IN VITRO INCORPORATION OF DEOXYRIBONUCLEOSIDE DIPHOSPHATES INTO DNA

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Summary: ADP replaces ATP in promoting the incorporation of deoxyribonucleoside di- and triphosphates into DNA in a bacterial in vitro system. Analysis of the nucleotides present in the cell lysate suggests that diphosphates are incorporated through intermediates different from deoxyribonucleoside triphosphates.

Introduction: Our earlier studies on the flow of thymine through intracellular nucleotide pools into DNA led to the conclusion that DNA replication and repair synthesis draw their precursors from separate pools (1,2). Unfortunately, these experiments did not allow us to determine whether replication uses precursors different from deoxyribonucleoside triphosphates, the known precursors of repair polymerases, or whether there exist two separate triphosphate pools, one for replication and one for repair. To distinguish between these two possibilities we studied the incorporation of deoxyribonucleotides into DNA in a cell-free replication system where compartmentation of nucleotide pools is unlikely. We used the cellophane disc system developed by Schaller et al. (3) which, like other in vitro DNA replication systems, requires ATP in addition to the four deoxyribonucleoside triphosphates (4,5,6). It has been shown that deoxyribonucleside diphosphates can replace the triphosphates as precursors of DNA in this system (3). To explain this fact it has generally been assumed that diphosphates are phosphorylated to triphosphates prior to their incorporation into DNA. In this paper we report studies on the conversion of di- into triphosphates in the cellophane disc system. Our results suggest that such a conversion to triphosphates, although occurring under certain conditions, is probably not required for the incorporation of diphosphates into DNA. It seems more likely that diphosphates and triphosphates have to be converted into another form of activated nucleotide prior to their incorporation into DNA.

Experimental: The in vitro replication system developed by Schaller et al. (3) was slightly modified and used throughout the experiments. E. coli DI10 (polA1$^-$, endoI$^-$, thy$^-$) was grown at 25°C to 2 x 10^8 cells/ml in Bacto-Penassay broth (Difco) supplemented with 2 μg/ml of thymine. Cultures were chilled to 0°C, and the cells centrifuged, washed with minimal medium (1) (containing 0.05 M tris instead of tricine), and resuspended at 5 x 10^{10} cells/ml in the same medium containing 0.33 M sucrose, 10 mM EGTA and 0.5% Brij-58 (Ruger Chemical Co.). 1 μl of the cell suspension was incubated at 2°C with 1 μl of egg white lysozyme (2 mg/ml) on a cellophane disc (12 mm diam., Kalle Einmach Cellophan), supported by an agar plate containing 2% agar (Difco), 20 mM MOPS, pH = 7.5 (Calbiochem), 5 mM $MgCl_2$, 10 mM EGTA, and 0.33 M sucrose. After 20 min the discs were transferred to a second agar plate containing only 20 mM MOPS and 5 mM $MgCl_2$ and incubated at 2°C for 50 min without a cover to allow the lysate to partially dry off. Immediately prior to the incorporation, discs were transferred for 2 min to an identical agar plate at 25°C. For incorporation of nucleotides the discs were transferred into a moisture-saturated chamber at 25°C, and layed on a drop of 10 μl of incorporation mixture containing: 20 mM MOPS pH 7.2, 100 mM KCl, 5 mM $MgCl_2$, 2 mM ATP or ADP, 100 μM thymidine, and 20 μM each of the four deoxyribonucleoside di- or triphosphates including [^{3}H]-TTP or [^{3}H]-TDP (1 C/mM) as radioactive label. Incorporation was terminated by placing the disc in a 10 ml beaker containing 0.5 ml of 0.5% sodium dodecyl sulfate (SDS), 0.1 M NaOH and 10% saturated $Na_4P_2O_7$. Samples were heated briefly to 80°C, cooled, diluted with saline, and after the addition of 100 μg of salmon sperm DNA as carrier, precipitated with 5% TCA. Precipitates were collected on HAWP filters (Millipore Corp.) and counted by liquid scintillation spectroscopy. Thymidine nucleotides labeled in the 5-methyl position were used exclusively.

