Influence of Cations at the Plasma Membrane in Controlling Polysaccharide Secretion from Sycamore Suspension Cells

By MARTIN R. MORRIS and D. H. NORTHCOTE

Department of Biochemistry, University of Cambridge, Cambridge CB2 1QW, U.K.

(Received 18 March 1977)

1. Three soluble polysaccharides and a soluble protein containing hydroxyproline were secreted by sycamore suspension cultures. L-[1-³H]Fucose was incorporated solely into the fucose of fucoxyloglucan and L-[1-¹⁴C]arabinose mainly into the arabinose of arabinogalactan. [U-¹⁴C]Glucose was a general precursor for soluble protein and polysaccharides. 2. The steady-state rate of secretion of all the polymers was increased within seconds of adding various electrolytes and polyelectrolytes to the growth medium. The increased secretion was induced by cations at the outer surface of the plasma membrane. It was brought about by a stimulation of the normal mechanisms of cell-wall polysaccharide secretion. It was partly inhibited by anaerobiosis or sodium arsenate and was unaffected by temperature changes in the range 0–35°C. 3. The precursor pool from which secretion was induced contained completely synthesized polysaccharides and was probably located in the Golgi-derived vesicles. The results indicated that the endoplasmic reticulum did not secrete polysaccharide directly to the cell exterior. 4. The various cations probably induced secretion by causing a depolarization of the negative electric potential of the cell surface, and this resulted in the fusion of vesicles with the plasma membrane. 5. Analogy with exocytosis and pinocytosis in various animal tissues suggested that the decreased surface potential brought about membrane fusion by causing an increase in plasma-membrane permeability to Ca²⁺. 6. The results showed that the fusion of vesicles with the plasma membrane was rate-limiting and a potential control point. Auxin-stimulated cell-wall deposition could be a result of a stimulated influx of Ca²⁺ causing vesicle fusion with the plasma membrane.

The growth and differentiation of plants is partly governed by the rate of polysaccharide secretion to the cell wall. Although the mechanisms of polysaccharide secretion by intracellular organelles have been investigated (Chrispeels, 1976; Northcote, 1974, 1977), the factors controlling secretion and the relationship of secretion to intracellular membrane movement are unknown. The aim of the present paper is to elucidate some of these factors and to propose a mechanism for controlling polysaccharide secretion at the level of fusion of vesicles with the plasma membrane.

Suspension cultures of higher plants secrete large quantities of soluble polysaccharides into the growth medium. All cultures so far examined probably secrete three polysaccharides containing the following sugars: (A) polygalacturonan containing galacturonic acid (approx. 70%) (partially as the methyl ester), arabinose, galactose and rhamnose; (B) A neutral highly branched arabinogalactan, (C) xyloglucan in dicotyledons and gymnosperms, or an arabinoxylan in monocotyledons (Olson *et al.*, 1969; Olson, 1971; Hawes & Adams, 1972; Liao & Boll, 1972; Mante & Boll, 1973; Wilder & Albersheim, 1973; Burke *et al.*, 1974). The structure and composition of the soluble polysaccharides secreted by sycamore cell cultures

have been thoroughly studied and shown to be similar to that of the non-cellulosic cell-wall polysaccharides; the xyloglucan contains fucose as a minor terminal sugar (Becker *et al.*, 1964; Aspinall *et al.*, 1969; Bauer *et al.*, 1973).

We have taken advantage of the ability of radioactive L-fucose and L-arabinose to be incorporated solely into certain components of sycamore polysaccharides (Stoddart & Northcote, 1967; Roberts, 1968; Rubery & Northcote, 1970) to measure the rate of soluble-polysaccharide secretion by sycamore cell cultures and to investigate possible control mechanisms.

Materials and Methods

Chemicals and radiochemicals

All chemicals were of analytical grade and glass-distilled water was used for all solutions. Poly-L-lysine hydrobromide (mol.wt. about 100 000) was obtained from Serva, Heidelberg, Germany, and N-Z amine type A, a casein hydrolysate, was from Sheffield Chemical, Norwich, NY, U.S.A. L-[1-³H]-Fucose (specific radioactivity 4.2 Ci/mmol), D-[U-¹⁴C]glucose (specific radioactivity 283 mCi/mmol) and L-[1-¹⁴C]arabinose (specific radioactivity 60 mCi/

mmol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

Tissue culture

Sycamore tissue (*Acer pseudoplatanus*), isolated in this laboratory in 1970 and designated S4 (Wright & Northcote, 1972), was maintained in suspension culture in 500 ml conical flasks at 25°C in the dark by using a rotary shaker at 110 rev./min. The tissue was subcultured every 7 days by using 80 ml of inoculum into 100 ml of PRL4 medium (Gamborg, 1966) containing 2 mg of naphth-1-ylacetic acid, 2 g of N-Z amine and 20 g of sucrose/litre. This routine resulted in a minimal lag phase of growth and rapid growth and division of the cells for about 5 days. Media and glassware were autoclaved at 120°C (103 kPa) for 30 min. Conditioned medium was prepared by growing the cells for the same period as that for the particular experiment in which it was used and separating the cells by centrifugation at 1000g for 0.5–1 min.

Radioactive labelling

Cells were used 2–5 days after subculture, except where otherwise stated. Incubations of greater duration than 3 h were under sterile conditions. The cells were allowed to settle for 10–15 min in 2.5 cm × 9 cm (30 ml) sterile containers (Sterilin, Teddington, Middx., U.K.) and the supernatant was decanted to give a cell volume of 35 ml for every 100 ml of suspension. Radioactive precursors were added as carrier-free aqueous solutions at a final radioactive concentration of 3 µCi/ml ([1-³H]fucose and [U-¹⁴C]-glucose) or 0.1 µCi/ml ([1-¹⁴C]arabinose). The cells (30–60 ml suspension) were incubated, in a closed 100 ml or 150 ml conical flask in the rotary shaker used for growing the cells, for 2.5 h ([1-³H]fucose and [U-¹⁴C]glucose) or 45–75 min ([1-¹⁴C]arabinose). After this time the polysaccharides secreted by the cells were radioactive and the cells were prelabelled for subsequent use. For experiments in which cells were prelabelled with [1-³H]fucose or [U-¹⁴C]glucose, they were washed with 2 vol. of conditioned growth medium, each equal to half the volume of the cell suspension, in order to decrease the initial concentration of radioactive soluble polymers already secreted into the medium. The shorter incubation period used with [1-¹⁴C]arabinose made this wash unnecessary. Samples of radioactive soluble polymers prepared for analysis were from cells incubated overnight with radioactive precursors.

Separation of polymers and analyses

The soluble polymers were separated by glass-fibre paper electrophoresis for 30 min at 2 kV, in either pyridine/acetic acid/water (100:3:1000, by vol.) (pH 6.5) or sodium borate (19 g/litre) (pH 9.4).

Samples of radioactive polymers were hydrolysed with 3% (w/v) H₂SO₄ at 120°C in an autoclave for 1 h after first dissolving the samples, dried on to Whatman 2.1 cm GF/C glass-fibre discs, in 72% (w/v) H₂SO₄. The hydrolysate was neutralized with BaCO₃, applied to Whatman no. 1 chromatography paper and electrophoresed in 8% (v/v) acetic acid/2% (v/v) formic acid, pH 2, at 4 kV for 40 min to separate amino acids from the sugars. A strip (6 cm) containing the radioactive sugars and uronic acids was sewn on to Whatman no. 1 paper and electrophoresed at pH 3.5 (pyridine/acetic acid/water, 1:10:89, by vol) to separate galacturonic acid and glucuronic acid. The non-mobile sugars were then eluted and separated by descending chromatography on Whatman no. 1 paper in ethyl acetate/pyridine/water (8:2:1, by vol.) for 12–60 h. Xylose and fucose were separated by chromatography in butanol/ethanol/water (5:1:4, by vol.) for 48 h. Chromatograms and electrophoretograms were cut into 4 cm × 1 cm strips and counted for radioactivity in toluene scintillant (Harris & Northcote, 1970) in a Philips liquid-scintillation counter with 22% efficiency (³H) or 34% efficiency (¹⁴C) (glass-fibre strips), or 3% efficiency (³H) or 25% efficiency (¹⁴C) (chromatography paper strips).

