

DETERMINATION OF RATES OF DNA SYNTHESIS IN CULTURED MAMMALIAN CELL POPULATIONS

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ABSTRACT

In cultures of a murine mastocytoma, endogenous synthesis of thymidine phosphates, as determined by the incorporation of [³H]deoxyuridine into DNA, was reduced within 15 min to less than 3% of control values by the addition of amethopterin (10 μM) in combination with hypoxanthine and glycine. If [³H]thymidine and unlabeled thymidine were added simultaneously with amethopterin, the increase with time of radioactivity in cellular DNA was linear at least between 30 and 90 min, while radioactivity in the acid-soluble nucleotide fraction remained constant during this time interval, indicating that intracellular thymidine nucleotides had the same specific activity as exogenously supplied [³H]thymidine. This permitted calculation of the amount of thymidine incorporated per hour into DNA of 10⁶ cells. In conjunction with the base composition of mouse DNA, these results were used to calculate rates of DNA synthesis. Cell proliferation rate, cell cycle time, and the duration of the S period were not affected to any appreciable extent by the addition of amethopterin and thymidine. Rates of DNA synthesis, as derived from thymidine incorporation rates, were in good agreement with those derived from the measured mean DNA content of exponentially multiplying cells and rates of cell proliferation.

INTRODUCTION

Since its introduction in 1957 (27, 29), labeled thymidine has been used extensively as a specific precursor of DNA in autoradiographic as well as in biochemical studies. Exogenously supplied thymidine, after its uptake by a cell, is converted by thymidine kinase to thymidine monophosphate. This intermediate is also synthesized from endogenous deoxyuridine monophosphate which usually is produced by synthetic reactions from simple precursors such as aspartic acid. Upon conversion to thymidine monophosphate, exogenous labeled thymidine is, therefore, diluted by endogenously

synthesized unlabeled thymidine monophosphate before being further phosphorylated and incorporated into DNA. Since the relative contribution of the two metabolic pathways converging at thymidine monophosphate usually is not known, it is not possible to calculate rates of DNA synthesis from the time-course of incorporation of labeled thymidine. In fact, discrepancies between incorporation of labeled thymidine into DNA and the rate of DNA synthesis have been observed in various cell culture systems (1, 25).

In the present communication, a method is

described which permits determination of average rates of DNA synthesis in cultured cell populations at intervals of 30–60 min. This method is based on the inhibition of endogenous synthesis of thymidine monophosphate by amethopterin, with provision of exogenous thymidine at a concentration sufficient for continued DNA synthesis. As previously reported, in mammalian cell cultures the inhibition of cell reproduction by the folic acid antagonist, amethopterin, is prevented by the addition of glycine, a purine, and thymidine to the culture medium (11, 21). If the exogenously supplied thymidine is labeled, its incorporation may be determined as a function of incubation time. It will be shown that under appropriate conditions, after addition of amethopterin, of unlabeled and of labeled thymidine to cultures supplemented with hypoxanthine and glycine, (a) synthesis of thymidine phosphate from deoxyuridine phosphate is reduced to a negligible level, (b) rates of DNA synthesis and of cell proliferation are nearly identical with those in the absence of amethopterin and exogenous thymidine, (c) radioactivity in DNA increases in a linear fashion at least between 30 and 90 min, (d) radioactivity of thymidine nucleotide pools remains essentially constant during this time interval, and (e) DNA synthesis rates, as calculated from thymidine incorporation rates and the base composition of DNA, closely correspond to those calculated from the chemically determined mean cellular DNA content and rates of cell proliferation.

MATERIALS AND METHODS

Cell Culture Techniques

Suspension cultures in vitro of a transplantable murine mastocytoma (cell line P-815-X2) (22) were used. The medium has been described as medium I (19) and contained 10% undialyzed horse serum. This medium was further supplemented with hypoxanthine (30 μ M) and glycine (100 μ M). In order to maintain highly reproducible culture conditions, the same lot of serum was used in all experiments reported in the present communication. The cultures were grown from frozen cells stored at -80°C which had been tested and found to be free of mycoplasma contamination.

