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PURINE DEOXYRIBONUCLEOSIDES COUNTERACT EFFECTS OF HYDROXYUREA ON DEOXYRIBONUCLEOSIDE TRIPHOSPHATE POOLS AND DNA SYNTHESIS

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Abstract—Inhibition of cell growth and DNA synthesis by hydroxyurea is thought to occur via an effect on the enzyme ribonucleotide reductase leading to a block of deoxyribonucleotide synthesis. Earlier attempts to bypass such a block by delivering deoxyribonucleosides to the medium of cultured cells have given equivocal results. Complications arise in such experiments from the specificity of the phosphorylating enzymes since 3 of the 4 deoxyribonucleosides are substrates for the same enzyme, with widely differing K_m values, and from allosteric effects exerted by deoxyribonucleotides. We simplify this situation by using a mutant hamster V79 line that lacks the enzyme dCMP deaminase. The cells contain a 20-fold enlarged dCTP pool and require thymidine for optimal growth. Concentrations of hydroxyurea (50 or 100 μM) that in short-term experiments inhibited DNA synthesis depleted the dATP pool without seriously affecting pyrimidine deoxyribonucleotide pools. The dATP pool could be restored by addition of deoxyadenosine but this depleted the dGTP pool. This depletion could be counteracted by the simultaneous addition of deoxyguanosine but then critically depended on the relative concentrations of the two purine deoxyribonucleosides, with optimal results at 1 μM deoxyadenosine + 100 μM deoxyguanosine. Under those conditions the inhibition of DNA synthesis by hydroxyurea was partially reversed.

Hydroxyurea (HU)† inhibits the growth of rapidly proliferating cells *in vivo* [1] and *in vitro* [2]. The drug is also active against human malignancies [3, 4] and is used in therapy. Inhibition of cell growth and DNA synthesis probably occurs via an inhibition of the enzyme ribonucleotide reductase by the drug [5, 6]. HU scavenges the tyrosyl free radical of the reductase and thereby inactivates the enzyme [7, 8]. This deprives cells of the deoxyribonucleoside triphosphates (dNTPs) required for the synthesis of DNA.

Mammalian cells contain kinases active in the phosphorylation of deoxyribonucleosides [9–12]. If the drug acts solely by depletion of dNTPs it should be possible in such cells to bypass the requirement for an active ribonucleotide reductase and reverse the inhibition of HU by providing a mixture of the four deoxyribonucleosides. Experiments along this line have, however, given contradictory results [13–16]. Lack of reversal [15, 16] has been taken to indicate that inhibition of DNA synthesis by HU is not due to inactivation of ribonucleotide reductase or that purine dNTPs obtained by phosphorylation of deoxyribonucleosides do not have access to the replication fork.

In most of these experiments intracellular dNTP pools were not measured and negative results might have been caused by inability to restore one or several of these pools. Deoxyribonucleosides are degraded by deaminases [17, 18] and phosphorylases [19] and may not be available for phosphorylation. Also, phosphorylation of deoxycytidine, deoxyguanosine and deoxyadenosine is catalyzed by one and the same enzyme and competition between substrates might pose a serious problem, in particular since the K_m of this kinase for deoxycytidine is much lower than that for the two purine deoxyribonucleosides [10]. Recently, the existence of a specific deoxyadenosine kinase has also been reported [20].

We now reopen this question by experiments in which dNTP pool sizes and DNA synthesis were measured accurately after addition of deoxyribonucleosides to cells. To this purpose we chose the hamster lung fibroblast V79 cell line (V79/dC) lacking the enzyme dCMP deaminase. The cells have a large dCTP pool and depend on thymidine in the medium for optimal growth also in the absence of HU [21]. In order to avoid some of the complications that might arise from the degradation of deoxyribonucleosides (and HU) we carried out short-term experiments lasting only a few hours. In this way the presence of thymidine in the medium and the high capacity of the cells to synthesize dCTP obviated further supplementation with pyrimidine deoxyribonucleosides and we could limit our experiments to study the effects of purine deoxyribonucleosides. Our results indicate that preincubation of V79/dC cells with deoxyadenosine + deoxyguanosine under the proper circumstances reversed the effects of low concentrations of HU on pool changes and on DNA synthesis.

