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Substrate Specificity of Human Ribonucleotide Reductase from Molt-4F Cells[1]

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ABSTRACT

Nucleoside triphosphates were examined as the activator for various nucleoside diphosphate reductions catalyzed by a highly purified ribonucleotide reductase obtained from Molt-4F cultured human cells. It was found that cytidine 5′-diphosphate and uridine diphosphate reductions are activated by adenosine 5′-triphosphate with apparent K_a's of 0.63 ± 0.03 (S.E.) and 1.25 ± 0.10 mM, respectively. Guanosine 5′ diphosphate reduction is activated by deoxythymidine 5′-triphosphate with an apparent K_a of 1.25 ± 0.11 μM, and adenosine 5′-diphosphate reduction is activated by guanosine 5′-triphosphate or deoxyguanosine 5′-triphosphate with an apparent K_a of 1.1 ± 0.09 or 1.1 ± 0.08 mM, respectively. In the presence of saturating amounts of their best activating nucleoside triphosphates, the K_m's of various nucleoside diphosphates for this purified enzyme were studied. Double reciprocal plots of velocity against substrate concentration were found to be linear for all four substrates in the concentration range tested and yielded apparent K_m's of 7 ± 0.3 μM for cytidine 5′-diphosphate, 80 ± 6.5 μM for adenosine 5′-diphosphate, 33 ± 3.1 μM for guanosine 5′-diphosphate, 50 ± 2.0 μM for uridine 5′-diphosphate. The reduction of one ribonucleoside diphosphate could be inhibited by other ribonucleoside diphosphates in a noncompetitive manner.

INTRODUCTION

Ribonucleotide reductase is the enzyme responsible for catalyzing the reduction of ribonucleotides to the corresponding deoxyribonucleotides. The kinetic behavior of the enzyme has been investigated with highly purified preparations from *Escherichia coli* (14, 15) and *Lactobacillus leichmanii* (1, 23). In contrast, the kinetic behavior of the enzyme obtained from mammalian systems could not be fully studied due to the difficulty involved in purification. However, the regulation of the partially purified enzyme obtained from Novikoff rat tumor (17), rat embryo (18), chick embryo (20), leukemic leukocytes (13), and Chinese hamster ovary cells (16) has been reported; the results obtained with these enzymes were similar. The activators required for the enzyme derived from *E. coli* (14, 15) are not completely the same as the enzyme obtained from mammalian systems; for instance, dTTP is the activator for pyrimidine nucleoside diphosphate reductase of *E. coli* but not for that enzyme obtained from mammalian sources (13, 17, 18). However, irrespective of its sources, the activity of the enzyme is controlled by nucleoside triphosphates acting as allosteric effectors.

Differences have been observed in properties of ribonucleotide reductase derived from various human sources (3). The highly purified enzyme recently obtained from Molt-4F cells (4) is composed of 2 components (Components A and B), and the CDP, UDP, ADP, and GDP reductase activities are associated throughout the entire purification procedure. We have studied the kinetic behavior of the enzymes with highly purified preparations which are devoid of contamination by interfering enzyme activities.

MATERIALS AND METHODS

Materials. The sodium salts of nucleoside diphosphates and nucleoside triphosphates dithiothreitol and *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid were all purchased from Sigma Chemical Co., St. Louis, Mo. Ammonium salts of all ^{14}C-labeled nucleotides were supplied by Amersham/Searle Corp., Arlington Heights, Ill. Dowex 1-Cl^- was obtained from Bio-Rad Laboratories, Richmond, Va. All materials required for cell cultures were from Grand Island Biological Co., Grand Island, N. Y. All other chemicals were of reagent grade.

Preparation of the Enzyme Components. The methods for culturing Molt-4F cells and the purification of Components A and B of this ribonucleotide reductase were described previously (4). The final preparations of Components A and B are not homogeneous as judged by electrophoretic techniques. However, they were purified to such an extent that nucleotide phosphatases and nucleoside diphosphate kinases which would interfere with kinetic studies of the enzyme were not present in the purified Components A and B. All the studies were performed by using the reconstituted enzyme from the components after the final step of purification (phenyl Sepharose for Component A and sucrose density gradient centrifugations for Component B).