Amino acid analysis (non-radioactive) was carried out on freeze-dried soluble polymer isolated from 100 ml of culture (2-day) by dialysis against water. Hydrolysis and analysis was as described by Heath & Northcote (1971). PRL4 growth medium (100 ml) was dialysed and analysed as a control.

A cell-wall fraction was obtained by washing and resuspending cells in ice-cold 0.5 M-potassium phosphate buffer (pH 7.0) and sonicating at 5 A for 4 min in 30 s bursts to break the walls (Soniprobe type 7530-1 A; Dawe Instruments, London W.3., U.K.). They were centrifuged in an MSE Minor bench centrifuge at 1000g for 5 min, washed twice with ice-cold buffer, resuspended in 72% (w/v) H₂SO₄ and hydrolysed in 3% (w/v) H₂SO₄ at 120°C for 1 h.

Cells were sometimes isolated free from medium by thoroughly draining the liquid away from them through a fine hypodermic needle attached to a syringe. This allowed the cells to be isolated, without centrifugation, as a compact mass relatively free from medium.

Cell incubation

A suspension of prelabelled cells was transferred to sterile containers (2.5 cm × 9 cm; 30 ml) and samples (1.35 ml) were transferred to 10 ml conical flasks. The flasks either already contained the added reagents as aqueous solutions (pH 6–7) added in a total volume of 0.15 ml, or the reagents were added later at the required time intervals. Radioactive precursors were added to the cell suspension immediately before transfer to the 10 ml conical flasks if prelabelled cells

were not used. Incubation was at 25°C in a reciprocating water bath at 150 strokes/min, stroke length 3 cm.

Samples (0.1–0.2 ml) of the cell suspension were withdrawn at various times and assayed for radioactive soluble polymers and cellular polysaccharides. Investigation of the early effect of CaCl_2 on secretion was carried out by rapid filtration of a cell suspension (1.5 ml) through 2.5 cm Whatman GF/C glass-fibre discs.

The results of addition of metabolic inhibitors and of 1 M-sorbitol on secretion in the presence and absence of Ca^{2+} were found by pipetting prelabelled cells (1.35 ml) into 10 ml flasks containing inhibitors (15 μl aqueous solutions) or sorbitol (solid) and assaying for radioactive soluble polysaccharide after 30 min. CaCl_2 (1 M; 0.15 ml) was immediately added and the suspension sampled again after a further 30 min. The influence of temperature on secretion was measured by pipetting prelabelled cells (1.35 ml) into 10 ml flasks, incubating at the required temperature for 10 min, then adding 0.15 ml of 0.2 M- CaCl_2 and measuring secretion after a further 10 min.

The influence of inhibitors and other reagents on O_2 uptake by 2 ml samples of suspension was measured by using an O_2 electrode with a pen-recorder output. The effect of anaerobiosis on polysaccharide secretion was measured by using 2 ml samples of prelabelled cells by allowing the suspension to reach anaerobiosis in the O_2 -electrode cell and adding 0.2 ml of a deaerated 1 M- CaCl_2 solution after 20 min. After stirring, the cells were allowed to settle, a sample of the medium was withdrawn by screwing down the central chamber of the electrode cell, the cells were aerated and another sample was taken after a further 10 min.

Pulse-chase experiments were carried out by incubating the cells in [$1\text{-}^{14}\text{C}$]arabinose for 15 min, adding non-radioactive arabinose to give a final concentration of 10 mM and then pipetting 1.35 ml samples at various times into 10 ml flasks containing 0.15 ml of 1 M- CaCl_2 or water. Samples of the suspension were taken for measurement of radioactive soluble polysaccharide after 5 min. Radioactive polysaccharides present within the cell and cell wall (cellular radioactive polysaccharide) were measured by thoroughly draining the medium from a 1.35 ml sample, adding 2 ml of 80% (v/v) ethanol and heating at 60°C for 30 min. The cells were again drained and washed twice with 2 ml of 80% (v/v) ethanol containing non-radioactive arabinose (10 mM) at 60°C for 30 min, resuspended in 1.35 ml of 3% H_2SO_4 and hydrolysed.

Protoplasts

Cells were used for protoplast preparation 3 days after subculture while in a state of rapid growth. A 50 ml suspension was allowed to settle in two

sterile containers (6 cm $\times$ 4 cm; 60 ml) (Sterilin), the medium decanted and the cells were washed once with 40 ml of PRL4 medium containing 0.3 M-sorbitol. After thorough draining, the cells were resuspended in a 60 ml sterile container with 4.5 ml of enzyme mixture. The mixture consisted of 5% (w/v) Driselase (mixture of pectinase and cellulases; Kyowa Hakko Kogyo Co., Tokyo, Japan) and 2% (w/v) pectinase (Sigma Chemical Co., St. Louis, MO, U.S.A.) dissolved in PRL4 medium containing 0.45 M-sorbitol (pH 5.5). The enzyme mixture was centrifuged to remove insoluble salts and sterilized by filtration through a Millipore disc (0.45 μm pore size) before addition to the cells. The cells were incubated at room temperature (20°C) in a reciprocal shaker (120 strokes/min) and protoplasts collected after 6.5 h by filtration through stainless-steel mesh (61 μm mesh) to remove whole cells and debris. The protoplasts were centrifuged (50 g, 10 min) and washed twice with 15 ml of PRL4 medium containing 0.45 M-sorbitol by resuspension and centrifugation. The protoplasts were then resuspended (7.5 h after addition of enzymes) in 7.5 ml of PRL4 medium containing 0.45 M-sorbitol, 20 mM-sucrose and 10 μCi of [$1\text{-}^{14}\text{C}$]arabinose. After a further 4.5 h incubation, 0.4 ml samples of the protoplast suspension were transferred to 4 cm $\times$ 1 cm flat-bottomed glass vials containing 0.1 ml of 0.25 M- CaCl_2 or 0.1 ml of 0.75 M-sorbitol (iso-osmotic concentrations) and returned to the shaker. The triplicate samples were assayed for radioactive soluble polysaccharide over the next 2 h.

Assay of radioactive soluble polymers

Samples (0.1–0.2 ml) of the cell suspension were withdrawn immediately after adding radioactivity and at subsequent time intervals. The samples were centrifuged in Durham tubes (27 mm $\times$ 6 mm) for at least 30 s at 1000 g to sediment the cells. Two methods were adopted to remove unincorporated radioactive sugar precursors from polysaccharides in the supernatant. In Method 1, duplicate samples (10 μl) were chromatographed overnight in ethyl acetate/pyridine/water (8:2:1, by vol.) containing the appropriate non-radioactive sugar at 10 mM; the polysaccharide remained at the origin. In Method 2, duplicate samples (50 μl) were applied to 2.1 cm Whatman GF/C glass-fibre discs. After drying at 60°C for approx. 30 min, each disc was washed once for approx. 60 min at 60°C in 2 ml of 80% ethanol and then with 2 ml of 80% ethanol containing the appropriate non-radioactive sugar at 10 mM for 30 min at 60°C. After drying, the paper strips or glass-fibre discs were counted for radioactivity. Uptake of radioactivity into the cells was calculated from the initial total radioactivity of the incubation medium, the final total radioactivity of the medium and radioactivity in secreted soluble polymers.

Each value given in the Figures and Tables is the mean from three samples of cells per treatment, $\pm$ S.E.M.

Microscopy

Cells and protoplasts were viewed on a Zeiss Ultraphot II equipped with Nomarski optics and u.v. lamp.

Results

The line of cells used in these experiments (S4) has improved growth and friability properties compared with the sycamore line isolated here in 1958 (Lamport & Northcote, 1960) and designated S2 (Wright & Northcote, 1972). The following experiments on the structure and composition of the soluble polysaccharides of S4 cells indicate that the two cell lines may be regarded as equivalent in terms of soluble-polysaccharide metabolism.

