Nucleoside Triphosphate Pools in Synchronous Cultures of *Escherichia coli*[1]

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Endogenous nucleoside triphosphate pools in synchronized cultures of *Escherichia coli* B/r/1 oscillate as a function of age. Purine nucleoside triphosphates show a gradual 50% increase from zero age to the time of subsequent division, immediately prior to division. In contrast, pyrimidine nucleoside triphosphates undergo a dramatic change of about 50% in the first half of the generation at a time coincident with the termination of a round of deoxyribonucleic acid replication. A 50 to 70% increase starts at the initiation of the next round of deoxyribonucleic acid replication and continues until cell division, in parallel with the purine nucleotides. The fluctuation of pyrimidines between zero age and the middle of the division cycle suggests a functional relationship for pyrimidine metabolism and the regulation of cell division.

The biochemical mechanism which couples deoxyribonucleic acid (DNA) replication and cell division is not understood, although some activities of enzymes involved in nucleic acid metabolism during the cell cycle of eucaryotic cells have been examined. Johnson and Schmidt (11) found periodic changes in thymidylate kinase in synchronous cultures of *Chlorella pyrenoidoso*. The enzyme reached maximal activity prior to the maximal rate of DNA synthesis. Turner et al. (22) found a strict correlation between ribonucleotide reductase levels and that fraction of L cells which were replicating their DNA. After DNA replication, there was a continuous decay of enzymatic activity, which decreased shortly after cell division in Chinese hamster fibroblast cells (20). Cleaver described the increase of thymidine enzyme activities at the onset of DNA replication and then a decrease in their activity as "DNA synthesis ceases" (4). He suggested that the close relationship of these enzymes with DNA synthesis may reflect control of initiation of DNA replication.

In bacteria, there is no information about the activity of enzymes involved in nucleic acid metabolism. Lark (12) examined the fluctuation of deoxyribonucleosides as a function of the cell cycle of *Salmonella typhimurium*. He noted an increase in the pool of thymidine coincident with the age at which there was increased synthesis of

DNA. He did not measure the purine deoxyribonucleosides. Since cell division is dependent upon DNA replication (3, 8, 13), and the end of a round of replication is required for cell division in normal cells (3, 9), it is possible that a signal for division could be activated as a result of the completion of the round of DNA replication. That signal could be directly or indirectly related to the precursors of DNA synthesis. This report describes fluctuations in the endogenous nucleoside triphosphate pools as a function of the growth cycle of *Escherichia coli* B/r.

MATERIALS AND METHODS

Bacterial strains and culture methods. *E. coli* B/r/1 (ATCC 12407), a prototroph, was used for synchronous cultures since it forms single cells. The cultures were maintained on nutrient agar slants and in liquid cultures of 0.05 M tris(hydroxymethyl)aminomethane (Tris)-glucose-mineral salts medium. A growth medium that was low in PO_4 was used to provide efficient incorporation of ^{32}Pi into nucleic acids. The low PO_4 media had the following composition: 0.05 M Tris, 2 × 10^{-5} M K_2HPO_4, 10^{-4} M $CaCl_2$, 2 × 10^{-3} M $MgCl_2$, 3 × 10^{-6} M $FeCl_2 \cdot 7H_2O$, 5.2 × 10^{-2} M NaCl, and 3 × 10^{-2} M $(NH_4)_2SO_4$ × 0.2% carbon source. Cultures were incubated at 37 C in a shaking water bath. Synchronization of the cells was accomplished by using the technique of Helmstetter and Cummings (7). Cell growth was measured with a modified Coulter counter (3).

Determination of acid-soluble nucleoside triphosphate pools. Samples were prepared by the method of Irr and Gallant (10). Chromatographic separation was carried out on polyethyleneimine (PEI)-impregnated cellulose thin layers prepared by the method of Randerath and

Randerath (18, 19). Preliminary washings of the thin layers and chromatography were carried out by the methods of Irr and Gallant (10). Slight modifications were made in the solvents. In the first direction, the first solvent was 1 N acetate-1 M LiCl (1:1, v/v) for 12 cm; the second solvent was 1 N acetate-1.5 M LiCl (1:1, v/v) for the remainder of 5.5 hr. The solvent of the second direction was 3 M NH$_4$ acetate plus 4.3% borate (pH 7) for 4.5 hr. To ensure distinct separation of deoxyribonucleotides from their corresponding ribonucleotides, wicks made of Whatman no. 3 paper were attached to the thin layer for each direction. Autoradiography and removal of nucleoside triphosphates were done by the methods of Irr and Gallant (10). The film was exposed to the radioactive chromatogram for 3 days. Counts per minute were corrected for isotope decay, counting efficiency, volume spotted, conversion factor for microcuries to disintegrations per minute, specific activity, and rate of mass increase to give nanomoles of ^{32}Pi per milliliter per nucleotide.

