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Effects of Nucleoside Triphosphates on Human Ribonucleotide Reductase from Molt-4F Cells[1]

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ABSTRACT

The effects of nucleoside triphosphates on various nucleoside diphosphate reductions catalyzed by a highly purified ribonucleotide reductase from Molt-4F cultured human cells were examined. It was found that deoxyadenosine 5′-triphosphate strongly inhibited all four reductions. The reduction of pyrimidine nucleoside diphosphate in the presence of an activator [adenosine 5′-triphosphate (ATP)] was inhibited in a noncompetitive manner with respect to ATP by deoxyguanosine 5′-triphosphate (dGTP) and deoxythymidine 5′-triphosphate (dTTP). For cytidine 5′-diphosphate reduction, the value of the K_i intercept for dGTP was 47 μM and for dTTP, it was 270 μM; the K_i slope was 25 μM for dGTP, 100 μM for dTTP. Similarly, for uridine diphosphate reduction, the K_i intercept was 4.3 μM for dGTP, 25 μM for dTTP. The K_i slope was 1.5 μM for dGTP and 9 μM for dTTP. The reduction of ADP in the presence of its activator (dGTP) was inhibited noncompetitively by dTTP. The values of K_i intercept and slope of dTTP for adenosine 5′-diphosphate (ADP) reduction were 1.8 and 0.9 mM, respectively. Although guanosine 5′-triphosphate (GTP) and dGTP were found to serve equally well as activators for ADP reductions with the same apparent K_a and V_{max}, the inhibition pattern of GTP and dGTP on the enzyme activity for cytidine 5′-diphosphate reduction was different. ATP was found to be an accessory activator for ADP reduction due to the fact that ATP at 1.0 or 0.3 mM concentration decreased the apparent K_a of GTP for ADP reduction from 1.1 to 0.14 or 0.08 mM, respectively. In the absence of ATP, the V_{max} for ADP reduction was increased 2-fold. ATP at a concentration of 1.0 or 0.3 mM also changed the apparent K_a of dTTP for guanosine 5′-diphosphate reduction from 1.25 to 0.9 or 0.6 μM, respectively, but V_{max} for guanosine 5′-diphosphate reduction was increased.

GTP at a concentration of 0.3 or 0.5 mM decreased the apparent K_a's of ATP for pyrimidine nucleoside reduction. At these concentrations of GTP, the V_{max} for cytidine 5′-diphosphate reduction was decreased 7 and 12%, and the V_{max} for uridine 5′-diphosphate reduction was decreased 33 and 45% when they were compared with those in the absence of GTP. Therefore, a change in any of these nucleotide concentrations could lead to changes in ribonucleotide reductase activity. ATP emerges as a most important factor in controlling the reduction of all four ribonucleoside diphosphates.

INTRODUCTION

The specificity of ribonucleotide reductase obtained from bacterial sources has been reported to be strongly influenced by different nucleoside triphosphates (1, 10, 11, 16). Reduction of pyrimidine ribonucleotides catalyzed by the enzyme system from *Escherichia coli* B was stimulated by ATP and dTTP. GDP reduction was stimulated by dTTP, and ADP reduction was stimulated by dGTP (10, 11). dATP strongly inhibited all 4 reductions. Moreover, CDP and UDP inhibited the reduction of each other in the enzyme system from *E. coli* B (10). The regulation of the reduction of ribonucleotides to deoxyribonucleotides has also been described for enzyme obtained from mammalian systems (12–14). The reduction of pyrimidine nucleoside diphosphates required ATP as activator for the enzyme system from Novikoff tumor (12). Reduction of ADP and GDP required, respectively, dGTP and dTTP as activators. Reduction of pyrimidine ribonucleoside diphosphates was inhibited by dATP, dGTP, dTTP, and dUTP, reduction of GDP was inhibited by dATP and dGTP, and reduction of ADP was inhibited by dATP. Similar results have been obtained with the enzyme of rat embryos (13) and chick embryo (14), and calf thymus (9).