Composition and structure of radioactive polysaccharides

Table 1 shows that [$U^{14}C$]glucose labels all the known sugars in the soluble polysaccharide secreted by the suspension callus cells (S4). Hydrolysis of the radioactive polymer and electrophoresis at pH2 separated a substantial quantity of amino acids from the sugars. We found no radioactive amino acids free in the incubation medium by electrophoresis at pH2.0, unlike Simpkins & Street (1970) who found

that S2 cells could release free amino acids. This difference may be due to the high concentration of amino acids in the PRL4 medium used for our cells.

Roberts (1968) found that L-[$1^{14}C$]fucose labelled only the fucose residues in cell-wall polysaccharides of sycamore cultures (S2). We found that L-[$1^{14}C$]fucose similarly labelled only the fucose residues in the soluble polysaccharides (S4). Radioactivity (about 10%) was also found in the cell wall. L-[$1^{14}C$]Arabinose also has a limited incorporation into walls of sycamore cells (S2), radioactivity being found mainly in the arabinose residues (Rubery & Northcote, 1970). We have found that this is also true for the soluble polysaccharides (S4) (Table 1).

[$U^{14}C$]Glucose can be used as a general label for the various components of the soluble polymers, whereas [1^3H]fucose and [$1^{14}C$]arabinose can be used to label individual sugars in the polysaccharides. Separation of the soluble polysaccharides labelled with [$U^{14}C$]glucose by electrophoresis in borate buffer yielded three components. The position of these components varied slightly with the individual electrophoretic run on the glass-fibre paper (Fig. 1). Electrophoresis at pH 6.5 gave two components, one weakly acidic and one neutral (Fig. 2). Only one of the peaks in both separations was labelled by [1^3H]fucose, whereas [$1^{14}C$]arabinose labelled one major and one minor peak on electrophoresis in borate, but three at pH 6.5. The components were probably a neutral fucoxyloglucan, possibly a neutral arabinogalactan, a weakly acidic arabinogalactan and an acidic polygalacturonan containing some arabinose.

Table 1. *Radioactive composition of soluble polymers secreted by cells prelabelled with [$U^{14}C$]glucose or [$1^{14}C$]arabinose and incubated in the presence or absence of $CaCl_2$*

Cells were incubated with [$1^{14}C$]arabinose (1.5 μ Ci/ml) for 18 h under sterile conditions or with [$U^{14}C$]glucose (3 μ Ci/ml) for 2.5 h. Samples (1.35 ml) of cells prelabelled with [$U^{14}C$]glucose were transferred to 10 ml flasks containing water or 1 M- $CaCl_2$ (0.15 ml) and incubated for 1 h. Soluble polymers were isolated from samples (50 μ l) of the media (Method 1 as described in the text), hydrolysed with 3% (w/v) H_2SO_4 , neutralized and the monomers separated by paper electrophoresis and chromatography as described in the Materials and Methods section. The radioactivity recovered in the amino acids was the sum of the radioactivity recovered in the separated amino acids on the electrophoretogram.

	Radioactivity (%)		
	After incorporation of [$U^{14}C$]glucose		After incorporation of [$1^{14}C$]arabinose
	Water	100 mM- $CaCl_2$	Water
Amino acids	23	18	0
Galacturonic acid	10	11	4
Glucuronic acid	0	0	0
Galactose	12	14	0
Glucose	25	22	0
Mannose	12	18	0
Arabinose	5	7	56
Xylose	4	4	38
Fucose	2	1	0
Rhamnose	7	5	0

Fig. 1. Separation of components of radioactively labelled soluble polysaccharides by electrophoresis in borate buffer (pH 9.4) (a) —, incorporation of $[1-^{14}\text{C}]$ arabinose; ----, incorporation of $[1-^3\text{H}]$ fucose; (b) —, incorporation of $[\text{U}-^{14}\text{C}]$ -glucose; ----, incorporation of $[1-^3\text{H}]$ fucose. Polysaccharides were prepared by incubation of cells with $[1-^{14}\text{C}]$ -arabinose ($1.5\ \mu\text{Ci/ml}$) and $[1-^3\text{H}]$ fucose ($15\ \mu\text{Ci/ml}$) (a) or in $[\text{U}-^{14}\text{C}]$ glucose ($1.5\ \mu\text{Ci/ml}$) and $[1-^3\text{H}]$ fucose ($15\ \mu\text{Ci/ml}$) (b) under sterile conditions for 18 h. The cell suspension was centrifuged and a $10\ \mu\text{l}$ sample of the supernatant applied to glass-fibre paper for electrophoresis (sodium borate buffer, pH 9.4, 30 min, 2 kV).

The reason for the low radioactivity of the acidic polygalacturonan may be that the precursors $[\text{U}-^{14}\text{C}]$ glucose or $[1-^{14}\text{C}]$ arabinose were rapidly incorporated into neutral polysaccharides before labelling of the polygalacturonic acid chains.

Presence of hydroxyproline in the secreted polymers

Amino acid analysis of the non-radioactive polymers found in the growth medium of cells as they were routinely cultured showed that they contained a significant amount of hydroxyproline (over 5 mol/

Fig. 2. Separation of components of radioactively labelled soluble polysaccharides by electrophoresis at pH 6.5 (a) —, Incorporation of $[1-^{14}\text{C}]$ arabinose; ----, incorporation of $[1-^3\text{H}]$ fucose; (b) —, incorporation of $[U-^{14}\text{C}]$ -glucose; ----, incorporation of $[1-^3\text{H}]$ fucose. Polysaccharides were prepared as described in the legend for Fig. 1. A $10\mu\text{l}$ sample was applied to glass-fibre paper for electrophoresis (pH 6.5, 30 min, 2 kV).

100 mol of amino acids). This was present in the media from which low-molecular-weight components had been removed by dialysis. The polymers contained large amounts of peptides derived from N-Z

amine included in the growth medium, so more quantitative data on amino acid composition were not obtained. The N-Z amine did not contain hydroxyproline.

Fig. 3. Effect of different concentrations of (a) CaCl₂, (b) KCl and (c) LaCl₃ on soluble-polysaccharide secretion. Cells were incubated with (a) [1-³H]fucose (3 μCi/ml) for 2.5 h or (b and c) [1-¹⁴C]arabinose (0.1 μCi/ml) for 1 h. Samples (1.35 ml) were then transferred to 10 ml flasks containing water or salts (0.15 ml) and secretion was measured after 1 h by using Method 1 (a) or Method 2 (b and c) as described in the text. The secretion induced by the presence of the salts is expressed as a percentage of that induced by 100 mM-CaCl₂.

Effects of CaCl₂ on polysaccharide secretion

Increasing the concentration of CaCl₂ by 5 mM in the incubation medium of cells prelabelled with [1-³H]fucose stimulated the secretion of radioactive soluble polysaccharide. An increase of 100 mM was found to be optimal (Fig. 3a). The CaCl₂ concentration in PRL4 growth medium was 1 mM (Table 2). The radioactivity was released as a pulse and the rate of

Table 2. Composition of PRL4 growth medium. The osmotic strength of the medium was 105 mosm.

Non-electrolytes (mm)	Sucrose 58.43	N-Z amine 5.00 (approx.)	meso-Inositol 0.55	Pyridoxine hydrochloride 0.005	Nicotinic acid 0.005	Thiamin 0.05	α-Naphthyl-1-acetic acid 0.01	Total
Cations	Na ⁺	K ⁺	NH ₄ ⁺	Ca ²⁺	Zn ²⁺	Cu ²⁺	Co ²⁺	Fe ²⁺ as FeEDTA
	1.08	13.91	4.12	1.00	0.01	0.001	0.001	64.0
Anions	Cl ⁻	I ⁻	NO ₃ ⁻	MoO ₄ ²⁻	BO ₃ ³⁻			Fe ²⁺ as FeEDTA
	6.02	0.004	9.89	0.001	0.05			20.5
(mequiv./litre)			3.14	0.86				EDTA ²⁻ as FeEDTA
(mequiv./litre)								0.07

Fig. 4. Kinetics of polysaccharide secretion induced by CaCl_2 from cells prelabelled with $[1\text{-}^3\text{H}]\text{fucose}$

Cells were incubated with $[1\text{-}^3\text{H}]\text{fucose}$ ($3\text{ }\mu\text{Ci/ml}$) for 2.5 h, samples (1.35 ml) transferred to 10 ml flasks containing water (■) or 1 M- CaCl_2 (●, 0.15 ml; final concn. 100 mM) and secretion was measured by using Method 1 as described in the text.