Chemicals. ^{32}Pi (orthophosphate-free) was obtained from Tracerlab; ^{3}H-thymidine was from Schwartz Bio Research, Inc.; unlabeled marker nucleotides were from Calbiochem; PEI was from Chemirad Corp.; and Cellulose MN 300 HR was from Canlab.

RESULTS

Growth curves in low phosphate concentrations. The concentration of deoxynucleotide and ribonucleotide precursors in bacteria is extremely low

(14). Since the yield of cells obtained for synchronous cultures is quite low (2 × 10^6 to 1 × 10^7 cells per ml) when the membrane technique for synchronization is used, a high specific activity of ^{32}Pi is required to obtain sufficient labeling of the precursors. Since ^{32}Pi emits beta rays which are deleterious for biological systems, a specific activity of ^{32}Pi must be obtained high enough to measure the pools yet low enough in total activity so as not to disturb the physiology of the cells. The optimum concentration of PO$_4$ and the amount of ^{32}Pi to be added to the cultures were determined by using a Coulter counter to monitor exponential growth of cell numbers and the size distribution of the population. A shift in the size distribution of a population was reflected by a change in the peak position of the size distribution curve. Such a change is considered an index of unbalanced growth (19). As seen in Fig. 1, a PO$_4$ concentration of 1 × 10^{-4} M allowed an exponential increase in cell number to a density of 7.5 × 10^8 cells per ml; a shift in the size distribution, however, occurred at 5 × 10^8 cells per ml. Similar analyses were applied to cultures growing at 2 × 10^{-5} and 5 × 10^{-5} M PO$_4$. Since 2 × 10^{-5} M PO$_4$ showed no change in balanced growth until a density of 2 × 10^8 cells per ml was reached (Fig. 2), this concentration

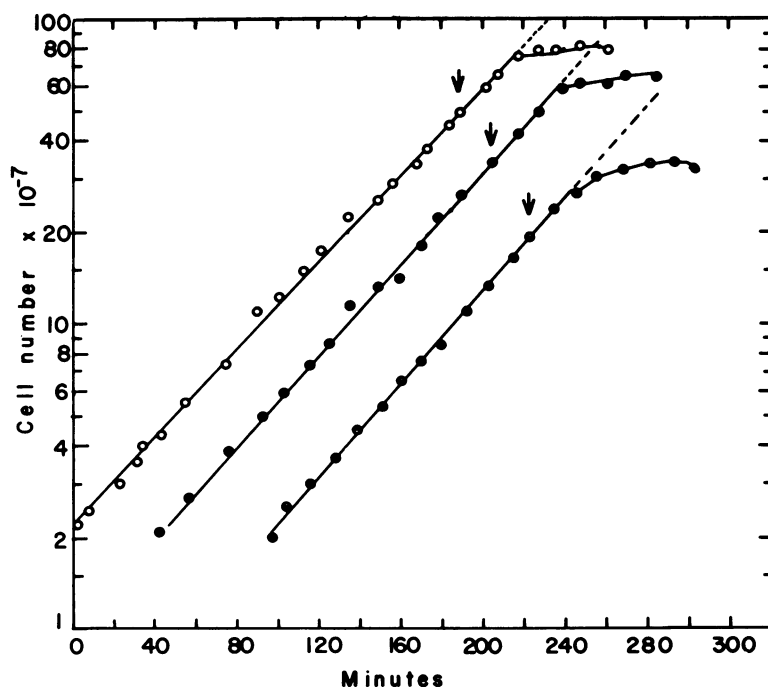FIG. 1. *Effect of PO$_4$ concentrations on the growth of Escherichia coli B/r/1. Cells grown for several generations in 10^{-4} M PO$_4$ (O), 5 × 10^{-5} M PO$_4$ (●), or 2 × 10^{-5} M PO$_4$ (⊖) were followed with a Coulter counter for several hours. The increase in cell number is plotted as a function of time. The arrow (↓) indicates the time at which a shift in the size distribution of the total population occurs.*

of PO_4 was chosen as the lower limit for subsequent experiments. Exponential increases of cell numbers continued almost to 3×10^8 cells per ml. Figure 2 shows a shift in the size distribution of a population limited for PO_4.