The regulation of the reduction of ribonucleotides to deoxyribonucleotides by the enzyme obtained from human Molt-4F cells has now been studied (3). In this communication, we have further studied the effects of nucleoside triphosphates on the enzyme from this cell line.

MATERIALS AND METHODS

Materials. All the materials used were same as those described in the preceding paper (5).

Preparation of the Enzyme Components. Molt-4F ribonucleotide reductase was prepared as described previously (4). All of the studies reported here were performed using the reconstituted enzyme from the components after the final step of purification (phenyl Sepharose for Component A and sucrose density gradient centrifugation for Component B).

Enzyme Assays. CDP reductase was assayed by the method of Steeper and Steuart (15), and ADP reductase activity was determined by the method of Cory *et al.* (8). The details for assaying ADP, CDP, UDP, and GDP were described in the preceding paper (5). Each experiment was repeated at least 3 times, and assays were done in duplicate.

Protein Determination. Protein concentrations were determined by the method of Bradford (2). Bovine serum albumin was used as the standard.

RESULTS

Effects of Nucleoside Triphosphates on CDP, UDP, ADP, and GDP Reductions in the Presence of the Best Activators.

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The effects of various nucleoside triphosphates at a concentration of 2.5 mM on the enzyme activities for CDP, UDP, ADP, and GDP reductions in the presence of the best activators are shown in Table 1. In the absence of nucleoside triphosphates, except for the best activators, the enzyme activities for all 4 reductions were determined and set as 100%. dATP was the most potent inhibitor among those nucleoside triphosphates tested. It inhibited the enzyme activities for all 4 reductions at a concentration of 2.5 mM. When the concentration of dATP was at 0.25 mM, as shown in Table 1 (numbers in parentheses), dATP was still the most potent inhibitor. dTTP inhibited the reduction of CDP, UDP, and ADP; dGTP inhibited the reduction of pyrimidine nucleoside diphosphates. However, ATP at a concentration of 2.5 mM slightly stimulated the reduction of GDP in the presence of the best activator, dTTP. Although GTP and dGTP served equally well as activators for ADP reduction, dGTP was a more potent nucleoside triphosphate than was GTP as an inhibitor of CDP reductase activity.

Different Effects of GTP and dGTP on the Enzyme Activity for CDP Reduction. The effects of GTP and dGTP on the enzyme activity for CDP reduction were different. The inhibition pattern of these nucleoside triphosphates is shown in Chart 1. When the concentration of GTP and dGTP was varied, and the enzyme activity for CDP reduction in the presence of activator (ATP) was measured, it was observed that dGTP at a concentration higher than 0.1 mM gave more than 50% inhibition of the enzyme activity. However, GTP at a concentration of between 0.1 and 0.3 mM slightly increased the enzyme activity. Inhibition was seen at GTP concentrations higher than 1.0 mM.

Inhibition Constants for dGTP on the Enzyme Activity for CDP and UDP Reductions. The inhibition constants for dGTP on the enzyme activity for pyrimidine nucleoside diphosphate reduction are shown in Chart 2. The pattern of inhibition and kinetic constants are determined according to the definition of Cleland (7). The concentration of ATP was varied for CDP or UDP reduction at several fixed concentrations of dGTP. By means of double reciprocal plots, it was demonstrated that dGTP behaved as a noncompetitive inhibitor with respect to ATP for CDP (Chart 2*A*) and UDP reduction (Chart 2*B*). The values of K_ii and of K_is for dGTP for CDP reduction were calculated based on the replot and gave the values of 47 and 25 μM, respectively (Chart 2*A*). The values of K_ii and of K_is for dGTP for UDP reduction were 4.3 and 1.5 μM, respectively (Chart 2*B*).

Table 1

Effects of various nucleoside triphosphates on ribonucleotide diphosphate reductions catalyzed by ribonucleotide reductase derived from Molt-4F cells in the presence of the best activator

The concentrations used for the activator are all at 2.5 mM. The best activator for CDP and UDP reduction is ATP; for ADP reduction, it is GTP; for GDP reduction, it is dTTP. The enzyme activity for CDP, UDP, ADP, and GDP reduction was 85, 110, 110 and 92 pmol/hr, respectively.

