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The Phosphorylation of Ribavirin by Deoxyadenosine Kinase from Rat Liver. Differentiation between Adenosine and Deoxyadenosine Kinase†

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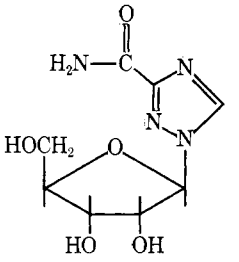
ABSTRACT: A nucleoside kinase activity has been partially purified 30-fold from rat liver which is capable of phosphorylating the synthetic nucleoside, ribavirin. This activity copurifies with both adenosine and deoxyadenosine kinase. The K_m values for the three substrates were: ribavirin, 3.2 mM; deoxyadenosine, 0.50 mM; and adenosine, 0.76 μM. The pH and $MgCl_2$ concentration for optimal enzyme activity were the same with either ribavirin or deoxyadenosine as substrates, but were much lower for adenosine phosphorylation. Both ribavirin and deoxyadenosine kinase exhibited the same sensitivity to inactivation by heat or *p*-chloromercuribenzoic acid, and to protection against heat inactivation by dithiothreitol. The adenosine kinase activity was less sensitive to inactivation by *p*-chloromercuribenzoic acid or heat and to protection by dithiothreitol. Kinetic studies revealed that ribavirin and deoxyadenosine competitively inhibited the phosphorylation of one another, while adenosine was a noncompetitive inhibitor of these two kinase activities. The results indicate that adenosine and deoxyadenosine kinase are separate enzyme activities and that the phosphorylation of ribavirin is associated with the latter activity.

The synthesis and antiviral activity of 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide (I, ribavirin)[1,2] have been



I, ribavirin

reported (Witkowski *et al.,* 1972; Sidwell *et al.,* 1972) demonstrating that this synthetic nucleoside is a potent inhibitor of a wide variety of RNA and DNA viruses. Studies on the mechanism of antiviral action of this drug in mammalian cell culture (Streeter *et al.,* 1973a) revealed that the 5′-phosphate of ribavirin was a potent inhibitor of the enzyme IMP dehydrogenase, and that the antiviral activity of ribavirin might be due to the inhibition of GMP biosynthesis in virus-infected cells. This would, in turn, result in the inhibition of virus nucleic acid synthesis. Some preliminary evidence that ribavirin could indeed be phosphorylated *in vivo* was presented (Streeter *et al.,* 1973a). Since the 5′-phosphate of ribavirin appears to be the active antiviral agent, the enzymic phosphorylation of ribavirin became of prime interest. Furthermore, both the mechanism of action studies, as well as X-ray crystallography studies on ribavirin (Prusiner and Sundaralingam, 1973), indicated a structural similarity to guanosine or inosine. It was therefore of interest to determine which naturally occurring nucleoside kinase activity was responsible for the phosphorylation of ribavirin, in order to further determine the structural analogy of ribavirin to naturally occurring nucleosides.

A nucleoside kinase activity has been found in crude extracts from rat liver (Streeter *et al.,* 1973b) which phosphorylates ribavirin, and it is the purpose of this paper to report the partial purification and characterization of this enzyme activity.

Experimental Section

Materials. ^{14}C- and ^{3}H-labeled purine and pyrimidine nucleosides were obtained from ICN Pharmaceuticals, Inc., Life Sciences Group. Ribavirin was synthesized according to the method of Witkowski *et al.* (1972). [^{3}H]Ribavirin was obtained by exchange of the unlabeled material in [^{3}H]H_2O. The exchanged material was separated from unexchanged tritium by repeated evaporation from water. The final product was recrystallized from ethanol. Nonradioactive nucleosides and deoxynucleosides, ATP, phosphoenol pyruvate (trisodium salt), and pyruvate kinase

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[1] Referred to in previous publications as ICN 1229 or by the ICN Pharmaceuticals, Inc. trademark: Virazole®.

[2] Abbreviations used are: RV, ribavirin; Ado, adenosine; dAdo, deoxyadenosine; PCMB, *p*-chloromercuribenzoate.

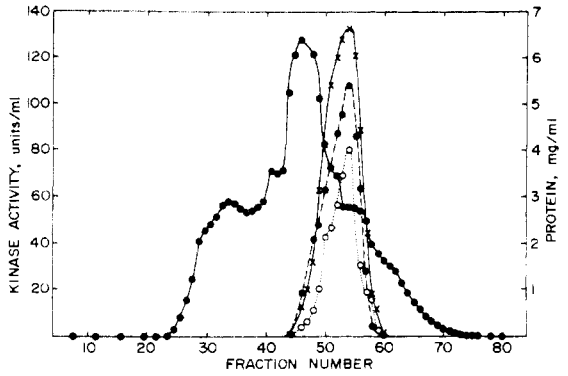

FIGURE 1: Chromatography of rat liver ribavirin kinase activity on Sephadex G-100; 5 ml of the ammonium sulfate fraction was applied to a column of Sephadex G-100 (4.7 cm^2 × 35 cm) equilibrated with 0.01 M Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 75 mM KCl, and 1 mM dithiothreitol. The column was eluted with the same buffer at a flow rate of 0.25 ml/min and collected in 4-ml fractions. The following assays were performed (see Experimental Section): (●—●) protein; (● - - ●) ribavirin kinase; (O · · · O) adenosine kinase; (X—X) deoxyadenosine kinase. Fractions 45–58 were pooled for further purification.