Effect of different amounts of [32]Pi. Growth curves based on viable cell number were constructed at different specific activities of [32]Pi for cells which were grown in the same concentration of PO_4 (2×10^{-5} M PO_4). A culture which had been growing for six generations and had reached a density of 10^7 cells per ml was diluted into culture vessels containing the following specific activities of [32]Pi: (i) 100 μCi of [32]Pi/μmole of PO_4; (ii) 250 μCi of [32]Pi/μmole of PO_4; (iii) 500 μCi of [32]Pi/μmole of PO_4; and (iv) a control with no radioactivity. At regular time intervals, samples were diluted and plated for viable counts. Figure 3 shows the growth rate obtained by plotting viable cells as a function of time. The doubling time for the cultures containing 100 and 250 μCi of [32]Pi/mole of PO_4 is identical to the control. On the other hand, the culture containing 500 μCi of [32]Pi/μmole of PO_4 showed a loss in viability after 60 min of incubation. This experiment was repeated by using optical density readings at 660 nm over a 3-hr period; the results were similar to those obtained when viability was measured. Thus the cell death in the presence of a specific activity of 500 μCi of [32]Pi/μmole of PO_4 was due to an immediate effect and not to some delayed action of the radioactive [32]Pi affecting a small clone of bacteria on a plate. A specific activity of 100 μCi of [32]Pi/μmole of PO_4 was chosen as the optimal working level.

In an effort to alleviate the deleterious effects of [32]Pi, similar experiments were repeated with [33]Pi. This isotope has a lower energy level (0.25

FIG. 3. *Effect of the specific activity of [32]Pi/PO_4 on the growth of Escherichia coli in 2×10^{-5} M PO_4 as indicated by viable cell number. Cultures grown for several generations in 2×10^{-5} M PO_4 received [32]Pi which gave a final specific activity of 100 μCi of [32]Pi/μmole of PO_4 (●); 250 μCi of [32]Pi/μmole of PO_4 (◑); or 500 μCi of [32]Pi/μmole of PO_4 (⊖). No radioactivity was added to a control culture (O). Viable cell number is plotted as a function of time.*

Mev, cf. 1.7 for [32]Pi) and a longer half-life (25 days, cf. 14.3 for [32]Pi; New England Nuclear Corp.). Presumably [33]Pi is safer to handle and would allow longer-term experiments. Much to our surprise, [33]Pi at the same specific activities used for [32]Pi showed loss of viability after only a few minutes in 100 μCi of [33]Pi/μmole of PO_4, and the cells at higher specific activities showed no viability at all.

Levels of endogenous nucleoside triphosphate pools in exponential cells. Control levels of nucleoside triphosphate pools were established in exponentially growing cultures of *E. coli* B/r/1 grown in a turbidostat. A sample rate was predetermined, and the culture volume was adjusted so that dilution rate would correspond to the specific growth rate (0.91/hr). A culture was grown for several generations to a density of 10^8 cells/ml, at which time [32]Pi was added to give a specific activity of 100 μCi of [32]Pi/μmole of PO_4, and dilution of the culture was started to maintain the steady state. The culture was di-
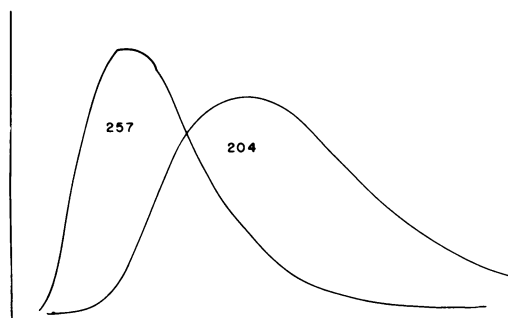
FIG. 2. *Shift in the size distribution of Escherichia coli as the culture stops growing in 2×10^{-5} M PO_4. The designations 204 and 257 indicate the time at which the size distribution is monitored for cells grown in 2×10^{-5} M PO_4 (●) from Fig. 1, and describes the size distribution of normal cells and cells starved of PO_4, respectively. Ordinate refers to relative cell number; abscissa is increasing cell size.*

