Effects of Thymidine on Deoxyribonucleoside Triphosphate Pools and Deoxyribonucleic Acid Synthesis in Chinese Hamster Ovary Cells*

GUNNAR BJURSELL AND PETER REICHARD

From the Medical Nobel Institute, Department of Biochemistry, Karolinska Institute, S-104 01 Stockholm, Sweden

SUMMARY

Cultured Chinese hamster ovary cells were synchronized by isoleucine starvation. Thymidine was added either during G1 or during S-phase and deoxyribonucleotide pools and the rate of DNA synthesis were measured.

Addition of 1 mM thymidine to G1 cells did not appreciably influence the entry of the cells into S-phase but inhibited the rate of DNA synthesis up to 90%. The pools of dTTP, dGTP, and dATP increased about 2.5, 10-, and 2-fold, respectively, while the dCTP pool decreased to about 10% of the controls. Inhibition of DNA synthesis was completely prevented when 5 μM deoxycytidine was added together with thymidine. The dCTP pool was then almost of normal size while the other three pools were expanded.

Addition of 1 mM thymidine to cells in S-phase gave similar changes in pool sizes and also inhibited DNA synthesis. The decline in the rate of DNA synthesis was correlated in time with the decrease of the dCTP pool. Addition of 5 μM deoxycytidine to thymidine-inhibited cells normalized both DNA synthesis and the dCTP pool without affecting the other three pools.

These results, as well as earlier data concerning pool sizes of deoxynucleoside triphosphates in synchronized cell populations, suggest the possibility that the size of the dCTP pool may have a regulatory function for the rate of DNA synthesis. Moreover, our present results show that the allosteric regulation of purified ribonucleoside diphosphate reductase is applicable to the situation in intact cells.

MATERIALS AND METHODS

Cells—The Chinese hamster ovary cells used were originally supplied by Dr. Robert Tobey, University of California, Los Alamos, New Mexico. They were cultured and synchronized by isoleucine starvation (6) as described previously (7). All manipulations were carried out at 37℃. The cultures were washed three times with prewarmed medium in order to remove exogenous thymidine.

Chemicals—[3H]Thymidine (6.7 Ci per mmole) was obtained from New England Nuclear Corp., tritiated deoxyribonucleoside triphosphates from Amersham, nonlabeled nucleosides and nucleotides from Calbiochem.

Measurements of DNA Synthesis—The fraction of cells synthesizing DNA was estimated by autoradiography in the following way. After incubation of the cells with [3H]thymidine (6.7 Ci per mmole, final medium concentration 1.5 μM) for 30 min, the medium was discarded and the monolayers were washed twice with ice-cold isotonic Tris-HCl buffer, pH 7.4 (9). The cells were then extracted with a solution of 95% ethanol and glacial acetic acid (9:1) and stained with orcein. The film (Kodac Strip plates, AR10) was mounted directly on the Petri dish. After exposure for 4 days at least 1000 nuclei were scored on each plate. A lower specific radioactivity of [3H]thymidine (20 mCi...
per mmole) was used in the experiments with thymidine-inhibited cell cultures. In this case the film was exposed for 30 days.

The rate of DNA synthesis was estimated from the incorporation of [3H]thymidine into DNA. Since we wanted to compare rates of DNA synthesis in the presence of widely differing concentrations of thymidine in the medium it was necessary to determine the specific activities of the dTTP pools. For this purpose cells on parallel 5-cm Petri dishes were incubated with identical concentrations of thymidine. One set of dishes contained nonlabeled thymidine and the cells were used for the determination of the size of the dTTP pool (10). The cells from the other set were incubated with [3H]thymidine and extracted with 60% methanol. Aliquots of the extracts were used for the determination of the total radioactivity of the dTTP pool. For this purpose dTTP was separated from other thymidine phosphates by paper electrophoresis (11). The residue after methanol extraction was used to determine the incorporation of [3H]thymidine into DNA (12). All values were normalized to 1 μg of DNA. The total amount of DNA on each plate was determined by the method of Burton (13).