MATERIALS AND METHODS

[^{3}H]thymidine (25 Ci/mmol) and ^{3}H and ^{32}P labeled dNTPs for pool measurements were obtained from Amersham Int., Amersham, UK. Freshly-

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† Abbreviations used: HU, hydroxyurea; dNTP, deoxyribonucleoside triphosphate; EHNA, erythro-9 (2-hydroxy-3-nonyl) adenine.

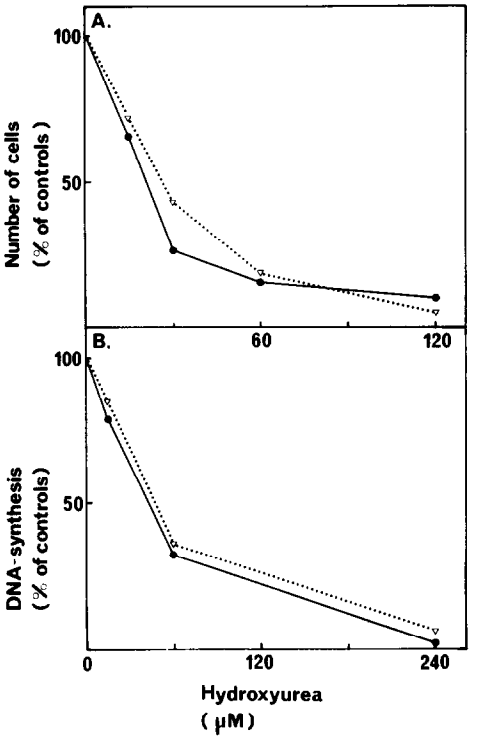

Fig. 1. Sensitivity of V79 and V79/dC cells towards inhibition by HU. Panel A: effect of HU on cell growth. Cells (50.000 V79 or 80.000 V79/dC) were seeded onto 5 cm dishes in the presence of varying amounts of HU. After 60 hr the number of cells on the dishes were counted. In the absence of HU 700.000 V79 and 200.000 V79/dC cells were recovered (=100%). Panel B: effect of HU on thymidine incorporation. V79 cells (1.1×10^6 cells/5 cm dish) and V79/dC cells (0.6×10^6 cells/5 cm dish) were pulsed on the third day of growth with 0.3 μM[^{3}H]thymidine. Various concentrations of HU were added together with the isotope. Incorporation of isotope into DNA and the specific radioactivity of the dTTP pool were determined after 30 and 60 min. The rate of DNA synthesis could then be calculated from the difference between the two incorporations and the constant specific activity of dTTP. 100% synthesis corresponded to the incorporation of 7.5 pmol/min and 6.4 pmol/min of dTMP into DNA of V79 and V79/dC cells, respectively. ▽ = V79 cells; ● = V79/dC cells.

prepared solutions of HU (Sigma) were used. EHNA was a gift from Burroughs Welcome Co. (Research Triangle Park, NC). Two lines of V79 cells were used, both provided by Dr. Vera Bianchi, Department of Biology, University of Padova, Italy and earlier characterized by her [21]. One line (V79/dC) lacked the enzyme dCMP deaminase while the other line was normal in that respect.

