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Dynamics of pyrimidine deoxynucleoside triphosphate pools in relationship to DNA synthesis in 3T6 mouse fibroblasts

(nucleoside incorporation/compartmentation/amethopterin block)

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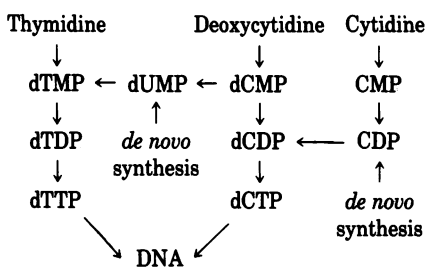
ABSTRACT **The ^{3}H-labeled nucleosides cytidine, deoxycytidine, and thymidine are rapidly incorporated into DNA via dCTP or dTTP pools. Between 30 and 60 min after addition of tracer amounts of a labeled nucleoside to the medium of rapidly growing 3T6 cells, dNTP pools attained a constant specific activity resulting from a steady-state equilibrium between incorporation of nucleoside, *de novo* synthesis, and linear incorporation of isotope into DNA. Removal of labeled deoxycytidine or thymidine depleted the dNTP pools of isotope within a few minutes and incorporation into DNA stopped. When *de novo* synthesis of dTTP was blocked with amethopterin, the intracellular dTTP pool rapidly reached the specific activity of thymidine of the medium and isotope incorporation into DNA then measured absolute rates of DNA synthesis. In experiments with and without amethopterin, we found no kinetic evidence for the existence of more than one dTTP pool and the decay of the pool suggested that all dTTP served as precursor of DNA. In contrast, experiments with deoxycytidine and cytidine suggested the presence of separate dCTP pools with preferential DNA synthesis from the pool labeled from cytidine.**

Incorporation of radioactive thymidine or, less frequently, other labeled nucleosides into DNA is often used to measure the rate of DNA synthesis. With thymidine and other *deoxy*nucleosides, incorporation involves a series of phosphorylation steps leading directly to dNTPs; with *ribo*nucleosides, reduction of the ribose moiety is required in addition. dNTPs are also formed *de novo* from small molecules. These interrelationships for the nucleosides thymidine, deoxycytidine, and cytidine are outlined below.



Clearly, incorporation of a labeled nucleoside into DNA depends on other factors in addition to the rate of the final polymerization of dNTPs.

In spite of this, incorporation of thymidine has been used widely, and often successfully, to monitor relative rates of DNA synthesis, neglecting possible errors introduced by changes in specific radioactivities of dTTP. A safer, but more cumbersome, approach would be to measure the specific activity of the immediate precursor dNTP and relate incorporation of radioactivity into DNA to this value (1, 2). Such a procedure becomes imperative when one wishes to distinguish between effects of different manipulations—e.g., pharmacological interference—on precursor synthesis and DNA replication. Knowing the specific activity of a dNTP under steady-state conditions may in addition permit determination of absolute rather than relative rates of DNA synthesis.

The possibility of compartmentation of dNTP pools poses additional complications (3). Fractionation of cells in nonaqueous media led to the suggestion of separate cytoplasmic and nuclear dNTP pools in Chinese hamster ovary cells (4). Kinetic experiment with HeLa cells were interpreted to indicate preferential incorporation of labeled thymidine into DNA via a dTTP pool that was not in equilibrium with dTTP formed *de novo* (5). The suggested channeling of DNA precursors via a postulated multienzyme "replitase" also leads to compartmentation of dNTPs (6).

