Isotope-Dilution Analysis of Rate-Limiting Steps and Pools Affecting the Incorporation of Thymidine and Deoxycytidine into Cultured Thymus Cells

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(Received 4 October 1973)

1. Rat thymus cells were incubated in homologous serum (10%) and medium 199. The effects of various quantities of thymidine or deoxycytidine (0–30 μM) on the radioactive labelling of cells with the corresponding radioactive deoxynucleoside were examined. From plots of the reciprocal of the radioactivity incorporated against the total deoxynucleoside concentration ('isotope-dilution plots'), values were obtained for (a) the V_{max} of the rate-limiting step governing incorporation of the deoxynucleoside, and (b) the concentration of the pool of compounds competing with the radioactive deoxynucleoside at that rate-limiting step. From changes in these values under different experimental conditions inferences were drawn on the position and control of the rate-limiting step within intact cells. 2. Isotope-dilution plots for deoxycytidine were linear, whereas plots for thymidine were bimodal, indicating an abrupt increase in both the V_{max} and pool concentration at a critical thymidine concentration (approx. 5 μM). The bimodality was removed by amethopterin. The V_{max} determined with deoxy[U- ^{14}C]cytidine was approximately equal to the sum of the V_{max} determined with deoxy[5- ^3H]cytidine and the V_{max} determined with [Me- ^3H]thymidine at thymidine concentrations above 5 μM . 3. The thymidine competitor pool at thymidine concentrations above 5 μM was approximately equal to the sum of the deoxycytidine competitor pool and the thymidine competitor pool at thymidine concentrations below 5 μM . The pools were independent of cell concentration and dependent on serum concentration. 4. These results were explained on the following basis. Deoxycytidine in serum (16 μM) is the major source of both cytosine and, by way of thymidylate synthetase, thymine, in the DNA of thymus cells. Serum deoxycytidine normally maintains a sufficient intracellular concentration of dTTP to inhibit partially the activity of thymidine kinase. When the dTTP concentration is lowered, either by decreasing the concentration of deoxycytidine or by inhibiting thymidylate synthetase, the activity of thymidine kinase increases. The activity of thymidine kinase may also be increased by concentrations of thymidine greater than 5 μM , which overcome the inhibition of the enzyme by dTTP. At concentrations of thymidine below 5 μM , thymidine kinase limits the rate of labelling with [Me- ^3H]thymidine and the radioactivity is diluted by a pool of unlabelled thymidine in serum (4 μM). At thymidine concentrations greater than 5 μM , the activity of DNA polymerase limits the rate of labelling and the radioactivity is diluted both by serum thymidine and, indirectly, by serum deoxycytidine.

What are the rate-limiting steps and regulatory mechanisms governing the formation of deoxynucleoside triphosphates for DNA synthesis in mammalian cells? The main biochemical approach to these problems has been at the subcellular level. Individual enzymes have been identified and purified and their properties examined (Maley & Maley, 1972; Henderson & Paterson, 1973). Attention is now turning to the problem of the relevance of the results obtained in subcellular systems to the situation in the intact cell (Reichard, 1972). This has involved study of the kinetics of incorporation of radioactive DNA precursors by cultured cells (Cleaver, 1967; Wittes & Kidwell, 1973) or

measurement of the concentrations of metabolic intermediates that can be extracted from the cells with cold acid (Adams *et al.*, 1971; Skoog & Nordenskjöld, 1971). In the present paper the problem is approached in another way by using isotope-dilution analysis (Forsdyke, 1971).

To perform isotope-dilution analysis a radioactively labelled precursor of a relatively stable molecule is added to cultured cells together with different quantities of the corresponding unlabelled precursor. Under ideal conditions, a plot of the reciprocal of the radioactivity incorporated into the molecule against concentration of the unlabelled precursor is a straight line. From the parameters

of this line, values can be obtained for (a) the $V_{\max}$ of the rate-limiting step in the incorporation pathway and (b) the pool of compounds within the system capable of competing with the labelled precursor. 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 positions of rate-limiting steps. We demonstrate here that differences in $V_{\max}$ and pool values under different experimental conditions can be interpreted in terms of the isotope-dilution theory to obtain information on the positions of rate-limiting steps, flow rates along converging and diverging metabolic pathways and regulatory mechanisms.

The paper is in three parts. First, isotope-dilution plots using deoxy[5- ^3H]cytidine and deoxy[U- ^{14}C]cytidine are shown to be linear and the effects of variations in cell and serum concentration on the plots are examined. Then similar studies are presented using [Me- ^3H]thymidine; it is reported that isotope-dilution plots for thymidine are bimodal containing two linear portions. Finally our interpretations of the data are examined by using inhibitors of thymidylate synthetase to decrease the flow between the deoxycytidine and thymidine incorporation pathways. Preliminary reports of this work have appeared elsewhere (Sjostrom & Forsdyke, 1972; Scott & Forsdyke, 1973).

