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EFFECTS OF METHOTREXATE ON THYMIDINE TRIPHOSPHATE LEVELS IN CHINESE HAMSTER CELL CULTURES

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Abstract—Chinese hamster ovary cells were incubated in medium containing hypoxanthine and glycine and supplied with 10^{-5} M methotrexate to inhibit the endogenous synthesis of thymidine nucleotides. Within 1–5 min after addition of the inhibitor, incorporation of [6-^{3}H]deoxyuridine into DNA was as low as 1 to 1.5 per cent of control values, indicating that endogenous synthesis of thymidine nucleotides was blocked rapidly and almost completely. The cellular dTTP content was determined under various culture conditions as a function of time after addition of methotrexate. If the medium used contained undialyzed fetal calf serum, dTTP levels decreased relatively slowly in asynchronous as well as in synchronous S-phase cell populations. Similar results were obtained with asynchronous cultures incubated in medium with dialyzed serum. In contrast, if synchronous cultures consisting predominantly of cells in S phase were incubated in medium containing dialyzed serum and supplied with methotrexate, dTTP levels decreased within 15 min from control values of 15–20 pmoles/10^6 cells to levels of 1.5 to 3 pmoles/10^6 cells or lower. The previously reported failure of methotrexate to cause a rapid depletion of cellular dTTP may reflect, therefore, maintenance of thymidine nucleotide pools by cells that are not in the S phase and/or uptake by cells of thymidine present in undialyzed serum used for preparation of culture media.

Methotrexate is a specific inhibitor of dihydrofolic acid reductase. The depletion of tetrahydrofolic acid derivatives induced by this antimetabolite results in impairment of 1-carbon transfer reactions which are required for the synthesis of various metabolites. In mammalian cell cultures, the inhibition of cell proliferation by methotrexate was shown to be prevented by the addition of glycine, a purine, and thymidine to the culture medium [1, 2]. The addition of methotrexate in combination with glycine and a purine may be expected, therefore, to result in a specific deficiency of thymidine nucleotides mediated by inhibition of the conversion of deoxyuridylate to thymidylate.

Contrary to this expectation, it has been reported that, after addition of methotrexate to HeLa cell cultures, the cellular dTTP content remained essentially constant for as long as 16 hr [3]. In conjunction with observations on the kinetics of [^{3}H]thymidine incorporation into DNA in the presence and absence of methotrexate, this finding was interpreted as reflecting the existence of two separate cellular pools of thymidine nucleotides synthesized *de novo* and from exogenous thymidine respectively [4]. In mouse lymphoma cells, a 50 per cent reduction of the dTTP pool was observed 1 hr after addition of methotrexate, but almost no further decrease occurred for up to 24 hr [5]. Similarly, after addition of methotrexate to cultures of human lymphocytic cells, the cellular dTTP content decreased within 1 hr by approximately 50 per cent; during further incubation in the presence of the inhibitor, however, cellular dTTP levels decreased at a rather slow rate, and 7 hr was required before 10 per cent of the control value was attained [6].

Amounts of dTTP within a cell are relatively small. For Chinese hamster ovary (CHO) cell cultures, it was shown that the cellular dTTP pool is sufficient to support DNA synthesis for approximately 5 min [7]. This supports the assumption of a rapid turnover of dTTP and is in contrast to the observations of a slow and/or limited decrease of cellular dTTP after inhibition of endogenous thymidine nucleotide synthesis by methotrexate.

The present work was undertaken to study various factors which might affect the extent and time course of changes in cellular dTTP content after addition of methotrexate to CHO cell cultures. It will be shown that, under appropriate experimental conditions, a rapid and nearly complete depletion of intracellular dTTP after addition of methotrexate may be observed.

MATERIALS AND METHODS

Cell line and culture techniques. Chinese hamster ovary cells [8] were grown as monolayer cultures in Eagle's minimal essential medium [9] supplemented with L-alanine, L-asparagine, L-aspartic acid, L-glutamic acid, L-proline, L-serine, glycine (all at 10^{-4} M), penicillin (50 units/ml), streptomycin (50 μg/ml), aureomycin (50 μg/ml), and 15% fetal calf serum (FCS). In one series of experiments, medium containing 15% dialyzed fetal calf serum was used. The serum was dialyzed at 4° for at least 24 hr against 10–15 vol. saline, and the saline was changed four times during dialysis. Cultures were tested frequently and found to be free of mycoplasma contamination (tests were kindly performed by G. Kronauer).