Enzyme Assays. CDP reductase was assayed by the method of Steeper and Steuart (22) with the use of Dowex 1-borate ion-exchange chromatography. Under "standard conditions," the assay mixture contained, in a final volume of 0.2 ml, [^{14}C]CDP (0.2 μCi; 0.15 mM), DTT (3 mM), $MgCl_2$ (6 mM), ATP (5 mM), and a specified amount of the enzyme. ADP reductase activity was determined by the method of Cory *et al.* (10). Under standard conditions, the assay mixture contained, in a final volume of 0.2 ml, [^{14}C]ADP (0.4 μCi; 0.3 mM), DTT (3 mM), $MgCl_2$ (6 mM), dGTP (5 mM), and a specified amount of the enzyme. An enzyme sample heated for 2 min in a boiling water bath prior to the addition of the labeled substrate served as the reaction blank. The incubation was at 37° for 60 min, and the reaction was linear with respect to time and enzyme concentration during this incubation period. UDP and GDP reductions

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were determined by a similar way as for ADP and CDP, but with some modifications. Under standard conditions, the assay mixture contained in a final volume of 0.2 ml: [^{14}C]UDP or [^{14}C]GDP (0.3 μCi; 0.15 mM); DTT (3 mM); $MgCl_2$ (6 mM); ATP or dTTP (5 mM), and a specific amount of the enzyme. After termination of the reaction by heating for 3 min in a boiling water bath, 50 μl of dUDP or dGDP (15 mg/ml), 75 μl of a solution containing 10 mM $MgCl_2$ and 5 mM Tris-Cl (pH 8.8), and 1.2 mg of crude snake venom from *Crotalus adamanteus* were added. The reactions were stopped by heating after an incubation of at least 2.5 hr at 37°. Water (0.6 ml) was added to each assay tube, and the tubes were mixed well and centrifuged at 1200 × *g* for 15 min. The supernatant was loaded on Dowex 1-borate column (60 x 5 mm for UDP reduction and 120 x 5 mm for GDP reduction) and allowed to drain. The column was then washed with 45 mM sodium borate (pH 9.2) for UDP reduction or washed with a mixture containing 95% alcohol:5 M ammonium acetate (pH 7.5):60 mM sodium borate:0.1 M EDTA (10:0.2:5:0.1) for GDP reduction. Each 2-ml fraction (Fractions 5 to 11 for deoxyuridine and Fractions 4 to 8 for deoxyguanosine as shown in Chart 1) was collected in a scintillation vial and scintillation fluid (ACS) was added for measuring the radioactivity. The absorbance of the effluent was taken spectrophotometrically at 260 nm. Each experiment was repeated at least 3 times, and assays were done in duplicate.

The results of UDP and GDP reduction assays by the Dowex 1-borate column method are shown in Chart 1. Deoxyuridine elutes between fifth and 11th fractions (Chart 1*A*). Deoxyguanosine elutes between fourth and eighth fractions (Chart 1*B*). Recovery of deoxyuridine and deoxyguanosine is more than 90%.

Protein Determination. Protein concentration was 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. The effects of various nucleoside triphosphates at a concentration of 5 mM on CDP, UDP, ADP, and GDP reductions are shown in Table 1. In the absence of

