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Regulation of Pyrimidine Deoxyribonucleotide Metabolism by Substrate Cycles in dCMP Deaminase-Deficient V79 Hamster Cells

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A mutant V79 hamster fibroblast cell line lacking the enzyme dCMP deaminase was used to study the regulation of deoxynucleoside triphosphate pools by substrate cycles between pyrimidine deoxyribosides and their 5′-phosphates. Such cycles were suggested earlier to set the rates of cellular import and export of deoxyribosides, thereby influencing pool sizes (V. Bianchi, E. Pontis, and P. Reichard, Proc. Natl. Acad. Sci. USA 83:986–990, 1986). While normal V79 cells derived more than 80% of their dTTP from CDP reduction via deamination of dCMP, the mutant cells had to rely completely on UDP reduction for de novo synthesis of dTTP, which became limiting for DNA synthesis. Because of the allosteric properties of ribonucleotide reductase, CDP reduction was not diminished, leading to a large expansion of the dCTP pool. The increase of this pool was kept in check by a shift in the balance of the deoxycytidine/dCMP cycle towards the deoxynucleoside, leading to massive excretion of deoxycytidine. In contrast, the balance of the deoxyuridine/dUMP cycle was shifted towards the nucleotide, facilitating import of extracellular deoxynucleosides.

A network of biosynthetic and catabolic reactions regulates the size of deoxyribonucleoside triphosphate (dNTP) pools in mammalian cells so that the production of DNA precursors is adjusted to their utilization during DNA replication and repair. It has been appreciated for some time that the enzyme ribonucleotide reductase plays a pivotal role in this connection. It not only directs the total flow of metabolites into DNA, but also, via an exquisite allosteric control mechanism, divides the flow into four separate channels so that proper amounts of each dNTP are available for DNA synthesis (14, 18a, 21). More recently it has become apparent that catabolic reactions also control the size of dNTP pools. This first became apparent in connection with two inborn errors of metabolism arising from the lack of adenosine deaminase or purine nucleoside phosphorylase, enzymes catabolizing purine nucleosides (16). In both instances purine dNTPs accumulate and, via disturbances of the allosteric control of ribonucleotide reductase, interfere with the synthesis of DNA of lymphocytes, resulting in immunodeficiency syndromes.

Catabolic reactions also contribute to the regulation of pyrimidine dNTP pools (2, 3, 18). Mouse 3T6 fibroblasts in tissue culture normally catabolize a fraction of pyrimidine dNTPs synthesized de novo to deoxynucleosides that are excreted into the medium. However, cells also use deoxynucleosides present in the medium for the synthesis of dNTPs. We proposed that the balance between import and export is set by the intracellular concentration of deoxynucleosides, which is regulated by substrate cycles. Such cycles operate via phosphorylation of deoxynucleosides by kinases and dephosphorylation of their 5′-phosphates by nucleotidases. The relative rates of these opposing reactions are influenced by both the rate of de novo synthesis of dNTPs and the rate of DNA synthesis. Inhibition of dNTP synthesis decreases nucleotidase activity and favors import of deoxynucleosides, while a slowdown of DNA synthesis favors their excretion.

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The present investigation is a first attempt to test this model with the aid of somatic-cell mutants. We used a V79 hamster fibroblast line lacking dCMP deaminase and investigated how the loss of this enzyme affects dNTP and DNA synthesis as well as the catabolism of dNTPs. In normal cells the reduction of CDP by ribonucleotide reductase provided all the dCTP and more than 80% of the dTTP required for DNA synthesis. dTTP was provided by dCMP deaminase, which regulates the size of both pyrimidine dNTP pools. In the mutant line, only UDP reduction was available for de novo synthesis of dTTP, resulting in a decreased dTTP pool which became limiting for DNA synthesis. Since the allosteric configuration of ribonucleotide reductase is sensitive to dTTP but not to dCTP, reduction of CDP was increased in the mutant cells, leading to an accumulation of dCTP. This shifted the balance of the deoxycytidine/dCMP cycle towards deoxycytidine, resulting in a large excretion of the nucleoside into the medium. In contrast, the balance of the deoxyuridine/dUMP cycle was shifted towards the nucleotide, favoring import of the nucleoside from the medium.

MATERIALS AND METHODS

Materials. Labeled nucleosides and nucleotides were obtained from Amersham Corp., and tetrahydrouridine was from Calbiochem. All other chemicals and reagents were of the purest quality available to us.

Two V79 cell lines were used; one, labeled V79, was from S. Bonatti, Laboratorio di Mutagenesi e Differenziamento del C.N.R., Pisa, Italy, and originated with C. F. Arlett, Brighton, England; the other, labeled V79/dC, was from D. Jenssen, Wallenberg Laboratory, University of Stockholm, Sweden, and originated with A. T. Natarajan, Leiden, The Netherlands. V79 served as a normal control. V79/dC lacks the enzyme dCMP deaminase.