Breakdown of thymidine to thymine might have complicated the interpretation of our data. However, after 30 hours incubation of cultures with either 0.1 μM or 1 mM [3H]thymidine no formation of [3H]thymine was observed by paper chromatography (14).

Pools of deoxynucleoside triphosphates were determined as described earlier (10, 15).

RESULTS

Tobey et al. (6, 16, 17) showed that starvation of a suspension culture of Chinese hamster ovary cells in an isoleucine-free medium resulted in an accumulation of G1 cells. Addition of isoleucine to such cultures gave rise to a parasynchronously growing population of cells. We found that 48 hours after addition of isoleucine-free medium to monolayers of Chinese hamster ovary cells, less than 1% of the cells was synthesizing DNA as judged by autoradiography. When complete medium was added at this time point (time 0) the cells began to synthesize DNA after a short lag period (Fig. 1). The peak of synthesis occurred reproducibly between 10 and 15 hours at which time about 80% of the cells were in S-phase. This time interval was chosen for most of our experiments.

The effect of different concentrations of thymidine on the synthesis of DNA was determined first. Thymidine was added at time 0 together with complete medium and the increase in the amount of DNA between 0 to 48 hours was determined colorimetrically (Fig. 2). In the controls the amount of DNA increased almost 4-fold. Addition of either 1 or 10 mM thymidine resulted in a strong inhibition of DNA synthesis while 0.1 mM thymidine only showed a limited effect. It is clear, however, that even at the highest thymidine concentration the amount of DNA in the culture increased from 6 to 10 μg during the 48-hour period.

This increase may either have resulted from DNA synthesis occurring at an essentially normal rate in a limited number of cells or from a decreased rate of DNA synthesis occurring in most cells. We used autoradiography to distinguish between these two main alternatives. [3H]Thymidine (20 μCi per mmole) was added at 0.1 μM, 0.1 mM, or 1 mM concentration to isoleucine-starved cultures together with complete medium. After 8 and 14 hours the percentages of labeled nuclei were determined. At all thymidine concentrations approximately 60% of the nuclei were labeled after 8 hours and almost 100% after 14 hours.

Furthermore, it was clear that the control nuclei (0.1 μM thymidine) had incorporated more radioactivity than the nuclei from the thymidine-inhibited cultures. These results indicate that the inhibition of DNA synthesis in cells growing at high concentrations of thymidine is due to a generally decreased rate of DNA synthesis and not a selective inhibition of the synthesis in a fraction of the cells.

Addition of extracellular thymidine greatly expanded the intracellular dTTP pool. This is illustrated by an experiment in which the dTTP pools of synchronized cultures were measured at 10 and 18 hours after addition of different concentrations of thymidine (Fig. 3). Thymidine was added at time 0 together with complete medium. The size of the dTTP pools varied considerably with the concentration of the nucleoside. At the lowest concentration studied (0.1 μM) no expansion occurred. However, 1 μM thymidine caused a 2 fold expansion, and addition of...
Cultures of isoleucine-starved cells were divided into two sets. One set received at time 0 complete medium and 1 mM thymidine, while the other received complete medium and served as a control. After 10 hours the experiment was started by addition of [3H]-thymidine (100 μCi to inhibited cultures and 5 μCi to controls). Parallel dishes were removed 15 to 240 min later and used for measurements of DNA, dTTP pools, and radioactivity in DNA and dTTP. For further details see the text.

Table I shows that in both sets of cultures the specific activities of DNA, dTTP pools, and radioactivity in DNA and dTTP. For further details see the text.

<table>
<thead>
<tr>
<th>Cultures</th>
<th>Time after addition of [3H]-thymidine</th>
<th>dTTP pool</th>
<th>DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>min</td>
<td>pmoles</td>
<td>cpm</td>
</tr>
<tr>
<td>Thymidine-inhibited</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>15</td>
<td>2.1</td>
<td>527</td>
</tr>
<tr>
<td>60</td>
<td>60</td>
<td>2.4</td>
<td>900</td>
</tr>
<tr>
<td>120</td>
<td>120</td>
<td>2.4</td>
<td>874</td>
</tr>
<tr>
<td>240</td>
<td>240</td>
<td>2.5</td>
<td>650</td>
</tr>
</tbody>
</table>

a All values are normalized for a culture containing 1 μg of DNA.

b This value gives the rate of DNA synthesis. It was calculated from the radioactivity of the DNA and the average specific activity of the dTTP pools (2.5 cpm per pmole in the inhibited cultures and 314 cpm per pmole in the controls).