NTP (2.5 mM)	CDP reduction[a]	% of activity UDP reduction[a]	ADP reduction[a]	GDP reduction[a]
None	100	100	100	100
ATP	100	100	ND[b]	130
UTP	68	ND	100	119
GTP	63	25 (79)	100	ND
CTP	ND	1 (52)	92	103
dATP	2 (28)[c]	0 (15)	0 (9)	42
dUTP	52 (100)	1 (44)	86	118
dTTP	31 (67)	0 (26)	26 (85)	111
dGTP	10 (30)	0 (15)	100	95
dCTP	70	22 (80)	100	104

[a] The purified Components A and B are used.
[b] ND, not determined.
[c] Numbers in parentheses, percentage of activity produced by various NTP at 0.25 mM concentration.

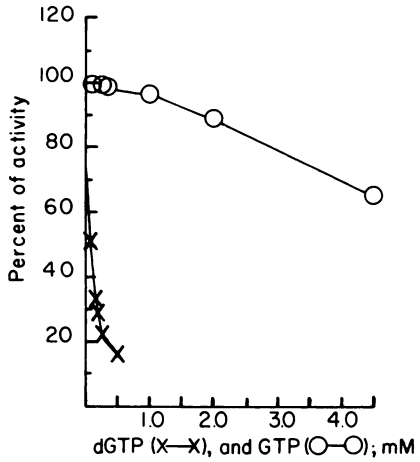

Chart 1. Effects of GTP and dGTP on the enzyme activity for CDP reduction. Standard incubation conditions for CDP reduction were used. Each reaction mixture contained 14 μg of purified Component A and 12 μg of purified Component B of ribonucleotide reductase obtained from Molt-4F cells.

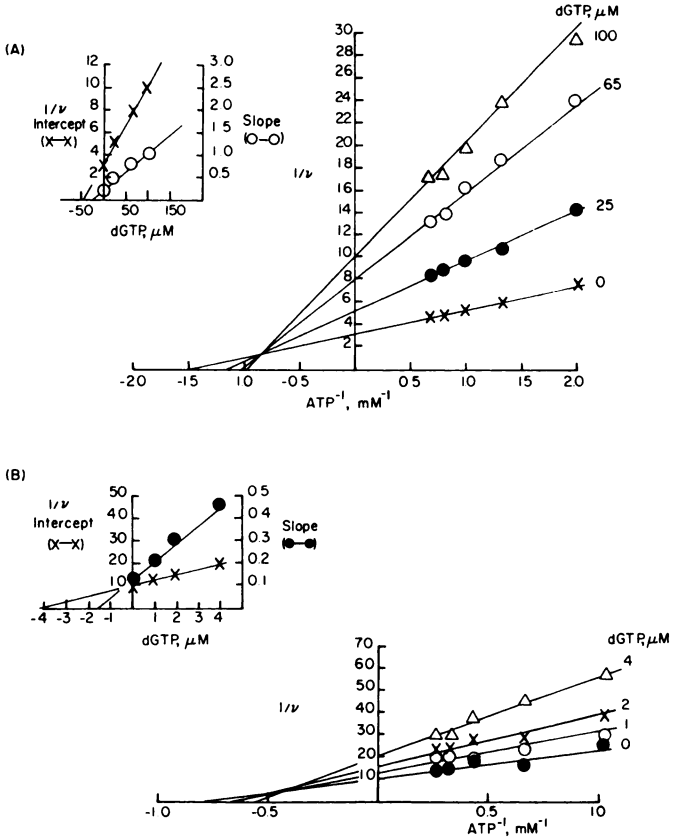

Chart 2. Effects of dGTP on the Lineweaver-Burk plots of the reciprocal of activator concentration with respect to the reciprocal of reaction velocity (nmol of deoxyribonucleotides produced in 1 hr). Standard incubation conditions were used except for addition of activator at the indicated concentration. Each assay contained 14 μg of purified Component A and 12 μg of purified Component B of ribonucleotide reductase obtained from Molt-4F cells. *A*, reduction of CDP; *B*, reduction of UDP.

