Cell Cycle-dependent Metabolism of Pyrimidine Deoxynucleoside Triphosphates in CEM Cells*

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We incorporated [3H]-labeled thymidine, deoxycytidine, or cytidine into dNTPs and DNA of exponentially growing CEM cells. G_1 and S phase cells were separated by centrifugal elutriation, and the size and specific activity of dNTP pools were determined to study the cell cycle-dependent regulation of specific dNTP synthesizing enzymes in their metabolic context. With [3H]thymidine, we confirm the earlier demonstrated S phase specificity of thymidine kinase. Incorporation of radioactivity from [5-3H]deoxycytidine into dCTP occurred almost exclusively in G_1 cells. During S phase, de novo synthesis by ribonucleotide reductase was switched on, resulting in a 70-fold dilution of [3H]dCTP, confirming that ribonucleotide reductase is an S phase-specific enzyme, whereas deoxycytidine kinase is not. [5-3H]Cytidine appeared in dCTP almost to the same extent in G_1 as in S phase, despite the S phase specificity of ribonucleotide reductase. During S phase, DNA replication greatly increased the turnover of dCTP, requiring a corresponding increase in ribonucleotide reductase activity. During G_1, the enzyme maintained activity to provide dNTPs for DNA repair and mitochondrial DNA synthesis. The poor incorporation of isotope from deoxycytidine into DNA earlier led to the suggestion that the nucleoside is used only for DNA repair (Xu, Y.-Z., Peng, H., and Plunkett, W. (1995) J. Biol. Chem. 270, 631–637). The poor phosphorylation of deoxycytidine in S phase provides a better explanation.

In mammalian cells, many of the enzymes required for the production of dNTPs are regulated during the cell cycle such that their activities increase during S phase, the time of DNA replication (1). In some instances the molecular mechanisms creating this phenomenon are known in detail and affect both transcription and translation (2–7). The magnitude of the effect varies greatly for different enzymes. Thymidine kinase (2–4) and ribonucleotide reductase (5–7) show low activity outside S phase, with large increases during S phase. Conversely, deoxycytidine kinase (8) is constitutively active, and also thymidylate synthase undergoes only minor variations during the cell cycle (9). It is noteworthy that the activity of enzymes belonging to the de novo pathway as well as enzymes of the salvage pathway may either vary greatly or not at all during the cell cycle.

This picture comes largely from experiments in which the activity of enzymes was measured either directly in cell extracts or by an in situ assay in intact cells. The latter method is of greater physiological relevance. It involves incubation of cells in culture with highly radioactive ribo- or deoxyribonucleosides, usually followed by an analysis of the time-dependent flow of isotope through the nucleotide pools into nucleic acids and, in appropriate cases, into excretion products in the medium. Inhibitors (9–12) or mutations (13, 14) affecting enzymes of dNTP synthesis influence the flow of isotope and can give information about the normal process. Two major pitfalls of this technology are all too often neglected (1): (i) heterogeneity in the cell population under study and (ii) changes in the specific radioactivity of the dNTP pools. The two factors may combine and distort the result to a point where they are misinterpreted (14). This was first recognized in experiments with Chinese hamster ovary cells (15) and further studied in more detail with V79 (16) and CEM (13) cells. Consider a population of cycling cells in culture in which the DNA is labeled from a nucleoside. Fig. 1 shows the series of reactions that, after the entry of a labeled nucleoside into the cell, transform the nucleoside into a labeled dNTP prior to incorporation into DNA. A deoxyribonucleoside, such as thymidine or deoxycytidine, is transformed directly via three phosphorylation steps, whereas a ribonucleoside, such as cytidine, is first phosphorylated to CDP, followed by reduction to dCDP by ribonucleotide reductase, and finally phosphorylated to dCTP. The radioactive nucleotides mix with nucleotides synthesized de novo, resulting in dilution of radioactivity. The specific radioactivity of a dNTP pool thus depends not only on the efficiency with which the nucleoside is transformed to the dNTP but also on the rate at which the dNTP is synthesized de novo. An additional important consideration is the rate with which the dNTP is removed, either by its catabolism or by its utilization for DNA synthesis. The specific activity of the dNTP gives an indication of the relative activities of all these processes.