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For experiments with asynchronous cultures, approximately 3×10^5 cells in 2 ml medium were incubated in Petri dishes (3 cm diameter, Nunc, Denmark) overnight at 37° in an atmosphere of 5% CO_2 and 95% air. For subsequent studies of the effects of methotrexate on deoxyuridine incorporation or on cellular dTTP content, the dishes were placed on the surface of a 37° water bath, and the medium was replaced by prewarmed fresh medium supplemented with 3×10^{-5} M hypoxanthine and 20 mM 4-(2-hydroxyethyl)-1-piperazine-ethane sulfonic acid (HEPES) buffer (pH 7.2) and containing 15% dialyzed or undialyzed FCS. The water bath was covered with a plastic foil, and the atmosphere above the water surface was saturated with a mixture of 5% CO_2 and 95% air to maintain the pH in the culture medium. At the time of the experiments, two Petri dishes were used for determination of cell numbers. Cells were removed from the dishes by trypsinization and counted with a hemocytometer.

Synchronous cell populations were obtained by selectively detaching mitotic cells from non-confluent monolayer cultures [10, 11]. Mitotic cells were kept at 0° for up to 3 hr, and during this time the procedure of detaching mitotic cells was repeated until sufficient cells had been collected [12]. After centrifugation, the mitotic cells were suspended, at a density of approximately 1.8×10^5 cells/ml, in conditioned medium containing 3×10^{-5} M hypoxanthine and 10 mM HEPES buffer (pH 7.2), and 2 ml of this suspension was pipetted into a series of Petri dishes and incubated at 37° in an atmosphere of 5% CO_2 and 95% air.

Determination of relative numbers of DNA-synthesizing cells. Cells were incubated for 15 min with [^{3}H]thymidine (1 μCi/ml of medium), removed from the Petri dishes, fixed with a mixture of ethanol and acetic acid (3:1), and the fixed cells were brought to microscope slides. For autoradiography, the slides were covered with Kodak NTB-2 emulsion. After the appropriate exposure time, the preparations were developed and stained with Giemsa. For determination of the percentage of labeled cells, 500 cells were scored per preparation.

Chemicals. [^{14}C]Thymidine (57 mCi/m-mole), [^{3}H]thymidine (20 Ci/m-mole), [6-^{3}H]deoxyuridine (14 Ci/m-mole), [2-^{14}C]deoxyuridine (58 mCi/m-mole), and [^{3}H]dATP (15–29 Ci/m-mole) were purchased from the Radiochemical Centre, Amersham, Bucks, England while [5-^{3}H]deoxyuridine (12 Ci/m-mole) was obtained from ICN Pharmaceuticals Inc., Irvine, CA. Methotrexate was kindly supplied by Cyanamid GmbH, München, West Germany. Unlabeled deoxyribonucleoside triphosphates were obtained from Serva, Heidelberg, West Germany. These nucleotides were dissolved in 0.02 M Tris–HCl buffer (pH 7.5), and their concentrations were determined by optical density measurements (at 267 nm for dTTP and at 259 nm for dATP). DNA polymerase (EC 2.7.7.7) from *Micrococcus luteus* was obtained from Miles Laboratories, Slough, England, with specific activity of approximately 600 units/mg of protein, and diluted to 200 units/ml with 0.05 M potassium phosphate buffer (pH 6.9) containing 0.01 M 2-mercaptoethanol and 30% ethylene glycol. Poly [d(A-T)] · [d(A-T)] was purchased from Boehringer, Mannheim, West Germany and dissolved in 0.01 M Tris–HCl (pH 7.5).