1 and 10 mM thymidine expanded the dTTP pools 25- and 150-fold, respectively.

One major purpose of the present investigation was to correlate changes in the rate of DNA synthesis with variations in deoxyribonucleotide pools caused by the addition of thymidine. Measurements of rates of DNA synthesis were therefore of critical importance. The colorimetric method used in the experiment illustrated in Fig. 2 does not measure small changes in the rate of DNA synthesis, expressed as picomoles of dTMP incorporated per μg of DNA per min, was then calculated as illustrated by the following example. In the control cultures the average specific activity of the dTTP pool was 314 cpm per pmole. The radioactivity incorporated into 1 μg of DNA was 22,599, and 31,320 cpm after 60 and 120 min, respectively. Thus the rate of incorporation between 60 and 120 min was on the average 479 cpm per min. This value corresponds to an incorporation of 479/314 = 1.52 pmol of dTPM per min and μg of DNA. In the controls, the rate of DNA synthesis increased by about 50% between 10 and 14 hours after isoleucine addition, in accordance with the data depicted in Fig. 1. In the thymidine-inhibited cultures no increase was observed and the rate of DNA synthesis was only between 10 and 20% of the controls. The measurements thus confirm the earlier conclusion that DNA synthesis is not completely abolished in the presence of 1 mM thymidine.

We then investigated the effect of 1 mM thymidine added at time 0 on the size of the four deoxyribonucleoside triphosphate pools between 6 and 24 hours (Fig. 4). The initial effect was on the dTTP pool which was increased about 10-fold at 6 hours and 25-fold at 24 hours. The dGTP pool expanded after the dTTP pool was increased 10-fold at 24 hours. The increase in the dATP pool occurred still later and was much smaller. In contrast to these three pools the dCTP pool was greatly diminished. The decrease was apparent at all time points but was most pronounced at 14 hours at which time the dCTP pool of the controls had its maximum. In the thymidine-inhibited cultures the dCTP pool was the smallest pool at 14 hours and later. It should be realized, however, that the size of the dGTP pool (2.0 pmol per μg of DNA) in the inhibited culture was larger than the size of the dGTP pool (0.8 pmole per μg of DNA) in the noninhibited culture.

In the previous experiment thymidine was added simultaneously with isoleucine at time 0 to G1 cells and thus was present when the cells were harvested for DNA synthesis. In the following experiment thymidine was added at 10 hours, i.e. at a time when the majority of cells had already entered G2-phase (cf. Fig. 1) and...
TABLE II

Rate of DNA synthesis after addition and removal of thymidine

The experiment included three sets of isoleucine-starved cultures. The first set received complete medium at time 0 and 1 mM [H]thyridine (100 μCi per culture) at 10 hours. The second set received complete medium and 1 mM nonlabeled thymidine at time 0 and was rinsed free of thymidine after 10 hours. A tracer amount of [H]thyridine (5 μCi per culture, final concentration 0.15 μM) was then added together with complete medium. The third set served as control and received complete medium at time 0 and 5 μCi of [H]thyridine after 10 hours. The rest of the experiment was conducted as described in Table I.