The dilution factor may vary widely between cells inside or outside S phase. Deoxycytidine kinase, the enzyme that phosphorylates not only deoxycytidine but also deoxyadenosine and deoxyguanosine, is not cell cycle-regulated (8). The nucleosides are therefore phosphorylated to the same extent in S phase and in G_1 or G_2 cells. In contrast, the de novo synthesis of unlabeled dNTPs via ribonucleotide reductase proceeds at a faster rate during S phase when this enzyme is turned on (5–7). The dilution of isotope from deoxycytidine to dCTP is therefore much larger in S phase. In an asynchronous cell population, the measured specific activity of the dCTP pool is an average for

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RNA   DNA

CTP   dCTP   dTTP

RNR

CDP   dCDP   dTDP

CMP   dCMP   dTMP

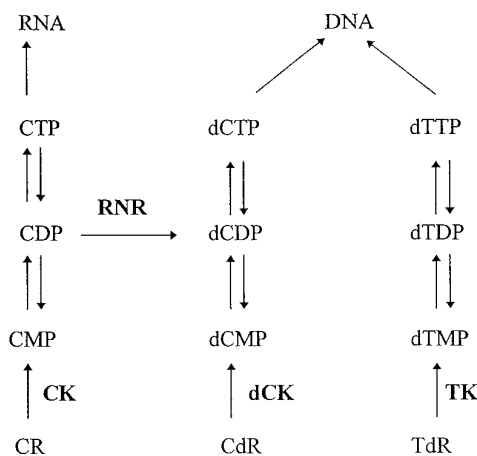**CK**   **dCK**   **TK**

CR   CdR   TdR

FIG. 1. **Pathways for incorporation of label from [*methyl*-³H]thymidine, [5-³H]cytidine, or [5-³H]deoxycytidine into nucleotides and nucleic acids.** Note that only cytidine requires the participation of ribonucleotide reductase (the *de novo* pathway) and that the label from the 5-position of cytosine is not incorporated into thymine of DNA. *CK*, cytidine kinase; *dCK*, deoxycytidine kinase; *TK*, thymidine kinase; *RNR*, ribonucleotide reductase.

dCTP from all cells. DNA replication utilizes only the pool of S phase cells that has a lower value. Calculating the rate of DNA replication from the average specific activity then gives too low a value (17). The situation differs for thymidine, since thymidine kinase is cell cycle-regulated and increases in S phase. In this case, the average specific activity of dTTP in a cycling population is close to that of S phase cells (17).

To obtain true measurements of the turnover of dNTP pools in relation to DNA replication, one must use cells whose stage in the cell cycle is defined. To achieve this, previous studies used cells synchronized by isoleucine (18) or serum starvation (19), or they employed inhibitors of DNA replication (9). The cells were then analyzed at different times after the release of the block as they moved more or less synchronously through the cell cycle.

In a different approach, cells from an asynchronous population can be separated by centrifugal elutriation (20, 21) and then analyzed for specific cell cycle-related activities. With this method, cells are separated according to size into fractions enriched in small G₁ cells, larger S phase cells, or still larger G₂ cells. Some overlap occurs, but by analyzing separate subpopulations with various proportions of G₁ and S phase cells, valuable information can be obtained on enzyme activities in specific phases of normal cells. In the present work, we combined centrifugal elutriation with the use of radioactive nucleosides to define the pool sizes and specific activities of dNTPs in G₁ and S cells labeled from thymidine, deoxycytidine, or cytidine. CEM cells from exponentially growing cultures labeled from nucleosides were separated, and subpopulations enriched in different phases of the cell cycle were obtained. The specific activities of appropriate pyrimidine dNTP pools of G₁ and S phase cells were determined, and the rate of DNA replication was calculated from the incorporation of isotope into DNA.

<center>EXPERIMENTAL PROCEDURES</center>

Materials—³H-Labeled nucleosides were obtained from Amersham Life Science, Inc., with the following specific activities (in Ci/mmol): [³H-methyl]thymidine, 20; [5-³H]deoxycytidine, 26; [5-³H]cytidine, 28. One µCi corresponded to 1.12 million cpm in our liquid scintillation counter. ³²P-Labeled dATP (for pool determinations) was also from Amersham. The human T lymphoblasts CEM CCL 119 line was obtained from the American Type Culture Collection. These cells are considered to be diploid but contain about 5% polyploid cells.

Cells and Incubations with Labeled Precursors—Cells were grown in Dulbecco's modified Eagle's medium, supplemented with 7.5% fetal calf serum, glutamine, and antibiotics, at 37 °C and 5% CO₂ in a humidified incubator. Under these conditions, the population doubling time was 24 h. Absence of Mycoplasma contamination in the cultures was ascertained periodically with a kit based on the detection of Mycoplasma-specific rRNA (Gen-probe Inc., San Diego, CA). For the experiments, cultures were started by inoculating 0.2×10^6 cells/ml in fresh medium. After 48 h, the cells were counted, and about 150×10^6 cells were transferred without change of medium to a 225-cm² flask, returned to the incubator, and incubated with the radioactive precursor for the appropriate time. The cells were handled in a climatized room at 37 °C to minimize interference with cell metabolism. Tritiated thymidine, deoxycytidine, and cytidine were used at 0.1 µM final concentration at the specific activities supplied by the manufacturers. At the end of the incubations, replicate samples of $1–2 \times 10^6$ cells were taken as controls and immediately processed for extraction of the soluble nucleotide pool and the macromolecular fraction, while the remaining cell suspension was separated by elutriation to obtain populations of cells enriched in different phases of the cell cycle.