Materials and Methods

Cell labelling with [^3H]thymidine

Details of materials and methods have been described previously (Forsdyke, 1971). [Me- ^3H]Thymidine (14.0–28.0 Ci/mmol), deoxy[5- ^3H]cytidine (15.8 Ci/mmol) and deoxy[U- ^{14}C]cytidine (495–512 mCi/mmol) were obtained from Amersham/Searle, Des Plaines, Ill., U.S.A. Unlabelled deoxynucleosides were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K.

Freshly prepared rat thymus cells suspended in medium 199 (Morgan *et al.*, 1950) were added directly to an appropriate mixture of fresh homologous serum (10%) and medium 199 (90%) to give a final cell concentration of 1×10^6 – 4×10^6 /ml. To initiate radioactive labelling 1 ml samples were pipetted into 12 ml round-bottomed glass centrifuge tubes containing the radioactive deoxynucleoside (1 μCi) and various quantities of the corresponding unlabelled deoxynucleoside in a total volume of 20 μl . After 4 h of incubation at 37°C in air+CO₂ (95:5), cells were pelleted by centrifugation (1200g, 4 min) and washed once in 4 ml of 0.14 M-NaCl (4°C).

The cell pellets were stored in ethanol (95%, v/v) at –20°C for 1–7 days before determination of radioactivity in cold-acid-precipitable material solubilized in Hyamine.

A Schmidt-Thannhauser fractionation of the radioactivity in cell pellets (Forsdyke, 1967) provided evidence that most of the radioactivity was in DNA. In the average experiment, of the total radioactivity (d.p.m.) precipitated by cold acid, approx. 10% was alkali-labile and in this respect resembled RNA, 80% was released from cells by incubation for 30 min at 70°C with 0.5 M-HClO₄ and in this respect resembled DNA, and 10% remained acid-precipitable (see also Goldspink & Goldberg, 1973). A similar distribution of radioactivity between these three acid-precipitable fractions was obtained with [Me- ^3H]thymidine, deoxy[5- ^3H]cytidine and deoxy-[U- ^{14}C]cytidine.

Studies with [Me- ^3H]thymidine, which are documented elsewhere (Sjostrom, 1972), established the following general characteristics of the system. The pool of radioactivity which could be extracted from cells with cold acid remained relatively constant between 1 h and 4 h of culture and then declined. The time-course of the incorporation of radioactivity into material precipitated by cold acid was linear for the first 4 h of culture and could be extrapolated back close to zero radioactivity at zero time; after 4 h the rate of labelling usually declined. Radioautographic studies showed (a) that 6–17% of the cultured cells were in S phase, with an average around 10%, and (b) that the decline in labelling after 4 h probably reflected the inability of cycling cells under culture conditions to initiate S phase at a rate matching the rate of termination of S phase. Approx. 10% of the total radioactivity in cultures (1 μCi) was incorporated into cells in a 4 h culture period. The remaining radioactivity probably remained as [^3H]thymidine in the culture medium (Cooper & Milton, 1964; Merey & Cox, 1972; Beltz *et al.*, 1973); media separated from cells at different times of culture (0–8 h) showed little difference in their ability to label freshly isolated thymus cells when compared with media containing freshly added [^3H]thymidine. The degree of labelling was a linear function of cell concentration over a range of 1×10^6 – 4×10^6 cells/ml; plots of radioactive labelling against cell concentration could be extrapolated back to zero radioactivity at zero cell concentration.

Inhibitors of thymidylate synthetase

Amethopterin (4-amino- N^{10} -methylpteroylglutamic acid) (Hitchings & Burchall, 1965) was obtained from Lederle Laboratories, London W.C.2, U.K. 5-Fluorodeoxyuridine (Heidelberger, 1965) was obtained from Dr. W. E. Scott, F. Hoffman-La Roche Inc., Nutley, N.J., U.S.A. The inhibitors were added

to cultures at approximately the same time as the radioactive deoxynucleoside. Preincubation of cells with amethopterin for 1 h did not alter its effects on labelling with [³H]thymidine (Sjostrom, 1972); this was in keeping with studies with radioactive amethopterin which show that it enters cells rapidly (Harrap *et al.*, 1971).

Isotope-dilution model

The theoretical basis for the isotope-dilution model used in this work was presented previously (Forsdyke, 1971). The theory requires that the pools in the system be of two types: (a) pools with which the radioactive precursor can rapidly equilibrate; these will dilute the radioactivity and may be directly detected by isotope-dilution analysis; (b) pools which are separated from the rapid-equilibrium pools by rate-limiting steps; these pools will not be directly detected by isotope-dilution analysis. In terms of the model, a plot of the reciprocal of the radioactivity incorporated (abscissa) from a constant quantity of a radioactively labelled deoxynucleoside, against the total concentration of added deoxynucleoside (labelled and unlabelled; ordinate), should be linear at all concentrations of deoxynucleoside other than those at which the concentration of the deoxynucleoside itself is rate-limiting. Such a plot is termed an isotope-dilution plot and is in essence a specialized application of the Dixon plot used in studies of enzyme inhibition (Dixon, 1953). The slope of an isotope-dilution plot provides a measure of the maximum velocity ($V_{\max}$) of the rate-limiting step in the biochemical pathway under study. The negative intercept at the ordinate provides a measure of the pool of compounds in the system competing with the radioactive precursor before that rate-limiting step.