<table>
<thead>
<tr>
<th>Cultures</th>
<th>Time after addition of [H]-thymidine</th>
<th>dTTP pool*</th>
<th>DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>min</td>
<td>pmolcs</td>
<td>cpm</td>
</tr>
<tr>
<td>Thymidine added</td>
<td>15</td>
<td>30</td>
<td>78</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>40</td>
<td>112</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>50</td>
<td>120</td>
</tr>
<tr>
<td></td>
<td>240</td>
<td>96</td>
<td>154</td>
</tr>
<tr>
<td>Thymidine washed away</td>
<td>15</td>
<td>17</td>
<td>68</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>8.4</td>
<td>213</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>8.1</td>
<td>213</td>
</tr>
<tr>
<td></td>
<td>240</td>
<td>5.3</td>
<td>163</td>
</tr>
<tr>
<td>Control</td>
<td>15</td>
<td>1.6</td>
<td>534</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>2.1</td>
<td>720</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>2.4</td>
<td>813</td>
</tr>
<tr>
<td></td>
<td>240</td>
<td>2.8</td>
<td>826</td>
</tr>
</tbody>
</table>

* All values are normalized for a culture containing 1 μg of DNA.

The following average values for the specific activities of the dTTP pools were used in the calculations: 2.7 cpm per pmole for cultures given 1 mM thymidine at 10 hours, 27.5 cpm per pmole for washed cultures, and 324 cpm per pmole for control cultures.

The effects on DNA synthesis and on pool sizes were studied between 10 and 14 hours. As a corollary we also made the reverse experiment in which thymidine was present between 0 and 10 hours and removed by washing at 10 hours.

When thymidine was added at 10 hours a gradual inhibition of DNA synthesis occurred. The inhibition increased between 11 and 14 hours (Table II) but was never as pronounced as when thymidine was present from time 0 (cf. Table I). On the other hand removal of thymidine at 10 hours resulted in a rapid normalization of the rate of DNA synthesis (Table II).

The effects on pool sizes are shown in Fig. 5. Addition of thymidine at 10 hours (Fig. 5A) resulted in changes similar to those found in cultures to which thymidine had been added at time 0. The dTTP and dGTP pools expanded very rapidly while the decrease in the dCTP pool was more gradual.

After removal of thymidine from inhibited cultures the pool changes showed a biphasic appearance (Fig. 5B). During the first 15 min a rapid contraction of the expanded dTTP pool and a similar rapid increase of the dCTP pool occurred, resulting in pools intermediate between normal and inhibited values. The second slower phase lasted for several hours and resulted in the attainment of almost normal pools for all deoxynucleoside triphosphates. Only during this second phase was there any decrease in the expanded dGTP pool. It seems quite possible that the biphasic character of the response depended on our inability to wash out all thymidine from the culture.

A comparison of Table II and Fig. 5A shows that the gradual decrease in the rate of DNA synthesis after addition of thymidine was not correlated in time with the rapid expansions of the dTTP and dGTP pools but might have been connected with the decrease in the dCTP pool. The rapid resumption of DNA synthesis after washing was paralleled by rapid changes in both the dTTP and dGTP pools (Fig. 5B).

We next investigated the effects of deoxycytidine on thymidine-inhibited cells. Addition of 5 μM deoxycytidine at time 0 completely prevented the inhibition by 1 mM thymidine when the increase in the amount of DNA with time was measured colorimetrically (data not given here). The effects on pool sizes are shown in Fig. 6 which gives pool measurements from three sets of parallel cultures treated at time 0 with (a) isoleucine, (b) isoleucine + 1 mM thymidine, and (c) isoleucine + 1 μM thymidine + 5 μM deoxycytidine.

Fig. 6 shows that addition of deoxycytidine together with thymidine had little effect on the dTTP, dGTP, and dATP pools. However, the dCTP pool was increased considerably even though the values at no time amounted to more than 50% of the noninhibited controls. After addition of deoxycytidine the dCTP pool peaked around 14 hours when the majority of the cells were in S-phase.

In a final experiment deoxycytidine (5 μM) was added at 10 hours to thymidine-inhibited cultures and the rate of DNA synthesis and pool sizes were measured between 10 and 14 hours. The data are summarized in Table III and Fig. 7.

The rate of DNA synthesis increased gradually and was normalized after 120 min. With respect to pool sizes (Fig. 7) we only observed major changes in the dCTP pool which increased during the first 120 min. At this time a plateau level was reached which, however, was lower than in the absence of added nucleosides.

