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Isotope-dilution analysis of the effects of deoxyguanosine and deoxyadenosine on the incorporation of thymidine and deoxycytidine by hydroxyurea-treated thymus cells

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It is presumed that the dGTP and dATP needed for replicative DNA synthesis can be formed by way of either 'salvage' pathways or biosynthesis *de novo*. This was examined by adding hydroxyurea to cultures of rat thymus cells to inhibit ribonucleoside diphosphate reductase, a key enzyme of the '*de novo*' pathway. Most of the inhibition of the incorporation of [*Me*-^{3}H]thymidine and deoxy[5-^{3}H]cytidine by low concentrations of hydroxyurea (100–500μM) was prevented by substrates of the salvage pathway (400μM-deoxyguanosine and, to a lesser extent, 200μM-deoxyadenosine). However, isotope-dilution studies indicated that the purine deoxyribonucleosides prevented inhibition by decreasing pyrimidine deoxyribonucleotide competitor pools. Evidence was obtained that a hydroxyurea-induced increase in the thymidine-competitor pool (probably dTTP) was prevented to an equal extent by deoxyguanosine and by the inhibitor of thymidylate synthase, deoxy-5-fluorouridine. These compounds had almost identical effects on hydroxyurea dose–response curves and on thymidine isotope-dilution plots. The evidence suggests that exogenous purine deoxyribonucleosides cannot prevent the inhibition by hydroxyurea of thymus-cell DNA synthesis. This could mean that, with respect to the metabolism of purine deoxyribonucleotides, ribonucleoside diphosphate reductase is tightly coupled to DNA polymerase in a multienzyme complex. The complex would not permit entry of exogenous metabolic intermediates into the '*de novo*' pathway, but would still be subject to the regulatory effects of these intermediates. Thus dGTP and dATP formed from exogenous purine deoxyribonucleosides by salvage pathways might deplete pyrimidine deoxyribonucleotide competitor pools by inhibiting relatively hydroxyurea-insensitive activities of ribonucleoside diphosphate reductase.

A key enzyme of the pathway of deoxyribonucleotide synthesis *de novo* is ribonucleoside diphosphate reductase, which converts CDP, UDP, GDP and ADP into the corresponding deoxyribonucleoside diphosphates (Thelander & Reichard, 1979). The latter are then converted into dCTP, dTTP, dGTP and dATP. With the exception of dCTP, these end-products feedback-regulate the enzyme with respect to its substrate specificity or overall activity. The enzyme is inhibited by elevated concentrations of dGTP or dATP, and this is believed to play a critical role in inhibition of DNA synthesis in the lymphoid tissues of patients with certain immunodeficiency diseases (Carson *et al.*, 1977, 1978).

Inhibition of ribonucleotide reductase by hydroxyurea results in a rapid fall in the quantity of dGTP and dATP that can be extracted from cells. The quantity of dCTP and dTTP is elevated (Skoog & Nordenskjöld, 1971; Adams *et al.*, 1971). Since a balanced supply of all four deoxyribonucleoside triphosphates is needed for normal replicative DNA synthesis, it is thought that hydroxyurea inhibits DNA synthesis primarily by decreasing the intracellular concentrations of dGTP and dATP, which are normally lower than those of dCTP and dTTP (Thelander & Reichard, 1979). In hydroxyurea-treated cells dCTP and dTTP would increase, because the rates of their formation would exceed the rates of their utilization for DNA synthesis or of

Abbreviation used: FdUrd, 2′-deoxy-5-fluorouridine.

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their degradation. Their formation might continue in the presence of hydroxyurea, either because of the 'salvage' of serum deoxycytidine, which is converted into both dCTP and dTTP (for references see Sjostrom & Forsdyke, 1974), or because the activities of ribonucleotide reductase towards CDP and UDP are relatively insensitive to hydroxyurea in intact cells.

In theory, the inhibition of DNA synthesis by hydroxyurea should be corrected by exogenous purine deoxyribonucleosides, which would be converted into dGTP and dATP by way of salvage pathways. However, attempts to show this have met with limited success (Adams & Lindsay, 1967; Young *et al.*, 1967; Yarbro, 1968; Bachetti & Whitmore, 1969). This could be due to the fact that non-lymphoid tissues have little ability to phosphorylate purine deoxyribonucleosides, which instead would be phosphorylysed to free bases (Snyder & Henderson, 1973; Plagemann & Erbe, 1974). It could also reflect a tight functional coupling between ribonucleotide reductase and DNA polymerase, which would stop salvage intermediates entering the '*de novo*' pathway (Mathews *et al.*, 1979).

Since thymus cells have highly active purine deoxyribonucleoside kinases (Durham & Ives, 1969; Carson *et al.*, 1977), this lymphoid tissue would appear most suitable for testing the ability of purine deoxyribonucleosides to prevent inhibition by hydroxyurea. The present paper extends previous studies of the inhibition by hydroxyurea of the incorporation of [*Me*-^{3}H]thymidine and deoxy[5-^{3}H]cytidine by cultured thymus cells (Scott & Forsdyke, 1976). In the first part of the paper we report the effects of various concentrations and combinations of hydroxyurea and purine deoxyribonucleosides. Particular attention is given to hydroxyurea concentrations that give sub-maximum inhibition. At these concentrations it was envisaged that the concentration of one of the purine deoxyribonucleoside triphosphates would limit DNA synthesis before the other. Under these conditions one purine deoxyribonucleoside alone might prevent the inhibition. We then report isotope-dilution studies which show that an apparent enhancement of DNA synthesis in hydroxyurea-treated cells by purine deoxyribonucleosides is due to a pool effect. Preliminary accounts of this work have appeared (Scott & Forsdyke, 1978*b*; Forsdyke & Scott, 1979). A fuller account is available (Scott, 1976).