The cells used for the experiments were preincubated under steady-state culture conditions (22) at a density of approximately 2×10^5 cells/ml. After an adaptation period of 3–5 days after the onset of steady-state incubation, the cell number doubling time remained at a constant level which always was between 8.4 and 9.4 h. Cell proliferation rates and cell number doubling times were determined as follows: at regular time intervals,

samples were withdrawn from the steady-state culture and used to determine the cell density (cells per milliliter) with a Coulter counter (Coulter Electronics, Inc., Fine Particle group, Hialeah, Fla.). Measured cell densities were multiplied by the cumulative dilution ratio of the steady-state culture for the time at which the respective sample was taken. Results thus obtained represented cell densities that would have been attained if the cells had multiplied at the same rate without any dilution of the culture with fresh medium (20).

Determination of Incorporation of Labeled Precursors into DNA

In order to measure the incorporation of labeled thymidine, samples were withdrawn from a steady-state culture and incubated at 37°C for different periods of time in the presence of [methyl- ^3H]thymidine (5.0 Ci/mmol, The Radiochemical Centre, Amersham), unlabeled thymidine, and amethopterin. Similarly, samples withdrawn before and at various time intervals after the addition of amethopterin to steady-state cultures were incubated for 15 min at 37°C in the presence of [6- ^3H]deoxyuridine (28.3 Ci/mmol, The Radiochemical Centre).

After incubation with the labeled precursor, the cell suspensions were immediately cooled in an ice bath and mixed with an equal volume of 10% cold trichloroacetic acid (TCA) and 0.06 g of homogenized rat liver suspended in 5% cold TCA, to serve as a carrier for the minute amounts of cellular material. Subsequently, the TCA precipitate was fractionated according to the method of Schmidt and Thannhauser (23) as modified by Schneider (24), and the percentage of radioactivity incorporated into DNA was determined as reported previously (18). After incubation of cells with [^3H]thymidine, radioactivities in the lipid, RNA, and protein fractions amounted to less than 2.5% of the label in the DNA hydrolysate, indicating that recovery of labeled DNA was essentially complete.

The radiochemical purity of tritiated thymidine was assessed by paper chromatography in two solvent systems (double-distilled water and *n*-butanol/acetic acid/water, 40:6:15, respectively), and compared with that of [^{14}C]thymidine (62 mCi/mmol, The Radiochemical Centre). Both [^3H]thymidine and [^{14}C]thymidine were mixed with unlabeled thymidine and thymine before being placed on the chromatography paper. After chromatography, the positions of thymidine and thymine were identified by their ultraviolet light absorption, and the chromatograms were cut into 1.0-cm strips which were subjected to liquid scintillation counting. The results indicate that the radiochemical purity of the preparation of tritiated thymidine used was above 96%.

Cell Cycle Analysis

Cells from steady-state cultures were pulse labeled by incubation for 20 min with 0.2 $\mu\text{Ci/ml}$ of [^3H]thymidine

as described previously (7) and reincubated under steady-state culture conditions in the presence of unlabeled thymidine (3 μ M). Subsequently, samples were withdrawn at intervals of 1 h, and cells were subjected to autoradiography (7) in order to determine variations with time of the mitotic labeling index.

Relative numbers of DNA-synthesizing cells were determined in samples withdrawn from steady-state cultures by incubation for 20 min at 37°C in the presence of 0.2 μ Ci/ml of [3 H]thymidine or with 3 μ M of unlabeled thymidine and 0.5 μ Ci/ml of [3 H]thymidine, respectively. Subsequently, the cells were processed as described above.

Determination of Mean Cellular DNA Content

Aliquots of 50 ml were withdrawn from steady-state cultures and centrifuged for 5 min at 490 g. The cell pellet was resuspended in 3.0 ml of culture medium, and 0.5 ml of this suspension was added to 4.5 ml of culture medium under gentle Vortex mixing. No cell clumps were seen in aliquots of this preparation when controlled in the microscope. After further dilution with saline, the cell density of this diluted cell suspension was determined with the Coulter counter.

Of the original 3.0 ml of cell suspension, an aliquot of 2.0 ml was cooled to 0°C and mixed with an equal volume of 10% cold TCA. Subsequent procedures for extraction of DNA were as described above, except that no carrier material was added and DNA was hydrolyzed with 10% perchloric acid (PCA) for 30 min at 70°C. The DNA hydrolysate was used for colorimetric determination of hydrolyzed DNA by the diphenylamine method of Burton (5) modified by Giles and Myers (9).

