The Synthesis of 3′-dATP and Its Use as an Inhibitor of ATP-Dependent DNA Synthesis in Toluene-Treated *Escherichia coli*†

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ABSTRACT: A structural analogue of ATP, 3′-deoxyadenosine triphosphate (3′-dATP), has been synthesized from cordycepin (3′-deoxyadenosine), characterized, and determined to be an inhibitor of ATP-dependent DNA synthesis in *Escherichia coli* cells which have been rendered permeable to nucleoside triphosphates by treatment with toluene. The analogue is a competitive inhibitor of ATP and it inhibits replicative DNA synthesis 50% at concentrations of ca. 0.15 mM in the presence of 1.0 mM ATP and 4×10^8 cells/ml. The degree of inhibition of a given amount of 3′-dATP is inversely related to the cell concentration in the reaction mixture. The analogue interferes with some function of ATP which is continuously required during the course of the reaction and does not irreversibly inactivate the cells' DNA synthesis apparatus. 3′-Deoxyadenosine triphosphate may prove useful in elucidating the roles of ATP in DNA synthesis in more purified replicating systems.

The treatment of exponential phase *Escherichia coli* cells with toluene allows the demonstration of DNA synthesis from deoxyribonucleoside triphosphate precursors (Moses and Richardson, 1970; Mordoh et al., 1970). Two types of DNA synthesis have been demonstrated in cells which have been rendered permeable to nucleotides by toluene (Moses and Richardson, 1970), ether (Vosberg and Hoffman-Berling, 1971), or sucrose (Wickner and Hurwitz, 1972).

One mode of synthesis is similar to in vivo DNA replication in that it is semiconservative (Moses and Richardson, 1970) and the DNA product is synthesized from the region of the chromosome about to be replicated at the time of toluene treatment (Burger, 1971). The synthesis is discontinuous and the fragments which are initially formed can be chased into high molecular weight DNA (Moses et al., 1971; Pisetsky et al., 1972). In addition, the synthesis is temperature sensitive in cells prepared from strains bearing the thermolabile "fast stop" DNA synthesis mutations *dna*B (Mordoh et al., 1970; Wickner and Hurwitz, 1972) and *dna*E (=*pol*C) or *dna*G (Wickner and Hurwitz, 1972). DNA is synthesized in treated cells at rates of the same order of magnitude as in vivo (Moses and Richardson, 1970; Mordoh et al., 1970; Burger, 1971) and the synthesis is inhibited by nalidixic acid (Pisetsky et al., 1972), edeine (Kurloborowska and Szer, 1971), and mitomycin C (Vosberg and Hoffman-Berling, 1971)—three inhibitors of in vivo replication. The synthesis also occurs in several *pol*A mutants which are defective in the polymerase activity of DNA polymerase I. *Bacillus subtilis* toluene-treated cells synthesize biologically active product and the replication fork moves in the same direction as it does in vivo (Matsushita et al., 1971). Replicative DNA synthesis in toluene-treated cells differs from in vivo replication primarily in that the initiation of new rounds of synthesis does not occur; i.e., no new replication forks are formed (Winston and Matsushita, 1975).

A second mode of DNA synthesis can be differentiated from the replicative type in permeable cells. This synthesis requires the polymerase portion of the *pol*A gene product, is dependent upon endogenous or exogenous nuclease activity, and is not semiconservative (Moses and Richardson, 1970; Vosberg and Hoffman-Berling, 1971; Wickner and Hurwitz, 1972). Neither is this synthesis thermolabile in toluene-treated, temperature-sensitive *dna* mutants (Moses and Richardson, 1970). This type of DNA synthesis resembles the repair synthesis which occurs in vivo as a result of ultraviolet light damage to the cellular DNA (Pettijohn and Hanawalt, 1964).

In permeable cells the two types of DNA synthesis can be readily distinguished from one another by the nearly stringent ATP requirement for the replicative synthesis (Pisetsky et al., 1972; Forterre and Kohiyama, 1974). Although an ATP-dependent repair synthesis has also been demonstrated in ultraviolet-irradiated toluene-treated *E. coli* (Masker and Hanawalt, 1973), this synthesis can be differentiated from replicative synthesis because it is more limited in extent, can be supported by other ribonucleoside triphosphates, does not require the *dna*B and *pol*C gene products, depends upon the *uvr*A gene product, and is insensitive to *ara*-CTP (Masker and Hanawalt, 1974).