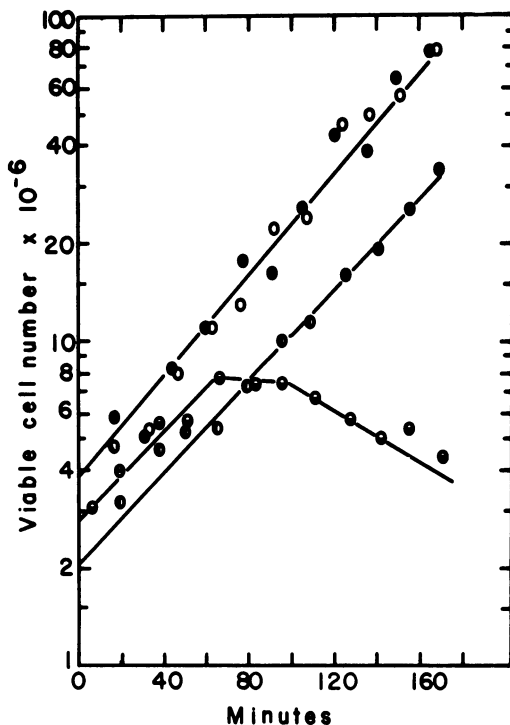luted with medium of the same PO_4 concentration and of the same specific activity of $^{32}Pi/PO_4$. In this manner, an exponential culture was maintained at 10^8 cells per ml in the presence of 100 μCi of $^{32}Pi/\mu$mole of PO_4. After a 30-min equilibration period, samples were withdrawn every 5 min for 2 hr and analyzed as described previously. A balanced steady state was evidenced by radioactive counts per minute per nucleotide which were almost identical in all samples. An example of the degree of fluctuation is illustrated in Fig. 4 for two of the eight nucleoside triphosphates, deoxythymidine triphosphate (dTTP) and uridine triphosphate (UTP). The highest fluctuation for any nucleoside triphosphate was less than 10%. The actual concentrations of the endogenous nucleoside triphosphates are listed in Table 1.

Nucleoside triphosphate pools as a function of the division cycle (cell age). Figure 5 shows the growth curve of a synchronous culture which was analyzed for nucleoside triphosphate concentrations. Also shown in Fig. 5 are three different
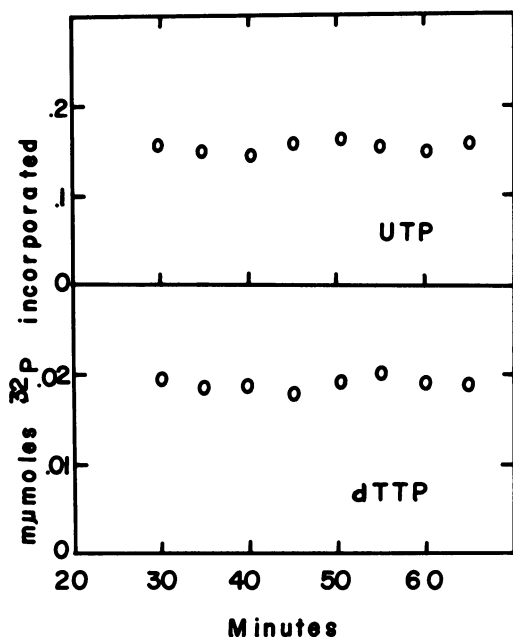
TABLE 1. *Nucleoside triphosphate pool levels in exponentially growing Escherichia coli B/r/1*

Nucleoside triphosphate[a]	Amt of ^{32}Pi[b]
GTP	0.212
ATP	0.559
CTP	0.151
UTP	0.153
dGTP	0.022
dATP	0.048
dCTP	0.020
dTTP	0.019

[a] Abbreviations: GTP, guanosine triphosphate; ATP, adenosine triphosphate; CTP, cytidine triphosphate; UTP, uridine triphosphate; d, deoxy.

[b] Expressed as nanomoles of ^{32}Pi per nucleotide per 10^8 cells per milliliter.

