Reduction in the level of intracellular *myo*-inositol in cultured soybean (*Glycine max*) cells inhibits cell division

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Although *myo*-inositol is included in media for the successful growth of plant tissues, the actual requirement of most tissues, including soybean (*Glycine max*) callus in suspension culture, for *myo*-inositol has not been demonstrated. We have made use of deoxyglucose to reduce intracellular levels of *myo*-inositol. Deoxyglucose is phosphorylated to deoxyglucose 6-phosphate, which inhibits L-*myo*-inositol 1-phosphate synthase, an important enzyme in the synthesis of *myo*-inositol. Addition of deoxyglucose to the medium resulted in a decrease in the intracellular level of *myo*-inositol that corresponded with a decrease in cell division. Cell viability was not affected. When *myo*-inositol was added to cells along with deoxyglucose, cell division was restored, as were intracellular levels of *myo*-inositol. Addition of *myo*-inositol had no effect on the uptake or metabolism of deoxyglucose. From these results we propose that *myo*-inositol has a role in maintaining cell division in soybean callus tissue in suspension culture.

INTRODUCTION

Active uptake of *myo*-inositol has been recorded in a number of plant systems, including germinating pollen (Kroh & Loewus, 1968), wheat (*Triticum aestivum*) seedlings (Maiti & Loewus, 1978) and cultured cells (Verma & Dougall, 1979). Plant cells metabolize *myo*-inositol via a number of pathways, leading to phytate production (Sasaki & Loewus, 1980), conjugation with plant growth substances (Bandurski, 1979), oxidation to glucuronate [which is incorporated into the pectin in the cell wall (Loewus *et al.*, 1973)] and formation of lipids. *myo*-Inositol-containing lipids include phosphatidylinositol, phosphatidylinositol 4-monophosphate and phosphatidylinositol 4,5-bisphosphate as well as other uncharacterized *myo*-inositol-containing phospholipids (Heim & Wagner, 1986; Drøbak *et al.*, 1988; Irvine *et al.*, 1989). The polyphosphoinositides have a role in signal transduction in animal cells (Michell *et al.*, 1981; Nishizuka, 1984; Berridge, 1986), and there is some evidence that they may play a similar role in plants (Ettlinger & Lehle, 1988).

Although plant cells take up and subsequently metabolize *myo*-inositol, when these cells are deprived of *myo*-inositol there is often no deleterious effect. This is because plant cells, like some animal tissues, have the capacity to synthesize *myo*-inositol from D-glucose (Loewus & Kelly, 1962) via the enzyme L-*myo*-inositol 1-phosphate synthase, which utilizes D-glucose 6-phosphate as substrate (Funkhouser & Loewus, 1975; Loewus & Loewus, 1973). The L-*myo*-inositol 1-phosphate formed is then dephosphorylated to yield *myo*-inositol. The level of synthase present in cells may be regulated by the availability of *myo*-inositol in the medium (Loewus & Loewus, 1973). Thus a decrease in extracellular *myo*-inositol could result in an increase in the amount of enzyme, enabling the cell to produce sufficient *myo*-inositol for its metabolic requirements. This makes it difficult to assess the importance of *myo*-inositol in plant

development, as depriving the cells of *myo*-inositol will not necessarily result in a decrease in intracellular *myo*-inositol.

To overcome this we attempted to inhibit the L-*myo*-inositol 1-phosphate synthase, reducing the capacity of the cell to synthesize *myo*-inositol. We made use of the metabolic inhibitor 2-deoxyglucose (dGlc), at concentrations which caused no loss in cell viability over the course of the experiments. In living tissue dGlc is readily phosphorylated, forming dGlc 6-phosphate, which is a competitive inhibitor of, though not a substrate for, the synthase in rat testes (Barnett *et al.*, 1970) and yeast (Charalampous & Chen, 1966).