All incubations were started with cells from one original clone of V79 cells as described earlier for 3T6 cells [22]. The cells were grown in Eagle's modification of Dulbeccos medium with 5% fetal calf serum. Except when stated otherwise, 100,000 V79/dC cells were seeded onto 20–60 parallel Petri dishes (5 cm) in 5 ml of complete medium. Care was taken to distribute an equal number of cells to each dish and to ascertain that the cells settled evenly. All comparable dishes within each experiment were kept on the same shelf in an incubator at 37° in 7.5% CO_2 atmosphere and handled simultaneously. After two-and-a-half days the cultures had reached a density of between 0.4 and 0.8×10^6 cells per dish. At this point variations in cell number should be less than 10 per cent. An experiment was started by replacing the medium with 2 ml of fresh medium containing 20 mM of Hepes buffer, pH 7.4, and 10 μM thymidine. In the experiment depicted in Fig. 1 and Table 1 thymidine was not added. Purine deoxyribonucleosides and EHNA (0.1 mM to inhibit adenosine deaminase) were added after 2 hr when indicated, and, after one additional hour, the cells were pulsed with 0.3 μM [^{3}H]thymidine (giving a final concentration of 10.3 μM thymidine). Where indicated, HU was added together with the isotope. All additions (10 or 20 μl) were made rapidly from automatic pipettes, with the dishes remaining inside the incubator.

Incubations were terminated and dNTP pools extracted with 2.5 ml of 60% methanol as described earlier [23]. Isotope incorporation into DNA was measured after dissolving the extracted cells remaining on the dish in 0.3 M NaOH. Relative rates of DNA synthesis were obtained directly from the incorporation of isotope into DNA. Absolute rates were calculated from the linear rate of isotope incorporation into DNA and the specific activity of the dTTP pool during steady state conditions, and expressed as pmol dTMP incorporated per min into DNA [22].

Pool sizes and the specific activities of dTTP were determined by the DNA-polymerase assay on the material present in the 60% methanol extract [24–26].

All analyses were made on duplicate dishes. With few exceptions results from such duplicates varied by less than 10 per cent. The data presented in Figs 4 and 5 show the kind of variations found when values obtained at different time points were compared. Strict adherence to the described protocol was required to obtain such data. Larger variations were

Table 1. Size of dNTP pools of V79 and V79/dC cells after addition of HU

	dATP		dCTP		dGTP		dTTP	
	V79	V79/dC	V79	V79/dC	V79	V79/dC	V79	V79/dC
Control	80	135	21	534	11	17	33	48
60 μM HU	33	76	18	312	12	13	53	79
240 μM HU	21	34	17	318	10	8	53	92

Pool analyses were made 60 min after addition of HU in the experiment described in the legend to Fig. 1B. All values give pmol/10^6 cells.

found between separate experiments since absolute pool sizes strongly depend on variations in growth conditions that are difficult to control.

RESULTS

Does a large dCTP pool protect cells from hydroxyurea?

We found earlier by accident a line of V79 cells that lacked the enzyme dCMP deaminase and therefore contained a large dCTP pool [21]. We now compared the sensitivity to HU of these cells and normal V79 cells containing the deaminase. To this purpose we carried out two kinds of experiments and measured (i) the inhibition of cell growth by HU and (ii) the effects of the drug on DNA synthesis, as measured by incorporation of labeled thymidine.

The first approach gave the results shown in Fig. 1A. Cells were grown for 60 hr with HU at concentrations ranging from 15 to 120 μM. It is evident that the various concentrations of the drug inhibited growth of the two cell lines to the same extent. In the second approach HU was added to cell cultures together with a tracer dose of [^{3}H]thymidine (Fig. 1B). DNA synthesis was measured from the incorporation of isotope into DNA between 30 and 60 min after addition of isotope, when the specific activity of the dTTP pool had attained a constant value with equal amounts of radioactivity entering the pool by phosphorylation of thymidine and leaving it for incorporation into DNA. From the amounts of radioactivity incorporated into DNA and the specific activity of dTTP we can determine absolute rates of DNA synthesis, expressed as pmol of [^{3}H]dTMP incorporated into DNA per minute and 10^6 cells. Furthermore, from the effect of various concentrations of HU on this value we obtain the percentage inhibition of DNA synthesis by HU. Figure 1B gives such values, plotted as a function of the concentration of the drug. Again, no difference was found between the two cell lines.