Results and Discussion: Schaller et al. had shown that deoxyribonucleoside diphosphates compete with triphosphates for incorporation into DNA (3). We have confirmed this observation and, in addition, have shown that in the presence of ATP the rate of incorporation of diphosphates into DNA is indistinguishable from that of triphosphates (Fig. 1a). In order to inhibit the phosphorylation of diphosphates to triphosphates we tried to substitute ADP for ATP, the known phosphate donor of the nucleoside diphosphate kinase. Previously, Pisetsky et al. had shown that in toluene-treated cells ADP can replace ATP in promoting the incorporation of triphosphates into DNA (7). Fig. 1b shows that the incorporation of deoxyribonucleoside diphosphates into DNA in the cellophane disc system is supported by ADP just as well as by ATP. As will be shown later, the rate of conversion of di- into triphosphates is drastically reduced under these conditions. After 20 min incubation we found only 6% of the ADP (2 mM) converted to ATP. The resulting concentration of ATP (120 μM) is by itself not sufficient to promote the rapid incorporation of nucleotides into DNA (K_m for ATP or ADP: 1.5 mM).

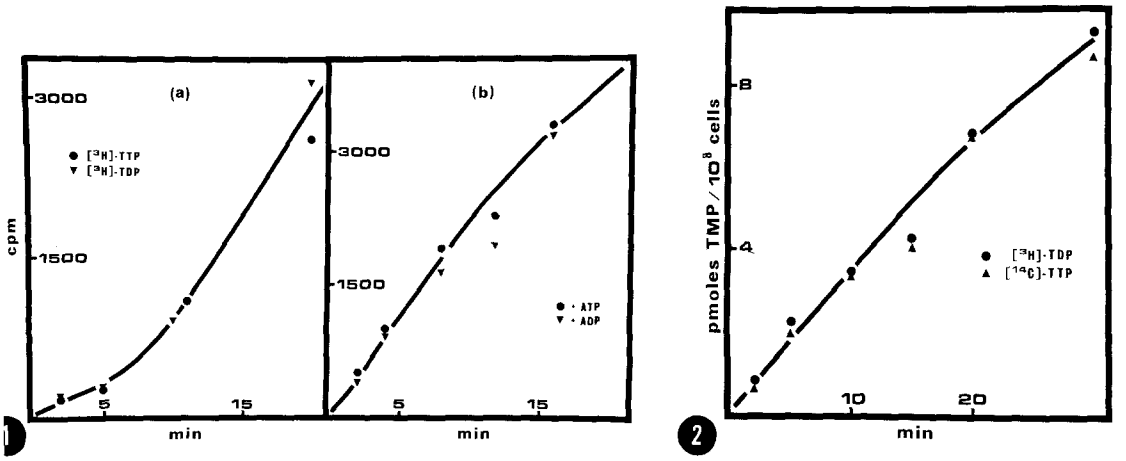
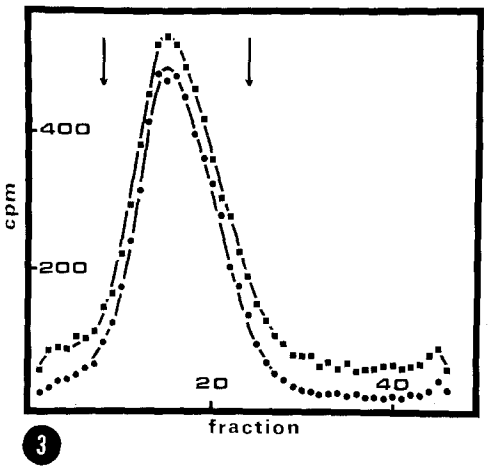


Figure 1. a) Incorporation of deoxyribonucleoside di- and triphosphates in the presence of ATP; b) Incorporation of deoxyribonucleoside diphosphates in the presence of ATP and ADP. (The initial lag in incorporation rate observed in a) was caused by omitting the 2 min incubation of the discs at 25°C prior to incorporation).

Figure 2. Simultaneous incorporation of deoxyribonucleoside di- and triphosphates in the presence of ADP. Bacterial lysates were incubated as described in the experimental section with 20 μM each of [^{3}H]-TDP, dADP, dCDP, dGDP, and [^{14}C]-TTP, dATP, dCTP, dGTP. (Relative counting efficiencies of [^{3}H]- and [^{14}C]-labeled DNA were determined by incorporating [^{3}H]-TTP and [^{14}C]-TTP in separate systems and comparing the counts incorporated).