Growth and incubation of cells. Both cell lines were routinely grown as monolayers at 37°C in a 7.5% CO_2 atmosphere in Dulbeccomodified Eagle medium (DMEM) containing 5% nondialyzed heat-inactivated fetal calf serum (FCS). Where indicated, extensively dialyzed (against 0.15 M NaCl) serum was used. Cell growth was determined on replicate cultures after trypsinization by counting cell sus-

pensions in an AI Cell Counter −134 (Analysinstrument, Stockholm).

Isotope experiments were performed with exponentially growing cultures approximately 48 h after 5×10^4 to 10×10^4 cells were seeded per 5-cm dish. At this time, each dish contained between 5×10^5 and 10×10^5 cells. The labeled nucleoside (10 to 30 Ci/mmol) was added at a concentration of 0.3 μM either directly to the medium or, when indicated, 1.5 h after the medium was replaced with fresh medium containing dialyzed FCS.

Analyses of DNA and dNTPs. Experiments were made as described earlier (2, 3). Briefly, isotope incorporation was terminated by removal of the labeled medium, followed by three rinses with ice-cold Tris-saline. Nucleotides were extracted with 60% methanol, and the remaining cell layer was dissolved in 2 ml of 0.3 M NaOH and used to measure isotope incorporation into DNA. The nucleotide fraction was evaporated to dryness in a vacuum, dissolved in 0.4 ml of 0.3 M perchloric acid, centrifuged, and extracted with Freon-octylamine to remove the acid (13). The size and specific activity of dNTP pools were determined by an enzymatic assay (9, 15, 20). CTP and UTP were analyzed after separation by high-pressure liquid chromatography on a Partisil SAX column by isocratic elution with 0.4 M ammonium phosphate, pH 3.3, containing 2.5% acetonitrile. The amount of each of the two nucleotides was obtained by comparing their peak heights with those of known standards. A linear correlation was found between peak heights and amount of nucleotide (50 to 400 pmol). dCTP was only partially resolved from CTP in this system. This became a problem only with V79/dC cells containing large dCTP pools. In this case correction factors were determined from chromatograms of known mixtures of CTP and dCTP. Since the amount of dCTP was known from the DNA polymerase assay, the appropriate corrections could be made. Radioactivity was determined by liquid scintillation counting of portions (0.2 ml, to avoid quenching) of each chromatographic fraction (1 ml). Specific activities were obtained by dividing the total radioactivity present in the peak with the amount of nucleotide determined from the peak height.

Measurements of thymidylate synthase activity and nucleoside excretion. The in situ activity of thymidylate synthase was determined from the release of ^3H_2O from cells incubated with 5-^{3}H-labeled nucleosides (19, 22) as described earlier (3).

To determine nucleoside excretion, the medium was precipitated with perchloric acid (final concentration, 1 M), centrifuged, neutralized with 4 M KOH, and centrifuged again to remove precipitated $KClO_4$. A portion of the supernatant solution was analyzed on a 25-cm Nucleosil C_{18} column (particle size, 5 μm). The nucleosides were separated by isocratic elution with 20 mM ammonium acetate, pH 5.0, at a flow rate of 1 ml/min, and identified from their coelution with known standards. Their radioactivity was determined as described above for UTP and CTP. All values in the figures and tables are normalized for 10^6 cells.

RESULTS

Effects of deoxynucleosides on the growth of V79 and V79/dC cells. A comparison of the growth properties of V79 (Fig. 1A) and V79/dC cells (Fig. 1B) shows that in DMEM supplemented with nondialyzed FCS, V79/dC cells had a longer generation time (19 versus 15 h) and also showed a more pronounced lag period. The use of dialyzed FCS further prolonged their generation time to 26 h. Addition of thymidine or deoxycytidine to the medium normalized the growth of the mutant cells, and optimal growth was observed at 4.8 μM thymidine or 48 μM deoxycytidine. The two deoxynucleosides did not affect the growth of V79 cells.

Since V79/dC cells cannot deaminate dCMP (see below), the ability of deoxycytidine to substitute for thymidine suggests that the cells contain an active deoxycytidine deaminase, converting deoxycytidine to deoxyuridine, which, after phosphorylation, can give rise to dTTP. Tetrahydrouridine is a powerful inhibitor of this deaminase, and we tested the effect of this drug on the growth of V79/dC cells. In the experiment described in Table 1, we measured over a 72-h period the increase in cell number of V79/dC cells grown in triplicate under different conditions. The presence of tetrahydrouridine did not significantly affect the growth of cells in nondialyzed serum, dialyzed serum, or dialyzed serum supplemented with thymidine. However, the drug counteracted the stimulatory effect of deoxycytidine, strongly implicating deamination of the nucleoside by cellular deoxycytidine deaminase. This enzyme was absent from our FCS (data not shown).

V79/dC cells are deficient in dCMP deaminase. The growth stimulation of V79/dC cells by pyrimidine deoxyribosides suggested that the cells were deficient in dTTP synthesis, possibly because of deficient deamination of dCMP. To test this, we measured the incorporation of tritium into water from [5-^{3}H]cytidine added to V79/dC cells, a reaction that requires an active dCMP deaminase (see below and Fig. 2).