Inhibition Constants for dTTP on the Enzyme Activities for CDP, UDP, and ADP Reduction. Results of an examination of inhibition by dTTP of the enzyme activities for CDP, UDP, and ADP reduction are shown in Chart 3. dTTP inhibited these reductions noncompetitively with respect to the activators (*i.e.*, ATP as activator for CDP and UDP reduction or dGTP as activator for ADP reduction). The values of K_i intercept and of K_i slope of dTTP with respect to dGTP for ADP reduction were 1.8 and 0.9 mM (Chart 3*C*). The values of K_i intercept and of K_i slope of dTTP with respect to ATP for CDP reduction were 270 and 100 μM (Chart 3*A*). The values of K_i intercept and of K_i slope of dTTP with respect to ATP for UDP reduction were 25 and 9 μM (Chart 3*B*). These K_i's of dTTP for UDP reduction were the lowest among those of inhibition constants of dTTP on these reductions tested.

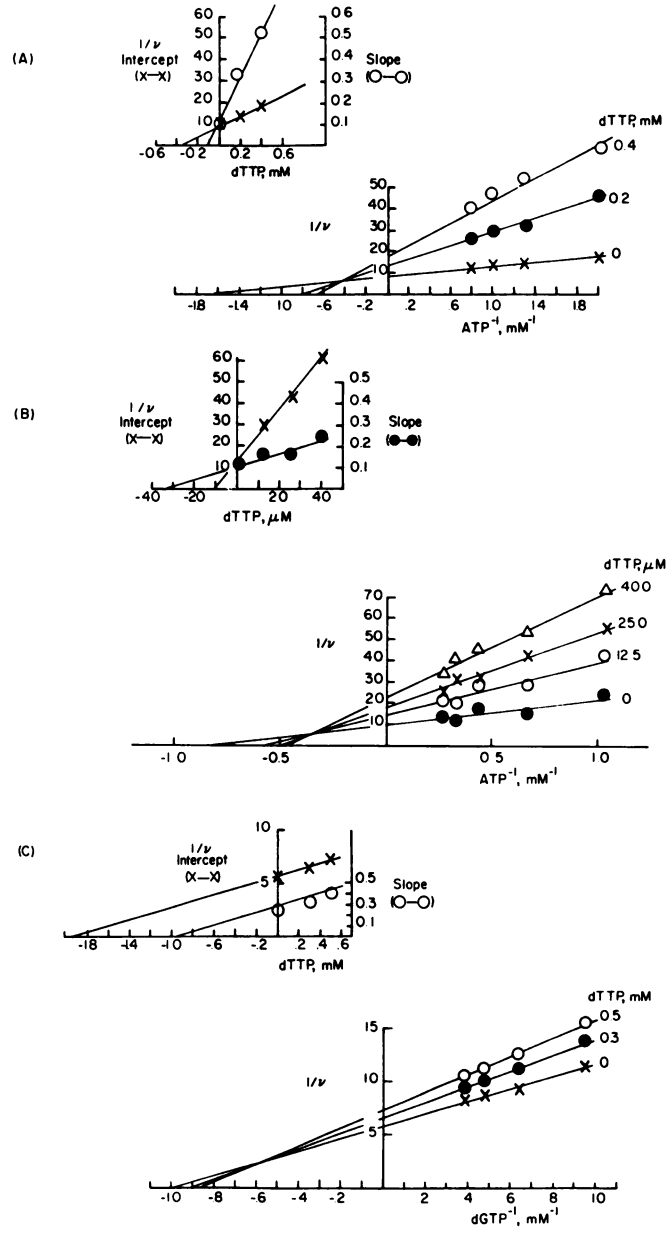

Chart 3. Effects of dTTP on the Lineweaver-Burk plots of the reciprocal of activator concentration with respect to the reciprocal of reaction velocity (nmol of deoxyribonucleotides produced in 1 hr). Standard incubation conditions were used except for addition of activator at the indicated concentration. Each assay contained 14 μg of purified Component A and 12 μg of purified Component B of ribonucleotide reductase obtained from Molt-4F cells. *A*, reduction of CDP; *B*, reduction of UDP; *C*, reduction of ADP.