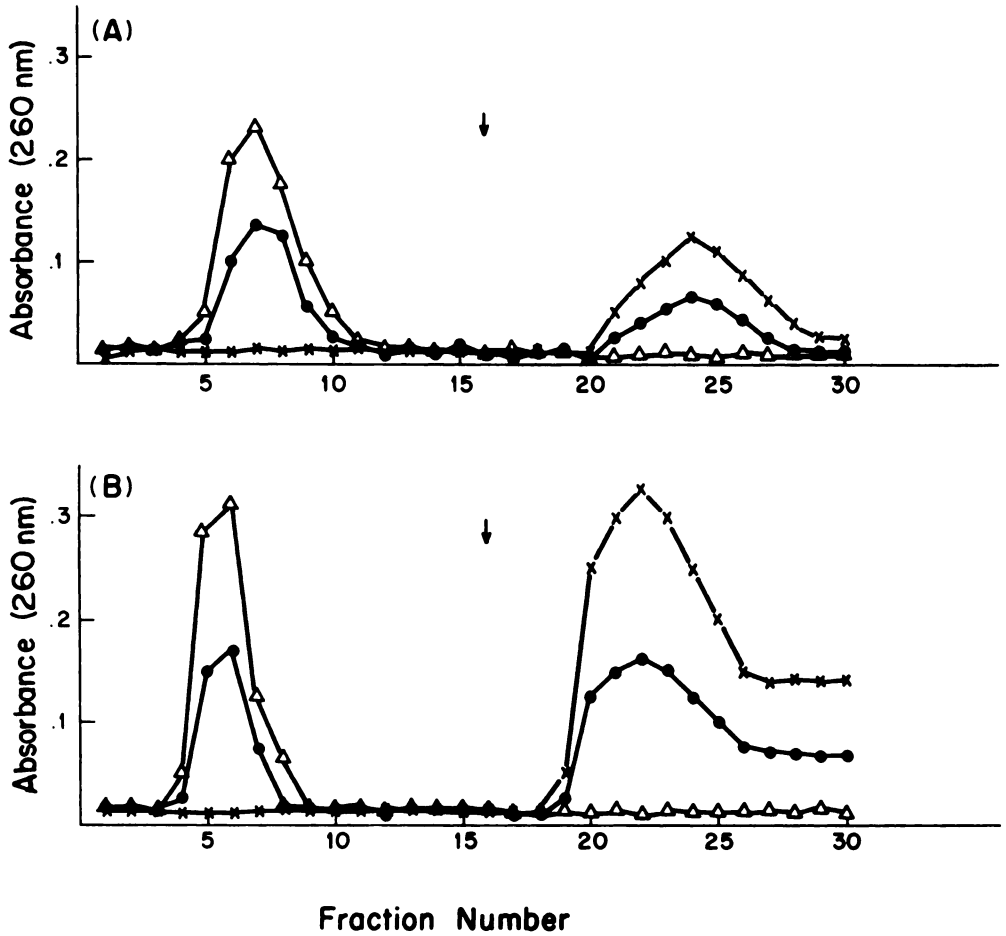

Chart 1. *A*, separation of uridine and deoxyuridine on Dowex 1-borate columns. The Dowex 1-borate column was prepared as described in "Materials and Methods." Deoxyuridine (△) at 0.2 nmol, uridine (×) at 0.2 nmol and a mixture (●) of uridine and deoxyuridine at 0.1 nmol each were applied to the column. The column eluted with 45 mM sodium borate and then with saturated sodium borate (*arrow*). Fractions (2 ml/fraction) were collected, and the absorbance of the effluent was measured at 260 nm. Uridine came off in Fractions 20 to 30; *B*, separation of guanosine and deoxyguanosine on Dowex 1-borate columns. Deoxyguanosine (△) at 0.2 nmol, guanosine (×) at 0.6 nmol, and a mixture (●) of deoxyguanosine (0.1 nmol) and guanosine (0.3 nmol) were applied to the column. The column eluted with a mixture containing 95% alcohol:5 M ammonium acetate (pH 7.5):60 mM sodium borate: 0.1 M EDTA (10:0.2:5:0.1) and then with a mixture containing 95% alcohol:5 M ammonium acetate (pH 7.5):60 mM sodium borate:0.1 M EDTA (10:2:5:0.1) (*arrow*). Fractions (2 ml/fraction) were collected, and the absorbance of the effluent was measured at 260 nm. Guanosine came off in Fractions 20 to 30.

