural evidence for endocytosis in protoplasts (Tanchak *et al.* 1984; Joachim and Robinson, 1984; Hillmer *et al.* 1986; Tanchak and Fowke, 1987), it is conceivable that the lack of fluorescent labelling in vacuoles of protoplasts following incubation with FD-70S3 or FD-20S3 was due to a faster rate of endocytosis in cells than protoplasts. Recent results have indicated that FD-70S3 is taken up into the vacuoles of protoplasts after an 18-h incubation period (Cole, unpublished results). It is possible that in the presence of FDs the osmotic conditions subjected to protoplasts, com-

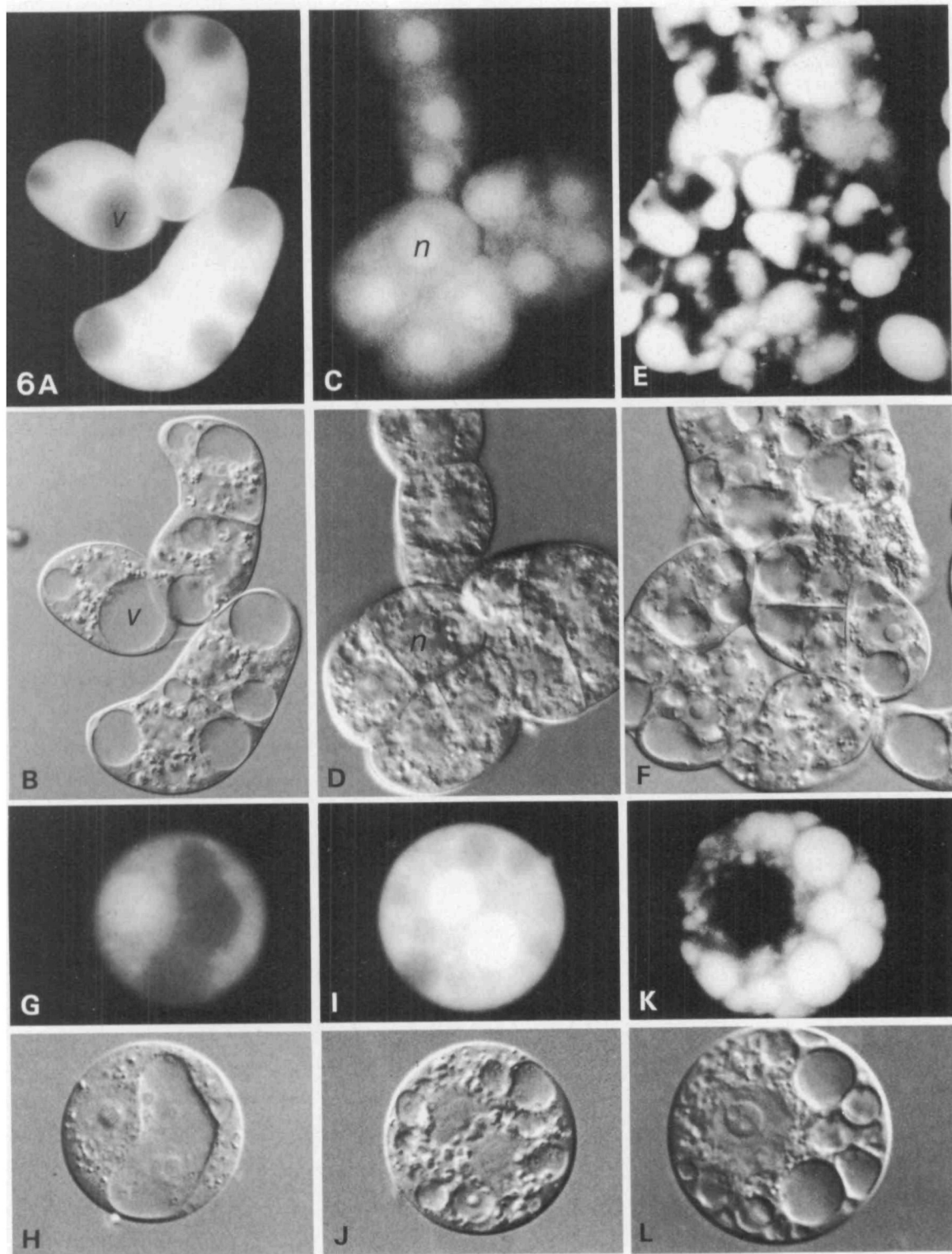


Fig. 6. Effect of the anion transport-inhibiting drug Probenecid on the uptake of FITC into cells and vacuoles. A,B. Cells were preincubated with Probenecid (1 mM) for 3 h prior to incubation with FITC (0.5 mg ml^{-1}) and Probenecid (2.5 mM) for 40 min. FITC fluorescence was observed in cytoplasm of cells but excluded from vacuoles (v). $\times 520$. C,D. FITC fluorescence accumulated in the nuclei (n) and cytoplasm of cells (5-day-old) that were preincubated with Probenecid (0.5 mM) for 17 h prior to FITC treatment (0.05 mg ml^{-1} , 5 min) in the presence of Probenecid (2.5 mM). $\times 790$. E,F. Cells preincubated as described in A,B prior to FITC treatment in the absence of Probenecid. FITC fluorescence reappeared in the vacuoles and was not observed in the cytoplasm or nuclei. $\times 700$. G–J. Protoplast preincubated with Probenecid (2.5 mM) for 1 h prior to FITC treatment (0.05 mg ml^{-1} , 30 min) in the presence of the drug. Note that FITC fluorescence is excluded from the vacuoles but present in the cytoplasm and nuclei. G,H. $\times 1100$. I,J. $\times 900$. K,L. Protoplast preincubated as in G–J, prior to FITC treatment in the absence of Probenecid. FITC fluorescence reappeared in the vacuoles and is excluded from the nucleus. K,L. $\times 1080$.

