Plant Physiol. (1976) 57, 241-244

Effect of Benzyladenine, 2,4-Dichlorophenoxyacetic Acid, and D-Glucose on *myo*-Inositol Metabolism in *Acer pseudoplatanus* L. Cells Grown in Suspension Culture[1]

Received for publication September 11, 1975 and in revised form October 27, 1975

DEVI C. VERMA,[2] JAMES TAVARES, AND FRANK A. LOEWUS[3]
Division of Cell and Molecular Biology, State University of New York at Buffalo, Buffalo, New York 14214

ABSTRACT

Suspension cultures of *Acer pseudoplatanus* L. cells grown for 15 days in medium (T. Murashige and F. Skoog. 1962. Physiol. Plant. 15: 473-497) contained 3% sucrose, 1 mg/l 6-benzylaminopurine (BA), and 0.1 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), referred to here as normal media, removed newly added *myo*-inositol-2-^{3}H up to 100 mg/l in 24 hours and utilized up to 20% of this cyclitol for pectin biosynthesis. When the BA content of the growth medium was raised 10-fold, uptake of *myo*-inositol was drastically reduced and very little was available for pectin biosynthesis. Neither cell growth as measured by packed cell volume or by dry weight, nor monomer composition of pectic polysaccharides was affected by the increased level of cytokinin. Increasing, 2,4-D 10-fold instead of BA had little or no effect on *myo*-inositol uptake, although it did reduce the amount of *myo*-inositol utilized for pectin biosynthesis. Cells grown 15 days in normal media failed to remove added *myo*-inositol if 3% D-glucose was included. The net result was similar to that found in cells grown in the high BA condition. If a trace amount of D-galactose-1-^{14}C was supplied to cells after 15 days of growth in normal, high BA, or high 2,4-D media, there was no significant variation in uptake and utilization of label among the three growth conditions.

Historical as well as practical reasons have singled out *Acer pseudoplatanus* L. cell cultures as a convenient system for the study of plant cell wall biosynthesis. Cell suspension cultures originally isolated by Lamport and Northcote (11) have been subcultured and subsequently utilized in a number of studies concerned with hormonal control of growth, carbohydrate metabolism, and cell wall biosynthesis (1, 5, 10, 13, 15, 21-25).

Plant growth regulators which influence growth and differentiation may affect the metabolism of cell wall precursors. The work of Street *et al.* (25) have indicated an effect of cytokinins on cell growth and carbohydrate metabolism in *Acer* suspension cultures. It has also been demonstrated that cultured *Acer* cells utilize MI[4] for pectin biosynthesis (15, 21).

The experiments reported here are a further study of the incorporation of MI into cell wall polysaccharides and, specifically, the effects of BA, 2,4-D, and D-glucose on the uptake and metabolism of labeled MI in *Acer pseudoplatanus* cells grown in suspension cultures.

MATERIALS AND METHODS

Labeled Compounds. MI-2-^{3}H (21 μCi/μmole) was prepared by reduction of *myo*-inosose-2 with sodium borohydride-^{3}H (20). D-Galactose-1-^{14}C (34.9 μCi/μmole) was purchased from Amersham/Searle.

Cell Cultures. *Acer pseudoplatanus* L. cells from a clonal cell line maintained by this laboratory since 1964 were used in this study (21). Cultures were grown routinely on Murashige and Skoog's agar medium (17), modified by omission of MI and by an increased level (0.4 mg/l) of thiamine (14). To initiate suspension cultures, 50 mg of callus fragments from a 15- to 20-day-old callus culture were suspended in 100 ml of freshly prepared liquid medium in a 250-ml flask and shaken continuously at 135 rpm on a gyrotary shaker at 26 C. Diffuse light of low intensity was supplied for 9 hr/day.