Determination of [6-^{3}H]*deoxyuridine incorporation into DNA.* These incorporation studies were performed as double labeling experiments. After incubation of cells in Petri dishes overnight with [^{14}C]thymidine (10^{-3} μCi/ml), the medium was replaced by fresh medium containing hypoxanthine and HEPES buffer, and 2 hr later the cultures were transferred to the 37° water bath and supplied with 10^{-5} M methotrexate. At various times after addition of the inhibitor, [6-^{3}H]deoxyuridine (final concn: 1 μCi/ml, 4×10^{-7} M) was added, and incubation with the labeled precursor was continued for 15 min. The cells were lysed with 0.5 ml of 1 N NaOH, and cellular RNA was hydrolyzed by incubation during 60 min at 37°. After addition of 200 μg of calf thymus DNA, the acid-insoluble material was precipitated with 1 ml of 1.5 N HCl containing 6% $Na_4P_2O_7$ and brought to Whatman GF/C filters. The filters were washed with a solution of 0.1 N HCl containing 0.5% $Na_4P_2O_7$ and dehydrated with ethanol and acetone. Radioactivities attributable to ^{14}C and ^{3}H were measured in a Packard scintillation spectrometer.

Determination of relative incorporation of deoxyuridine into dTTP and dUTP. Replicate cultures were incubated with medium containing hypoxanthine, HEPES buffer and 15% dialyzed FCS. After 45 min, one group of cultures was supplied with 10^{-5} M methotrexate and 15 min later a mixture of [5-^{3}H]deoxyuridine and [2-^{14}C]deoxyuridine (final concn: 6 μCi/ml and 0.1 μCi/ml respectively) was added. After further incubation for 15 or 60 min, the cultures were washed with an ice-cold solution of 0.15 M NaCl and 0.015 M sodium citrate, 2 ml of cold (approximately −20°) 66% ethanol was added, and the Petri dishes were maintained at −20° for 5–20 hr. Subsequently, the extracts were centrifuged (3200 rev/min, 0°), and the clear supernatant fraction was decanted, lyophilized, and stored at −20°. For separation of nucleotides by thin-layer chromatography [13], the lyophilized extracts were dissolved in 0.1 ml of 60% methanol, and aliquots were applied to PEI-cellulose plates in addition to a marker solution containing unlabeled deoxyuridine, thymidine, dTMP, dTDP and dTTP. The plates were developed with 50% methanol, followed by 0.38 M LiCl in 1 N formic acid–sodium formate buffer (pH 2.0) without intermediate drying. The spot containing dUTP and dTTP was scraped off, and after elution with 2 ml of 0.02 M Tris–HCl buffer (pH 6.5) containing 0.7 M $MgCl_2$, 18 ml of a xylene–Triton X-100 mixture was added, and radioactivities attributable to ^{3}H and ^{14}C were determined. While [2-^{14}C]deoxyuridine is a precursor for both dUTP and dTTP, [5-^{3}H]deoxyuridine may be assumed to be incorporated into dUTP only, since the tritium atom is removed during dTMP synthesis.

Extraction and assay of thymidine triphosphate. Cultures containing $6–9 \times 10^5$ cells were washed with an ice-cold solution of 0.15 M NaCl and 0.015 M sodium citrate, extracted with cold 66% ethanol, and the extracts were lyophilized as described above. For determination of dTTP, the lyophilized extracts were dissolved in 0.1 ml of 0.02 M Tris–HCl buffer (pH 7.5), and 0.01 ml was used for the assay. More than 90 per cent of cellular dTTP was recovered by this extraction procedure, and the extent of hydrolysis of dTTP during extraction and preparation of extracts for the assay was below 10 per cent.