Materials and methods

Materials

[*Me*-^{3}H]Thymidine (18.4–22.0 Ci/mmol) and deoxy[5-^{3}H]cytidine (15.8–21.2 Ci/mmol) were obtained from Amersham Corp., Arlington Heights, IL, U.S.A. Nucleosides and hydroxyurea were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. The nucleosides showed u.v.-absorption spectra characteristic of the pure compounds. FdUrd was obtained from Dr. W. E. Scott of F. Hoffman–La Roche, Nutley, NJ, U.S.A. Culture medium 199 (Morgan *et al.*, 1950) containing a salts base (Earle, 1943) with 15 mM-$NaHCO_3$ was obtained from Grand Island Biological Co., Grand Island, NY, U.S.A. Female Wistar rats, weighing approx. 200 g, were from Biobreeding Laboratories, Ottawa, Ontario, Canada.

Cell culture and determination of radioactivity

These were as described previously (Forsdyke, 1971; Sjostrom & Forsdyke, 1974; Scott & Forsdyke, 1976). Rat thymus cells were suspended at a concentration of 2×10^6/ml in medium 199 (90%) and fresh homologous serum (10%). Radioactive labelling of DNA was initiated by adding [^{3}H]thymidine or deoxy[^{3}H]cytidine to a final concentration of 1 μCi/ml. Then 1 ml volumes were immediately pipetted into 12 ml round-bottomed glass centrifuge tubes containing various quantities of unlabelled deoxyribonucleosides or inhibitors. After 4 h of incubation at 38°C in air/CO_2 (19:1), cells were pelleted by centrifugation (1200 *g*, 4 min) and washed once in 2 ml of 0.14 M-NaCl (4°C). The cell pellets were stored in ethanol (95%) at −20°C for 1–7 days before determination of radioactivity in cold-$HClO_4$-precipitable material solubilized in Hyamine. Data values, when averaged, were expressed as the arithmetic mean (±S.E.M.). Significance was assessed by Student's *t* test. Lines in Figures were fitted to data points by eye. In previous work a non-linear-regression computer program was used to calculate the parameters of isotope-dilution plots directly from data by reiterative curve fitting (Forsdyke, 1971). However, the improved accuracy obtained with this procedure was not sufficient to warrant its routine use in the present study.

Isotope-dilution model

The theoretical basis of the isotope-dilution model has been discussed in detail elsewhere (Forsdyke, 1971; Sjostrom & Forsdyke, 1974; Scott & Forsdyke, 1976, 1978*a*). A constant quantity of a radioactively labelled DNA precursor is added to cultured cells together with various quantities of the corresponding unlabelled precursor. The reciprocal of the radioactivity (c.p.m.) incorporated into DNA (abscissa) is plotted against the total concentration of the added precursor (labelled and unlabelled; ordinate). The plot is linear at all concentrations of precursor other than those at which the concentration of the precursor is itself rate-limiting. The slope of the plot provides a measure of the maximum

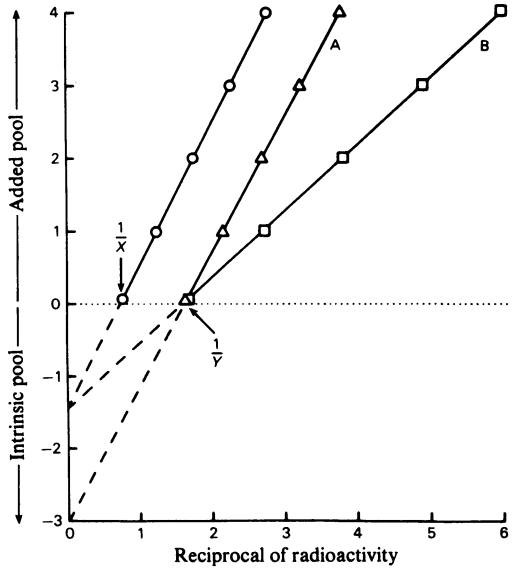

Fig. 1. *Ideal isotope-dilution plots showing the effects of inhibitors of the incorporation of a radioactive precursor into a stable macromolecule*

In the absence of inhibitors (○), the macromolecule acquires X units of radioactivity in a given time. In the presence of inhibitor A (△), the macromolecule acquires only Y units of radioactivity in the same time. In the presence of inhibitor B (□), the macromolecule also happens to acquire only Y units of radioactivity. These three values, X, Y and Y, are expressed as reciprocals and plotted along the abscissa against the concentration of the added radioactive precursor (ordinate). If the added precursor is of high specific radioactivity, the actual concentration of the added radioactive precursor may be very low. Further sets of three reciprocal values are generated when the specific radioactivity of the precursor is progressively decreased by carrying out the incorporation in the presence of increasing quantities of unlabelled precursor. From the resulting lines ('isotope-dilution plots'), the nature of the inhibitory effects of A and B can be distinguished. A acts by increasing the value of the negative intercept at the ordinate. There is no change in slope. This indicates that A is inhibiting incorporation of the radioactive precursor solely by increasing the intrinsic pool of compounds capable of competing with the precursor for incorporation at the rate-limiting step. B acts by decreasing the slope, but does not change the intercept at the ordinate. This indicates that B is decreasing incorporation of the precursor solely by decreasing the V of the rate-limiting step.

velocity (V) of the rate-limiting step in the biochemical pathway under study. Assuming a uniform cell population, at any one time there is likely to be only one such rate-limiting step. If the step is due to the activity of a rate-limiting enzyme, then the V value represents a kinetic parameter of the enzyme when in its natural environment within an intact cell. The negative intercept at the ordinate of isotope-dilution plots provides a measure of the pool of compounds in the system competing with the radioactive precursor before the rate-limiting step. Isotope-dilution theory requires that this pool be equal to the sum of those pools with which the added radioactive precursor can rapidly reach chemical equilibrium. These rapid-equilibrium pools are separated from non-equilibrium pools by rate-limiting steps. The non-equilibrium pools can be converted into equilibrium pools by changes in the position of rate-limiting steps. Fig. 1 shows the use of isotope-dilution plots to distinguish between two inhibitors of the incorporation of a radioactive precursor into DNA.