One experimental approach toward understanding ATP promoted replicative synthesis has been to use ATP analogues to determine how the synthesis is affected by defined changes in the structure of the coenzyme. None of the other common ribo- or deoxyribonucleoside triphosphates can substitute for ATP in their ability to support replicative synthesis (Pisetsky et al., 1972). The phosphonate analogues of ATP, α,β-methylene adenosine triphosphate and β,γ-methylene adenosine triphosphate, do not substitute and are, in fact, inhibitors as is α,β-methylene adenosine diphosphate (Vosberg and Hoffmann-Berling, 1971; Pisetsky et al., 1972; Forterre and Kohiyama, 1974).

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We have synthesized and examined an ATP analogue in which the 3′-hydroxyl group has been replaced with a hydrogen atom for its effects upon DNA synthesis in toluene-treated *E. coli*. We find that 3′-dATP is an inhibitor of replicative synthesis and that it has little effect upon repair synthesis. 3′-dATP can therefore be used to differentiate between these two modes of synthesis. It is a competitive inhibitor of ATP ($K_I \simeq$ 30 μM) and does not irreversibly inactivate the replicative apparatus of the cell whether or not DNA synthesis is occurring while it is present. This compound may be useful in dissecting out the roles of ATP in DNA replication when used in more purified replicating systems.

Experimental Section

Materials. [*methyl*-^{3}H]dTTP at a specific activity of approximately 50 Ci/mmol was obtained from New England Nuclear. *ara*-ATP and *ara*-CTP were purchased from Terra Marine Bioresearch and deoxyribonucleoside triphosphates, ATP, and dThd were from P-L Laboratories, Inc. Bacto-yeast extract and Bactotryptone were obtained from Difco Laboratories and thiamine was from Sigma Chemical Co. Amberlite XAD-4 was from Rohm and Haas Co. All other chemicals were of reagent grade.

Isolation of 3′-Deoxyadenosine (Cordycepin). 3′-Deoxyadenosine was isolated from 24 day old cultures of *Cordyceps militaris* as described (Suhadolnik et al., 1968). The physical and chemical properties (ir, NMR, uv, melting point) all showed that the 3′-deoxyadenosine was pure and free of any adenosine (Suhadolnik, 1970).

Synthesis of 3′-dAMP. The 5′-monophosphate of 3′-deoxyadenosine was synthesized by a modification of the method of Yoshikawa et al. (1967). 3′-Deoxyadenosine (0.8 mmol) was suspended by stirring at 0 °C in a 5-ml round-bottomed flask containing 2 ml of trimethyl phosphate, 160 μl of phosphorus oxychloride were added dropwise, and the stoppered flask was incubated in an ice bath. After 5 min of stirring, the 3′-deoxyadenosine went into solution. The homogeneous reaction mixture was monitored for the formation of the 5′-monophosphate by taking 5-μl samples every 30 min, spotting, and developing them on Eastman chromagrams (No. 13255 cellulose) using isobutyric acid–concentrated ammonium hydroxide–H_2O (66:1:32, v/v/v). After 4.5 h very little cordycepin remained. Eight grams of chopped ice was added slowly and the reaction mixture stirred at 0 °C for an additional 30 min. Two milliliters of concentrated ammonium hydroxide was added dropwise over a 2-min period. After 5 min stirring, the alkaline solution was transferred to a separatory funnel and 5 ml of ether was added to extract the trimethyl phosphate. The ether extraction was repeated twice. The aqueous phase was taken to dryness on a rotary evaporator at 40 °C under vacuum. One milliliter of water was added and the solution was applied to an Amberlite XAD-4 column (20 g, 200 mesh, 2.5 × 1.5 cm) that had been previously washed with methanol, water, and finally with 100 ml of 0.05 M triethylammonium bicarbonate (pH 7.4). The inorganic phosphate, 3′-dAMP, and 3′-deoxyadenosine were eluted from the column with a 400-ml gradient of 0.1–0.4 M triethylammonium bicarbonate (pH 7.4) as shown in Figure 1. The yield of 3′-dAMP was 0.6 mmol (75%), and 0.11 mmol (14%) of the 3′-deoxyadenosine was recovered. The fractions containing the 3′-dAMP were combined and evaporated to dryness on a rotary evaporator at 45 °C under vacuum. Ten milliliters of ethanol was added and the suspension was taken to dryness to remove the buffer. The 3′-dAMP was shown to be the pure 5′-phosphorylated product by its complete hydrolysis with snake venom 5′-nucleotidase in 0.1 M Tris-HCl[1] (pH 8.5) at 37 °C (Suhadolnik et al., 1968).