In studies reported here, a 5-ml aliquot of 15-day-old suspension-cultured cells was transferred to 50 ml of test medium in a 125-ml flask. Three test media were used: normal (BA, 4.4 μM and 2,4-D, 0.45 μM), high 2,4-D (BA, 4.4 μM and 2,4-D, 4.5 μM), and high BA (BA, 44 μM and 2,4-D, 0.45 μM).

In the growth study, PCV was measured after centrifugation at 2,000*g* for 10 min in graduated 50-ml tubes. In other experiments, the PCV was estimated from 10-ml aliquots of the cell suspension after centrifugation in graduated 12-ml tubes. Dry weights were obtained on PCV pellets after 24 hr at 70 C.

Labeled Experiments. To sterile, screw-capped, 50-ml flasks containing the labeled compound, were added 10-ml aliquots of a 15-day-old cell suspension in appropriate media. Aliquots were taken from cell suspensions that had been subcultured at least once in liquid media. Samples were shaken for 24 hr and harvested by centrifugation at 1,300*g* for 10 min at 25 C. Cells were twice resuspended in distilled H_2O and centrifuged to remove labeled media. Washed cells were suspended in four times the PCV of 95% ethyl alcohol and ground in a motor-driven glass homogenizer at 25 C. Residues were recovered by centrifugation at 10,000*g* for 10 min and extracted several times with 80%, (v/v) ethyl alcohol to remove all soluble radioactivity. Extracted residues were dried at reduced pressure over KOH pellets.

Paper Chromatography. All separations were done by descending chromatography on Whatman No. 1 paper. Three solvent systems were used for sugar separations: (A) ethyl acetate-acetic acid-pyridine-water (50:12:18:10, v/v) (7) to separate sucrose from monosaccharides; (B) ethyl acetate-pyridine-water (10:6:5, v/v) to separate MI from sugars; and (C) ethyl

[1] This investigation was supported by National Institutes of Health Grant GM-12422 from the National Institute of General Medical Sciences. This research was taken in part from a thesis submitted by D. C. V. in partial fulfillment of his requirements for the Ph.D., SUNY/Buffalo, 1975. A preliminary report of this work has appeared elsewhere (29).

[2] Present address: W. Alton Jones Cell Science Center, Lake Placid, New York 12946.

[3] Present address: Department of Agricultural Chemistry, Washington State University, Pullman, Wash. 99163.

[4] Abbreviations: MI: *myo*-inositol; PCV: packed cell volume; TFAA: trifluoroacetic acid.

acetate-pyridine-water (8:2:1, v/v) to separate simple sugars. Acidic components were separated in solvent system D, ethyl acetate-water-acetic acid-formic acid (18:4:3:1, v/v). Sugars were detected with periodate-permanganate (12) or alkaline silver nitrate reagent (26).

Hydrolysis of Ethyl Alcohol-insoluble Residues. Ten-mg samples were hydrolyzed with 1 ml of 0.2 or 2 N TFAA for 1 hr at 121 C in sealed 2-ml glass ampoules. TFAA was removed by flash evaporation, and samples were stored over KOH pellets at reduced pressure to remove final traces of acid. Residues from 0.2 N TFAA hydrolysates were further hydrolyzed with fungal carbohydrases from *Sclerotium rolfsii* Sac (28). A 0.25% (w/v) solution of lyophilized enzyme in 10 mM Na acetate buffer, pH 4.5, was dialyzed against the same buffer overnight prior to use. Two ml of the dialyzed solution containing 125 μg/ml of protein (16) were added to each sample and hydrolysis continued for 24 hr at 26 C. A few drops of toluene were added to each digest to retard microbial growth. Each enzymic digest was centrifuged at 10,000*g* for 20 min to pellet insoluble residues. Residues were washed with 1-ml portions of distilled H_2O to remove all traces of soluble radioactivity. Washed residues were resuspended in 1 ml of water and assayed for radioactivity in a thixatopic liquid scintillation mixture.