Elutriation Procedure—The cell suspension was chilled on ice, and cells were collected by centrifugation in a refrigerated centrifuge (10 min at $500 \times g$) and then resuspended in 8 ml of ice-cold Dulbecco's modified Eagle's medium containing 5% fetal calf serum. They were immediately injected in the elutriation chamber of a Beckman Avanti J-25 high performance centrifuge equipped with a JE-6B rotor and a standard chamber and a Cole-Parmer system model 7553-75 pump. The centrifuge was run continuously at 2500 rpm. Eight separate fractions were collected by pumping ice-cold Dulbecco's modified Eagle's medium with 5% fetal calf serum through the chamber at increasing flow rates (18, 18, 20, 22, 24, 26, 28, and 30 ml/min). The first two fractions were 50 ml each, and the others were 100 ml. The first and last fractions were discarded. The latter contained a high percentage of polyploid cells.

Each elutriated fraction was immediately centrifuged at 4 °C for 10 min at $500 \times g$, and the sedimented cells were suspended in 5 ml of cold Hanks' saline, sedimented again by 5-min centrifugation at $300 \times g$, and resuspended in 5 ml of cold saline. Cells were counted in a Coulter Z1 counter, portions (10^6 cells) were taken for flow cytometric analysis and autoradiography, and the rest was used for nucleotide pool and DNA analyses.

The distribution of cells in the different phases of the cell cycle was determined by flow cytometry in an Epics-Elite flow cytometer (Coulter), equipped with a 488-nm argon ion laser running at 15 milliwatts. The percentage of S phase cells was also measured by autoradiography, since flow cytometry did not score very early S phase cells whose DNA content had only increased minimally. The slides were prepared as described previously (22), covered with autoradiographic emulsion (K2 Ilford, Eastman Kodak Co.), exposed for 15 days at 4 °C in the dark, developed, fixed, and stained with 10% Giemsa. Labeling indexes were determined by counting 1500 cells/slide. The values from autoradiography, rather than from flow cytometry, were used for the calculations of S phase.

Analyses of Nucleotide Pools—Extraction of the soluble nucleotide pool and determination of dNTP pool size and specific activity were performed as described (23, 24). Substances interfering with the analyses in experiments involving labeling from cytidine were removed by acid treatment (11). The specific activity of CTP in the cells labeled from cytidine was determined by high pressure liquid chromatography on Partisil-10 SAX, using isocratic elution with 0.4 M NH₄H₂PO₄, pH 2.9 (25).

Calculation of dNTP-specific Activities of G₁ and S Cells—The dNTP determinations gave the size and specific activity of dNTP pools of subpopulations enriched with respect to G₁, S, or G₂ cells. They did not give the values of pure cell cycle populations. Such values could be calculated from data sets of elutriated enriched subpopulations as shown by the following example; two fractions devoid of G₂ cells (fractions 1–5 in Fig. 2) were used for two sets of two equations. The first set served to determine pool sizes in S phase (a) and G₁ (b) from the following equation that uses the experimental data obtained from one subpopulation rich in G₁ cells (*e.g.* fraction 2 in Fig. 2) and one rich in S phase cells (*e.g.* fraction 5 in Fig. 2): ($a \times$ % cells in S) + ($b \times$ % cells in G₁) = 100 × dNTP pool size$_{det}$. Once a and b are known, the second two-equation system can be solved to determine x (the specific activity in S) and y (the specific activity in G₁): ($a \times x \times$ % cells in S) + ($b \times y \times$ % cells in G₁) = 100 × dNTP$_{size}$ × specific activity$_{det}$. Values of a, b, x, and y obtained by combining the data from different pairs of elutriated cell populations were averaged. The specific activity for the dNTP pools of S phase cells could then be used to measure the true rate of DNA synthesis.

Determination of the Rate of DNA Synthesis—The cells remaining

TABLE I
Rate of DNA replication calculated from the specific activity of dNTPs in cycling cells

Precursor	dNTP	DNA	
		Radioactivity	Rate of synthesis
	$cpm/pmol$	$cpm/min/10^6\ cells$	$pmol/min/10^6\ cells$
Thymidine	1150	3200	2.8
Deoxycytidine	6150	730	0.12
Cytidine	910	1500	1.6

after extraction of the soluble pool with methanol were dissolved in 1 ml of 0.3 M NaOH, and the radioactivity in DNA was determined (10). The rate of DNA synthesis is expressed as pmol of dNMP incorporated per min by dividing the increase in radioactivity (cpm/min) during a time window by the averaged specific activity (cpm/pmol) of the dNTP during the same window. Cytidine and deoxycytidine were labeled with tritium in the 5-position of the pyrimidine ring. The isotope equilibrates with water during the methylation of dUMP to dTMP, and the label is therefore not incorporated into thymine of DNA.