The slopes and intercepts of the linear reciprocal plots were calculated directly from lines fitted to data points by eye. In previous work a non-linear regression computer program was used to calculate parameters directly from data by curve fitting (Forsdyke, 1971). However, the improved accuracy obtained with this procedure was not sufficient to warrant its routine use in the present study. The $V_{\max}$ of deoxynucleoside incorporation by a given number of cells was calculated from the expression $Z/(D \cdot E \cdot H \cdot C \cdot T)$ where Z was the slope of the isotope-dilution plot (quantity of unlabelled nucleoside/reciprocal unit), D was the number of radioactive disintegrations corresponding to 1 μ Ci (2.2×10^6), E was the counting efficiency (approx. 0.34 for ³H and 0.91 for ¹⁴C), H was the quantity of radioactivity added (μ Ci), C was the cell number and T was the time of incubation. This is a simplified form of the equation used previously.

Results and Discussion

Rat thymus cells were incubated for 4 h 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 results obtained with deoxycytidine were the most easily interpreted and are presented first.

Higher $V_{\max}$ for deoxy[U-¹⁴C]cytidine than for deoxy[5-³H]cytidine

Addition of increasing quantities of unlabelled deoxycytidine to cultures progressively depressed the radioactive labelling obtained with deoxy[5-³H]cytidine or deoxy[U-¹⁴C]cytidine. Fig. 1 shows the data, with each individual radioactivity value expressed as a reciprocal ('isotope-dilution plot'). Each set of experimental points could be described

Fig. 1. Isotope-dilution plots with either deoxy[5-³H]cytidine or deoxy[U-¹⁴C]cytidine

Cultures (1 ml) contained 2×10^6 cells, homologous serum (10%), medium 199 (90%), various quantities of unlabelled deoxycytidine and either deoxy[5-³H]cytidine (○; 1 μ Ci, sp. radioactivity 21.2 Ci/mmol) or deoxy[U-¹⁴C]cytidine (●; 0.167 μ Ci, sp. radioactivity 0.512 Ci/mmol). After incubation at 37°C for 4 h the cells were harvested by centrifugation for the determination of the acid-precipitable radioactivity incorporated as described in the Materials and Methods section. Each individual radioactivity value is expressed as a reciprocal. $V_{\max}$ values calculated from the slopes of the plots were 21.6 pmol/h per 10^6 cells (deoxy[U-¹⁴C]cytidine) and 7.7 pmol/h per 10^6 cells (deoxy[5-³H]cytidine). The magnitude of this difference is not apparent on direct inspection of the slopes, since the ¹⁴C-labelled compound differed from the ³H-labelled compound in respect of quantity used, specific radioactivity and counting efficiency.

by a single straight line. The lines could be extrapolated to meet at the ordinate below the abscissa. The $V_{\max}$ (slope) calculated for deoxy-[U- ^{14}C]cytidine was greater than that calculated for deoxy[5- ^3H]cytidine by a factor of 3.4 (S.E.M. ± 0.5 ; 3 expts.).

The latter finding is in keeping with the following observations. (a) When deoxy[5,6- ^3H]cytidine is injected into rats, radioactivity is rapidly incorporated both into the cytosine and the thymine of thymus DNA (Sugino *et al.*, 1963). (b) The rat thymus has a high activity of deoxycytidylate deaminase converting dCMP into dUMP (Maley & Maley, 1959). (c) Conversion of [5- ^3H]dUMP into dTMP by way of thymidylate synthetase involves the loss of ^3H in the 5-position (Lomax & Greenberg, 1967). Thus only cytosine residues in DNA would be labelled by deoxy[5- ^3H]cytidine whereas both cytosine and thymine would be labelled by deoxy[U- ^{14}C]cytidine.

A serum pool of deoxycytidine

In Fig. 1 the negative intercept at the ordinate demonstrated the existence of a pool in the system. The concentration of this pool was independent of cell concentration (Fig. 2a), but was dependent on serum concentration (Fig. 2b). On the other hand the $V_{\max}$ was directly dependent on cell concentration (Fig. 2a) and was only slightly influenced by serum concentration (i.e. there was a small increase in slope on increasing the serum concentration from 10 to 30%; Fig. 2b). From plots of the reciprocal

of the radioactivity against serum concentration (Forsdyke, 1971) it was calculated that the pool was contributed by both serum and medium 199; serum had a competitive ability towards radioactive deoxycytidine equivalent to $16.1\mu\text{M}$ -deoxycytidine, whereas medium 199 had a competitive ability equivalent to $2.2\mu\text{M}$ -deoxycytidine. The former value is in close agreement with the finding of Guri *et al.* (1969) using a different experimental approach, that rat serum contains deoxycytidine at a concentration of $19.8\mu\text{M}$. Thus it is likely that the competing activity in serum was in fact due to deoxycytidine. Medium 199 (Morgan *et al.*, 1950) contains small quantities of deoxyribose ($3.7\mu\text{M}$), uracil ($2.7\mu\text{M}$) and thymine ($2.4\mu\text{M}$), and these may be responsible for the competitive activity of medium 199.