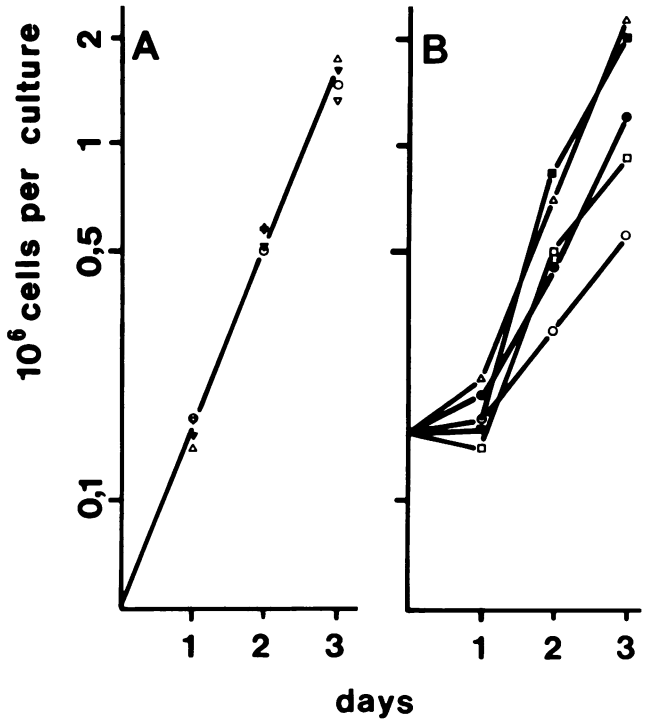

FIG. 1. Effect of deoxynucleosides on the growth of V79 and V79/dC cells. (A) V79 cells were seeded at 5×10^4 cells per plate in DMEM supplemented with dialyzed FCS only (○) or with 1.6 (▽), 4.8 (△), or 16 (▼) μM thymidine. Cell growth was determined on replicate cultures at 24-h intervals for 3 days after seeding. (B) V79/dC cells were seeded at 1.6×10^5 cells per plate in DMEM with FCS (●) or dialyzed FCS (○) only or in medium containing dialyzed FCS and 4.8 μM deoxythymidine (△) or 4.8 (□) or 48 μM (■) deoxycytidine. The average generation time in the two lines was calculated from the linear portion of the growth curves.

TABLE 1. Effect of tetrahydrouridine on the growth of V79/dC cells in different media[a]

Medium ingredients	Growth (10^6 cells ± SD)	% of control growth
FCS (control)	1.82 ± 0.06	100
FCS + THU	1.66 ± 0.03	91
DFCS	0.39 ± 0.03	21
DFCS + THU	0.34 ± 0.05	19
DFCS + TdR	1.96 ± 0.09	108
DFCS + THU + TdR	1.69 ± 0.12	93
DFCS + CdR	1.63 ± 0.08	89
DFCS + THU + CdR	0.77 ± 0.03	42

[a] V79/dC cells were seeded at 10^5 cells per plate in the presence or absence of 1 mM tetrahydrouridine (THU) in medium containing FCS (control) or dialyzed FCS (DFCS) alone or supplemented with 4.8 μM thymidine (TdR) or 16 μM deoxycytidine (CdR). Cell numbers were counted after 72 h in triplicate cultures. The mean cell number ± standard deviation is indicated for each set of cultures.

In contrast to V79 cells, the V79/dC cells did not release isotope into the water.

Consequences of the absence of dCMP deaminase. Parallel sets of V79 and V79/dC cells were incubated with [5-^{3}H]cytidine or [6-^{3}H]deoxyuridine to measure the effect of dCMP deaminase deficiency on the flow of isotope through deoxynucleotide pools into DNA. Figure 2 outlines the relevant metabolic pathways. After entering the cells, both nucleosides are phosphorylated, and labeled dUMP formed from [6-^{3}H]deoxyuridine is then quite directly incorporated into dTTP and DNA. With CMP labeled from cytidine, the situation is more complicated. The main incorporation of isotope occurs into RNA via CTP, but ribonucleotide reductase also provides a shunt to dCTP and DNA. dCTP is incorporated as such into DNA and is normally also the main source of dTTP. This requires deamination of dCMP to dUMP, followed by methylation to dTMP. During the methylation, tritium is lost from the 5-position of the pyrimidine ring and transferred to ^3H_2O. The appearance of tritium in the medium is thus a simple and convenient measure for the combined in vivo activities of dCMP deamination and dUMP methylation (19, 22).