The soluble portion from the enzymic hydrolysis and washes of the insoluble residue were combined and then separated by ion exchange chromatography into neutral and acidic fractions (21). Each fraction was analyzed further by paper chromatography and radiochromatogram scanning. Reducing sugar content was estimated with the arsenomolybdate procedure of Nelson (3). Radioactive samples were counted in a liquid scintillation spectrometer using toluene-Triton X-100 (27).

Gas-Liquid Chromatography. The monosaccharide composition of the neutral sugar fraction from 2 N TFAA hydrolyses of 80% ethyl alcohol-insoluble cell residues was determined by the method of Albersheim *et al.* (2). Acidic components, which are destroyed in part by the 2 N TFAA treatment (9), were released by partial hydrolysis with 0.2 N TFAA, followed by enzymic digestion as described above and then analyzed (8). Following conversion of sugar components to their alditol acetates, excess acetic anhydride was removed by aeration and residues were redissolved in ethyl acetate to eliminate solvent peak interference with compounds of low retention time. Separations were made on a Packard Model 7821 gas-liquid chromatograph equipped for flame-ionization detection. Conditions were as follows: flame gases: H_2, 60 ml/min, air, 600 ml/min; carrier gas: N_2, 35 ml/min; column: glass "cobra" coil, 122 × 0.635 cm; packing: 3% ECNSS-M on Gas-chrom Q, 100/120 mesh; temperature: inlet, 225 C; column programmed from 175 C to 190 C at 1 C/min for alditol acetates and isothermal at 190 C for aldonolactone acetates; outlet, 230 C and detector, 220 C. Areas under each peak were measured with an Autolab Model 6300 integrator. Retention times were determined by reference to internal standards, MI hexacetate for alditol acetates, and mannonolactone pentaacetate for aldonolactone acetates.

RESULTS

A cytokinin in the medium was essential for growth of *Acer* cells used in this study. At 4 to 5 μM, kinetin or BA were equally effective but at 45 μM, kinetin repressed growth whereas BA did not alter the growth curve. For this reason, BA was used in all experiments reported here. An exogenous source of auxin or 2,4-D was not required for growth and its presence in the medium at 0.45 or 4.5 μM did not alter cell yields as determined by PCV or dry weight. The higher concentration of 2,4-D was included in this study to test earlier reports (4, 22) that *Acer* cells grown in its presence exhibited an altered pattern of MI metabolism. The cell line used here had been maintained through many transfers on media lacking MI.

Cell yields, as measured by PCV and dry weight, were very similar among all sets of conditions used, namely, normal, high BA-, or high 2,4-D-grown cells. After 16 days of growth in suspension culture, a 50-ml suspension yielded 25 ± 1 ml of PCV cells with a dry weight of 780 ± 40 mg. Addition of 0.55 mM MI to the growth media did not affect PCV or dry weight significantly. It should be noted that cells were near the end of the exponential growth phase when harvested at 16 days. Other experiments not described here showed that the concentrations of growth regulators and MI used in this study did not alter the shape of the growth curve significantly.

MI-2-^{3}H Metabolism. Ten-ml aliquots from suspension cultures which had been grown for 15 days under the three sets of conditions described under "Materials and Methods" were transferred to sterile flasks containing MI-2-^{3}H and shaken for 24 hr at 26 C. Distribution of label between media and cells for three such experiments is summarized in Table I. Growth, measured as PCV or dry weight, gave values similar to results described above. In experiment 1, the MI concentration at the start of labeling was 0.7 mM, while in experiments 2 and 3 this value was 0.09 mM. In normal media, cells removed 88 to 96% of the MI supplied during the 24-hr period and most, if not all, of this label was accounted for in the ethyl alcohol-soluble and -insoluble fractions. In experiment 1, uptake was 8.3 mg of MI/g dry weight of cells, and incorporation into the ethyl alcohol-insoluble fraction was 0.36 mg/g. In experiment 2, the corresponding values were 0.8 mg/g and 0.1 mg/g, respectively.

