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Communication

Response of Cytosolic-Isozyme and Plastid-Isozyme Levels of 3-Deoxy-D-*arabino*-Heptulosonate 7-Phosphate Synthase to Physiological State of *Nicotiana silvestris* in Suspension Culture[1]

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ABSTRACT

Two isozymes of 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthase are partitioned into plastid (DS-Mn) and cytosolic (DS-Co) compartments of at least several higher plants (RA Jensen 1986 Rec Adv Phytochem 20: 257–258). Differential variation of isozyme levels and in the timing of their expression was observed during growth of *Nicotiana silvestris* in suspension culture. The ratio of DS-Co to DS-Mn varied about fivefold in comparison of the different physiological stages of growth. Cultures maintained in exponential phase for >10 generations (EE cells) possessed balanced-growth properties and did not exhibit the considerable variation of isozyme levels found during the initial 2 to 3 generations of exponential growth (E cells) that followed subculture of stationary-phase cultures. The plastid isozyme level declined substantially in stationary phase, responded immediately to subculture, and reached a peak in early exponential growth similar to the steady-state level of DS-Mn in EE cells. In contrast, the cytosolic isozyme level peaked in late exponential growth. A recent history of stationary-phase physiology appeared to foster elevated synthesis of DS-Co since the steady-state level of DS-Co in EE cells was much lower than in E cells.

Cell populations from higher plants grown in suspension culture offer attractive opportunities for study of the response of enzyme levels to transitions from one physiological stage of growth to another. A particularly intriguing dimension of analysis is the comparison of isozymes catalyzing the same reaction, but known to occupy different intracellular compartments. The initial enzyme of aromatic amino acid biosynthesis, DAHP[3] synthase, exists as a plastid-localized isozyme (DS-Mn) and as a cytosolic isozyme (DS-Co) in *Nicotiana silvestris* (3, 7). A rationale and supporting evidence that part or all of the aromatic amino acid pathway is present in both subcellular locations of higher plants have been presented (9, 10).

A common subculture routine for fast-growing plant cells in suspension culture is a regimen in which stationary-phase cells are diluted fivefold into fresh medium to initiate a new growth cycle. A lag phase of 1 to 2 d is followed by about 3 d of exponential growth with a doubling time of about 42 h for *N. silvestris* at room temperature. In contrast to growth cycles routinely obtained with microorganisms where many generations of balanced exponential growth follow high dilution subculturing, less than two generations of truly exponential growth separate lag and stationary phases in the low dilution routine for plant cell subculture. This procedural difference is primarily because microorganisms do not exhibit the minimal-density requirement for growth that is characteristic of cultured plant cells. Thus, in plant subcultures the carryover of enzymes synthesized during stationary and/or lag phase may produce flawed characterizations of the apparent nature of exponential-phase cells.

Separate populations of cells, derived from line ANS-1 of *N. silvestris* (2), and denoted EE cells have been maintained in a routine of uninterrupted exponential growth for up to 2 years. Such EE cells are expected to be free of significant carryover of enzymes and metabolites that may be preferentially formed in stationary-phase physiology, thereby approaching steady-state levels of macromolecules that define balanced growth. In the present study we have determined the levels of plastid and cytosolic isozymes of 3-deoxy-D-*arabino*-heptulosonate 7-P synthase during the progression of cells through lag, exponential, and stationary phases of a growth cycle, and in turn compared these levels to the quasisteady-state levels present in cells maintained continuously in exponential phase.

MATERIALS AND METHODS

Growth Conditions and Cell Populations. Suspension-cultured cells were grown as previously described (2) in Murashige and Skoog medium. Cells were illuminated with 15 to 20 $\mu E/m^2 \cdot s$ of white fluorescent light and grown heterotrophically with 3% sucrose. The population size in stationary-phase cultures levels off at about 3.0×10^6 cells/ml. In the regimen chosen to obtain and maintain EE cells, a mid-exponential phase population of 1.6×10^6 cells/ml is diluted 16-fold into fresh medium. After four generations (about 7 d), another subculture is carried out via a 16-fold dilution. In this subculture routine EE-cell popu-

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[3] Abbreviations: DAHP, 3-deoxy-D-*arabino*-heptulosonate 7-phosphate; DS, DAHP synthase. EE cells, cell populations in suspension culture that have been maintained continuously in exponential phase for at least 10 generations. S cells, cell populations in the stationary phase of growth. L cells, cell populations in the lag phase of growth following subculture from an S-cell culture. E cells, cell populations in the exponential phase of growth (for less than three generations) following subculture of S-cell cultures in the standard subculture protocol.

lations range between 1×10^5 and 1.6×10^6 cells/ml.