In this experiment we also determined the effect of HU on the size of the four dNTP pools (Table 1). In both cell lines the drug caused a major decrease of the dATP pool as well as minor decreases in the size of the dCTP and dGTP pools, while the dTTP pool increased. These results are similar to earlier data obtained with 3T6 cells [23, 27]. The V79/dC cells lacking dCMP deaminase activity had a more than 20-fold larger dCTP pool than the normal line. This pool remained large also after addition of hydroxyurea. Note that in this experiment thymidine was not added to the cells. Since thymidine affects the size of all dNTP pools the values for V79/dC cells in Table 2 differ greatly from later values. The results of Fig. 1 and Table 1 demonstrate that the large dCTP pool does not affect the inhibition of DNA synthesis or cell growth by HU.

Reversal of hydroxyurea inhibition by purine deoxyribonucleosides

The data shown in Table 1 show that the principal effect of HU on dNTP pools of V79/dC cells was to deplete the cells of dATP. Addition of deoxyadenosine to the medium of cells expands the intracellular dATP pool and in the following experiments we attempted to reverse the inhibition of DNA synthesis by hydroxyurea with deoxyadenosine. The presence of a large dCTP pool in the V79/dC cells made the cell line particularly useful in this connection since a high capacity for dCTP synthesis should decrease the sensitivity of cells to the well-known inhibitory effect of deoxyadenosine on pyrimidine dNTP synthesis [28].

In the experiment depicted in Figs 2 and 3 one set of cells was pretreated for one hour with concentrations of deoxyadenosine varying between 1 and 4 μM while a second set was treated with 100 μM deoxyguanosine in addition to deoxyadenosine. Both sets of cells were then labelled with [^{3}H]thymidine for 30 or 120 min and labeling was carried out in the presence and absence of HU. Isotope incorporation into DNA was measured at the two time points and the rate of DNA synthesis was calculated as the difference between these two values. Also the size of the dATP and dGTP pools was determined at 120 min after addition of isotope (3 hr after addition of purine deoxyribonucleosides).

Figure 2 shows the effects of increasing concentrations of deoxyadenosine on pool sizes in the presence and absence of HU. In the experiments depicted in panels A and C only deoxyadenosine was added to the medium while the cultures represented by panels B and D in addition contained 100 μM deoxyguanosine. Addition of deoxyadenosine expanded the dATP pool (panels A and B) considerably, both with and without HU. Addition of HU to the cells in the absence of deoxyadenosine had decreased the dATP pool roughly three-fold, but in the presence of as little as 1 μM deoxyadenosine the pool size remained at the control level. Higher concentrations of deoxyadenosine resulted in a further expansion of the dAMP pool that was only marginally affected by HU.

Turning now to the dGTP pool one finds that the size of this pool was very sensitive to the addition of deoxyadenosine in the absence of HU. As little as 1 μM of the deoxyribonucleoside decreased the dGTP pool by more than 25% and at 4 μM the dGTP pool had all but disappeared. In the presence of HU this effect was still more pronounced. Addition of 100 μM deoxyguanosine, together with deoxyadenosine, counteracted the contraction of the dGTP pool (Fig. 2D). One μM deoxyadenosine now had no effect, but at higher concentrations the dGTP pool again decreased in size, Altogether the data shown in Fig. 2 demonstrate that addition of deoxyadenosine prevented the contraction of the dATP pool brought about by HU but instead led to a decrease in the size of the dGTP pool, probably by inhibition of ribonucleotide reductase. At 1 μM deoxyadenosine this decrease could be counteracted by the simultaneous addition of a large excess of deoxyguanosine.

The effect of deoxyadenosine on the inhibition of DNA synthesis by hydroxyurea is shown in Fig. 3A (deoxyadenosine alone) and 3B (deoxyadenosine + 100 μM deoxyguanonsine). DNA synthesis is measured by incorporation of labeled thymidine without corrections for possible variations in the specific activity of dTTP. In a subsequent experiment we will show that this omission does not introduce an appreciable error.