Standard curves for DNA determination were obtained using calf thymus DNA (Fluka AG, Buchs, Switzerland) or herring DNA (Fluka AG) which were subjected to the extraction procedure in parallel with the cellular material. Aliquots of the hot PCA extract containing the hydrolyzed DNA were used for DNA determination (a) by the diphenylamine method and (b) by phosphorus determination. For phosphorus determination, samples were subjected to wet ashing by mixing with 2.5 ml of 10 N sulfuric acid and boiling to a final volume of approximately 1.2 ml (6). This solution was brought to 10.0 ml with distilled water, and the phosphorus content was determined by the colorimetric method of Gomori (10), using a solution of KH_2PO_4 as standard. H_2SO_4 was omitted from the molybdate solution for the color reaction with the DNA hydrolysate to compensate for the sulfuric acid content of the latter.

The phosphorus content was used to calculate the concentration of hydrolyzed calf thymus or herring DNA in the hot PCA extract. This extract in turn served as standard in the colorimetric determination of cellular DNA content by the diphenylamine method.

Determination of Radioactivity in Acid-Soluble Nucleotide Pools

A culture under steady-state conditions was supplied with amethopterin (10 μ M), thymidine (3 μ M), and [3 H]thymidine (1 μ Ci/ml), and at the same time the inflow of fresh medium to the culture was interrupted. At various times thereafter, samples were withdrawn from the cell suspension, cooled to 0°C, centrifuged, and the cells were washed four times with medium. Subsequently, the cells were extracted with cold 0.5 M PCA, and the radioactivity in this extract was determined by liquid scintillation counting.

RESULTS

Effect of Amethopterin on the Incorporation of [3 H]Deoxyuridine into DNA

From a steady-state culture, a sample was withdrawn and incubated for 15 min with 10 μ Ci/ml of [3 H]deoxyuridine. Subsequently, the cell suspension in the steady-state culture was used to prepare two parallel cultures, and a sample of each of these cultures was again incubated for 15 min with [3 H]deoxyuridine. At the end of this 15 min period, the two cultures were supplemented with amethopterin at a concentration of 0.1 or 10 μ M, respectively, and every 15 min samples were withdrawn from both cultures and incubated with [3 H]deoxyuridine during the subsequent 15-min interval. Immediately after incubation with labeled deoxyuridine, samples were processed for determination of radioactivity incorporated into DNA as described under Materials and Methods.

In a first series of experiments, the effect of amethopterin on deoxyuridine incorporation was studied without addition of thymidine. The results of a typical experiment are illustrated in Fig. 1. It is seen that incorporation of deoxyuridine under steady-state conditions (–30 to –15 min on the time scale) was not significantly different from that after dividing the steady-state culture (–15 to 0 min). After addition of amethopterin at a concentration of 0.1 μ M, a relatively slow decrease of [3 H]deoxyuridine incorporation was observed, and 45 min elapsed before a value of less than 20% of the control was attained. In contrast, amethopterin at a concentration of 10 μ M caused a rapid and more pronounced reduction of [3 H]deoxyuridine incorporation into DNA, and after 15 min, incorporation was less than 3% of the control.

In a second series of experiments, thymidine (3 μ M) was added simultaneously with amethopterin

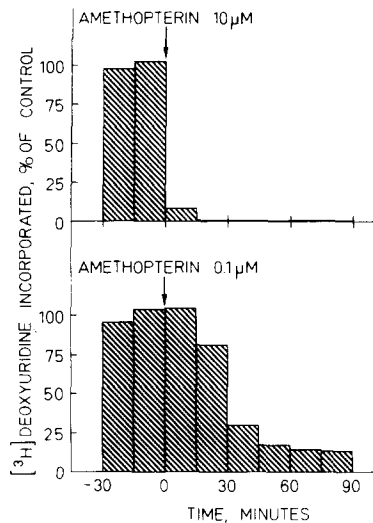


FIGURE 1 Incorporation of [³H]deoxyuridine during 15-min intervals as a function of time after addition of amethopterin at two different concentrations.

(10 μM). Under these conditions, incorporation of [³H]deoxyuridine into DNA was reduced as rapidly and to the same extent as without addition of thymidine.

These results indicate that after addition of amethopterin at a concentration of 10 μM, conversion of deoxyuridine monophosphate to thymidine monophosphate, i.e., endogenous synthesis of thymidine nucleotides, is blocked within 15 min with more than 97% efficiency.