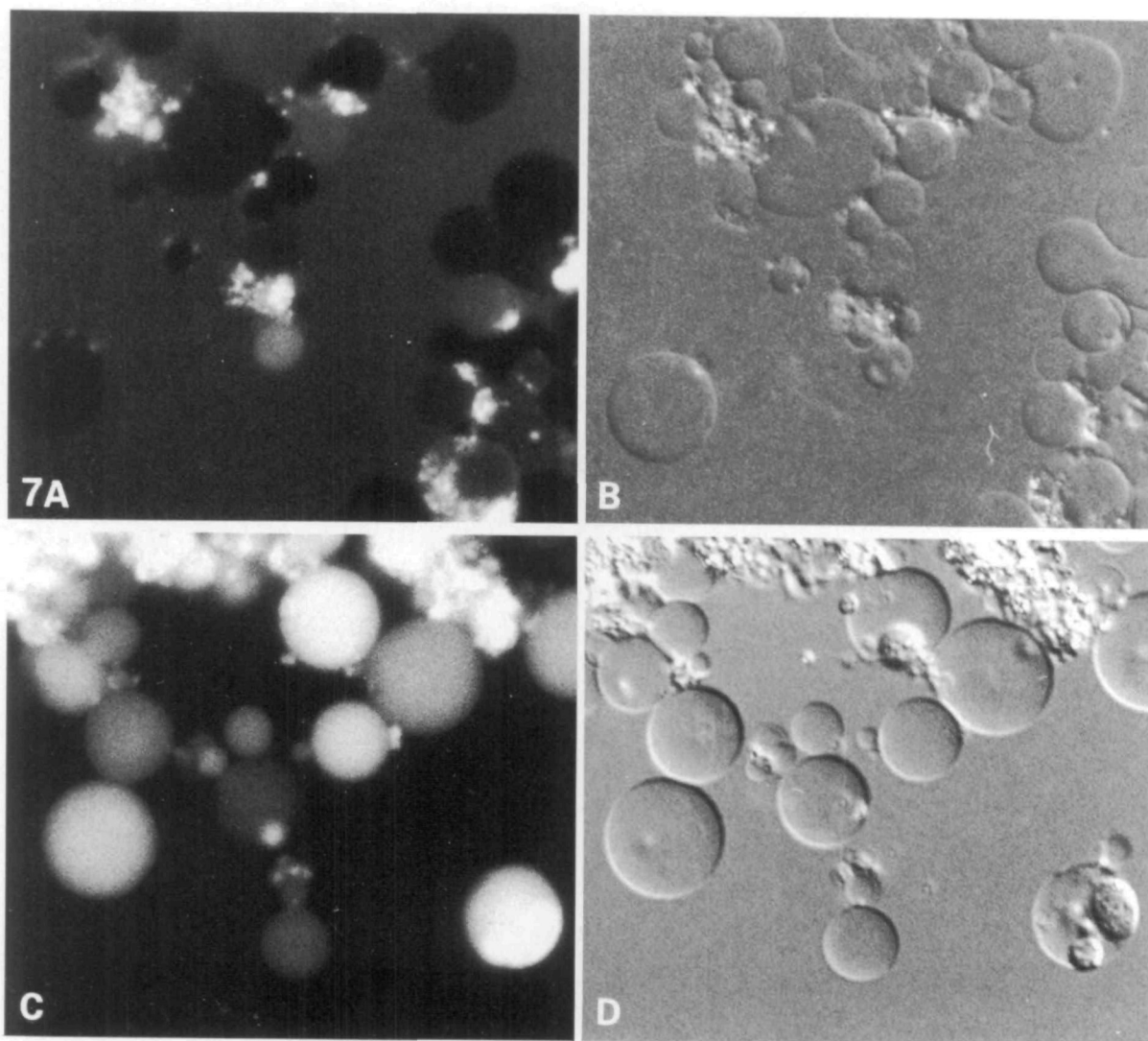


Fig. 7. Sequestration of FITC into isolated vacuoles. A,B. Vacuoles incubated with FITC (0.05 mg ml^{-1}) for 30 min. Little or no FITC fluorescence was observed in vacuoles. $\times 1200$. C,D. Vacuoles incubated with FITC (as in A,B) in the presence of 5 mM Mg-ATP. The probe is rapidly incorporated into the vacuoles. $\times 1100$.

pared to cells in suspension culture, preclude a fast rate of endocytosis. To date, no comparative quantitative data exist on endocytosis by plant cells and protoplasts in varying environmental conditions and further experimentation is required. The observation that cells appeared to take up FD-20S3 faster than FD-70S3 may reflect the difference in the molecular size of the FDs, since it has been shown that FDs of molecular size equal to or less than 20K can rapidly penetrate the plant cell wall (Baron-Epel *et al.* 1988).

An alternative conclusion is that fluorescence observed in vacuoles of cells resulted from small amounts of low molecular weight compounds released from the purified dextrans by some hydrolytic activity in the cell wall. This would account for lack of fluorescent labelling in the protoplasts. The observed difference between FD-20S3 and FD-70S3 in cells could be determined by the greater facility with which FD-20S3 permeates the cell wall. However, there is as yet no evidence to support this hypothesis. In the only other report on FD uptake by plant cells, Griffing (1988) showed that a 76K FD was internalised into soybean suspension cell protoplasts after

5 min, but was not present in the central vacuole. Recently, the specific uptake of FITC-conjugated polygalacturonic acid by cultured soybean cells has been reported (Horn *et al.* 1989). These researchers concluded that this uptake was receptor-mediated endocytosis, since non-specific proteins, e.g. FITC-labelled insulin (M_r 5.7K) and FITC-labelled BSA (M_r 60K), were not taken up even after 8 h of incubation, although some fluid-phase uptake of these probes could have been expected.

