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THE CHANGING NUCLEOTIDE PATTERN OF SYCAMORE CELLS DURING CULTURE IN SUSPENSION

E. G. BROWN and K. C. SHORT*

Department of Botany, University College, Swansea, Glamorgan

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Abstract—The nucleotide components of cells of *Acer pseudoplatanus* L., grown in a large-scale suspension culture (4·5 l.), have been examined and fluctuations in their individual concentrations assessed during the three phases of growth, i.e. lag phase, period of rapid cell division, and stationary phase. Initially, the predominant nucleotide is ATP but, by the end of the lag phase, its place has been taken by UDP-glucose. The most marked change in nucleotide pattern occurs between days 0 and 4 (lag phase) when a relatively large increase is observed in the concentration of all the components. During this stage, there is a 46-fold increase in UDP-glucose concentration and a 7-fold increase in that of UTP. By day 8 (period of rapid cell division) the individual concentrations of ATP, the guanosine nucleotides, UTP and UDP-glucose, have started to fall and are then at positions intermediate to those of days 0 and 4. In contrast, the NAD concentration shows a marked increase at day 8. Traces of a UDP-uronic acid, tentatively identified as UDP-galacturonic acid, and an unidentified guanosine nucleotide of the type GDP-X were observed. The results reveal a high peak of metabolic activity in *Acer* suspension cultures immediately before the onset of the period of rapid cell division. No nucleotides could be detected in the culture medium at the end of the 3-week growth period.

INTRODUCTION

IN RECENT years, rapid advances have been made in techniques for aseptically culturing higher plant cells in suspension. These advances and the experimental significance of such cultures have been reviewed by Street, Henshaw and Buiatti.[1] However, despite increasing utilization of suspension cultures of higher plant cells for metabolic and growth studies, little is known of their nucleotide constituents. Yet, because of their multifarious roles as (i) coenzymes and constituent moieties of coenzymes, (ii) allosteric effectors (see reviews by Stadman[2] and Atkinson[3]), and (iii) precursors of nucleic acids, nucleotides occupy a central position in metabolism and its regulation. Furthermore, as most nucleotides are present in limiting amounts, fluctuations in their respective concentrations have, therefore, an important directional influence on metabolism. In consequence, the nucleotide pattern of a tissue provides a convenient index to the type and extent of the predominant metabolic activity of that tissue (see, for example, Schmitz,[4] Brown,[5–7] Mandel[8]). This approach has been utilized in the present work in an attempt to obtain indications of the major metabolic trends associated

* Present address: The Biological Laboratories, Harvard University, Massachusetts, U.S.A.

[1] H. E. STREET, G. G. HENSHAW and M. C. BUIATTI, *Chem. Ind.* **27**, (1965).
[2] E. R. STADTMAN, *Advan. Enzymol.* **28**, 41 (1966).
[3] D. E. ATKINSON, *Ann. Rev. Biochem.* **35**, 85 (1966).
[4] H. SCHMITZ, in *Zur Bedeutung der Freien Nucleotide*, p. 1, Springer-Verlag, Berlin (1961).
[5] E. G. BROWN, *Biochem. J.* **85**, 633 (1962).
[6] E. G. BROWN, *Biochem. J.* **88**, 498 (1963).
[7] E. G. BROWN, *Biochem. J.* **95**, 509 (1965).
[8] P. MANDEL, *Prog. Nucl. Acid Res.* **3**, 299 (1964).

with the different phases of growth in suspension cultures of sycamore (*Acer pseudoplatanus* L.).

