Biochemical Pharmacology, Vol. 31, No. 19, pp. 3107–3113, 1982.
Printed in Great Britain.

0006-2952/82/193107-07 $03.00/0

EFFECT OF HYDROXYUREA AND 5-FLUORODEOXYURIDINE ON DEOXYRIBONUCLEOSIDE TRIPHOSPHATE POOLS EARLY IN PHYTOHEMAGGLUTININ-STIMULATED HUMAN LYMPHOCYTES

GERDA TYRSTED
Department of Biochemistry C, University of Copenhagen, Panum Institute, 2200 Copenhagen N, Denmark

(*Received* 18 *December* 1981; *accepted* 8 *April* 1982)

Abstract—The induction of deoxyribonucleoside triphosphate pools was studied in phytohemagglutinin-stimulated human lymphocytes in the presence of metabolic inhibitors. The dATP pool was completely inhibited in cells treated with hydroxyurea, in contrast to the dTTP pool. However, 1-formylisoquinoline thiosemicarbazone inhibited the formation of these pools equally. During approximately 3 hr of treatment of stimulated cells with hydroxyurea, the dATP, dGTP and dCTP pools were depleted to the base levels observed in the cells before the pools were induced. The base level of the dTTP pool was achieved only in the presence of 5-fluorodeoxyuridine, but the inhibition was completely prevented by addition of thymidine. It is suggested that, when resting lymphocytes were stimulated to enter the growth cycle, the formation of deoxyribonucleoside triphosphates in the early transformation was due to the *de novo* pathway.

Human lymphocytes are normally non-proliferating quiescent cells which can be transformed by phytohemagglutinin [1] and then begin the cell cycle. We have observed very small pools of all four deoxyribonucleoside triphosphates in non-stimulated cells [2, 3] and very low activities of CDP reductase (ribonucleoside diphosphate reductase; EC 1.17.4.1) and thymidine kinase (ATP:thymidine 5′-phosphotransferase, EC 2.7.1.21) [4, 5]. During transformation, the pool sizes and enzyme activities increase dramatically, followed by an increase in cellular DNA content after about 40 hr [4]. Further, we have observed simultaneous increases of DNA polymerase (deoxynucleosidetriphosphate:DNA deoxynucleotidyl transferase, EC 2.7.7.7) and thymidine kinase activities after 15 hr of stimulation. The pool sizes of dTTP and dCTP expand about 2 hr later and those of dATP and dGTP 4 hr later [5]. The incorporation of labeled thymidine into DNA shows a biphasic exponential increase which contrasts with the incorporation of other labeled deoxyribonucleosides [5]. Furthermore, a new type of thymidine kinase appears in stimulated lymphocytes [6]. As a consequence of these observations, it was of interest to study whether cells, when shifted from the resting state into active growth, use the *de novo* pathway or the salvage pathway early in transformation for formation of deoxyribonucleoside triphosphates and dTTP in particular.

To investigate these two biosynthetic pathways, each of them in turn, was blocked with suitable metabolic inhibitors. Hydroxyurea blocks the *de novo* pathway since it exerts its primary biochemical effect by inhibiting ribonucleoside diphosphate reductase [7, 8]. Recent reports indicate that this drug inactivates the enzyme by destroying the free tyrosine radical in the iron-containing B2 subunit [9, 10]. Thus, hydroxyurea is a potent inhibitor of replicative DNA synthesis, due to the depletion of the deoxyribonucleoside triphosphate pools [11, 12], whereas hydroxyurea has little effect on DNA repair synthesis [13].

Similar metabolic effects are produced by some isoquinoline derivatives, such as 1-formylisoquinoline thiosemicarbazone, which inhibit tumor growth, cell proliferation and DNA synthesis [14–16] by inhibiting ribonucleoside diphosphate reductase. However, these two metabolic inhibitors inhibit the enzyme by different mechanism, since 1-formylisoquinoline thiosemicarbazone exerts its effect through its great affinity for iron and other metal ions [16, 17].

To study whether the dTTP pool was synthesized from metabolites in the *de novo* pathway or from thymidine in the salvage pathway, the formation of dTMP from dUMP, as catalyzed by thymidylate synthetase (EC 2.1.1.45), was turned off by the pyrimidine analoque, 5-fluorodeoxyuridine. In the cells, this analogue is phosphorylated to the active enzyme inhibitor, 5-fluorodeoxyuridylate [18, 19].