When the BA concentration of growth media was increased 10-fold (high BA experiments), very little MI was taken up and incorporation of label into the ethyl alcohol-insoluble fraction was very low. Cells grown in media containing 2,4-D at 10-fold the normal level (high 2,4-D) removed 6.2 mg of MI/g dry weight of cells in experiment 1, almost as much as cells grown in normal media. Incorporation of label into cell wall residues was somewhat lower, 0.15 mg/g, than that found in cells from normal media. Reducing the amount of MI supplied, as in experiment 3, did not alter the general pattern of uptake and incorporation of label by cells in high 2,4-D media as compared to cells in normal media.

Table I. *Distribution of Tritium from MI-2-^{3}H in Acer Cells Grown in Normal, High BA or High 2,4-D Medium*

Ten ml aliquots from 15-day-old cultures were incubated with MI-2-^{3}H (7 μmoles in experiment 1 and 0.9 μmole in experiments 2 and 3) for 24 hr.

Experiment	Radioactivity Recovered		
	Medium	Ethyl alcohol-soluble fraction	Ethyl alcohol-insoluble fraction
	%		
1. Normal	3.8	92.5	4.2
High BA	92.1	6.6	0.2
High 2,4-D	25.7	71.3	1.8
2. Normal	8.1	70.2	9.9
High BA	79.5	10.7	0.3
3. Normal	12.1	57.2	19.2
Normal + 3% Glc	73.0	20.1	0.7
High BA	78.9	16.2	0.3
High BA + 3% Glc	80.0	7.7	0.2
High 2,4-D	5.2	77.3	10.2
High 2,4-D + 3% Glc	66.4	19.5	0.6

Essentially all label remaining in spent media of cells grown with high BA was identified as MI by paper chromatography and by trapping label with carrier MI, followed by recrystallization. Of the much lower amounts of label remaining in spent media after growing cells in normal or high 2,4-D conditions, one-half or less was MI. The remainder appeared to be saccharides of higher mol wt which failed to move from the origin after paper chromatography in solvent system B. Further study of this material was not made.

In the ethyl alcohol-soluble fraction of cells recovered from labeled media (Table I), radioactivity was primarily MI under all conditions of growth.

Ethyl alcohol-insoluble fractions from each experiment were hydrolyzed by the 0.2 N TFAA/*S. rolfsii* enzyme procedure and separated into neutral and acidic fractions by ion-exchange chromatography. Distribution of radioactivity between these two fractions is given in Table II. Incorporation into cell wall substance at 0.7 mM MI (experiment 1) was, at best, only twice that obtained at 0.09 mM (experiments 2 and 3) for cells grown in normal media. In high 2,4-D-treated cells, a similar pattern of labeling was found, although the amount of label per unit weight incorporated was only about 50% of normal conditions. Very little label appeared in cells grown under high BA conditions, but the distribution between neutral and acidic fractions reflected that obtained under normal or high 2,4-D conditions.

Over 99% of the tritium contained in ethyl alcohol-insoluble residue was released by the 0.2 N TFAA/*S. rolfsii* enzyme treatment. This label appeared in galacturonic acid, arabinose, and xylose, primarily the first two compounds. Although most of the label was released by this hydrolytic procedure, only 60% of the total ethyl alcohol-insoluble residue was solubilized. Analysis by gas-liquid chromatography of soluble products from experiment 1 gave the following distribution: fucose and/or rhamnose, 4%; arabinose, 14%, xylose, 5%; galactose, 9%; glucose, 4%; and galacturonic acid, 21%.