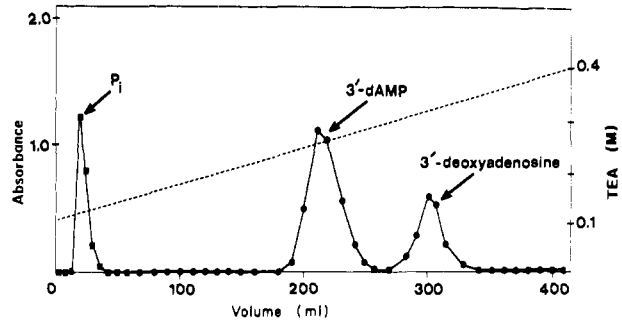

FIGURE 1: Separation of 3′-dAMP from inorganic phosphate and cordycepin by XAD-4 column chromatography. Chromatography was as described in Experimental Section and 5-ml fractions were collected. The elution of cordycepin and 3′-dAMP was monitored by the absorbance at 259 nm (●—●). Inorganic phosphate was determined by the method of Ames (1966) and the absorbance at 820 nm is plotted (■—■).

Synthesis of 3′-dATP. The 3′-dAMP (0.5 mmol) was converted to 3′-dATP by the method of Smith (1961). To isolate the 3′-dATP, the same XAD-4 column-gradient procedure described for the purification of 3′-dAMP was used except that a 500-ml gradient was employed. Further details on the use of XAD-4 column chromatography for the separation and purification of nucleosides and nucleotides can be found in Uematsu and Suhadolnik (1976). 3′-dATP was eluted from 420 to 490 ml, and the yield was 0.11 mmol (22%). Acid labile and total phosphate analyses were in agreement with pure 3′-dATP (Suhadolnik et al., 1968).

Bacterial Strains. Escherichia coli strains P3478 (*thy*-A36*pol*A1*dra*-2,λ$^-$) and W3110 its *pol*A$^+$ parent (DeLucia and Cairns, 1969) were obtained from P. Carl. *E. coli* strain MM387 (*pol*A12*rec*B21 *rha*$^-$*lac*$^-$*str-r*) (Monk and Kinross, 1972) was provided by P. Modrich. Strains carrying a *pol*A mutation were routinely checked for this phenotype by incubation at the appropriate temperature on agar plates containing 0.04% methylmethanesulfonate.

Preparation of Toluene-Treated Cells. Cells were grown in a medium containing (g/l.) Bactotryptone (10), NaCl (10), glucose (10), Bactoyeast extract (1), deoxythymidine (10^{-3}), and thiamine (10^{-5}) at 37 °C, except for strain MM387, which was grown at 33 °C. When the cells had reached a concentration of 3.5×10^8/ml, they were harvested by centrifugation at 8000*g* for 2 min at 4 °C. All subsequent steps were performed at 0–4 °C. The cells were resuspended in 50 mM KP_i (pH 7.5) to a concentration of 3.5×10^9/ml, resedimented, and resuspended at a concentration of 2×10^{10}/ml in the same buffer. An equal volume of 50 mM KP_i (pH 7.5) containing 2% toluene, which had been suspended in the buffer by brief sonication, was added to the cells, and the mixture was continuously agitated by means of a Vortex mixer for 3 min. The cells were resedimented as described, the top of the pellet was gently rinsed with 50 mM KP_i (pH 7.5), and the cells were suspended to a concentration of 9.5×10^{10}/ml in the same buffer, quick frozen in a dry ice–isopropyl alcohol bath, and stored at −70 °C in small portions. This method is a modifi-

[1] Abbreviations following the IUPAC-IUB Commission on Biochemical Nomenclature (1971) (*J. Mol. Biol. 55*, 229) are used throughout. Other abbreviations are: dTTP, 2′-deoxythymidine 5′-triphosphate; dTMP, 2′-deoxythymidine 5′-monophosphate; KP_i, potassium phosphate; ir, infrared; uv, ultraviolet; NMR, nuclear magnetic resonance; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; TEA, triethylammonium bicarbonate.

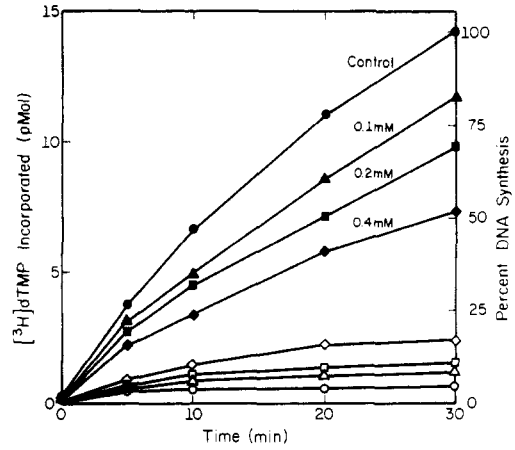

FIGURE 2: The kinetics of DNA synthesis in the presence of increasing amounts of 3′-dATP. ATP-dependent (filled symbols) and ATP-independent (open symbols) syntheses were tested. Reaction mixtures (275 μl) were as described in Experimental Section and contained 7.9×10^8 cells/ml. Where present, the ATP concentration was 1.0 mM. The specific activity of the [^{3}H]dTTP was 545 cpm/pmol and 100% incorporation represents 14.2 pmol of [^{3}H]dTMP incorporated per 50 μl of reaction mixture. Control with no 3′-dATP (●, ○), 0.1 mM 3′-dATP (▲, △), 0.2 mM 3′-dATP (■, □) and 0.4 mM 3′-dATP (♦, ◇).