Table 1

Effects of various nucleoside triphosphates on ribonucleoside diphosphate reductions catalyzed by ribonucleotide reductase derived from Molt-4F cells

NTP (5 mM)	Substrates[a] (pmol/hr)			
	CDP	UDP	ADP	GDP
None	11	8	0	4
ATP	185	110	7	25
UTP	18	ND[b]	10	42
GTP	29	0	110	ND
CTP	ND	0	6	23
dATP	6	0	3	3
dUTP	29	0	24	87
dTTP	15	0	11	92
dGTP	35	0	110	79
dCTP	47	10	6	40

[a] The purified components, A and B, of ribonucleotide reductase were used. The amount of enzyme used gave an activity of 185 pmol/hr for CDP reduction.
[b] ND, not determined.

an activating nucleoside triphosphate, there was a minimal reduction of each of the substrates, and no activity was detected for ADP reduction. CDP and UDP reductions were found to be best activated by ATP. For ADP reduction, GTP and dGTP were found to be the best activators, whereas dTTP, dUTP, and dGTP all can serve as the activators for GDP reduction.

Initial Velocity Studies with Respect to Substrate. Initial velocity studies were performed for each substrate reduction at a saturating level (5 mM) of its best activator. The results are shown in Chart 2. When the purified reconstituted enzyme is used, linear plots are obtained in the concentration range studied when the reciprocal of reaction velocity is plotted against the reciprocal of the initial substrate concentration. The apparent K_m for ADP calculated from 3 experiments is 80 ± 6.5 μM (S.E.). The apparent K_m for GDP is 33 ± 3.1 μM. The apparent K_m's of CDP and UDP calculated from 3 experiments are 7 ± 0.3 and 50 ± 2.0 M, respectively.

Kinetic Constant of the Best Activator for Various Ribonucleoside Diphosphates Reduction. The K_a's of the best activators were determined by using the purified reconstituted enzyme in the presence of 0.15 mM ribonucleoside diphosphate substrates except ADP with 0.3 mM (Chart 3; Table 2). An apparent K_a of GTP for ADP reduction is 1.1 ± 0.1 mM, and the K_a of dGTP for ADP reduction is almost the same as that of GTP. For GDP reduction, the apparent K_a of dTTP is 1.25 ± 0.11 μM. The apparent K_a's of ATP for CDP and UDP reductions were determined to be 0.63 ± 0.03 and 1.25 ± 0.10 mM, respectively.

Effects of Other Nucleoside Diphosphates on ADP, GDP, CDP, and UDP Reduction. The addition of CDP, UDP, or GDP inhibited ADP reduction by the enzyme. This is shown in Chart 2*A*. Similarly, the reduction of GDP, CDP, and UDP could also be inhibited by the addition of other ribonucleoside diphosphates in a noncompetitive manner, as shown in Chart 2, *B* to *D*. The K_i's are summarized in Table 3. The pattern of inhibition and kinetic constants are determined according to the definition of Cleland (7). In general, K_{ii}'s[3] are much higher than are K_{is}' for ribonucleoside diphosphates used as inhibitors, no matter which ribonucleoside diphosphate reduction is studied with the exception of ADP in the CDP reduction system, where the K_{ii} is the same as the K_{is}. Moreover, the K_{ii}'s or K_{is}' of the nucleoside diphosphates for each type of reduction are much higher than are the K_m's when the same diphosphates are utilized as substrates, except that the K_{is} for CDP in the UDP reduction is about the same as the K_m of CDP, and the K_{is} for UDP in the CDP reduction is about the same as the K_m of UDP. This is not surprising because both the CDP and the UDP reductions were studied with ATP as the activator.

[3] The abbreviations used are: DTT, dithiothreitol; K_{ii}, K_i of intercept; K_{is}, K_i of slope.