The aim of the present work was to relate the synthesis and breakdown of pyrimidine dNTP pools to DNA synthesis in 3T6 cells. When dTTP was labeled by using thymidine and *de novo* synthesis was blocked with amethopterin, the specific activity of the pool rapidly approached that of the radioactive thymidine in the medium, permitting determination of absolute rates of DNA synthesis. In parallel experiments, we measured incorporation of labeled thymidine, deoxycytidine, or cytidine into DNA and dNTP pools in the absence of amethopterin. From data obtained under steady-state conditions with thymidine and cytidine, we could calculate rates of DNA synthesis very close to those obtained with thymidine in the presence of amethopterin, while the results with labeled deoxycytidine gave much lower values. These experiments suggest that, under steady-state conditions, DNA in 3T6 cells is synthesized from dTTP and dCTP pools in rapid equilibrium with labeled thymidine or cytidine. On the other hand, isotope from deoxycytidine appears to be preferentially incorporated into a dCTP pool that is not used directly for DNA synthesis. We also found that the decay of the labeled dTTP pool closely matched the rate of DNA synthesis.

MATERIALS AND METHODS

Materials. [5-^{3}H]Cytidine, [5-^{3}H]deoxycytidine, and [*methyl*-^{3}H]thymidine with specific activities of 20–30 Ci/mmol (1 Ci = 3.7 GBq) and ^{3}H- and α-^{32}P-labeled dNTPs were obtained from Amersham. Care was taken to use the radioactive compounds shortly after delivery or to redetermine their specific activity by HPLC.

Incorporation of ^{3}H-Labeled Nucleosides into Cells. 3T6 mouse fibroblasts (10^5 cells) were explanted onto 5-cm dishes and grown for 44–48 hr at 37°C in 5 ml of Dulbecco's modified Eagle's medium/10% inactivated horse serum to a density of

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Abbreviation: HAT, hypoxanthine/amethopterin/thymidine.

0.6–1.0 × 10^6 cells. Three hours before addition of isotope, the medium was reduced to 2.5 ml and buffered with 1 M Hepes (pH 7.4) (final concentration, 25 μM). In experiments measuring the decay of dNTPs, the cells were transferred in a warm (37°C) room from the incubator to tightly sealed boxes containing 5% CO_2/95% air atmosphere and all further manipulations in the warm room outside the boxes were as brief as possible. A ^{3}H-labeled nucleoside without dilution was added to the medium to a final concentration of about 0.3 μM and incubation was continued for the indicated times. To measure the decay of label, medium was removed by aspiration and immediately replaced with 2.5 ml of conditioned medium (from parallel cultures without isotope) containing 0.3 μM unlabeled nucleoside.

Incubations were terminated and isotope incorporation into DNA was determined as described (7) except that 2 ml of cold 60% methanol (8) was used in place of $HClO_4$. The mixture was centrifuged at 22,000 × g for 45 min, the supernatant was evaporated to dryness at reduced pressure, and the residue so obtained was dissolved in 0.2 ml of 10 mM Tris buffer (pH 7.0) and used for the determination of dNTPs (8, 9) and their specific activity (refs. 1, 2; unpublished data).

All values in the figures and tables are normalized to 10^6 cells.

RESULTS

Attainment of Steady-State Conditions. Tracer amounts of highly ^{3}H-labeled thymidine, deoxycytidine, and cytidine were added to separate sets of cultures of rapidly growing 3T6 cells. After 60 min at 37°C, equilibrium was attained in each case (Fig. 1). The total acid-soluble radioactivity, as well as the specific activity of the labeled dNTP pools, had reached a plateau value and isotope incorporation into DNA was occurring at a linear rate. The observed specific activity of a given dNTP is the result of a balance between phosphorylation of the labeled nucleoside and *de novo* synthesis. The rate of DNA synthesis can be calculated from the incorporation at 60–120 min. After that time (arrow in Fig. 1), radioactive medium was replaced with prewarmed conditioned medium containing 0.3 μM unlabeled nucleoside. With [^{3}H]thymidine and [^{3}H]deoxycytidine, this resulted in rapid depletion of the intracellular acid-soluble radioactivity, indicating rapid turnover of the dNTP pools, with half-lives of about 5 min. With [^{3}H]cytidine, about 90% of the acid-soluble radioactivity was present as CTP and, after medium change, isotope continued to flow from this pool into the much smaller dCTP pool. In this case, we could therefore not determine the turnover of the dCTP pool.