Preparation of Enzyme Extracts. Cells harvested by vacuum filtration on Miracloth discs were resuspended in 50 mM K-phosphate, 0.1% 2-mercaptoethanol (pH 7.2) at a ratio of 1 g cells per ml. The resulting slurry was placed in an ice-chilled French pressure cell, and disruption was accomplished by expulsion at 16,000 p.s.i. The extract was clarified by centrifugation at 38,000*g* for 15 min at 4°C. The supernatant was desalted on Sephadex G-25 (PD-10) columns equilibrated with 10 mM K phosphate (pH 7.2) and assayed immediately.

The presence of high mercaptoethanol concentrations during extract preparation has been found to be crucial for determination of consistent levels of DS-Mn activity. In the absence of mercaptoethanol, extracts originating from L cells or S cells are especially labile. Data shown in ref. 10 illustrate this lability.

Enzyme Assays. The two isozymes of DAHP synthase were selectively assayed in crude extracts as previously described (4). Specific activity is expressed as nmol DAHP/min·mg protein at 37°C. Units of enzyme activity are given as μmol DAHP/min at 37°C.

Protein Determination. Protein concentrations were determined by the method of Bradford (1) with BSA (fraction V) as a standard.

Materials. Prepacked Sephadex G-25 (PD-10) columns were obtained from Pharmacia. Murashige and Skoog salt base was obtained from KC Biologicals (Lexena, KS). Substrates and other biochemicals were obtained from Sigma. Protein assay reagent was from Bio-Rad (Richmond, CA). Other chemicals were obtained from commercial sources and were reagent grade or better.

RESULTS AND DISCUSSION

Most suspension cultures of *N. silvestris* are maintained in our laboratory by subculture every 7 d. This routine entails a fivefold dilution of 7-d cell populations in suspension culture. Figure 1 shows the growth curve obtained from samples taken in the experiment done to monitor isozyme levels throughout the growth cycle. In individual growth cycles the lag phase is of variable duration, 0.5 to 2.0 d. In different subcultures, cells harvested at 3.0 d of growth prove reliably to be E cells. EE cells

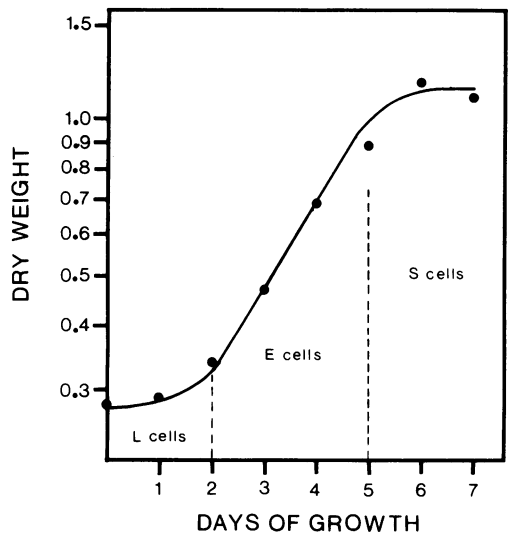

FIG. 1. Growth curve of *N. silvestris* in suspension culture. A population of 7-d S cells was diluted fivefold and aliquoted into seven 500-ml flasks, one of these then used as the d-0 sample. Dry weight (g) per 200 ml of culture is plotted on a logarithmic scale as a function of days of growth following subculture. The three physiological phases of growth (lag, exponential, and stationary) obtained are indicated.

grow at about the same rate as E cells, exhibiting a typical doubling time of 42 h. E cells (256×10^6 cells/g dry weight) are larger and less aggregated than EE cells (393×10^6 cells/g dry weight), this itself reflecting a carryover feature of cell enlargement during stationary-phase physiology (CA Bonner, RA Jensen, unpublished data).