Deoxyadenosine by itself inhibited DNA syn-

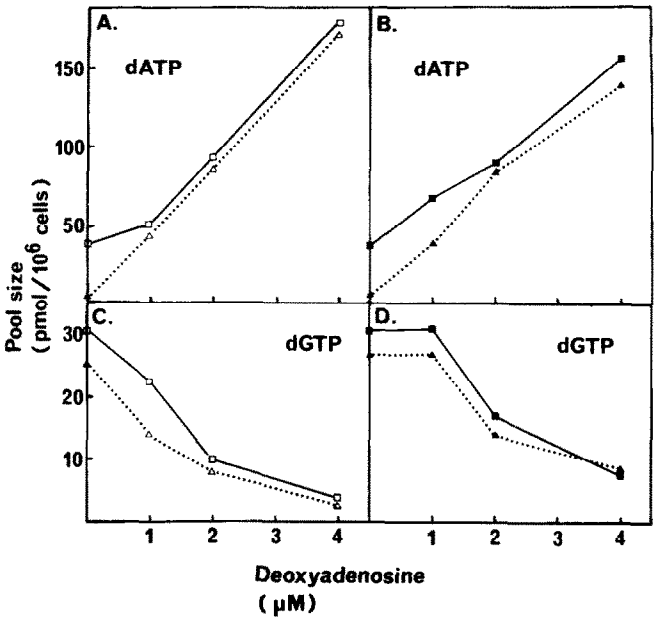

Fig. 2. Effects of deoxyadenosine and deoxyguanosine addition on the size of the dATP and dGTP pools. Various concentrations of deoxyadenosine alone (panels A and C) or deoxyadenosine together with 100 μM deoxyguanosine (panels B and D) were added to growing V79/dC cells 1 hr before addition of 0.3 μM [^{3}H]thymidine (to measure DNA synthesis, see Fig. 3) with or without 100 μM HU. After 120 min the cells were extracted with 60% methanol and the dATP (panels A and B) and the dGTP (panels C and D) pools were determined. □, ■ = no HU; △, ▲ = 100 μM HU.

thesis. In the absence of HU only 30% of DNA synthesis remained after addition of 4 μM deoxyadenosine. When 100 μM deoxyguanosine was added together with deoxyadenosine DNA synthesis amounted to 65% of the control. Thus deoxyguanosine could partially, but not completely, relieve the inhibition of DNA synthesis caused by deoxyadenosine alone. The inhibition of DNA synthesis by HU was counteracted by deoxyadenosine, both alone and in combination with deoxyguanosine. Best reversal was found at 1–2 μM deoxyadenosine + 100 μM deoxyguanosine.

The next experiment is a more detailed study of the reversal of hydroxyurea inhibition by the combination of 1 μM deoxyadenosine and 100 μM deoxyguanosine. One group of cells was preincubated with the two deoxyribonucleosides for one hour before addition of labeled thymidine while a second group, without added deoxynucleosides, served as a control. For the addition of labeled thymidine each group was further divided into 3 subsets: the first received 50 μM hydroxyurea together with [^{3}H]thymidine, the second received 100 μM and a final subset served as a non-inhibited control. From this point on, duplicate samples from each of the 6 subsets were analysed at time intervals during the ensuing 3 hours.

Changes in pool size of the 4 dNTPs elicited by the presence of the two concentrations of hydroxyurea are given in Table 2. The data are the mean values from 4 determinations made between 60 and 180 min after addition of HU when the pools had attained constant levels. In the absence of HU the addition of purine deoxyribonucleosides increased the dATP pool slightly while the dCTP pool decreased to approximately one third of its normal size. The dTTP and dGTP pools were marginally or not at all affected. Addition of HU alone resulted in the usual increase of dTTP and a decrease of dATP, dGTP and