Rates of Cell Proliferation and Labeling Indices in the Presence and Absence of Amethopterin and Thymidine

Cells were cultured under steady-state conditions until a constant cell number doubling time was maintained for at least 51 h. Subsequently, amethopterin (10 μM) and thymidine (3 μM) were added, and rates of cell proliferation were again determined by repeated measurements of cell density during the following 51 h. The results of a typical experiment are presented in Fig. 2. It is seen that after addition of amethopterin and thymidine, the cell proliferation rate was nearly identical with that under control conditions. Regression lines were calculated from each of the 10 experimentally determined points before and after addition of amethopterin and thymidine. Results on cell multiplication in the presence and absence of amethopterin and thymidine, as calculated from

pairs of regression lines of three independent experiments, are presented in Table I. They indicate that rates of cell multiplication in different experiments were not quite as reproducible as within individual experiments. On the other hand, after addition of amethopterin and thymidine a small, but significant decrease in cell proliferation rate was regularly observed.

In view of the possibility of changes in cell cycle characteristics as a result of the addition of amethopterin and thymidine, aliquots of cell suspension were withdrawn from steady-state cultures at regular time intervals, incubated for 20 min with [³H]thymidine, and subsequently the cells were processed for autoradiography. The average labeling indices of the cell population, as derived from autoradiographic analysis of samples withdrawn at five different times during 51 h before and 51 h after addition of amethopterin and thymidine, are listed in Table I. It is seen that the labeling index was not affected to a significant extent by blocking endogenous synthesis of thymidine nucleotides.

Cell Cycle Time and Duration of the S Period in the Presence and Absence of Amethopterin

Cells from a steady-state culture were pulse labeled with [³H]thymidine as described under Materials and Methods. After washing, the cell suspension was divided to obtain two parallel steady-state cultures. One of these was supplemented with amethopterin (10 μM) and thymidine (3 μM), while the other steady-state culture contained thymidine, but no amethopterin. Samples were withdrawn from both cultures at intervals of

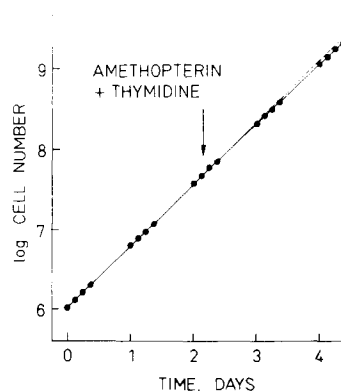


FIGURE 2 Rate of cell proliferation before and after addition of amethopterin (10 μM) and thymidine (3 μM).

TABLE I
Cell Multiplication and Labeling Indices in Steady-State Cultures in the Presence and Absence of Amethopterin (10 μ M) and Thymidine (3 μ M)

Experiment no.	Cell multiplication during 10 h*			Average labeling index (%)§	
	Control	Amethopterin + thymidine	Difference	Control	Amethopterin + thymidine
1	2.104	2.030	0.074	66.7 \pm 2.2	68.7 \pm 1.1
2	2.089	2.042	0.047		
3	2.256	2.133	0.123	67.4 \pm 1.9	67.9 \pm 0.3
Mean with standard error	2.15 \pm 0.053	2.07 \pm 0.033	0.08 \pm 0.022		

Data of exp. 1 are presented in Fig. 2.

* Relative to cell number at the beginning of respective 10-h interval; data are derived from regression lines as illustrated in Fig. 2.

§ With standard error.

1 h for autoradiographic determination of relative numbers of labeled mitotic cells.

The results of a typical experiment are illustrated in Fig. 3. It is seen that variations with time of the mitotic labeling index were not significantly different in the two cultures. The cell cycle time and the duration of the S period were derived from the curves by determining respective time intervals between points at which the curves cross the 50% level as originally proposed by Quastler and Sherman (17). The results obtained by this procedure in two independent experiments are summarized in Table II. They indicate that cell cycle parameters as obtained in different experiments exhibit a certain degree of variability. On the other hand, no consistent effect of amethopterin on cell cycle time is detectable, whereas the duration of the S period appears to be prolonged by 0.2–0.3 h in both experiments.