The results of this experiment are summarized in Table 2 and Fig. 3. The table compares the two cell lines with respect to pyrimidine dNTP pools, rate of DNA synthesis, and excretion of ^3H_2O and deoxynucleosides into the medium. Three major differences immediately became apparent: V79/dC cells produced no ^3H_2O, contained a much larger dCTP pool, and excreted much more deoxycytidine into the medium. The large dCTP pool and, coupled to this, the large excretion of deoxycytidine were in all probability secondary to the loss of dCMP deaminase. Table 2 also shows calculations of the absolute rates of DNA synthesis determined

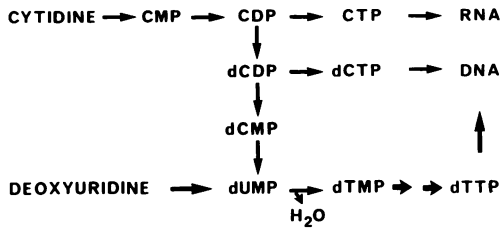

FIG. 2. Pathways of nucleic acid labeling from cytidine and deoxyuridine. When [5-^{3}H]cytidine is used, the isotope enters DNA via dCTP and is released in ^3H_2O during methylation of dUMP to dTMP.

from the incorporation of cytidine and deoxyuridine. Rates were calculated by dividing the amount of isotope incorporated into DNA between 60 and 120 min by the specific activities of the respective dNTP pools. The dTTP pools of both cell lines and the dCTP pool of the V79 cells had reached a steady state after 60 min (Fig. 3). In contrast, the specific activity of the large dCTP pool of V79/dC cells continued to increase between 60 and 120 min, and in this case we averaged the two values for the calculation. Note also that the specific activity of the dTTP pool in V79/dC cells was much larger than in V79 cells, indicating more efficient utilization of deoxyuridine.

DNA synthesis could be calculated independently from the incorporation of either dTMP ([6-^{3}H]deoxyuridine) or dCMP ([5-^{3}H]cytidine). Both cell lines incorporated 1.3 times more dTMP into DNA than dCMP, faithfully reflecting the ratio between thymine and cytosine in mammalian DNA. DNA synthesis was approximately 1.4 times faster in V79 cells than in V79/dC cells, in accordance with their relative growth rates. This result suggests that the slower growth of V79/dC cells depended on a longer S phase.

In V79 cells the amount of isotope appearing in water was a measure of the total amount of dTMP synthesized by deamination of dCMP and possibly deoxycytidine. This value corresponded to 6.8 pmol/min and should be compared with the total amount of dTTP synthesized (7.4 pmol for DNA synthesis plus 0.45 pmol excreted as thymidine into the medium), suggesting that dCMP deamination accounted for 86% of total dTTP synthesis. Deamination of deoxycytidine was probably not significant, since V79/dC cells that had lost dCMP deaminase transferred little isotope to water. We will demonstrate below that the remaining synthesis occurred largely by reduction of UDP.

Table 2 also summarizes the excretion of deoxynucleosides into the medium. Both cell lines excreted very little deoxyuridine. This finding is different from earlier results with rapidly growing 3T6 cells, in which the excretion of deoxyuridine corresponded to approximately one-fourth of

TABLE 2. Comparison of pool sizes, DNA synthesis, and deoxynucleoside excretion of V79 and V79/dC cells[a]

Cells	Pool size (pmol)		Release of ^3H_2O (pmol/min)	Incorporation (pmol/min) into DNA[b]		Nucleoside excretion (pmol/min)			Total reduction of CDP (pmol/min)
	dTTP	dCTP		dTMP	dCMP	CdR	UdR	TdR	
V79	44	27	6.8	7.4	5.5	0.44	0.05	0.45	12
V79/dC	34	568	0.09	5.4	4.1	11	0.09	0.20	15

[a] Cells were grown for 2 days in DMEM with nondialyzed FCS. The experiment was started by adding 0.3 μM [5-^{3}H]cytidine or [6-^{3}H]deoxyuridine directly to the medium. Dishes were removed after 0, 30, 60, and 120 min and analyzed as described in Materials and Methods. Pool sizes represent average values from the four time points. Rates of release of ^3H_2O, excretion of nucleosides, and DNA synthesis were calculated from the increase in isotope content between 60 and 120 min and the specific activities of dCTP or dTTP, as reported in Fig. 3. The total reduction of CDP was obtained from the sum of the incorporation of dCMP into DNA, the release of ^3H_2O, and the excretion of deoxycytidine (CdR) and deoxyuridine (UdR) in the experiment with [5-^{3}H]cytidine. TdR, Thymidine.

[b] dTMP from deoxyuridine labeling, dCMP from cytidine labeling.