Results

Rat thymus cells were incubated for 4h with radioactive thymidine or deoxycytidine in serum (10%) and medium 199 (90%). The incorporation of radioactivity into material precipitated by cold acid was measured. The general characteristics of the system have been described (Sjostrom & Forsdyke, 1974).

Effects of purine deoxyribonucleosides on the incorporation of [^{3}H]thymidine and deoxy[^{3}H]cytidine

Low concentrations of deoxyguanosine and deoxyadenosine enhanced the incorporation of deoxy[^{3}H]cytidine, but did not consistently enhance the incorporation of [^{3}H]thymidine (Fig. 2). With deoxyguanosine (100μM) the enhancement of deoxy[^{3}H]cytidine incorporation was 19.3% (±3.0%; $P < 0.05$, $n = 3$). With deoxyadenosine (40μM) the enhancement was 8.2% (±3%; $P < 0.05$, $n = 3$). Higher concentrations of the purine deoxyribonucleosides were inhibitory. Deoxyguanosine inhibited the incorporation of [^{3}H]thymidine more than the incorporation of deoxy[^{3}H]cytidine (Fig. 2*a*). However, this differential susceptibility to inhibition was not found when deoxyadenosine was employed (Fig. 2*b*). On a molar basis, deoxyadenosine was more inhibitory than deoxyguanosine.

Purine deoxyribonucleosides decrease inhibition by hydroxyurea

Concentrations of hydroxyurea were chosen to produce approx. 70% inhibition of the incorporation of the ^{3}H-labelled pyrimidine deoxyribonucleosides: with [^{3}H]thymidine 100μM-hydroxyurea was used, and with deoxy[^{3}H]cytidine 500μM-hydroxyurea was used. Fig. 3 shows purine deoxyribonucleoside dose–response curves in the presence

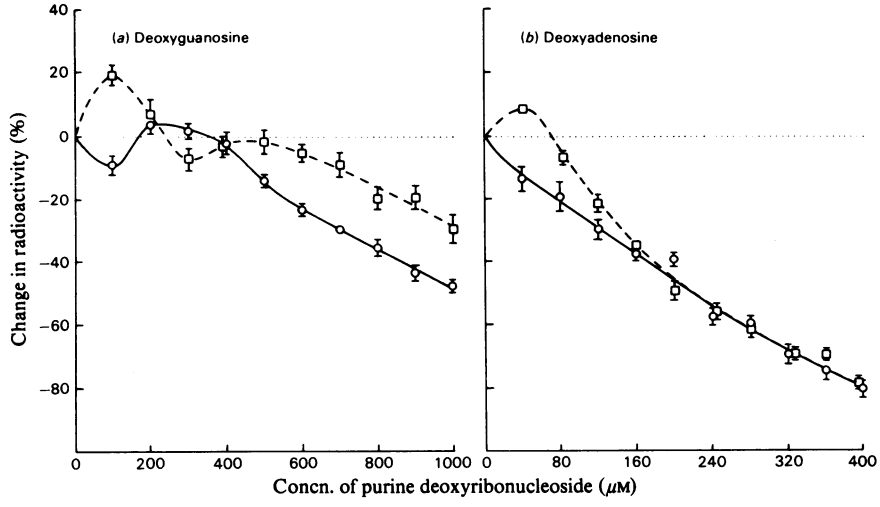

Fig. 2. *Effects of various concentrations of (a) deoxyguanosine and (b) deoxyadenosine on the incorporation of [Me-^{3}H]thymidine (○) and deoxy[5-^{3}H]cytidine (□) by rat thymus cells*

The 1 ml cultures each contained 2×10^6 cells, fresh homologous serum (10%), medium 199 (90%) and 1 μCi of radioactive DNA precursor (approx. 20 Ci/mmol). Acid-precipitable radioactivity in cells was determined after a 4 h incubation. Each point represents the arithmetical mean (±S.E.M.) of six percentage values (from three separate experiments performed with duplicate cultures at each concentration of added purine deoxyribonucleoside). In the three experiments that provided data for (*a*), the mean values for radioactive labelling (c.p.m.) in control cultures without added deoxyguanosine (quadruplicate cultures in each experiment) were 24 995 ± 1598 ([^{3}H]thymidine) and 3694 ± 232 (deoxy[^{3}H]cytidine). In the three experiments that provided data for (*b*), the mean values for controls without added deoxyadenosine were 20 125 ± 2483 ([^{3}H]thymidine) and 2934 ± 299 (deoxy[^{3}H]cytidine). There is less incorporation of deoxy[^{3}H]cytidine because of the pool of deoxycytidine in serum (Sjostrom & Forsdyke, 1974).