MATERIALS AND METHODS

Chemicals. Unlabeled deoxy- and ribonucleotides, Dowex-1-Cl (8% cross-linked dry mesh 200–400), potato apyrase (grade 1), hydroxyurea and 5-fluorodeoxyuridine were obtained from the Sigma Chemical Co., St. Louis, MO, U.S.A. Alkaline phosphatase from calf intestine (grade 1) was from Boehringer, Mannheim, West Germany. [^{3}H]dCTP (sp. act. 25.5 Ci/mmole), [^{3}H]dGTP (sp. act. 12 Ci/mmole, [^{3}H]dATP (sp. act. 22 Ci/mmole), [^{3}H]dTTP (sp. act. 30 Ci/mmole, [^{14}C]CDP (sp. act. 436–470 mCi/mmole), [Me-^{3}H]thymidine (sp. act. 2 Ci/mmole), and [6-^{3}H]2′-deoxyuridine (sp. act. 18 Ci/

mmole) were purchased from the Radiochemical Centre Amersham, U.K. Penicillin, streptomycin and Fischer's medium for leukemic cells of mice were purchased from the Grand Island Biological Co., Grand Island, NY (GIBCO). Phytohemagglutinin (PHA-P) was from Difco Laboratories, Detroit, MI. Ficoll was from Pharmacia, Uppsala, Sweden and Isopaque from Nygård & Co., Oslo, Norway.

All unlabeled deoxyribonucleotides were purified as described [4]. The large fragment of *Escherichia coli* DNA polymerase [20], which contains polymerase and 3′-5′-exonuclease activities, was donated by Dr. H. Klenow. 1-Formylisoquinoline thiosemicarbazone was a gift from Dr. A. C. Sartorelli. This inhibitor was solubilized in dimethyl sulfoxide. The highest solvent concentration did not exceed 0.15%. This concentration, added to cells without the inhibitor, did not interfere with formation of deoxyribonucleoside triphosphates, compared to control cells.

Isolation and incubation of human lymphocytes. Peripheral blood was obtained from medical students, and the lymphocytes were isolated by the Isopaque–Ficoll gradient centrifugation technique [2]. The cells were cultured at a cell density of 10^6 cells/ml in donor's serum and Fischer's tissue culture medium [2, 5] and stimulated with phytohemagglutinin as previously described [21].

Other methods. The enzymes were prepared at 4°, and the activities were determined immediately thereafter. The enzyme preparations and the assays for CDP reductase and thymidine kinase activities were carried out as described elsewhere [4, 5].

The pool sizes of deoxyribonucleoside triphosphates were determined by using synthetic copolymers in a reaction catalyzed by DNA polymerase [2, 3]. The rate of DNA synthesis was determined by labeling cell aliquots with [6-^{3}H]2′-deoxyuridine (15 μCi/ml cell suspension, final concentration 8.4×10^{-7} M). After incubation for the indicated times with the isotope, the amounts of DNA and radioactivity were determined in duplicate 5-ml aliquots. DNA was isolated, and radioactivity was measured as described previously [4, 5]. The amount of DNA was determined according to Burton [22] with calf thymus DNA as standard. The cellular DNA content is related to the number of cells initially placed in the cultures at 0 hr

All the determinations of the pool sizes and DNA content were obtained as an average of duplicate samples. The enzyme activities were based on initial velocities determined from four time samples.

RESULTS

Preliminary examinations of the effect of hydroxyurea on stimulated human lymphocytes are shown in Table 1. The CDP reductase activity, DNA content and deoxyribonucleoside triphosphate pools were determined in cells grown in the continuous presence of phytohemagglutinin and hydroxyurea for 28 hr. The calculations were based on an average DNA content of 6.8 μg DNA/10^6 cells. Hydroxyurea treatment caused a decrease of about 70% in CDP reductase activity but it had no effect on cellular DNA content as compared to the control cells. A pronounced difference in the effect of hydroxyurea

Table 1. Effect of hydroxyurea on CDP reductase activity, DNA content and deoxyribonucleoside triphosphate pools in lymphocytes stimulated for 28 hr.

Treatment	CDP reductase* (pmoles/hr per mg DNA)	DNA (μg/10^6 cells)	Deoxyribonucleoside triphosphate content† (pmoles/mg DNA)			
			dTTP	dCTP	dATP	dGTP
None	41.6	6.5	243	44	60	26
10^{-5} M Hydroxyurea	42.7 (103)‡	6.6	251 (103)	47 (107)	53 (88)	27 (104)
10^{-4} M Hydroxyurea	40.9 (98)	7.0	181 (74)	34 (77)	12 (20)	17 (65)
5×10^{-4} M Hydroxyurea	26.4 (63)	6.7	134 (55)	19 (43)	7 (12)	12 (46)
10^{-3} M hydroxyurea	13.3 (32)	7.1	134 (55)	17 (39)	8 (13)	11 (42)

* The average CDP reductase activity in non-stimulated cells was 10 pmoles/hr per mg DNA [4].