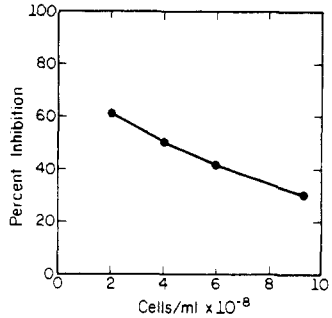

FIGURE 3: The effect of cell number upon the ability of a fixed concentration of 3′-dATP to inhibit DNA synthesis in the presence of 1.0 mM ATP. Reaction mixtures (100 μl), which were run in triplicate, were as described in Experimental Section and, in addition, contained 0.2 mM 3′-dATP. The cell concentration was varied from 2.0×10^8 to 9.3×10^8/ml. Reactions were run for 30 min at 37 °C. Percent inhibition was calculated by taking the rate of ATP-dependent DNA synthesis for each cell concentration in the absence of 3′-dATP as the 100% value.

cation of that of Moses (1974). Cells prepared and stored in this manner lost little ATP-dependent DNA synthesis activity over a 2-month period.

Assay for DNA Synthesis. Assay conditions were essentially those of Moses and Richardson (1970) with minor changes in the concentrations of some components and with the omission of dithiothreitol. Assays contained 40 μM each of dATP, dCTP, dGTP, 40 μM [*methyl*-^{3}H]dTTP at a specific activity of from 450 to 550 cpm/pmol, 70 mM KP_i (pH 7.5), 10 mM $MgCl_2$, and 1.0 mM ATP in 100 μl. Controls were run in the absence of ATP in order to determine the level of repair synthesis. Reactions were incubated at 37 °C for 30 min and terminated by the addition of 2 ml of cold 10% trichloroacetic acid, 0.1 M sodium pyrophosphate. The acid precipitated reaction mixtures were held at 0 °C for at least 10 min before collection on Whatmann GF/C glass-fiber filters, washing three times with cold 5% trichloroacetic acid, 0.01 M sodium pyrophosphate, three times with cold 1 M HCl, and two times with cold 95% ethanol. The filters were dried under an infrared lamp and were counted in 3 ml of a scintillation fluid containing 0.6% 2,5-diphenyloxazole and 0.025% 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene in toluene in a Nuclear

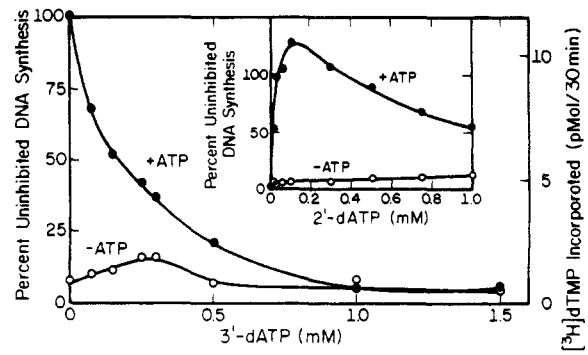

FIGURE 4: The effect of increasing concentrations of 3′-dATP upon DNA synthesis in the presence (●) and absence (○) of 1.0 mM ATP. Reaction mixtures (100 μl) run in triplicate were as described in Experimental Section and contained 4×10^8 cells/ml. When present, ATP was at 1.0 mM. The specific activity of the [^{3}H]dTTP was 525 cpm/pmol and 100% incorporation represents 11.4 pmol of [^{3}H]dTMP. The insert shows the effect of increasing concentrations of 2′-dATP upon DNA synthesis in the presence (●) and absence (○) of 1.0 mM ATP under conditions similar to those described for the 3′-dATP experiment. dCTP, dGTP, and [^{3}H]dTTP were always present at 40 μM, and 100% represents the incorporation at 40 μM 2′-dATP.

Chicago Isocap 300. When the kinetics of DNA synthesis were examined, appropriately larger volumes of reaction mixtures were run, and 50-μl portions were removed at the indicated times, added to 2 ml of 10% trichloroacetic acid, 0.1 M sodium pyrophosphate, and treated as described.