We report that, in medium lacking *myo*-inositol, addition of 1 mM-dGlc resulted in a decrease in cellular content of *myo*-inositol and a reduction in cell division and growth. The presence of *myo*-inositol in the medium reversed the effect on cell division, suggesting a requirement for *myo*-inositol in maintaining cell division in soybean callus suspension cells.

EXPERIMENTAL

Methods

Cell culture. Soybean [*Glycine max* (L.) Merr. cv. Acme] cells were cultured in the dark in 500 ml Ehrlenmeyer flasks using the medium and conditions previously described (Connett & Hanke, 1986). The plant growth substances naphthyl-1-acetic acid (11 μ M) and kinetin (2.3 μ M) were present in all culture media used. The level of *myo*-inositol, when present, was 0.5 mM in the fresh medium at the time of subculturing. Subsequent incubations were set up by subculturing 4 ml of the 7-day-old stock culture cells into 20 ml of medium in 100 ml Ehrlenmeyer flasks under aseptic conditions. The flasks were stoppered with a cottonwool bung, covered in aluminium foil and incubated at 25 °C on an orbital shaker set at 100 rev./min.

Abbreviations used: dGlc, 2-deoxy-D-glucose; Ins (in media), *myo*-inositol.

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Cell counts, yield and viability. Liquid suspension of cells, 0.5 ml, was added to 1 ml of 15 % (w/v) chromium trioxide and heated at 70 °C for 30 min. Water (1 ml) was added, the cells passed twice through a hypodermic needle (internal diam. 0.8 mm) and counted on a modified Fuchs–Rosenthal haemocytometer with a clearance of 0.2 mm. The density of stock cells at 7 days prior to subculturing was $(1.6\text{--}2.0) \times 10^6$ cells $\cdot$ ml $^{-1}$.

The fresh cell mass was determined by pipetting a known volume of cells on to glass-fibre paper (Whatman GF/A) in a Buchner flask under vacuum. The cell pellet on the filter paper was weighed and the yield presented in units of mg of fresh mass/ml of suspension.

Cell viability was based on exclusion of the dye phenosafranin (Widholm, 1972). By this method, cell viability in all the described experiments was between 90 and 95 %.

Radiolabelling with [$1\text{-}^{14}\text{C}$]dGlc. Cell suspension (4 ml) was subcultured into 20 ml of medium containing 2 μ Ci of [$1\text{-}^{14}\text{C}$]dGlc. Total cellular uptake was measured by harvesting cells (1 ml samples) as for fresh-mass determinations. The cell pellet was washed with ice-cold sucrose solution (20 g $\cdot$ litre $^{-1}$) to remove radiolabel not taken up into the cells. Cells were then scraped into vials, lysed with 80 μ l of 10 % (v/v) Triton X-100 solution before scintillation fluid (Optiphase 'Hisafe' II) was added and the radioactivity quantified in a Packard 2000 Tri-Carb liquid-scintillation analyser.

Incorporation of [$1\text{-}^{14}\text{C}$]dGlc into 80 %-(v/v)-ethanol-soluble fraction. To release the 80 %-ethanol-soluble fraction, 1 ml of cells was harvested on glass-fibre paper under suction, washed in sucrose (20 g $\cdot$ litre $^{-1}$), and added to 1 ml of 80 % ethanol in water and left at room temperature for 10 min. The insoluble material was pelleted by centrifugation in a Beckman Microfuge B for 1 min. The supernatant was removed, dried under vacuum and, following resuspension in water, the radioactivity was determined.

Separation of sugars. Ethanol (80 %) extracts were resuspended in water, spotted on to Whatman no. 3 paper and developed overnight by descending chromatography [mobile phase ethyl acetate/pyridine/water (12:5:4, by vol.)]. The paper was air-dried and the standards visualized with silver stain. Sample tracks (unstained) were cut up, put into scintillation vials and the radioactivity determined.