† The pool sizes (pmoles/mg DNA) are from stimulated cells before induction of the nucleotides took place [5]. The results are from eight to ten different experiments and N is the number of observations. Preinduction pool sizes (pmoles/mg DNA): dTTP 12.8 (S.D. 4.0, N = 13); dCTP 17.0 (S.D. 4.8, N = 13); dATP 16.1 (S.D. 5.3, N = 11); and dGTP 13.7 (S.D. 2.7, N = 12).

‡ Numbers in parentheses indicate values of the hydroxyurea-treated cells as percentages of the control values.

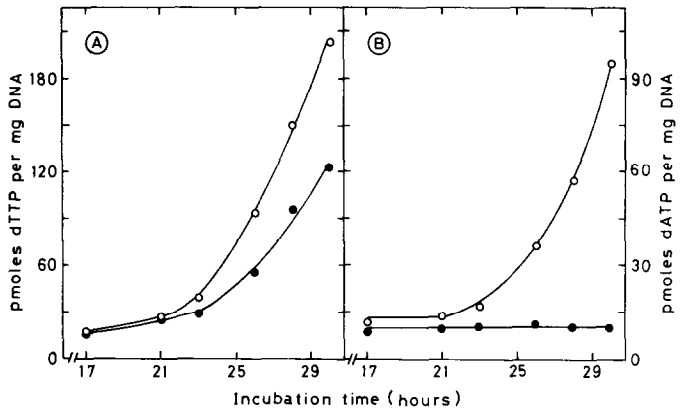

Fig. 1. Effect of 10^{-3} M hydroxyurea on the induction of the dTTP pool (Fig. 1A) and the dATP pool (Fig. 1B) in stimulated cells. The expansion of the pool sizes was measured in control cultures (○ — ○) and in hydroxyurea-treated cultures (● — ●). Hydroxyurea and phytohemagglutinin were added to the cell cultures at 0 hr, and the cells were harvested at the indicated times. The DNA content ($\mu g/10^6$ cells; see Materials and Methods) was 6.9 (S.D. = 0.27, N = 6) in the controls and 6.8 (S.D. = 0.30, N = 6) in the hydroxyurea-treated cells. These mean values were used in the calculations of the pool sizes.

on pool size was observed at concentrations higher than 10^{-5} M, the dATP pool was reduced by about 85% and the other three pools by about 40–60% as compared to the controls. However, the concentrations of dATP and dGTP in the presence of 10^{-4} M hydroxyurea and that of dCTP in the presence of 5×10^{-4} M hydroxyurea were all in the range of the concentrations observed in stimulated cells before expansion of these pools took place (Table 1, footnote), i.e. after stimulation for about 17 hr [5]. This indicates that the increases in the dATP, dGTP and dCTP pools were completely inhibited in the presence of hydroxyurea. In contrast, the dTTP pool was still about ten times higher than that observed in stimulated cells before induction occurred, even in the presence of 10^{-3} M hydroxyurea. It seems likely that 10^{-3} M hydroxyurea inhibited the CDP reductase activity to the base activity, since both the dCTP pool which was reduced to base level and the enzyme activity which was observed in the hydroxyurea-treated cells (Table 1) were of the same magnitude as observed in untreated non-stimulated cells [4].

The different inhibitory effects on accumulation of the various deoxyribonucleoside triphosphates, as observed in stimulated cells cultured for 28 hr in the presence of hydroxyurea, were unexpected. Therefore, the time-courses of both dTTP and dATP formation in stimulated cells were examined in the presence and the absence of 10^{-3} M hydroxyurea (Fig. 1). The dTTP pool (Fig. 1A) began to increase after 17 hr of incubation in the control cells as well as in hydroxyurea-treated cells. After about 23 hr, the accumulation of dTTP was less in hydroxyurea-treated cells than in control cells, resulting in 40% inhibition after 30 hr. However, in contrast to the dTTP pool, the expansion of the