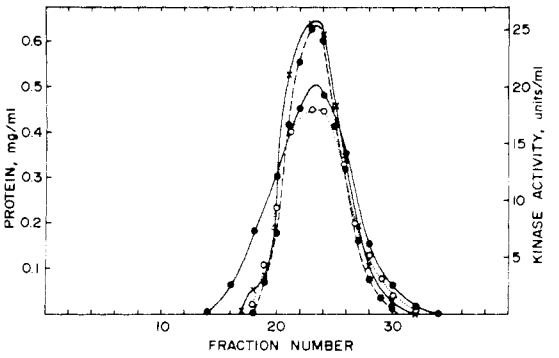

FIGURE 3: Chromatography of ribavirin kinase on Sephadex G-75. The pooled fractions from DE-52 chromatography containing ribavirin kinase activity were concentrated by ammonium sulfate precipitation (see text) and applied to a column of Sephadex G-75 (4.7 cm^2 × 35 cm) previously equilibrated in 0.01 M Tris-HCl (pH 7.5), 0.01 M $MgCl_2$, 0.075 M KCl, 0.001 M dithiothreitol, and containing 20% glycerol. The column was eluted with the same buffer at a flow rate of 0.5 ml/min and 4-ml fractions were collected. The following assays were performed: (●—●) protein; (● - - ●) ribavirin kinase; (X—X) deoxyadenosine kinase; (O · · · O) adenosine kinase. Fractions 20–27 were pooled and stored at −20°.

were obtained from Sigma Chemical Co. Whatman DE-52 and DE-81 filter discs (2.4 cm in diameter) were obtained from Reeve Angel and Co. *p*-Chloromercuribenzoic acid was obtained from ICN Nutritional Biochemicals, and dithiothreitol from ICN-K & K Laboratories.

Enzyme Assays. The standard assay for various nucleoside kinase activities contained, in a final volume of 0.15 ml: 10 μmol of Tris-HCl (pH 7.5); 0.21 μmol of $MgCl_2$; 0.20 μmol of ATP; 0.38 μmol of phosphoenol pyruvate; 0.75 unit of pyruvate kinase; 0.11 μmol of NaF; 3.8 μmol of KCl; 0.045 μmol of dithiothreitol; and enzyme and substrates at the indicated concentrations. Reactions were incubated at 37° for 2–30 min, depending on the substrate, at which time 0.05 ml of the reactions was transferred to DE-81 discs. The discs were air-dried and washed several times in 2 mM ammonium formate (pH 7), to remove unphosphorylated nucleosides, then, successively, in ethanol–water, ethanol, and ether, and air-dried. The discs were counted in 3 ml of toluscint scintillation fluid (ICN Pharmaceuticals, Inc., Life Sciences Group), using a Packard TriCarb Model 3320 liquid scintillation counter. One unit of enzyme activity is defined as the amount catalyzing the conversion of 1 nmol of nucleoside to nucleoside 5′-phosphate in 1 min, under the assay conditions described. Protein was determined by the method of Lowry *et al.* (1951), or Warburg and Christian (1941). For the assay of adenosine deaminase the reaction mixture contained, in a final volume of 2.5 ml: Tris-HCl (pH 7.5), 7.5 μmol; adenosine, 0.1 μmol; and 0.1 ml of the enzyme fraction in a quartz cuvet. The reaction was monitored by following the decrease in absorbance at 265 nm against a blank cuvet containing no enzyme, using a Cary Model 15 spectrophotometer.