Bimodal thymidine isotope-dilution plots

In contrast with the isotope-dilution plots obtained with deoxycytidine, which were linear at all deoxycytidine concentrations studied (Fig. 1), isotope-dilution plots with thymidine showed an abrupt transition at approx. $5\mu\text{M}$ -thymidine, so that the experimental points were most easily described by two straight lines (Fig. 3). Thus at a critical thymidine concentration the $V_{\max}$ and the pool increased to new values.

The bimodal plots are most easily interpreted as showing that at $5\mu\text{M}$ -thymidine the position of the step, which limits the rate of [^3H]thymidine incorporation into DNA at lower thymidine

Fig. 2. Effect of (a) cell concentration and (b) serum concentration on deoxy[5- ^3H]cytidine isotope-dilution plots

In Fig. 2(a) cultures contained either 2×10^6 cells (○) or 4×10^6 cells (□) and 10% serum. Fig. 2(b) shows data from a separate experiment in which cultures contained 2×10^6 cells and either 10% serum (○) or 30% serum (Δ). Other details are as in Fig. 1.

Fig. 3. Thymidine isotope-dilution plots at different cell concentrations

Cultures (1ml) contained 10% serum, 1 μ Ci of [³H]-thymidine (20.4 Ci/mmol), various quantities of unlabelled thymidine and either 1.25×10^6 cells (○) or 2.5×10^6 cells (□). All data points are plotted except at low thymidine concentrations, where the reciprocal of the mean of triplicate radioactivity determinations is plotted. For other details see the text.

concentrations, is shifted so that a subsequent step on the pathway becomes rate-limiting. This shift would permit a pool of compounds entering the pathway between the two rate-limiting steps to compete with exogenous thymidine. Thus concomitant with the change in slope of the plot ($V_{\max}$), there would be a change in the intercept at the ordinate (pool). At low thymidine concentrations the first rate-limiting step would be limiting and only the pool of compounds competing before that step would be detected. The data may be compared with the [³H]uridine isotope-dilution plots obtained previously from pig lymphocyte cultures (Forsdyke, 1968); at a critical uridine concentration the $V_{\max}$ changed but there was no change in the pool.

Obvious sites for the two rate-limiting steps in intact cells would be on either side of the point of entry of the product of thymidylate synthetase (dTMP) into the thymidine incorporation pathway and evidence supporting this is presented below. At low thymidine concentrations a step involved with permeation or the phosphorylation of thymidine to dTMP would be rate-limiting. It is known that dTTP exerts a feed-back inhibition on thymidine kinase activity in extracts of various rat tissues, including the thymus, and that thymidine can prevent this inhibition (Ives *et al.*, 1963). It is possible

that, at a critical concentration, thymidine overcomes a feed-back inhibition by dTTP of thymidine kinase so that the $V_{\max}$ of the enzyme increases to an extent such that a subsequent step on the pathway then becomes rate-limiting. A change in $V_{\max}$ at a critical substrate concentration demonstrated by the bimodal isotope-dilution plots (Fig. 3) has also been inferred from bimodal Lineweaver-Burk plots obtained with purified bacterial thymidine kinase (Okazaki & Kornberg, 1964) and with mammalian deoxycytidine kinase (Kozai *et al.*, 1972); the bimodality was noted in the absence of the corresponding deoxynucleoside triphosphates. Abrupt transitions in bimodal Lineweaver-Burk plots have been interpreted as showing a change from negative co-operativity at low substrate concentrations to positive co-operativity at high substrate concentrations (Engel & Ferdinand, 1973).

The rate-limiting step at high thymidine concentrations could be involved with the further phosphorylation of dTMP to dTTP, or the incorporation of dTTP into DNA (DNA polymerase). The latter possibility seems most likely in view of the specific requirement for dTTP in the feed-back inhibition of pathways involved in dTTP formation (Maley & Maley, 1972) and the high intracellular concentration of dTTP compared with dTMP and dTDP (Cleaver, 1967).

