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The Rate of Deoxyribonucleic Acid Synthesis by Cultured Chinese-Hamster Ovary Cells

AN APPLICATION OF ISOTOPE-DILUTION ANALYSIS

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The rate of DNA synthesis in exponentially growing cells was determined by isotope-dilution analysis of the incorporation of [*Me*-^{3}H]thymidine. Thymidine concentrations greater than 7 μM were used so that the rate-limiting step governing incorporation would be at the level of DNA polymerase rather than at the level of thymidine kinase [Sjostrom & Forsdyke (1974) *Biochem. J.* **138**, 253–262]. In early exponential phase the rate determined by isotope-dilution analysis closely correlated with the rates calculated either from growth curves or from known cell-cycle parameters. However, in late-exponential phase the rate calculated from the growth curve was less than that determined by isotope-dilution analysis. We conclude that, under certain conditions, the pool-corrected rate of incorporation of [*Me*-^{3}H]thymidine, as determined by isotope-dilution analysis, can accurately reflect the rate of DNA synthesis. Discrepancies between the observed rate of DNA synthesis and increase in cell number could reflect an exponential degeneration of post-S-phase cells.

There is increasing evidence that a significant degree of organization exists among apparently soluble enzyme systems within intact cells (Mowbray & Moses, 1976). Thus information obtained under conditions used in classical enzymology may not necessarily be of physiological significance (Masters, 1977). For DNA synthesis there are indications that enzymes catalysing formation of the deoxyribonucleoside triphosphate precursors of DNA may be physically associated with the enzymes involved in polymerization of the precursors to form macromolecular DNA (Baril *et al.*, 1974; Chiu *et al.*, 1976; Reddy *et al.*, 1977). Thus it is necessary to examine the process of DNA synthesis within the context of the intact cell (Reichard, 1972).

Several experimental approaches have been used. Studies of the kinetics of incorporation of radioactive DNA precursors by cultured cells have been informative (Cleaver, 1967; Wittes & Kidwell, 1973). However, analysis of the data in terms of mathematical models was not easy. Pools of deoxyribonucleotides which can be extracted from cells under different conditions have been measured (Walters & Ratliff, 1975; Meuth *et al.*, 1976). However, the extent to which such pools are functional can be questioned, since there is evidence for compartmentation of intracellular pools (Kuebbing & Werner, 1975). In non-synchronized populations, pools would also be extracted from cells that are not in S-phase. An approach used in our laboratory has been isotope-dilution analysis (Forsdyke, 1971; Sjostrom & Forsdyke, 1974; Scott & Forsdyke, 1976*a*). This approach is relatively simple and provides values for (*a*) the maximum velocity of the rate-limiting step affecting incorporation of a radioactive precursor, and (*b*) the pool of compounds functionally competing with the precursor for incorporation at that rate-limiting step.

Previous studies with freshly prepared rat thymus-cell suspensions demonstrated that the value for the maximum velocity of DNA synthesis as determined by isotope-dilution analysis was only one-sixth of the value calculated from known cell-cycle parameters (Sjostrom & Forsdyke, 1974). The poor viability of the cells did not permit long-term culture, and hence a correlation of the rate of DNA synthesis with increase in cell number was not possible. In the present paper we report similar studies of DNA synthesis in exponentially growing Chinese-hamster ovary cells. The problem of the degree to which the incorporation of [*Me*-^{3}H]thymidine can reflect the rate of DNA synthesis was examined by comparing maximum velocities calculated from (i) isotope-dilution analysis, (ii) growth curves and (iii) known cell-cycle parameters. A preliminary report of this work has been given (Scott & Forsdyke, 1976*b*). A fuller account is available (Scott, 1976).

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Materials and Methods

Materials

[*Me*-^{3}H]Thymidine (17Ci/mmol) was obtained from Amersham Corp., Arlington Heights, IL, U.S.A. Thymidine was obtained from Sigma Chemical Co., St Louis, MO, U.S.A. The α-minimum essential Eagle's medium (Stanners *et al.*, 1971) with Earle's salts and glutamine (2mM), but lacking deoxyribonucleosides and ribonucleosides, was obtained from Flow Laboratories, Rockville, MD, U.S.A. Penicillin-G (sodium salt) and streptomycin sulphate were obtained from Glaxo–Allenburys, Toronto, Ontario, Canada, and used in final concentrations of 400 international units and 0.1 mg respectively per ml of culture medium. Foetal calf serum was obtained from Grand Island Biological Co., Grand Island, NY, U.S.A.

