SHORT COMMUNICATIONS

The Pool of Ribonucleoside Triphosphates in Synchronized *Tetrahymena pyriformis*

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INTRODUCTION

Measurements of the molar concentration of ATP in the cell are of interest either for evaluating possible interactions of ATP with other kinds of molecule (for example, chelating and allosteric interactions) or for estimating the energy potential of the cell. The aim of this work, supplemented by results to be presented in a later paper, was primarily to provide data on the above-mentioned interactions and, particularly, to study the possible regulatory effect of changes in nucleoside triphosphate (NTP) concentration on the translational level of protein synthesis, as a continuation of earlier studies (Plesner, 1964; Leick & Plesner, 1968).

This paper describes the use of a sensitive thin-layer chromatography method for the determination of all four ribonucleoside triphosphates. The results confirm and extend earlier results obtained by other workers and ourselves (Scherbaum *et al.*, 1962; Plesner, 1964) and show that substantial changes in the cellular concentrations of all the four nucleotides occur during the division cycle. The variations are of such a magnitude that it seems justified to suggest that the changes in NTP concentration might act as regulatory signals for cell division.

METHODS

Materials and media. Carrier-free [32 P]orthophosphate was purchased from Risø, Denmark; [8- 14 C]-adenosine 5'-triphosphate, ammonium salt (40 mCi mol $^{-1}$) from The Radiochemical Centre, Amersham; and the common ribo- and deoxyribonucleotides from Sigma. Cellulose powder MN300 was from Machery and Nagel, Düren, Germany; polyethyleneimine (PEI; 50 %, w/v, in water) from Fluka, Buchs, Switzerland; and bakelite rigid vinyl sheets from Holm and Halby, Rødovre, Denmark. The growth medium (PPY) contained: 0.75 % (w/v) proteose peptone (Difco); 0.75 % (w/v) yeast extract (Difco); 1.5 % (w/v) glucose; 1 mM-MgSO $_4$; 0.05 mM-CaCl $_2$; 0.1 mM-ferric citrate; 0.5 mM-KH $_2$ PO $_4$. The medium was autoclaved in bottles with rubber stoppers. Cell counting solution contained 0.1 % (v/v) glutaraldehyde and 0.2 % (w/v) NaCl. Liquid scintillation counting solution consisted of 0.07 g 1,4-bis-(5-phenyloxazol-2-yl)benzene (POPOP) and 3.5 g 2,5-diphenyloxazole (PPO) in 700 ml toluene plus 300 ml 96 % (v/v) ethanol.

Culture. *Tetrahymena pyriformis* GL (amicronucleate) was used in all experiments. The cells were grown in 40 to 50 ml of PPY medium containing 32 P, in either 100 or 200 ml bottles as described by Hjelm (1970). A 4 h synchronizing treatment of alternate 30 min periods at 34 and 28 °C was programmed to start when the cells had grown for over six generations. Population densities varied between 10 6 and 5 $\times$ 10 6 cells ml $^{-1}$. The synchronized cell divisions occurred at 80 min and 175 min after the end of the synchronizing heat treatment (EH).

Sampling. Samples (2 ml) were taken at intervals of 20 min from EH to EH+80 min during the first synchronous division cycle, and at intervals of 15 min from EH+80 to EH+170 min during the second division cycle. They were transferred to pre-chilled centrifuge tubes and sedimented at 20000 g at 4 °C for 2 min to form a hard pellet.

Cell counts. These were made on duplicate samples. Each culture sample (1 ml) was mixed with 9 ml of the counting solution, stirred and counted in a Coulter counter, model B.

Extraction of acid-soluble nucleotides. All operations were performed at 0 to 4 °C. Ice-cold 0.4 M-perchloric

acid (PCA; 2 ml) was added to the pellets obtained as described above. Tubes were shaken briefly, allowed to stand for 30 min at 0 °C, and then centrifuged at 20000 *g* for 10 min. A portion (1 ml) of the supernatant was neutralized to about pH 6 with 6 M-KOH containing 0.5 M-EDTA, and allowed to stand for 30 min. The KClO₄ precipitate was separated by centrifuging at 20000 *g* for 5 min. The resultant supernatant was used immediately or deep-frozen for later use.

Chromatography. PEI-cellulose thin-layer plastic plates (20 × 20 cm) were prepared as described by Randerath & Randerath (1964). Extract (200 μl) was mixed with 10 μl of marker solution (containing 1 μmol of each of the following: GTP, dGTP; ATP, dATP; UTP, dTTP; CTP, dCTP) and applied to the origin with a motorized Hamilton syringe. The plates were dried in a current of cold air, washed in anhydrous methanol (5 min), dried and chromatographed stepwise in two dimensions using Neuhaard's solvents (Neuhaard, 1967). Before chromatography in the second direction, the plates were treated with Tris/methanol and anhydrous methanol as described by Randerath & Randerath (1964). Chromatograms were radioautographed on Ilford or Kodak X-ray films (exposure 18 h), and then spots were cut out, placed into plastic vials and dried at 60 °C for 90 min. The vials were cooled for 10 min before adding 5 ml of the scintillation solution, and then counted in a Beckman liquid scintillation counter.

Quantification. The nucleotides were quantified by measuring their ³²P content after isotopic equilibrium had been reached (usually after the sixth generation). [³²P]Orthophosphate (2 to 5 μCi mol⁻¹) was aseptically added to the medium just before inoculation. A sample (10 μl) of the medium was pipetted on to (1.5 × 1.5 cm) PEI thin-layer chromatography sheets in plastic vials for the determination of the specific activity of the medium based on its inorganic phosphate content as determined by Peel & Loughman (1963). Corrections for radioactive decay and recovery were made.