RESULTS

Comparison of Thymidine, Deoxycytidine, and Cytidine as Precursors for DNA in Cycling Cells—Thymidine and deoxycytidine are both efficiently phosphorylated to deoxynucleotides by mammalian cells, more so than purine deoxynucleosides, which are rapidly degraded by catabolic enzymes. In lymphoid cells, deoxycytidine kinase is highly expressed, and these cells phosphorylate deoxycytidine more actively than most other cell types (8). However, in exponentially growing CEM cells, the incorporation of radioactivity from deoxycytidine into DNA is low compared with that of thymidine (Table I). The cultures were incubated for either 30 or 60 min with the labeled deoxynucleoside, after which time the incorporation of radioactivity into DNA and dCTP or dTTP was measured. Whereas the specific activity of dCTP labeled from deoxycytidine is 5–6-fold higher than that of dTTP labeled from thymidine, the incorporation is reversed, with 5 times more radioactivity in DNA in the presence of thymidine. When these data are used to calculate the rate of DNA synthesis from the apparent incorporation of each dNMP into DNA during the time window as described under "Experimental Procedures," the value in the deoxycytidine experiment is only 5% compared with the thymidine experiment. Table I also gives the results from a parallel experiment in which dCTP and DNA were labeled from radioactive cytidine via the *de novo* pathway. The rate of DNA synthesis is now close to that found in the thymidine experiment.

Separation of G_1 and S Phase Cells by Centrifugal Elutriation—The poor incorporation of deoxycytidine into DNA agrees with previous data (10, 17). It suggests compartmentation of the dCTP pool, either intracellular or intercellular. To distinguish between these two possibilities, we separated CEM cells, labeled during exponential growth from nucleosides, by centrifugal elutriation to determine separately the labeling of S phase and G_1 cells. The procedure gives subpopulations of cells enriched in different phases of the cell cycle. Each can then be analyzed separately, and the results can be used to calculate dNTP pool sizes and specific activities of G_1 and S phase cells as described under "Experimental Procedures." Fig. 2 shows results concerning cell distribution among different cycle phases in the various subpopulation determined by flow cytometry and autoradiography. In this experiment, the cells were labeled for 60 min with thymidine, but closely similar separations were obtained in all other experiments reported here. G_1 cells dominated in fractions 1 and 2, and S phase cells dominated in fractions 4–6, with fraction 3 taking an intermediate position (Fig. 2). G_2 cells started to appear in fractions 5 and 6 and were abundant in fraction 7. For our calculations, we always paired two fractions with opposite extreme values for

G_1 and S phase cells and lacking G_2 cells. Fraction 1 often contained too few cells to give reliable results.

The discrepancy in the amount of S phase cells determined by flow cytometry and autoradiography was expected. The latter method always gave a larger proportion of S phase cells. The effect was much more pronounced in the early fractions that contain small cells than in later ones with larger cells. A correlation between the two methods is given in Fig. 3 with data from several experiments using either labeled thymidine or deoxycytidine. The same correlation was found with both labeled nucleosides, although cells in the deoxycytidine experiments were less intensely labeled, suggesting that the two deoxynucleosides labeled the same cell population. In the equations described under "Experimental Procedures," we used the values from autoradiography, since they include all S phase cells. Since only subfractions lacking G_2 cells were used, the fraction of G_1 cells could be calculated by the difference. In the cytidine experiment, autoradiography was not feasible, since cytidine is extensively incorporated into RNA. Fig. 3 was used to correct the data from flow cytometry.

Incorporation of Nucleosides into dNTPs of Separated Subpopulations—Incubations were carried out with a 0.1 μM concentration of each highly labeled nucleoside for two time periods (30 and 60 min for deoxynucleosides, 60 and 120 min for cytidine). The two time points served to measure the increase of isotope in DNA from which we calculated the rate of DNA synthesis. The time window was chosen such that the specific activity of the dNTP approached a steady-state value. Cells were immediately separated by centrifugal elutriation into fractions such as shown in Fig. 2, and the dNTP pools of the subpopulations were analyzed. Cell isolation involved a series of centrifugations in the cold, and we cannot rule out some breakdown of dNTPs during this process. This would not affect determinations of specific activities but would influence measurements of pool sizes. A portion of the cells before elutriation, representing the exponentially growing culture was used to determine isotope incorporation into DNA. Fig. 4 shows results of dNTP pool analyses in fractions from CEM cells separated by elutriation after 60 min of incubation with tritiated thymidine (Fig. 4A) or deoxycytidine (Fig. 4B) or 120 min with cytidine (Fig. 4C). Subpopulations are numbered as in Fig. 2. Similar results were obtained in each experiment also at the earlier time point (data not shown). All fractions were analyzed by flow cytometry, and in the thymidine and deoxycytidine experiments also by autoradiography, to determine the distribution of cells along the cell cycle. These data, which are not shown here, were used for the equations to calculate pool sizes and specific activities.