RESULTS

Sycamore cells cultured in suspension on a large scale (4·5 l. per culture vessel) in the apparatus described by Short, Brown and Street[9] pass through three distinct phases of growth from inoculation to maturity (Fig. 1). Inoculation is followed by a lag phase lasting

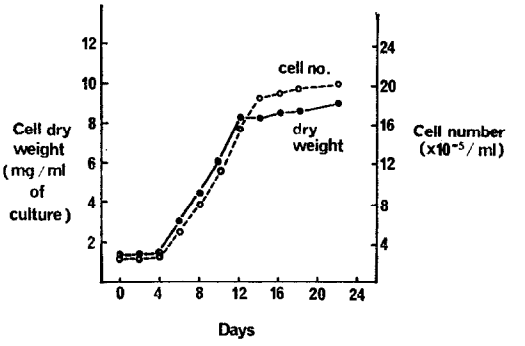

FIG. 1. GROWTH OF A LARGE-SCALE SUSPENSION CULTURE (4·5 l.) OF *Acer* CELLS. CONDITIONS WERE AS DESCRIBED IN THE TEXT.

Cell number —○—○; dry weight —●—●

about 4 days, during which little or no growth occurs. This gives way to a period of rapid cell division until about day 14 when a stationary phase begins. Mature cells at day 21 are used as inoculum for subsequent cultures and may thus be compared directly to cells at day 0. Nucleotide analyses were carried out during all three phases of culture, cells from days 0 (inoculum), 4, 8 and 21 being used for this purpose. The nucleotide patterns of sycamore

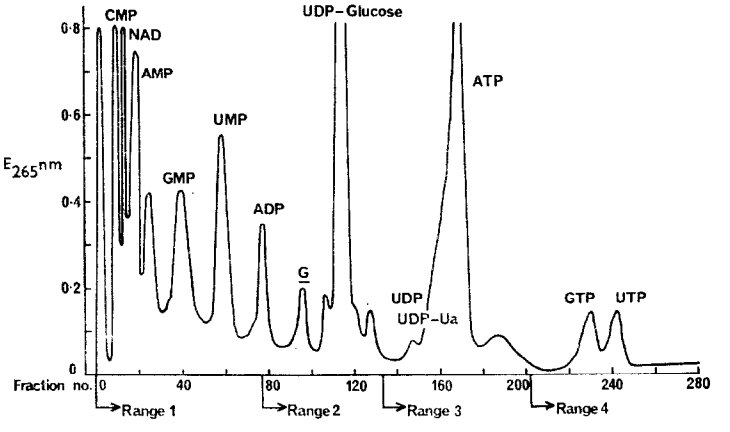

FIG. 2. ANION-EXCHANGE CHROMATOGRAM OF ACID-SOLUBLE NUCLEOTIDES OF *Acer* CELLS AT DAY 4. (DOWEX 1-FORMATE; ×8; LINEAR FORMATE GRADIENTS[5].)

UDP-Ua = a UDP-uronic acid; *G* = unidentified guanosine nucleotide.

[9] K. C. SHORT, E. G. BROWN and H. E. STREET, *J. Exp. Botany* (in press).

cells at these times were qualitatively similar and, in consequence, only one anion-exchange chromatogram is illustrated as representative of them all (Fig. 2). Although, however, the same nucleotide components were present at each stage (Table 1), the individual concentrations of these substances showed striking fluctuations (Table 2).

TABLE 1. MAJOR FREE NUCLEOTIDES OF SYCAMORE CELLS DURING CULTURE IN SUSPENSION

Nucleotide	Content in cells (μmoles/10^9cells) Day 0	Day 4	Day 8	Day 21
CMP	0·03	0·17	0·12	0·05
NAD	0·03	0·26	0·84	0·03
AMP	0·45	3·43	3·40	0·42
GMP*	1·30	4·05	2·75	1·34
UMP	1·52	6·89	8·11	1·47
ADP	0·68	2·06	2·82	0·65
G†	0·26	1·22	0·41	0·27
UDP-glucose‡	0·45	20·71	10·69	0·51
ATP§	2·85	11·33	8·08	2·78
GTP	0·32	1·35	0·50	0·32
UTP	0·22	1·55	0·29	0·17

* Contains trace of NADP.
† Estimated as GDP.
‡ Contains trace of UDP-galactose.
§ Contains trace of UDP and, at days 0 and 4, trace of a UDP-uronic acid.