Results

Inhibition of Replicative DNA Synthesis by 3′-dATP. The ability of 3′-dATP to inhibit ATP-dependent DNA synthesis as measured by [^{3}H]dTMP incorporation into acid insoluble material in toluene-treated *E. coli* P3478 is shown in Figure 2. The ATP-dependent synthesis is inhibited in a concentration-dependent fashion with 50% inhibition occurring at 30 min with 0.4 mM 3′-dATP in reaction mixtures containing 1.0 mM ATP and 7.9×10^8 cells/ml. In the absence of ATP, 3′-dATP stimulates the incorporation of [^{3}H]dTMP into DNA (Figure 2). The ability of 3′-dATP to stimulate DNA synthesis is limited, depends upon its concentration (Figure 2), and is not as great as the stimulation due to ATP. The DNA synthesis which is stimulated by 3′-dATP in the absence of ATP is completely abolished by 1.5 mM *N*-ethylmaleimide, suggesting that it is replicative in nature (Moses and Richardson, 1970) and that 3′-dATP is in some way partially substituting for an ATP function.

The degree of inhibition of ATP-dependent synthesis by a given concentration of 3′-dATP is a function of the concentration of cells in the reaction mixture. When the cells are increased from 2×10^8 to 9.3×10^8/ml, the inhibition caused by 0.2 mM 3′-dATP decreases from 61% to 30% (Figure 3). At each cell concentration tested, the incorporation of [^{3}H]-dTMP into DNA in the absence of ATP was less than 10% of that in its presence and the rate of DNA synthesis over this range of cell concentrations was directly proportional to the cell number. Because of the strong dependence of inhibition upon cell number, all the remaining experiments were carried out at 4×10^8 cells/ml in order to facilitate comparisons.

In reaction mixtures containing 4×10^8 cells/ml and 1.0 mM ATP, ca. 0.15 mM 3′-dATP was sufficient to 50% inhibit ATP-dependent synthesis and concentrations greater than 0.9 mM completely inhibited the synthesis (Figure 4). The ATP-independent synthesis is stimulated by 3′-dATP in the concentration range up to ca. 0.4 mM and is inhibited at higher

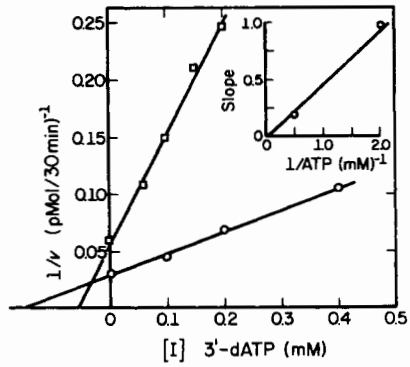

FIGURE 5: A Dixon plot of the reciprocal of the velocity of DNA synthesis at two ATP concentrations as a function of 3′-dATP concentration. Reaction mixtures (100 μl), which were run in triplicate, were as described in Experimental Section except that the ATP concentration was either 0.5 mM (□) or 2.0 mM (○) and, in addition, contained the indicated amounts of 3′-dATP. The specific activity of the [^{3}H]dTTP was 500 cpm/pmol. Lines were fit to the data points by a least-squares analysis. The insert is a plot of the slopes of the two curves vs. the reciprocal of the ATP concentration.

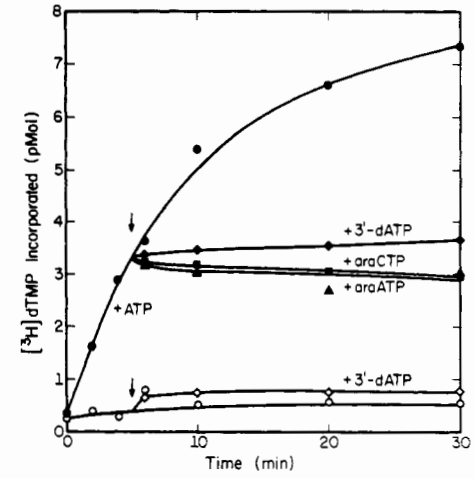

FIGURE 6: The effect upon DNA synthesis of adding inhibitors to a reaction in progress. A 1.075-ml reaction mixture as described in Experimental Section with [^{3}H]dTTP at 500 cpm/pmol and containing 1.0 mM ATP (●) was started by adding cells to a final concentration of 4 × 10^8/ml. At 5 min, 225-μl aliquots were removed and added to 5 μl of H_2O (●), 5 μl of a solution containing sufficient 3′-dATP to give a final concentration of 1.0 mM (♦), 5 μl of *ara*-CTP to give a 0.25 mM final concentration (■), or 5 μl of *ara*-ATP to give a final concentration of 0.25 mM (▲). Analogously a similar reaction mixture containing no ATP (○) was started by the addition of cells and, at 5 min, 225-μl portions were added to 5 μl of H_2O (○) or to 5 μl of a solution containing sufficient 3′-dATP (◇) to give a final concentration of 1.0 mM. At the indicated times 50-μl portions were removed and acid precipitated as described in Experimental Section.