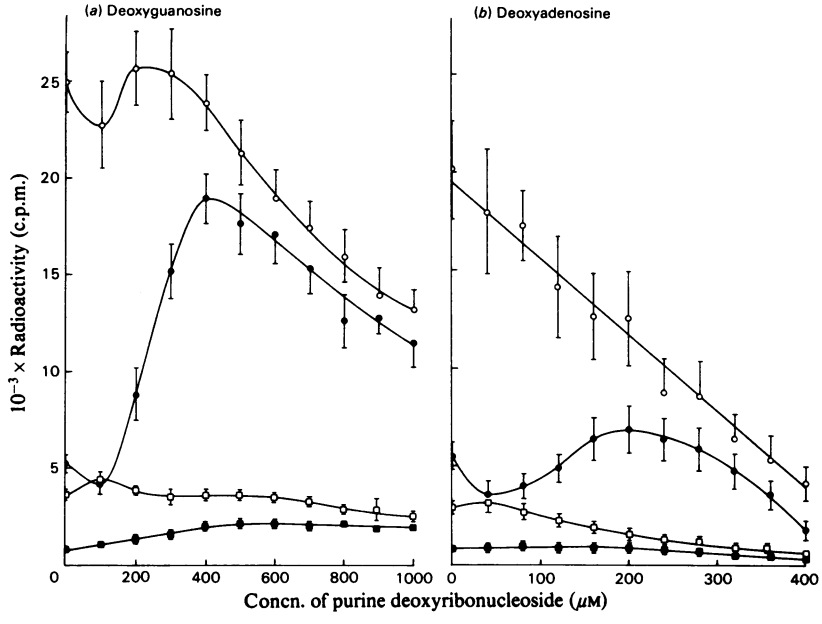

Fig. 3. *Decrease by (a) deoxyguanosine and (b) deoxyadenosine of the inhibition by hydroxyurea*

The cultures contained 1 μCi of either [^{3}H]thymidine (○, ●) or deoxy[^{3}H]cytidine (□, ■). Filled symbols indicate the presence of hydroxyurea (100 μM for cultures containing [^{3}H]thymidine and 500 μM for cultures containing deoxy[^{3}H]cytidine). Each point represents the mean of six radioactivity values (from three separate experiments with duplicate cultures at each concentration of added purine deoxyribonucleoside).

and absence of hydroxyurea. Inhibition of [^{3}H]-thymidine incorporation by hydroxyurea was slightly increased by low concentrations (100μM) of deoxyguanosine ($P < 0.02$, $n = 4$) and by low concentrations (40μM) of deoxyadenosine ($P < 0.001$, $n = 6$). Higher concentrations of deoxyguanosine were able to prevent most of the inhibition of [^{3}H]thymidine incorporation by hydroxyurea (Fig. 3*a*). The optimum deoxyguanosine concentration (400μM) was not inhibitory for control cultures without hydroxyurea. Higher deoxyguanosine concentrations were inhibitory in both the presence and the absence of hydroxyurea.

In the presence of hydroxyurea, 200μM-deoxyadenosine increased [^{3}H]thymidine incorporation relative to the inhibition observed at 40μM-deoxyadenosine (Fig. 3*b*). However, 200μM-deoxyadenosine did not prevent inhibition by hydroxyurea. Thus the [^{3}H]thymidine incorporation observed in the presence of hydroxyurea alone was not significantly different from that observed in the presence of hydroxyurea and 200μM-deoxyadenosine together.

Inhibition of deoxy[^{3}H]cytidine incorporation by hydroxyurea was partially prevented by deoxyguanosine (Fig. 3*a*). In the presence of hydroxyurea, quantities of deoxyguanosine up to 500μM progressively increased deoxy[^{3}H]cytidine incorporation. Higher deoxyguanosine concentrations produced no further change in incorporation. Deoxyadenosine, at a concentration that was inhibitory in the absence of hydroxyurea (200μM), did not affect the inhibition of deoxy[^{3}H]cytidine incorporation by hydroxyurea (Fig. 3*b*). Higher deoxyadenosine concentrations were inhibitory in both the presence and the absence of hydroxyurea.

Effects of purine deoxyribonucleosides depend on the hydroxyurea concentration

Fig. 4 shows changes in the incorporation of [^{3}H]thymidine and deoxy[^{3}H]cytidine produced by different concentrations of hydroxyurea. As reported previously (Scott & Forsdyke, 1976), the curve for inhibition of [^{3}H]thymidine incorporation was to the left of the curve for inhibition of deoxy[^{3}H]cytidine incorporation. Deoxyguanosine (400μM) was found to decrease the increased sensitivity of [^{3}H]-thymidine incorporation to inhibition by hydroxyurea (Fig. 4*a*). Thus the curve for inhibition of [^{3}H]thymidine incorporation was shifted to the right by deoxyguanosine. Except at high hydroxyurea concentrations, the curve was similar to the curve for inhibition of deoxy[^{3}H]cytidine incorporation by hydroxyurea (in the absence of deoxyguanosine).

Hydroxyurea dose–response curves for deoxy[^{3}H]cytidine incorporation showed a small stimulation by low hydroxyurea concentrations (50μM), a steep descending limb and then a shoulder at higher hydroxyurea concentrations (Scott & Forsdyke, 1976). Deoxyguanosine (400μM) increased both the stimulation at low hydroxyurea concentrations and the shoulder at higher hydroxyurea concentrations (Fig. 4*a*). The steep descending limb was shifted to the right of the control curve without deoxyguanosine. A similar, though less clear-cut, pattern of curves was obtained when deoxyadenosine (200μM) was used (Fig. 4*b*).