Recovery. Cells were killed with formaldehyde, precipitated by centrifugation, and resuspended in PCA. [¹⁴C]ATP (5 μCi mmol⁻¹) was added, a 10 μl sample was taken and the cells were extracted as described. A portion (10 μl) of the extract was taken and counted along with 10 μl of unextracted sample. Recovery from the extraction procedure alone was 98.5 to 100 %. Recovery from live cells was determined by a similar procedure but with omission of the formaldehyde treatment. Subsequent chromatography of the neutralized PCA extract showed that the recovery from the chromatography step alone was 85 to 95 %. The final recovery ranged between 83 and 94 %.

RESULTS AND DISCUSSION

Autoradiography of the chromatograms revealed eight spots, six of which were identified as ATP, dATP, GTP, UTP, CTP and dTTP. Two spots, X and Y, were unidentified. X ran slower than GTP, being very close to, but below xanthosine triphosphate. From its brilliant blue fluorescence under HCl fumes, X must be a derivative of guanine. Comparison of one-dimensional chromatograms of formic acid extracts of *Escherichia coli* 15 TAU^{-A} with PCA and formic acid extracts of heat-synchronized *Tetrahymena* revealed that X was not ppGpp (Cashel, 1969; Buckel & Bock, 1973). Spot Y was not seen under ultraviolet light. Thus four ribonucleoside and two deoxyribonucleoside triphosphates were found in synchronized *Tetrahymena pyriformis*. If more cell material was used for analysis, or if the specific activity of [³²P]orthophosphate was increased, dGTP and dCTP were also found. ATP, GTP, UTP, CTP and X were present throughout the division cycles, but dATP and dTTP appeared only between EH+20 and EH+60 min and from EH+110 min to EH+155 min. They were not detectable at other times during the two division cycles.

Pool sizes

The sizes of the pools and their variation with time are shown in Fig. 1. The total amount of nucleotides (Fig. 1*a*) increased from EH and reached a maximum at about EH+60 min, 20 min before the first synchronized division which occurred at EH+80 min; the amounts then decreased to a minimum at EH+110 to 125 min, and rose to a second maximum at EH+155 min before the second division which occurred at EH+175 min. This is in accordance with earlier findings (Plesner, 1964), except that the nucleotide content did not fall from its high level at EH before increasing to the maximum at 60 min. The purine nucleotides ATP and GTP formed 90 to 95 % of the total pool, ATP alone forming about 90 % of the purine nucleotides. CTP and UTP comprised 5 to 10 % of the total nucleotides, UTP forming about 67 % of the pyrimidine nucleotides.

Fig. 1. Amounts of ribonucleoside triphosphates in *Tetrahymena pyriformis* during the first and second synchronous division cycles. Cells were grown exponentially in PPY medium containing [32 P]orthophosphate (2 to 5 μ Ci mol $^{-1}$) for over six generations to achieve isotopic equilibrium, then induced for synchrony of divisions. At intervals after the end of the synchronizing heat treatment (EH), samples were taken, chromatographed, and the nucleotides were quantified as described in Methods. Results shown are the average from five experiments. (a) Sums of ribonucleoside triphosphates: $\square$, ATP+GTP+CTP+UTP; $\triangle$, ATP+GTP; $\circ$, UTP+CTP. (b) Individual ribonucleoside triphosphates: $\bullet$, ATP; $\triangle$, UTP; $\circ$, GTP; $\square$, CTP.

Figure 1(b) shows the variation of the individual nucleotides during the division cycles. In the first division cycle, ATP, GTP and CTP reached a maximum at EH + 60 min while UTP peaked 20 min earlier. At the peaks, the contents of ATP, GTP and CTP were increased threefold and UTP was increased fourfold. During the second division cycle, the levels of all the nucleotides fell to a minimum at about EH + 125 min (except UTP at EH + 110 min) and rose to a maximum at EH + 155 min. The content of the nucleotides was lower than during the first division cycle. If the peak at EH + 155 min corresponds to the first peak at EH + 60 min, then its values are correspondingly lower than those of the first peak. This would show that desynchronization was occurring.

The very low content of pyrimidine nucleoside triphosphates explains why earlier investigations using less sensitive methods failed to detect these compounds.

Earlier Plesner (1963) suggested that variations in the NTP contents reflected changes in the rate of protein synthesis. Sachsenmaier *et al.* (1969) also interpreted the division-related increase in NTP in *Physarum polycephalum* as caused by a decrease in macromolecular synthesis, but argued that changes in nucleic acid metabolism were more likely to be the

main cause. An investigation of the effect of actinomycin D on the NTP levels (Echetebeu, 1972) supports this.

It seems that the division-related increase in NTP content is a passive or secondary change resulting from a decrease in the functional intensity of the protein-synthesizing apparatus, which is taken to include transcriptional and translational processes.

Although a peak concentration of energy-rich nucleotides is found immediately prior to cell cleavage, there is no reason for identifying this as an energy reservoir for cell division because its extent is too small (Echetebeu, unpublished). This, together with the conclusion that NTP accumulates passively as a result of a decreased production of macromolecules, encourages us to abandon the teleonomic concept of a cell which accumulates energy in anticipation of an expenditure of energy (during cleavage).

Thus there seems to be good reason to abandon the energy reservoir theories, but the peak content of NTP might still have an important regulatory function.

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