TABLE 2. FLUCTUATIONS IN THE NUCLEOTIDE CONTENT OF SYCAMORE CELLS DURING THEIR CULTURE IN SUSPENSION

Nucleotide	Net changes (μmoles/10^9 cells) Period Days 0–4	Period Days 4–8	Period Days 8–21
CMP	+ 0·14	− 0·05	− 0·07
NAD	+ 0·23	+ 0·58	− 0·81
AMP	+ 2·98	− 0·03	− 2·98
GMP*	+ 2·75	− 1·30	− 1·41
UMP	+ 5·37	+ 1·22	− 6·64
ADP	+ 1·38	+ 0·76	− 2·17
G†	+ 0·96	− 0·81	− 0·14
UDP-glucose‡	+20·26	−10·02	−10·18
ATP§	+ 8·48	− 3·25	− 5·30
GTP	+ 1·03	− 0·85	− 0·18
UTP	+ 1·33	− 1·26	− 0·12

* Contains trace of NADP.
† Estimated as GDP.
‡ Contains trace of UDP-galactose.
§ Contains trace of UDP and, at days 0 and 4, trace of a UDP-uronic acid.

The most noticeable feature of the changing nucleotide patterns was the sharp contrast between those obtained at days 0 and 4 (Table 1). A general increase in the concentration of all the nucleotides was seen during this, the lag phase (Table 2). Most significant, however, was the large, specific build-up in concentration of UDP-glucose and UTP which showed, respectively, a 46-fold and a 7-fold increase (Table 1). In a freshly inoculated culture (day 0), the predominant nucleotide was ATP but, although the concentration of this substance increased approximately 4-fold between days 0 and 4 (Table 1), it was superseded in quantitative importance at day 4 by this relatively massive accumulation of UDP-glucose.

The UDP-glucose peak from cells at days 0 and 4 was found to contain traces of UDP-galactose, too small for estimation. Another trace nucleotide component was found in the first few fractions of the ATP peak from the same cells. Insufficient of this material was available for detailed examination or estimation but paper chromatography of the appropriate fractions revealed an u.v. light-absorbing substance which gave a positive carbazole reaction for uronic acids.[10] The material exhibited u.v. absorption spectra typical at both pH 2 and pH 12 of a uridine nucleotide and upon hydrolysis with 0·05 N HCl at 100° for 15 min, it yielded UDP. The latter was identified by chromatographic and electrophoretic comparison with authentic samples. Too small an amount of the uronic acid was available for positive identification, but as it migrated with a galacturonic acid marker in two solvent systems (i), isopropanol/water (4:1, v/v), and (ii) *n*-butanol/pyridine/water (2:2:1, v/v/v) which clearly separate this sugar, it was inferred that the original nucleotide was UDP-galacturonic acid. In system (ii) both the reference sample and the suspected UDP-galacturonic acid produced two spots.

Between days 4 and 8, a period which covers the onset of the phase of rapid cell proliferation (Fig. 1), there was a fall in the concentration of all three nucleoside triphosphates, ATP, GTP and UTP (Table 2). This was accompanied by a decrease in the concentration of the other guanosine nucleotides and UDP-glucose. During this period, the ADP content of the cells increased slightly whilst the AMP concentration remained virtually unchanged. The most striking feature of the changing nucleotide pattern at this stage is, however, the notable increase in concentration shown by NAD which rose to a value twenty-eight times higher than that originally present in the inoculum (Table 1). By day 21, the culture was in the stationary phase (Fig. 1) and the concentration of each of the major free nucleotides fell to a level comparable to that seen at day 0 (Table 1).