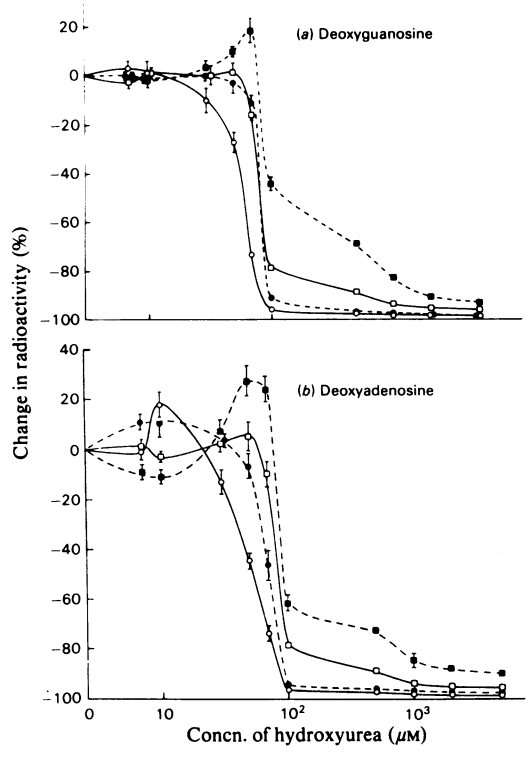

Fig. 4. *Effects of (a) deoxyguanosine (400μM) and (b) deoxyadenosine (200μM) on hydroxyurea dose–response curves*

The cultures contained 1μCi of either [^{3}H]-thymidine (○, ●) or deoxy[^{3}H]cytidine (□, ■). Filled symbols indicate the presence of added purine deoxyribonucleoside. Each point represents the mean of six percentage values (from three separate experiments with duplicate cultures at each concentration of hydroxyurea). In (*a*) the mean values (c.p.m.) for radioactive labelling in control cultures without hydroxyurea (quadruplicate cultures in each experiment) were 22270 ± 1511 ([^{3}H]thymidine), 21718 ± 2967 ([^{3}H]thymidine + deoxyguanosine), 5835 ± 1128 (deoxy[^{3}H]cytidine) and 5020 ± 885 (deoxy[^{3}H]cytidine + deoxyguanosine). Corresponding values for the three experiments that generated the data for (*b*) were 35293 ± 2802 ([^{3}H]-thymidine), 18207 ± 1958 ([^{3}H]thymidine + deoxyadenosine), 6478 ± 788 (deoxy[^{3}H]cytidine) and 3526 ± 453 (deoxy[^{3}H]cytidine + deoxyadenosine).

Isotope-dilution analysis of the decrease of hydroxyurea inhibition by FdUrd and purine deoxyribonucleosides

Fig. 5 shows the effects of FdUrd and hydroxyurea, separately and in combination, on thymidine isotope-dilution plots. As reported previously (Sjostrom & Forsdyke, 1974), thymidine isotope-dilution plots were bimodal, showing two linear portions which met at a point corresponding to approx. 7μM-thymidine. Each line could be extrapolated to the ordinate, which was crossed below the abscissa. In the presence of FdUrd, thymidine isotope-dilution plots were unimodal, with a slope similar to that of the upper limb of the bimodal control plots and an intercept at the ordinate the same as that for the lower limb of the control plots. In the presence of hydroxyurea, the single kink between the two limbs of thymidine isotope-dilution plots was no longer evident and the experimental points were described by fitting three linear portions (Scott & Forsdyke, 1976). Hydroxyurea greatly decreased the slope of the lower limb of the plots, but the change in slope of the upper limb was much less. The major effect of hydroxyurea was a great increase in the negative value of the intercept at the ordinate of the upper limb of the plots (value obtained by extrapolation).

For cultures containing both FdUrd and hydroxyurea, thymidine isotope-dilution plots were unimodal. The slope was less than that of the lower limb of the bimodal control plots, but greatly exceeded that of the lower limb of the plots derived from cultures treated with hydroxyurea alone. The intercept at the ordinate of the unimodal plot for cultures containing FdUrd and hydroxyurea was the same as the intercept for the lower limb of the bimodal control plots.

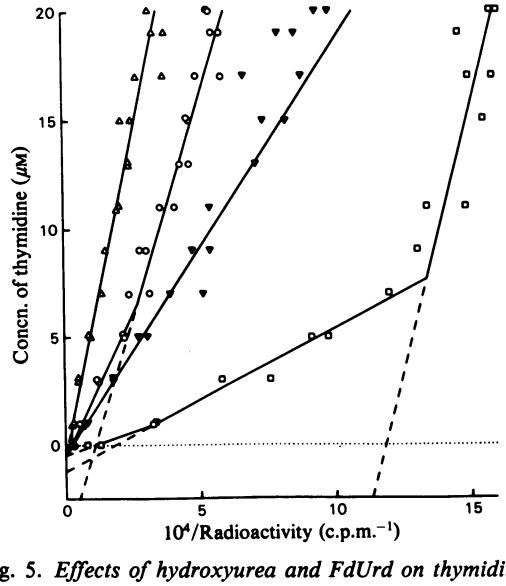

Fig. 5. *Effects of hydroxyurea and FdUrd on thymidine isotope-dilution plots*

O, Control cultures; △, +FdURD (1μM); □, +hydroxyurea (100μM); ▼, +FdURD+hydroxyurea. For details see the Materials and methods section.

The purine deoxyribonucleosides produced changes in thymidine isotope-dilution plots (Fig. 6) that were similar to those produced by FdUrd. In the absence of hydroxyurea, deoxyguanosine (400μM) increased the slope of the lower limb of the plots, thus tending to make them unimodal (Fig. 6*a*). There was a small increase in the negative value of the intercept at the ordinate of the lower limb, but this was not significant ($P<0.05$, $n=3$). The great decrease in the slope of the lower limb of the plots by hydroxyurea was almost completely corrected by deoxyguanosine. The latter had no significant effect on the small decrease in the slope of the upper limbs of the plots by hydroxyurea, but there was a great decrease in the hydroxyurea-induced increase in the negative value of the intercept at the ordinate.