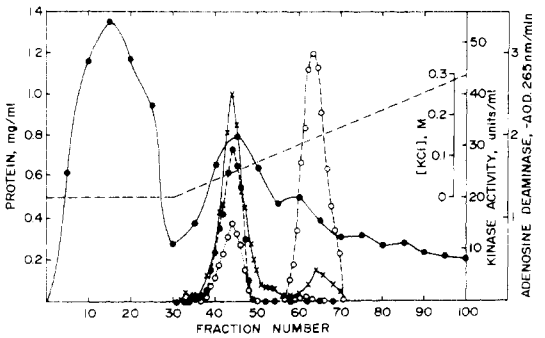

FIGURE 2: Chromatography of ribavirin kinase on DEAE-cellulose. The pooled fractions containing ribavirin kinase activity, after Sephadex G-100 chromatography, were diluted with an equal volume of distilled water and applied to a column of Whatman DE-52 (7.1 cm^2 × 20 cm) which had been equilibrated in 0.01 M Tris-HCl (pH 7.5) buffer containing 0.01 M $MgCl_2$, and 0.001 M dithiothreitol. The column was immediately eluted with a linear gradient consisting of 150 ml of the above buffer and 150 ml of the same buffer + 0.3 M KCl. The flow rate was 2 ml/min and 4-ml fractions were collected. The following assays were performed (see Experimental Section): (●—●) protein, (● - - ●) ribavirin kinase; (X—X) deoxyadenosine kinase; (O · · · O) adenosine kinase; (O - - O) adenosine deaminase. Fractions 38–49 were pooled for further purification.

Results

Purification of Enzyme

Crude Extract. Two or three large rats (200–250 g) were killed by decapitation and the livers perfused through the portal vein with saline and then removed to yield about 30 g of tissue. The following steps were performed at 3–5°. The tissue was minced in 3 ml/g of tissue of buffer A: 10 mM Tris-HCl (pH 7.5); 10 mM $MgCl_2$; 1 mM dithiothreitol; and 0.075 M KCl. The minced tissue was homogenized in a Kontes Teflon–glass homogenizer. The homogenate was centrifuged at 37,000*g* for 30 min and filtered through glass wool. The filtered supernate was recentrifuged at 144,000*g* for 90 min in a Spinco Model L2-65B ultracentrifuge. The clear supernate was carefully removed from beneath the lipid overlay.

Ammonium Sulfate Fractionation. To this supernate, solid ammonium sulfate was added over a 15-min period to 55% of saturation. The solution was stirred an additional 15 min and centrifuged 30 min at 30,000*g*. The pellet was discarded. To this supernate, ammonium sulfate was again added to 90% of saturation. The solution was again stirred and centrifuged, and the pellet was dissolved in 4–5 ml of buffer A.

Chromatography on Sephadex G-100. The ammonium sulfate fraction was then applied to a column of Sephadex G-100 (4.7 cm^2 × 35 cm) equilibrated in buffer A. The column was eluted with buffer A at a flow rate of 0.25 ml/

TABLE I: Purification of Ribavirin Kinase.[a]

Fraction	Total Protein (mg)	Specific Activity (units/mg)	Total Activity (units)
I. 144,000*g* supernate	1750	1.9	3320
II. Ammonium sulfate	314	5.8	1820
III. Sephadex G-100	144	12	1680
IV. DEAE-cellulose	35	21	745
V. Sephadex G-75	7	60	420

[a] The purification was followed by the standard assay described in the text with [^{3}H]ribavirin at a concentration of 0.3 mM. The assays were performed on the pooled fractions of each step in the purification.

TABLE II: Nucleoside Acceptor Specificity of Purified Rat Liver Ribavirin Kinase.[a]

Substrate	Nucleotide Formed (nmol min^{-1} mg of protein^{-1})
Ribavirin	60
Adenosine	37
Deoxyadenosine	52
Guanosine	0.0
Deoxyguanosine	0.5
Cytidine	0.9
Deoxycytidine	0.0
Uridine	0.9
Thymidine	0.0

[a] The standard assay described in the text was used. The reaction contained 6 μg of fraction V as the enzyme source, and 50 nmol of ^{3}H nucleoside (10 μCi). The assays were incubated at 37° for 5 min.

min. It was repeatedly observed that both adenosine kinase and deoxyadenosine kinase cochromatographed on G-100 with the ribavirin kinase activity (Figure 1). No other areas of the elution profile contained any of the three activities. The fractions containing ribavirin kinase activity were pooled (Figure 1). The only other nucleoside kinase activities present in the pooled G-100 fraction were those for uridine and cytidine.

Chromatography on DEAE-Cellulose (DE-52). The pooled G-100 fractions were diluted twofold with water and applied to a column of DEAE-cellulose Whatman DE-52 (7.1 cm^2 × 20 cm), equilibrated in buffer A without KCl. The column was then eluted with a linear gradient consisting of 150 ml of buffer A without KCl and 150 ml of buffer A containing 0.3 M KCl at a flow rate of 2 ml/min. Here again, the adenosine, deoxyadenosine, and ribavirin kinase activities cochromatographed (Figure 2). The fractions were also assayed for adenosine deaminase activity which was cleanly separated in this step from the major peak of kinase activity. A minor second peak of deoxyadenosine kinase activity was found within the adenosine deaminase peak, but no adenosine or ribavirin kinase activity was detected in these fractions. The fractions containing ribavirin kinase were again pooled and concentrated by addition of solid ammonium sulfate to 95% of saturation. The solution was stirred for 15 min and centrifuged at 30,000*g* for 30 min. The pellet was dissolved in 2–3 ml of buffer B: 10 mM Tris-HCl (pH 7.5); 10 mM $MgCl_2$; 1 mM dithiothreitol; and 20% glycerol.