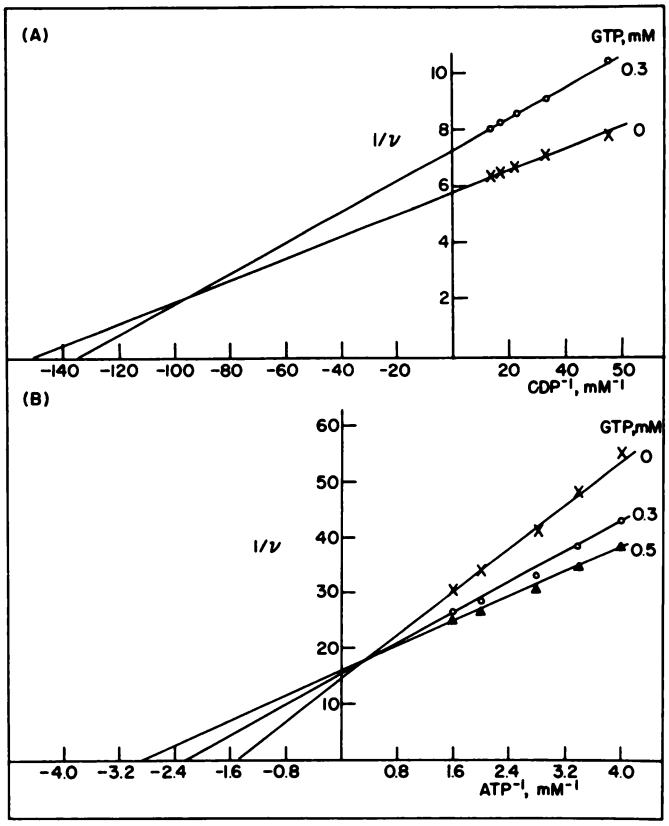

Chart 4. Effects of GTP on the kinetic constants of (*A*) substrate (CDP) and (*B*) activator (ATP) for CDP reduction. The unit of reaction velocity is nmol of deoxyribonucleotides produced in 1 hr. Standard incubation conditions were used except for (*A*) substrate and (*B*) activator concentration. Each assay contained 14 μg of purified Component A and 12 μg of purified Component B of ribonucleotide reductase obtained from Molt-4F cells.

Effects of GTP on the Kinetic Constants of Substrates and Activators for Pyrimidine Nucleoside Diphosphate Reduction. It was shown in Chart 1 that GTP at a concentration less than 0.5 mM slightly stimulated the enzyme activity for CDP reduction. This observation led us to study the influence of GTP on the kinetic constants of CDP and of ATP for CDP reduction. The results are shown in Chart 4. GTP at a concentration of 0.3 mM did not change the K_m and V_{max} of CDP for CDP reduction substantially (Chart 4*A*). However, GTP at a concentration of 0.3 or 0.5 mM decreased the apparent K_a of ATP from 0.63 ± 0.03 (S.E.) mM to 0.45 ± 0.05 or 0.35 ± 0.02 mM, respectively (Chart 4*B*). The V_{max} for CDP reduction was also decreased by GTP at those concentrations tested.

Since ATP is the common activator for CDP and UDP reduction, the effects of GTP on apparent K_a of ATP and K_m of UDP for UDP reduction were also examined. The results are shown in Chart 5. As was the case for CDP reduction, GTP did not change the K_m of CDP or the V_{max} for CDP reduction substantially (Chart 5*A*). However, GTP at a concentration of 0.3 or 0.5 mM significantly decreased the apparent K_a of ATP from 1.25 ± 0.10 to 0.27 ± 0.01 and 0.3 ± 0.01 mM, respectively (Chart 5*B*). The V_{max}'s for UDP reduction were also decreased.