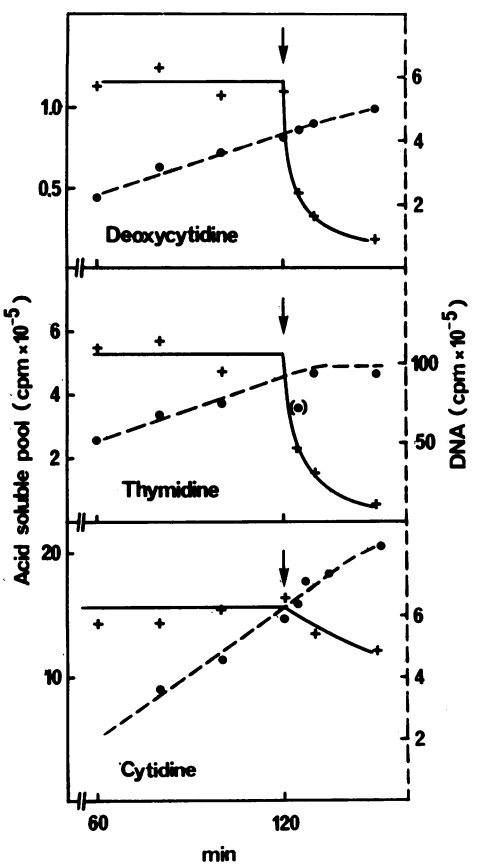

FIG. 1. Time course of incorporation of labeled nucleoside into acid-soluble nucleotide (+——+) and DNA (●---●).

Decay of dNTP Pools. A more careful analysis of the events during the first 10 min of the decay of dTTP and dCTP pools labeled by using thymidine or deoxycytidine, respectively, was made in a second experiment. In Fig. 2 the logarithms of the specific activities of the two dNTP pools are plotted as a function of time. The linearity of the first part of the curves suggests that, during this part of the experiment, the reaction followed first-order kinetics with half-lives of 5.5 (dTTP) and 6 (dCTP) min. From these values and the pool sizes for dTTP (80 pmol) and dCTP (115 pmol), one can calculate that the initial decay of radioactivity corresponded to turnover rates of 10 and 13 pmol/min per 10^6 cells, respectively.

Thymidine Incorporation Measures the True Rate of DNA Synthesis. Rates of DNA synthesis during brief experiments can be computed from the incorporation of isotope into DNA and the specific activity of a dNTP pool labeled under steady-state conditions as described in Fig. 1. This approach assumes that the measured value for the dNTP pool represents the specific activity of the immediate precursor for DNA synthesis. However, a given dNTP may be present in more than one pool and have a different specific activity in each. If then DNA is synthesized preferentially from only one such pool—derived, e.g., by *de novo* synthesis via reduction of ribonucleotides—the specific activity of a given dNTP pool labeled by using thymidine or deoxycytidine via the salvage pathway may not represent the relevant value for measurement of the rate of DNA synthesis.

We investigated this question by an experiment in which the *de novo* pathway was blocked and the cells were forced to syn-

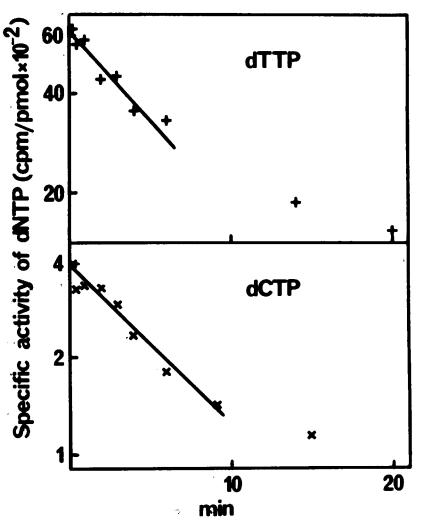

FIG. 2. Decay of radioactivity of dNTP pools labeled with thymidine or deoxycytidine. 3T6 cells were grown in radioactive medium for 60 min, medium was changed, and samples were removed for analysis of acid-soluble pools.