Dilution of [³H]thymidine by thymidine at low thymidine concentrations and by both thymidine and deoxycytidine at high thymidine concentrations

Fig. 3 also shows that both the $V_{\max}$ below 5μ M-thymidine and the $V_{\max}$ above 5μ M-thymidine were directly dependent on cell concentration, whereas the corresponding pools were independent of cell concentration. Isotope-dilution plots at two different serum concentrations (Fig. 4) showed that at thymidine concentrations below 5μ M increasing the serum concentration decreased the $V_{\max}$ and increased the pool (intercept at the ordinate). At thymidine concentrations above 5μ M increasing the serum concentration increased the pool, but usually did not change the $V_{\max}$.

At thymidine concentrations below 5μ M it was calculated that serum had a competitive ability equivalent to 4.3μ M-thymidine whereas medium 199 had a competitive ability equivalent to 0.48μ M-thymidine. The former value is in remarkable agreement with the finding of Christine *et al.* (1972), using a different experimental approach, that mouse serum contains thymidine at a concentration of 3.4μ M. Thus it is likely that the serum competitive activity towards [³H]thymidine, which was diffusible (Sjostrom, 1972), was actually due to thymidine.

However, at thymidine concentrations above

Fig. 4. Thymidine isotope-dilution plots at different serum concentrations

Cultures (1 ml) each contained 1.8×10^6 cells, $1 \mu\text{Ci}$ of $[^3\text{H}]$ thymidine (22.0 Ci/mmol) and either 10% (○) or 30% (Δ) serum. For other details see the text.

Fig. 5. Thymidine isotope-dilution plots at different deoxycytidine concentrations

Cultures (1 ml) each contained 1.5×10^6 cells, $1 \mu\text{Ci}$ of $[^3\text{H}]$ thymidine (18.6 Ci/mmol) and 10% serum. ○, No added deoxycytidine; ●, $10 \mu\text{M}$ -deoxycytidine added. At low thymidine concentrations the reciprocal of the mean of the triplicate radioactivity determinations is plotted. For other details see the text.

$5 \mu\text{M}$ it was calculated that the competitive activity of serum was equivalent to approx. $19 \mu\text{M}$ -thymidine. The increase in pool size at high thymidine concentration would be explained if $[^3\text{H}]$ thymidine was then competing both with serum thymidine ($4.3 \mu\text{M}$) and with serum deoxycytidine ($16.1 \mu\text{M}$), which entered the thymidine kinase pathway by way of the thymidylate synthetase pathway. These two pools would have additive competitive effects. This would imply that at high thymidine concentrations the serum thymidine and deoxycytidine pools were in equilibrium by way of the thymidylate synthetase pathway. This in turn would imply that, under the experimental conditions used, the two pools were not separated by any rate-limiting steps.

The decrease in slope of isotope-dilution plots at thymidine concentrations below $5 \mu\text{M}$, produced by increasing the serum concentrations from 10 to 30% (Fig. 4), could be reproduced by adding deoxycytidine to medium containing 10% serum (Fig. 5). The effect would be explained if the deoxycytidine concentration in culture medium containing 10% serum was insufficient to maintain the intracellular dTTP concentration to an extent capable of exerting the maximum possible inhibition of thymidine kinase. Provided that an enzyme in the thymidylate synthetase pathway or in the pathway converting dTMP into dTTP was not rate-limiting, increasing the serum concentration (and hence the deoxycytidine content of the culture medium) would increase the dTTP concentration, which would decrease the V_{max} of thymidine kinase.

The inhibition of $[^3\text{H}]$ thymidine labelling was produced mainly by low concentrations of added deoxycytidine (Table 1). Increasing the deoxycytidine concentration above a critical value (between $1.5 \mu\text{M}$ and $6 \mu\text{M}$) did not decrease labelling much more. This indicates that above the critical concentration, either (a) a step in the thymidylate synthetase pathway or in the pathway converting dTMP into dTTP, had become rate-limiting in the formation of dTTP from deoxycytidine, or (b) that thymidine kinase was not responsive to a further increase in the intracellular dTTP concentration. In contrast with the inhibition of labelling with $[^3\text{H}]$ thymidine by deoxycytidine, labelling with deoxy[5- ^3H]cytidine was slightly enhanced by thymidine (Table 1; see also Maley & Maley, 1962).

Fig. 5 also shows that the increase in intercept of isotope-dilution plots at thymidine concentrations above $5 \mu\text{M}$, produced by increasing the serum concentration from 10 to 30% (Fig. 4), could be reproduced by adding deoxycytidine to medium containing 10% serum. This is in accordance with the above proposal that at such thymidine concentrations, thymidine kinase is not rate-limiting and the pool is mainly contributed by deoxycytidine.

Table 1. *Effects of deoxycytidine on labelling with [Me-³H]thymidine and of thymidine on labelling with deoxy[5-³H]cytidine*

Cells (2×10^6 /ml) were incubated for 4 h in medium containing serum (10%), 1 μ Ci of either deoxy[5-³H]cytidine (15.8 Ci/mmol) or [Me-³H]thymidine (15.1 Ci/mmol) and various quantities of either deoxycytidine or thymidine. Each radioactivity value is the mean of triplicate cultures $\pm$ S.E.M.