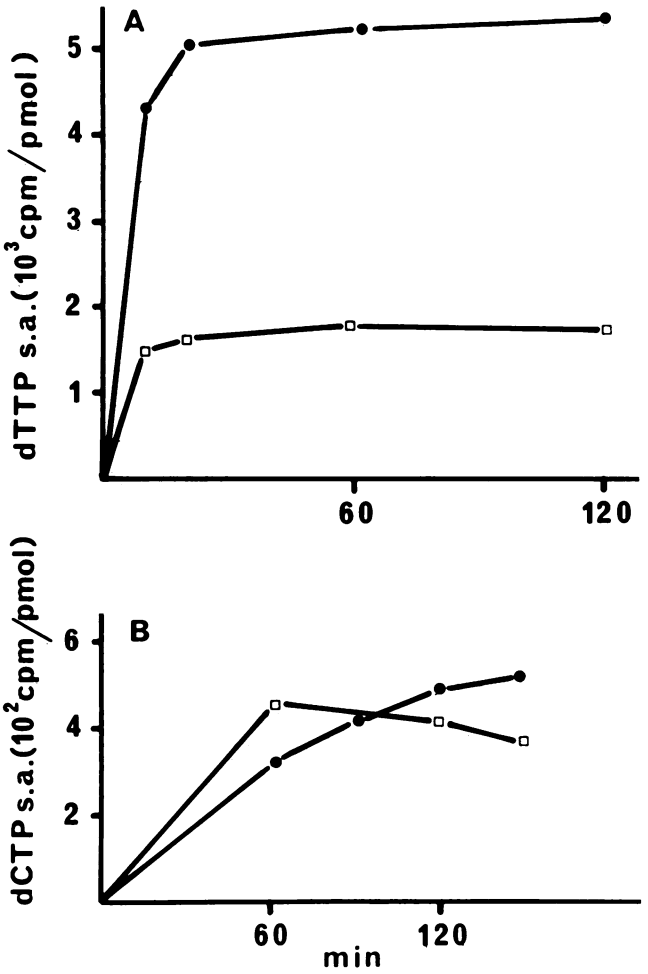

FIG. 3. Specific activities (s.a.) of dTTP and dCTP of V79 and V79/dC cells incubated with [6-^{3}H]deoxyuridine or [5-^{3}H]cytidine. V79 cells (□) and V79/dC cells (●) were grown for 2 days in DMEM with FCS. The experiment was started by the addition of 0.3 μM [6-^{3}H]deoxyuridine (A) or [5-^{3}H]cytidine (B) to the conditioned growth medium. At the indicated times, the specific activities of dTTP and dCTP, respectively, were measured.

the total amount of dCDP synthesis (18). Excretion of thymidine was moderate, about twice as much as in V79 cells. Deoxycytidine was the major excretion product of V79/dC cells and accounted for more than 95% of the total deoxynucleoside excretion. In V79 cells the excretion of deoxycytidine was much smaller and equalled that of thymidine.

Finally, the last column of Table 2 gives the in situ activity of CDP reduction in the two cell lines. This value was obtained by summation of the rates for dCMP incorporation into DNA, ^3H_2O production, and excretion of deoxycytidine and deoxyuridine. V79 cells require reduction of CDP for the synthesis of both dCTP and dTTP, while in V79/dC cells this reaction only provides dCTP. In spite of this, more CDP was reduced in the latter cell line. The difference becomes still more apparent when CDP reduction was related to the rate of DNA synthesis, since this rate is slower in V79/dC cells. In these cells two-thirds of the dCDP produced by CDP reduction was degraded and excreted into the medium.

Synthesis of dTTP via reduction of UDP in V79 and V79/dC cells. In most cells the deamination of dCMP appears to be the major pathway for the synthesis of dTTP (11, 18). In rapidly growing 3T6 cells, between 80 and 90% of dTTP originated from dCMP, and the rest was accounted for by salvage of deoxyribosides from the medium or reduction of UDP (18). V79/dC cells cannot deaminate dCMP but can salvage deoxynucleosides present in the medium (Fig. 1 and 3) for dTTP synthesis. However, these cells can also grow, albeit slowly, in the absence of added deoxynucleosides (Fig. 1). Under those circumstances, UDP reduction appears to be the only source for the synthesis of dTTP.

To compare the efficiency of UDP reduction in V79/dC and V79 cells, we incubated parallel sets of each line with a trace amount of [6-^{3}H]uridine and determined the incorporation of isotope into NTPs and DNA. Ribo- and deoxyribonucleoside triphosphate pools were analyzed after 120 and 150 or 180 min (Table 3). The size of the ribonucleotide pools was the same in the two cell lines, while, as demonstrated earlier, the dCTP pool of V79/dC cells was increased approximately 20-fold and the dTTP pool decreased to 75% of the corresponding pools in V79 cells.

All four NTPs of V79/dC cells had a slightly higher specific radioactivity than those of V79 cells. The difference was small, however, and far from that observed for the dTTP pools labeled with [6-^{3}H]deoxyuridine (Fig. 3). Within each cell line, the specific activity of dCTP was identical to that of CTP, demonstrating that isotope equilibrium had been established between cytosine nucleotides. However, this was not the case for UTP and dTTP. There, the specific activity of dTTP was always lower than that of UTP; in V79 cells it was less than 40%, in V79/dC cells close to 50%. However, in both lines the cells had reached a steady state, since the ratio between the specific activities of UTP and dTTP was the same at the two time points. This indicates that UDP reduction satisfied only part of the requirement for dTTP. The fraction of dTTP that was obtained by reduction of UDP can be estimated by making the reasonable assumption that UDP had the same specific activity as UTP.