thesize DNA from dTTP obtained exclusively via the salvage pathway from added radioactive thymidine. For this purpose, amethopterin was included in the culture medium (10) and the cells were supplied with exogenous hypoxanthine and thymidine (HAT medium). Such cells, as well as parallel cultures in normal medium, were pulsed with [^{3}H]thymidine and the amount of radioactivity incorporated into DNA and the specific activity of the dTTP pool were determined at various times after addition of the radioactive nucleoside. Two separate experiments with HAT medium were made: (*i*) HAT medium was present from the start of the culture—i.e., for 48 hr before addition of [^{3}H]thymidine (HAT 1)—and (*ii*) HAT medium was introduced into the culture medium only 2 hr before addition of isotope (HAT 2). After 48 hr, the HAT 1 cultures (1×10^6 cells per dish) had reached almost exactly the same density as the control cells (0.8×10^6 cells per dish), showing that the growth rates were similar.

Incorporation of isotope into DNA (Fig. 3) was linear 30–90 min after addition of [^{3}H]thymidine in both normal and HAT cultures [where the enlarged dTTP pools (see Table 1) might delay equilibration with [^{3}H]thymidine]. The amount of radioactivity incorporated per unit time was about 20-fold higher in the control cultures. However, as shown in Table 1, this difference is matched by a corresponding difference in the specific activities of the dTTP pools. The latter values had already plateaued by the 30-min time point; the values given in Table 1 represent averages from four determinations between 30 and 90 min. All three cultures synthesized DNA at identical rates (expressed as pmol of dTMP incorporated). In the two HAT experiments, the specific activities of the dTTP pools were almost identical to the specific activity of the [^{3}H]thymidine of the medium. In contrast, the specific activity of the dTTP pool of the control culture was only 39% of the specific activity of the precursor thymidine, demonstrating dilution by endogenous dTTP synthesis.

In conclusion, this experiment showed that the true rate of DNA synthesis of 3T6 cells can be calculated from the incorporation of labeled thymidine into DNA, provided that the specific activity of the dTTP pool is determined under steady-state conditions.

DNA Synthesis from [^{3}H]Deoxycytidine and [^{3}H]Cytidine. Can the rate of DNA synthesis be similarily computed from the incorporation of deoxycytidine or cytidine? To decide this question, the incorporation of these nucleosides into DNA was compared with the incorporation of thymidine in parallel sets of cultures. Thymidine incorporation then gave the correct rate of DNA synthesis with which the rates computed from the incorporation of other nucleosides could be compared. The results of three such experiments are summarized in Table 2. In each case, several sets of cultures were pulsed with tracer amounts of nucleoside and the linear incorporation of isotope into DNA and the specific activities of the corresponding dNTP pools were determined, similar to the experiment described in Fig. 1 (experiment 2 of Table 2 is in effect identical to the one described in Fig. 1).

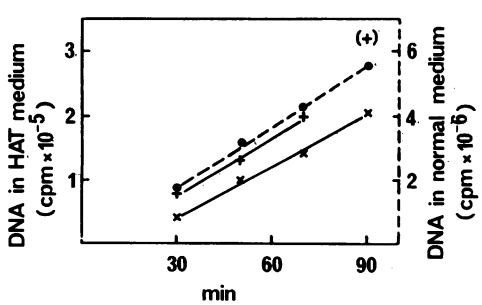

FIG. 3. Effect of HAT medium on incorporation of [^{3}H]thymidine into DNA. Labeled thymidine was added to cultures in normal medium (●) or in medium to which HAT medium had been added 2 (×) or 48 (+) hr earlier. The specific activity of thymidine in normal medium (0.3 μM) was 15,200 cpm/pmol and that in HAT medium (16.3 μM) was 286 cpm/pmol.