concentrations (0.5 mM and greater) to levels equivalent to the incorporation which occurs in the absence of either ATP or 3′-dATP. The 3′-dATP-stimulated synthesis at all concentrations is *N*-ethylmaleimide (1.5 mM) sensitive. In addition, toluene-treated *E. coli* MM387 cells, which lack a functional *rec*BC nuclease (exonuclease V), show the same stimulation of ATP-independent synthesis by 3′-dATP. This result indicates that 3′-dATP is not activating exonuclease V to promote the repair type of synthesis which has been observed in some systems (Buttin and Kornberg, 1966). *E. coli* W3110, the *pol*A$^+$ parent of P3478, is also similarly inhibited by 3′-dATP; however, the presence of more DNA polymerase I promotes greater ATP-independent DNA synthesis.

That the inhibition of ATP-dependent DNA synthesis is due to some specific effect of 3′-dATP and not to a general increase in the level of dNTP is indicated by the insert in Figure 4. At levels up to ca. 0.4 mM, 2′-dATP does not inhibit ATP-dependent synthesis and, although it does inhibit at higher concentrations (50% inhibition at ca. 1.0 mM), it is clearly much less effective than 3′-dATP. This experiment also confirms the previous observations (Pisetsky et al., 1972) that 2′-dATP is unable to substitute for ATP in stimulating DNA synthesis in toluene-treated cells.

The Inhibitor Is Competitive with ATP. The inhibitory effect of 3′-dATP upon ATP-dependent DNA synthesis can be overcome by increasing the concentration of ATP. Increasing amounts of 3′-dATP were tested at two ATP concentrations and the results plotted according to the method of Dixon and Webb (1964) with the reciprocal of the velocity vs. the inhibitor concentration (Figure 5). These data generate two straight lines which intersect above the abscissa in the second quadrant. This type of plot indicates that the inhibition is either purely competitive or of a mixed variety (Segel, 1975). The insert shows that a plot of the slopes of the lines of the Dixon plot vs. the reciprocal of the ATP concentration passes through the origin indicating the competitive nature of the inhibition (Segel, 1975). Lineweaver–Burke plots of the reciprocal of the rate of the ATP-dependent synthesis vs. the reciprocal of the ATP concentration at different 3′-dATP concentrations generated lines which intersected on the ordinate also suggesting that the inhibition was partially or purely competitive. A purely competitive inhibitor can be distinguished from a mixed type of inhibitor by the Cornish-Bowden plot of [S]/v vs. [S] (Cornish-Bowden, 1974) in which competitive inhibitors generate a set of parallel lines while mixed-type inhibitors give a series of lines which intersect in the third quadrant. When the data used to give Figure 5 were plotted in this manner, two lines which were nearly parallel but did tend to intersect in the first quadrant resulted (data not shown). Since the curves showed no tendency to intersect in the second or third quadrants, this further indicates the purely competitive nature, with respect to ATP, of the inhibition by 3′-dATP. The intersection of the two lines in the Dixon plot yields a K_I of ca. 30 μM for 3′-dATP, assuming that the inhibition is of a purely competitive type. We wish to point out that the data shown in Figure 5 are for the best single experiment that we were able to do in several attempts. There is generally a good deal of scatter in the data at the lower levels of incorporation which makes it difficult to obtain accurate measurements of the velocities at either high inhibitor or low ATP concentrations. However, the experiments taken together suggest that the inhibition is competitive. These experiments also indicated that the apparent K_M for ATP is ca. 6×10^{-4} M. Experiments attempting to relate 3′-dATP inhibition to 2′-dATP yielded curved lines in the various plots which were uninterpretable. Control experiments indicated that *ara*-CTP and *ara*-ATP were competitive inhibitors of 2′-dCTP and 2′-dATP, respectively.

The Inhibitor Rapidly Stops a Reaction in Progress. The addition of a quantity of 3′-dATP (1.0 mM), which is sufficient to completely inhibit ATP-dependent DNA synthesis if it is present before the addition of the cells, very rapidly stops the synthesis if it is added after the reaction is started (Figure 6). It is similar to *ara*-ATP and *ara*-CTP in this respect (Figure 6). This result indicates that 3′-dATP either interferes with some function of ATP which is continuously required during synthesis or that it in some fashion inactivates the replicative machinery. The addition of 3′-dATP in the presence of ATP