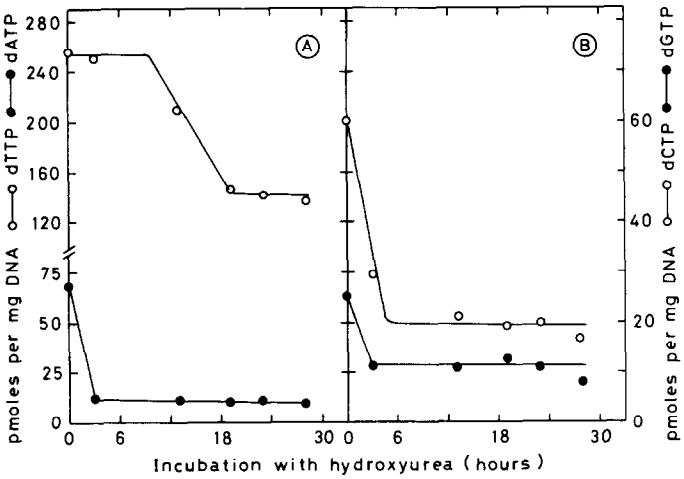

Fig. 2. Effect of 10^{-3} M hydroxyurea on deoxyribo-nucleoside triphosphate pools in stimulated cells. (A) dTTP (○ — ○) and dATP (● — ●) pools. (B) dCTP (○ — ○) and dGTP (● — ●) pools. The DNA content ($\mu g/10^6$ cells) in the control cells was 6.9 (mean of duplicates), and in the hydroxyurea-treated cells it was 6.9 (S.D. 0.21, N = 5). These mean values were used in the calculation of the pool sizes.

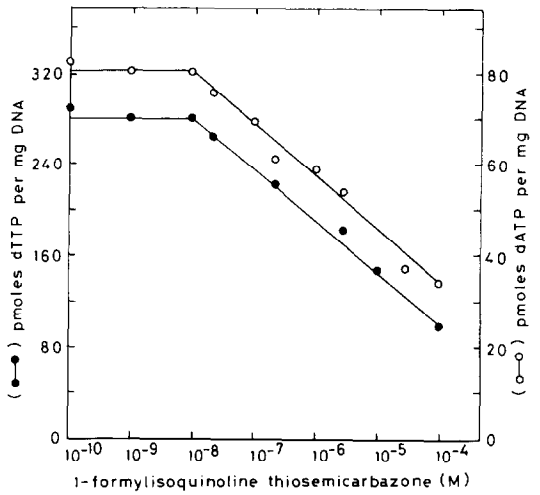

Fig. 3. dATP (○ — ○) and dTTP (● — ●) pools in stimulated lymphocytes in the presence of different 1-formylisoquinoline thiosemicarbazone concentrations. The inhibitor and phytohemagglutinin were added to the cell cultures at 0 hr, and all the cultures were harvested after 28 hr of incubation. The pool sizes in the stimulated cells in the absence of the inhibitor were 83 and 294 pmoles/mg DNA for dATP and dTTP respectively. The DNA content (μg/10^6 cells) in the control cells was 6.6 (mean of duplicates). In the presence of the inhibitor, it was 6.4 (S.D. 0.40, N = 10). These mean values were used in the calculation of the pool sizes.

dATP pool (Fig. 1B) was completely prevented by the presence of hydroxyurea, since dATP remained at the base level whereas an expansion of the pool in the control cells was observed after 21 hr.

To obtain enough of all four deoxyribonucleoside triphosphates for an experiment such as that shown in Fig. 1, more cells were required than could be obtained from one donor. To solve this technical problem, another experiment was designed as shown in Fig. 2. Phytohemagglutinin was added to all the cultures (0 hr), and hydroxyurea (10^{-3} M) was added at different times (0–25 hr) during the transformation period. After 28 hr of incubation all the cultures were harvested. Stimulated cells, incubated in the absence of hydroxyurea, served as control cells. As illustrated, the dATP and dGTP pools (Fig. 2, A and B) were depleted after 3 hr of treatment with hydroxyurea, and the dCTP (Fig. 2B) pools after about 4 hr. All three deoxyribonucleoside triphosphates reached a base level with mean values of 11.1, 10.8 and 19.0 pmoles/mg DNA for the dATP, dGTP and dCTP pools respectively. However, the dTTP pool (Fig. 2A) remained constant during treatment for 3 hr with hydroxyurea while a decrease of 18% in the pool size was observed after 13 hr. The dTTP pool reached a plateau after 19 hr of incubation with hydroxyurea, corresponding to 43% inhibition as compared to the pool in the control cells.