Extraction and separation of phospholipids. The method was based on that of Schacht (1981). Cells were harvested on filter paper and added to 3 ml of chloroform/methanol (1:2, v/v). Further additions were chloroform (1.0 ml), 2.4 M-HCl (1.0 ml) and water (a total of 0.8 ml); the amount was adjusted to take account of the water in the cells [approx. 95 % (w/w)]. After being mixed vigorously the sample was centrifuged in a MSE Centaur centrifuge for 5 min at 1120 g_{max} to aid phase separation. The lower phase was pipetted off and the upper phase re-extracted with 1.6 ml of chloroform. The combined lower phases were dried under a stream of N_2 . The phospholipids were resuspended in chloroform/methanol (2:1, v/v) and spotted on to silica-gel G t.l.c. plates previously sprayed with potassium oxalate (10 g $\cdot$ litre $^{-1}$) in water/methanol (3:2, v/v) and air-dried. The

plates were developed by ascending chromatography in chloroform/acetone/methanol/acetic acid/water (40:15:13:12:8, by vol.) (Heim & Wagner, 1986) and the phospholipids revealed by Molybdenum Blue spray. The t.l.c. plate was cut up into 5 mm strips, scintillation fluid was added and radioactivity was determined after 24 h.

Inositol determination. The method was based on that of Fry (1988). Cell suspension (12 ml) was harvested on Whatman GF/A paper and washed thoroughly with ice-cold sucrose (20 g $\cdot$ litre $^{-1}$). Cells were transferred to boiling tubes and ethanol added to 80 % (taking into account the intracellular water). At this stage 10000 d.p.m. of [^3H]inositol was added to enable determination of *myo*-inositol recovery. Tubes were placed in a boiling-water bath and boiled vigorously for 15 s before cooling and centrifuging for 5 min at 1120 g_{max} . The supernatant was poured into a boiling tube, made 0.1 M with respect to H_2SO_4 and placed in a boiling-water bath for another 20 min. After cooling, saturated $\text{Ba}(\text{OH})_2$ solution was added until the mixture was alkaline, and the tube returned to the boiling-water bath for a further 30 min. When cool, 25 μ l of 1 % Bromothymol Blue was added and the solution neutralized by the addition of solid CO_2 . After centrifugation to remove the insoluble BaSO_4 and BaCO_3 the supernatant was passed through Dowex-50 (H^+ form), followed by Dowex-1 (OH^- form) and the eluate dried. The sample was dissolved by heating at 80 °C in 0.5 ml of anhydrous pyridine. When cool, 0.1 ml of hexamethyldisilazane and 0.05 ml of chlorotrimethylsilane were added to convert the *myo*-inositol into the trimethylsilyl derivative. Samples (2 μ l) were injected into a Pye–Unicam PU4500 gas chromatograph with dual flame-ionization detectors and fitted with 1.52 m-long, 3 mm-internal-diameter columns packed with 2 % (w/w) OV-17 on diatomite (80–120 mesh). The output was recorded on a Pye–Unicam CDP4 computing integrator, and peak heights were quantified. A linear response was detected for injection of 0–35 nmol of *myo*-inositol standards. Overall recovery of [^3H]inositol was about 60 %.

Invertase treatment. Invertase (300 units) was incubated with the sample in a 50 mM- KH_2PO_4 solution, pH 4.5, for 1 h at 37 °C in a total volume of 0.4 ml. Incubations were terminated by the addition of 0.8 ml of ethanol and the samples were dried under vacuum.

Inositol uptake. Incubations were initiated by addition of 4 ml of 7-day-old cells into 20 ml medium containing 0.12–2 mM-*myo*-inositol as well as 2 μ Ci of *myo*-[$2\text{-}^3\text{H}$]inositol. At 0, 2, 5, 7 and 10 h cells were harvested and total incorporation was determined. Incorporation of label was linear for up to 10 h.

Materials

[$1\text{-}^{14}\text{C}$]dGlc, invertase and all chemicals were obtained from Sigma Chemical Co., Poole, Dorset, U.K. Optiphase HiSafe II was from FSA, Loughborough, Leics., U.K. *myo*-[$2\text{-}^3\text{H}$]inositol was from Amersham International.