Cell culture

The Chinese-hamster ovary cells were a gift from Dr. R. Mankovitz of the Department of Pathology, Queen's University. He obtained the cells from Dr. L. Thompson of the Ontario Cancer Institute, Toronto (see Thompson & Baker, 1973). The cells were maintained at 37°C in 250ml spinner flasks (Bellco Glass Co., Vineland, NJ, U.S.A.) and were found to be free of mycoplasma contamination. The culture medium contained 90% Eagle's α-minimum essential medium and 10% foetal calf serum. Every 3–4 days the cells were subcultured by centrifuging (400*g*, 3min) a volume of cell suspension containing 10^6 cells. The pelleted cells were then resuspended in 100ml of fresh medium that had been prewarmed to 37°C and equilibrated with air/CO_2 (19:1). The gas phase in the flasks was equilibrated with air/CO_2 before sealing. At appropriate times cells were counted in an improved double Neubauer counting chamber.

There was a lag phase of 19.0h (S.E.M. ±2.4) (five expts.) before exponential growth was resumed. Cells then proliferated with a doubling time of 15.0±1.6h (five expts.), in agreement with the results of Thompson & Baker (1973). Exponential growth ceased after 85–100h. Incorporation of [5-^{3}H]uridine during the early hours of culture was improved if the foetal calf serum was preheated at 56°C for 30min. This suggested that the lag phase was partly due to a complement-dependent inactivation of a proportion of the cultured cells.

Radioactive labelling

To initiate radioactive labelling, 1ml volumes were taken from the master cultures and pipetted into 12ml round-bottomed glass centrifuge tubes containing [*Me*-^{3}H]thymidine (1μCi) and various quantities of unlabelled thymidine. The tubes were placed in an air/CO_2 incubator (19:1) at 37°C for 1h. Cells were then pelleted by centrifugation (1200*g*, 4min), washed once in 2ml of 0.14M-NaCl (4°C) and stored in ethanol (95%) at −20°C for 1–7 days. Radioactivity in cold-acid-precipitable material was determined with Hyamine as described by Forsdyke (1971).

Isotope-dilution model

The theoretical basis for the isotope-dilution model used in this work has been discussed in detail elsewhere (Forsdyke, 1971; Sjostrom & Forsdyke, 1974; Scott & Forsdyke, 1976*a*). Briefly, a constant amount of radioactive precursor (i.e. [*Me*-^{3}H]-thymidine) is added to cultured cells together with various quantities of the corresponding unlabelled precursor. The reciprocal of the radioactivity incorporated (c.p.m.) 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 precursor itself is rate-limiting. The slope of the plot provides a measure of the maximum velocity 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 maximum velocity represents a kinetic parameter of the enzyme when in its natural environment in the intact cell.

The maximum velocity of precursor incorporation by a given number of cells is calculated from the expression $Z/(D{\cdot}E{\cdot}H{\cdot}C{\cdot}T)$, where Z is the slope of the isotope-dilution plot, D is the number of radioactive disintegrations corresponding to 1μCi (2.2×10^6), E is the counting efficiency (approx. 0.34 for ^{3}H), H is the quantity of radioactivity added (μCi), C is the cell number and T is the time of incubation (h).

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 should 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 position of rate-limiting steps.

Results and Discussion

Maximum velocity of thymidine incorporation into DNA determined by isotope-dilution analysis

Isotope-dilution plots of radioactive labelling with [*Me*-^{3}H]thymidine at various stages of culture were