Fig. 5. Polysaccharide secretion induced by CaCl_2 from cells prelabelled with $[U\text{-}^{14}\text{C}]\text{glucose}$

Cells were incubated with $[U\text{-}^{14}\text{C}]\text{glucose}$ ($3\text{ }\mu\text{Ci/ml}$) for 2.5 h, samples (1.35 ml) transferred to 10 ml flasks containing water (■) or 1 M- CaCl_2 (●, 0.15 ml; final concn. 100 mM) and secretion was measured by using Method 1 as described in the text.

secretion of radioactive polysaccharide decreased after the first hour (Fig. 4). The increase produced by Ca^{2+} was not confined to the secretion of fucoxyloglucan, as it was also found with cells prelabelled with $[U\text{-}^{14}\text{C}]\text{glucose}$ (Fig. 5) and the radioactive composition of the secreted polymers was unchanged (Table 1). This showed that the secretion of all components of the soluble polymers, including protein, was increased to an equal extent.

A similar effect was also found with cells prelabelled with $[1\text{-}^{14}\text{C}]\text{arabinose}$, and since this sugar was taken up and metabolized much more rapidly than $[1\text{-}^3\text{H}]\text{fucose}$ it was used for all subsequent experiments. As a routine CaCl_2 was added at 100 mM in these experiments, although a significant effect was observed at much lower concentrations (Fig. 3a). It was possible that this high concentration of Ca^{2+} might in some way have been toxic. However, the addition of CaCl_2 had no effect on the O_2 uptake of the cells, and they were able to grow in PRL4 medium with added 100 mM- CaCl_2 at a similar rate to those in normal PRL4 medium. These cells have now been grown through several subcultures in medium containing high amounts of Ca^{2+} . Apart from being less highly aggregated, the cultures had a normal appearance in the optical microscope and were able to take up $[1\text{-}^{14}\text{C}]\text{arabinose}$ and secrete radioactive soluble polysaccharide.

The pulse of radioactive polysaccharide may have been released from the cell wall rather than from within the plasma membrane. However, plasmolysing the cells with 1 M-sorbitol inhibited the secretion induced by 100 mM- CaCl_2 by 50%. Similarly addition of sorbitol at a concentration at which plasmolysis was incipient (300 mM) inhibited secretion in the absence of CaCl_2 by 35%. If release was from the

cell wall, plasmolysis would be unlikely to have affected polysaccharide secretion. The inhibitory effect of plasmolysis also demonstrated that the effect of CaCl_2 on secretion cannot be a consequence of osmotic effects on the cell; furthermore it explained the decreased stimulation of secretion at CaCl_2 concentrations higher than 100 mM (Fig. 3a). Plasmolysis was incipient in 5–10% of the cells at 100 mM- CaCl_2 (in growth medium), and at 150 mM and above more than 90% of the cells were clearly plasmolysed.

Addition of metabolic inhibitors or anaerobic conditions might affect the secretion induced by CaCl_2 if release was from within the plasma membrane, but not if release was from the cell wall. The secretion induced by CaCl_2 was decreased in the presence of arsenate and also by the absence of O_2 . If release were from the cell wall, it would not be induced by addition of CaCl_2 to prelabelled protoplasts lacking a cell wall. We found that secretion could be stimulated 2–3-fold from protoplasts by adding 50 mM- CaCl_2 (see Fig. 11).

The cells were probably in a state of metabolic shock owing to pipetting them immediately before adding CaCl_2 or during the subsequent centrifugation. Metabolic shock in sycamore cells (S2), which may be induced simply by shaking the culture, has been associated with changes in membrane permeability (Doree *et al.*, 1972; Thoirion *et al.*, 1974). We therefore allowed the cells to recover by pipetting them 18 h before adding radioactive arabinose. Samples of the medium, free of cells, were taken direct from the incubation vessels in a pipette covered with a double layer of muslin, thereby avoiding the need for centrifugation. A stimulation of similar magnitude to that obtained in routine experiments was observed.

Fig. 6. *Early kinetics of polysaccharide secretion induced by CaCl_2 from cells prelabelled with $[1-^{14}\text{C}]$ arabinose* Cells were incubated with $[1-^{14}\text{C}]$ arabinose ($0.1 \mu\text{Ci/ml}$) for 1 h, samples (1.35 ml) transferred to 10 ml flasks, water (■) or 1 M-CaCl_2 (●, 0.15 ml ; final concn. 100 mM) was added with shaking and the suspension filtered rapidly through 2.5 cm glass-fibre discs at the appropriate times. Radioactive polysaccharides in the filtrate were assayed by using Method 1 as described in the text.

Fig. 7. *Effect of CaCl_2 on the steady-state rate of polysaccharide secretion*

$[1-^{14}\text{C}]$ Arabinose was added to a cell suspension ($0.1 \mu\text{Ci/ml}$) and samples (1.35 ml) were immediately transferred to 10 ml flasks containing water (■) or CaCl_2 (●, 0.15 ml ; final concn. 100 mM). Secretion was measured using Method 2 as described in the text.

Kinetics and magnitude of the effects of CaCl_2 on secretion

Radioactive polysaccharide was released rapidly from prelabelled cells as a pulse on addition of CaCl_2 . The early timing of this pulse was investigated by using rapid filtration to separate the cells from their medium (Fig. 6). The secretion was substantial within 5 s from the addition of 100 mM-CaCl_2 and nearly complete after 10 s. The secretion induced by CaCl_2 was considerable, varying from 30 to 45% of the total radioactivity that was present in the cells before the CaCl_2 was added (radioactivity in the medium initially minus that present at the time the CaCl_2 was added). This suggested that the decreased rate of secretion of radioactive polysaccharide after the initial pulse was a consequence of decreased incorporation of label into polysaccharide from the diminished pool of radioactive precursors in the cell rather than an actual decrease in the rate of secretion of total soluble polysaccharides.

The effect of CaCl_2 on the rate of secretion of radioactive soluble polysaccharides when added at the same time as $[1-^{14}\text{C}]$ arabinose was investigated. As there was no radioactive polysaccharide synthesized when Ca^{2+} was added, a pulse of secretion was not found, but instead a large stimulation of the steady-state rate of secretion was observed (Fig. 7). As uptake of $[1-^{14}\text{C}]$ arabinose was 80% complete within 15 min and complete at 30 min in both the treated and control samples, the effect on secretion could not have been a consequence of different rates of radioisotope uptake. This conclusion was

substantiated by measuring the rate of secretion of labelled polysaccharide by a culture grown with added 100 mM-CaCl_2 . These cells had a lower rate of $[1-^{14}\text{C}]$ arabinose uptake than normal cells, and uptake was continuous during the experiment. The cells had a much higher rate of secretion than normal cells, 50% of the total radioactivity of the cells being secreted after 1 h compared with not more than 7% in experiments with normal cells. This high rate of secretion remained constant as long as radioactive arabinose was present (Fig. 8). This confirmed that the pulse found with normal prelabelled cells was merely a consequence of exhaustion of the radioactive-precursor pools in the cells. The experiments on the early kinetics of the secretion pulse, however, indicated that the increased steady-state rate of secretion was established within 10 s of the addition of CaCl_2 .

The experiments were normally carried out on rapidly dividing cells. However, similar results were obtained by using a culture that had reached the stationary phase of growth 15 days after subculture. As these cells had a high rate of $[^{14}\text{C}]$ arabinose uptake a pulse of secretion was obtained on addition of 100 mM-CaCl_2 that amounted to 22% of the total radioactivity of the cells, compared with 0.7% in the control samples.

Effects of other electrolytes on polysaccharide secretion

The effects of various inorganic salts on secretion were investigated and the results are summarized in

Table 3. Their order of effectiveness in inducing secretion varied over at least one order of magnitude, and as in most cases the anion (chloride) was the same, it was the cations that were active. This was further shown by comparing the optimal concentrations of CaCl_2 , KCl and LaCl_3 (Figs. 3a, 3b and 3c) for inducing secretion.