DISCUSSION

A sensitive method for assaying UDP or GDP reductase activity was developed. By using this method and the assay for ADP and CDP reduction, the kinetic properties of the reconstituted ribonucleotide reductase from its purified components derived from human Molt-4F culture cells were investigated with all 4 naturally occurring ribonucleoside diphosphophosphates as the substrate. Grossly, the specificity of a nucleoside triphosphate serving as the activator for a given ribonucleoside diphosphate reduction resembled those enzymes obtained from other mammalian sources (13, 17, 18, 20). Pyrimidine nucleoside diphosphate reduction was best activated by the addition of ATP. dTTP was not an activator for the reduction of either of the pyrimidine nucleoside diphosphates. The result is different from that of the enzyme isolated from *E. coli*, in which dTTP or ATP could activate the reaction to the same extent (14, 15). For ADP reduction, GTP or dGTP served as the best activator. They gave the same maximal velocity and with the same K_a's for the enzyme. GDP reduction was best stimulated by dTTP, dUTP, or dGTP.

Double reciprocal plots of the enhanced velocity against the activator concentration were linear for all reductions in the concentration range examined. The apparent K_a's of ATP for CDP and UDP reduction are 0.63 ± 0.03 and 1.25 ± 0.10 mM, respectively. This difference suggests that possibly more than one type of ATP binding site could be present on these enzymes, although other explanations are also feasible. Further studies will be required to explore this possibility. It is important to note that the apparent K_a's of ATP for both pyrimidine nucleoside diphosphate reductions are within the physiological concentration of ATP (about 3 mM) in cells (21). The apparent K_a of GTP or dGTP for ADP reduction is 1.1 mM. This value is higher than the concentration of GTP (about 0.3 mM) or dGTP (about 4 μM) in cells (6, 21). Whether there is an additional activator to lower the apparent K_a's of GTP and dGTP for ADP reduction to the physiological concentration of GTP or dGTP inside the cells is under current investigation. The apparent K_a of dTTP for GDP reduction is the lowest found for an activator of this class of enzymes. This low K_a might reflect to the lower concentration of dTTP relative to ribonucleoside triphosphate that is present in cells engaged in DNA synthesis.

Double reciprocal plots of velocity against substrate concentration in the presence of saturating amounts of the activator were also linear in the concentration range studied for the 4 ribonucleoside diphosphate reductions. The apparent K_m's were calculated to be 7 ± 0.3 μM for CDP, 80 ± 6.5 μM for ADP, 33 ± 3.1 μM for CDP, and 50 ± 2.0 μM for UDP. The apparent K_m for CDP is much lower than are those of the other 3 ribonucleoside diphosphates. This low K_m might be related to the fact that smaller amounts of CDP are present in cells than are those of other substrates (14).