Unlabelled nucleoside	Concn. of unlabelled nucleoside (μ M)	10 ⁻³ $\times$ Radioactivity (c.p.m.)	
		Deoxy[³ H]cytidine	[³ H]Thymidine
Deoxycytidine	0	5.5 $\pm$ 0.5	70.9 $\pm$ 0.6
	1.5	4.6 $\pm$ 0.4	64.1 $\pm$ 1.3
	6.2	2.2 $\pm$ 0.1	38.8 $\pm$ 8.2
	15.4	1.0 $\pm$ 0.0	36.7 $\pm$ 4.9
	24.7	0.9 $\pm$ 0.2	39.1 $\pm$ 2.1
Thymidine	0	5.5 $\pm$ 0.5	70.9 $\pm$ 0.6
	1.3	—	29.3 $\pm$ 4.6
	3.3	6.7 $\pm$ 0.4	12.6 $\pm$ 1.1
	13.2	7.4 $\pm$ 0.7	5.4 $\pm$ 0.2
	33.0	8.4 $\pm$ 0.6	2.2 $\pm$ 0.2

Amethopterin makes thymidine isotope-dilution plots unimodal

Further evidence for the above interpretations of our data was obtained from studies with agents that directly or indirectly inhibit thymidylate synthetase.

Table 2 shows the effects of various concentrations of amethopterin and 5-fluorodeoxyuridine on cell labelling with [Me-³H]thymidine in the absence of added unlabelled thymidine. Both inhibitors enhanced the labelling. The maximum enhancement was the same with each inhibitor, indicating that they were both affecting the system in the same manner. The enhancement could be due to an increase in the $V_{\max}$ of the rate-limiting step governing incorporation at the low thymidine concentrations used (thymidine kinase), or a decrease in

the corresponding competitor pool. As discussed above, this pool would be formed mainly by serum thymidine and it is difficult to imagine how an inhibitor of thymidylate synthetase could decrease serum thymidine *in vitro* under our culture conditions. A decrease in the contribution of thymidylate synthetase to the dTTP pool (Adams *et al.*, 1971) would not directly influence the degree of labelling when thymidine kinase was rate-limiting, since the isotope-dilution model requires that a pool can only compete before the rate-limiting step. However, the feed-back inhibition of thymidine kinase by dTTP would be decreased, so that the $V_{\max}$ could increase and a subsequent step on the pathway (e.g. DNA polymerase) could then become rate-limiting.

These considerations led to a number of experimental predictions which were supported by the data shown in Fig. 6. In the presence of inhibitors of thymidylate synthetase, (a) the points on [³H]-thymidine isotope-dilution plots should be described by a single straight line indicating only one rate-limiting step at both high and low thymidine concentrations, (b) the slope of the line should be the same as that obtained in the absence of inhibitors of thymidylate synthetase at high thymidine concentrations, indicating that the rate-limiting step was at the level of DNA polymerase, and (c) the plot should extrapolate to demonstrate a pool of a concentration equal to that obtained in the absence of inhibitors of thymidylate synthetase at low thymidine concentrations; this would indicate a contribution mainly from serum thymidine and the absence of a contribution by way of the thymidylate synthetase pathway.

The only exception to these predictions was a loss in linearity of isotope-dilution plots at very low

Table 2. *Effects of various concentrations of amethopterin and 5-fluorodeoxyuridine on the labelling of thymus cells with [³H]thymidine*

Cells (3.4×10^6) were incubated for 4 h in 1 ml of medium containing 15% serum, 1 μ Ci of [³H]thymidine (27.8 Ci/mmol) and various concentrations of each inhibitor. Each radioactivity value is the mean of triplicate cultures $\pm$ S.E.M.

Concn. of inhibitor (M)	10 ⁻³ $\times$ Radioactivity (c.p.m.)	
	Amethopterin	Fluorodeoxyuridine
0	133.2 $\pm$ 0.4	144.4 $\pm$ 4.2
10 ⁻⁸	131.8 $\pm$ 1.9	174.7 $\pm$ 5.4
10 ⁻⁷	180.3 $\pm$ 8.1	190.5 $\pm$ 8.4
10 ⁻⁶	193.1 $\pm$ 1.4	185.9 $\pm$ 9.9
10 ⁻⁵	194.5 $\pm$ 7.9	175.2 $\pm$ 6.8
10 ⁻⁴	175.1 $\pm$ 2.7	82.0 $\pm$ 14.2

thymidine concentrations in the presence of amethopterin or fluorodeoxyuridine (Fig. 6). The points curved down towards the abscissa, indicating that such low thymidine concentrations were unable to maintain the dTTP concentration to an extent sufficient to saturate DNA polymerase. Thus substrate concentration was rate-limiting and DNA polymerase was not at its $V_{\max}$. In keeping with this,

Fig. 6. Effects of concentrations of amethopterin and 5-fluorodeoxyuridine producing an optimum enhancement of labelling with $[^3\text{H}]$ thymidine on isotope-dilution plots

Cultures contained 3.3×10^6 cells, 10% serum, $1 \mu\text{Ci}$ of $[^3\text{H}]$ thymidine (22.7 Ci/mmol), various quantities of unlabelled thymidine and either amethopterin ($20 \mu\text{M}$; Δ), or 5-fluorodeoxyuridine ($0.1 \mu\text{M}$; $\blacksquare$). Control cultures contained neither of these compounds ($\circ$). At low thymidine concentrations the reciprocal of the mean of the duplicate radioactivity determinations is plotted. For other details see the text.