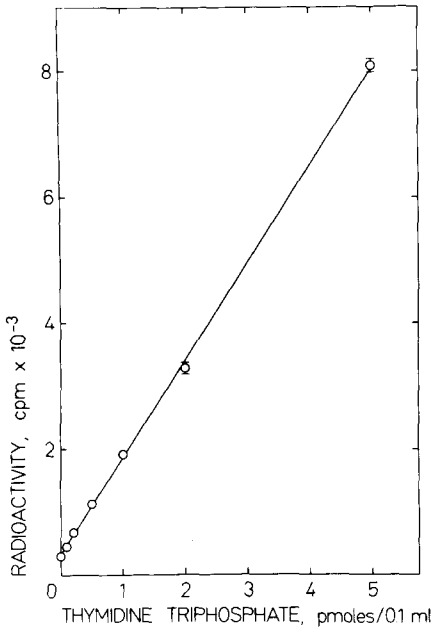

Fig. 1. Incorporation of [^{3}H]dATP into acid-insoluble material as a function of dTTP concentration in a reaction mixture containing DNA polymerase and poly [d(A-T) · d(A-T)] under standard assay conditions. Bars indicate ranges of duplicate measurements, unless these were smaller than diameter of circles.

To determine dTTP, the method of Lindberg and Skoog [14] was used. The standard reaction mixture (usually 0.1 ml) had the following composition: 60 mM Tris–HCl buffer (pH 7.5); 2 mM $MgCl_2$; 0.3 mM 2-mercaptoethanol; [^{3}H]dATP, 1–5 μCi/ml, 10^{-6} M; poly [d(A-T) · d(A-T)], 4.5 to 23 × 10^{-3} E_{260} units; DNA polymerase, 7 units/ml; and 1 to 50 × 10^{-9} M dTTP (for obtaining reference curve) or cell extract respectively. This mixture was incubated at 37° for 60 min. To stop the reaction, the tubes were placed in an ice-water bath, and 2 ml of ice-cold 0.2 N NaOH containing 200 μg/ml of calf thymus DNA was added. Subsequently, the acid-insoluble materials were precipitated with 1 ml of 1.5 N HCl containing 6% $Na_4P_2O_7$, quantitatively transferred to Whatman GF/C filters, and processed for liquid scintillation counting as described above for cellular DNA. For calibration, a standard curve was established with known amounts of dTTP in parallel with each assay.

As shown in Fig. 1, incorporation of radioactivity increased in a linear fashion with dTTP concentrations up to 5 pmoles/0.1 ml of reaction mixture, and amounts of dTTP as small as 0.1 pmole, which corresponds to approximately 1.3 pmoles/10^6 cells, were detectable.

If dUTP was added to the reaction mixture in place of dTTP, essentially the same concentration-dependent incorporation of [^{3}H]dATP into acid-insoluble material was observed. This indicates that the assay had a limited specificity and did not permit us to distinguish between dTTP and dUTP.

Table 1. Effect of methotrexate on incorporation of [6-^{3}H]deoxyuridine (1 μCi/ml of medium, 4 × 10^{-7} M) into DNA*

Time after addition of methotrexate (min)	Incorporation† (% of control)
1	3.3 ± 1.2
5	1.1 ± 0.2
10	1.4 ± 0.2
30	1.0 ± 0.2

* At various times after addition of methotrexate (10^{-5} M) to asynchronous CHO cultures, the cells were incubated with the labeled precursor for 15 min.

† Means of results obtained in two experiments each, with ranges.

RESULTS

Effect of methotrexate on incorporation of [6-^{3}H]*deoxyuridine into DNA.* To asynchronous cultures containing hypoxanthine, methotrexate was added to obtain a concentration of 10^{-5} M. At different times after the addition of the inhibitor, cultures were supplied with [6-^{3}H]deoxyuridine, and incubation was stopped 15 min later to determine incorporation of the precursor into DNA. The results are presented in Table 1. If deoxyuridine was added 1 min after methotrexate, incorporation during the subsequent 15-min period was less than 5 per cent of the control value, and addition of deoxyuridine from 5 to 30 min after methotrexate resulted in an incorporation which was approximately 1