The compound *G*, listed in Fig. 2 and Tables 1 and 2, is an unidentified nucleotide exhibiting u.v. absorption spectra indicative of a guanosine derivative. At pH 2, λ_{max} was 257 nm and λ_{min} 228 nm, at pH 12 the corresponding values were 256 and 230 nm respectively. Hydrolysis in 0·05 N HCl at 100° for 10 min in a sealed tube yielded GDP together with a trace of GMP, which were identified by chromatographic comparison with authentic samples. These observations, coupled with the position of *G* in the elution sequence during anion-exchange chromatography (Fig. 2), suggest that it is a sugar nucleotide of the type GDP-*X*.

In order to examine for secretion or leaching of nucleotides from the sycamore cells into the culture medium, samples of the cell suspension were taken at the end of the culture period (21 days) and freed from cells, as described in the Materials and Methods section; the small amount of protein and fine cell-debris present was also removed. Indications of four peaks were just discernible in the first twenty fractions of range 1 but no significant amount of nucleotide material could be detected in them. The remainder of the elution ranges showed no trace of u.v. light-absorbing material.

[10] Z. DISCHE, *J. Biol. Chem.* **167**, 189 (1947).

DISCUSSION

The changing nucleotide pattern of *Acer* cells grown in suspension culture shows a number of significant features in relation to the growth behaviour of the culture (Fig. 1). As shown in Table 2, from inoculation to the onset of the phase of rapid cell proliferation (days 0–4) each of the major nucleotide components increased in concentration. The total rise was very large; calculated from Table 2, it is 44·91 μmoles/10^9 cells as compared to the original nucleotide content of the inoculum (day 0, Table 1) of 8·11 μmoles/10^9 cells. The magnitude of this increase is mainly a reflection of the very large rise in concentration of UDP-glucose and ATP, especially the former (Table 2). The general increase in nucleotide content observed during the lag phase is compatible with the increase in total soluble nitrogenous compounds reported to occur during this period in *Acer* cell suspension cultures.[11]

The 4-fold increase in concentration of ATP seen in *Acer* cells during the lag phase (Table 1) is similar in timing and extent to that observed by Plesner[12] in synchronously dividing cultures of *Tetrahymena pyriformis*. With both types of cells, this may be interpreted as an accumulation of biologically available energy for the endergonic processes of cell division. Plesner considered his findings to be more likely due to a small decrease in utilization of ATP rather than an increase in its production.

The massive increase in the concentration of UDP-glucose, and the related compound, UTP, during the lag phase is almost certainly also preparatory to the ensuing period of rapid cell division which would necessitate the synthesis, via nucleotide-glycosyl intermediates, of relatively large amounts of polysaccharide cell-wall material. Whether the immediate precursor of cellulose is UDP-glucose or GDP-glucose is still a matter of controversy although Hassid[13] considers that the weight of evidence is in favour of the guanosine nucleotide. Recently however, Ordin and Hall[14] have presented data which they interpret as showing that, in oat coleoptiles, UDP-glucose is utilized to form different β-linked polysaccharides, one of which is cellulose. In preparations of higher plants, UDP-glucose is dehydrogenated to UDP-glucuronic acid[15] which can then be enzymically epimerized to UDP-galacturonic acid, a precursor of the polygalacturonide component of pectin.[16] The large amount of UDP-glucose found in *Acer* cells at day 4 suggests that the concomitant trace of UDP-uronic acid, tentatively identified as UDP-galacturonic acid, arises in this way. The guanosine nucleotide compound *G*, although present in cells from cultures at all three stages of growth, rose maximally in concentration during the lag phase, i.e. days 0–4 (Table 2). Its increased concentration in lag phase cells, considered in relation to its chemical nature, suggests that it, too, is connected with polysaccharide synthesis. It is likely that *G* is GDP-glucose, a known precursor of β,1:4-glucans including, probably, cellulose (see Discussion above).