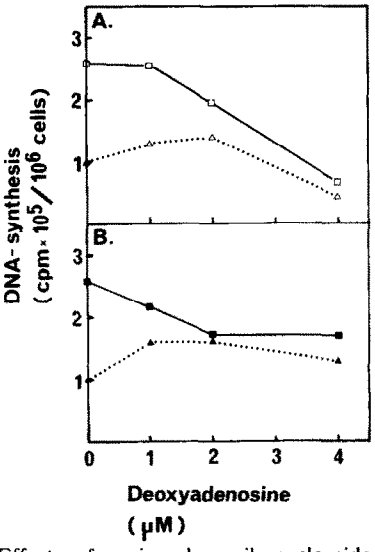

Fig. 3. Effects of purine deoxyribonucleosides on the inhibition of DNA synthesis of V79/dC cells by HU. The experiment is described in the legend to Fig. 2. Panel A: incorporation of [^{3}H]thymidine between 30 and 120 min after addition of isotope, effect of addition of increasing amounts of deoxyadenosine alone. Panel B: 100 μM deoxyguanosine + increasing deoxyadenosine. □, ■ = no HU; △, ▲ = +100 μM HU.

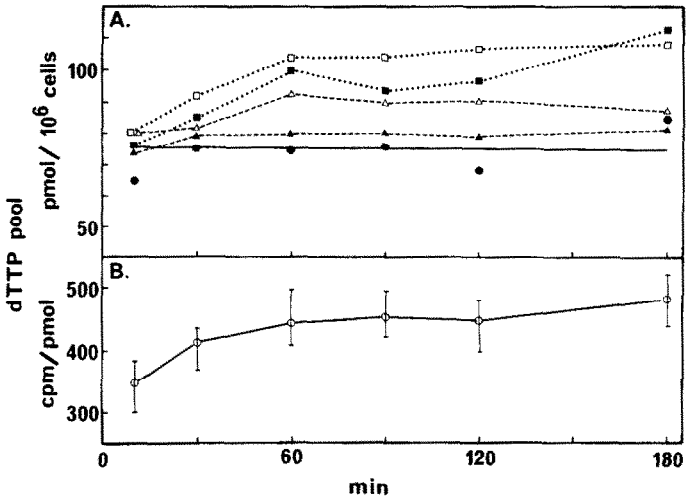

Fig. 4. Purine deoxyribonucleosides revert the effects of HU on the size of the dTTP pool. V79/dC cells were pretreated with deoxyadenosine (1 μM) + deoxyguanosine (100 μM) for 1 hr before addition of 0.3 μM[^{3}H]thymidine with and without 50 or 100 μM HU. Samples were removed thereafter at the indicated time intervals and analyzed. Panel A: size of dTTP pools: ●——●, control no HU, with or without deoxyribonucleosides; △- - - -△, 50 μM HU, no deoxyribonucleosides; □.....□, 100 μM HU, no deoxyribonucleosides; ▲- - - -▲, 50 μM HU + deoxyribonucleosides; ■.....■, 100 μM HU + deoxyribonucleosides. Panel B: specific activities of dTTP pools, average values from all 6 sets. The bars give the extreme values of the determinations.

dCTP. Addtion of HU in the presence of purine deoxyribonucleosides gave smaller changes in the size of both dTTP and purine dNTP pools but further decreased the dCTP pool. Of particular interest is the finding that the increase in the size of the dTTP pool brought about by hydroxyurea was counteracted by the purine deoxyribonucleosides.

This last effect is illustrated in more detail in Fig. 4A which gives the size of the dTTP pools at different times after addition of HU in the presence and absence of purine deoxynucleosides. In their absence, the dTTP pool increased by more than 50% during the first hour, but in the presence of purine deoxyribonucleosides this increase was much smaller. In contrast, purine deoxyribonucleosides did not affect the specific activity of the dTTP pool. In all subsets this value increased from 350 to 450 cpm/pmol between 10 and 60 min after addition of HU and then remained constant (Fig. 4B).