In the absence of hydroxyurea, deoxyadenosine (200μM) had no effect on the slopes of the lower limbs of thymidine isotope-dilution plots (Fig. 6*b*). However, there was an increase in the negative value of the intercept at the ordinate ($P<0.02$, $n=3$). In two out of three experiments the slope of the upper limb of the plots was decreased by deoxyadenosine and the negative value of the intercept at the ordinate decreased. Thus, as with deoxyguanosine, there was a tendency for the bimodal isotope-dilution plots to become unimodal. The decrease in the slope of the lower limb of the plots by hydroxyurea was partially relieved by deoxyadenosine. Although in the presence of hydroxyurea deoxyadenosine further decreased the slope of the upper limb of the plots, there was a great decrease in the hydroxyurea-induced increase in the negative value of the intercept at the ordinate (Fig. 6*b*).

Deoxycytidine isotope-dilution plots

These were unimodal (Sjostrom & Forsdyke, 1974). Hydroxyurea at concentrations up to 100μM decreased both the slope and the negative value of the intercept at the ordinate (Scott & Forsdyke, 1976). Hydroxyurea at a concentration of 500μM sustained the decrease in the negative value of the intercept at the ordinate and produced a further decrease in the slope (Fig. 7). In some experiments, at this concentration of hydroxyurea, the curve showed an upward inflection at a point corresponding to a deoxycytidine concentration of approx. 11μM. After this, the original slope was regained at higher deoxycytidine concentrations. A similar

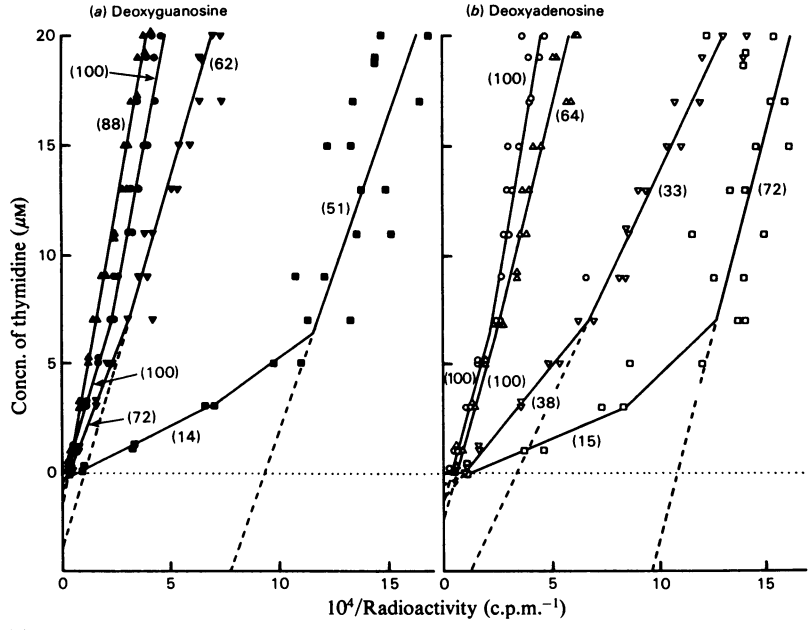

Fig. 6. *Effects of (a) deoxyguanosine and (b) deoxyadenosine on thymidine isotope-dilution plots in the presence and absence of hydroxyurea (100 μM)*

●, ○, Controls; ■, □, + hydroxyurea; ▲, + deoxyguanosine (400 μM); ▼, + deoxyguanosine + hydroxyurea; △, + deoxyadenosine; ▽, + deoxyadenosine + hydroxyurea. Numbers in parentheses indicate the slopes of the plots relative to the slope of the control plot (100).

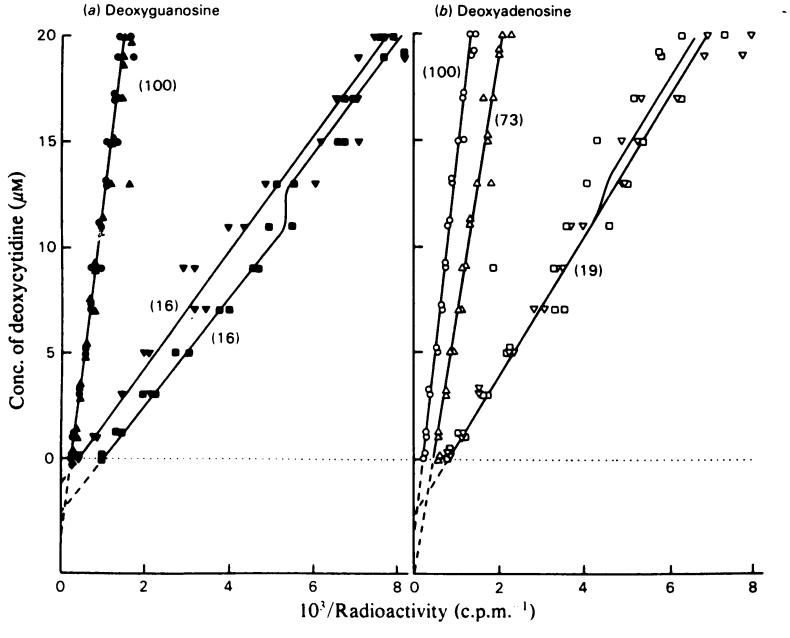

Fig. 7. *Effects of (a) deoxyguanosine and (b) deoxyadenosine on deoxycytidine isotope-dilution plots in the presence and absence of hydroxyurea (500 μM)*

Symbols are as in Fig. 6. Numbers in parentheses indicate the slopes of the plots relative to the slope of the control plot (100).

inflection was reported previously for cultures treated with FdUrd (Scott & Forsdyke, 1976).

In the absence of hydroxyurea, deoxyguanosine had no effect on deoxycytidine isotope-dilution plots (Fig. 7*a*). In the presence of hydroxyurea, deoxyguanosine decreased the negative value of the intercept at the ordinate, but the slope of the plot was not changed. In the absence of hydroxyurea, deoxyadenosine produced a small decrease in the slope of the plots and increased the negative value of the intercept at the ordinate (Fig. 7*b*). Deoxyadenosine did not in any consistent way affect either the slope or intercept of the plots in the presence of hydroxyurea.