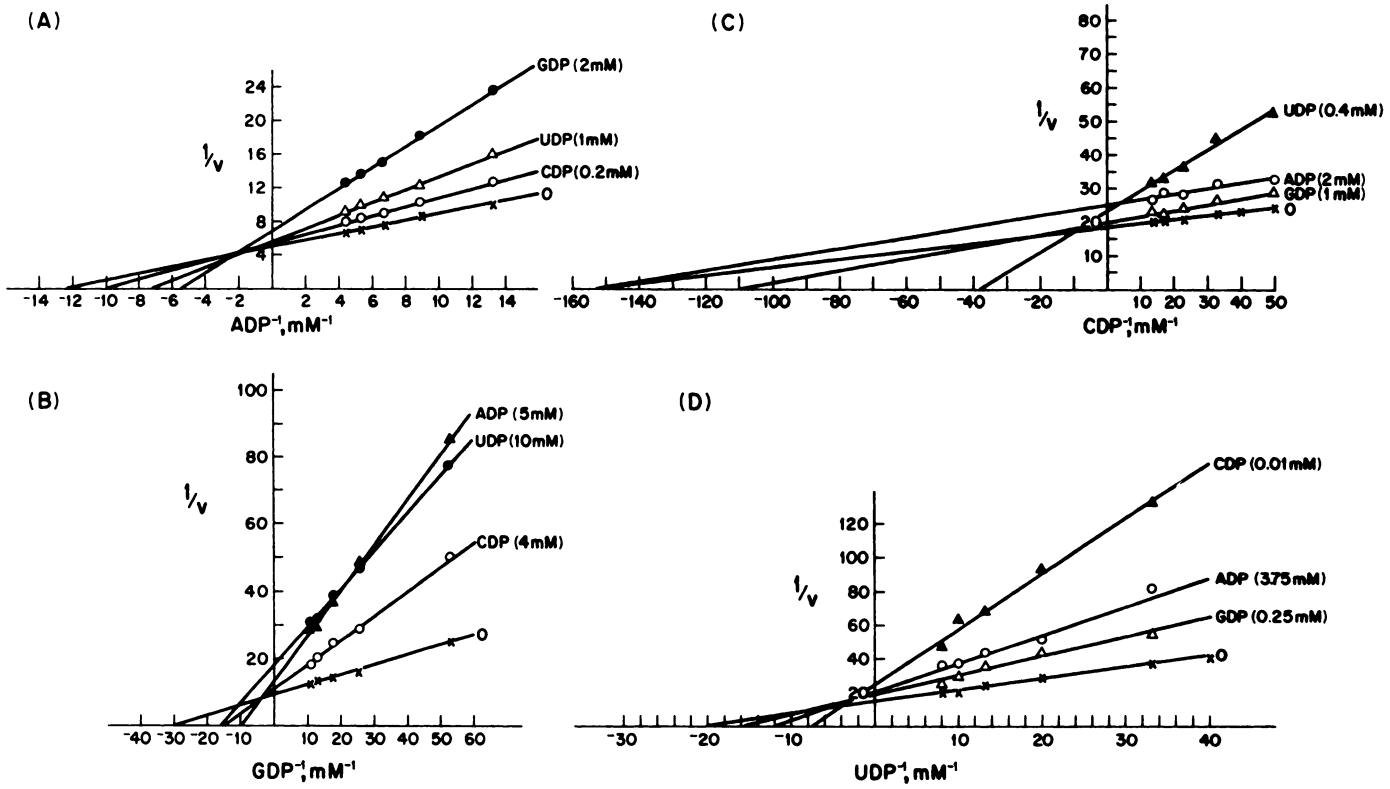

Chart 2. Effects of ribonucleoside diphosphates on the Lineweaver-Burk plots of the reciprocal of substrate concentration with respect to the reciprocal of reaction velocity (nmol of deoxyribonucleotides produced in 1 hr). Standard incubation conditions were used except for substrate concentration. Each 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 ADP with dGTP as activator; *B*, reduction of GDP with dTTP as activator; *C*, reduction of CDP with ATP as activator; and *D*, reduction of UDP with ATP as activator.