A striking feature of the fluorescent labelling observed when cells were incubated with commercial FDs was the degree of compartmentalisation involved. Similar compartmentalisation of fluorescence was observed when either cells or protoplasts were incubated with FITC for 5–30 min. FITC was incorporated rapidly into a network of fine tubules and small vesicles/vacuoles or into large vacuole(s). The pleiomorphic vacuolar system observed here appears to be similar to that described previously in suspension-cultured carrot cells by Hillmer *et al.* (1989) after Lucifer Yellow CH internalisation and also as confirmed by our own unpublished results. Conventional fluorescence microscopy of FITC-treated cells has shown

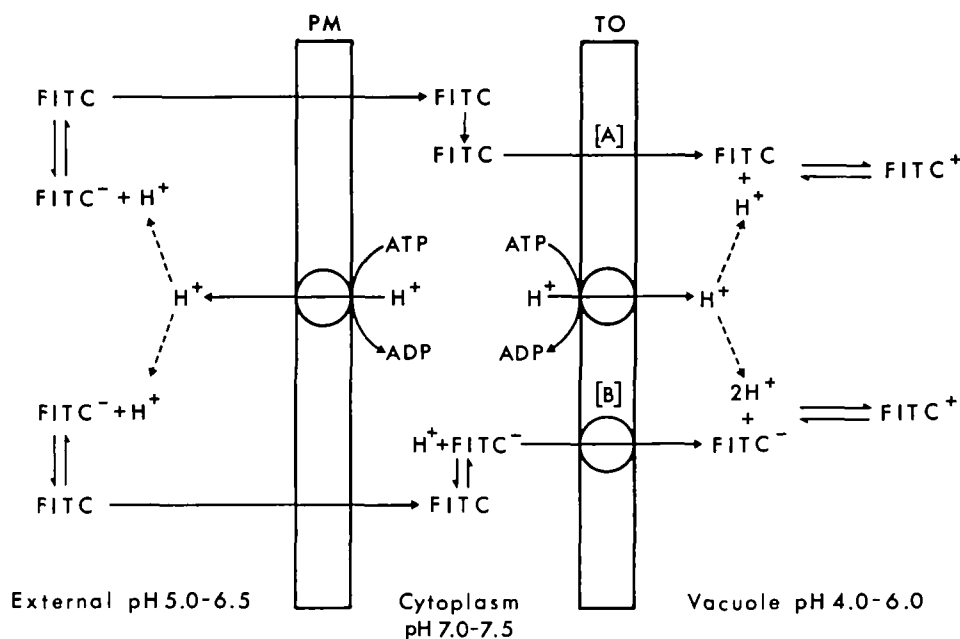


Fig. 8. Overview of the possible pathways for FITC transport in carrot cell systems. At external pH 5.0–6.5, the neutral form of FITC enters the cell by diffusion across the plasma membrane (PM). In the cytoplasm (pH 7.0–7.5) the majority of the neutral FITC dissociates into its anionic form. From the cytoplasm there are two possible pathways by which the probe may be transported into the vacuole: [A] neutral FITC diffuses rapidly across the tonoplast (TO); or [B] FITC anion is transported across the tonoplast by an organic anion transporter. Within the vacuole (pH 4.0–6.0), both the neutral and anionic forms become trapped as FITC cations. The tonoplast H⁺-ATPase may facilitate the trans-tonoplast transport of both neutral and anionic forms of FITC.

that FITC highlighted the dynamic system of tubules and vacuoles that exhibited considerable saltatory movement, budding and fusing with each other. Confocal laser scanning fluorescence microscopy confirmed that FITC was present within the lumen of such compartments and not merely bound to the cytosolic face of their membranes. We propose that the fine tubular compartments are vacuolar in nature and may be precursors of the central vacuole. Furthermore, if FDs and Lucifer Yellow CH are internalised *via* a fluid-phase endocytotic pathway, then it can be concluded that FITC is an excellent molecular probe for rapidly labelling the compartments along this pathway.