The soluble protein content was determined on each day after stationary-phase cells were diluted into fresh medium. As has been found elsewhere (10), a marked change in the protein content occurred late in the lag phase (Fig. 2A), increasing from 88 mg protein/g dry weight at the time of dilution to 176 mg/g on d 2. During this time interval the cells must synthesize macromolecular constituents necessary for the transition to exponential-phase physiology (Fig. 1). Throughout exponential growth, the protein content remained at a high level and then decreased as cells neared stationary phase. The protein content of EE cells was similar to the high levels found in E cells at d 2 to 3.

The specific activity of the plastid isozyme, DS-Mn (Fig. 2B) increased nearly two-fold from 23 to 42 nmol/min·mg protein during the first 24 h after stationary-phase cells were transferred to fresh medium. The increase in DS-Mn activity preceded the large increase in soluble protein observed during the transition to exponential phase. The total activity of DS-Mn increased steadily throughout 5 d (panel D). Between d 1 and 2 of the lag phase (Fig. 1), DS-Mn rises if it is related to dry weight and falls if it is related to soluble protein (specific activity). Thus, DS-Mn synthesis begins early. Peak levels are reached at d 2 with respect to dry weight, but are reached at d 1 with respect to specific activity. This indicates that DS-Mn synthesis makes up relatively little of the burst of protein synthesis occurring between d 1 and 2. Both the specific activity and units/g dry weight of DS-Mn of EE cells resembled those found in E cells (d 3–5).

From subculture until d 1, DS-Co levels declined whether related to soluble protein or to dry weight (panel C), probably the consequence of slight dilution by growth since total DS-Co (panel D) remained level. The synthesis of DS-Co between d 1 and 2 resulted in an increased level when related to dry weight, but a decreased level when expressed as specific activity. As with DS-Mn, this indicates that DS-Co is a relatively small proportion of the total soluble protein made in the burst of synthesis between days 1 and 2. However, throughout midexponential phase and early-stationary phase (d 3–6) DS-Co increased in specific activity. Since DS-Co levels did not increase when related to dry weight, this indicates that DS-Co must have become a relatively large proportion of the soluble protein synthesized between days 3 and 6. Note that total cumulative units of DS-Co increased steadily through at least d 5.

The level of DS-Co in EE cells was found to be lower than seen at any physiological stage of a growth cycle initiated with stationary-phase cells. This observation applied whether isozyme activity was related to soluble protein or to dry weight. We suggest that the increasing specific activity of DS-Co from d 3 is triggered by a regulatory mechanism that senses impending stationary-phase physiology. That DS-Co in E cells fails to decline to the level which is truly characteristic of exponential-phase physiology must be due to a phenomenon of carryover. Once exponential-phase cells are ten generations or more removed from a history of stationary-phase physiology (EE cells), isozyme DS-Co exhibits a reproducibly low level of activity that may be the basal activity characteristic of exponentially dividing cells during balanced growth.

The nonparallel relationships shown in Figure 2 during the interval between d 2 and 5 with respect to soluble protein, dry weight, and the activities of DS-Co and DS-Mn illustrate relationships clearly contrary to expectations for balanced growth. The ratios of DS-Co/DS-Mn were calculated and are plotted in