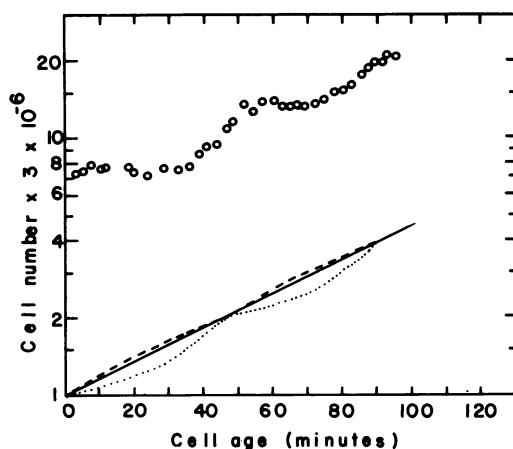FIG. 5. *Relationship between the increase in mass and the increase in cell number during synchronous growth of Escherichia coli. The increase in cell number during synchronous growth in 2 × 10⁻⁵ M PO_4 is plotted as function of time (O). The increase in cell mass is plotted as a function of time assuming: (i) an exponential increase in cell volume (solid line); (ii) a linear increase in cell volume (dashed line); or (iii) accelerated growth just prior to cell division (dotted line).*

FIG. 4. *Level of uridine triphosphate (UTP) and deoxythymidine triphosphate (dTTP) as established by measurements of ^{32}Pi incorporation during the steady-stage growth of Escherichia coli in a turbidostat. Cells were maintained at 10^8 cells per ml in 2 × 10⁻⁵ M PO_4 with a specific activity of 100 μCi of $^{32}Pi/\mu$mole of PO_4. The concentration of ^{32}Pi incorporated is plotted as a function of time. Similar measurements were done for all of the nucleoside triphosphates; UTP and dTTP are given as examples. A summary is presented in Table 1.*

curves, drawn to illustrate three possible types of mass increases during synchronous growth: linear, exponential, and a slow to rapid increase in growth rate. Since nucleoside triphosphates are expressed as concentrations, corrections are necessary on a per mass basis rather than on a per number basis. Nucleoside triphosphate concentrations were corrected assuming the Painter-Marr model for mass increase (16), in which cells exhibit the slowest rate of mass increase immediately after cell division, and those just beyond a half generation grow at the maximal

rate. There is supporting evidence for this model from time-lapse photography studies (1) of single cells of *E. coli*.

The synchronous culture was grown for one generation in the presence of ^{32}Pi before sampling for analysis of nucleotides. Samples were taken at 3-min intervals during the period of the second generation of growth, and were analyzed for nucleoside triphosphates as described above. The fluctuation in the nucleotide levels is indicated in Fig. 6, during a full generation of 42 min. Bearing in mind that the data have been corrected for mass increase, the purine nucleoside triphosphates increased in concentration, reached a maximum at 75 min, and dropped sharply as cell division occurred (Fig. 6A and 6C). There was an exaggerated effect on deoxyadenosine triphosphate (dATP), but clearly the maximal concentration of purine nucleoside triphosphates occurred between 60 and 75 min. In all cases, there was approximately a 50% increase in the concentration near the time of cell division as compared to the levels at zero time (42 min). The pyrimidine nucleotides all exhibited a double-peak pattern during the division cycle between the time of cell division and termination of DNA replication, and again just before the following cell division (Fig. 6B and 6D). The purine deoxyribonucleoside triphosphate concentrations showed almost parallel fluctuation patterns (Fig. 6C), whereas the pyrimidine ribonucleotides, cytidine triphosphate (CTP) and UTP, oscillated in opposite directions (Fig. 6B). The information in Fig. 6 was computed according to percentage of change and is presented in Fig. 7A and B. Purine ribo- and deoxyribonucleoside triphosphates are shown in Fig. 7A. In Fig. 7B, the data for pyrimidine ribo- and deoxyribonucleoside triphosphates are presented in a similar manner.

DISCUSSION

In our examination of endogenous nucleoside triphosphates during the growth cycle of *E. coli*, we made a concerted effort to maintain balanced growth of a synchronous culture. Figure 3 indicates that a concentration of 2×10^{-5} M PO$_4$ maintains balanced growth to 2×10^8 cells per ml. Since our cell density never exceeded 1×10^8 cells per ml, we felt that 2×10^{-5} M PO$_4$ offered sufficient margin of safety that the cells would not be adversely affected by the concentration of PO$_4$ we employed.