Kinetic studies of FITC uptake have shown that there are at least two components to the process, a pH-sensitive and a temperature-sensitive component. We suggest that the former represents the passive diffusion of FITC, in its neutral form, across the plasma membrane of cells and protoplasts. This component would be sensitive to the external pH of the ambient fluid, which would determine the state of dissociation of FITC. The temperature-sensitive component may represent the process that regulates the passage of FITC into the vacuole.

A proposed mechanism by which FITC is taken up and compartmentalised into vacuoles of suspension-cultured carrot cells and isolated protoplasts is summarised in Fig. 8. In the external medium (pH 5.0–5.6) FITC is presented to the cell predominantly as the neutral molecule ($pK=4.4$). In this form FITC can traverse the cell wall and plasma membrane by diffusion. The influx of FITC would be facilitated by the acidic pH outside the cell and the alkaline pH in the cytosol. This ΔpH would be maintained by the activity of the H⁺-pumping ATPase in the plasma membrane.

From the cytoplasm, there are two possible pathways by which FITC may be transported to the vacuolar compartment: diffusion of neutral FITC across the vacuolar membrane and its accumulation as the cation form at the low pH of the vacuole (proposal [A] in Fig. 8) or transport of anionic FITC across the vacuolar membrane by an anionic transporter and its subsequent accumulation as the cation at the low pH of the vacuole (proposal [B]). The activity of the H⁺-pumping ATPase and PPase (inorganic pyrophosphatase) in the vacuolar membrane maintains the low pH of the vacuole (Rea and Sanders, 1987). We assume that similar activities would be present in the membranes of the tubular compartments observed by fluorescence microscopy.

In addition our results suggest that anionic forms of FITC may be rapidly transported across the tonoplast by a Probenecid-sensitive organic anion transporter. Although this possibility may seem unlikely, in view of the fact that FITC is a non-physiological molecule, Probenecid, which has been used as an inhibitor of organic anion transport in animal cell systems (Steinberg *et al.* 1987, 1988), reversibly inhibited the sequestration of FITC into vacuoles of carrot cells. At the moment, the precise site of Probenecid inhibition in carrot cells and protoplasts is unknown, and sites of action other than on organic anion transport cannot be discounted.

The observation that isolated vacuoles accumulate FITC from the surrounding medium, by a mechanism that is enhanced by Mg-ATP, is in keeping with both proposals [A] and [B] shown in Fig. 8. In the case of [A], the ΔpH generated by the tonoplast ATPase would lead to the trapping of FITC⁺ at the acid pH of the vacuole, whereas in [B] the membrane potential ($\Delta \Psi$) generated by the ATPase would drive the uptake of FITC by an organic anion transporter.

In summary, our results have shown that the use of FDs as fluorescent substrates for endocytosis can be complicated by FITC and/or low molecular weight FITC derivatives that may be present in commercial batches of FDs. Initial experiments with purified FDs indicated that there may be an endocytotic pathway from the outside of the cell to the central vacuole, as suggested by previous studies. In addition, the capacity for endocytosis in carrot protoplasts was lower than that of cells. Since both purified FDs and non-conjugated FITC labelled similar intracellular compartments in cells and protoplasts, the latter proved to be a useful fluorophore for rapidly labelling putative compartments of the endocytotic pathway, including the pleiomorphic vacuolar system of carrot cells. We have also indicated by kinetic studies that FITC enters the cell by diffusion in the form of the neutral molecule. However,

further research on both the Probenecid-sensitive, Mg-ATP-enhanced transport of FITC from the cytoplasm to the vacuole and the FITC-trapping mechanism apparent in the vacuolar lumen of suspension-cultured carrot cells and protoplasts is required.

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