Thymidine Salvage Pathway in Normal and Leukemic Leukocytes with Effects of ATP on Enzyme Control

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SYNTHESIS of DNA and subsequent cell division is dependent on the availability of all four nucleotide 5'-triphosphate building blocks. The synthesis of thymidine 5'-triphosphate (TTP) has been of particular interest because of the specificity of the thymine base for DNA. Several feedback mechanisms have been demonstrated which affect the pathways of TTP synthesis and, therefore, have a potential for control of DNA synthesis (reviewed by Cleaver). The conversion of thymidine (TdR) to thymidine 5'-phosphate (TMP) by thymidine kinase is thus inhibited by TTP the distal end product of the pathway. Other factors which may affect enzymes in the pathways of TTP synthesis in vitro include the concentrations of Mg++ and of ATP.

Most cells do not require TdR for DNA synthesis, but utilize TMP synthetase to convert deoxyuridine 5'-phosphate (dUMP) to TMP. Cells almost universally, nevertheless, contain TdR kinase which appears to function in a salvage pathway for the reutilization of TdR after the breakdown of cells. This might be especially important in bone marrow for the salvage of DNA from nuclei extruded from RBC as well as from dead leukocytes.

The present study was designed to investigate the relative activities of enzymes (Fig. 1) involved in the metabolism of thymidine in normal and leukemic leukocytes separated on glass bead columns. As the study progressed it became increasingly apparent that the concentration of ATP used in the reactions was a crucial factor in determining the nature of the end products found after incubation of TdR or TMP with cellular enzyme extracts. Special emphasis accordingly was placed on the effects of different ATP concentrations upon the enzyme pathways competing for buildup to TTP and for breakdown to thymine (T) and dihydrothymine (DHT) in an effort to assay the importance of ATP as an adjunct to feedback control mechanisms.

MATERIALS AND METHODS

The Blood. Normal blood was obtained from medical students and laboratory technicians. Leukemic blood was drawn from patients at the Veterans Administration Hospital, Hines, Illinois. This investigation was supported in part by U.S.P.H.S. Research Grant CA06504-08 from the National Cancer Institute.

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Fig. 1.—Enzymes of the thymidine (TdR) salvage pathway which gave evidence of their presence in most of the cells studied:

1. dihydro-uracil dehydrogenase, EC 1.3.1.1
2. thymidine phosphorylase, EC 2.4.2.4
3. thymidine kinase, EC 2.7.1.21
4. thymidine monophosphate kinase, EC 2.7.4.9
5. thymidine diphosphate kinase
6. pyrophosphatase, EC 3.6.1.8
7. 5'-nucleotidase, EC 3.1.3.5

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Leukocyte Collection, Separation and Storage. The separation of normal and leukemic leukocytes on glass bead columns was previously described. Contaminating RBC were removed by hemolysis with 0.83 per cent ammonium chloride or exposure to distilled water for 15 seconds. The cells were washed twice with Hank BSS, quick-frozen, and stored at -98 C.

Enzyme Preparation. Cells were sonicated for 5 minutes in 0.05 M Tris buffer, pH 7.5. Supernatants, after centrifugation at 10,000 g for 20 minutes at 4 C., were used as the enzyme preparations.

Enzyme Incubation. The enzyme reactions were carried out in total volumes of 30 µl, which contained the enzyme preparation and in final concentration: 0.1 M Tris buffer, pH 7.5, MgCl₂ 1.33 mM, K₂HPO₄ 1.33 mM, phosphoenolpyruvate (PEP) (Calbiochem) 2.33 mM, pyruvate kinase (PK) (Calbiochem) 1.5 µg, (c-4.5 EU), ATP 0.67 mM (Sigma), mercaptoethanol 0.0015 µl., and either 0.083 mmoles of TdR-H₃ (Schwarz, Sp. Act. 6 c/mmole), or 2.66 mmoles of TMP-C (Schwarz, Sp. Act. 7.5 mc/mmole).