In Fig. 4A (thymidine experiment) the specific activity of dTTP increased progressively from the early fractions rich in G_1 cells to later ones, rich in S phase cells. The size of the dTTP pool also increased.

In Fig. 4B (deoxycytidine experiment) the specific activity of dCTP follows the opposite pattern; a progressive decline occurs from high values in the first fractions to a minimum in the S phase-rich fractions 4 and 5. The size of the dCTP pool increased only marginally or not at all, which differs from the behavior of the dCTP pool in the cytidine experiment (see below). In this experiment, fraction 1 also contained sufficient cells to be used for our calculations. Fraction 5, on the other hand, contained 25% G_2 cells and was excluded.

In Fig. 4C (cytidine experiment) the specific activity of dCTP shows small variations and increases at most 20% from the G_1 phase-rich fraction 2 to the S phase-rich fractions 3–5. Fraction 5 again contained too many G_2 cells to be used for the calculations. Comparing Fig. 4, B and C, we note that the dCTP pool

FIG. 2. **Separation of exponentially growing CEM cells by centrifugal elutriation.** The cells were labeled for 60 min from [³H]thymidine. Fractions 1–7 described under "Experimental Procedures" were analyzed by flow cytometry to give the percentage of G_1 (■), S phase (□), and G_2 (▲) cells. In addition, fractions 2–7 were analyzed by autoradiography to give the percentage of S phase cells (○). The *right part* of the *figure* shows the patterns of the fluorometric analyses of fractions 2, 5, and 7.
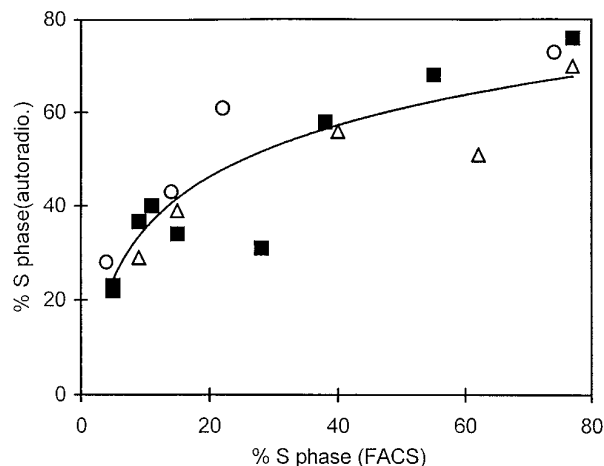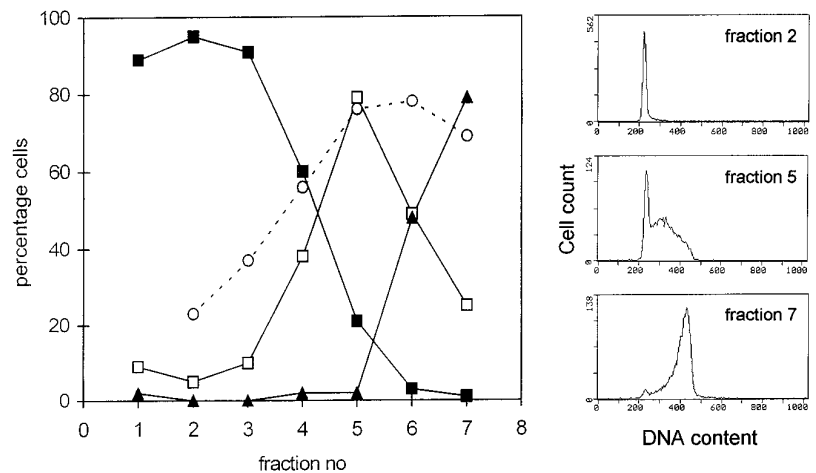

FIG. 3. **Correlation between the percentages of S phase cells obtained by flow cytometry and by autoradiography.** The data are from two separate experiments with thymidine (■ and ○) and from one with deoxycytidine (△).

is more heavily labeled from deoxycytidine than from cytidine, 20 times more in fraction 2 (G_1 cells), and 5 times more in fraction 5 (S phase cells), reflecting the large dilution of radioactivity in CTP by *de novo* synthesis from nonlabeled precursors. The size of the CTP pool did not vary between fractions 2 and 5, and its specific activity was within experimental error the same as that of dCTP, both after 60 and 120 min (data not shown). Note also that the size of the dCTP pool in the cytidine experiment was smaller than in the deoxycytidine experiment. In the latter case, the dCTP pool was expanded due to the presence of deoxycytidine in the medium. In separate experiments not involving elutriation, the addition of as little as 0.1 μM deoxycytidine to the medium of CEM cells led to a 50% expansion of the dCTP pool after 1 h (data not shown). A final difference concerning dCTP is the 3-fold increase in pool size moving from fraction 2 to 5 in Fig. 4C, demonstrating the presence of a larger dCTP pool in S phase cells.