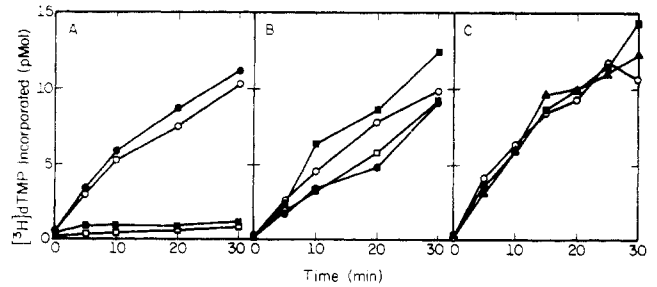

FIGURE 7: The effect of various pretreatments of toluene-treated cells upon subsequent DNA synthesis. (A) Cells (4×10^8/ml) were incubated in 70 mM KP_i (pH 7.5) and 10 mM $MgCl_2$ in the presence (■, ●) or absence (□, ○) of 1.0 mM ATP for 2.5 min at 37 °C. The reaction mixtures were centrifuged for 4 min at 25 °C at 15 000*g* in an Eppendorf microcentrifuge, resuspended in reaction mixtures containing 40 μM each of the dNTPs with [^{3}H]dTTP at a specific activity of 500 cpm/pmol, 70 mM KP_i (pH 7.5), and 10 mM $MgCl_2$ in the presence of (○, ●) or absence (□, ■) of 1.0 mM ATP and incubated at 37 °C. At the indicated times, 50-μl portions were removed from the 550-μl reaction mixtures and precipitated as described in Experimental Section. (B) Cells (4×10^8/ml) were incubated in 70 mM KP_i (pH 7.5) and 10 mM $MgCl_2$, in the presence (■, ●) or absence (□, ○) of 80 μM each of the unlabeled dNTPs and in the presence (○, ●) or absence (□, ■) of 1.0 mM ATP for 2.5 min at 37 °C. The reaction mixtures (275 μl) were centrifuged as described and resuspended in a solution containing 40 μM each of the dNTPs with [^{3}H]dTTP at a specific activity of 500 cpm/pmol, 70 mM KP_i (pH 7.5), 10 mM $MgCl_2$, and 1.0 mM ATP, incubated at 37 °C and 50-μl portions precipitated at the indicated times as described. (C) Cells (4×10^8/ml) were incubated in 70 mM KP_i (pH 7.5), 10 mM $MgCl_2$, and 40 μM each of the unlabeled dNTPs and in addition 1.0 mM ATP (○, ▲) or 1.0 mM 3′-dATP (▲, ■) for 2.5 min at 37 °C. The cells were centrifuged as described and resuspended in 375-μl reaction mixtures containing 40 μM each of the dNTPs with [^{3}H]dTTP at a specific activity of 500 cpm/pmol, 70 mM KP_i (pH 7.5), 10 mM $MgCl_2$, and 1.0 mM ATP. Aliquots were removed at the indicated times and precipitated as described.

does not cause the degradation of previously synthesized DNA indicating that no significant amount of DNase is activated by the inhibitor.

When 3′-dATP is added to an ongoing reaction in the absence of ATP, there is a slight increase in the incorporation of [^{3}H]dTMP but it rapidly reaches a plateau which is not much higher than that of the reaction in the absence of the inhibitor (Figure 6).

The Inhibitor Does Not Irreversibly Inactivate the DNA Synthetic Machinery. In order to determine whether 3′-dATP inactivated the synthetic apparatus in an irreversible fashion, e.g., by being stably incorporated into a growing nucleic acid chain and acting as a terminator, we performed an experiment in which toluene-treated cells were incubated in the presence of 3′-dATP under conditions which either allowed (+ATP) or prevented (−ATP) replicative DNA synthesis. After a short incubation in the presence of the four unlabeled dNTPs and either ATP, 3′-dATP, or both, the cells were pelleted and resuspended in a reaction mixture which would allow ATP-dependent synthesis to be determined. Figure 7C shows that whether or not DNA synthesis occurred in the pretreatment, i.e., whether or not ATP was present with the four dNTPs, 3′-dATP had no effect upon the subsequent ability of the cells to carry out ATP-dependent synthesis. Figure 7A is a control experiment which shows that the preincubation treatment with or without ATP present neither activates a subsequent repair-like synthesis nor does it impair the ability of the cells to make DNA after they are resuspended into a complete reaction mixture containing ATP (compare the 10 pmol incorporated in this experiment with the 7 pmol synthesized in the experiment illustrated in Figure 6 in which there was no preincubation of the cells and the conditions were otherwise similar). Figure 7B is a further control which indicates that preincubation in the absence of the four dNTPs with or without ATP does not allow as much subsequent synthesis as does preincubation in their presence, but that the cells are still capable of synthesizing near normal amounts of DNA in the regular reaction mixture augmented with ATP.

This experiment shows that no irreversible damage is done by 3′-dATP to the cell's ability to carry out replicative DNA synthesis whether or not the inhibitor is present under conditions where DNA can be synthesized. This suggests that 3′-dATP interferes with some function of ATP which is continuously required during the course of the reaction and that it does so in a reversible way.