DISCUSSION

Our experiments concern the effect of the addition of thymidine to cell populations either in G1 or in S-phase. In both instances...
Fig. 6. Effect of deoxycytidine on deoxyribonucleoside triphosphate pools in the presence of thymidine. Deoxycytidine (5 μM) was added together with thymidine (1 mM) and complete medium at time 0 to isoleucine-starved cells and pools were measured between 10 to 24 hours (∇—∇). Control cultures received either complete medium alone (▲—▲) or complete medium and thymidine (○—○).

Table III
Rate of DNA synthesis after addition of deoxycytidine to thymidine-inhibited cultures

<table>
<thead>
<tr>
<th>Time after addition of [H]thymidine</th>
<th>dTTP pool</th>
<th>DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>min</td>
<td>pmol/mg</td>
<td>cpm/mg</td>
</tr>
<tr>
<td>15</td>
<td>36</td>
<td>97</td>
</tr>
<tr>
<td>60</td>
<td>45</td>
<td>99</td>
</tr>
<tr>
<td>120</td>
<td>57</td>
<td>108</td>
</tr>
<tr>
<td>240</td>
<td>70</td>
<td>148</td>
</tr>
</tbody>
</table>

* All values are normalized for a culture containing 1 μg of DNA.

The addition of thymidine resulted in striking expansions of the dTTP and dGTP pools. The size of the dCTP pool on the other hand was greatly reduced as compared to control cultures. When thymidine was present from the beginning of the experiment, i.e. during G1, the dCTP pool started out very low and had increased about 3-fold after 10 hours, i.e. at a time when the cells were entering S-phase. It did not show the pronounced peak at 14 hours apparent in the noninhibited controls. Similarly, addition of thymidine at 10 hours resulted in a substantial decrease of the size of the dCTP pool.

Are any of these changes in pool sizes connected with the slowing down of the rate of DNA synthesis? The gradual decrease of DNA synthesis after addition of thymidine at 10 hours (Table II and Fig. 5A) is more closely related in time to the gradual decrease in the dCTP pool than to the rapid increase in the dTTP and dGTP pools. More decisive evidence comes from experiments in which the inhibition by thymidine was completely reversed by the simultaneous addition of deoxycytidine. The only effect on pool sizes was a large increase of the dCTP pool. Addition of deoxycytidine at 10 hours to thymidine-inhibited cultures again only led to an increase of the dCTP pool. We conclude that the size of the dCTP pool may be of critical importance for the rate of DNA synthesis. Our data suggest that DNA synthesis was not limited at values above 4 to 8 pmol of dCTP per μg of DNA. This conclusion is based on the results of the “washing experiment” (Table II and Fig. 5B) and the deoxycytidine reversal experiments (Table III and Fig. 7). On the other hand, the rate of DNA synthesis was inhibited by about 90% when the
dCTP pool was 1 to 2 pmol (Table I and Fig. 4). Clearly our data are still very crude and it would in particular be desirable to know the concentration of dCTP at the locus of DNA synthesis or at least to determine the distribution of the nucleotide between the cell sap and the cell nucleus.

Is the size of the dCTP pool connected with the rate of DNA synthesis also during the normal cell cycle? Measurements of the size of the dCTP pool taken place. This was followed by a more gradual increase in the dATP pool. The results were again in accordance with the behavior of the enzyme in the test tube: the initial expansion of the dGTP pool was caused by the enlarged dTTP pool and the secondary increase in the dATP pool was a consequence of the enlarged dGTP pool.

It thus appears that the complicated allosteric regulatory mechanism of ribonucleoside diphosphate reductase observed in vitro indeed is relevant for the situation in intact cells.

REFERENCES
Effects of Thymidine on Deoxyribonucleoside Triphosphate Pools and Deoxyribonucleic Acid Synthesis in Chinese Hamster Ovary Cells
Gunnar Bjursell and Peter Reichard


Access the most updated version of this article at http://www.jbc.org/content/248/11/3904

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/248/11/3904.full.html#ref-list-1