The effects of increasing the specific activity indicated that a specific activity of 100 and 250 μCi of ^{32}Pi per μmole of PO$_4$ did not adversely affect the growth of the culture as determined by viable counts and by the size distribu-

tion as a function of time (Fig. 4). On the other hand, the viability drops sharply when the cells have grown for just over one generation in the presence of 500 μCi of ^{32}Pi per μmole of PO$_4$ (Fig. 4). By combining these two results (Fig. 3 and Fig. 4), the lower limits of PO$_4$ concentration and the upper limit of the specific activity of ^{32}Pi per μmole of PO$_4$ were established at 2×10^{-5} M PO$_4$ and 100 μCi of ^{32}Pi per μmole of PO$_4$, respectively.

From the summary of the steady-state concentrations given in Table 1, the concentration of ribonucleoside triphosphates is found to be very nearly 10 times greater than their deoxyribonucleotide counterparts. The concentration of the ribonucleoside triphosphates decrease in the order adenosine triphosphate (ATP), guanosine triphosphate (GTP), and CTP = UTP. Likewise, the concentration of the deoxyribonucleoside triphosphates decreases in the order dATP, dGTP, and dCTP = dTTP. These results are in agreement with those of others (5, 14).

The concentrations of nucleoside triphosphates as a function of the cell cycle are shown in Fig. 6A and 6B. The percentage of change of the purine and pyrimidine nucleoside triphosphates is given in Fig. 7A and 7B, respectively. It is clear that both purines and pyrimidines increase just before cell division. On the other hand, dTTP and UTP also increase at or about the middle of the division cycle. There is a slight increase in dCTP about the same time, but it is not clear that the increase is significant. Alternatively, dCTP decreases just slightly after the increase in dTTP. Clearly, the decrease in dCTP must occur from either decreased synthesis or increased conversion of dCTP. The increase in dTTP could occur due to an increased rate of synthesis or a decreased rate of conversion of dTTP to the product (presumeably DNA).