Discussion

ATP may play a number of different roles in replicative DNA synthesis in *E. coli*. The existence of RNA on the 5′-termini of some nascent DNA fragments (Kurosawa et al., 1975), the involvement of the *dna*G gene product in the initiation of these fragments (Lark, 1972), and the identification of the *dna*G protein as a DNA-dependent RNA polymerase (Bouché et al., 1975) suggest that ATP may serve as one of the ribonucleoside triphosphate substrates required for the synthesis of a segment of primer RNA. The ability of 3′-dATP to be incorporated into a growing RNA chain and to serve as a chain terminator in reactions catalyzed by rifamycin-sensitive DNA-dependent RNA polymerases (Shigura and Boxer, 1964; Shigura and Gordon, 1965; Sentenac et al., 1968) indicates that one possible mechanism by which this analogue might interfere with DNA synthesis is at the initiation step involving the synthesis of the primer RNA. The reversibility of the inhibition, which will be discussed below, does not exclude this mechanism of inhibition by 3′-dATP because there may be repair processes which rapidly excise the terminating nucleotide.

There are also a number of other potential sites at which the inhibitor might be acting. The *pol*C protein is a part of the physiologically active form of DNA polymerase III and the active enzyme complex requires ATP to initiate DNA synthesis on an artificially primed DNA molecule (Wickner and Kornberg, 1973; Hurwitz and Wickner, 1974) and in order to elongate DNA chains complementary to long single strand regions of DNA (Wickner and Kornberg, 1974). The purified *dna*B protein has a specific ribonucleoside triphosphatase activity which is markedly stimulated by DNA, although the triphosphatase activity has not been linked to DNA synthesis (Wickner et al., 1974). Purified *dna*B and *dna*C gene products interact to form a complex in vitro and the interaction specifically requires ATP (Wickner and Hurwitz, 1975a). One of the 11 purified *E. coli* proteins (factor Y) required to convert φX174 DNA to a double strand form in a reconstituted system is a φX174 DNA-dependent ATPase (Wickner and Hurwitz, 1975b). This ATPase activity has not been specifically related to DNA synthesis. See Wickner and Hurwitz (1975b) for a discussion of ATP and DNA synthesis. There may also be other functions of ATP which are affected by 3′-dATP.

While we have been able to demonstrate that 3′-dATP is an inhibitor of replicative DNA synthesis in permeable *E. coli* cells, we have been unable to define the point or points in the process at which it interferes with ATP. A part of this inability arises from the fact that the potency of inhibition is inversely related to the cell number in the reaction mixture (Figure 3). This has precluded us from performing velocity or equilibrium sedimentation analyses of pulse-chase experiments in which we could have determined if 3′-dATP inhibited DNA chain initiation, elongation, or both. The cell concentration (10^{10}/ml)

necessary to detect 15 to 30 s of [^{3}H]dTMP incorporation was not appreciably inhibited by 3.5 mM 3′-dATP. The ratio of molecules to cells under these conditions is nearly the same (ca. 2×10^8) as it was in the kinetic experiments described where ca. 0.15 mM 3′-dATP inhibited 4×10^8 cells/ml by 50%. The near linearity of the ATP-dependent DNA synthesis at several 3′-dATP concentrations (Figure 2) suggests that gross degradation of the inhibitor by the cells is not occurring. We are unable to explain the strong reciprocal dependence of inhibition upon cell number.

Varying the concentration of 3′-dATP in the presence of two unsaturating levels of ATP has demonstrated that the inhibitor is probably a purely competitive one ($K_I \simeq 30\ \mu M$) with respect to ATP (Figure 5). This finding indicates that 3′-dATP is most likely exerting its primary effect by interfering with some function of ATP and not of 2′-dATP.

The ability of 3′-dATP to effect the rapid termination of DNA synthesis upon addition to a progressing reaction while not stimulating the degradation of previously synthesized product (Figure 6) indicates that it is interfering with a function of ATP which is continuously required and that it does not activate appreciable nucleolytic activity. The competitive nature of the inhibition and the capacity of cells which have been incubated in the presence of the inhibitor, under conditions which would have allowed ATP-dependent DNA synthesis, to subsequently carry out such synthesis (Figure 7) indicate that 3′-dATP does not irreversibly inactivate the replicative synthetic machinery.

3′-dATP can specifically inhibit replicative DNA synthesis in toluene-treated *E. coli* by acting as a competitive inhibitor of ATP. It may prove to be useful in defining the functions of ATP in DNA replication if used in the purified reconstituted replication systems which are considered to be models of *E. coli* chromosomal replication (Wickner and Hurwitz, 1974; Schekman et al., 1975).

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