Concerning the dTTP pool, the results obtained so far indicate that it was impossible in the presence of hydroxyurea to deplete this pool to the base level. This could mean that hydroxyurea exerts a differential inhibitory effect on the ribonucleotide reductase system. The use of another enzyme inhibitor such as 1-formylisoquinoline thiosemicarbazone, which operates through a different mechanism, might elucidate this problem. The insufficent inhibition of the dTTP pool (40%) by hydroxyurea could also mean that dTMP, a key metabolite in dTTP formation, is synthesized from both the *de novo* pathway and through salvage of the pyrimidine deoxyribonucleosides. By employing 5-fluorodeoxyuridine as a metabolic inhibitor of dTMP synthetase, only thymidine is utilized in dTTP formation, whereas the utilization of deoxyuridine and deoxycytidine is blocked.

To test these hypotheses, the effect of 1-formylisoquinoline thiosemicarbazone on the dATP and dTTP pools was examined. The inhibitor was added to lymphocyte cultures at the same time as phytohemagglutinin, and all the cultures were harvested after 28 hr of incubation. As illustrated (Fig. 3), both pool sizes remained at control levels in the presence of inhibitor concentrations from 10^{-10} M to 10^{-8} M, whereas at higher concentrations a decrease was observed in both pools. Furthermore, in contrast to the effect of hydroxyurea on the dATP and dTTP pools (Table 1), 1-formylisoquinoline thiosemicarbazone inhibited these pool sizes to the same extent (Fig. 3), since an inhibitor concentration of about 10^{-5} M decreased both pool sizes by 50% in comparison with the control pools. However, it was impossible to deplete the dATP and dTTP pools to base levels in the presence of 10^{-4} M 1-formylisoquinoline thiosemicarbazone, and higher concentrations gave a large variation in cellular DNA content,

Table 2. Effect of 5-fluorodeoxyuridine and thymidine on dTTP pool size and DNA content in lymphocytes stimulated for 28 hr

Treatment with 5-fluorodeoxyuridine	dTTP pool size (pmoles/mg DNA)	DNA content (μg/10^6 cells)
None	139	6.2
10^{-8} M	81 (58)*	6.1
10^{-7} M	41 (29)	6.1
10^{-5} M	11 (8)	6.0
10^{-4} M	5 (4)	6.0
10^{-4} M plus 1 μM Thymidine	64 (46)	5.9
10^{-4} M plus 10 μM Thymidine	121 (87)	6.4
10^{-4} M plus 100 μM Thymidine	284 (204)	6.0

* Numbers in parentheses indicate values of the drug-treated cells as percentages of the control.

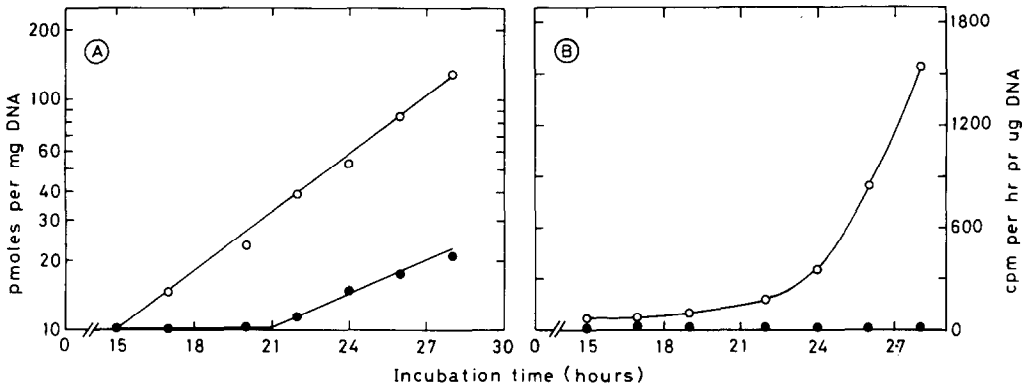

Fig. 4. Effect of 10^{-5} M 5-fluorodeoxyuridine on the induction of the dTTP pool in stimulated cells (Fig. 4A). The expansion of the dTTP pool was measured in the control cultures (○ — ○) and in cultures in the presence of 5-fluorodeoxyuridine (● — ●). In Fig. 4B is shown the incorporation of [6-^{3}H] 2'-deoxyuridine into DNA in the control cultures (○ — ○) and in cultures in the presence of the inhibitor (● — ●). Phytohemagglutinin and 5-fluorodeoxyuridine were added to the cell cultures at 0 hr, and the cells were harvested at the indicated times. The DNA content (μg/10^6 cells) in the controls was 6.3 (S.D. 0.25, N = 7) and in the presence of the inhibitor 6.2 (S.D. 0.27, N = 7). The calculations were performed with these mean values.

indicating cell death. As a consequence of these observations, the contribution of thymidine to the dTTP formation by blocking dTMP synthetase with 5-fluorodeoxyuridine was examined.