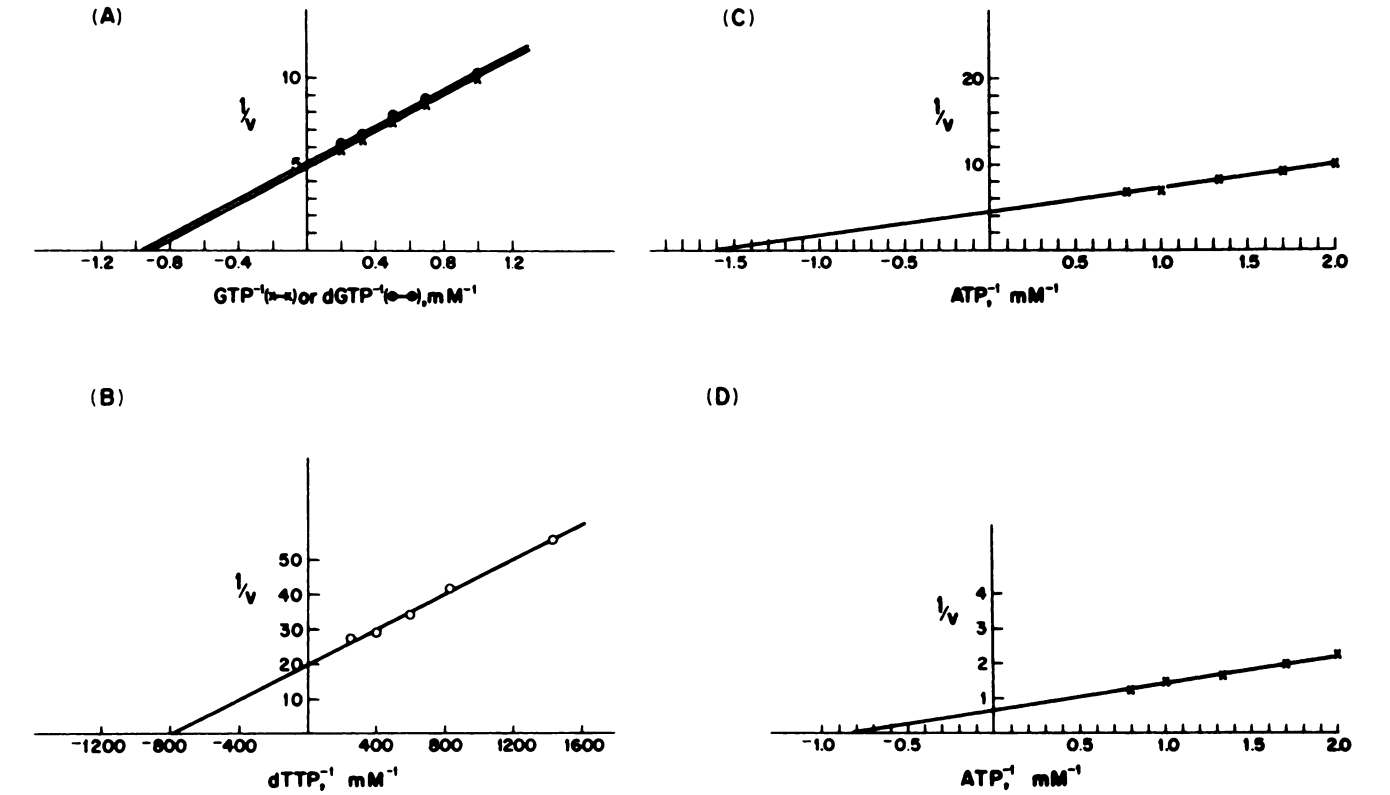

Chart 3. Double reciprocal plots of initial activator concentration with respect to the reaction velocity (nmol of deoxyribonucleotides produced in 1 hr). Standard incubation conditions were used except for activators. Each tube contained 14 μg of Component A and 12 μg of Component B of ribonucleotide reductase obtained from Molt-4F cells. *A*, reduction of ADP; *B*, reduction of GDP; *C*, reduction of CDP; *D*, reduction of UDP.

The K_m's for CDP, UDP, and GDP reduction obtained from calf thymus enzyme (12) are about 4, 2, and 1.7 times those values for CDP, UDP, and GDP reduction obtained from the Molt-4F enzyme. However, the K_m for ADP reduction catalyzed by the Molt-4F enzyme is 2 times that value for ADP reduction catalyzed by the calf thymus enzyme, but the value is similar to that obtained from the hepatoma enzyme (17). The K_m's for UDP reduction in the presence of 2.1 mM ATP and GDP reduction in the presence of 0.075 mM dTTP obtained from the hepatoma enzyme are also similar to those values obtained from Molt-4F enzyme. However, the K_m for CDP reduction (11 to 16 × 10^{-6} M in the presence of 0.1 to 0.3 mM ATP and 15 to 27 × 10^{-6} M in the presence of 2.1 mM ATP) obtained from the hepatoma enzyme is higher than that value obtained from Molt-4F enzyme. The enzyme preparation in their studies had the contamination of nucleoside diphosphate kinase activity (12, 17).