RESULTS

myo-Inositol uptake into pollen tubes has been shown

Fig. 1. Kinetics of *myo*-inositol uptake by soybean callus cells in suspension culture

A 4 ml portion of a 7-day suspension culture of soybean cells was added to 20 ml of medium in 100 ml Ehrlenmeyer flasks, producing an initial cell density of 0.25×10^6 cells/ml. Also present was $2 \mu\text{Ci}$ of *myo*-[^3H]inositol at various concentrations of unlabelled *myo*-inositol. Samples, from duplicate incubations, were taken at 0, 2, 5, 7 and 10 h and the uptake of *myo*-[^3H]inositol was plotted against time. For each concentration of *myo*-inositol the mean value of the slope of this line was taken to calculate the amount of *myo*-inositol taken up/h. These values were then used for the double-reciprocal plot.

to be non-saturable (Loewus *et al.*, 1973), so the characteristics of uptake into soybean callus cells in suspension culture were examined (Fig. 1). Accumulation showed Michaelis-Menten kinetics with a K_m of 1.25 mM and a $V_{\max}$ of 154 pmol of *myo*-inositol $\cdot \text{mg}^{-1}$ of fresh mass $\cdot \text{h}^{-1}$. Although these parameters may not necessarily represent those of the *myo*-inositol carrier, they indicate that the cells are competent at actively taking up *myo*-inositol from the medium.

Although capable of utilizing *myo*-inositol from the medium, soybean callus suspension still grows and divides in the absence of *myo*-inositol. Several lines of soybean callus have been grown in our laboratory in *myo*-inositol-free medium for over a year, with no morphological or developmental differences compared with cells maintained in *myo*-inositol-containing medium. This apparent independence of exogenous *myo*-inositol reflects the cells ability to synthesize *myo*-inositol from glucose 6-phosphate under conditions of *myo*-inositol deprivation. This was supported by detection of [^{14}C]inositol in cells which had been incubated with [^{14}C]glucose (results not shown).

In an attempt to lower the intracellular level of *myo*-inositol, soybean cells were incubated in medium containing 1 mM-dGlc, which, when phosphorylated to its 6-phosphate, has been shown to inhibit competitively L-*myo*-inositol 1-phosphate synthase from other tissues (Barnett *et al.*, 1970; Charalampous & Chen, 1966). A concentration of 1 mM-dGlc was selected, as this did not affect cell viability over the 2 weeks of the experiments, whereas addition of dGlc to 5 mM resulted in a decline in

Table 1. Effect of *myo*-inositol and dGlc on cell division and yield of soybean callus

Cells were incubated for 7 days in culture medium in the presence (+) or absence (–) of either 0.5 mM-*myo*-inositol or 1 mM-dGlc. Cell number and fresh mass were then determined. (a) is the result of subculturing the stock culture cells into media shown such that the initial cell density was 0.3×10^6 cells $\cdot \text{ml}^{-1}$. Results are means $\pm$ S.E.M. for four separate incubations. (b) Cells were incubated for 7 days in $\text{Ins}^-/\text{dGlc}^+$ medium before being subcultured into the media shown at an initial cell density of 0.3×10^6 cells $\cdot \text{ml}^{-1}$. Results are means $\pm$ S.E.M. for four separate incubations.