Fig. 4 gives only data from the later time point, but similar results were found with each of the three nucleosides also at the earlier time point (data not shown).

Size and Specific Activity of dNTP Pools in G_1 and S Phase Cells—The data from the individual elutriated fractions were used to calculate the size and specific activity of dNTP pools of G_1 and S phase cells. For all calculations, we used pairs of subpopulations, with one member containing a majority of G_1 cells and the other a majority of S phase cells. Subpopulations with more than 8% G_2 cells were excluded.

Table II uses the data from the thymidine experiment for

such a calculation. A comparison of the results obtained from the two combinations of pairs shows good agreement for S phase cells but larger variations for G_1 cells. It is, however, evident that the specific activity of dTTP is approximately 10 times higher in S phase than in G_1, both after 30 and 60 min. This result agrees with the well known S phase specificity of thymidine kinase. It is also clear that the pool had not yet reached a steady state after 30 min, since its specific activity was 50% higher after 60 min. This, together with the low specific activity of the pool shows that CEM cells phophorylate thymidine poorly compared with 3T6 (10) or 3T3 cells (26). The pool size of dTTP is 2.5–3.5-fold larger in S phase than in G_1. In the nonseparated cycling cells, most of dTTP is therefore present in S phase cells. As a consequence, the specific activity of dTTP in cycling cells is close to that of S phase cells.

Similar calculations for the cells labeled from deoxycytidine give a completely different picture (Table III). The average specific activity of the dCTP pool is now approximately 30 times higher in G_1 than in S phase. For G_1 cells, the duplicates from the combinations of pairs are in good agreement. There is a doubtful slight increase of the specific activity between 30 and 60 min. The values for S phase cells agree rather poorly, similar to the G_1 values of Table II. When large differences occur between the specific activities of G_1 and S phase the calculation of the low values is very sensitive to small experimental errors. The size of the dCTP pool was more than 2-fold larger during S phase. The specific activity of the S phase dCTP pool amounted to only 7% of the value in nonseparated, cycling cells (Table I). In the cytidine experiment, dCTP had a higher specific activity in S phase than in G_1, but the difference was small, less than a factor of 2 (Table IV). The duplicate values from the pair combinations agreed quite well, and for S phase values they showed a small increase between 60 and 120 min. The dCTP pool was very small in G_1 but increased 3-fold during S phase.

DNA Replication Calculated from the Specific Activities of dNTPs in S Phase—The specific activities of the respective dNTP pools in S phase represent the true specific activities of the DNA precursors in cells synthesizing DNA. In Table I we used the mean specific activities of the dNTPs in cycling cells to calculate DNA synthesis. In Table V we use S phase values. Since in the thymidine and cytidine experiments the specific activities of the dNTPs in S phase were close to the values for the general population, it is not surprising to find that the rates changed only little. In the deoxycytidine experiment, however, the change is dramatic, with the rate of DNA synthesis increasing almost 15-fold. As pointed out earlier, the S phase values from this experiment cannot be considered to be very accurate. Nevertheless, it is obvious that when using the S phase value
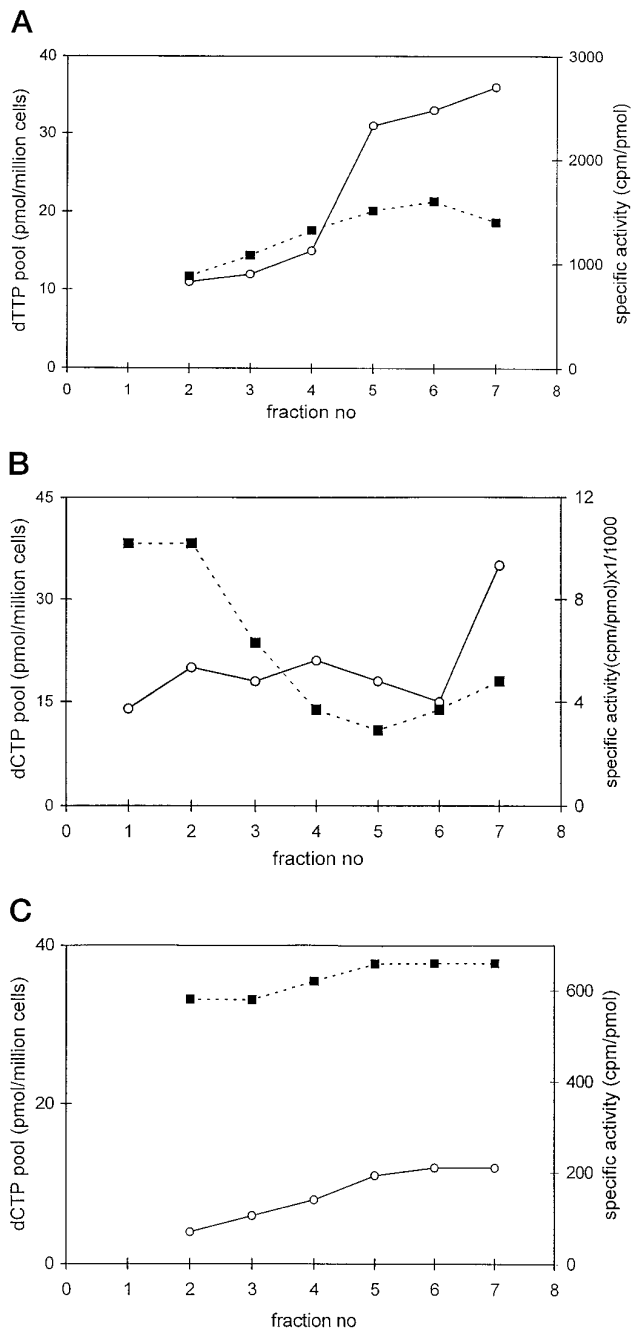
A