The tubes were incubated at 37.5 C. and the reactions terminated by immersion in boiling water for 3 minutes. After cooling in ice water the tubes were centrifuged at 10,000 g for 20 minutes at 4 C. The pellets were washed once with 20 µl. of Tris 0.05 M, pH 7.5. The supernatants were combined and brought to a volume of 50 µl. Ten µl aliquots were spotted on Whatman 1 MM filter paper for chromatography in several solvent systems.

Control studies performed with each experimental run consistently showed that no alteration in the nucleotides occurred either in tubes without enzyme carried through the incubation and boiling procedure, or in tubes with added enzyme in which the added reaction was stopped at zero time.

Paper Chromatography. The solvents used were: (A) isobutyric acid, 1N NH₄OH and 0.1 M EDTA (50:30:1); and (B) ethyl acetate, H₂O and formic acid (60:35:5). Ascending chromatography for 16 hours with solvent A separated TTP, TDP, dUMP, TMP, DHT and dU, but gave a single spot for TdR and T. Ascending chromatography for 6 hours with solvent B, on the other hand, separated TdR, T, dU and DHT from the other compounds which migrated together.

Cold standards of these compounds were chromatographed with the samples. DHT was
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identified by spraying the dried chromatogram first with 0.5 N NaOH and then, when dry with p-dimethylaminobenzaldehyde. The other compounds were located with a mineralite ultraviolet lamp.

The spots were cut out and placed into vials with 1 ml. of Hydroxide of Hyamine (Packard) and 19 ml. of scintillation mixture (toluene 1,000 ml., POPOP 50 mg., PPO 2 Cm.) for counting with a Nuclear-Chicago Mark I liquid scintillation counter. All parts of the chromatogram not included in the identifiable spots were also counted so that no major unidentified area of radioactivity could be overlooked. Of approximately 200,000 CPM of TdR-H3, or 24,000 CPM of TMP-C14 added to each reaction tube, almost 100 per cent were recovered.

Protein was determined by the method of Lowry et al. (1951).

RESULTS

The supernatant cell extracts when incubated with TdR-H3 or TMP-C14 gave evidence of the presence of several enzymes involved in the anabolism and catabolism of TdR (Fig. 1). The activities of these enzymes were indicated by the identification of their products with paper chromatography. Results of typical experiments with representative cells of different types are shown graphically in Figures 2–7. Portrayed are the proportions of the end-products of the mixed enzyme reactions found at the time of termination of incubation in each reaction tube. TdR and its breakdown products are shown above zero base line, while the phosphorylated nucleotides are below it. These end products represent the net balance of the conflicting activities of the enzymes present.

Most of the cells studied, both normal and leukemic, showed evidence of the presence of all the enzymes listed in Figure 1, although in different proportions. The end products after incubation thus varied considerably with the cell type present. Cells of the same type usually gave similar results. Disparate results found occasionally appeared to depend on differences in cell size and total protein content. Thus very large blasts of the type used in the experiments shown in Figure 5 gave evidence of much greater TdR kinase activity than did smaller blasts.

Although distinct differences in enzyme content of cells of different types were found, it became apparent that the end products could be altered by changing the incubation conditions, especially the concentration of ATP in the reaction. It was found advantageous to compare the enzyme activities of the different cells at the concentrations of ATP which were optimum for maximum TTP formation. Optimum ATP ranged between 0.67 mM (1×) for small lymphocytes and 6.7 mM (10×) for large blasts and appeared to be a function of cell size and enzyme content.

Lymphocytes (Fig. 2) from a case of chronic lymphatic leukemia (CLL-L) and monoblasts (Fig. 5) demonstrated some findings which were generally applicable to all of the cells studied. The reaction tubes illustrated in Figure 2 were selected from those with concentrations of ATP which gave the maximum per cent of TTP. The optimum ATP concentration was greater with larger cell numbers (i.e., more total enzyme). The amount of TTP formed increased with the number of cells and with time. This was also true in this case for the breakdown product T. Similarly (Fig. 5) an increase in optimum
ATP from 2.7 mM to 6.7 mM occurred when monoblasts were increased from 2 to $9.5 \times 10^6$ cells per reaction tube.