Effect of D-Glucose on Uptake and Metabolism of MI-2-^{3}H. All cells used in this study were grown on Murashige and Skoog's medium with 3% sucrose as the carbon source. After 16 days of growth in normal or high 2,4-D media, neither sucrose, glucose, nor fructose were detected in aliquots of spent media. The analytical procedure used, paper chromatography followed by periodate-permanganate detection, would have revealed amounts greater than 0.01%. In samples grown on high BA media, about 0.2% of the carbon source remained as sucrose, glucose, and fructose. It was the presence of reducing sugars in media of cells grown on high BA that prompted the second half of experiment 3, in which 3% D-glucose, (w/v) was added along with MI-2-^{3}H. Cells from all three conditions of growth increased in dry weight about 10% during the 24-hr labeling period, but there was no detectable change in PCV. Results are listed in Tables I and II as experiment 3. Uptake of MI was drastically reduced in normal and high 2,4-D-grown cells to about the same level as high BA cells when 3% glucose was present, but distribution of MI label between neutral and acidic fractions of cell wall constituents from glucose-treated cells was similar to that of untreated cells.

In order to determine whether the reduced incorporation of MI-2-^{3}H in the presence of high BA was entirely caused by the reducing sugars present in the 15-day-old medium, a labeling experiment was done on washed cells. Prior to labeling, cells were washed 3 times with freshly prepared, sterile media containing appropriate BA and 2,4-D concentrations. Sucrose was deleted from this wash media, but 3% glucose was present in media used to wash cells that were later incubated in the presence of glucose. Cells were incubated in fresh media containing MI-2-^{3}H for 24 hr, harvested, washed, homogenized, and extracted with 80%, (v/v) ethyl alcohol. The ethyl alcohol-insoluble residues were dried and assayed for radioactivity after combustion in a sample oxidizer. Results from two experiments are given in Table III. High BA-blocked MI-2-^{3}H uptake and utilization by 75 to 80% as compared to normal (low BA) cells. Addition of 3% glucose to the incubation medium blocked incorporation of MI-2-^{3}H into 80% ethyl alcohol-insoluble material under all three conditions.

Uptake and Metabolism of D-Galactose-1-^{14}C. An experiment was run to determine the effect of high BA and high 2,4-D on uptake and incorporation of this sugar. No significant decrease in uptake or utilization of D-galactose occurred when growth regulator levels were increased. Three per cent MI added at the time of labeling reduced uptake slightly, but did not repress incorporation of label into acid-hydrolyzable material, as would be expected if the label had entered the MI oxidation pathway. Analysis of the 2 N TFAA hydrolysate by ion exchange and paper chromatography revealed that galactose, glucose, and arabinose accounted for most of the label in neutral sugar residues, although some label appeared in xylose, rhamnose, and mannose. In the acidic fraction, most of the label appeared in galacturonic acid. More label appeared in galacturonic acid

Table II. *Distribution of Radioactivity in Neutral and Acidic Components of Ethyl alcohol-insoluble Fractions of Acer Cells*

Experiment	Radioactivity Hydrolyzed from Ethyl Alcohol-insoluble Fraction	
	Neutral fraction	Acidic fraction
	cpm/mg dry weight	
1. Normal	33,500	14,800
High BA	1,600	1,000
High 2,4-D	15,400	7,500
2. Normal	8,700	6,500
High BA	240	200
3. Normal	18,600	8,900
Normal + 3% Glc	670	200
High BA	280	170
High BA + 3% Glc	130	80
High 2,4-D	8,100	4,400
High 2,4-D + 3% Glc	433	170

Table III. *Incorporation of Tritium from MI-2-^{3}H into Acer Cells Grown in Normal, High 2,4-D, and High BA Medium*

Cells from 15-day-old cultures were washed 3 times with fresh, sterile medium, and a 10-ml aliquot of cells, suspended in the last wash, was incubated with the label for 24 hr.

Treatment	Radioactivity from Ethyl Alcohol-insoluble Fraction	
	Experiment 1	Experiment 2
	cpm/mg[1]	
Normal	11,923	10,551
High 2,4-D	6,156	8,445
High BA	2,395	2,902
Normal + 3% Glc	132	268
High 2,4-D + 3% Glc	213	179
High BA + 3% Glc	160	167

[1] Total radioactivity was measured by oxidizing 30 mg dry weight samples of the 80% ethyl alcohol-insoluble material and is expressed here as cpm/mg dry weight. A Packard sample oxidizer, Model 306, was used to prepare the samples in Packard Monophase 40 scintillation fluid.

residues from high 2,4-D-grown cells than was found in normal or high BA-grown cells.