	Inositol and dGlc content of the medium		$10^{-6} \times$ Cell density (cells $\cdot \text{ml}^{-1}$)	Fresh mass (mg $\cdot \text{ml}^{-1}$)
	Ins	dGlc		
(a)	+	–	1.7 ± 0.1	104 ± 6
	–	–	1.9 ± 0.2	99 ± 6
	+	+	1.9 ± 0.2	103 ± 2
	–	+	1.3 ± 0.1	78 ± 2
(b)	+	–	1.3 ± 0.2	62 ± 1
	–	–	1.1 ± 0.1	60 ± 2
	+	+	1.0 ± 0.1	38 ± 2
	–	+	0.5 ± 0.1	21 ± 1

cell viability to 50% within 1 week. Table 1 shows that, over 7 days, omission of *myo*-inositol from the medium had no effect on cell number or yield of fresh mass. Similarly, inclusion of 1 mM-dGlc had no effect on these parameters when *myo*-inositol was present, though in medium lacking *myo*-inositol and in the presence of dGlc the cell number was reduced by 32% and the fresh mass was reduced by 25% relative to that of cells in medium containing *myo*-inositol and dGlc.

To examine this effect further, cells were incubated in *myo*-inositol-free medium containing 1 mM-dGlc ($\text{Ins}^-/\text{dGlc}^+$) for 1 week. These cells were then subcultured into the media shown in Table 1(b). In $\text{Ins}^-/\text{dGlc}^+$ medium the final cell number was half that of cells in $\text{Ins}^-/\text{dGlc}^-$, $\text{Ins}^+/\text{dGlc}^+$ or $\text{Ins}^+/\text{dGlc}^-$ medium. The fresh mass after 7 days was lowest in $\text{Ins}^-/\text{dGlc}^+$ medium (being about half that in $\text{Ins}^+/\text{dGlc}^+$ medium). However, the fresh mass of the cells in dGlc-containing medium after 7 days was significantly less than that of cells incubated without dGlc, even though the cell density in $\text{Ins}^+/\text{dGlc}^+$ medium was the same as that for cells in dGlc-free medium. This supports the contention that *myo*-inositol is necessary for the maintenance of cell division. The decreased fresh mass yield in both dGlc-containing media suggests that dGlc had two effects: one is upon the maintenance of cell division and is inositol-reversed, whereas the second effect is on tissue mass, is inositol-independent and is only observed after treatment with dGlc for longer than 1 week. Further subculturing of cells into $\text{Ins}^-/\text{dGlc}^+$ from $\text{Ins}^-/\text{dGlc}^+$ for a third week resulted in decreased cell viability. However, if *myo*-inositol was present with dGlc, then the cells continued undergoing weekly subcultures for 4 weeks in the same medium ($\text{Ins}^+/\text{dGlc}^+$) before the cell viability decreased to below 85%. This showed that 1 mM-dGlc was not toxic when present in incubations for 2 weeks or less.

Fig. 2. Profile of radioactivity in 80%-ethanol-extracted ^{14}C -labelled metabolites separated by paper chromatography

Cells, at a density of 0.3×10^6 cells/ml, were incubated with $2 \mu\text{Ci}$ of $[1-^{14}\text{C}]\text{dGlc}$ at a final concentration of 1 mM. After 5 days the cells were extracted in 80% ethanol, the extract dried under vacuum and analysed by descending paper chromatography in ethyl acetate/pyridine/water (12:5:4, by vol.) overnight. After locating the standards with silver stain the paper chromatogram was cut into strips and the radioactivity determined. The strips were 2 cm wide, except for the region between 18 and 25 cm in (a), where 1 cm strips were taken to improve resolution of the peaks. The position of standards run alongside the samples is shown. (a) The profile of radioactivity in ^{14}C -labelled metabolites extracted from 10 mg of fresh mass of cells incubated in *myo*-inositol-free medium (○) or medium supplemented with 0.5 mM-*myo*-inositol (●). The result is representative of five such determinations. (b) The profile of radioactivity on a paper chromatogram generated after running samples of the 80%-ethanol extract after incubation with (●) or without (○) invertase.

As dGlc is a metabolic inhibitor, its incorporation into the cells was measured to determine whether cells in $\text{Ins}^-/\text{dGlc}^+$ medium took up more dGlc than did cells in $\text{Ins}^+/\text{dGlc}^+$. An increased uptake of dGlc could result in a decreased energy charge in these cells through inhibition of glycolysis. It should be noted that the cells are subcultured into a medium containing 30 g of sucrose/litre, so the hydrolysis of sucrose would yield 88 mM-glucose and 88 mM-fructose. This means that fructose was also available for glycolysis and its metabolism would not be directly affected by dGlc.