A limiting role for TdR kinase in the salvage pathway may be indicated by the fact that different cell types varied greatly in their production of TTP when the incubations were made with TMP-C$^{14}$ rather than TdR-H$^3$. Normal and leukemic lymphocytes both yielded less TTP from TdR-H$^3$ (Figs. 3A and 4A) than from TMP-C$^{14}$ (Figs. 3B and 4B). In the case of monoblasts (Fig. 5) and myeloblasts (not illustrated) high levels of TdR kinase were suggested by results showing considerable amounts of TTP after incubation with either TdR-H$^3$ or TMP-C$^{14}$. With granulocytes, on the other hand, little or no TTP was formed from TdR-H$^3$ (Fig. 6A), although some was produced from TMP-C$^{14}$ (Fig. 6B). Normal granulocytes thus appeared to have little or no TdR kinase while retaining the enzymes to phosphorylate TMP to TTP. Red blood cells showed no evidence of ability to convert TdR-H$^3$ to T, yet were able to phosphorylate (Fig. 7) more than 40 per cent of TMP-C$^{14}$ to TTP. Low activity of 5'-phosphatase may have contributed to these results, since there was little production of TdR and T from TMP-C$^{14}$ (Fig. 7). In other experiments, however, it was demonstrated that increasing the ATP to $10\times$ (6.7 mM) resulted in an increase in TdR from TMP to 25 per cent.

**Effects of Varied ATP Concentration**

Results obtained with the different cells depended on the proportions of
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N - LYMPHOCYTES

(A) + THYMIDINE-H³ (B) + TMP-C¹⁴

Fig. 3.—Effect of ATP concentration on the distribution of radioactivity after incubation of extracts of 10 × 10⁶ normal lymphocytes with (A) TdR-H³ and (B) TMP-C¹⁴ for one hour.

the enzymes present and the ATP concentration when other factors were kept constant. The results perhaps depended greatly on different effects of ATP on the activities of the constituent enzymes. Maximum TTP production for any ATP concentration was found when Mg concentration was made equimolar with the ATP.

No ATP. In the absence of added ATP and without an ATP-regenerating system maximum production of T occurred, while phosphorylation was negligible with all the cells studied. Without added ATP, but with an ATP-regenerating system some cells with a low optimum ATP level (Figs. 4A and 4B) had sufficient endogenous ATP to permit some TTP production and reduction in the amount of T formed. Lower proportions of T were produced from TMP-C¹⁴ than from TdR-H³ perhaps as the results of the additional phosphatase step and the activities of the competing enzymes.

Sub-optimal ATP. At sub-optimal ATP concentrations TMP tended to persist since TTP production was less than optimum while T was reduced from the levels seen without ATP (Figs. 5A and 5B). In some instances sub-optimal ATP was associated with a greater accumulation of TDP than TTP. Normal lymphocytes (Fig. 3B), for example, showed an accumulation of TDP with 1× (0.67 mM) ATP while optimum TTP production from TMP-C¹⁴ occurred with 4× (2.7 mM) ATP. Such results suggested that a lower ATP concentration was required by TMP than TDP kinase.

Optimum ATP. At optimum ATP concentrations (0.67–2.7 mM) both normal and CLL lymphocytes yielded high proportions of T with only moderate
Fig. 4.—Effect of ATP concentration on the distribution of radioactivity after incubation of extracts of 10 X 10⁶ chronic lymphatic leukemic lymphocytes with (A) TdR-H³ and (B) TMP-C¹⁴ for one hour.

amounts of TTP from either TdR-H³ or TMP-C¹⁴ (Figs. 2–4). These results might be due to either low activity of the anabolic enzymes, or high levels of the catabolic enzymes. Monoblasts (Figs. 5A and 5B), lymphoblasts and myeloblasts (not illustrated) yielded considerably greater proportions of TTP and much less T at optimum ATP concentrations (6.7 mM) than did lymphocytes. High levels of TdR, TMP and TDP kinases in these cells were indicated,
Fig. 5.—Effect of ATP concentration and cell count on the distribution of radioactivity after incubation of extracts of 9.5 × 10⁶ monoblasts with (A) TdR-H³ and (B) TMP-C¹⁴ for one hour.

although low activities of the catabolic enzymes could have contributed to the results obtained. Mature normal granulocytes, on the other hand, produced little or no TTP from TdR-H³ while yielding high proportions of both T (76 per cent) and DHT (11.2 per cent) after one hour incubation in a typical run. Results (Fig. 6A and 6B) of incubation of PMN with TMP-C¹⁴ as the initial compound differed considerably from those with TdR-H³. At optimum ATP (2.7 mM), 10 × 10⁶ cells and one hour incubation (not illustrated) 59 per cent of radioactivity was found in TTP and only 11.4 per cent in T.