Tables 1 and 2 indicate that in *Acer* cells, a high peak in metabolic activity occurs immediately before the onset of the phase of rapid cell proliferation (Fig. 1). This observation is in accord with those of Givan and Collin[11] who, studying the same strain of cells grown on a smaller scale in the same culture medium, found a pronounced increase in respiratory rate and cell nitrogen-content at this time. Short, Brown and Street[9] have shown that the nucleic

[11] C. V. Givan and H. A. Collin, *J. Exp. Botany* **18**, 321 (1967).
[12] P. Plesner, *Compt. Rend. Lab. Carlsberg.* **34**, 1 (1964).
[13] W. Z. Hassid, *Ann. Rev. Plant Physiol.* **18**, 253 (1967).
[14] L. Ordin and M. A. Hall, *Plant Physiol.* **43**, 473 (1968).
[15] J. L. Strominger, and L. W. Mapson, *Biochem. J.* **66**, 567 (1957).
[16] C. L. Villemez, T. S. Lin and W. Z. Hassid, *Proc. Natl Acad. Sci. U.S.* **54**, 1626 (1965).

acid content of *Acer* cell suspensions, grown as described in this paper, also mirrors this rise in metabolic activity. DNA was shown to double in concentration and RNA to increase by a factor of about four just before the onset of the rapid growth phase.

The period between days 4 and 8 which represent the onset of the period of rapid cell division (Fig. 1) is characterized by a general decline in nucleotide concentration (Table 2). It should, however, be emphasized that the individual concentrations of most of the nucleotides are still relatively high and indicative of continuing, if declining, metabolic activity. It is interesting that the only nucleotides showing an increase in concentration during this period are ADP and NAD both of which are intimately associated with respiratory activity and are capable of undergoing further phosphorylation. It is likely that the rapid decline in the respiratory activity of *Acer* cells at this phase of culture[11] leads to a short-lived accumulation of substrates that would normally be further phosphorylated. By day 21, the concentration of each has returned to the lower level characteristic of the stationary phase.

Since Becker, Hui and Albersheim[17] had shown the presence of galacturonide-containing polysaccharides in the culture medium of sycamore cells in suspension culture, it was of interest to examine the medium at the end of the 21-day culture period for the presence of nucleotides, particularly sugar nucleotides. No evidence was, however, obtained for any significant degree of secretion or leaching of nucleotides from *Acer* cells.

Present results suggest that the large-scale suspension cultures of sycamore cells utilized in the work provide an excellent system for studies of polysaccharide synthesis and of metabolic control.

MATERIALS AND METHODS

Suspension Cultures

Sycamore cells were cultured on a large scale at 27° in a medium based on Heller's inorganic salt mixture supplemented with sucrose, thiamine, 2,4-dichlorophenoxyacetic acid, Fe-EDTA and coconut milk.[11] The apparatus and techniques used in inoculating, culturing and sampling sycamore cells on a large scale (4·5 l. of cell suspension per 10 l. culture vessel) were those described by Short, Brown and Street.[9] Cells for analysis (approximately 250 g fresh weight) were harvested from sample suspensions by filtration through nylon gauze and washed in cold distilled water. Washing was effected by transferring the cells to a measuring cylinder and re-suspending them to a final volume of 1 l. A sample (100 ml) of this washed suspension was removed separately for determination of cell number and fresh weight of tissue by the techniques of Henshaw *et al.*[18] The remainder of the suspension (900 ml) was filtered through nylon gauze and the cell mass taken for nucleotide analysis.

Extraction of Cells and Preparation of the Extract

The cell mass filtered from the 900 ml of washed cell suspension was homogenized for 5 min in 300 ml of ice-cold 0·75 N $HClO_4$ using a Waring blender. After standing for a further 3 min at 4°, the homogenate was centrifuged at 25,000 ***g*** for 15 min at the same temperature. The supernatant was decanted, its pH adjusted to 7·2 and the resulting precipitate of $KClO_4$ removed by centrifuging at 2500 ***g*** for 15 min. Using a rotary film evaporator, the extract was evaporated *in vacuo* to about 100 ml and its pH adjusted to 3·5 with HOAc. As

[17] G. E. BECKER, P. A. HUI and P. ALBERSHEIM, *Plant Physiol.* **39**, 913 (1964).
[18] G. G. HENSHAW, K. K. JHA, A. R. MEHTA, D. J. SHAKESHAFT and H. E. STREET, *J. Exp. Botany* **17**, 362 (1966).