Discussion

Rate-limiting steps affecting thymidine incorporation

With a knowledge of the properties of the enzymes of the thymidine-incorporation pathway, it was deduced from bimodal isotope-dilution plots that thymidine kinase was normally rate-limiting at low thymidine concentrations (0.04–7.0μM; Sjostrom & Forsdyke, 1974; Marz *et al.*, 1977). When studied by means of Lineweaver–Burk or Hofstee plots, purified thymidine kinase also demonstrated bimodal kinetics, with an inflection between the two limbs of the plots at a point corresponding to approx. 7μM-thymidine (Kizer & Holman, 1974; Nawata & Kamiya, 1975; Munch-Petersen & Tyrsted, 1977). The K_m for lymphocyte thymidine kinase at low thymidine concentrations is 0.14μM (Munch-Petersen & Tyrsted, 1977). This activity of thymidine kinase might be responsible for the V value that was observed over the range 0.04–7.0μM-thymidine (lower limb of isotope-dilution plots). The K_m for thymidine kinase at high substrate concentrations is 83μM. Thus, over the concentration range 7–20μM (upper limb of isotope-dilution plots), it is unlikely that this activity of thymidine kinase would be rate-limiting for the incorporation of [^{3}H]thymidine into DNA. It was suggested that at these thymidine concentrations a subsequent step in the pathway, at the level of DNA polymerase, was rate-limiting (Sjostrom & Forsdyke, 1974).

Hydroxyurea increases the dTTP pool

From thymidine isotope-dilution plots it was deduced that hydroxyurea causes a great increase in the dTTP pool (Scott & Forsdyke, 1976). This was in keeping with the direct measurements of pools that could be extracted from hydroxyurea-treated cells (Skoog & Nordenskjöld, 1971; Adams *et al.*, 1971). The accumulation of dTTP would reflect the continued formation of dTTP at a greater rate than it could be degraded or utilized for DNA synthesis. Continued formation could reflect the transformation of deoxycytidine present in serum to dTTP by way of the dCMP deaminase pathway. However, dCMP deaminase is very sensitive to feedback inhibition by dTTP (Maley & Maley, 1972). In these circumstances the continued formation of dTTP could reflect some residual function of ribonucleotide reductase. This enzyme differs from dCMP deaminase in being (i) less sensitive to feedback inhibition by dTTP (Jackson, 1978) and (ii) susceptible to regulation by dGTP and dATP. Since dGTP and dATP appear to prevent the dTTP-pool increase (discussed below), this argues for a primary role of the reductase, rather than of dCMP deaminase, in the pool increase.

The intracellular concentrations of dGTP and dATP are normally lower than those of dTTP and dCTP, so that the primary effect of inhibition of ribonucleotide reductase by hydroxyurea would be a depletion of the pools of dGTP and dATP (Skoog & Nordenskjöld, 1971; Adams *et al.*, 1971). Since dGTP and dATP inhibit the activity of ribonucleotide reductase towards UDP and CDP, the fall in the concentration of dGTP and dATP would tend to oppose an inhibitory effect of hydroxyurea on the reduction of UDP and CDP. Thus, although inhibited by hydroxyurea, ribonucleotide reductase might still be capable of reducing the pyrimidine ribonucleoside diphosphates.

Purine deoxyribonucleosides decrease competitor pools

To attempt to prevent inhibition of DNA synthesis by hydroxyurea, purine deoxyribonucleosides were added to cultures. It is likely that in thymus cells a significant proportion of the added molecules would be converted into the corresponding deoxyribonucleoside triphosphates (Tattersall *et al.*, 1975; Mitchell *et al.*, 1978). These would be expected to inhibit ribonucleotide reductase and produce effects similar to those produced by hydroxyurea (Carson *et al.*, 1978). The following evidence was consistent with this. (i) It was found previously that at low hydroxyurea concentrations (50μM) the incorporation of deoxy[^{3}H]cytidine showed a small enhancement (owing to a decrease in competitor pool), but the incorporation of [^{3}H]thymidine was not consistently affected (Scott & Forsdyke, 1976). Similar results were found at low concentrations of deoxyguanosine and deoxyadenosine (Fig. 2). (ii) At higher hydroxyurea concentrations (100–500μM) the incorporation of [^{3}H]thymidine was inhibited more than the incorporation of deoxy[^{3}H]cytidine (Scott & Forsdyke, 1976). This differential susceptibility to inhibition also applied to inhibition by high concentrations of deoxyguanosine (Fig. 2*a*). (iii) Low concentrations of purine deoxyribonucleosides further inhibited [^{3}H]thymidine incorporation

in cultures that were sub-maximally inhibited by hydroxyurea (Fig. 3).

Ostensibly, the notion that the dGTP and dATP formed from the added purine deoxyribonucleosides could by-pass the inhibition of ribonucleotide reductase by hydroxyurea was supported by the observation that high concentrations of individual deoxyribonucleosides decreased the inhibition by hydroxyurea (100–500 μM; Fig. 3). However, the effects of purine deoxyribonucleosides on hydroxyurea dose–response curves (Fig. 4) and on isotope-dilution plots (Fig. 6) indicated that the decrease in the inhibitory effect of hydroxyurea was due to a pool-size change rather than to an actual increase in the rate of DNA synthesis. Thus the purine deoxyribonucleosides produced the same effect on hydroxyurea dose–response curves as that previously reported to be caused by FdUrd (Scott & Forsdyke, 1976). Deoxyguanosine abolished the greater inhibitory effect of hydroxyurea on [^{3}H]-thymidine incorporation, as shown by the shift of the curve to the right so that it closely followed the descending limb of the curve for inhibition of deoxy[^{3}H]cytidine incorporation by hydroxyurea (Fig. 4). It appeared that, like FdUrd, the purine deoxyribonucleosides were preventing the hydroxyurea-induced increase in the dTTP pool (which would feedback-inhibit thymidine kinase) and thus were removing the differential susceptibility of [^{3}H]thymidine incorporation to inhibition by hydroxyurea.