Chromatography on Sephadex G-75. The concentrated DE-52 fraction was applied to a column of Sephadex G-75 (4.7 cm^2 × 35 cm) equilibrated in buffer B. The column was eluted with the same buffer at a flow rate of ~0.5 ml/min. The protein and the three kinase activities each demonstrated symmetrical and overlapping elution profiles (Figure 3). Fractions containing 20% or more of the peak fraction of ribavirin kinase were pooled and stored in aliquots at −20°. The stepwise purification of ribavirin kinase is summarized in Table I.

The specific activity of the final fraction represents a 30-fold purification over the original supernate and a recovery of 13% of the initial activity. The purified enzyme loses its activity very rapidly if stored frozen in the absence of glycerol (from a preparation in which glycerol was omitted from buffer B). In addition, the presence of dithiothreitol markedly increased the stability of the enzyme during storage. The final preparation (fraction V) was substantially free of both phosphatase and nucleoside phosphorylase activity. Adenosine deaminase was completely removed by DEAE-cellulose chromatography (Figure 2).

Properties of Partially Purified Enzyme

Substrate Specificity. When preparations of fraction V were assayed with all the common nucleosides and deoxynucleosides as substrates, only adenosine and deoxyadenosine showed significant activity (Table II). This is consistent with the data shown in Figures 1–3 which demonstrates that these two kinase activities cochromatograph with ribavirin kinase through Sephadex G-100, DEAE-cellulose, and Sephadex G-75 chromatography. Table III further com-

TABLE III: Nucleoside Acceptor Specificity of Ribavirin Kinase during Purification.[a]

Purification Step	Purification Factor		
	Ribavirin	Deoxyadenosine	Adenosine
I. 144,000*g* supernate	1	1	1
II. Ammonium sulfate	3.1	2.6	2.3
III. Sephadex G-100	6.2	4.0	3.9
IV. DEAE-cellulose	11	6.1	5.6
V. Sephadex G-75	32	17	29

[a] The purification was followed by the standard assay described in the text. Ribavirin and deoxyadenosine were assayed at a concentration of 0.3 mM and adenosine at 30 μM.

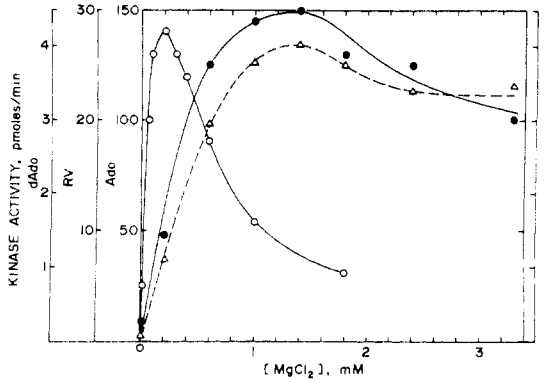

FIGURE 4: Effect of $MgCl_2$ on the phosphorylation of ribavirin, adenosine, and deoxyadenosine. The standard reaction described in the text was followed using 5 μg of enzyme that was prepared in the absence of $MgCl_2$ in the final purification step (Sephadex G-75 chromatography). The following symbols and substrate concentrations were used: (●—●) ribavirin, 0.3 mM; (○—○) adenosine, 30 μM; (Δ - - Δ) deoxyadenosine, 30 μM.

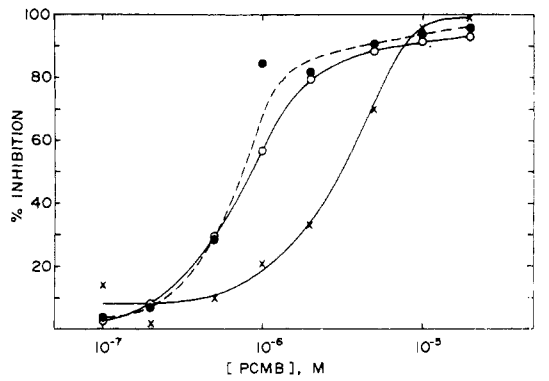

FIGURE 6: Effect of PCMB on the phosphorylation of ribavirin, adenosine, and deoxyadenosine. The standard assay was followed using 10 μg of enzyme which had been prepared in the absence of $MgCl_2$ and dithiothreitol in the final purification step (Sephadex G-75 chromatography). The enzyme and PCMB were preincubated at 0° for 10 min prior to starting the reaction by addition of the labeled substrate and other incubation components at 37°. The substrate concentrations used were: (○—○) ribavirin, 0.67 mM; (● - - ●) deoxyadenosine, 0.13 mM; (X—X) adenosine, 0.3 μM.

pares the fold purification of these three kinase activities throughout the enzyme preparation. The increase in specific activities was similar for all three activities through the first three steps, but was higher for ribavirin kinase at the fourth step. The overall fold purification of fraction V was virtually the same for ribavirin and adenosine, but was about a factor or 2 less for deoxyadenosine. It is apparent from the data in Tables I and II that all three enzyme activities are unstable to purification, particularly in the last two steps (only 13% of the original ribavirin kinase activity was pooled in the final fraction). Thus, despite the relatively high protein purification from DEAE-cellulose treatment (Figure 2), the purification factor for each of the three kinase activities increases only slightly at this step.