B

C

FIG. 4. **dNTP pool size (○) and specific radioactivity of dNTP (■) in the different fractions obtained by centrifugal elutriation in the experiments with thymidine (dTTP pool, 60 min (*A*)), deoxycytidine (dCTP pool, 60 min (*B*)), and cytidine (dCTP pool, 120 min (*C*)).**

the discrepancy between deoxycytidine and the other precursors seen in Table I becomes insignificant.

DISCUSSION

Many earlier experiments measuring the flow of isotope from labeled nucleosides into dNTP pools and DNA were made in cycling cells in culture (10–13). Cell heterogeneity may complicate the interpretation of such experiments (15), since DNA replication is limited to cells in S phase, whereas dNTP pools are present in all cells. Later work with synchronized cell cultures avoided some of these problems (16, 19, 26). Cell synchronization may, however, introduce new complications, that we avoid in the present work by carrying out experiments with cycling cells that were separated into G_1 and S phase

fractions by centrifugal elutriation after isotope incubation. Separation was not complete. Fractions 1 and 2 contained approximately 75% G_1 and 25% S phase cells, whereas fractions 4 and 5 showed the opposite distribution, 65–80% in S phase, the rest mostly in G_1. By combining pool data from two extreme fractions, we calculated theoretical values for pool sizes and specific activities of pools in homogeneous G_1 and S phase cell populations.

The limitations of this approach should be recognized. A basic flaw of the procedure is that it does not differentiate between early and late S phase cells. A second, practical problem is that small errors in the determination of specific activities are magnified if the values for G_1 and S phase cells are very different. This affects the value of the pool with the low specific activity. The large variations in Tables II and III for the G_1 dTTP pool and S phase dCTP pool, respectively, bear witness of this problem. A third problem comes from the prolonged manipulation of the cells, during which the dNTPs can undergo some degradation. This does not affect their specific activity but causes some uncertainty about pool size data. Once these limitations are realized, we believe that this approach provides reliable information concerning differences in the synthesis of dNTPs during G_1 and S phase. This information in turn reflects on the regulation of the enzymes involved in dNTP synthesis. Enzyme regulation is usually studied with isolated proteins or their genes, but here we can observe their activity in their metabolic context, together with other related enzymes. In general, our data confirm the earlier known S phase-dependent activation of the enzymatic machinery required for the synthesis of DNA precursors. In addition, we now provide evidence that *de novo* synthesis of dNTPs also occurs outside S phase. Below we will discuss separately results obtained with each of the three labeled nucleosides.

The thymidine experiment indicates that the nucleoside is utilized rather poorly for dTTP synthesis by CEM cells, compared with several other cell lines. In 3T3 cells, dTTP attained its final high specific activity already during 15 min, with the radioactivity being only 2-fold diluted from thymidine (26). Synchronized G_1 and S phase cells reached isotope equilibrium equally rapidly, but due to DNA replication the turnover of dTTP was 200 times faster in S phase (26), indicating that thymidine phosphorylation by the kinase also occurred 200 times more rapidly. In the present experiment with CEM cells, the dilution from thymidine to dTTP was at least 10-fold during S phase and 100-fold in G_1. Thymidine kinase was essentially inactive during G_1, in agreement with many earlier experiments demonstrating the S phase dependence of the activity of this enzyme. However, in CEM cells, thymidine kinase also showed low activity in S phase and competed poorly for the synthesis of dTTP with the *de novo* pathway.

The deoxycytidine experiment provides very different results. The dCTP pool attained isotope equilibrium with the nucleoside already after 30 min with a dilution factor of 5 in the average cell population and 2 in G_1 cells. In comparison with 3T3 or 3T6 fibroblasts (10, 19), CEM cells use deoxycytidine very efficiently. This also appears from the finding that the presence of 0.1 μM deoxycytidine in the medium slightly expands the dCTP pool, which is not the case with the fibroblastic cell lines. Most striking is, however, the low labeling of dCTP from deoxycytidine during S phase. The specific activity of dCTP then amounts to only 3% of that in G_1 cells. The low radioactivity of dCTP during S phase is caused by the rapid, cell cycle-dependent *de novo* synthesis of dCTP from nonlabeled precursors that dilutes the specific activity of dCTP (*cf.* Fig. 1).