High ATP. All the cells studied showed reduction and finally inhibition of synthesis of TTP from either TdR-H³ or TMP-C¹⁴ when ATP concentrations were progressively increased above optimum levels. Inhibition persisted when Mg⁺⁺ in the reactions was increased to levels which were equimolar with the ATP. The amount of ATP which inhibited TTP production was higher (Fig. 5B) in cells with high optimum ATP levels than in those with low optimum ATP levels (Figs. 3B, 4B and 7). The concentration of ATP needed to either enhance or inhibit TTP production appeared to be related to cell size, cell protein, cell type and the level of enzyme activity in the cell.

Progressive reduction in the amount of T produced from TdR with increased ATP is shown in Figures 4A, 5A and 6A indicating that TdR phosphorylase is also inhibited by high ATP levels.

Persistence of TMP in the reactions was promoted by high ATP levels (Figs. 3, and 5-7). Results such as those shown in Figures 3A and 4A suggested that TMP and TDP kinases were inhibited by lower concentrations of ATP than was TdR kinase, since production of TTP decreased appreciably at 10× (6.7 mM) ATP while TMP production from TdR continued. Persis-
Fig. 6.—Effect of ATP concentration on the distribution of radioactivity after incubation of extracts of 10 × 10⁶ normal granulocytes with (A) TdR-H³ and (B) TMP-C¹⁴ for 5 minutes.

tence of TMP with high ATP levels perhaps represented a combination of inhibition of 5'-nucleotidases and of the kinases phosphorylating TMP.

TdR kinase required high levels of ATP for inhibition (Figs. 3A, 4A, 5A and 6A). Monoblasts extracts (Fig. 5A) with their high TdR kinase content continued to produce TMP from TdR even with 40× ATP. CLL-L (Fig. 4A) with lower enzyme levels did show considerable reduction in TdR kinase activity at high ATP concentrations.

Effects of Added 5'AMP, Cyclic 3'5'AMP, GTP, or TTP on the Enzyme Activities

A series of experiments were done with additions to the reactions of several reagents which have been shown to exert controlling effects on enzymes of nucleotide metabolism or on other enzymes. Typical results were obtained when these reagents were added to reaction tubes containing extracts from 10 × 10⁶ CLL-L cells (from same donor as used for experiments shown in Figs. 4A and 4B) and incubated for one hour at 37 C.
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**Fig. 7.—Effect of ATP concentration on the distribution of radioactivity after incubation of extracts of 10 × 10⁶ RBC with TMP-C¹⁴ for one hour.**

**TTP.** Addition of 0.1 mM TTP to tubes with ATP at a concentration of 2.7 mM resulted in a reduction of TTP-H³ produced from TdR-H³ from 11.8 per cent to 0.5 per cent while the counts in TMP + TDP + TTP decreased from 20.4 per cent to 3.0 per cent. End product or feedback inhibition of TdR kinase by TTP was thus indicated. When the initial radioactivity was TMP-C¹⁴, TTP production fell from 32.2 per cent to 23.7 per cent. The lesser production of TTP-C¹⁴ in this case was perhaps due to block of resynthesis of TMP after dephosphorylation to TdR.

**Cyclic 3'5'AMP.** Addition of cyclic 3'5'AMP in concentrations of 0.1 mM or 1.0 mM to tubes containing 2.7 mM or 6.7 mM ATP did not alter the results seen in the control tubes.