An increase in the concentration of the total salts in the PRL4 medium was effective in inducing secretion (Table 3). Successive addition of different cations to prelabelled cells induced successive secretion pulses; successive addition of Ca^{2+} alone had a similar effect (Fig. 9). This indicated that the different cations were affecting a common active site. This was confirmed by an experiment with prelabelled cells grown in PRL4 medium with added 100mM- CaCl_2 . Addition of KCl or CaCl_2 to these cells inhibited rather than induced secretion (Fig. 8). LaCl_3 , the most effective salt when used on normal cells (Table 3), had only a slight stimulatory effect. Ca^{2+} therefore blocked the stimulatory effect of other cations so that Ca^{2+} competed with other cations for the same site of action. We suggest that this site was an anionic group and that it thereby interacted electrically and possibly chemically with cations. Further evidence in favour of this hypothesis was that the polyvalent organic cation poly-L-lysine was able to induce secretion with greater effectiveness than many inorganic cations and that basic amino acids at a pH (5.5) at which they carried a net positive charge could also induce secretion (Table 4).

If the active site is anionic it is most likely to arise by the ionization of an acidic group. H^+ ions would be effective at suppressing the ionization of the group and thereby at inducing secretion. This was found (Fig. 10). H^+ at 2.8mM (pH2.5) was more effective than Ca^{2+} at 100mM and almost as effective as La^{3+} at 10mM (Table 3). Low pH values might have had deleterious effects on the cells, and polysaccharide could have been released by non-specific means such as increased permeability of the plasma membrane or cell breakage. However, the cells had a normal appearance in the optical microscope and cell break-

age was not observed. Cells could be grown slowly at pH2.7, and if then subcultured into normal medium gradually reverted to a normal growth rate even though the pH of the growth medium did not return to a normal value (pH 5.5) for three or four subcultures.

The reason for the induction of secretion at high pH (Fig. 10) was not clear. Fischer & Albersheim (1974) provided evidence that alkali stimulated an H^+ ion pump at the plasma membrane of sycamore cells (S2). Activation of this pump might have affected ion transport across the plasma membrane and thereby induced secretion. Fischer & Albersheim (1974)

Fig. 8. Effect of inorganic salts on polysaccharide secretion by cells grown in 100mM- CaCl_2 medium and prelabelled with $[1-^{14}\text{C}]$ arabinose

Cells were incubated with $[1-^{14}\text{C}]$ arabinose ($0.1 \mu\text{Ci/ml}$) for 1 h, samples (1.35 ml) were transferred to 10 ml flasks containing salts or water (0.15 ml) and secretion was measured by Method 2 as described in the text. Δ , 10mM- LaCl_3 ; $\blacksquare$, water; $\circ$, 20mM- CaCl_2 ; $\square$, 100mM- KCl ; $\bullet$, 100mM- CaCl_2 .

Table 3. Induction of polysaccharide secretion from cells prelabelled with $[1-^{14}\text{C}]$ arabinose by various inorganic salts. Cells were incubated with $[1-^{14}\text{C}]$ arabinose ($0.1 \mu\text{Ci/ml}$) for 1 h, samples (1.35 ml) transferred to 10 ml flasks containing water or salts (0.15 ml) and secretion was measured after 1 h by Method 1 (cations at 100 mequiv./litre) or Method 2 (cations at 10 mequiv./litre) as described in the text.

Induced secretion (%) (cations 10mequiv./litre)	Salt ...	LaCl ₃	Cr(NO ₃) ₃	AlCl ₃	FeCl ₃	CaCl ₂		
		199 ± 2.6	191 ± 11.8	184 ± 4.2	152 ± 2.4	100 ± 17.4		
Induced secretion (%) (cations 100 mequiv./litre)	Salt ...	CaCl ₂	PRL4 salts	MnCl ₂	NH ₄ Cl	NaCl	KNO ₃	MgCl ₂
		100 ± 1.7	98.9 ± 3.7	79.0 ± 9.9	76.7 ± 1.9	71.3 ± 6.2	68.2 ± 3.7	53.5 ± 11.3

Fig. 9. Induction of polysaccharide secretion from cells prelabelled with [1-¹⁴C]arabinose by successive additions of CaCl₂, KCl and LaCl₃

Cells were incubated with [1-¹⁴C]arabinose (0.1 μCi/ml) for 1.25 h, samples (1.35 ml) transferred to 10 ml flasks and CaCl₂, KCl, LaCl₃ or water (0.05 ml) added at various times to the concentrations indicated. In (a) 15 mM-CaCl₂ was added at 5 min and polysaccharide secreted (■) was measured after 10 min. Additional 25 mM-CaCl₂ was added at 25 min and polysaccharide secreted (▤) was measured after 30 min. Additional 50 mM-CaCl₂ was added at 45 min and polysaccharide secreted (□) was measured after 50 min. In (b) 15 mM-CaCl₂ was added at 5 min and polysaccharide secreted (■) was measured after 10 min. Additional 50 mM-KCl was added at 25 min and polysaccharide secreted (▤) was measured after 30 min. Additional 2 mM-LaCl₃ was added at 45 min and polysaccharide secreted (□) was measured after 50 min. In (c) water was added at 5 min and polysaccharide secreted (□) measured after 50 min. Secretion was measured by Method 2 as described in the text.

conducted their experiments on cells in aqueous sucrose media and therefore certainly in a state of metabolic shock. However, we have confirmed their results using sycamore cells (S4) suspended in their own growth medium. If small quantities (<1 mM) of KOH were added, the cells maintained the growth

Fig. 10. Effect of pH on polysaccharide secretion by cells pre-labelled with [1-¹⁴C]arabinose

Cells were incubated with [1-¹⁴C]arabinose (0.1 μCi/ml) for 1.25 h and samples (1.35 ml) transferred to 10 ml flasks containing the solutions (0.15 ml). The solutions for pH 4.40, 3.50 and 2.55 contained 6.7 mM-, 27 mM- and 100 mM-HCl respectively; those for pH 7.80 and 10.75 contained 13 mM- and 100 mM-KOH. Secretion was measured after 1 h by Method 1 as described in the text (●). Secretions at pH 5.55 in the presence of 10 mM-KCl (□) and 100 mM-CaCl₂ (■) are shown.

medium at its normal pH of 5.5, after a transient (1–2 s) increase in pH. As the opposite effect was not found on acidification, the presence of an alkali-stimulated acid pump at the plasma membrane was indicated.

In view of the effects of changes in pH on secretion the effect of electrolytes used in earlier experiments on the pH of the cell suspension was investigated. No pH changes were found, except for a slight increase on addition of poly-L-lysine (Table 4). This increase was insufficient to account for the resulting secretion.

Location of the active anionic site

It seems unlikely that a heavy-metal ion such as La³⁺ could have penetrated the plasma membrane as rapidly as, for instance, K⁺, yet La³⁺ was by far the more effective ion at inducing secretion. Together with the rapidity of the response of the cells to Ca²⁺ (Fig. 6), this suggested that the active site was outside the plasma membrane.

Table 4. Induction of secretion by poly-L-lysine and basic amino acids from cells prelabelled with [1-¹⁴C]arabinose
Cells were incubated with [1-¹⁴C]arabinose for 1 h, samples (1.35 ml) transferred to 10 ml flasks containing water or reagent (0.15 ml), the pH was immediately measured and secretion measured after 1 h by Method 2 as described in the text.

Reagent	Induced secretion (%)	pH of medium
10 mM-CaCl ₂	100 ± 17.4	5.50
Poly-L-lysine hydrobromide (2.5 mg/ml)	100 ± 2.1	5.70
10 mM-L-Arginine hydrochloride	10.1 ± 2.1	5.50
10 mM-L-Lysine hydrochloride	9.2 ± 5.3	5.50
10 mM-L-Glutamine hydrochloride	3.8 ± 2.4	5.50

The polyvalent cation poly-L-lysine has a mol.wt. of about 100000, so it is unlikely to have penetrated the plasma membrane. As it is positively charged at the pH of the medium (5.7), this macromolecule should have induced secretion only if the active anionic site was external to the plasma membrane. A substantial induction was obtained (Table 4).