Effects of ATP on the Kinetic Constants of Substrates and

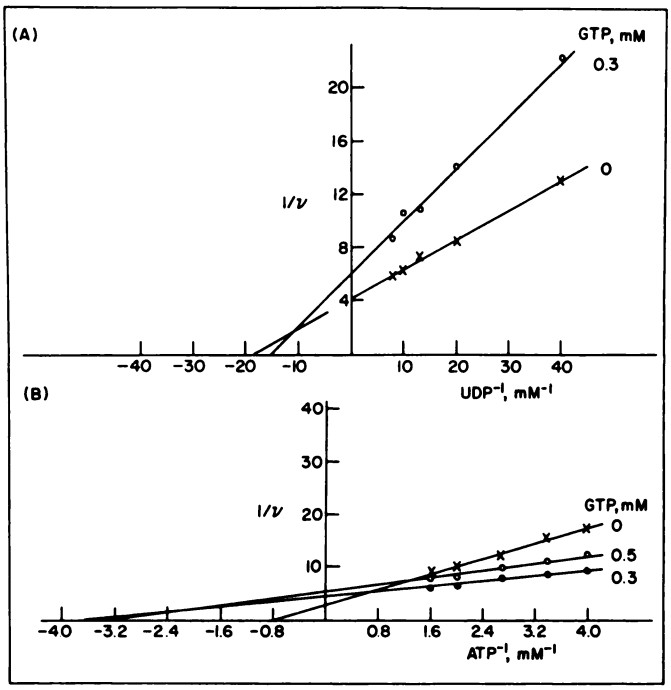

Chart 5. Effects of GTP on the kinetic constants of (*A*) substrate (UDP) and (*B*) activator (ATP) for UDP reduction. The unit of reaction velocity is nmol of deoxyribonucleotides produced in 1 hr. Standard incubation conditions were used except for (*A*) substrate and (*B*) activator concentration. Each assay contained 14 μg of purified Component A and 12 μg of purified Component B of ribonucleotide reductase obtained from Molt-4F cells.

Activators for Purine Nucleoside Diphosphate Reduction. ATP slightly stimulated GDP reduction in the presence of the activator, dTTP, as shown in Table 1. The effects of ATP on GDP reduction in the presence of dTTP were further studied based on the change of the kinetic constants of GDP and dTTP for GDP reduction. The results are shown in Chart 6. ATP at a concentration of 1 mM produced a small decrease in the K_m's of GDP but increased the V_{max} for GDP reduction (Chart 6*A*). However, ATP at a concentration of 0.3 or 1 mM changed the apparent K_a of dTTP from 1.25 ± 0.11 to 0.6 ± 0.1 or 0.9 ± 0.05 μM, respectively, but V_{max}'s for GDP reduction were increased by those concentrations of ATP tested (Chart 6*B*).

ATP at a concentration of 1 or 0.3 mM also changed the apparent K_a of GTP for ADP reduction from 1.1 ± 0.09 to 0.14 ± 0.04 or 0.08 ± 0.01 mM, respectively, as shown in Chart 7*B*, a result similar to the effects of ATP on GDP reduction. However, the V_{max}'s were decreased by the addition of ATP at those concentrations tested. ATP at a concentration of 1 mM did not decrease the K_m of ADP for ADP reduction as shown in Chart 7*A*.

DISCUSSION

Several reports have indicated that ribonucleotide reductase from *E. coli* and from mammalian cells is highly regulated by nucleoside triphosphates (1, 10–14). The effects of nucleoside triphosphates on the enzyme activity of human Molt-4F cells in the presence of the best activator for each specific reduction were investigated. The reduction of pyrimidine nucleoside diphosphates was inhibited by dATP, dGTP, and dTTP; the reduction of ADP was inhibited by dATP and by dTTP; the reduction of GDP was inhibited by dATP. The results obtained are similar to those using the enzyme system from Novikoff tumor (12), rat embryo (13), and chick embryo (14). A summary of the inhibition constants of dATP, dGTP, and dTTP on each ribonucleoside diphosphate reduction is shown in Table 2. They all behaved as noncompetitive inhibitors with respect to the activator of the reduction studied. Some of the inhibitors studied could act also as activators for other ribonucleoside diphosphate reductions, and this suggested that the activator for reduction of a given ribonucleoside diphosphate may not bind to the enzyme at the same site or to the enzyme in the same conformation as it does when acting as inhibitor.