One of the combinations which seems attractive to us is a reduced conversion of dTTP by incorporation into DNA as rounds of DNA replication are completed. This is sufficient to explain the increase in dTTP but does not explain the decrease in dCTP and CTP (Fig. 7B). An increase in dTTP could account for a concomitant decrease in CTP and dCTP, since low dTTP generally is correlated with an increase in CTP and dCTP (2, 16, 17). It has been shown that dTTP is a universal activator of ribonucleoside diphosphate reductase (2). Increased activity of the reductase would decrease the level of CTP, as observed in Fig. 7B. If CTP is rapidly converted to dCTP, one would anticipate an immediate increase in dCTP. As CTP decreases, one would anticipate that dCTP would eventually decrease as the precursor (CTP) supply is ex-
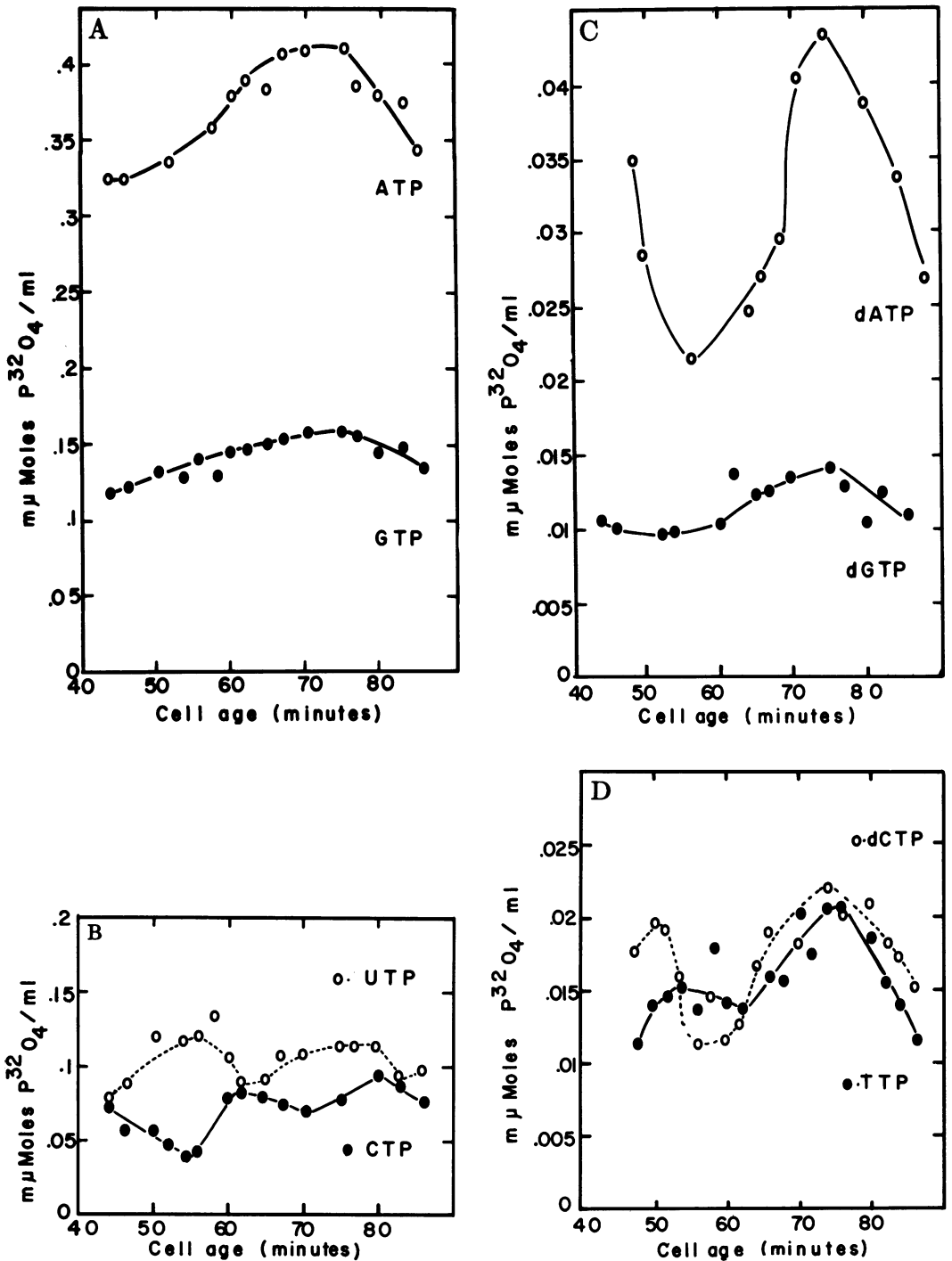
FIG. 6. *Concentration of deoxy- and ribonucleoside triphosphates during synchronous growth of Escherichia coli B/r/1. Synchronous cultures were grown at a density of approximately* 2×10^7 *cells per ml in* 2×10^{-5} M PO_4 *with a specific activity of 100* μCi *of* $^{32}Pi/\mu mole$ *of* PO_4. *Samples were taken from 45 to 90 min, which corresponds to the beginning and to the end of the second cell division cycle. A, Fluctuation of purine ribonucleotides ATP* (O) *and GTP* (●). *B, Fluctuation of pyrimidine ribonucleotides UTP* (O) *and CTP* (●). *C, Fluctuation of purine deoxyribonucleotides dATP* (O) *and dGTP* (●). *D, Fluctuation of pyrimidine deoxyribonucleotides dCTP* (O) *and dTTP* (●). *Abbreviations used in Fig. 6 and 7: ATP, adenosine triphosphate; GTP, guanosine triphosphate; CTP, cytidine triphosphate; dATP, deoxyadenosine triphosphate; dGTP, deoxyguanosinetriphosphate; dCTP, deoxycytidine triphosphate; dTTP, deoxythimidine triphosphate.*
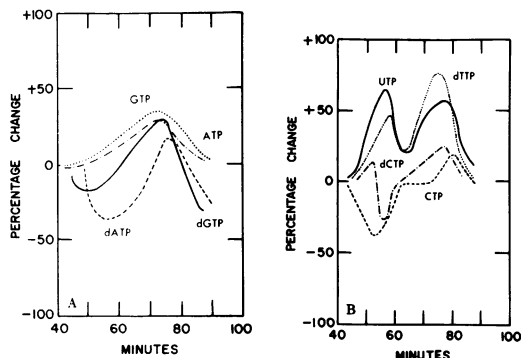
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FIG. 7. Data from Fig. 6 converted to percentage of increase or decrease relative to the first sample taken at 45 min and plotted as a function of the cell age of the culture. A, Percentage of increase or decrease of purine ribo- and deoxyribonucleoside triphosphates; ATP (dash-and-dot line), GTP (dotted line), dATP (dashed line), and dGTP (solid line). B, Percentage of increase or decrease of pyrimidine nucleoside triphosphates; CTP (dashed line), UTP (solid line), dCTP (dash-and-dot line), and dTTP (dotted line).

hausted, providing dCTP is continuously converted to dUTP. This seems to be exactly the case as seen in Fig. 7B. As CTP decreases coincident with the increase in dTTP, there is a short burst of dCTP and then the dCTP concentration falls to a minimum after the decrease in CTP.