Radioactivities in Acid-Soluble Nucleotide Pools in the Presence of Amethopterin and Thymidine

A steady-state culture was supplemented with amethopterin (10 μ M), thymidine (3 μ M), and [3 H]thymidine, and after 30, 60, and 90 min, samples were withdrawn for determination of radioactivity in the acid-soluble fraction. From the specific activity of [3 H]thymidine in the culture medium, the number of picomoles of thymidine nucleotides formed from exogenous thymidine per 10^6 cells was calculated. The results obtained in three independent experiments are shown in Table

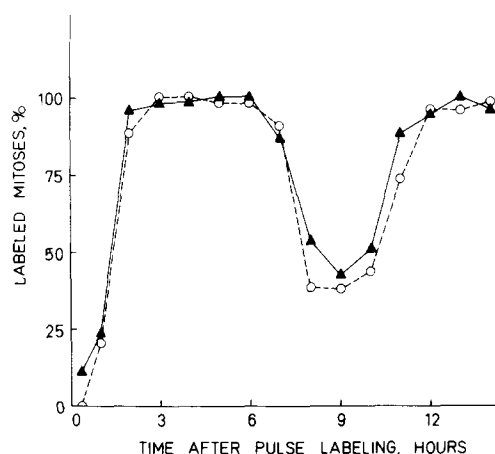


FIGURE 3 Variations with time of the mitotic labeling index in the presence of amethopterin (10 μ M) and thymidine (3 μ M) (\blacktriangle — \blacktriangle), and of thymidine alone (\circ — \circ).

III. It is seen that the size of the acid-soluble, labeled thymidine nucleotide pool remained essentially constant during the observation period of 1 h.

Incorporation of [3 H]Thymidine into DNA in the Presence of Amethopterin and Thymidine

From a steady-state culture, samples were withdrawn, supplemented with amethopterin, thymidine, and [3 H]thymidine, and incubated at 37°C. The effects of amethopterin at a concentration of 0.1 μ M were compared with those at 10 μ M, while

TABLE II
Cell Cycle Characteristics of Steady-State Cultures Containing Thymidine in the Presence and Absence of Amethopterin

Experiment no.	Cell cycle time			Duration of S period		
	Control	Amethopterin	Difference	Control	Amethopterin	Difference
	<i>h</i>	<i>h</i>	<i>h</i>	<i>h</i>	<i>h</i>	<i>h</i>
1	8.75	8.65	-0.10	6.4	6.6	+0.2
2	8.20	8.35	+0.15	5.7	6.0	+0.3

Data of exp. 1 are presented in Fig. 3.

TABLE III
Size of Acid-Soluble Thymidine Nucleotide Pool Derived from Exogenous Thymidine at Different Times after Addition of Amethopterin (10 μM) and Thymidine (3 μM)

Experiment no.	Pool size (pmol/10 ⁶ cells)		
	30 min	60 min	90 min
1	50.2	50.9	57.8
2	44.5	49.4	44.4
3	48.3	47.1	48.1
Mean with standard error	47.7 ± 1.7	49.1 ± 1.1	50.1 ± 4.0

the concentrations of thymidine were 3 and 30 μM, respectively. [³H]Thymidine was added at 1.0 μCi/ml or 10 μCi/ml to obtain a specific activity of 333 μCi/μmol in the culture medium.

At various times after the addition of amethopterin, thymidine, and [³H]thymidine, incubation was stopped and radioactivities incorporated into DNA were determined. From measured radioactivities and the specific activity of [³H]thymidine in the culture medium, nanomoles of thymidine incorporated into DNA were calculated and divided by the number of cells per sample as determined at the time of addition of [³H]thymidine. The exponential increase in cell number during incubation of samples with amethopterin, thymidine, and [³H]thymidine with the concomitant increase in the rate of incorporation of [³H]thymidine was accounted for by the following correction:

$$\frac{M_{\text{corr}}(t)}{N_o} = \frac{M_{\text{meas}}(t)}{N_o} \times \frac{t/T_2 \times \ln 2}{2^{t/T_2} - 1}$$

where N_o = cell number per sample at the time of addition of amethopterin, thymidine, and

[³H]thymidine, $M_{\text{meas}}(t)$ = nanomoles of thymidine incorporated during time t , t = time of incubation with amethopterin, thymidine, and [³H]thymidine, T_2 = cell number doubling time. $M_{\text{corr}}(t)$ thus represents nanomoles of thymidine incorporated during time t if cell number and rate of DNA synthesis had remained constant during the incubation, and $M_{\text{corr}}(t)$ should, in theory, show a linear increase with time.