**5'AMP.** Addition of AMP in increasing concentrations, in the absence of the ATP-regenerating-system, to tubes containing 2.7 mM ATP resulted in a progressive fall in TTP produced from TdR-H³ from 9.9 per cent without added AMP to 0.3 per cent with 13.4 mM AMP, while TMP increased from 4.6 to 10.4 per cent and T decreased from 60.0 to 44.1 per cent. When AMP was increased from zero to 13.4 mM with TMP-C¹⁴ as the initial radioactivity, TTP decreased from 25.2 to 0.1 per cent, TdR increased from 9.8 to 20.4 per cent, T decreased from 13.2 to 6.1 per cent, while residual TMP increased from 37.9 to 62.1 per cent. These changes resembled those obtained when ATP was correspondingly increased in concentration. These results might be due to AMP acting at the same allosteric sites as ATP or as a competitive inhibitor with ATP, or to a controlling influence of altered AMP/ATP ratio.

**GTP.** Addition of 2.7 mM GTP instead of ATP to the complete reaction gave results with either TdR-H³ or TMP-C¹⁴ which indicated only a limited ability to replace ATP in the reactions. Results obtained were similar to those at sub-optimal concentrations of ATP.
Discussion

Study of cells separated on glass-bead columns permitted differences in enzyme content of individual cell types to become apparent. The ability of most of the cells studied, including RBC, to phosphorylate TMP was consistent with the findings of Bianchi, Farina et al. TdR kinase activity was negligible in mature granulocytes and RBC, low in normal lymphocytes and CLL-L but very high in all types of blast-cells. Marsh and Perry had shown that CLL-L had a high TdR phosphorylase content. We found that this was also true for normal lymphocytes and granulocytes. Since these cells also produced large amounts of T from TMP it was evident that they also had considerable 5'-nucleotidase activity. Blast-cells, on the other hand, appeared to have relatively low levels of TdR phosphorylase and of phosphatase, since little T was produced from either TdR or TMP except in the absence of ATP. High levels of TdR kinase with low phosphatase and TdR phosphorylase activity found in blast-cells was in keeping with findings in other actively dividing cells, while low TdR kinase activity with high levels of TdR phosphorylase and phosphatase activity, on the other hand, was observed in cells which were either not dividing (lymphocytes), or lacked a potential for division (granulocytes). Such findings have been interpreted as indicating that these enzymes, particularly phosphatase and TdR kinase have a potential for control of DNA synthesis by limiting the amount of TTP produced.

Our results showed that the ATP concentration available could modify greatly the end products obtained with enzyme extracts from the different cell types. Influence of ATP concentration on the activities of enzymes of the TdR salvage pathway has been noted previously. There appears to be a close relationship between ATP effects and Mg concentration. Brookes found that equimolar Mg and ATP gave optimum TdR kinase activity. High concentrations of ATP inhibited TdR kinase, but the block was removed by the addition of Mg in concentration equimolar to the ATP. Ives et al. reported that feedback inhibition of TdR kinase by TTP was reduced by high Mg-ATP concentrations. Both Mg and ATP are required for production of TTP from TMP. This was clearly demonstrated in our results in control tubes without addition of ATP, or without added Mg++ but with EDTA. In our experiments TdR kinase was less effectively inhibited by high ATP than were TMP and TDP kinases. Addition of equimolar Mg failed to block the inhibition of TTP production from TMP by high concentrations of ATP.

Ives et al. suggested that increased phosphatase activity found in the absence of ATP resulted from the presence of free Mg++ unchelated by ATP. With Chang liver cells Eker found that optimum Mg concentration for phosphatase activity was 5 mM, while excess Mg was inhibitory. In our experiments increased phosphatase activity in the absence of ATP was evident, since it was an essential step in the increased breakdown of TMP to T which was observed. In experiments with large molar excesses of ATP over Mg reduced 5'-phosphatase activity was shown by the accumulation of TMP. In our control experiments dephosphorylation of TMP ceased when Mg++
was chelated with EDTA, but activity of TdR phosphorylase with production of T from TdR continued.