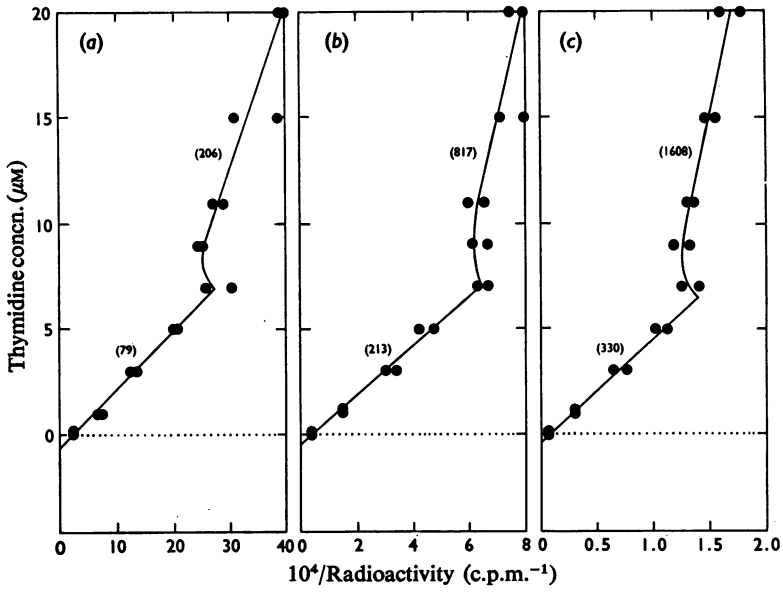

Fig. 1. *Isotope-dilution plots with [Me-³H]thymidine at (a) 24, (b) 49 and (c) 78 h of culture*
Samples (1 ml) containing (*a*) 4.8×10^4, (*b*) 7.8×10^4 and (*c*) 2.2×10^5 Chinese-hamster ovary cells were taken from a master culture and incubated for 1 h with [*Me*-³H]thymidine (1 μCi; 17 Ci/mmol) which had been diluted with increasing quantities of unlabelled thymidine. The radioactivity incorporated into cold-acid-precipitable material was measured. Each value plotted is the reciprocal of the radioactivity (c.p.m.) incorporated into a single 1 ml culture. Numbers in parentheses indicate maximum velocities of thymidine incorporation (pmol/h per 10^6 cells) determined from the slopes of the plots.

bimodal (Fig. 1). The linear plots showed an inflection at thymidine concentrations of approx. 7 μM. Thus at this critical thymidine concentration there was an increase both in the maximum velocity of incorporation (slope) and in the competitor pool (negative intercept at the ordinate; not shown).

A similar result was reported for cultured rat thymus cells (Sjostrom & Forsdyke, 1974). The plots were interpreted as showing that thymidine kinase was rate-limiting for incorporation of [*Me*-³H]thymidine into DNA at thymidine concentrations below 7 μM. That thymidine kinase is rate-limiting in mammalian cells under most experimental conditions is now generally recognized (Schuster & Hare, 1971; Wohlhueter *et al.*, 1977). Our studies with various inhibitors suggested that concentrations of thymidine above 7 μM were capable of preventing feedback inhibition of thymidine kinase by dTTP (Sjostrom & Forsdyke, 1974; Scott & Forsdyke, 1976*a*). This had been demonstrated with partially purified thymidine kinase (Ives *et al.*, 1963). The lack of feedback inhibition would permit the maximum velocity of the enzyme to increase so that a subsequent step on the incorporation pathway could become rate limiting. A pool entering the pathway between the two rate-limiting steps would then become detectable, and isotope-dilution plots would show a concomitant change in both maximum velocity and pool. A change in maximum velocity at a critical substrate concentration has also been inferred from bimodal Lineweaver–Burk plots obtained with purified bacterial and mammalian thymidine kinases (Okazaki & Kornberg, 1964; Kizer & Holman, 1974; Nawata & Kamiya, 1975). Since the pool increase at thymidine concentrations above 7 μM was prevented by inhibitors of thymidylate synthetase it was inferred that the new contribution to the pool was at the level of dTMP (Sjostrom & Forsdyke, 1974).

These considerations suggested that measurement of the maximum velocity of thymidine incorporation at low (below 7 μM) thymidine concentrations would not provide a measure of the maximum velocity of the incorporation of thymine into DNA. Thymidylate formed by the thymidylate synthetase pathway would also contribute to DNA thymine. However, this contribution to the thymidylate pool would compete with exogenous [*Me*-³H]thymidine if the rate-limiting step was shifted from thymidine kinase to a point higher up the incorporation pathway, such as at the level of DNA polymerase. If so, the maximum velocity of thymidine incorporation at high (above 7 μM) thymidine concentrations should provide a