The results of a typical experiment are illustrated in Fig. 4. It is seen that incorporation of thymidine into DNA exhibited an increase with time which under all three conditions was essen-

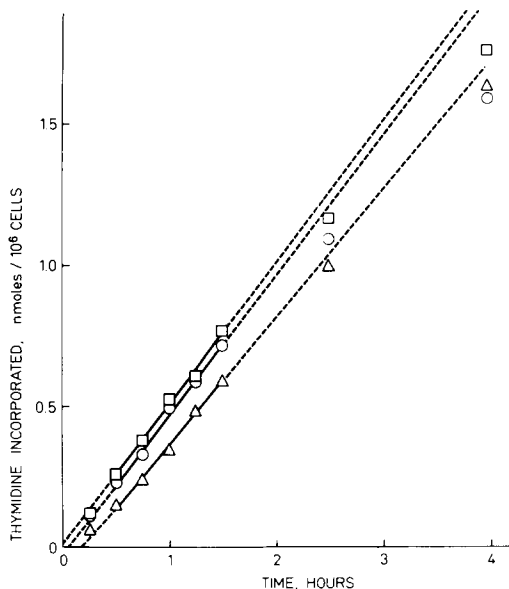


FIGURE 4 Incorporation of thymidine into DNA as a function of time after addition of amethopterin, thymidine, and [³H]thymidine. □—□, amethopterin 10 μM, thymidine 30 μM; ○—○, amethopterin 10 μM, thymidine 3 μM; Δ—Δ, amethopterin 0.1 μM, thymidine 3 μM.

tially linear from 0.5 to 1.5 h after addition of amethopterin, thymidine, and [^3H]thymidine. The solid lines represent regression lines based on the five points from 0.5 to 1.5 h, and the dotted lines were obtained by extrapolation. In the presence of amethopterin at a concentration of $10\ \mu\text{M}$, thymidine added to the cultures at a level of $3\ \mu\text{M}$ was incorporated into DNA at nearly the same rate as at $30\ \mu\text{M}$. On the other hand, in the presence of amethopterin at a concentration of $0.1\ \mu\text{M}$, the rate of incorporation of thymidine was somewhat lower. In addition, a lag period, as expressed by the intercept of the extrapolated regression line with the abscissa, was evident in the presence of amethopterin at $0.1\ \mu\text{M}$, while no significant lag was observed after addition of the inhibitor at the level of $10\ \mu\text{M}$. These findings are in good agreement with the observation that amethopterin at the lower concentration inhibited the endogenous synthesis of thymidine nucleotides, as measured by the incorporation of [^3H]deoxyuridine, more slowly than at the concentration of $10\ \mu\text{M}$. After 1.5 h, the rate of incorporation of thymidine tended to decrease under all three conditions studied. This may be attributable, at least in part, to a change in culture conditions, since the cell suspension was not agitated any more after addition of amethopterin, thymidine, and [^3H]thymidine. In addition, the observed decrease in thymidine incorporation rate may reflect the decrease of the concentration of thymidine in the culture

medium resulting from the uptake of this precursor by the cells.

The results of a series of experiments with respect to the incorporation of thymidine between 30 and 90 min after addition of amethopterin, thymidine, and [^3H]thymidine are summarized in Table IV. They indicate that in the presence of amethopterin at a concentration of $0.1\ \mu\text{M}$, the rate of incorporation of thymidine regularly was somewhat lower than after addition of amethopterin at the $10\text{-}\mu\text{M}$ level. Furthermore, at the higher inhibitor concentration (cultures II and III), the rate of incorporation of thymidine added at a concentration of $3\ \mu\text{M}$ was, on the average, lower by approximately 8% than that at the level of $30\ \mu\text{M}$. After addition of amethopterin at the $10\text{-}\mu\text{M}$ level, thymidine incorporation measured during the first 60 min was found to be nearly identical with that measured between 30 and 90 min.

In two additional experiments, the rate of incorporation of exogenous labeled thymidine between 30 and 90 min after addition of amethopterin ($10\ \mu\text{M}$) was compared with that in the absence of amethopterin. The results indicate that in the absence of the inhibitor, 66–68% of thymidine nucleotides incorporated into DNA were derived from exogenous thymidine if this precursor was supplied at a concentration of $3\ \mu\text{M}$, while the contribution of exogenous thymidine added at the level of $30\ \mu\text{M}$ was 71–72%.