Table 2. Effects of deoxyadenosine (1 μM) + deoxyguanosine (100 μM) on change of dNTP pools of V79/dC cells caused by HU

HU (μM)	Purine deoxyribosides	dATP	dCTP	dGTP	dTTP
			pmol/10^6 cells		
0	–	71	163	30	76
0	+	100	56	34	76
50	–	58	145	30	100
50	+	85	49	31	81
100	–	43	126	24	105
100	+	63	38	28	90

HU (0.50 or 100 μM) was added together with [^{3}H]-thymidine to two sets of cultures, one of which had been preincubated for one hr with 1 μM deoxyadenosine + 100 μM deoxyguanosine. During the ensuing 3 hr the pool sizes of the 4 dNTPs were determined at the time intervals apparent in Figs 4 and 5. The Table gives average values of pool sizes from four determinations after addition of HU. Figures 4 and 5 give time curves for dTTP pools and DNA synthesis from the same experiment.

The inhibition of DNA synthesis by HU and the reversal of inhibition by purine deoxyribonucleosides is illustrated in Fig. 5, with panel A depicting the inhibition by HU in the absence of deoxyribonucleosides and panel B in their presence. The rate of DNA synthesis was linear during the 3 hr experiment and from the slope of the curves we can calculate that the addition of 50 and 100 μM of hydroxyurea in the absence of purine deoxyribonucleosides inhibited DNA synthesis to 54 and 35% respectively, while the corresponding inhibition in the presence of deoxyribonucleosides only amounted to 76 and 54%. Thus purine deoxyribonucleosides partially reversed the inhibition of DNA synthesis by HU.

DISCUSSION

Earlier evidence suggested that the inhibition of cell growth by HU is due to the destruction of the tyrosyl free radical of ribonucleotide reductase by the drug observed *in vitro*. The presence of a mutated ribonucleotide reductase in *E. coli* and mammalian cells often confers an altered sensitivity for HU to the cells [29, 30]. Furthermore organisms containing a cobalamine-dependent reductase [31, 32], an enzyme lacking the tyrosyl free radical, are resistant to inhibition by the drug.

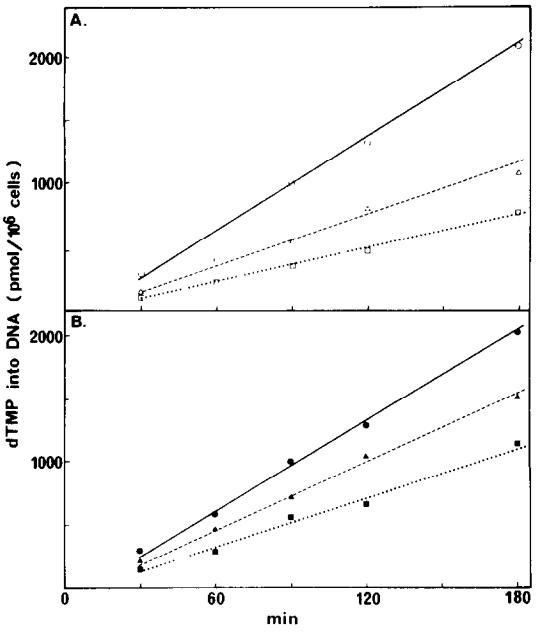

Fig. 5. Reversal of HU effect on DNA synthesis of V79/dC cells by purine deoxyribonucleosides. The experiment is described in the legend to Fig. 4. The incorporation of dTMP into DNA was calculated for each time interval from the incorporation of radioactivity into DNA of 2 adjacent time points and the average specific activity of the dTTP pool during the time interval (data in Fig. 4B). The rate of DNA synthesis can be calculated from the slopes of the curves. Panel A shows data from experiments in the absence of deoxyribonucleosides, panel B from experiments in their presence. ○, ● = no HU; △, ▲ = +50 μM HU; □, ■ = +100 μM HU.