Fig. 3. Incorporation of radioactivity from $[1-^{14}\text{C}]\text{dGlc}$ into phospholipids of cultured soybean cells

Suspension culture of soybean callus was incubated for 5 days with $2 \mu\text{Ci}$ of $[1-^{14}\text{C}]\text{dGlc}$ in 24 ml medium at a final concentration of 1 mM-dGlc in *myo*-inositol-free (○) or *myo*-inositol-containing (●) medium. Phospholipids were extracted and separated as described in the Experimental section. The solvent front on the thin-layer chromatogram was at 14.5 cm. Standards of phosphatidylinositol (PI) and phosphatidic acid (PA) were chromatographed at the same time. The result is corrected to 100 mg fresh mass in both cases and is representative of five such experiments.

Soybean cells were incubated with $2 \mu\text{Ci}$ of $[1-^{14}\text{C}]\text{dGlc}$ at 1 mM for 5 days and the radioactivity incorporated into the cells was determined. The mean value obtained from six separate incubations for the accumulation of $[1-^{14}\text{C}]\text{dGlc}$ was 1112 ± 38 d.p.m. $\cdot \text{mg}^{-1}$ of fresh mass in the absence of *myo*-inositol compared with 1081 ± 25 d.p.m. $\cdot \text{mg}^{-1}$ of fresh mass in medium with added *myo*-inositol. Therefore accumulation of $[1-^{14}\text{C}]\text{dGlc}$ into the cells was identical irrespective of the presence of *myo*-inositol in the medium. The distribution of radiolabel in the cells was further examined to determine whether the presence of *myo*-inositol altered the metabolism of dGlc in the cells. The incorporation of ^{14}C from $[^{14}\text{C}]\text{dGlc}$ into the fraction that remained after extraction of 80%-ethanol-soluble material and phospholipids (i.e. the macromolecular component containing the cell wall, proteins and nucleic acids) was similarly unaffected by the inclusion of *myo*-inositol in the medium. In $\text{Ins}^+/\text{dGlc}^+$ medium 177 ± 9 d.p.m. $\cdot \text{mg}^{-1}$ of fresh mass was incorporated into this macromolecular fraction compared with 176 ± 10 d.p.m. $\cdot \text{mg}^{-1}$ of fresh mass incorporated in $\text{Ins}^-/\text{dGlc}^+$ medium.

The radiolabel incorporated from $[1-^{14}\text{C}]\text{dGlc}$ into the 80%-ethanol-soluble fraction appeared mainly in one peak, which migrated slightly more slowly than glucose on paper chromatography (Fig. 2a). The peak near the origin may be dGlc 6-phosphate, and the small fraction of $[1-^{14}\text{C}]\text{dGlc}$ in the ethanol-soluble fraction indicated that dGlc did not accumulate in the cells. The major peak was $12 \pm 3\%$ smaller in $\text{Ins}^-/\text{dGlc}^+$ medium than in

Table 2. Inositol content of soybean cells

Cells were incubated for 7 days in culture medium in the presence (+) or absence (−) of either 0.5 mM-*myo*-inositol or 1 mM-dGlc. After 7 days incubation in the indicated media, soybean cells were harvested and the *myo*-inositol extracted and measured as described in the Experimental section. The results are from duplicate incubations and the mean and total range are presented. The *myo*-inositol peak was identified and quantified by comparison with standards containing known amounts of *myo*-inositol. (a) Subcultured from cells in Ins⁺/dGlc[−] media. (b) Grown in Ins[−]/dGlc⁺ medium for 7 days before being subcultured into the indicated media.