TABLE IV
Rate of Incorporation of Thymidine into DNA at Different Concentrations of Amethopterin (A) and of Thymidine (T)

Experiment no.	Cell number doubling time <i>h</i>	Rate of incorporation of thymidine (pmol/h $\times 10^6$ cells)		
		I A $0.1\ \mu\text{M}$ T $3\ \mu\text{M}$	II A $10\ \mu\text{M}$ T $3\ \mu\text{M}$	III A $10\ \mu\text{M}$ T $30\ \mu\text{M}$
1	8.79	443	488	492
2	8.88	473	521	586
3	8.85	458	499	565
4	8.81		505	
5	8.91		477	
6	8.88		545	
Mean* (exps. 1–3)	8.84 ± 0.03	458 ± 9	503 ± 10	548 ± 28
Mean* (all exps.)	8.85 ± 0.02		506 ± 10	

Data of exp. 1 are presented in Fig. 4.

* With standard error.

Comparison of Rates of DNA Synthesis Derived from Thymidine Incorporation with Those Derived from Mean Cellular DNA Content and Rates of Cell Proliferation

Rates of incorporation of thymidine in the presence of amethopterin (10 μ M), thymidine (3 μ M), and [3 H]thymidine were used to calculate rates of DNA synthesis of the cultured cell population. This calculation was based on a base composition of mouse DNA corresponding to 58% thymine-adenine base pairs (26). In addition, rates of DNA synthesis were calculated from cell number doubling times and measured DNA contents of 10^6 cells. The results are presented in Table V. It is seen that rates of DNA synthesis, as determined by the two independent methods, are in good agreement with each other. In fact, the average rate of DNA synthesis derived from the incorporation of [3 H]thymidine corresponds to more than 97% of the DNA synthesis rate derived from the measured DNA content and the rate of cell multiplication.

DISCUSSION

Several lines of evidence indicate that the method presented in this communication is suitable for determining true rates of DNA synthesis in the culture system used. Optimal results appear to be

obtained if [3 H]thymidine incorporation is determined from 30 to 90 min or, alternatively, during the first 60-90 min after the addition of amethopterin at a concentration of 10 μ M and of thymidine at concentrations in the range from 3 to 30 μ M. It should be noted that rates of DNA synthesis, as determined under these conditions, represent average values for the cultured cell population and do not apply to individual cells, since in the asynchronous cultures used, only a fraction of the cell population is in the S period at any one time.

As seen in Fig. 1, endogenous synthesis of thymidine nucleotides, as estimated from the incorporation of [3 H]deoxyuridine into DNA, is blocked rapidly and almost completely by amethopterin at the level of 10 μ M. The contribution of this pathway becomes, therefore, negligible as compared with synthesis of thymidine nucleotides from exogenous thymidine. Inhibition of deoxyuridine incorporation was as pronounced in the absence as in the presence of thymidine in the culture medium. This excludes the possibility that the observed effects on deoxyuridine incorporation may be attributable to competition between deoxyuridine and thymidine at the level of transport across the cell membrane and/or phosphorylation by thymidine kinase.

Instead of amethopterin, 5-fluoro-2'-deoxyuridine (dFUrd) may be also considered for use as an inhibitor of thymidylate synthesis from deoxy-

TABLE V
Rates of DNA Synthesis as Determined by Two Different Methods

Experiment no.	Cell number doubling time <i>h</i>	DNA content μ g/ 10^6 cells	Rate of DNA synthesis (ng/h \times 10^6 cells)	
			Eased on thymidine incorporation	Based on DNA content and rate of cell proliferation
1	8.79		520	
2	8.88		555	
3	8.85		531	
4	8.81		538	
5	8.91		508	
6	8.88		580	
7	9.04	6.81		522
8	8.69	7.01		559
9	8.59	7.19		580
Mean with standard error		7.0 \pm 0.11	539 \pm 11	554 \pm 17

Exps. 1-6 are the same as those presented in Table IV.

uridylylate. Unlike amethopterin, however, dFURd may possibly compete with thymidine for transport across the cell membrane and/or for thymidine kinase. Autoradiographic studies have indicated that after addition of dFURd to suspensions of bone marrow cells, incorporation of labeled thymidine, after a time lag of 1–2 min, was linear for 6–10 min only (4).