The inhibitor, thymidine, and phytohemagglutinin were added at the same time to the cells and all the cultures were harvested after 28 hr of incubation. As it appears from the results in Table 2, the dTTP pool decreased more than 92% in the presence of the inhibitor and reached the dTTP level observed in cells before induction takes place (Table 1 footnote). The combination of 5-fluorodeoxyuridine (10^{-4} M) and thymidine showed that the presence of about 10 μM thymidine in the cultures almost re-established the dTTP pool to control level. An increase of about 2-fold in the pool size was obtained in the presence of 100 μM thymidine. An average DNA content of 6.1 μg/10^6 cells was used in the calculations.

The effect of 5-fluorodeoxyuridine on the dTTP pool was studied in more detail. Thus, the time course of dTTP formation in stimulated cells was examined in the presence and the absence of 5-fluorodeoxyuridine (10^{-5} M). The results are shown on a semilogarithmic scale in Fig. 4A. The dTTP pool increased exponentially in the control cells from 15 hr until 28 hr after stimulation. However, the dTTP pool in cells treated with 5-fluorodeoxyuridine remained at the base level from 15 hr until about 21 hr, followed by an exponential increase in the pool size for the next 7 hr. However, at 21 hr, when the pool size still was on the base level in the 5-fluorodeoxyuridine-treated cells, an enhancement of about 3-fold occurred in the control cells. The dTTP pool increased 13-fold in the control cells from 15 hr until 28 hr while only a 2-fold increase was observed in the presence of the inhibitor. The incorporation of labeled deoxyuridine into DNA is shown in Fig. 4B. A smaller enhancement in the incorporation in the controls during the first 22 hr was followed by a steep increase, whereas the incorporation into DNA was completely blocked in the presence of the inhibitor.

In the experiment presented, long-term incubation was performed in the presence of 5-fluorodeoxyuridine, which might cause minor cell damage. Therefore, the effect of the inhibitor was examined for a shorter period of time. All the cultures were stimulated (0 hr), and 5-fluorodeoxyuridine (10^{-4} M) was added at different times (0–25 hr) during the 28-hr transformation period. After 28 hr of incubation, all the cultures were harvested. Stimulated cells, incubated in the absence of 5-fluorodeoxyuridine, served as controls. The results were plotted on a semilogarithmic scale (Fig. 5) and, as illustrated, the dTTP pool decreased about 11-fold during a 3-hr incubation with 5-fluorodeoxyuridine. Furthermore, in the presence of the inhibitor, the pool size reached a base level with the average of 13.8 pmoles/mg DNA (12.8 to 15.2) corresponding

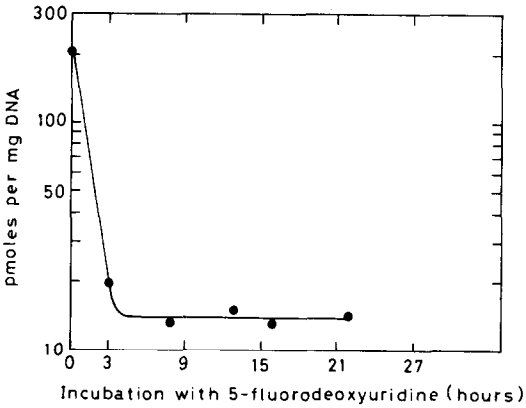

Fig. 5. Effect of 10^{-4} M 5-fluorodeoxyuridine on the dTTP pool in stimulated cells. The DNA content (μg/10^6 cells) in the control cells was 6.8 (mean of duplicates). In the presence of the inhibitor it was 6.6 (S.D. 0.35, N = 5). The calculations were performed with these mean values.

to the base level observed in stimulated cells before induction of dTTP occurs.

DISCUSSION

The variation in the deoxyribonucleoside triphosphate pools in stimulated lymphocyte cultures following exposure to hydroxyurea indicates a differential inhibition of the pool sizes in early transformation. In the presence of 10^{-4} M hydroxyurea for 28 hr, the pool sizes of dATP and dGTP were reduced (Table 1) to the base level observed in stimulated cells before these pools are induced [5]. However, the dCTP pool and CDP reductase activity were only inhibited to base level at hydroxyurea concentrations higher than 10^{-4} M. Thus, in the presence of 10^{-3} M hydroxyurea, the CDP reductase activity was of the same magnitude as the average activity observed in non-stimulated cells [4].