	Inositol and dGlc content of the medium		<i>myo</i> -Inositol content of cells (μg of <i>myo</i> -inositol · g ^{−1} of fresh mass)
	Ins	dGlc	
(a)	+	−	342 ± 16
	−	−	206 ± 10
	+	+	410 ± 10
	−	+	50 ± 2
(b)	+	+	571 ± 62
	−	+	37 ± 4

Ins⁺/dGlc⁺. Fig. 2(b) shows that this major peak was most probably deoxysucrose, since after treatment with invertase the radioactive compound co-migrated with dGlc when analysed by paper chromatography. Conversion of dGlc into deoxysucrose has been observed in tobacco (*Nicotiana tabacum*) and horseradish (*Armoracia rusticana*) (Fischer & Weidemann, 1966).

The incorporation of [1-¹⁴C]dGlc into phospholipids was also examined (Fig. 3). Numerous peaks were detected, though none were specific to, or enhanced in, cells grown in Ins[−]/dGlc⁺. The peaks presumably correspond to [1-¹⁴C]dGlc-containing, or even possibly [¹⁴C]deoxygalactose-containing, phospholipids. The incorporation of [¹⁴C]dGlc into five phospholipids has been reported in yeast (Steiner & Lester, 1972). Upon mild acid hydrolysis of the yeast lipids the [¹⁴C]dGlc was released intact.

Incorporation of [1-¹⁴C]dGlc by cells in Ins[−]/dGlc⁺ medium did not result in increased dGlc uptake or generation of any detectable new compounds compared with cells incubated in Ins⁺/dGlc⁺ medium. Therefore any such potential source of interference in cell division by dGlc can be discounted. Intracellular levels of *myo*-inositol were then measured to determine whether the effect of dGlc on cell division correlated with inhibition of the L-*myo*-inositol 1-phosphate synthase. Incubation in Ins[−] medium resulted in a 40% decrease in *myo*-inositol levels in soybean callus, whereas in Ins[−]/dGlc⁺ medium there was an 85% reduction in intracellular *myo*-inositol (Table 2a). However, if *myo*-inositol was included with dGlc in the medium, the cellular *myo*-inositol level did not fall.

Similarly, when the cellular *myo*-inositol level had fallen over 1 week in Ins[−]/dGlc⁺ medium, the level of cellular *myo*-inositol was restored by incubation for a further week in Ins⁺/dGlc⁺ medium (Table 2b). Therefore incubation of soybean cells with dGlc only resulted in a decrease in cellular *myo*-inositol when *myo*-inositol was

omitted from the medium. Under these conditions there was reduced cell division, which was also restored by inclusion of *myo*-inositol in the medium (Table 1). It therefore appeared that *myo*-inositol was necessary for cell division in soybean cell cultures.

DISCUSSION

myo-Inositol is a staple ingredient of media used to grow plant tissues, though the role that *myo*-inositol plays in the successful growth of the tissues has not been determined. Various effects of exogenously supplied *myo*-inositol on plant tissue growth and differentiation have been recorded. These include increased root and shoot formation in *Haworthia* tissue (Kaul & Sabharwal, 1975), development of the radicular meristem of the embryo axes of *Prunus* species (Abou-Zeid, 1973) and increased growth of explants from carrot (*Daucus carota*) roots (Shantz *et al.*, 1967). Ash (*Fraxinus*) callus tissue growth was shown by Wolter & Murmanis (1974) to be dependent upon a supply of exogenous *myo*-inositol, whereas *myo*-inositol was necessary to overcome the inhibitory effect of LiCl on cell plate formation in *Tradescantia* (Chen & Wolniak, 1987). Therefore *myo*-inositol appears to have a role in cell growth/differentiation in a number of tissues, though addition of *myo*-inositol, in the absence of dGlc, to our soybean callus cells had no effect on cell division (Table 1). The above observations suggest that cells require *myo*-inositol, which may be taken up from the medium or it may be synthesized by the *myo*-inositol-1-phosphate synthase. To reduce the level of endogenous *myo*-inositol it may not be sufficient solely to remove the extracellular *myo*-inositol but *de novo* synthesis of *myo*-inositol may also need to be inhibited.