The decrease in CTP with a decrease in dCTP following shortly thereafter, implies that the conversion of UTP to CTP is blocked. There is no known mechanism for blocking CTP synthetase which is compatible with our results, but such a block would explain the accumulation of UTP and the depletion of CTP seen in the middle of the cell cycle (Fig. 7B).

Lark (12) observed an increase in thymidine coincident with an increase in DNA synthesis. Although we have observed an increase in dTTP at or near the start of a round of replication, we think our results are supportive, in spite of the fact that Lark examined nucleosides and we examined nucleotides. From additional experiments, Lark concluded that there was no causal relationship between the initiation of DNA replication and the fluctuation in nucleoside pools. From our experiments, we cannot establish any causal relationship between the fluctuation of the nucleotide pools and the initiation of DNA replication. Indeed, at this growth rate (generation time = 42 min), the end of the round of DNA replication and the beginning of a new round of replication both occur at a cell age of approximately 20 min. Therefore, it is impossible to correlate the increase in dTTP with one or the other. To make such a conclusion, additional

experiments should be made with generation times in which the ends of the rounds at the initiation of DNA replication are not coincident.

Recent experiments by Werner (23) cast some suspicion upon the actual precursor of DNA synthesis. His experiments are interpreted to mean that deoxythymidine monophosphate (dTMP), and not dTTP, is the immediate precursor of DNA replication. If so, one can legitimately question the relevancy of fluctuations in the deoxyribonucleoside triphosphates to the replication cycle of DNA. We can only state: (i) that there is a dramatic fluctuation in the nucleoside triphosphates over the cell division cycle (Fig. 7A and Fig. 7B); and (ii) whether or not dTTP is the immediate precursor of DNA, the fluctuations in dTTP may be indirectly, but nevertheless precisely, related to the replication cycle.

If a dependence relationship exists between DNA replication and cell division, one would anticipate that cell division is dependent upon the completion of DNA replication. Clearly, there is a severe penalty for cells which divide in the absence of DNA synthesis, but it is not obvious that cells which continue to replicate DNA in the absence of cell division, i.e., those cells which form filaments, are at any severe disadvantage. It seems logical that the coordination of cell division and DNA replication would be mediated by a mechanism which has its origin and its dependence upon the state of DNA replication. Experimental data support this interpretation (3). The nucleotide precursor for DNA synthesis might provide a biochemical mechanism by which DNA replication and cell division are coupled. Since dTTP is unique to DNA, it or a closely related compound might serve as the signal to coordinate cell division and DNA replication. Upon completion of DNA replication, the reduction in growing point would lead to an accumulation of dTTP or a closely related compound. Increase to an active threshold level would activate those division processes required to effect or to complete cell division. This cannot be a sufficient explanation, since dTTP fluctuates twice during the cell cycle. On the other hand, the two situations are distinct in that the ratio of all nucleoside triphosphates is constant near division but is vastly different at or near the end of the round. Thus, a combination of, say, high dTTP and low dCTP at the end of a round of DNA replication may be required as the signal for cell division. In fact, the condition of low dCTP or CTP which occurs only at the end of the round might be the controlling effector.

Since cyclic AMP (6, 17) and ppGpp (21) appear to interact with the CAP protein and the Psi factor for the ribosomal locus, respectively, we find it tempting to suggest that dCTP or

CTP, or a combination of high dTTP and low dCTP, interacts with a Psi factor for cell division. For example, dCTP or CTP might inhibit transcription of division genes by interaction with a Psi factor analogous to ppGpp and Psi (ribosomal) during most of the cell cycle. At the end of a round of replication, when dCTP or CTP is low, dissociation from the Psi (division) would occur and transcription of a group of distal division genes would ensue.

ACKNOWLEDGMENTS

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