In V79 cells dTTP was labeled from two sources: from dCTP by deamination of dCMP and from UTP by reduction of UDP. Salvage of deoxynucleosides was low and was

TABLE 3. Size and specific activities of pyrimidine NTP pools of V79 and V79/dC cells labeled with [6-^{3}H]uridine[a]

Cells	Incubation time (min)	CTP		dCTP		UTP		dTTP	
		Pool size (pmol)	Sp act (cpm/pmol)	Pool size (pmol)	Sp act (cpm/pmol)	Pool size (pmol)	Sp act (cpm/pmol)	Pool size (pmol)	Sp act (cpm/pmol)
V79	120	1,084	235	20	235	966	839	32	372
	150	1,258	272	20	295	1,021	898	34	378
V79/dC	120	833	318	374	335	1,147	1,007	21	505
	180	1,019	250	400	372	1,243	672	27	394

[a] The cells were grown for 2 days in DMEM with nondialyzed FCS. The experiment was started by addition of 0.3 μM [6-^{3}H]uridine to the conditioned growth medium. Dishes were removed at the indicated times and analyzed as described in Materials and Methods.

disregarded in our calculation. Assuming that the specific activity of dCMP was identical to that of dCTP (265 cpm/pmol), we can calculate that 82% of dTTP (specific activity, 375 cpm/pmol) was formed by deamination of dCMP and 18% by reduction of UDP (specific activity, 870 cpm/pmol). This value compares favorably with the result from the previous experiment (Table 2) in which dCMP deamination accounted for 86% of total dTTP synthesis.

In V79/dC cells, dCMP deamination does not take place, and all isotope incorporation into dTTP occurs via UDP reduction. The low specific activity of dTTP compared with UTP indicates isotope dilution by synthesis of dTTP via salvage of deoxynucleosides from the medium. Deamination of intracellular deoxycytidine, derived from breakdown of dCTP, was insignificant and did not contribute to dTTP synthesis. This is apparent from the fact that V79/dC cells did not excrete any ^3H_aO (Table 2). Since the average specific activity of dTTP (450 cpm/pmol) was 54% of the specific activity of UTP (840 cpm/pmol), the data indicate that 54% of dTTP was synthesized via reduction of UDP.

Experiment 1 of Table 4 gives data on the rates of DNA synthesis from the same experiment. Isotope from [6-^{3}H]uridine reached DNA via both the dCTP and the dTTP pools. In our calculations (detailed in Table 4, footnote *a*), we considered that mammalian DNA contained 1.3 times more thymine than cytosine. In V79 cells, dTMP incorporation then amounted to 5.6 pmol/min, in V79/dC cells it amounted to 4.6 pmol/min. From these values we estimate that UDP reduction amounted to 1.0 pmol/min (18% of 5.6) in V79 cells and to 2.5 pmol/min (54% of 4.6) in V79/dC cells. In these calculations we neglected the small amounts of thymidine and deoxyuridine (cf. Table 1) excreted into the medium. In both cell lines, UDP reduction was small compared with CDP reduction.

In a final experiment we compared the utilization of labeled uridine by V79/dC cells under different growth conditions. Cells were first grown in medium containing nondialyzed FCS for 2 days and then divided into two groups. One was maintained in the same medium, and the second was shifted to fresh medium with dialyzed FCS. At 90 min after the shift, a tracer dose of [6-^{3}H]uridine was added to each group, and the specific activities of the dCTP and dTTP pools were measured during the ensuing 150 min (Fig. 4). In addition, we determined the sizes of the two pools, the rates of DNA synthesis, and the excretion of nucleosides into the medium (experiment 2 of Table 4 and Table 5).

The specific activity of dTTP reached a plateau value after 90 min, while incorporation into dCTP continued to increase during the whole experiment (Fig. 4), suggesting that only the dTTP pool had reached a steady state at 120 min. Both pools had a higher specific activity in cells growing with dialyzed FCS. This effect was more pronounced for the dTTP pool. In conditioned medium, a sizable fraction of