Table 3. Effect of amethopterin on labelling of thymus cells with radioactive deoxycytidine and thymidine

Cells ($2.9 \times 10^6/\text{ml}$) were incubated either with or without amethopterin ($20 \mu\text{M}$) in medium containing 20% serum and $1 \mu\text{Ci}$ of either deoxy $[5\text{-}^3\text{H}]$ cytidine or $[\text{Me-}^3\text{H}]$ thymidine. The values (c.p.m.) of duplicate cultures are shown.

Radioactive precursor	$10^{-3} \times \text{Radioactivity (c.p.m.)}$	
	Control	+ Amethopterin
Deoxy $[5\text{-}^3\text{H}]$ cytidine	13.6	1.3
	13.1	1.2
$[\text{Me-}^3\text{H}]$ Thymidine	96.2	148.6
	98.9	121.4

the labelling of cells with deoxy $[5\text{-}^3\text{H}]$ cytidine was dramatically decreased by amethopterin in the absence of added thymidine (Table 3) and preliminary experiments show that this decrease was due to a decrease in $V_{\max}$. Since deoxycytidine labelled with ^3H in the 5-position would not label DNA thymine (Lomax & Greenberg, 1967), the incorporation of label would not be directly affected by inhibitors of thymidylate synthetase.

The inhibition of labelling with $[\text{Me-}^3\text{H}]$ thymidine by very low concentrations of deoxycytidine (Table 1) was not observed in the presence of amethopterin (Table 4; see also Friedkin & Wood, 1956). This supports the above proposal that the inhibition required the formation of dTTP from deoxycytidine. Table 5, which summarizes the results obtained from isotope-dilution plots, shows that the $V_{\max}$, determined with deoxy $[\text{U-}^{14}\text{C}]$ cytidine was approximately equal to the sum of the $V_{\max}$, determined with deoxy $[5\text{-}^3\text{H}]$ cytidine and the $V_{\max}$, determined with $[\text{Me-}^3\text{H}]$ thymidine either at high thymidine concentrations or in the presence of amethopterin. This is in keeping with deoxy $[\text{U-}^{14}\text{C}]$ cytidine being able to label both cytosine and thymine residues in DNA, whereas deoxy $[5\text{-}^3\text{H}]$ cytidine would label only DNA cytosine and $[\text{Me-}^3\text{H}]$ thymidine would label only DNA thymine.

Enhancement by amethopterin of labelling with $[^3\text{H}]$ thymidine depends on the serum concentration

Fig. 7 shows the effect of varying the serum concentration on the radioactive labelling of cells with $[\text{Me-}^3\text{H}]$ thymidine. In the absence of amethopterin maximum labelling was obtained at approx. 5% serum and higher serum concentrations inhibited labelling. Serum at 5% concentration was also optimum for labelling with deoxy $[5\text{-}^3\text{H}]$ cytidine. The stimulation of labelling with $[^3\text{H}]$ thymidine by amethopterin (Table 2) only occurred at serum concentrations above 5%. In the presence of amethopterin maximum labelling was reached at

Table 4. Effect of amethopterin on the inhibition by deoxycytidine of labelling with $[\text{Me-}^3\text{H}]$ thymidine

Cells ($3.5 \times 10^6/\text{ml}$) were incubated for 4 h in the presence or absence of amethopterin ($20 \mu\text{M}$) in medium containing serum (10%), $1 \mu\text{Ci}$ of $[\text{Me-}^3\text{H}]$ thymidine (20.4 Ci/mmol), and various quantities of deoxycytidine. Each value (c.p.m.) is the mean of triplicate cultures ($\pm \text{S.E.M.}$)

Concn. of deoxycytidine (μM)	$10^{-3} \times \text{Radioactivity (c.p.m.)}$	
	Control	+ Amethopterin
0	84.4 ± 1.0	125.1 ± 1.6
2.6	66.1 ± 2.1	124.5 ± 2.7
5.2	64.2 ± 1.7	127.7 ± 4.5

Table 5. Summary of maximum rates of incorporation and pool sizes obtained from isotope-dilution plots

Values in parentheses refer to the number of experiments used for the calculations of the mean and standard error.