With most extracts of higher plant material required for nucleotide analysis, it was found necessary to introduce a preliminary purification step in order to remove the bulk of non-nucleotide material which otherwise interferes with the ensuing anion-exchange chromatography. The charcoal adsorption technique described by Brown[5] was utilized for this purpose. It was convenient to divide the extract into two approximately equal portions for treatment on separate adsorption columns (3 cm internal diameter) each containing a mixture of Celite Hyflo Super-cel (4 g) and Norit OL charcoal (2·5 g). Norit OL was prepared for use as previously described.[5] Elution was effected from each column with 100 ml of 25% (v/v) ethanol containing 0·5% (v/v) NH_4OH (sp. gr. 0·88): the eluates were combined and concentrated *in vacuo*.

Anion-exchange Chromatography

The prepared extract was chromatographed on a column (19 cm × 0·9 cm) of Dowex 1 (formate; X8; 200–400 mesh) employing the elution system of linear formate gradients described by Brown.[5] The eluate from the column (flow rate 0·5 ml/min) was collected in 10-ml fractions and $E_{265\ nm}$ monitored by a Vanguard Automatic u.v. Analyser.

Examination of Culture Medium for Nucleotides

Samples of cell-free culture medium (2 l.) were obtained by filtering appropriate volumes of the suspension culture through nylon gauze. The small amount of particulate matter remaining, mainly fine cell-debris, was removed by centrifuging at 25,000 *g* for 15 min. The supernatant was cooled to 4° and $HClO_4$ added to a final concentration of 0·3 M. After standing at 4° for 30 min, a small amount of protein precipitated and was removed by centrifuging at 25,000 *g* for 15 min. Following removal of ClO_4^- by precipitation with KOH, as described for the cell extract, the volume of the sample was reduced to about 500 ml. After adjusting the pH to 3·5 with HOAc, the sample was subjected to a charcoal adsorption step similar to that described above but this time elution of the charcoal column with ethanolic ammonia was followed by elution with an equal volume of aqueous pyridine (20% v/v); the two eluates were combined and evaporated to dryness *in vacuo*. Having ensured the complete removal of pyridine which would otherwise interfere with subsequent u.v. absorption examination, the residue was redissolved in water, the pH adjusted to 8–10 with ammonia and the extract introduced onto the anion-exchange column for analysis as before.

Identification and Estimation of Nucleotides

Constituents of u.v. light-absorbing peaks obtained during anion-exchange chromatography were examined by paper chromatography, high-voltage electrophoresis and u.v. spectrophotometry. Paper chromatographic separations were effected initially using isobutyric acid–ammonia–water (57:4:39 by volume; Pabst Laboratories[19]). Bands were eluted and rechromatographed using the solvent systems described by Brown.[5] UDP-sugars were hydrolysed in 0·05 N HCl at 100° for 15 min and the hydrolysate examined for sugars by paper chromatography and high-voltage electrophoresis in borate buffers as described by Brown and Mangat.[20]

[19] Pabst Laboratories, Circular OR-17, Pabst Laboratories, Milwaukee, Winsconsin, U.S.A. (1961).
[20] E. G. BROWN and B. S. MANGAT, *Biochim. biophys. Acta* **148**, 350 (1967).

Nucleotides separated as in Fig. 2 were estimated, for homogeneous peaks, by computation of $E_{265\,nm}$ of the fractions comprising the peak, allowance being made automatically by the monitoring instrument for the $E_{265\,nm}$ of the eluent; substances in heterogeneous peaks were also estimated by spectrophotometry but only after they had been resolved by paper chromatography or electrophoresis. With mixtures composed entirely of two defined nucleotides, differential spectrophotometry was applied directly.