By examining the effects of ribonucleoside diphosphates on each other's reduction, it was found that reduction of any ribonucleoside diphosphate examined could be noncompetitively inhibited by other ribonucleoside diphosphates. This observation suggests that more than one type of ribonucleoside diphosphate could bind to the enzyme and be reduced depending on what kind of activator is used. Even CDP and UDP, both of which require the same activator (ATP) for the activation, could act as noncompetitive inhibitors of the reduction of each other. With the exception of CDP, which has the same K_{ii}'s and K_{is}' for ADP reduction, the higher K_{ii}'s with respect to K_{is}' of different ribonucleoside diphosphates as inhibitors of other ribonucleoside diphosphate reduction, indicate that binding of one ribonucleoside diphosphate could interfere with the binding of another type of ribonucleoside diphosphate. This could be explained on the basis of 2 possible binding sites for each ribonucleoside diphosphate. Those sites are not necessary to be different for each ribonucleoside diphosphate. The binding of a ribonucleoside diphosphate to the first site could change the enzyme conformation to favor the binding of the same ribonucleoside diphosphate to the second site. The K_i for each ribonucleoside diphosphate is much higher than its K_m, which was estimated in the presence of its appropriate activator. This strongly suggests that at least one role of the specific activator for each ribonucleoside diphosphate reduction is to alter the conformation of the enzyme so that a better affinity for the appropriate substrate is achieved.

The interaction of the reduction of various ribonucleoside diphosphates with each other, together with our previous observation of the copurification of various ribonucleoside diphosphate reductases at each stage of the purification procedure (3, 4) and of the fluctuation of ADP and CDP reduction activities in the same fashion through the cell cycle of HeLa cells (5), strongly suggests that the same components of reductase derived from human Molt-4F cells are responsible for the reduction of all 4 ribonucleotides. Eriksson *et al.* (12) have also recently indicated that the same enzymes purified from calf thymus can also reduce all 4 ribonucleotides. This is in disagreement with the suggestion made by other investigators that ADP reductase and CDP reductase may be separate entities in mammalian cells (8, 9, 19).

Table 2

The kinetic constants of various substrates and activators on ribonucleoside diphosphate reductions catalyzed by reconstituted ribonucleotide reductase derived from Molt-4F cells

Substrates	K_m[a] (μM)	Activators	K_a[a] (mM)
ADP	80 ± 6.5[b]	GTP or dGTP	1.1 ± 0.09 or 1.1 ± 0.08
GDP	33 ± 3.1	dTTP	0.00125 ± 0.00011
CDP	7 ± 0.3	ATP	0.63 ± 0.03
UDP	50 ± 2.0	ATP	1.25 ± 0.10

[a] Calculations based on 3 experiments.
[b] Mean ± S.D.

Table 3

The kinetic constants of various ribonucleoside diphosphates on ribonucleoside diphosphate reductions catalyzed by reconstituted ribonucleotide reductase derived from Molt-4F cells in the presence of excess amount of best activators (5 mM)

	Inhibitors (mM)							
	ADP		GDP		CDP		UDP	
Substrates	K_{ii}[a]	K_{is}	K_{ii}	K_{is}	K_{ii}	K_{is}	K_{ii}	K_{is}
ADP			5.1	0.9	2.3	0.4	10.0	1.2
GDP	11.0	1.4			17.7	2.4	9.8	3.5
CDP	5.0	5.0	8.0	1.6			1.3	0.1
UDP	8.2	2.8	0.8	0.4	0.0124	0.0026		

[a] The kinetic constants (K_{ii} and K_{is}) are obtained by the replots of intercepts and slopes *versus* inhibitors as shown in Chart 2. Three concentrations of each inhibitor were used.

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