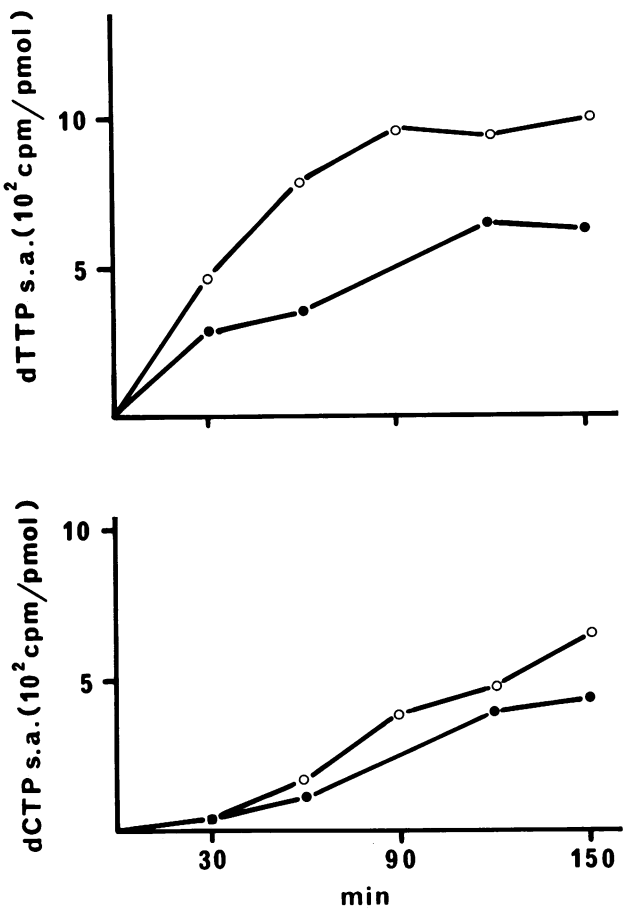

FIG. 4. Effect of nucleoside-free medium on the specific activities (s.a.) of dTTP and dCTP in V79/dC cells labeled with uridine. V79/dC cells were grown for 2 days in DMEM with nondialyzed FCS. Half of the cultures were shifted to fresh medium with dialyzed FCS, and 90 min later all the cultures received 0.3 μM [6-^{3}H]uridine. dTTP and dCTP pools were analyzed during the ensuing 150 min in conditioned medium (●) or in fresh medium with dialyzed FCS (○).

TABLE 4. DNA synthesis in V79 and V79/dC cells labeled with [6-^{3}H]uridine[a]

Expt no.	Cells or medium	Incubation time interval (min)	Sp act (cpm/pmol)		DNA synthesis (pmol/min) from:	
			dTTP	dCTP	dTMP	dCMP
1 (conditioned medium)	V79	120–150	375	265	5.6	4.3
	V79/dC	120–180	462	337	4.6	3.5
2 (V79/dC cells)	Conditioned medium	60–150	571	344	5.7	4.4
	Fresh medium + DFCS	60–150	929	432	4.2	3.2

[a] Experiment 1 is identical to the experiment in Table 3 in which 0.3 μM [6-^{3}H]uridine was added directly to the medium of either V79 or V79/dC cells after 2 days of growth. In experiment 2 only V79/dC cells were used, but half the cultures were shifted to fresh medium containing dialyzed FCS (DFCS) before addition of 0.3 μM [6-^{3}H]uridine. At two indicated time points, samples were removed and analyzed. The specific activities of dTTP and dCTP are the average values from the two points (cf. Table 3). Incorporation of dCMP (*a*) and dTMP (1.3*a*) into DNA was calculated from the equation $(C \times a) + (T \times 1.3a) = D$, where C is the specific activity of dCTP, T is the specific activity of dTTP, and D is the total radioactivity incorporated into DNA during 1 min. Our calculation is based on a thymine/cytosine ratio of 1.3 in hamster DNA.

TABLE 5. Excretion of nucleosides by V79/dC cells and comparison of CDP and UDP reduction[a]

Medium	Nucleoside excretion (pmol/min)			Ribonucleotide reduction[b] (pmol/min)	
	Cytidine	Deoxycytidine	Deoxyuridine	CDP	UDP
Conditioned	0.26	11.5	0.06	15.9	2.8
Dialyzed FCS	0.22	9.1	0.09	12.3	4.2

[a] Data are from experiment 2 of Table 4.

[b] The total amount of ribonucleotide reduction was calculated from the sum of the incorporation of dCMP into DNA (Table 4) plus the excretion of deoxycytidine (CDP) or from the incorporation of dTMP into DNA (UDP).

dTTP was derived from cold deoxynucleosides, while all dTTP had to be synthesized via reduction of UDP in cells growing with dialyzed FCS.

The rates of DNA synthesis in this experiment are shown as experiment 2 in Table 4. Results were calculated from the incorporation of isotope into DNA between 60 and 150 min and the average specific activities of dTTP and dCTP as outlined in footnote *a*. Nucleotide incorporation was 1.4 times faster in conditioned medium. In medium containing dialyzed FCS, all dTTP synthesis occurred by reduction of UDP and amounted to 4.2 pmol/min.

Finally, we also determined the excretion of nucleosides in this experiment and could then calculate the total amount of UDP and CDP reduction (Table 5). As earlier, V79/dC cells excreted large amounts of deoxycytidine. In contrast, only small quantities of labeled cytidine and deoxyuridine were recovered in the medium. Degradation of dCTP exceeded that of CTP almost 50-fold even though the size of the CTP pool exceeded that of the dCTP pool. Table 5 also gives the calculated rates of CDP and UDP reductions. The values for CDP were obtained by adding up the incorporation of dCMP into DNA (Table 4) and the excretion of deoxycytidine (Table 5). The values for UDP reduction come from dTMP incorporation into DNA (Table 4). In dialyzed FCS, all dTTP had to be synthesized by reduction of UDP, while in conditioned medium dTTP synthesis was assumed to be divided equally between salvage of deoxynucleosides and UDP reduction (Table 3). The data clearly show that V79/dC cells reduced CDP much more efficiently than UDP.