The external active site could either have been on the exterior surface of the plasma membrane or located at the cell wall. Only if the site were on the plasma membrane would secretion have been induced by Ca^{2+} from cells lacking a wall. Protoplasts secreted radioactive polysaccharide, and the steady-state rate of secretion was stimulated 2–3-fold by addition of 50mM- CaCl_2 (Fig. 11). However, a quantitative comparison cannot be made between this experiment and those on intact cells, and an additional active site at the cell wall may be present.

Effects of metabolic inhibitors, temperature and anaerobic conditions

Incubation of the cells with sodium arsenate (5mM) for 30min caused a 50% decrease in the amount of secretion induced by 100mM- CaCl_2 . The O_2 uptake of the cells was stimulated 1.5-fold, so its effect was at least partly that of an uncoupler of oxidative phosphorylation.

Maintenance of the cells under anaerobic conditions for 20min similarly caused a 50% decrease in Ca^{2+} -induced secretion. However, iodoacetic acid had no inhibitory effect on induced secretion at a concentration (5mM) at which it inhibited O_2 uptake by 80%. These experiments indicated that induced secretion was at least partially dependent on metabolic energy. This energy was derived from oxidative and probably also glycolytic metabolism.

Fig. 11. *Effect of CaCl_2 on polysaccharide secretion by protoplasts prelabelled with $[1-^{14}\text{C}]$ arabinose*

Protoplasts were prepared as described in the text, incubated for 4.5 h with $[1-^{14}\text{C}]$ arabinose (1.3 $\mu\text{Ci}/\text{ml}$) and samples (0.4ml) then transferred to glass vials (4cm $\times$ 1cm) containing 0.25M- CaCl_2 (0.1ml; ●, final concn. 50mM) or 0.75M-sorbitol (0.1ml; ■, final concn. 150M). Secretion was measured by Method 1 as described in the text.

Fig. 12. *Influence of temperature on polysaccharide secretion induced by CaCl_2 from cells prelabelled with $[1-^{14}\text{C}]$ arabinose*

Cells were incubated with $[1-^{14}\text{C}]$ arabinose (0.1 $\mu\text{Ci}/\text{ml}$) for 1.25h, samples (1.35ml) transferred to 10ml flasks at the appropriate temperature and water or CaCl_2 (0.15ml, 0.2M) was added after 10min. Induced secretion (difference in secretion between CaCl_2 and water) was measured after a further 10min by Method 2 as described in the text.

The following reagents had no measurable effect on the O_2 uptake of the cells within 15–20min of application: 5mM- NaF ; 5mM- Na_3N ; 10mM-KCN. As expected, none of these was able to inhibit induced and non-induced secretion. 2,4-Dinitrophenol (2.5mM) had a slight stimulatory effect on O_2 uptake, indicating uncoupling of respiration, and inhibited induced secretion by 15%. 2,4-Dinitrophenol did not prevent cytoplasmic streaming. None of the electrolytes had a measurable effect on O_2 uptake (Table 3).

Secretion induced by 100mM- CaCl_2 was almost insensitive to temperature (Fig. 12); substantial secretion was obtained even at 0°C

Kinetics of the intracellular precursor pools

The magnitude of the secretion pulse induced by Ca^{2+} from cells prelabelled with $[1-^{14}\text{C}]$ arabinose and subsequently incubated with non-radioactive arabinose for various times was investigated. Radioactive polysaccharide appeared in the compartment from which secretion was induced over the first 30min of the incubation. The amount of radioactive polysaccharide that could be released from this pool then gradually declined over the next 4h in parallel with the accumulation of secreted soluble radioactive polysaccharide in the medium (Fig. 13). This showed that the compartment from which secretion was induced by Ca^{2+} was part of the normal mechanism of polysaccharide synthesis and secretion such as that used for the assembly of the wall.

The substantial increase in radioactivity in the compartment over the first 30min of the incubation

Fig. 13. Polysaccharide secretion from cells prelabeled with $[1-^{14}\text{C}]$ arabinose and incubated in non-radioactive arabinose for various times

Cells were incubated with $[1-^{14}\text{C}]$ arabinose ($0.1 \mu\text{Ci/ml}$) for 15 min, non-radioactive arabinose (10 mM) was added and samples (1.35 ml) were transferred to 10 ml flasks containing water (■) or CaCl_2 (0.15 M ; ●, final concn. 100 mM) at various times. Secretion was measured after 5 min by Method 2 as described in the text.

Fig. 14. Kinetics of intracellular polysaccharide pools in cells prelabeled with $[1-^{14}\text{C}]$ arabinose and incubated in non-radioactive arabinose for various times

Cells were incubated with $[1-^{14}\text{C}]$ arabinose ($0.1 \mu\text{Ci/ml}$) for 15 min, non-radioactive arabinose (10 mM) was added and samples (1.35 ml) were transferred to 10 ml flasks containing water or 1 M-CaCl_2 (0.15 M ; final concn. 100 mM) at various times. After 5 min, secretion was measured in both control and CaCl_2 -treated samples (●) by Method 2 as described in the text. Cellular radioactive polysaccharide (▲) was measured in control samples by using ethanol extraction as described in the text.

radioactive polysaccharide were measured (Fig. 14). There was a similar increase in radioactivity of both the cellular polysaccharide and that released by the addition of Ca^{2+} during the first 20 min of the chase. After this time there was a lag between the decline of total cellular radioactive polysaccharide and that in the pool from which secretion was induced. This suggested that intracellular polysaccharide (part of the cellular radioactive polysaccharide) was being synthesized before being transferred to the compartment that was involved in the induced secretion.

Discussion

The results show that suspension-cultured sycamore cells (S4) used in this work secrete soluble protein and three soluble polysaccharides of similar structure and composition to the polysaccharides previously reported to be secreted by sycamore cultures (S2). The soluble protein contains a significant amount of hydroxyproline; the presence of this soluble protein in sycamore cultures has not been previously reported. A hydroxyproline-rich soluble protein is similarly associated with the soluble polysaccharides secreted by tobacco suspension cultures (Olson *et al.*, 1969).

The steady-state rate of secretion of all three polysaccharides and protein was substantially and very rapidly increased by the addition of various electrolytes and polyelectrolytes to the cells. The increased secretion was induced from within the cell by the presence of the cations, primarily at the outer surface of the plasma membrane, but possibly also at the cell wall. The final stages of secretion induced by CaCl_2 were at least partially dependent on metabolic energy derived from oxidative and probably also glycolytic metabolism. Induced secretion was relatively insensitive to temperature changes.

The increase in the rate of secretion was brought about by a stimulation of the normal mechanisms of polysaccharide secretion. Cell-wall breakdown was only a minor source of the soluble polysaccharides, and the secretion mechanism must be the same as for the non-cellulosic polysaccharides of the cell wall. This mechanism involves flow of polysaccharide through the endoplasmic reticulum, dictyosomes and finally Golgi-derived vesicles before secretion takes place by fusion of the vesicles with the plasma membrane (Northcote, 1977; Chrispeels, 1976). Both endoplasmic reticulum and dictyosomes are probably sites of polysaccharide synthesis (Bowles & Northcote, 1972), yet the pool from which secretion was induced contained completely synthesized polysaccharides. This evidence, together with the rapidity of the ion effects, indicated that secretion was being induced from Golgi-derived vesicles rather than from the endoplasmic reticulum or the Golgi cisternae where active synthesis occurred. This lends weight to

indicated that presynthesized radioactive polysaccharide was being transferred to it. This was investigated by a similar pulse-chase experiment in which the early kinetics of the production of cellular

the idea that the endoplasmic reticulum does not secrete polysaccharide directly to the cell exterior (O'Brien, 1972; Chrispeels, 1976; Northcote, 1977), although it is probably involved in polysaccharide synthesis.