The behavior, however, of the dTTP pool in hydroxyurea-treated cells was quite different in comparison with the three other deoxyribonucleotide pools. Thus, the dTTP pool in cells treated with 10^{-3} M hydroxyurea was always about ten times higher than the base level observed for this pool (Table 1 and Fig. 1A). Furthermore, the expansion of the dTTP pool occurred in hydroxyurea-treated cells at the same time as in the control cells. If it is assumed that the pyrimidine deoxyribonucleosides are not contributing to the formation of the dTTP pool, then this experiment indicates induction of functional reductase activity capable of UDP reduction in the presence of hydroxyurea. In contrast to the dTTP pool, the expansion of the dATP pool was entirely inhibited in the presence of hydroxyurea (Fig. 1B). Similar results were obtained by Walters *et al.* [23] since they found that accumulation of dATP was completely inhibited in cells treated with hydroxyurea (10^{-3} M) in the G_1 phase. We have not been able to measure ADP reductase activity in non-stimulated lymphocytes due to limitation of cell material and very low enzyme activity, but at maximum transformation the ADP reductase activity (unpublished results) was about ten times lower than the CDP reductase activity [4].

The differential inhibition of the formation of dTTP and dATP observed in stimulated lymphocytes treated with hydroxyurea was not observed in cells treated with 1-formylisoquinoline thiosemicarbazone since the pools of dTTP and dATP were inhibited to the same extent (Fig. 3). Similar observations were reported by Sartorelli and coworkers [17], using partially purified ribonucleoside reductase from rodent tumor cells. They observed that the reduction of CDP, UDP and GDP was inhibited to approximately the same extent by 1-formylisoquinoline thiosemicarbazone. Even though the two metabolic inhibitors have ribonucleoside diphosphate reductase as their target enzyme, it is known that different inhibition mechanisms are involved [9, 10, 16, 17], and it seems likely that this might reflect a different effect on the deoxyribonucleoside triphosphate pools. It is noticed that the inhibitory effect of 1-formylisoquinoline thiosemicarbazone is less pronounced in human lymphocytes than in rodent tumor cells [17].

To determine whether the initial expansion of deoxyribonucleoside triphosphates in early transformation is caused by reduction of ribonucleoside diphosphates and not utilization of pyrimidine deoxyribonucleosides, it should be possible to deplete the induced pool sizes to their base levels by hydroxyurea. As shown in Fig. 2, addition of hydroxyurea to 25-hr stimulated cultures (i.e. S-phase cells) resulted in a depletion of dATP and dGTP pools to the base level during 3 hr of incubation with hydroxyurea. Similar results were obtained by Skoog and Nordenskjöld [24] using S-phase mouse embryo cells, since hydroxyurea-treated cells depleted the dGTP pool rapidly and the dATP pool more slowly, whereas the dTTP and dCTP pools were increased. In contrast to Skoog and Nordenskjöld [24], we found that the dCTP pool in stimulated lymphocytes was depleted to the base level in hydroxyurea-treated cells (Fig. 2), whereas it was impossible to inhibit the expansion (Fig. 1) or to deplete the dTTP pool (Fig. 2) in stimulated lymphocytes by hydroxyurea. However, the concentration of dTTP was so low that a feed-back inhibition of CDP reductase is unlikely [4].

The effect of hydroxyurea on the dTTP pool could indicate different mechanisms, such as no turnover of the pool in hydroxyurea-treated lymphocytes as found in mouse cells [24], or differential effect of hydroxyurea on formation of deoxyribonucleoside triphosphates as observed in Chinese hamster cells [23]. However, in stimulated lymphocytes the pyrimidine deoxyribonucleosides, especially thymidine, could contribute to the formation of the dTTP pool since a new type of thymidine kinase is induced in these cells [6].

Therefore, 5-fluorodeoxyuridine was employed for blocking the dTMP synthetase activity. The complete inhibition of labeled deoxyuridine incorporation into DNA in the presence of the inhibitor (Fig. 4B) indicates that dTMP synthetase was inhibited, although an isotopic dilution caused by dUMP accumulation [25] cannot be ruled out. However, the dTTP pool was reduced to the base level in the cells treated with 5-fluorodeoxyuridine, indicating that the formation of this pool did not occur through salvage of thymidine (Table 2 and Fig. 5). The utilization of deoxycytidine and the purine deoxyribonucleosides for the formation of deoxyribonucleoside triphosphates is unlikely or insignificant since the dCTP, dATP and dGTP pools were not maintained in cells treated with hydroxyurea. The utilization of deoxyuridine cannot be ruled out but it seems unlikely that deoxyuridine should be the only deoxyribonucleoside which contributed to the dTTP pool in the early transformation.