Arguments against this have been: (i) that inhibition of cultured cells by HU only depeletes purine dNTP pools, while the dTTP pool actually increases; and (ii) that addition of purine deoxyribonucleosides to the medium of such cells does not reverse the inhibition. With respect to the first argument, it was recently found that HU increases the uptake of pyrimidine deoxyribonucleosides from the extracellular medium [27] and that this effect, together with a continued deamination of dCMP [23] is responsible for the increase in dTTP. The second argument is addressed by the experiments reported here.

A single enzyme has the ability to catalyze the phosphorylation of deoxycytidine and purine deoxyribonucleosides in mammalian cells [10]. This kinase has a much lower K_m for deoxycytidine than for purine deoxyribonucleosides and is strongly allosterically inhibited by dCTP. It can therefore not be taken for granted that the addition of a mixture of deoxyribonucleosides to cells will lead to a sufficient supply of all 4 dNTPs. Additional complications arise from the allosteric regulation of the reductase [28]. In particular the synthesis of dCTP is highly sensitive to inhibition by other dNTPs. Attempts to manipulate dNTP pools in the presence of HU by administration of deoxyribonucleosides should therefore be monitored with pool analyses. These aspects were often neglected in earlier work.

We attempted to circumvent some of the complications by using a cell line (V79/dC) that lacks the enzyme dCMP deaminase and therefore contains a large dCTP pool. We expected that in short term experiments with concentrations of HU that only partially inhibit DNA synthesis, addition of purine deoxyribonucleosides might suffice to reverse the inhibition by the drug. We found that the depletion of the dATP pool by HU in these cells could indeed be prevented by addition of deoxyadenosine to the medium. However, this resulted in a depletion of the dGTP pool, also in the absence of HU, and led to an inhibition of DNA synthesis. To some extent the depletion of dGTP was counteracted by supplying the cells with deoxyguanosine together with deoxyadenosine. At the lowest concentration of deoxyadenosine, just sufficient to replenish the dATP pool in the presence of HU, addition of a 100-fold excess of deoxyguanosine almost normalized the dGTP pool. Clearly a delicate balance between the two purine deoxyribonucleosides was required to obtain optimal reconstitution, as demonstrated by the results depicted in Figs 3 and 4.

With 1 μM deoxyadenosine + 100 μM deoxyguanosine the inhibition by HU was reversed. This became apparent not only when the rate of DNA synthesis was measured, but also from measurements of pool sizes. The dTTP pool is a sensitive indicator of the effect of HU and expands in parallel with the inhibition of DNA synthesis. Addition of the two purine deoxyribonucleosides clearly counteracted the HU-induced expansion (Fig. 4A).

Reversion was never complete, in particular not at higher concentrations of HU. This is perhaps not surprising, since it proved impossible to correct completely the pool changes caused by HU. Compartmentation of dNTP pools might further complicate the interpretation of our results. One kind of compartmentation derives from the heterogeneity of the cell population in our experiments, with only 50–60% of the cells in S-phase. These cells contain larger dNTP pools than cells in G1-phase and we consider therefore that this kind of compartmentation represents only a minor complication. However, S-phase cells appear to also contain kinetically compartmentized dCTP [33] and dGTP [34] pools. In each case only a fraction of the total dNTP appeared to participate in DNA replication. Our pool measurements with necessity determine the size of the total dNTP pools.

In spite of these reservations the present results demonstrate that manipulations of dNTP pools aimed at reversing pool changes brought about by HU reverted the inhibition of DNA synthesis by the drug. A similar conclusion was recently reached from experiments in which the size of the purine dNTP pools was maintained in HU inhibited cells by manipulation of the allosteric control of ribonucleotide reductase [35].

Finally, our results also demonstrate that dNTPs obtained by phosphorylation of purine deoxyribonucleosides do have access to the replication fork. Such a conclusion does of course not negate the existence of compartmentation of dNTPs, for which there exists considerable evidence. It suggests, instead, that communication between the compartments occurred in our experiments.

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