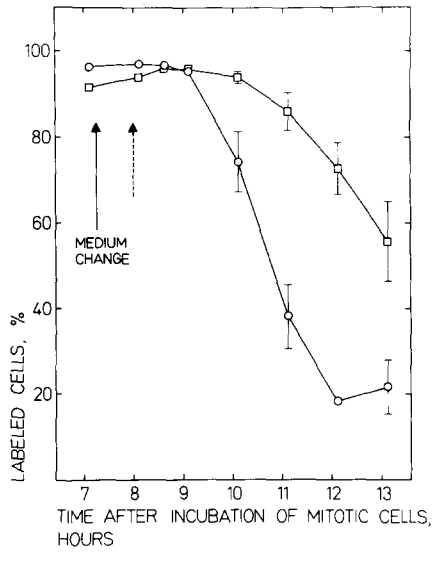

Fig. 2. Synchrony of cell populations obtained by collection of mitotic cells, as evaluated by time-dependent variations of the labeling index. At 7.25 hr (solid arrow), the medium was replaced by fresh medium containing 15% undialyzed FCS (O——O) or 15% dialyzed FCS (□——□). The broken line arrow indicates the time of addition of methotrexate (10^{-5} M) to cultures used for determination of dTTP content. Symbols represent means of values obtained in three independent experiments each, with standard errors, unless these were smaller than the diameter of the symbols.

per cent of that observed in the absence of the inhibitor. These results support the conclusion that inhibition of endogenous synthesis of thymidine nucleotides was nearly complete at time intervals as short as 1–5 min after addition of methotrexate at the concentration of 10^{-5} M.

Characterization of asynchronous and synchronous cell populations. In asynchronous cultures, relative numbers of DNA-synthesizing cells, as determined by autoradiography, were 63–75 per cent.

To characterize the synchrony of cell populations obtained by mechanical detachment of mitotic cells, cultures were labeled for 15 min with [^{3}H]thymidine at different times after reincubation of mitotic cells. If the labeled precursor was added 30 min after reincubation, less than 3 per cent of the total cell population was found to be labeled. Variations of the labeling index from 7 to 13 hr after reincubation of mitotic cells are illustrated in Fig. 2. It is seen that at 8–9 hr, labeling indices were 94–97 per cent. In order to study the effects of methotrexate on as pure S-phase cell populations as possible, the following experimental design was chosen. At 7.25 hr after incubation of mitotic cells (solid arrow in Fig. 2), the medium was replaced by fresh medium containing hypoxanthine and HEPES buffer. In one series of experiments, this medium contained 15% undialyzed FCS, while in a second series of experiments, the medium contained 15% dialyzed FCS. At 8 hr, as indicated by the broken line arrow, methotrexate was added to all cultures with the exception of those used for subsequent determinations of labeling indices. Changes with time of cellular dTTP content were studied during the 60-min period after addition of the inhibitor. As seen in Fig. 2, at times exceeding 9 hr after incubation of mitotic cells, a decrease of relative numbers of labeled cells was observed. In medium with 15% dialyzed FCS, this decrease occurred more slowly than in medium containing 15% undialyzed FCS. This supports the assumption that the duration of the S phase was longer in cell populations that had been transferred to medium containing dialyzed serum.

Effects of methotrexate on cellular dTTP content. In a first series of experiments, medium containing 15% undialyzed FCS was used. At the time of transfer to the water bath, asynchronous or synchronous cultures were supplied with prewarmed fresh medium containing hypoxanthine and HEPES buffer. Methotrexate was added 45 min later to obtain a concentration of 10^{-5} M, and at various times, two cultures each were used for determination of cellular dTTP content. The results of three experiments with asynchronous and three experiments with synchronous cultures are presented in Fig. 3. In asynchronous cell populations, the dTTP content decreased by a factor of two within 15 min after addition of the inhibitor. During further incubation, dTTP levels continued to decrease, although at a slower rate, and at 60 min, cellular dTTP content was, on the average, still 6–7 pmoles/10^6 cells.

Similar results were obtained if methotrexate was added to synchronous cell populations consisting predominantly of S-phase cells, i.e. at 8 hr after incubation of mitotic cells (broken line arrow in Fig. 2). The initial dTTP content of S-phase cells was rather variable. This may be atributed to small differences in cell cycle progression between individual experiments and/or to different serum batches used in the three experiments. On the average, somewhat smaller initial dTTP levels were observed in S-phase cells, as compared to those in asynchronous cell populations. Within the first 15 min