We observed that GTP and dGTP served equally well as activators for ADP reduction. The apparent K_a of GTP or dGTP for ADP reduction was the same, 1.1 mM (4). However, the effects of GTP and dGTP on the enzyme activity for CDP reduction with ATP as the activator were different. dGTP was a more potent inhibitor than GTP for the enzyme activity of CDP reduction. This suggests that the binding behaviors of GTP and dGTP to the enzyme are not the same. Different nucleoside diphosphates change the binding behavior of dGTP, as indicated by the observations that the K_i of dGTP for UDP reduction is much lower than the K_i of dGTP for CDP reduction. In the

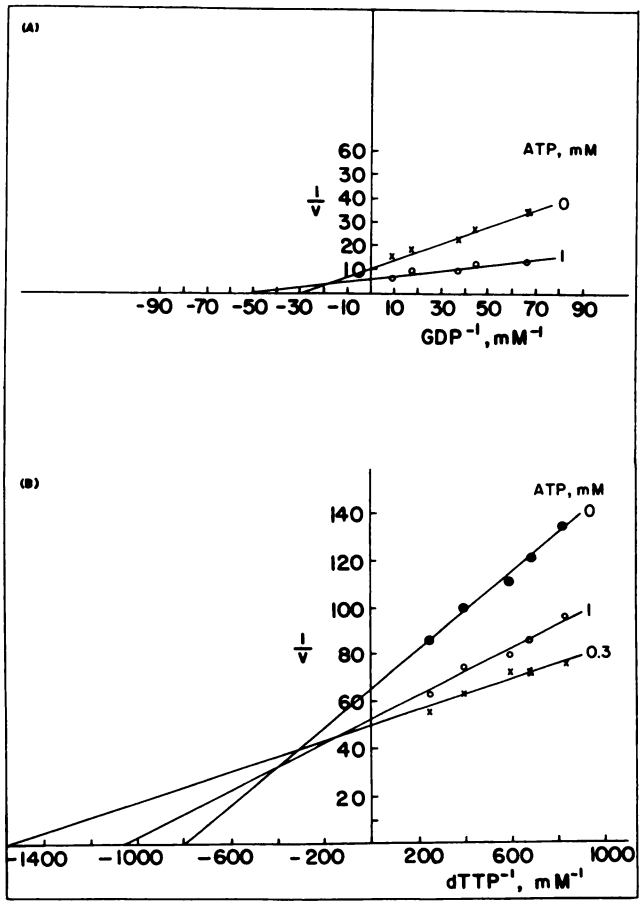

Chart 6. Effects of ATP on the kinetic constants of (*A*) substrate (GDP) and (*B*) activator (dTTP) for GDP reduction. The unit of reaction velocity is nmol of deoxyribonucleotides produced in 1 hr. Standard incubation conditions were used except for (*A*) substrate and (*B*) activator concentration. Each assay contained 14 μg of purified Component A and 12 μg of purified Component B of ribonucleotide reductase obtained from Molt-4F cells.