DISCUSSION

From earlier work with mouse 3T6 cells, we concluded that substrate cycles involving pyrimidine deoxynucleosides and their 5'-phosphates participated in the regulation of dNTP pools (2, 3, 18). To investigate whether similar effects also occurred in other mammalian cells, we used hamster V79 lung cells, which should make it easier to study how mutation of the enzymes of dNTP synthesis affects regulation by substrate cycles. We found that two V79 lines obtained from separate sources differed greatly in the size of their dCTP pools and that the line with the large pool (now named V79/dC) lacked the ability to deaminate dCMP. Several earlier reports have demonstrated the absence or low activity of the enzyme dCMP deaminase in hamster cells (4, 5, 12).

Our chance observation provided us with a tool to investigate how the loss of dCMP deaminase affects the operation of the substrate cycles. We cannot of course be certain that the two cell lines did not differ in other respects. A chromosome analysis, kindly made by Lore Zech of the Karolinska Institute, showed that the two lines had minor differences at three chromosomes but otherwise had identical karyotypes. It seems unlikely that the effects on pyrimidine metabolism discussed below are not related to dCMP deaminase.

The most striking difference between the two lines was the size of their dCTP pools. Greatly enlarged dCTP pools were earlier found in cells lacking dCMP deaminase or in cells with a deficient allosteric control of the enzyme CTP synthase (1, 4, 5, 12, 17, 23, 24). The CTP pool of our V79/dC cells was normal, and the increased dCTP pool should therefore be caused by the absence of dCMP deaminase. Loss of this enzyme also explains the decreased size of the dTTP pool of V79/dC cells as a consequence of deficient synthesis of dUMP. In line with this, the decreased growth rate of V79/dC cells was corrected by addition of thymidine or high concentrations of deoxycytidine. The latter effect required deamination at the nucleoside level, since tetrahydrouridine, an inhibitor of deoxycytidine deaminase, abolished growth stimulation by deoxycytidine.

A second striking consequence of the loss of dCMP deaminase was the huge excretion of deoxycytidine by V79/dC cells, in agreement with an earlier report of increased excretion of the deoxynucleoside by cells containing a large dCTP pool (7). We related deoxycytidine excretion to total CDP reduction in both V79 and V79/dC cells (Table 2) and could determine how the loss of dCMP deaminase affected the specificity of ribonucleotide reductase towards CDP. In V79 cells, CDP reduction supplies both dCTP and dTTP for DNA synthesis; in V79/dC cells, its function is limited to the supply of dCTP. Nevertheless, V79/dC cells reduced relatively more CDP than V79 cells. This implies that the huge dCTP pool arising from the loss of dCMP deaminase does not allosterically decrease CDP reduction and agrees with the known allosteric regulation of the enzyme: dCTP does not affect ribonucleotide reductase; instead, dTTP is a negative effector that turns off CDP (and UDP) reduction (21). In the present situation, the dTTP pool is in effect decreased, resulting in increased reduction of CDP (Table 2).

Instead, the increase in the size of the dCTP pool in V79/dC cells is kept in check by the deoxycytidine/dCMP substrate cycle. The deoxynucleotidase postulated as part of this cycle (2, 3) has a high K_m for dCMP (8) and is therefore sensitive to changes in deoxynucleotide concentration. Deoxycytidine kinase, the other enzyme of the cycle, is allosterically inhibited by dCTP (10). The increased dCTP pool thus exerts a dual effect: it inhibits deoxycytidine kinase and, via the concomitant increase in dCMP, increases the dephosphorylation of the nucleotide. Together, the two effects shift the intracellular balance of the cycle to deoxycytidine, which is excreted.

The dTTP pool is also affected in V79/dC cells by its corresponding substrate cycle. This is suggested from the data presented in Fig. 3, showing more efficient use of deoxyuridine for dTTP synthesis in V79/dC cells. We interpret this to be due to more efficient net phosphorylation of deoxyuridine, depending on a shift in the balance of the deoxyuridine/dUMP cycle. The decreased dTTP pool leads to an increase in the activity of thymidine/deoxyuridine kinase and a decrease in nucleotidase activity.

One final point concerns the contribution of UDP reduction to dTTP synthesis in the two cell lines. In V79 cells more than 80% of dTTP was synthesized from dCMP while the rest was derived from the reduction of UDP. In V79/dC cells lacking dCMP deaminase, UDP reduction became the main pathway for dTTP synthesis and limited DNA synthe-

sis. V79/dC cells grown with dialyzed FCS had almost twice the generation time of V79/dC cells. Addition of either thymidine or deoxycytidine (which is used after deamination of deoxyuridine) restored the growth rate of V79/dC cells. Ribonucleotide reductase could not provide the cells with a mechanism to increase the reduction of UDP at the expense of CDP reduction, since the same allosteric configuration of the enzyme handles both ribonucleotides. Since the K_m for UDP is almost an order of magnitude higher than the K_m for CDP (6), reduction of CDP is always favored. In V79/dC cells the enzyme cannot satisfy the demand for dTTP by reduction of UDP because it is busily reducing an excess of CDP, which will then be degraded by the substrate cycle and excreted into the medium.

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