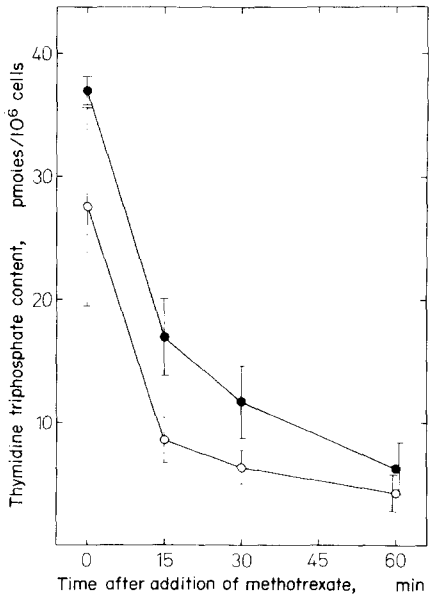

Fig. 3. Effect of methotrexate on cellular dTTP content of asynchronous cultures (●——●) and S-phase cell populations (○——○) incubated in medium containing 15% undialyzed FCS. Symbols represent means of values obtained in three independent experiments each, with standard errors.

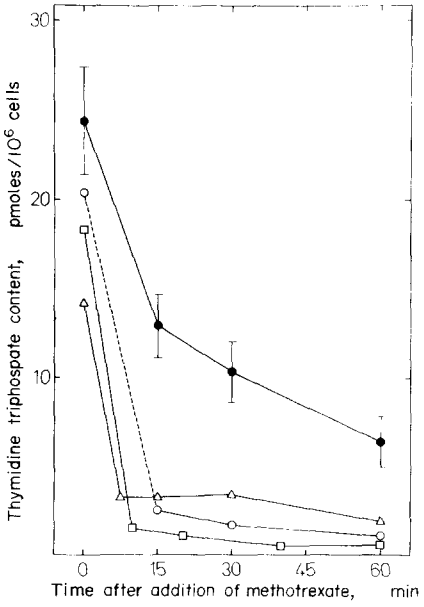

Fig. 4. Effect of methotrexate on cellular dTTP content of asynchronous cultures (●——●) and S-phase cell populations (open symbols) incubated in medium containing 15% dialyzed FCS. Filled circles represent means of values obtained in three independent experiments, with standard errors; open symbols represent values obtained in three individual experiments.

Table 2. Cellular dTTP content as a function of time after replacement of normal culture medium with medium containing 15% dialyzed FCS

Time after medium change (min)	dTTP content* (pmoles/10^6 cells)
0	31.2 ± 0.5
15	17.3 ± 0.6
30	14.9 ± 0.8
60	15.2 ± 0.3
120	15.9 ± 1.3

* Means of values obtained with two cultures each, with ranges.

after addition of the inhibitor to S-phase cells, cellular dTTP decreased by a factor of three. During the following 45 min, a slow but continued decrease of cellular dTTP was observed, and at 60 min, the average dTTP content still was above 4 pmoles/10^6 cells.

In a second series of experiments, the normal culture medium containing undialyzed serum was replaced by medium containing 15% dialyzed FCS, hypoxanthine and HEPES buffer at 45 min before the addition of methotrexate. The changes in cellular dTTP content of asynchronous and synchronous cultures exposed to methotrexate for 60 min are illustrated in Fig. 4. In comparison with cultures incubated in medium containing undialyzed serum (Fig. 3), the average dTTP content under control conditions, i.e. at the time of addition of methotrexate, was somewhat lower in both asynchronous and synchronous cell populations. As shown in Table 2, after replacing the medium containing undialyzed FCS by medium with dialyzed FCS, the cellular dTTP content decreased during 15–30 min and then remained essentially constant for the following 90 min. After addition of methotrexate, the decrease of cellular dTTP in asynchronous cell populations exhibited a time course similar to that observed in cultures incubated with medium containing undialyzed FCS. On the other hand, after addition of methotrexate to cell populations consisting predominantly of S-phase cells, the cellular dTTP content decreased within 10 min to levels as low as 1.5 to 3 pmoles/10^6 cells. During the following 50 min, a further slow decrease was observed, and at 60 min, cellular dTTP levels were 0.5 to 2 pmoles/10^6 cells.