Additional purification therefore appeared to be an unprofitable means of further distinguishing between these three kinase activities. It was decided, rather, to further compare these activities in terms of: (1) requirements for optimal enzyme activity; (2) sensitivity to inactivation by heat or PCMB; and (3) inhibition of each of the enzyme activities by the other substrates (substrate competition).

$MgCl_2$ Requirement. Mg^{2+} was required for optimal activity with all three substrates (Figure 4). The Mg^{2+} dependence of ribavirin and deoxyadenosine was virtually identical with an optimum around 1.4 mM. Adenosine exhibited a much sharper and much lower optimum at 0.2 mM Mg^{2+}.

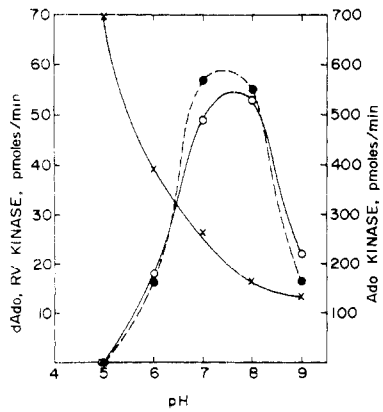

FIGURE 5: Effect of pH on the phosphorylation of ribavirin, adenosine, and deoxyadenosine. The standard assay was followed in 80 mM Tris-maleate buffer at the indicated pH's; 6.5 μg of enzyme was used with ribavirin and deoxyadenosine as the substrates, and 1.3 μg of enzyme with adenosine. The substrate concentrations were: (○—○) ribavirin, 0.3 mM; (● - - ●) deoxyadenosine, 0.15 mM; (X—X) adenosine, 30 μM.

pH Optimum. Again, ribavirin and deoxyadenosine showed a very similar pH dependence in Tris-maleate buffer with an optimum of around 7.5 (Figure 5). A similar optimum has been reported for the deoxyadenosine kinase purified from calf thymus (Krygier and Momparler, 1971a). The pH optimum for adenosine, however, is at or below pH 5 where neither ribavirin nor deoxyadenosine shows detectable activity. This is similar to the pH optimum reported for adenosine kinase from sarcoma 180 cells (Divekar and Hakala, 1971).

PCMB Inhibition. The increased stability of fraction V when stored in the presence of dithiothreitol suggested that sulfhydryl groups were necessary for activity. Therefore, the sensitivity of each of the three kinase activities present in fraction V to inactivation by PCMB was examined. The enzyme used in this assay was prepared in the absence of dithiothreitol in the final purification step. As seen in Figure 6, the inactivation of both ribavirin and deoxyadenosine kinase activities by PCMB was virtually identical, with 50% inhibition occurring at about 0.7 μM. Adenosine kinase was significantly less sensitive to PCMB inhibition, with 50% inhibition occurring at about 3 μM.

Heat Inactivation and Protection by Dithiothreitol. The sensitivity of the three kinase activities to heat inactivation was also determined by preincubating the enzyme, containing no dithiothreitol at various temperatures prior to the assay with each substrate at 37°. Table IV compares the first-order rate constants of inactivation for each of the three kinase activities as the temperature is increased from 38 to 42°. It is apparent that the rate constants for both ribavirin and deoxyadenosine show greater increases with increasing temperature than for adenosine, indicating that the latter activity was less sensitive to heat inactivation than the first two. In addition, the inactivation at 42° can be prevented to some degree by dithiothreitol (Table V). It is apparent that the protective effect of dithiothreitol is greater with ribavirin and deoxyadenosine kinase than with adenosine kinase, in accordance with the greater sensitivity of the former two activities to heat inactivation.

TABLE IV: Heat Inactivation of Kinase Activities.[a]

Temp (°C)	First-Order Rate Constant of Inactivation ($10^2 \times min^{-1}$) RV	dAdo	Ado
38	2.41	2.62	2.21
40	5.18	5.25	3.85
42	14.2	13.3	7.70

[a] The assay was performed in two stages. In the first stage, the enzyme (30 μg of a G-75 fraction prepared in the absence of dithiothreitol, but containing 10 mM $MgCl_2$) was preincubated in a final volume of 0.075 ml containing 50 mM Tris-HCl (pH 7.5); 1.3 mM $MgCl_2$. Duplicate sets of preincubation mixtures were incubated for 0, 5, 10, 15, 20, or 30 min at the indicated temperatures. In the second stage of the assay, all the additional components of the standard enzyme assay (see Experimental Section) were added and the mixtures were incubated for 4 min at 37° to determine the amount of enzyme activity remaining in each tube. The substrate concentrations used were: ribavirin and deoxyadenosine, 0.3 mM; adenosine, 30 μM. The log of the per cent remaining activity *vs.* the time of preincubation was plotted to determine the first-order rate constant of inactivation at each temperature.