The effects of the purine deoxyribonucleosides on thymidine isotope-dilution plots (Fig. 6) were also very similar to those of FdUrd (Fig. 5). Thus, in the absence of hydroxyurea, FdUrd and deoxyguanosine both increased the V of thymidine kinase (increased slope of lower limb of the plots) and decreased the competitor pool at the level of DNA polymerase (decreased negative value of the intercept formed by extrapolation of the upper limb of the plots to the ordinate). This indicated again that both were decreasing the dTTP pool and hence were decreasing feedback inhibition of thymidine kinase. By preventing the pool increase caused by hydroxyurea, FdUrd and the purine deoxyribonucleosides were able to reverse substantially the inhibitory effect of hydroxyurea (100 μM) on the incorporation of [^{3}H]thymidine (Figs. 5 and 6). FdUrd would prevent the pool increase by inhibiting thymidylate synthase. The purine deoxyribonucleosides would prevent the pool increase by being converted into the corresponding deoxyribonucleoside triphosphates, which would inhibit residual activity of ribonucleotide reductase towards UDP (discussed above).

For the combination of hydroxyurea and deoxyadenosine, the V at the level of DNA polymerase was decreased further than the decrease produced by hydroxyurea alone. However, the effect of the dTTP-pool decrease was so great that deoxyadenosine produced a substantial stimulation of [^{3}H]-thymidine incorporation relative to cultures with hydroxyurea alone (Fig. 6*b*). It should be noted that, in the absence of additional unlabelled thymidine to decrease the specific radioactivity of the [^{3}H]-thymidine, this ability of deoxyadenosine to decrease the inhibition by hydroxyurea would not have been detected. This was because the increase in V of thymidine kinase (tending to increase the observed incorporation of radioactivity) was compensated for by an increase in the pool competing before thymidine kinase (tending to decrease the observed incorporation of radioactivity; Fig. 6*b*). Thus the observed incorporation of [^{3}H]thymidine in the presence of deoxyadenosine (200 μM) and hydroxyurea (100 μM) was no different from the incorporation in the presence of hydroxyurea alone (Fig. 3*b*).

Deoxycytidine isotope-dilution plots

It was suggested that the rate-limiting step for deoxycytidine incorporation in thymus cells was at the level of DNA polymerase. Hydroxyurea (50–500 μM) and thymidine (19 μM) were shown to decrease the deoxycytidine competitor pool (Scott & Forsdyke, 1976), presumably by inhibiting entry to the pool by way of ribonucleotide reductase.

If the added purine deoxyribonucleosides were being converted into dGTP and dATP, which themselves inhibit ribonucleotide reductase, then a decrease in the deoxycytidine competitor pool would be predicted. The results were not as expected. Deoxyadenosine increased the deoxycytidine competitor pool (Fig. 7*b*). Deoxyguanosine did not change the pool (Fig. 7*a*). However, in the presence of hydroxyurea, deoxyguanosine produced effects that were consistent with the notion that dGTP was inhibiting ribonucleotide reductase. Thus the ability of deoxyguanosine (400 μM) partially to reverse the inhibition of deoxy[^{3}H]cytidine incorporation by hydroxyurea (500 μM; Fig. 3*a*) was entirely due to the ability of deoxyguanosine to decrease the competitor pool (Fig. 7*a*).

Inhibition of high hydroxyurea concentrations

Explanations for the inhibition by high hydroxyurea concentrations (>1 mM), which was only partially responsive to exogenous deoxyguanosine or deoxyadenosine (Fig. 4), include the following. (i) Hydroxyurea indirectly inhibits DNA synthesis by primarily inhibiting some other cellular process (Hawtrey *et al.*, 1974). This was not further examined. (ii) In the presence of hydroxyurea both dGTP and dATP pools are depleted, and exogenous deoxyguanosine or deoxyadenosine alone cannot act as a source of both DNA precursors (Maley & Maley, 1961). Preliminary experiments with combinations of deoxyguanosine and deoxyadenosine did

not provide evidence that the combination was much more effective than the individual purine deoxyribonucleosides alone (F. W. Scott & D. R. Forsdyke, unpublished work). This was consistent with the failure of others substantially to reverse the inhibitory effects of high concentrations of hydroxyurea with combinations of deoxyribonucleosides (Young *et al.*, 1967; Yarbro, 1968; Bachetti & Whitmore, 1969). (iii) Neither dGDP, dGTP, dADP nor dATP, when formed by way of purine deoxyribonucleoside salvage pathways, can enter the multienzyme complex between ribonucleotide reductase, purine deoxyribonucleoside diphosphate kinase and DNA polymerase (Mathews *et al.*, 1979; Forsdyke & Scott, 1979). Thus the 'salvage' and '*de novo*' pathways of purine deoxyribonucleotide synthesis would not converge.

Our present studies are generally consistent with the latter hypothesis. When normal replicative DNA synthesis slowed, owing to an imbalance in the concentration of deoxyribonucleoside triphosphates or to the need for DNA repair, the enzyme complex would release deoxyribonucleotides (see, e.g., Brutlag & Kornberg, 1972). These could feedback-regulate ribonucleotide reductase to correct the imbalance, but would not be substrates for the DNA polymerase involved in replicative DNA synthesis.

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