Soybean callus cells in suspension culture were incubated with dGlc, as in other systems uptake of this compound and its subsequent phosphorylation had been shown to result in dGlc 6-phosphate, which inhibits *myo*-inositol-1-phosphate synthase (Barnett *et al.*, 1970; Charalampous & Chen, 1966). In male mice, intraperitoneal injection of dGlc resulted in a decrease in the content of *myo*-inositol in the liver and testes, though the level of lipid-bound *myo*-inositol in these tissues was unchanged (Burton & Wells, 1977). As dGlc 6-phosphate may also inhibit glucose phosphate isomerase, it is necessary to consider the possibility that the inhibition of cell division by dGlc is a consequence of an inhibition of glycolysis by this compound. This possibility is rejected for the following reasons.

(1) If dGlc were inhibiting glycolysis, then a decrease in cell viability would be expected; this was not observed.

(2) At a concentration of 1 mM, dGlc has been shown not to affect glycolysis adversely in several tissues. In yeast dGlc has a greater effect on inhibiting growth than it does on inhibiting glycolysis (Heredia *et al.*, 1964), and dGlc has no effect in yeast on protein synthesis, a parameter which would be expected to be sensitive to cellular levels of ATP (Farkas *et al.*, 1970). When *Haworthia* tissue was incubated with dGlc the dry weight yield was unaffected, though differentiation was inhibited (Kaul & Sabharwal, 1975).

(3) Addition of *myo*-inositol to the culture medium completely prevents any inhibitory effect of dGlc on cell division. This is strong evidence that the inhibition of cell division is a consequence solely of the reduction in the level of endogenous *myo*-inositol.

(4) Fructose was available as an alternative carbon source.

Our results show that the presence of dGlc led to a large decrease in the level of cellular *myo*-inositol when *myo*-inositol was omitted from the media, and this correlated with a decrease in cell number. Similarly, in *myo*-inositol-requiring strains of baker's yeast (*Saccharomyces cerevisiae*) lack of exogenous *myo*-inositol resulted in decreased cell division and ultimately cell death (Becker & Lester, 1977). *myo*-Inositol is metabolized to xylose, arabinose and galacturonic acid, which are incorporated into the pectin cell-wall fraction. Oligosaccharide fragments from plant cell walls generated by partial acid hydrolysis have a number of effects, which include regulation of morphogenesis in tobacco explants (Darvill *et al.*, 1985), mediation of the hypersensitivity response (Yamazaki *et al.*, 1983) and, in soybean, a galacturonic acid-rich oligosaccharide elicits phytoalexin accumulation (Hahn *et al.*, 1981). *myo*-Inositol is also readily incorporated into inositol-containing phospholipids, which may be involved in cellular signalling in plants (Ettlinger & Lehle, 1988; Irvine *et al.*, 1989). As it appears a certain level of cellular *myo*-inositol is necessary for maintaining cell division, this raises the question of the role of *myo*-inositol in cell division. From our observations above we cannot determine whether the decrease in cellular *myo*-inositol results in a general impairment of processes necessary for the completion of the cell-division cycle and so gradually reduces the rate of cell division, or whether the decreased cellular inositol levels results in arrest at some specific point or points in the cell cycle. We also cannot yet decide on the chemical nature of the inositol derivative whose depletion or absence is responsible for the decreased cell division. An inositol-containing lipid, or phosphate ester, or a cell-wall polysaccharide are all still feasible candidates.

Although it has been shown that changes in the pattern of labelling of phosphoinositides are associated with the early stages of responses to cellular signals, there is still no clear evidence that these changes are necessary for the subsequent developmental response. Although our results do not identify the inositol-requiring step, they do demonstrate a functional dependency of cell division on inositol levels which is separate from the maintenance of viability. They therefore indicate that some feature of inositol metabolism is causally connected with the process of cell division in soybean callus cells in suspension culture.

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