When deoxyribonucleoside di- and triphosphates are offered in equimolar amounts, including [^{3}H]-TDP and [^{14}C]-TTP as radioactive labels, the nucleotides are incorporated into DNA in equimolar proportions (Fig. 2). The lack or preference for either nucleotide suggests either that the two nucleotides are interconverted very rapidly or that they are used for two separate reactions, perhaps occurring on the two DNA strands. To test the latter possibility we repeated the experiment replacing [^{14}C]-TTP and dCTP by BUTP and [^{32}P]-dCTP. The labelled DNA was isolated, sheared, denatured and subjected to density analysis in CsCl gradients. Our results show that both labels exhibit the same buoyant density halfway between that of light and heavy marker DNA (Fig. 3), indicating that di- and triphosphates had been incorporated to the same extent into both DNA strands. Still, it is possible that di- and triphosphates are incorporated by two different reactions occurring on the same DNA strands producing short alternating stretches of



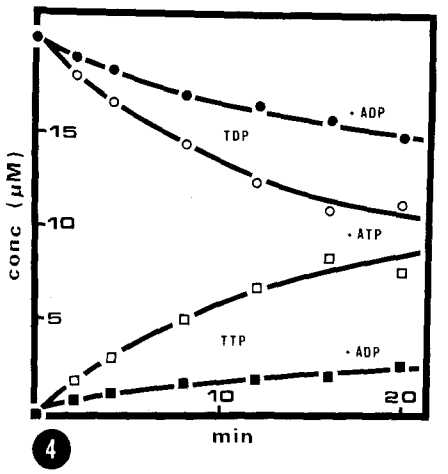


Figure 3. Density analysis in CsCl of DNA labeled simultaneously with 20 μM each of [^{3}H]-TDP, dADP, dCDP, dGDP and [^{32}P]-dCTP, BUTP, dATP, dGTP, in the presence of 1 mM ADP. Two discs (27 mm diam.) containing 10 μl of cells each were incubated with 50 μl of incorportion mixture as described in the experimental section. The reaction was terminated after 10 min by the addition of 75% ethanol containing 2% phenol, and the precipitate collected by centrifugation, resuspended in 0.6 ml of 0.05 M tris - 0.01 M EDTA, pH 7.4, and incubated with 30 μl of 20% sarkosyl and 60 μl of pronase (1 mg/ml, nuclease-free, Calbiochem) at 37°C for 30 min. The lysate was treated for 10 min with 0.1 M NaOH at 80°C, dialyzed against 0.05 M tris - 0.01 EDTA, pH 7.4, sheared by brief exposure to ultrasound and mixed with 2.2 ml of saturated CsCl to a final volume of 5.05 ml. Gradients were centrifuged at 34,000 rpm in a Spinco SW 50.1 rotor for 70 hr at 5°C. Fractions were collected from the bottom of the tubes onto filter paper circles, dried, and counted by liquid scintillation spectroscopy. The positions of light and heavy marker DNA are indicated by arrows, [^{3}H]-counts, circles; [^{32}P]-counts, squares.

Figure 4. Conversion of deoxyribonucleoside diphosphates to triphosphates in vitro. Cell lysates were incubated at 25°C with 20 μM each of [^{3}H]-TDP, dADP, dCDP, and dGDP in the presence of either ATP or ADP under conditions described in Fig. 1. The reaction was terminated at the indicated time by the addition of 100 μl of 5% TCA. The precipitate was removed by centrifugation, and the supernatant, after extraction of the TCA with ether, was chromatographed on PEI sheets (Brinkman, Westbury, N.Y.). TDP, circles; TTP squares. +ATP open symbols, +ADP closed symbols.

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light and heavy DNA that were not resolved in our experiment. An alternative interpretation of our results would be that the two nucleotides are rapidly interconverted. This interpretation is also supported by the determination of the K_m values. Within the limits of error both K_m and V_{max} values were identical for di- and triphosphates, whether in the presence of ADP ro ATP, the apparent K_m being about 12 μM.