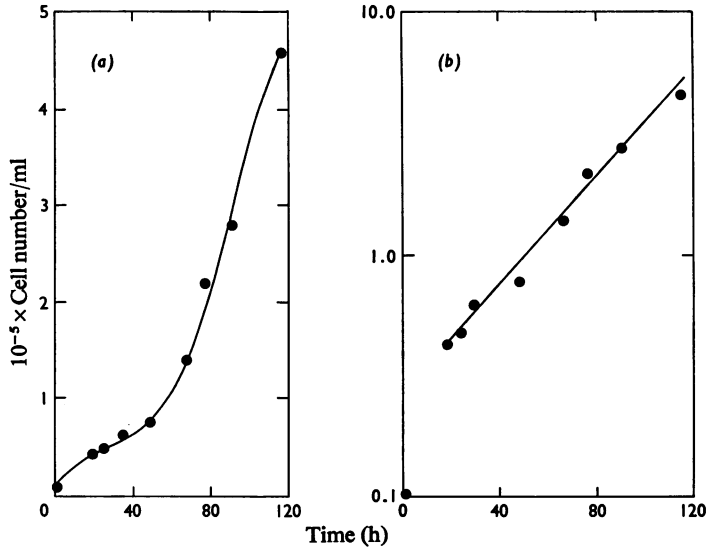

Fig. 2. *Growth curves for Chinese-hamster ovary cells*
In (*b*) the data in (*a*) are plotted with cell number on a logarithmic scale.

measure of the maximum velocity of thymine incorporation into DNA.

In the early exponential phase of culture the maximum velocity of thymidine incorporation calculated from the upper limb of isotope-dilution plots (Fig. 1*a*) was 208±17pmol/h per 10^6 cells (three expts.). At later time points (Figs. 1*b*, 1*c*) much higher values were obtained.

Maximum velocity of thymidine incorporation into DNA determined from growth curves

Fig. 2(*a*) shows a typical growth curve for cultured Chinese-hamster ovary cells. Fig. 2(*b*) shows a semi-logarithmic plot of the same data with cell concentration plotted on a logarithmic scale. After the first day of culture the experimental points could be fitted to a straight line, indicating exponential growth. This could be described by the equation:

$$\log y = a + mt \quad (1)$$

where y is cell number at time t and a and m are constants. A value for the latter constant was calculated as the slope of Fig. 2(*b*) by the method of least squares.

From eqn. (1):

$$y = 10^a \cdot 10^{mt} \quad (2)$$

and

$$y = A \cdot 10^{mt} \quad (3)$$

where A is a new constant. The rate of increase in cell number is given by:

$$dy/dt = m \cdot y \cdot \ln 10 \quad (4)$$

Thus the rate per 10^6 cells $= m \cdot 10^6 \cdot \ln 10$ (5)

Having calculated the rate of increase in cell number from growth curves, the rate of incorporation of thymidine into DNA was calculated from the assumptions that (i) 28.6% of the bases in Chinese-hamster ovary-cell DNA are thymine (Walters & Ratliff, 1975), (ii) the DNA of a diploid mammalian cell contains 5.5×10^9 nucleotide pairs (Vendredy & Vendredy, 1949) and (iii) Avagadro's number is 6.02×10^{23}. Thus the maximum velocity of thymidine incorporation (mol/h per 10^6 cells) was

$$(m \cdot 10^6 \cdot \ln 10)\,(11 \times 10^9 \times 0.286)/(6.02 \times 10^{23}) \quad (6)$$

Calculated in this way the maximum velocity was found to be 222±41 pmol of thymidine/h per 10^6 cells (five expts.). This value would apply throughout the period corresponding to the linear part of the logarithmic growth curve (Fig. 2*b*).

Theoretical maximum velocity of thymidine incorporation into DNA determined from known cell-cycle parameters

If the duration of S-phase is 7.4h (Bostock *et al.*, 1971), DNA would duplicate at a rate of 1.49×10^{15} nucleotides/h per 10^6 cells. If 28.6% of the bases are

thymine and 41% of the cells are in S-phase (Walters & Ratliff, 1975) this duplication rate would require 287 pmol of thymidine/h per 10^6 cells. The calculation shares some of the assumptions made when determining the maximum velocity from growth curves.

General discussion

These results suggest that (i) the upper limb of thymidine isotope-dilution plots can represent a rate-limiting step at the level of DNA polymerase and (ii) the maximum velocity calculated from this line can provide a valid measure of the rate of DNA synthesis, at least in early exponential phase (Fig. 1*a*). The high values obtained in mid- and late-exponential phases (Figs. 1*b*, 1*c*) could reflect an exponential decay of a proportion of the post-S-phase cells, so that the rate of DNA synthesis would be greater than expected from the observed increase in cell number. This would indicate that the constant m in eqn. (3) has both positive and negative components (Batchelet, 1976). In rat thymus cells, the rate of DNA synthesis determined by isotope-dilution analysis was much less than the theoretical maximum velocity (Sjostrom & Forsdyke, 1974). In the light of the present work it seems likely that this was due to a decreased rate of DNA synthesis under the culture conditions used.

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