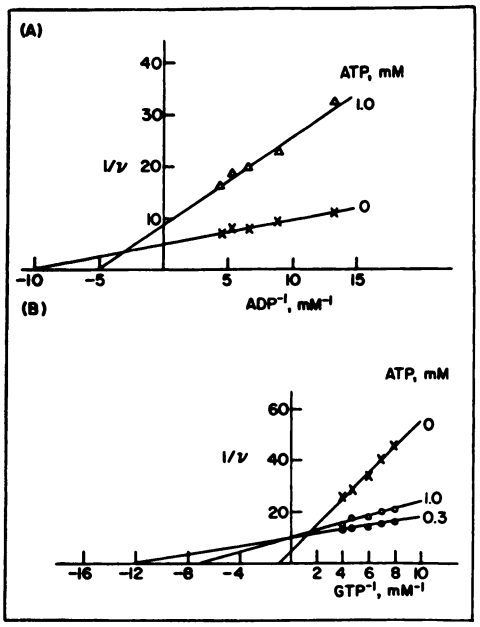

Chart 7. Effects of ATP on the kinetic constants of (*A*) substrate (ADP) and (*B*) activator (GTP) for ADP reduction. The unit of reaction velocity is nmol of deoxyribonucleotides produced in 1 hr. Standard incubation conditions were used except for (*A*) substrate and (*B*) activator concentration. Each assay contained 14 μg of purified Component A and 12 μg of purified Component B of ribonucleotide reductase obtained from Molt-4F cells.

Table 2

The inhibition constants of various deoxynucleoside triphosphates on ribonucleoside diphosphate reductions catalyzed by ribonucleotide reductase from Molt-4F cells in the presence of excess amount of substrate

The kinetic constants (K_i's of intercept and slope) are obtained by the replots of intercepts and slopes *versus* inhibitors as shown in Charts 2 and 3.

		Inhibitor (μM)					
		dTTP		dGTP		dATP	
Substrate	Variable activator	K_{is}[a]	K_{ii}	K_{is}	K_{ii}	K_{is}	K_{ii}
CDP	ATP	100	270	25	47	40	40
UDP	ATP	9	25	1.5	4.3	55	55
GDP	dTTP					1500	1500
ADP	dGTP	900	1800			4	4

[a] K_{is}, K_i of slope.
[b] K_{ii}, K_i of intercept.

reduction of CDP and UDP, the binding of dGTP to the enzyme is affected by the presence of ATP since the value of the K_i intercept is different from that of the K_i slope. The influence of the binding behavior of nucleoside triphosphates by other nucleoside triphosphates or diphosphates also is evident in the observations that the same nucleoside triphosphate has different K_i slopes and K_i intercepts for different ribonucleoside diphosphate reductions (Table 2).

Moore and Hurlbert (12) have reported that ATP at a lower concentration (about 0.15 mM) activates the reduction of GDP when dTTP is also present. In this laboratory, it was also found that ATP at a higher concentration (2.5 mM) slightly stimulated the enzyme activity for GDP reduction in the presence of dTTP, as shown in Table 1. Results of a kinetic study revealed that ATP increased the V_{max} and decreased the apparent K_a of dTTP for GDP reduction. Moreover, ATP not only decreased the apparent K_a of dTTP for GDP reduction, but it also decreased substantially the apparent K_a of GTP for ADP reduction. The apparent K_a of GTP was 1 mM, which is higher than the concentration of GTP (about 0.3 mM) in cells (6). The addition of ATP at 0.3 or 1 mM concentration, which is a physiological concentration, decreased the apparent K_a for GTP to a physiological level of about 0.08 or 0.14 mM, respectively. These results indicate that ATP may serve as an accessory activator to lower the apparent K_a of GTP for ADP reduction within the cell. Although the apparent K_a of dTTP for GDP reduction is within physiological concentration, the addition of ATP to decrease K_a and increase V_{max} of dTTP for GDP reduction appears to be required to drive the reduction more efficiently. Moreover, the addition of GTP also decreased the apparent K_a of ATP. Although the apparent K_a's of ATP for pyrimidine nucleoside diphosphate reduction are within the intracellular concentration range, it seems that GTP also served to decrease the apparent K_a of ATP for CDP and UDP reduction to accomplish more efficient reduction of the substrate. The decrease of apparent K_a's of activators can be considered as a consequence of potentiation by ATP or GTP of the binding affinity of activators for the enzyme. Results obtained in this study support the concept that intracellular ribonucleotide reductase activity is regulated by the steady state level of various nucleotides. A change in any of these nucleotide concentrations could lead to changes in ribonucleotide reductase activity. ATP emerges as an important factor in controlling the reduction of all 4 ribonucleoside diphosphates.

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