Radioactive precursor	Concn. of added nucleoside (μM)	Concn. of amethopterin (μM)	$V_{\max}$. (pmol/h per 10 ⁶ cell)	Pool concn. (μM)*
[Me- ³ H]Thymidine	0.04–5.0	0	6.4 ± 0.5 (15)	0.86 ± 0.06 (15)
	5.00–30.0	0	11.5 ± 0.6 (7)	5.31 ± 0.59 (7)
	0.04–30.0	20	13.5 ± 1.0 (9)	0.89 ± 0.08 (9)
Deoxy[5- ³ H]cytidine	0.06–30.0	0	8.1 ± 0.5 (5)	3.58 ± 0.44 (5)
Deoxy[U- ¹⁴ C]cytidine	0.30–30.0	0	25.2 ± 2.1 (3)	3.70 ± 0.85 (3)

* Total pool in the system containing serum (10%) and medium 199 (90%).

Fig. 7. Effect of serum concentration on the labelling of thymus cells with [³H]thymidine in the presence (Δ) or absence (○) of amethopterin

Cultures contained 3.5×10^6 cells, 1 μCi of [³H]thymidine (20.4 Ci/mmol), amethopterin (20 μM) and various volumes of serum. Each point is the mean of triplicate cultures (± S.E.M.; where no S.E.M. is shown the limits fell within or close to the area covered by the point).

serum concentrations around 20%; this was followed by a variable degree of inhibition at higher serum concentrations.

The amethopterin-insensitive stimulation of labelling by low serum concentrations required mainly non-diffusible serum molecules and also some diffusible molecules (Sjostrom, 1972; see also Sibatini, 1973). Thus the stimulation could be only partly due to an increased supply of nucleic acid precursors, which would expand the size of the intracellular deoxynucleotide pool (Skoog & Nordenskjöld, 1971). The amethopterin-sensitive inhibition of labelling by serum concentrations above 5% was due to diffusible serum molecules (Sjostrom, 1972). Plots of the reciprocal of the radioactivity against serum concentration (Forsdyke, 1971) indicated that the inhibition was mainly competitive, thus reflecting more the diluting effect of thymidine in serum than the indirect

depression of the $V_{\max}$ of thymidine kinase by serum deoxycytidine (Figs. 4 and 5). The shift by amethopterin of the serum concentration supporting maximum labelling (Fig. 7) would indicate that the rate-limiting step in the absence of amethopterin (thymidine kinase) is saturated at lower serum concentrations than the rate-limiting step in the presence of amethopterin (DNA polymerase).

Rate of DNA synthesis

The only known pathways for the formation of dTTP are the thymidine kinase pathway and the pathways leading to thymidylate synthetase. If the rate-limiting step at high thymidine concentrations and in the presence of amethopterin is at the level of DNA polymerase, as suggested above, then it would follow that the rate of thymidine incorporation under these conditions, determined from the slopes of isotope-dilution plots (Table 5), should accurately reflect the rate of DNA synthesis.

The rate of incorporation of deoxycytidine determined with deoxy[5-³H]cytidine, relative to the rate of thymidine incorporation at high thymidine concentrations (Table 5), was of an order expected from the base composition of rat DNA (C = 21.5%; T = 28.5%; Wyatt, 1951), suggesting that DNA polymerase might be rate-limiting for deoxycytidine incorporation into DNA cytosine. Reports of high intracellular dCTP pools (Adams *et al.*, 1971; Skoog & Nordenskjöld, 1971) would support this view, as would our inference above that, under conditions in which thymidine kinase was not rate-limiting, the pools of thymidine and deoxycytidine in the medium were in equilibrium. It would follow in our system of intact cells that deoxycytidine kinase was not rate-limiting. However, dCTP can feedback-inhibit purified thymus deoxycytidine kinase (Kozai *et al.*, 1972) so that the enzyme has the potential to become rate-limiting under some conditions.

Theoretical values for the $V_{\max}$ of incorporation of thymidine and deoxycytidine can be calculated from a knowledge of (a) the base composition of rat DNA, (b) the percentage of cultured cells in S

phase (10%; Sjostrom, 1972), (c) the average duration of S phase (7h; Bryant, 1972) and (d) the DNA content of a diploid cell (Vendredy & Vendredy, 1949) which is equal to approx. 5.5×10^9 nucleotide pairs. The theoretical rate for thymidine is 74 pm/h per 10^6 cells, whereas the rate for deoxycytidine is 56 pm/h per 10^6 cells. These values are approximately 6-fold greater than the experimental values (Table 5). The magnitude of this discrepancy is not easily explained by errors in our assumptions and indicates either that the rate of DNA synthesis by thymus cells is decreased under our culture conditions or that *de novo* pathways make contributions not detected by our techniques. Current studies with hydroxyurea may shed further light on this problem (Scott & Forsdyke, 1973).

We thank Mrs. Z. Vasa and Mrs. D. Lis for technical help at various stages of this work, which was supported by grants from the Medical Research Council of Canada and the National Cancer Institute of Canada.

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