Table 1. Effect of HAT medium on dTTP pools and rate of DNA synthesis

Medium	dTTP pool		DNA synthesis	
	pmol	cpm/pmol	Radioactivity incorporated, cpm/min	dTMP incorporated, pmol/min
Normal	90	5,900	77,000	13
HAT 1	150	280	3,400	12
HAT 2	172	220	3,000	14

In each experiment, the rate of DNA synthesis computed from the incorporation of [^{3}H]deoxycytidine was much less than the rate calculated from incorporation of thymidine or cytidine. This indicates that the specific activity of the dCTP pool labeled by using deoxycytidine was not identical to the specific activity of dCTP used for DNA replication. With [^{3}H]cytidine, however, the computed rates of DNA synthesis were similar to those found with [^{3}H]thymidine. Taken together, our data indicate compartmentation of the dCTP pool.

This question was further investigated by measuring the effect of HAT medium on the incorporation of deoxycytidine into DNA. HAT increases the size of the dTTP pool (Table 1), which inhibits the reduction of CDP (11) and decreases the size of the dCTP pool to one-fourth (data not shown). Because deoxycytidine kinase is feedback inhibited by dCTP (12), HAT medium facilitated the entry of labeled deoxycytidine into the dCTP pool, resulting in an almost 6-fold increase in its specific activity and an even greater increase in incorporation of isotope into DNA (Table 3). The rate of DNA synthesis calculated from deoxycytidine incorporation in the presence of HAT was equal to the rate calculated from thymidine (Table 3).

Comparison of dNTP Pool Turnover and Rate of DNA Synthesis. The data in Fig. 2 and experiment 1 of Table 2 derive from the same sets of cultures and permit direct correlation of the decay of the radioactive dNTP pools with the rate of DNA synthesis.

With dTTP, the decay [(10 pmol/min)/10^6 cells] closely matched the rate of DNA synthesis (11 pmol/min per 10^6 cells), indicating that, under steady-state conditions, all dTTP is used for DNA replication. This conclusion is further strengthened by our finding that little or no radioactivity was excreted into the medium during the decay period (data not shown). With

Table 2. Rates of DNA synthesis as determined from specific activities of dNTP pools labeled by using thymidine, deoxycytidine, or cytidine

Exp.	Radioactive nucleoside	Specific activity of dNTP, cpm/pmol	Labeled dNMP in DNA, pmol/min
1	Thymidine	6,800	11
	Deoxycytidine	390	4
2	Thymidine	3,900	14
	Deoxycytidine	580	6
	Cytidine	380	16
3	Deoxycytidine	950	5
	Cytidine	770	12

Table 3. Effect of HAT medium on apparent rate of DNA synthesis as determined by incorporation of deoxycytidine or thymidine

System	dNTP pool, cpm/pmol	Labeled dNMP in DNA, pmol/min
Deoxycytidine	380	4
Deoxycytidine/HAT	2,200	11
Thymidine	3,700	12
Thymidine/HAT	380	11

HAT was added to the medium 2 hr before addition of isotope.

dCTP labeled by using deoxycytidine, the decay (13 pmol) clearly exceeds the computed rate of DNA synthesis (4 pmol).

DISCUSSION

We have for some time been interested in the interrelationship between the synthesis of deoxyribonucleotides and their utilization for DNA replication (13). We have carried out measurements of dNTP pool sizes under various conditions of cell growth and shown that there are large increases in the sizes of all four pools during the transition of cells from G_1 to S phase (14, 15). In particular, the size of the dCTP pool appeared to show considerable correlation with the percentage of cells in S phase.