The minor increase in the dTTP pool in cells exposed to 5-fluorodeoxyuridine for more than 21 hr (Fig. 4A) is not clear. Cell death and degradation of cellular DNA were not extensive since the DNA content Fig. 4), as well as the dATP pool and thymidine kinase activity determined in the same experiment (results not shown), were similar in the absence and the presence of 5-fluorodeoxyuridine. The fact that the induced pool size of dTTP decreased to the base level in cells incubated for a short period of time (Fig. 5) indicates that neither exogenous nor

endogenous thymidine was present in significant amounts.

In conclusion, we suggest (a) that hydroxyurea, in contrast to 1-formylisoquinoline thiosemicarbazone, exhibits a differential inhibition of the reduction of ADP and UDP and (b) that all four deoxyribonucleoside triphosphates are synthesized from the *de novo* pathway in the early transformation.

Acknowledgements—The author is grateful to Dr. Birgitte Munch-Petersen for valuable comments and review of the manuscript. The skillful technical assistance of Mrs. Lisbeth Cloos is gratefully acknowledged. I wish to thank Miss Birthe Rasmussen for making the drawings. The work was supported in part by grants from Statens lægevidenskabelige Forskningsråd and from Landsforeningen til Kræftens Bekæmpelse.

REFERENCES

1. P. C. Nowell, *Cancer Res.* **20**, 462 (1960).
2. B. Munch-Petersen, G. Tyrsted and B. Dupont, *Expl. Cell Res.* **79**, 249 (1973).
3. G. Tyrsted, *Expl Cell Res.* **91**, 429 (1975).
4. G. Tyrsted and V. Gamulin, *Nucleic Acids Res.* **6**, 305 (1979).
5. G. Tyrsted and B. Munch-Petersen, *Nucleic Acids Res.* **4**, 2713 (1977).
6. B. Munch-Petersen and G. Tyrsted, *Biochim. biophys. Acta* **478**, 364 (1977).
7. I. H. Krakoff, N. C. Brown and P. Reichard, *Cancer Res.* **28**, 1559 (1968).
8. E. C. Moore, *Cancer Res.* **29**, 291 (1969).
9. B. M. Sjøberg, P. Reichard, A. Gräslund and A. Ehrenberg, *J. biol. Chem.* **252**, 536 (1977).
10. P. Reichard, *Fedn. Proc.* **37**, 9 (1978).
11. C. W. Young and S. Hodas, *Scienc* **146**, 1172 (1964).
12. J. E. Cleaver, *Radiat. Res.* **37**, 334 (1969).
13. W. N. Brandt, W. G. Flamm and N. J. Bernheim, *Chem. Biol. Interact.* **5**, 327 (1972).
14. F. A. French and E. J. Blanz, Jr., *J. med. Chem.* **9**, 585 (1966).
15. A. C. Sartorelli, *Biochem. biophys. Res. Commun.* **27**, 26 (1967).
16. A. C. Sartorelli, M. S. Zedeck, K. C. Agrawal and E. C. Moore, *Fedn Proc.* **27**, 650 (1968).
17. E. C. Moore, M. S. Zedeck, K. C. Agrawal and A. C. Sartorelli, *Biochemistry* **9**, 4492 (1970).
18. R. J. Langenbach, P. V. Danenberg and C. Heidelberger, *Biochem. biophys. Res. Commun.* **48**, 1565 (1972).
19. D. V. Santi, C. S. McHenry and H. Sommer, *Biochemistry* **13**, 471 (1974).
20. H. Klenow and K. Overgaard-Hansen, *Fedn Eur. Biochem. Soc. Lett.* **6**, 25 (1970).
21. G. Tyrsted, B. Munch-Petersen and L. Cloos, *Expl Cell Res.* **77**, 415 (1973).
22. K. J. Burton, *Biochem. J.* **62**, 315 (1956).
23. R. A. Walters, R. A. Tobey and R. L. Ratliff, *Biochim. biophys. Acta* **319**, 336 (1973).
24. L. Skoog and B. Nordenskjöld, *Eur. J. Biochem.* **19**, 81 (1971).
25. R. C. Jackson, *J. biol. Chem.* **253**, 7440 (1978).