TABLE V: Protection by Dithiothreitol against Heat Inactivation of Kinase Activities.[a]

Dithiothreitol Concn (mM)	First-Order Rate Constant of Inactivation at 42° ($10^2 \times min^{-1}$) RV	dAdo	Ado
0	14.0	13.4	7.94
0.5	13.9	13.2	7.89
1.0	11.5	11.1	7.61
5.0	7.23	7.55	6.40
10.0	6.82	6.90	5.52
50.0	6.80	6.80	5.45

[a] A two-stage assay similar to that described in Table IV was used. In the first stage, 22 μg of a G-75 fraction was preincubated with 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, and the various indicated dithiothreitol concentrations in a final volume of 0.02 ml. After preincubation at 0, 5, 10, 15, or 25 min at 42°, all the additional components of the standard assay (see Experimental Section) were added, with the exception that the final dithiothreitol concentration in the second stage was 10 mM, and the mixtures were incubated for 5 min at 37° to determine the amount of enzyme activity remaining in each tube. The first-order rate constants of inactivation were determined as described in Table IV.

Kinetics of Substrate Competition

In order to further investigate the behavior of the three nucleoside substrates with the partially purified nucleoside kinase activity (fraction V), competition experiments were performed in which the substrate dependence of each labeled nucleoside was studied in the absence and in the presence of each of the other unlabeled nucleosides. These data were analyzed by the method of Lineweaver and Burk (1934) and are summarized in Table VI.

The substrate dependence of both ribavirin and deoxyadenosine was determined at the optimal Mg^{2+} concentration of 1.4 mM. When adenosine phosphorylation was studied under these conditions, the unusual results shown in Figure 7 were obtained. The reaction was inhibited by adenosine concentrations as low as 2 μM and showed only slight substrate dependence below that concentration. The specific activity of the [^{14}C]adenosine (50 Ci/mol) does not permit the accurate monitoring of the reaction below ~0.5 μM. When the experiment was performed at the optimal Mg^{2+} concentration for adenosine phosphorylation (0.2 mM), normal, saturating substrate dependence was observed with adenosine up to at least 5 μM (Figure 8). The data for adenosine phosphorylation summarized in Table VI was determined at 0.2 mM Mg^{2+}. The inhibition of adenosine phosphorylation by deoxyadenosine was of the mixed type (Dixon and Webb, 1964) and a K_i was not determined in this case.

The V_{max} values for the three substrates were remarkably similar. The K_m for ribavirin was approximately an order of magnitude greater than the K_m for deoxyadenosine. On the other hand, the K_m for ribavirin was approximately 10^4 greater than the K_m for adenosine. A comparison of the K_i values shows that adenosine was better than deoxyadenosine as an inhibitor of ribavirin phosphorylation; likewise, adenosine was better than ribavirin as an inhibitor of deoxyadenosine phosphorylation. In addition, the K_i for ribavirin inhibiting adenosine phosphorylation is an order of magnitude greater than the K_i for ribavirin inhibiting deoxyadenosine phosphorylation.

Discussion

The nucleoside kinase activity from rat liver capable of phosphorylating the synthetic nucleoside ribavirin has been purified to a stage in which only two naturally occurring nucleosides, adenosine and deoxyadenosine, can be significantly phosphorylated by this fraction. This raises the question as to whether the three nucleosides are phosphorylated by (1) the same enzyme, (2) different enzymes, or (3) the same enzyme with different catalytic sites. A comparison of the properties of the enzyme activities reported here with similar activities from other sources might shed some light

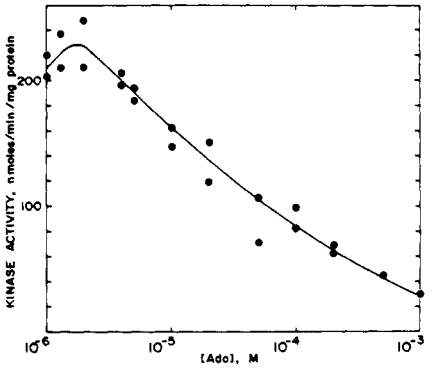

FIGURE 7: The phosphorylation of adenosine as a function of adenosine concentration: effect of high Mg^{2+} concentration. The standard assay described in the text was used. The curve represents a composite of four assays: (1) 1–10 μM adenosine using 0.75 μg of protein, (2) 5–50 μM adenosine using 1.5 μg of enzyme; (3) 20–200 μM adenosine using 3.0 μg of enzyme; and (4) 0.1–1 mM adenosine using 7.5 μg of enzyme. The Mg^{2+} concentration was 1.4 mM and the assays were incubated at 37° for 2 min.