With the present experiments, we begin to analyze the dynamics of pyrimidine dNTP turnover. We used cultures of rapidly growing 3T6 mouse fibroblasts with a generation time of about 12 hr. From autoradiography with [^{3}H]thymidine, we estimate that, during our experiments, about 70% of the cells were in different stages of S phase. Even though the heterogeneity of the cell population poses problems in the interpretation of our results, we believe that most of the effects described here relate to interrelationships between pyrimidine dNTP pools and DNA synthesis in S-phase cells.

dCTP and dTTP pools of 3T6 cells were labeled by addition of radioactive nucleosides to the growth medium. Earlier work by Plagemann *et al.* (16) has shown a rapid transport of labeled nucleosides from the medium into cultured cells followed by rate-limiting phosphorylation steps as outlined in the Introduction. The trace amounts of nucleosides used in our experiments caused minimal perturbation of the sizes of the dNTP pools, not measurable by our method. After an initial lag period, the specific activities of the dNTP pools attained constant values and isotope incorporation into DNA became linear. From data obtained during this steady-state period, we could calculate a rate of DNA synthesis, expressed as pmol of dNMP incorporated, assuming that the determined specific activity of the dNTP pool was identical with the specific activity of the substrate of the polymerase.

This assumption was validated for the dTTP pool by an experiment in which the pool was labeled with thymidine under two sets of conditions: (*i*) tracer amounts of labeled thymidine and (*ii*) excess labeled thymidine in HAT medium. In the second case, the radioactivity of the intracellular dTTP pool approached the specific activity of the external thymidine after 30 min and represented the specific activity of the substrate of the polymerase. Rates of DNA synthesis calculated from the two parallel sets of cultures were identical, showing that the experiment with the trace amount of labeled thymidine also lead to the correct value.

The turnover rate of the dTTP pool was determined after replacement of radioactive medium with medium containing unlabeled thymidine. From the decay of isotope, we could calculate a turnover of dTTP that closely matched the incorporation of dTMP into DNA calculated from the incorporation of radioactivity under steady-state labeling conditions. The stoichiometry between the amount of radioactivity leaving the dTTP pool and the amount of radioactivity entering DNA supports the existence of a single dTTP pool, exclusively involved in DNA synthesis. Our data do of course not exclude the existence of minor separate dTTP pools—e.g., in mitochondria (17)—or of several major pools in rapid equilibrium with each other.

The dCTP pool behaved differently. When the pool and DNA were labeled by using deoxycytidine, calculated rates of DNA synthesis were lower than rates calculated from incorporation of thymidine. On the other hand, cytidine labeling gave values similar to those obtained with thymidine. In these comparisons, one must include the reported thymidine/deoxycytidine ratio of 1.3 for mouse DNA (18). Deoxycytidine incorporation thus underestimated the rate of DNA synthesis while cytidine incorporation gave slightly high values. This suggests that deoxycytidine and cytidine introduce isotope into separate dCTP pools and that the pool labeled by using cytidine was more directly related to DNA synthesis. These pools need not necessarily be present in the same cell and we cannot exclude the possibility that deoxycytidine preferentially labeled dCTP present in cells not involved in DNA synthesis while cytidine labeled dCTP in cells in S phase. It is more likely, however, that both nucleosides were mainly incorporated into cells in S phase. Cytidine then labeled a dCTP pool directly connected with DNA synthesis while deoxycytidine labeled a second pool that reached equilibrium with the first one slowly. This explanation is favored by the stimulation of deoxycytidine incorporation by HAT medium (Table 3), an effect that can be ascribed to the contraction of the first dCTP pool formed by the reduction of CDP coupled to an increase of isotope incorporation from deoxycytidine into the second dCTP pool.

Earlier results (4) suggested that the dCTP pool is compartmentalized between the cytoplasm and the cell nucleus while the dTTP pool is exclusively in the nucleus. Our present data could relate to this result: the nuclear dNTPs then represent the proximal precursor pools and are labeled from thymidine and cytidine while deoxycytidine preferentially labels the cytoplasmic dCTP pool.

The existence of more than one dCTP pool makes it difficult to analyze the decay of isotope from dCTP labeled by using deoxycytidine. dCTP also serves as a substrate for dTMP formation via deamination of dCMP to dUMP (see the Introduction). During this reaction sequence, all isotope is lost to the medium because both deoxycytidine and cytidine were labeled in the 5′ position of the pyrimidine ring. Further work is required to clarify this situation.

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