Comparative incorporation of [5-^{3}H]deoxyuridine and [2-^{14}C]deoxyuridine into the nucleoside triphosphate fraction. Cultures pretreated for 15 min with methotrexate, as well as control cultures without inhibitor, were supplied with [5-^{3}H]deoxyuridine and [2-^{14}C]deoxyuridine, and radioactivities incorporated into the nucleoside triphosphate fraction were determined. As seen in Table 3, under control conditions [2-^{14}C]deoxyuridine was incorporated more efficiently than [5-^{3}H]deoxyuridine, resulting in a ^{3}H/^{14}C ratio of less than 1, as compared to the ratio of 60 for deoxyuridine added to the medium. In contrast, ^{3}H-radioactivity in the nucleoside triphosphate fraction of cultures treated with methotrexate was higher, while ^{14}C-radioactivity was markedly lower than in control cultures. Since radioactivity from [5-^{3}H]deoxyuridine may be expected to be incorporated into deoxyuridine phosphates, but not into thymidine phosphates, these results indicate that, in the presence of methotrexate, a significant portion of the ^{14}C-radioactivity appearing in the nucleoside triphosphate fraction is attributable to conversion of deoxyuridine to dUTP.

DISCUSSION

Studies on L-cells incubated with methotrexate in combination with various purine and pyrimidine nucleosides have indicated that the cytocidal action of the folic acid analog results from the inhibition of dTMP synthesis from dUMP [15]. The changes of intracellular thymidine nucleotide levels induced by methotrexate appear, therefore, to be of interest with respect to the mode of action of this inhibitor on proliferating cell populations.

In the studies presented in this paper, a rapid decrease of intracellular dTTP to 10–50 per cent of control levels was found to occur during the first 15 min after addition of methotrexate under all four experimental conditions used. On the other hand, in cultures of mouse lymphoma and human lymphocytic cells, cellular dTTP levels decreased more slowly to reach approximately 50 per cent of control values at 30–60 min after addition of the inhibitor [5, 6], while

Table 3. Effects of methotrexate on incorporation of doubly labeled deoxyuridine into the nucleoside triphosphate fraction*

Methotrexate concn (M)	Time of incubation with labeled deoxyuridine (min)	Radioactivity in dUTP–dTTP fraction†		^{3}H/^{14}C of dUTP–dTTP fraction
		^{3}H (pCi/10^6 cells)	^{14}C (pCi/10^6 cells)	
0	15	176 ± 29	220 ± 14	0.82
10^{-5}	15	436 ± 196	17 ± 7	25
10^{-5}	60	667 ± 90	18 ± 4	38

* Replicate cultures were supplied with 10^{-5} M methotrexate, and 15 min later a mixture of [5-^{3}H]deoxyuridine and [2-^{14}C]deoxyuridine (final concn: 6 μCi/ml and 0.1 μCi/ml respectively) was added. After further incubation for 15 or 60 min, nucleotides were extracted, separated by thin-layer chromatography, and ^{3}H- and ^{14}C-radioactivities in the nucleoside triphosphate fraction were determined.

† Means of results obtained with three cultures each, with standard errors.

the dTTP content of HeLa cells treated with methotrexate was reported to remain essentially constant [3]. The comparatively high initial rate of depletion of cellular dTTP observed for CHO cells may be attributable to the cell cycle characteristics of the cell line. In asynchronous cultures, 63–75 per cent of the cell population was found to be in S phase. Alternatively, the rapid initial decrease of cellular dTTP described in the present report may be due to a more rapid onset of action of methotrexate which was used at a concentration of 10^{-5} M, as compared to 10^{-6} M [3, 5] or 5×10^{-7} M [6]. Even at 10^{-5} M, the effect of methotrexate on endogenous synthesis of thymidine nucleotides was specific. This was demonstrated by comparing cell cycle characteristics, including the duration of the S phase, of CHO cells in the presence of 10^{-5} M methotrexate, hypoxanthine, thymidine and glycine with those in the absence of the inhibitor [16], as shown previously for P-815 murine mastocytoma cultures [17].