In these experiments the active components inducing secretion were exclusively cations. The outer surface of the plasma membrane of eukaryotic cells is known to have a considerable negative electrostatic charge by virtue of fixed carboxylate groups attached to proteins and polysaccharide chains and of the phosphate groups of phospholipids (Curtis, 1967; Nelson & McQuarrie, 1975). The outer surface of the plasma membrane of sycamore (S2) suspension cells has been shown to have a measured electric potential of between -14 and -18 mV (Morvan, 1972; Heller *et al.*, 1974). Ca^{2+} ions have a high affinity for binding to surface carboxylate groups, losing some water molecules of hydration in the process, and may also bind to specific surface phosphate groups (Williams, 1976). These bound Ca^{2+} ions will cause a decrease in the electrostatic charge density of the cell surface according to the following equation, as has been shown experimentally for binding of Ca^{2+} ions to the surface of blood cells (Seaman *et al.*, 1969):

$$\frac{1}{\Delta\sigma} = \frac{1}{2eN_a} + \left(\frac{1}{C} \cdot \frac{1}{2eN_aK_b} \right) \quad (1)$$

where σ is surface-charge density, e is electronic charge, N_a is the number of available counterion-binding sites, C is the molar concentration of Ca^{2+} and K_b is the equilibrium constant for counterion binding. This decrease in surface-charge density will in turn cause a decrease in the electrostatic surface potential of the cell. The effect is given by a linear approximation equation which holds for surface potentials of less than 25 mV (Gingell, 1971; Dean, 1975):

$$\psi_0 = \frac{4\pi\sigma}{D\kappa} \quad (2)$$

where ψ_0 is surface potential, D is dielectric constant of the growth medium and $\frac{1}{\kappa}$ is the Debye screening length.

Sycamore (S2) cell walls and plasma membrane have a high affinity for Ca^{2+} ions (Heller *et al.*, 1973), so Ca^{2+} ions probably cause a decrease in surface potential by directly binding to the cell surface. Other inorganic cations, however, are not bound tightly by the surface of cultured sycamore cells (Heller *et al.*, 1973), and the generality of the cation effect on secretion, particularly with the univalent alkali-metal ions and the organic poly-cations, suggests that a less-specific electrical interaction is also involved.

There are more mobile cations near a negatively charged surface than at distances further away, giving

rise to an electrical double layer a few angstroms from the anionic sites; the presence of hydrating water molecules prevents more specific binding (Nürnberg, 1974). The average width of the double layer is expressed in terms of the Debye screening length, $\frac{1}{\kappa}$;

an increase in the ionic concentration of the medium will increase the cationic concentration of the double layer and decrease the screening length according to the Poisson-Boltzmann equation of Debye-Hückel theory (Delahay, 1965; Dean, 1975).

$$\kappa^2 = \frac{4\pi e^2 N}{D\kappa T 1000} \sum_i C_i Z_i^2 \quad (3)$$

(N , Avogadro number; k , Boltzmann's constant; T , absolute temperature; C_i , molar concentration of the cation of the i^{th} species; Z_i , valency of the cation of the i^{th} species).

The decrease in Debye screening length will in turn cause a decrease in the electrostatic surface potential of the cell according to eqn. (2). It has been shown experimentally that increasing concentrations of KCl cause a 10–15 mV depolarization of the surface potential of sycamore (S2) cells (Heller *et al.*, 1974). Eqn. (3) predicts that tervalent ions will be particularly effective at decreasing surface potential, and this is found experimentally (Matthews, 1970; Matthews *et al.*, 1972). The high activity of tervalent cations in our experiments strongly suggests that a decrease in surface potential is involved. We postulate that this decrease eventually brings about the fusion of Golgi-derived vesicles with the plasma membrane.

Electrolytes and multivalent cations can induce pinocytosis in amoebae (Schumaker, 1958; Brandt & Pappas, 1960; Chapman-Andresen, 1962) and a contraction of the plasma membrane of *Xenopus* eggs (Gingell & Palmer, 1968), probably by decreasing the surface membrane potential of the cells (Gingell, 1971). The decreased surface membrane potential may cause a transitory increase in the electric potential across the plasma membrane (Gingell, 1971), which results in an increase in ionic permeability (Brandt & Freeman, 1967; Gingell & Palmer, 1968). The resulting Ca^{2+} influx causes the membrane contraction in *Xenopus* eggs (Gingell *et al.*, 1970) and probably pinocytosis in amoebae (Gingell, 1971).

Induced exocytosis in animal cells is preceded by membrane depolarization and is usually dependent on the presence of extracellular Ca^{2+} ions (Smith & Winkler, 1972; Poste & Allison, 1973; Douglas, 1974; Foreman *et al.*, 1976). Fusion of vesicles with the plasma membrane depends on the resulting Ca^{2+} influx from the extracellular medium or sometimes from an intracellular compartment (Matthews, 1970; Douglas, 1974; Foreman *et al.*, 1976). The fusion process may be initially facilitated by membrane-bound Ca^{2+} ions decreasing the electrical

repulsion between negatively charged vesicles and the negatively charged inner surface of the plasma membrane (Dean, 1975). The resulting closer approach of vesicles to the membrane leads to membrane contact by van der Waals forces and finally membrane fusion. The fusion process may involve displacement of Ca^{2+} ions from the inner membrane surface, the resulting change in membrane structure and fluidity making fusion an energetically more favourable process (Woodin & Wienecke, 1964; Poste & Allison, 1973).

Induced vesicle fusion in sycamore cells may occur by a similar mechanism to that involved in exocytosis in animal cells. The decrease in surface potential could bring about an influx of extracellular Ca^{2+} ions by changing membrane structure and permeability, as found with amoebae and *Xenopus* eggs. The change in ion distribution in the region of plasma-membrane ion carriers could also lead to a Ca^{2+} influx (Nelson & McQuarrie, 1975). The resulting increase in cytoplasmic Ca^{2+} concentrations could lead to vesicle discharge in the same way as other exocytotic processes.

Exocytosis in animal tissues requires metabolic energy and a direct role of ATP in mediating fusion of the vesicle membranes with the plasma membrane has been suggested (Woodin & Wienecke, 1964; Jamieson & Palade, 1971; Poste & Allison, 1973). Our experiments indicate that exocytosis in plant tissues has a similar energy requirement; this is in accord with experiments showing that plant tissues require energy for secretion (Schnepf, 1963; Morré *et al.*, 1967; Doerschug & Chrispeels, 1970; Varner & Mense, 1972). The cells in our experiments had a surprising resistance to certain metabolic inhibitors, which may be due to a lack of penetration through the plasma membrane. The cell wall may play a role in limiting penetration by certain reagents.

It has been suggested that membrane fluidity is important for membrane fusion to be energetically favourable (Poste & Allison, 1973; Fisher, 1974). However, the lack of temperature effects on secretion in our experiments makes it unlikely that changes of fluidity within the temperature range 0–35°C are important for membrane fusion in sycamore cells. This also appears to be true for pinocytosis in amoebae (Schumaker, 1958).

It was shown that Ca^{2+} induces a substantial increase in the steady-state rate of polysaccharide secretion by sycamore cells. As the major point of stimulation is at the fusion of vesicles with the plasma membrane, it follows that this step is rate-limiting in polysaccharide secretion and a potential control point. Effects of plant hormones on the synthetic enzymes of plant secretory products have been found, but the possibility that the secretion process itself may be under hormonal control appears to have been neglected (Chrispeels, 1976).

Effects of auxin on the ultrastructure of the Golgi apparatus of plant cells have been found (Gawlick & Shen-Miller, 1974). Auxins rapidly stimulate an outwardly directed plasma-membrane acid pump (Rayle & Cleland, 1970; Hager *et al.*, 1971; Rayle, 1973), and this may be dependent on a Ca^{2+} influx (Cohen & Nadler, 1976). These ion fluxes affect the surface and intracellular electric potentials of the cells (Jenkinson, 1962; Etherton, 1970; Tanada, 1973a,b). An increase in cytoplasmic Ca^{2+} could stimulate polysaccharide secretion and result in the increased cell-wall deposition found after treatment of the tissue with auxin (Albersheim, 1963; Baker & Ray, 1965a,b; Ray & Baker, 1965).

We thank Mr. M. J. Wilkinson of this Department for the information about the method to prepare sycamore protoplasts. M. R. M. thanks the Science Research Council for a grant during the tenure of which this work was carried out.