TABLE VI: A Summary of the Substrate Competition Experiments with Rat Liver Ribavirin Kinase.[a]

Substrate	Inhibitor	Type of Inhibition[b]	K_m (mM)	V_{max}[c]	K_i (mM)
RV			3.2	130	
RV	dAdo	C			0.97
RV	Ado	N			0.057
dAdo			0.50	155	
dAdo	RV	C			2.5
dAdo	Ado	N			0.035
Ado			0.00076	190	
Ado	RV	C			26

[a] The standard assay described in the text was followed using a Mg^{2+} concentration of 1.4 mM for ribavirin and deoxyadenosine phosphorylation, and 0.2 mM for adenosine phosphorylation. Ribavirin phosphorylation was determined over a concentration range from 1.0 to 10 mM using 5 μg of enzyme. Deoxyadenosine inhibition was assayed at 3.3 mM and adenosine inhibition at 30 μM. Deoxyadenosine phosphorylation was determined over a concentration range from 0.1 to 1.0 mM using 5 μg of enzyme. Ribavirin inhibition was assayed at 10 mM and adenosine inhibition at 38 μM. Adenosine phosphorylation was determined over a concentration range from 0.5 to 5 μM using 0.25 μg of enzyme. Ribavirin inhibition was assayed at 50 mM and deoxyadenosine inhibition at 1 mM. [b] C, competitive inhibition; N, noncompetitive inhibition. [c] Expressed as nmol of nucleotide formed per mg of protein per min.

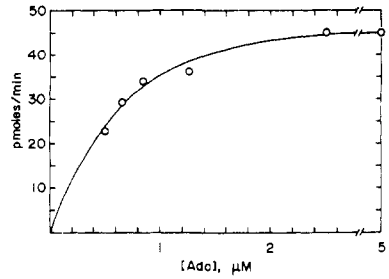

FIGURE 8: The phosphorylation of adenosine as a function of adenosine concentration: effect of low Mg^{2+} concentration. The standard assay described in the text was used. The Mg^{2+} concentration was 0.2 mM and 0.25 μg of enzyme was used per assay. The reaction was incubated at 37° for 2 min.

on the above question. Adenosine kinase has been partially purified and characterized from a number of mammalian sources including rabbit liver (Lindberg *et al.*, 1967), Ehrlich ascites tumor cells (Lindberg *et al.*, 1967; Murray, 1968; Henderson *et al.*, 1972), HeP-2 tumor cells (Schenebli *et al.*, 1967), and sarcoma 180 cells (Divekar and Hakala, 1971). Deoxyadenosine kinase has been well characterized from at least one source, calf thymus (Krygier and Momparler, 1971a,b, 1968). Despite the fact that in a few of these studies it was apparent that both adenosine and deoxyadenosine could be phosphorylated by the same enzyme fraction (Lindberg *et al.*, 1967; Schenebli *et al.*, 1967), there was no compelling evidence as to whether the same or different enzymes were involved. In none of the previous studies, however, were the properties of the enzyme activities determined using both adenosine and deoxyadenosine as substrates. In the present studies, the three nucleoside kinase activities of fraction V (adenosine, deoxyadenosine, and ribavirin) were compared in three categories: (1) copurification (Figures 1–3; Tables II and III), (2) requirements for optimal enzyme activity (Figures 4 and 5), and (3) sensitivity to inactivation (Figure 6; Tables IV and V). It is clear from these data that the ribavirin and deoxyadenosine kinase activities are virtually identical in all three categories of experiments. The adenosine kinase activity is significantly different from the former two activities with regard to requirements for optimal activity and sensitivity to inactivation.

The kinetics of inhibition of each substrate by the other substrates is also consistent with the view that ribavirin is phosphorylated by the deoxyadenosine kinase activity present in fraction V. Deoxyadenosine competitively inhibits ribavirin phosphorylation and *vice versa*. In addition, the K_i for deoxyadenosine inhibition of ribavirin phosphorylation is within a half-order of magnitude of the K_m for deoxyadenosine. Likewise, the K_i for ribavirin inhibition of deoxyadenosine phosphorylation is essentially the same as the K_m for ribavirin.

The kinetic data also show significant differences between ribavirin, deoxyadenosine, and adenosine. Adenosine is a noncompetitive inhibitor of either ribavirin or deoxyadenosine phosphorylation, while deoxyadenosine shows mixed inhibition of adenosine phosphorylation. In contrast, ribavirin is a competitive inhibitor of adenosine phosphorylation, but the K_i is relatively large (26 mM), about an order of magnitude greater than the K_m for ribavirin phosphorylation.