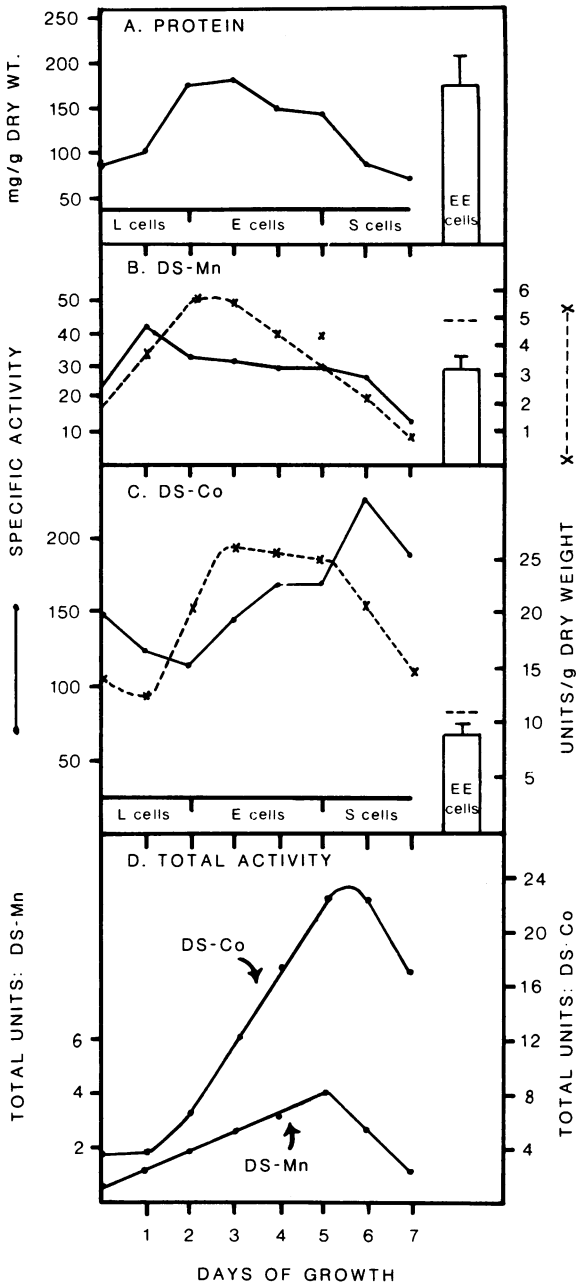

FIG. 2. Soluble protein content and activities of DAHP synthase isozymes assayed in samples harvested daily throughout the growth curve shown in Figure 1. The histograms shown on the right of panels A to C give mean values (with standard deviations indicated) of determinations made from six different cultures of EE cells. A, Soluble protein per gram dry weight. B, Changing activity of DS-Mn expressed as nmol DAHP/min/mg protein (specific activity) or as units (μmol DAHP/min) per g dry weight. C, Changing activity of DS-Co expressed as specific activity or as units/g dry weight. D, Total cumulative activities of DS-Mn and DS-Co.

Figure 3 as a function of time following subculture. The ratio was about 2.5 in EE cells, regardless of when sampling was carried out during the subculture period. Only L cells at d 1 exhibited a ratio this low, and the ratio progressively increased to 14 in extracts from 7-d S cells. The pattern followed by DS-Co during the growth cycle was similar to that found for proteases and both acid and alkaline phosphatases (CA Bonner, unpublished data). These latter activities were dramatically lower in EE

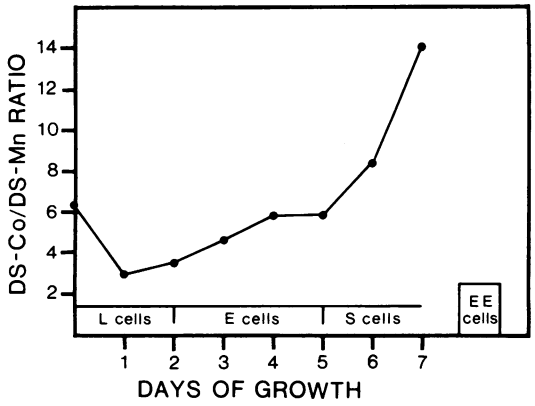

FIG. 3. Variation in the ratio of isozyme DS-Co to isozyme DS-Mn followed at the indicated sampling times throughout the growth curve of *N. silvestris* in suspension culture. The isozyme ratio of the mean values obtained for EE cells is indicated by the histogram on the right.

cells than in E cells.

The independent variation in isozyme levels during growth of cultured cells of *N. silvestris* suggests some form of control whereby the relative levels of the two isozymes are programmed by the physiological state of the cell population. Under conditions of active primary metabolism and protein synthesis (*i.e.*) late in the lag phase), the plastid-localized DS-Mn was at its highest level. The cytosolic isozymes of both chorismate mutase and DAHP synthase are proposed to function within a biochemical network that provides precursors for secondary metabolism in addition to supporting primary protein synthesis in the cytosol (6). The increased levels of DS-Co in stationary phase would be in line with this hypothesis. The basal level of DS-Co in EE cells may reflect the level appropriate for support of protein synthesis in the cytosol during active primary metabolism, whereas the higher levels observed in E cells may reflect an additional increment supporting the demands of secondary metabolism. In this connection it is suggestive that the level of cytosolic chorismate mutase was much higher in organismal tissue than in exponentially growing cells (4). The phenomenon of secondary metabolism is thought to occur in organismal tissue under physiological conditions that resemble the stationary phase of the growth cycle with cultured cells (5, 8, 9, 11, 12). If the elevation of DS-Co does indeed reflect an event of preparation for secondary metabolism, the timing in late exponential/early/ stationary growth seems appropriate.

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