References

- Albersheim, P. (1963) *J. Biol. Chem.* **238**, 1608–1610
- Aspinall, G. O., Molloy, J. A. & Craig, J. W. T. (1969) *Can. J. Biochem.* **47**, 1063–1070
- Baker, D. B. & Ray, P. M. (1965a) *Plant Physiol.* **40**, 345–352
- Baker, D. B. & Ray, P. M. (1965b) *Plant Physiol.* **40**, 360–368
- Bauer, W. D., Talmadge, K. W., Keegstra, K. & Albersheim, P. (1973) *Plant Physiol.* **51**, 174–187
- Becker, G. E., Hui, P. A. & Albersheim, P. (1964) *Plant Physiol.* **39**, 913–920
- Bowles, D. J. & Northcote, D. H. (1972) *Biochem. J.* **130**, 1133–1145
- Brandt, P. W. & Freeman, A. R. (1967) *Science* **155**, 582–585
- Brandt, P. W. & Pappas, G. D. (1960) *J. Biophys. Biochem. Cytol.* **8**, 675–687
- Burke, D., Kaufman, P., McNeil, M. & Albersheim, P. (1974) *Plant Physiol.* **54**, 109–115
- Chapman-Andresen, C. (1962) *C. R. Trav. Lab. Carlsberg* **33**, 73–264
- Chrispeels, M. J. (1976) *Annu. Rev. Plant Physiol.* **27**, 19–38
- Cohen, J. D. & Nadler, K. D. (1976) *Plant Physiol.* **57**, 347–350
- Curtis, A. S. G. (1967) *The Cell Surface*, Logos Press, London
- Dean, P. M. (1975) *J. Theor. Biol.* **54**, 289–308
- Delahay, P. (1965) *Double Layer and Electrode Kinetics*, Interscience, New York
- Doerschug, M. R. & Chrispeels, M. J. (1970) *Plant Physiol.* **46**, 363–366
- Doree, M., Leguay, J. J. & Terrine, C. (1972) *Physiol. Vég.* **10**, 115–131
- Douglas, W. W. (1974) *Biochem. Soc. Symp.* **39**, 1–28
- Etherton, B. (1970) *Plant Physiol.* **45**, 527–528
- Fischer, M. L. & Albersheim, P. (1974) *Plant Physiol.* **53**, 464–468

- Fisher, D. (1974) in *Biomembranes: Lipids, Proteins and Receptors* (Burton, R. M. & Packer, L., eds.), pp. 75–93, Burton International Science Publications, Webster Groves, MO
- Foreman, J. C., Garland, L. G. & Mongar, J. L. (1976) *Symp. Soc. Exp. Biol.* **30**, 193–218
- Gamborg, O. L. (1966) *Can J. Biochem.* **44**, 791–799
- Gawlick, S. R. & Shen-Miller, J. (1974) *Plant Physiol.* **54**, 217–221
- Gingell, D. (1971) in *Membranes and Ion Transport* (Bittar, E. E., Ed.), pp. 317–357, Wiley-Interscience, London
- Gingell, D. & Palmer, J. F. (1968) *Nature (London)* **217**, 98–102
- Gingell, D., Garrod, D. R. & Palmer, J. F. (1970) in *Calcium and Cellular Function* (Cuthbert, A. W., ed.), pp. 59–64, Macmillan, London
- Hager, A., Menzel, H. & Krauss, A. (1971) *Planta* **100**, 47–75
- Harris, P. J. & Northcote, D. H. (1970) *Biochem. J.* **120**, 479–491
- Hawes, G. B. & Adams, G. A. (1972) *Phytochemistry* **11**, 1461–1465
- Heath, M. F. & Northcote, D. H. (1971) *Biochem. J.* **125**, 953–961
- Heller, R., Grignon, C. & Scheidecker, D. (1973) in *Ion Transport in Plants* (Anderson, W. P., ed.), pp. 337–353, Academic Press, London
- Heller, R., Grignon, C. & Rona, J.-P. (1974) in *Membrane Transport in Plants* (Zimmermann, U. & Dainty, J., eds.), pp. 239–243 Springer-Verlag, Berlin
- Jamieson, J. D. & Palade, G. E. (1971) *J. Cell Biol.* **48**, 503–522
- Jenkinson, I. S. (1962) *Aust. J. Biol. Sci.* **15**, 101–125
- Lamport, D. T. A. & Northcote, D. H. (1960) *Biochem. J.* **76**, 52P
- Liau, D. F. & Boll, W. G. (1972) *Can. J. Bot.* **50**, 2031–2037
- Mante, S. D. & Boll, W. G. (1973) *Plant Physiol.* **51**, (Suppl.), 60
- Matthews, E. K. (1970) in *Calcium and Cellular Function* (Cuthbert, A. W., ed.), pp. 163–182, Macmillan, London
- Matthews, E. K., Evans, R. J. & Dean, P. M. (1972) *Biochem. J.* **130**, 825–832
- Morré, D. J., Jones, D. D. & Mollenhauer, H. H. (1967) *Planta* **74**, 286–301
- Morvan, H. (1972) *C. R. Hebd. Séances Acad. Sci. Ser. D* **274**, 2541–2544
- Nelson, A. P. & McQuarrie, D. A. (1975) *J. Theor. Biol.* **55**, 13–27
- Northcote, D. H. (1974) *Philos. Trans. R. Soc. London Ser. B* **268**, 119–128
- Northcote, D. H. (1977) in *Cell Surface Reviews* (Hillier, J. G., ed.), vol. 4, pp. 1205–1225, Elsevier, Amsterdam
- Nürnberg, H. W. (1974) in *Membrane Transport in Plants* (Zimmermann, U. & Dainty, J., eds.), pp. 48–53, Springer-Verlag, Berlin
- O'Brien, T. P. (1972) *Bot. Rev.* **38**, 87–118
- Olson, A. C. (1971) in *Les Cultures des Tissus de Plantes*, pp. 411–420, Colloques Internationaux du C.N.R.S., no. 193, Paris
- Olson, A. C., Evans, J. J., Frederick, D. P. & Jansen, E. F. (1969) *Plant Physiol.* **44**, 1594–1600
- Poste, G. & Allison, A. C. (1973) *Biochim. Biophys. Acta* **300**, 421–465
- Ray, P. M. & Baker, D. B. (1965) *Plant Physiol.* **40**, 353–360
- Rayle, D. L. (1973) *Planta* **114**, 63–73
- Rayle, D. L. & Cleland, R. (1970) *Plant Physiol.* **46**, 250–253
- Roberts, R. M. (1968) *Arch. Biochem. Biophys.* **128**, 818–820
- Rubery, P. H. & Northcote, D. H. (1970) *Biochim. Biophys. Acta* **222**, 95–108
- Schnepf, E. (1963) *Flora* **153**, 23–48
- Schumaker, V. N. (1958) *Exp. Cell Res.* **15**, 314–331
- Seaman, G. V. F., Vassar, P. S. & Kendal, M. J. (1969) *Experientia* **25**, 1259–1260
- Simpkins, I. & Street, H. E. (1970) *J. Exp. Bot.* **21**, 170–185
- Smith, A. D. & Winkler, H. (1972) *Handb. Exp. Pharmacol.* **33**, 538–617
- Stoddart, R. W. & Northcote, D. H. (1967) *Biochem. J.* **105**, 45–59
- Tanada, T. (1973a) *Plant Physiol.* **51**, 150–153
- Tanada, T. (1973b) *Plant Physiol.* **51**, 154–157
- Thoirion, A., Thoirion, B., Le Guél, J., Guern, J. & Thellier, M. (1974) in *Membrane Transport in Plants* (Zimmermann, U. & Dainty, J., eds.), pp. 234–238, Springer-Verlag, Berlin
- Varner, J. E. & Mense, R. M. (1972) *Plant Physiol.* **49**, 187–189
- Wilder, B. M. & Albersheim, P. (1973) *Plant Physiol.* **51**, 889–893
- Williams, R. J. P. (1976) *Symp. Soc. Exp. Biol.* **30**, 1–17
- Woodin, A. M. & Wienecke, A. A. (1964) *Biochem. J.* **90**, 498–509
- Wright, K. & Northcote, D. H. (1972) *J. Cell Sci.* **11**, 319–337