In conclusion, the results suggest that at least different catalytic sites, if not different enzymes, are involved in the phosphorylation of adenosine and deoxyadenosine by fraction V, and that the phosphorylation of ribavirin is associated with the deoxyadenosine kinase activity. The copurification data suggest that a single protein species is involved in the phosphorylation of all three nucleosides; however, the possibility of a protein aggregate or a subunit structure cannot be discounted at this time. No evidence has been reported for such a subunit structure in the various studies of adenosine kinase to date.

The possible effect of the Mg-ATP ratio must also be considered in these interpretations. Murray (1968) reported that free ATP rather than a Mg-ATP complex was a better substrate for adenosine kinase from Ehrlich ascites cells, and that free Mg^{2+} was in fact an inhibitor of the enzyme. This might account for the unusual kinetics of adenosine dependence observed in these studies at high (1.4 mM) *vs.* low (0.2 mM) Mg^{2+} concentrations.

Acknowledgment

The authors thank Mieka Scholten and Anita Beck for their able technical assistance in this study.

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Differential Inhibition of the Exchange Reactions Associated with Oxidative Phosphorylation†

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ABSTRACT: Two analogs of adenosine triphosphate (ATP), adenylyl imidodiphosphate (AMP-PNP) and adenylyl methylenediphosphonate (AMP-PCP), were used to investigate the ATP $\rightleftharpoons$ HOH exchange catalyzed by rat liver mitochondria and submitochondrial particles. Replacement of the terminal bridge oxygen of the triphosphate chain of ATP in these analogs prevents enzymatic cleavage by a wide variety of enzymes, including mitochondrial ATPase, while structural characteristics of ATP are retained (Dueè, E. D. and Vignais, P. V. (1968), *Biochem. Biophys. Res. Commun. 30,* 420; Yount *et al.* (1971a), *Biochemistry 10,* 2484). No incorporation of ^{18}O from water into AMP-PNP or AMP-PCP could be detected under conditions where the ATP $\rightleftharpoons$ HOH exchange was extensive. Both AMP-PNP and AMP-PCP inhibit ATPase activity in submitochondrial particles. The AMP-PNP and AMP-PCP analogs inhibit P_i $\rightleftharpoons$ ATP and P_i $\rightleftharpoons$ H_2O exchanges with or without ADP present but not the ATP $\rightleftharpoons$ H_2O exchange. This suggests there are separate sites for ATP in the facilitation of P_i binding and P_i $\rightleftharpoons$ H_2O exchange and for ATP directly involved in the ATP $\rightleftharpoons$ H_2O exchange.

Mitochondria catalyze a rapid exchange of water oxygen with the terminal phosphate oxygens of ATP (Cohn and Drysdale, 1955; Boyer *et al.,* 1956). This exchange is unique to the oxidative phosphorylation system and can exceed considerably the rates of the P_i $\rightleftharpoons$ ATP and ADP $\rightleftharpoons$ ATP exchanges (Boyer, 1967).

Difficulties encountered in explaining the very rapid rates of both the P_i $\rightleftharpoons$ HOH and ATP $\rightleftharpoons$ HOH exchange relative to the P_i $\rightleftharpoons$ ATP and ADP $\rightleftharpoons$ ATP exchanges may be overcome by proposing the existence of separate catalytic sites for the exchanges or by invoking the participation of covalent intermediates in ATP synthesis.

However, Boyer and his coworkers have suggested that all the exchanges could occur by the overall reversal of ATP formation and that the observed differences in exchange rates are consistent with a concerted mechanism for ATP synthesis and a single mode of entry of water oxygen, provided that substrate dissociation steps rather than covalent bond-forming and -breaking steps are rate limiting in the exchange (Mitchell *et al.,* 1967; Boyer, 1967; Boyer and Silverstein, 1963). Mitchell *et al.* (1967) have pointed out that the experimentally observed rates of P_i $\rightleftharpoons$ HOH and ATP $\rightleftharpoons$ HOH exchanges are simultaneously so rapid under some conditions that it is necessary to propose the existence of a reaction separate from the reversal of ATP synthesis to account for all of the observed exchange rates in terms of a single mode of entry of water oxygen. In support of this, Mitchell *et al.* (1967) found an uncoupler-resistant P_i $\rightleftharpoons$ HOH exchange in submitochondrial particles. Boyer *et al.* (1973) have confirmed and extended these findings and propose that the separate P_i $\rightleftharpoons$ HOH exchange may result from the reversible formation of very tightly bound ATP which is unable to exchange with ATP free in the medium.

An alternative explanation of the exchange reactions associated with oxidative phosphorylation has been advanced by Korman and McLick (1970, 1972, 1973). These authors also propose that ATP synthesis involves the direct union of ADP and phosphate to form ATP by a concerted mecha-

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