As seen in Table 1, within 5 min after addition of methotrexate at a concentration of 10^{-5} M, incorporation of [6-^{3}H]deoxyuridine into DNA decreased to approximately 1 per cent control values, indicating that endogenous synthesis of thymidine nucleotides was blocked rapidly and almost completely. Furthermore, it was shown previously that, in CHO cells, the size of the dTTP pool is sufficient to support DNA synthesis for a time period of less than 6 min [7]. On the other hand, in three of the four series of experiments that are summarized in Figs. 3 and 4, the depletion of cellular dTTP observed during the first 15 min after the addition of methotrexate was rather incomplete, and substantial amounts of dTTP were detected after incubation with the inhibitor for as long as 30–60 min. A rapid decrease of cellular dTTP content to very low levels was observed, however, when synchronous S-phase cell populations in medium containing dialyzed serum were supplied with methotrexate.

These findings may be interpreted as follows. In asynchronous cell populations, cells not engaged in DNA synthesis also contain dTTP. As reported for CHO cells [7, 18] and HeLa cells [19], dTTP levels during the G2 phase of the cell cycle were even higher than those in DNA-synthesizing cells. The results presented in Fig. 4 favor the assumption that rates of turnover of dTTP in cells not engaged in DNA synthesis were considerably lower than those of cells in S phase.

The results also demonstrate that, if methotrexate was added to S-phase cell populations cultured in medium with undialyzed serum (Fig. 3), cellular dTTP levels decreased more slowly than in S-phase cells incubated in medium with dialyzed serum (Fig. 4). This suggests that, in the presence of undialyzed serum, the cells had the possibility of synthesizing small amounts of dTTP from exogenous thymidine. In fact, it was shown that FCS may contain thymidine at concentrations of 8 to 20×10^{-7} M, depending on the serum batch [20]. In medium containing undialyzed FCS, sufficient thymidine may, therefore, be present to sustain cellular synthesis of thymidine nucleotides at substantial rates. Despite the essentially complete inhibition of endogenous synthesis, appreciable intracellular dTTP levels may thus be observed. It should be noted that, in previous studies of the effects of methotrexate on cellular dTTP content, culture media containing undialyzed bovine serum [3] or fetal calf serum [5, 6] were used. On the other hand, methotrexate was shown to inhibit the proliferation of chicken fibroblasts in medium containing chicken plasma, but not in medium containing serum with a thymidine content more than ten times higher than that of plasma [21].

As seen in Fig. 4, although the addition of methotrexate to S-phase cells in medium with dialyzed serum resulted in a rapid decrease of cellular dTTP, low levels of up to 3 pmoles/10^6 cells were observed for at least 30–60 min. This residual dTTP content may be attributed, at least in part, to the presence of 3–6 per cent of cells not engaged in DNA synthesis in the synchronous cell populations used. Furthermore, possibilities such as incomplete inhibition of the endogenous synthesis of thymidine nucleotides by methotrexate, and/or incomplete removal of thymidine from the serum by dialysis, should be considered. In fact, it was reported that incorporation of labeled deoxycytidine into DNA occurred at a reduced rate for several hours after addition of methotrexate to human lymphoblasts cultured in medium with dialyzed serum [22]. On the other hand, the results obtained on the incorporation of [5-^{3}H]deoxyuridine and [2-^{14}C]deoxyuridine into the nucleoside triphosphate fraction suggest that cells treated with methotrexate may contain significant amounts of dUTP. Due to the limited specificity of the dTTP assay, the residual dTTP levels observed following the first 15 min after the addition of methotrexate to S-phase cells (Fig. 4) thus may represent, at least in part, cellular dUTP.

In conclusion, failure of methotrexate to cause a rapid depletion of cellular dTTP may reflect maintenance of thymidine nucleotide pools by cells that are not in S phase and/or uptake by cells of thymidine present in the culture medium. Under appropriate experimental conditions, a decrease of cellular dTTP to near-zero levels was observed within time periods as short as 10–15 min after addition of the inhibitor. Thus, the results presented do not support the assumption of two separate dTTP pools, and are compatible with the hypothesis that the cytocidal action of methotrexate is attributable to depletion of cellular dTTP as substrate for DNA replication.

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