After addition of amethopterin at a concentration of 10 μM , [^3H]thymidine incorporation into DNA increased in a linear fashion for at least 90 min with a negligible lag period. This indicates that preexisting thymidine nucleotide pools were sufficient for DNA synthesis for not more than a few minutes. Furthermore, radioactivity of intracellular acid-soluble thymidine derivatives did not change between 30 and 90 min after addition of amethopterin, thymidine, and [^3H]thymidine, indicating that replacement of unlabeled by labeled thymidine nucleotides was essentially complete after the first 30 min of incubation.

In order to obtain meaningful results, it is essential that the rate of DNA synthesis of the cell population is not changed to any significant extent under the conditions used, i.e. by the addition of amethopterin, thymidine, and [^3H]thymidine. In agreement with previous reports (13, 14), preliminary experiments indicated that thymidine was nontoxic to the cells at concentrations up to 30 μM , while cell proliferation was inhibited after addition of thymidine at concentrations of 100 μM and higher. After addition of amethopterin and thymidine, rates of cell proliferation were lower by approximately 5% than control values if plotted on a semilogarithmic scale. Similar results have been reported for rat hepatoma cell cultures (8). No significant differences were observed between cell cycle times in the presence of amethopterin and thymidine, as compared with those after addition of thymidine alone. The duration of the S period which is inversely related to the rate of DNA synthesis, was prolonged by approximately 4%. This effect may be even smaller during the first 90 min after the addition of amethopterin and thymidine. In fact, rates of DNA synthesis derived from incorporation of thymidine in the presence of amethopterin are in good agreement with those based on mean cellular DNA content and rates of cell proliferation in the absence of amethopterin and thymidine, the difference of approximately 3% being within the limits of precision of the analytical procedures used.

As seen in Fig. 1, endogenous synthesis of

thymidylylate, as assessed by the incorporation of [^3H]deoxyuridine into DNA, was inhibited rapidly and efficiently by amethopterin at a concentration of 10 μM , whereas at the level of 0.1 μM , inhibition was incomplete. These findings are in agreement with previous observations on L-cell cultures (3). With respect to thymidine concentration, the amounts of thymidine incorporated at the level of 3 μM tended to be somewhat lower than those at the level of 30 μM . The significance of this difference of 7–8% is, however, questionable, and a thymidine concentration of 3 μM was felt to be preferable because (a) the concentration of 30 μM is at the borderline of toxic thymidine levels, and (b) at the concentration of 3 μM , smaller amounts of [^3H]thymidine are needed to measure its incorporation into DNA. Concentrations of thymidine lower than 3 μM may be expected to be too small to support DNA synthesis at a normal rate (8), possibly because of a limited transport of thymidine across the cell membrane (16).

In evaluating the reliability of average DNA synthesis rates obtained by the method presented, it should be considered that the serum used for preparation of culture media may itself contain some thymidine. This would result in an underestimation of DNA synthesis rates. Since DNA synthesis rates obtained at thymidine concentrations of 3 and 30 μM differed little from each other and agreed well with DNA synthesis rates calculated from the mean cellular DNA content and rates of cell proliferation, it may be concluded that horse serum did not contribute significantly to the thymidine concentration in the culture medium.

The good agreement between DNA synthesis rates calculated from rates of thymidine incorporation and those based on the mean cellular DNA content and rates of cell proliferation indicates that [*methyl*- ^3H]thymidine is incorporated by the cultured cells with essentially the same efficiency as unlabeled thymidine. Isotope effects resulting from replacement of a hydrogen by a tritium atom are, therefore, too small to be detectable by the methods employed. The results also indicate that in the cell culture system used, DNA exhibits a high degree of metabolic stability. This is in agreement with previous reports based on the stability of label incorporated into DNA (2, 12, 28). On the other hand, the concept of metabolic turnover of DNA (15) is not supported by the results presented in this communication.

The determination of thymidine incorporation in aliquots withdrawn from suspension cultures

allows for repeated measurements of rates of DNA synthesis in a cultured cell population. Variations in DNA synthesis rates under changing culture conditions may thus be studied at intervals as short as 30 min with a rather high degree of accuracy. The method is, however, not applicable to studies of the effects on DNA synthesis of inhibitors of the formation of thymidine nucleotides, such as dFurd or folic acid antagonists. On the other hand, the method presented in this communication may, for instance, be used with good success for measuring time-dependent variations of DNA synthesis rates in synchronous culture systems. Similarly, it may be applied to study changes in the rate of DNA synthesis after the addition of inhibitors of RNA or protein synthesis, or after interfering with various other cellular activities.

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