Adenosine nucleosides may serve as metabolic regulators since they have the ability to activate or inhibit a number of enzymes (reviewed by Atkinson\textsuperscript{29,30}). Inhibitory or stimulatory effects of ATP, ADP, AMP, or cyclic 3'5'-AMP perhaps depend on the modification of the affected enzyme at allosteric sites.\textsuperscript{29,31} In some instances nucleosides have antagonistic effects (i.e., ATP inhibits phosphofructokinase, while AMP stimulates it\textsuperscript{30}). Changes in ATP/AMP ratio may thus be regulatory.\textsuperscript{29} In our preliminary experiments, however, ATP/AMP ratios were not clearly shown to be important in control mechanisms in the TdR salvage pathway. High concentrations of AMP were inhibitory, perhaps acting at the same inhibitory sites as ATP. ADP was able to replace ATP to an extent in the reactions. This occurred without an ATP regenerating system.

The end products obtained after incubation with cell extracts of different types represented the net balance of the activities of the several enzymes present. It was shown that in these complex enzyme systems changes in concentrations of certain small molecules particularly ATP could affect the enzyme balance and alter the end products formed, probably by affecting the individual enzymes in different ways. These results suggested that changes in ATP concentration could exert a controlling influence in the TdR salvage pathway. ATP functions in these reactions to supply energy, as a coenzyme, as a phosphate donor, as an enzyme activator and as an enzyme inhibitor.\textsuperscript{5,29,30} These activities are in some instances in conflict with each other even with respect to the same enzyme. The concentration of ATP appears to determine which function will predominate. Changes in ATP levels in cell loci may thus serve to control thymidine metabolism and thereby regulate DNA synthesis.

**Summary**

The enzymes of the “salvage” pathway for thymidine in normal and leukemic leukocytes separated on glass-bead-columns were studied. Levels of thymidine kinase activity in blast cells were high, but were low in granulocytes, normal and leukemic lymphocytes, and in RBC. Phosphatase and thymidine phosphorylase activity was high in granulocytes and in normal and leukemic lymphocytes, but became apparent in blast cells only in the absence of ATP. All the cells studied could phosphorylate TMP to some extent, but the amount of TTP formed depended greatly on the presence of an optimum ATP concentration. Without added ATP breakdown to thymine was at its peak, while phosphorylation of TMP was negligible. Excess ATP reduced or inhibited the activity of most of the enzymes, preventing both phosphorylation and breakdown. Concentrations of Mg equimolar with the ATP gave maximum TTP production, while inhibition by high ATP concentrations occurred despite equimolar Mg concentrations. Both TTP and thymine production ceased in the absence of Mg. Changes in ATP levels in cell loci may function in regulating thymidine metabolism.
SUMMARIO IN INTERLINGUA

Esseva studiate le enzymas del circuito de "salvage" pro thymidina in leucocytos normal e leucemic, separate in coluna de perlas de vitro. Le nivello de activitate de kinase de thymidina in blastocytos esseva alte. In granulocytos, in lymphocytos normal e leucemic, e in erythrocytos, del altere latere, illos esseva basse. Le activitate de phosphatase e de phosphorylase de thymidina esseva alte in granulocytos e in lymphocytos normal e leucemic, sed in blastocytos illo deveniva apparente solo in le absentia de triphosphato de adenosina. Omne le cellulas studiate esseva capace a phosphorylar monophosphato de thymidina usque ad un certe grado, sed le quantitate de triphosphato de thymidina formate dependeva grandemente del presentia de un concentration optime de triphosphato de adenosina. Sin le addition de triphosphato de adenosina, le decomposition ad in thymina esseva maximal, durante que le phosphorylation de monophosphato de thymidina esseva neglectibile. Le presentia de un excesso de triphosphato de adenosina reduceva o inhibeva le activitate del majoritate del enzymas, prevenue le phosphorylation e le decomposition. Concentrationes de magnesium equimolar con le triphosphato de adenosina resultava in un production maximal de triphosphato de thymidina, sed le inhibition causate per alte concentrationes de triphosphato de adenosina occurreva mesmo in le presentia de concentrationes equimolar de magnesium. Le production tanto de triphosphato de thymidina e de thymina cessava in le absentia de magnesium. Alterationes del nivello de triphosphato de adenosina in locos cellular exerce possibilemente un function in le regulation del metabolismo de thymidina.

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REFERENCES


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