DISCUSSION

Uptake of MI by suspension-cultured *Acer* cells and conversion of this cyclitol to cell wall pectin components is dramatically blocked when the cytokinin level of the medium as represented by BA is increased from 4.4 to 44 μM. This effect is not accompanied by a reduction in growth rate of the cells. Street *et al.* (25) have reported that 46.5 μM kinetin reduced *Acer* cell growth in liquid culture as measured by PCV and dry weight. Two other cytokinins, 45.6 μM zeatin and 49.3 μM 6-γ,γ-dimethylallylaminopurine did not have this inhibitory effect. These results suggest that the inhibitory effect on growth by high levels of kinetin is not characteristic of all cytokinins at similar concentrations. This difference has made it difficult to compare our results using BA with previous *Acer* studies in which kinetin was used (24).

Street *et al.* (25) found that 11.6 μM kinetin depressed growth drastically when glucose was supplied as the carbon source. Growth was much less affected when sucrose or fructose were used as the carbon source. More recently, Simpkins and Street (23) have shown that this level of kinetin did not inhibit utilization of glucose by the cells, but did inhibit respiration. Measurement of O_2 uptake by cells to provide direct evidence as to whether high BA did or did not inhibit respiration was not made in the current study; indirect evidence suggests that such inhibition did not occur. High BA-treated cells grew just as well as the cells grown in normal level, and as far as cell wall formation is concerned, the carbon requirement in these cells was fulfilled.

When sucrose is used as the carbon source, both glucose and fructose appear in the medium of *Acer* suspension cultures (24). In our experiments, spent media from high BA-treated cells contained glucose, fructose, and sucrose, while media from normal or high 2,4-D-treated cells were virtually devoid of these sugars at 16 days. A cytokinin effect resembling these results has been reported by Gilad *et al.* (6) using detached dark-grown sunflower cotyledons. Two-day-old cotyledons incubated in kinetin solution showed an increased concentration of reducing sugar over that of the control.

Our experiments on the metabolism of MI-2-^{3}H in the presence of 3% D-glucose indicates that this reducing sugar affects transport of MI into the cells. The presence of reducing sugars, glucose and fructose, in the media under high BA conditions can partly explain the reduced uptake and incorporation of MI-2-^{3}H in the presence of high BA. The incorporation of MI-2-^{3}H into washed cells (Table III) demonstrated a direct effect of high BA on MI-2-^{3}H metabolism in addition to the indirect effect of high BA on the uptake of MI-2-^{3}H due to the presence of glucose.

The glucose effect described here may be the result of direct competition between glucose and MI for transport sites into the cell. It may also reflect a phenomenon already well known in bacterial metabolism (18). Little is known regarding the role of cyclic AMP in plant growth regulation (19), virtually nothing as regards *Acer* cell metabolism, so speculation at this time is not possible.

Preliminary experiments on D-galactose metabolism suggest that it readily enters the hexose phosphate pool and is utilized in the biosynthesis of glucose, galacturonic acid, and pentose residues, as well as galactosyl units in cell wall polysaccharides. The presence of a high concentration of *myo*-inositol did not greatly reduce this conversion, suggesting that galactose utilization proceeds primarily through UDP-D-glucose and its oxidation product, UDP-D-glucuronic acid. Of primary interest to the present study is the fact that high BA-grown cells readily utilized labeled galactose, but not *myo*-inositol. This selective effect may have a significant, but yet unknown, role in growth regulation.

Acknowledgment—The culture of *Sclerotium rolfsii* Sac used in this study was generously provided by D. F. Bateman, Cornell University.

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