The first two enzymes involved in the transformation of cytidine to dCTP and DNA are cytidine kinase and CMP kinase

TABLE II
Calculated size and specific activity of dTTP pools in G_1 and S phase cells from the thymidine experiment

Fraction pairs	G_1		S	
	Size	Specific activity	Size	Specific activity
	pmol/10^6 cells	*cpm/pmol*	*pmol/10^6 cells*	*cpm/pmol*
30 min				
2 + 4	20	70	23	1090
2 + 5	16	−60	49	900
60 min				
2 + 4	8	200	22	1550
2 + 5	2	170	41	1540
Average	12	150	34	1270

TABLE III
Calculated size and specific activity of dCTP pools of G_1 and S phase cells from the deoxycytidine experiment

Fraction pairs	G_1		S	
	Size	Specific activity	Size	Specific activity
	pmol/10^6 cells	*cpm/pmol*	*pmol/10^6 cells*	*cpm/pmol*
30 min				
1 + 4	13	13,700	23	0
1 + 3	12	15,300	27	−870
2 + 4	8	13,200	25	2100
60 min				
1 + 4	11	15,400	26	1320
1 + 3	10	17,600	30	245
2 + 4	19	13,700	32	−200
Average	12	14,800	27	430

TABLE IV
Calculated size and specific activity of dCTP pools in G_1 and S phase cells from the cytidine experiment

Fraction pairs	G_1		S	
	Size	Specific activity	Size	Specific activity
	pmol/10^6 cells	*cpm/pmol*	*pmol/10^6 cells*	*cpm/pmol*
60 min				
2 + 4	4	325	4	640
2 + 3	2	390	9	450
120 min				
2 + 4	2	270	12	750
2 + 3	3	390	9	680
Average	3	340	9	630

TABLE V
Rate of DNA replication calculated from the specific activity of S phase dNTPs

Precursor	dNTP	DNA	
		Radioactivity	Rate of synthesis
	cpm/pmol	*cpm/min*	*pmol/min*
Thymidine	1270	3200	2.5
Deoxycytidine	430	730	1.7
Cytidine	630	1500	2.4

(see Fig. 1). Our results suggest that neither enzyme is cell cycle-regulated. Incorporation of isotope from cytidine to CDP suffered a large dilution from *de novo* synthesis from nonlabeled precursors, but there was little difference between G_1 and S phase. The next step, reduction of CDP to dCDP then leads to no further dilution, either in G_1 or S phase. However, only in S phase is there a rapid turnover of dCTP (19) connected with DNA replication, requiring continuous replenishment via ribonucleotide reduction. Thus, the activity of ribonucleotide reductase must be greatly increased in S phase, in agreement with earlier results (5–7). The present finding that the dCTP pool of G_1 cells is labeled from cytidine almost equally well as the pool in S phase cells reflects the remaining ribonucleotide reductase activity, providing dNTPs for mitochondrial DNA synthesis and nuclear DNA repair.

Our work reemphasizes the importance of recognizing intercellular compartmentation when interpreting isotope incorporation data. With respect to dCTP, there is earlier evidence also for intracellular compartmentation. S phase-synchronized 3T3 cells contain a dCTP pool labeled from deoxycytidine, preferentially used for liponucleotide synthesis (19, 27). This pool is, however, in rapid equilibrium with the dCTP pool arising from *de novo* synthesis and labeled from cytidine. It is therefore also used for DNA replication (19). It has actually been long accepted knowledge that deoxycytidine is extensively incorporated into DNA during replication, both *in vivo* (28) and *in vitro* (19). It was therefore surprising to read in a recent report (29) that deoxycytidine is primarily used for DNA repair by CEM cells but not for replication. In support of this were experiments showing that deoxycytidine was poorly used for DNA synthesis during S phase, compared with thymidine, whereas the reverse occurred during repair in G_1. The authors explained this by referring to an earlier postulated multienzyme complex involved in DNA replication built from enzymes involved both in dNTP and DNA synthesis (replitase) (30). For some reason, deoxycytidine would not have access to this complex. The present results give a different explanation. dCTP, labeled from deoxycytidine, has a high specific activity in G_1 and a low specific activity during S phase; the reverse occurs with dTTP, labeled from thymidine.

Ever since it was originally proposed, the replitase has been an appealing device to "explain" isotope data that appear difficult to understand. In our opinion there is no evidence for such a complex, and, whenever invoked, a careful analysis of

the data has shown that they can be explained without it. The present case is one example. Another example is the demonstration that thymidylate synthase does not form part of such a complex (9), as claimed originally (31).

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