The extent of conversion of diphosphates into triphosphates in the

cellophane disc system was determined by extracting the nucleotides with trichloroacetic acid (TCA) and separating them by two-dimensional thin-layer chromatography (2,8). Fig. 4 shows that in the presence of ATP a rapid conversion of TDP into TTP takes place until, after about 20 min, an equilibrium is reached consisting of about 55% TDP and 40% TTP. In the presence of ADP, on the other hand, the rate of conversion of diphosphates into triphosphates is drastically reduced, the equilibrium concentration consisting of 75% diphosphates, 10% triphosphates and 15% monophosphates. These results seem to rule out the possibility that diphosphates have to be converted to triphosphates before they can be incorporated into DNA, because the rate of incorporation of diphosphates is maximal within less than a minute at which time the concentration of triphosphates is almost negligible. However, the cell lysate on top of the cellophane disc constitutes only a small fraction of the total reaction volume. Since all reactions take place in this fraction, a concentration gradient may exist across the cellophane disc resulting in completely different concentrations of individual nucleotides in the cell lysate as compared to the whole system. It was important, therefore, to determine the nucleotide composition in the cell lysate rather than in the total reaction mixture. This was accomplished by quickly pipetting off the lysate from the cellophane disc with a micro pipet and transferring it into TCA. The nucleotide analyses of cell lysates that had been incubated with different incorporation mixtures are shown in Table I.

The relative concentrations of TDP and TTP in the cell lysate are largely determined by the presence of ATP or ADP independently of the composition of the original incorporation mixture (nucleoside di- or triphosphates). In the presence of ADP the replication system contains 3-4 times more TDP than TTP, whereas in the presence of ATP the two nucleotides are present in about equimolar amounts. Since the rate of DNA synthesis is the same in both conditions the individual concentrations of either deoxyribonucleoside di- or triphosphates cannot be the controlling factor.

TABLE I

Incorporation Mixture	Concentration of nucleotides in cell lysate		
	TMP	TDP	TTP
(a) 3 minutes			
20 μMTDP + ADP	2.4 μM (12%)	13.8 μM (69%)	3.2 μM (16%)
20 μMTDP + ATP	1.6 μM (8%)	8.0 μM (40%)	7.0 μM (35%)
20 μMTTP + ADP	2.6 μM (13%)	12.2 μM (61%)	4.0 μM (20%)
20 μMTTP + ATP	1.8 μM (9%)	8.0 μM (40%)	6.6 μM (33%)
(b) 10 minutes			
20 μMTDP + ADP	5.6 μM (27%)	11.5 μM (55%)	3.0 μM (14%)
20 μMTDP + ATP	3.4 μM (14%)	9.2 μM (38%)	7.3 μM (31%)
20 μMTTP + ADP	5.6 μM (27%)	11.4 μM (55%)	3.4 μM (16%)
20 μMTTP + ATP	3.0 μM (12%)	8.0 μM (33%)	9.0 μM (37%)

Table I. Nucleotide concentrations in the cell lysate layer of the in vitro system. Cell lysates were incubated for 3 min (a) or 10 min (b) with 20 μM each of [^{3}H]-TDP, dADP, dCDP, dGDP and 2 mM of either ADP or ATP, or with 20 μM each of [^{3}H]-TTP, dATP, dCTP, dGTP, and 2 mM of either ADP or ATP. The lysate layer was pipetted from the top of the cellophane disc with a micropipet and transferred into 100 μl of 5% TCA. The nucleotide composition of this extract was determined as described in Fig. 4. Each value is the average obtained from three discs. The sum of mono-, di-, and triphosphates does not equal 100% because a fourth radioactive spot, probably representing TDP-rhamnose (9), was included in the calculation. The total nucleotide concentration in the cell lysate layer was determined by including [^{14}C]-labeled aspartate or [^{14}C]-thymine in the incorporation mixture and measuring the ratio of [^{3}H]- to [^{14}C]-counts in both the incorporation mixture and the cell lysate. The sum of all nucleotides in the cell lysate equals the nucleotide concentration in the incorporation mixture (20 μM).

Instead, the results of our nucleotide analysis as well as the K_m measurements indicate that the rate of incorporation is determined by the sum of the concentrations of di- and triphosphates.

One possible interpretation of these results is that neither di- nor triphosphates are the immediate precursors of DNA, but have to be converted into yet another form of activated nucleotide to serve as substrates in the replication process. If the rate of polymerization is slow compared to the rates of conversion of di- and triphosphates into this novel precursor, it would be controlled by the sum of the concentrations of di- and triphosphates rather than by the concentrations of either nucleotide alone. Such a hypothetical novel DNA precursor would have to be TCA-insoluble because no nucleotides other than mono-, di-, and triphosphates as well as some TDP-

rhamnose can be detected in TCA extracts. We had speculated earlier on the existence of a macromolecular carrier for nucleotides that would facilitate the "crystallization" of nucleotides upon the template strands (2). An alternative explanation may be that both di- and triphosphates are capable of entering the replication complex and that the diphosphates are phosphorylated in situ. We are currently investigating this possibility.

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