maintaining the active enzyme conformation separately from that concerned directly with shaping the active site.

Luciferases from AK-6, -20, -18, and -1H have been purified without difficulty. In the purification of luciferases from TSL-11, -4H, -9, and -12 losses have occurred, but they have not been prohibitive. The properties of the purified mutant enzymes appear not to differ from those in crude extracts.

Many features of the luciferase system, including the ease with which a large number and variety of mutants can be generated, and the ability to follow enzyme activity *in vivo* at the permissive and restrictive temperatures, make it ideally suited as a system for investigation of the mode of action of enzymatic lesions that cause increased thermal sensitivity. However, if the potential advantages of this system are to be realized, we must understand the chemistry of the luminescence reaction. The altered-kinetics mutants should be especially useful in this latter problem.

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Nucleoside Diphosphokinase from Beef Heart Cytosol. I. Physical and Kinetic Properties†

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ABSTRACT: Nucleoside diphosphokinase (NDP-kinase) from beef heart cytosol has been purified about 200-fold. With this preparation the following molecular characteristics were assessed by sucrose gradient centrifugation, gel filtration on Bio-Gel P300, and electrofocusing: s_{20} = 5.9 S, mol wt 108,000, and isoelectric point of a major component = 8.5. The kinetic properties of beef heart cytosol NDP-kinase were consistent with a Ping-Pong mechanism. The ATP–ADP exchange catalyzed by the enzyme was three times faster than the UTP–ADP exchange and five to seven times faster than the CTP–ADP and GTP–ADP exchange. The rate of exchange depended essentially on the nature of the nucleoside triphosphate, but not on that of the nucleoside diphosphate. Also, the affinity was found to vary according to the nucleoside triphosphate; for instance, the K_M for UTP was three times as high as the K_M for ATP. By contrast, the K_M for various nucleoside diphosphates, ADP, UDP, CDP, GDP, were in the same range from 0.04 to 0.06 mM. The K_M for ATP was roughly six times as high as the K_M for ADP. Mg-ATP and Mg-ADP, and not the free nucleotides, were the true substrates; this contrasts with the mitochondrial beef heart NDP-kinase for which free ADP was preferred to Mg-ADP. Excess Mg-ADP was inhibitory and the K_i for excess Mg-ADP acting as inhibitor was only twice as high as the K_M for Mg-ADP acting as substrate. AMP was a competitive inhibitor both with respect to ADP and ATP. The K_i for AMP was 2.5 times higher than the K_M for ATP and 100 times higher than the K_M for ADP. The possible involvement of NDP-kinase in the regulation of nucleoside triphosphate synthesis is discussed.

The attention which has been paid in the past few years to the mitochondrial nucleoside diphosphokinase,[1] an enzyme catalyzing an ADP–ATP exchange, stems mainly from data (for review, *cf.* Lehninger and Wadkins, 1962) relating the mitochondrial ADP–ATP-exchange reaction to the mechanism of oxidative phosphorylation. In this laboratory, prop-

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[1] Abbreviations used are: NDP-kinase, nucleoside diphosphokinase or nucleoside triphosphate:nucleoside diphosphate transphosphorylase, EC 2.7.4.6; (Mg), free magnesium concentration; AOPCP, adenosine 5′-methylenediphosphonate.

erties of NDP-kinase from beef heart mitochondria have been analyzed and compared to those of other mitochondrial NDP-kinases. Evidence was provided from kinetic studies (Colomb *et al.*, 1969) and the demonstration of a stable phosphoenzyme derivative (Colomb *et al.*, 1968) that the enzyme from beef heart mitochondria catalyzes a transphosphorylation reaction with a Ping-Pong mechanism.

Since a large part of the NDP-kinase activity (more than 90%) is located in the cytosol fraction of beef heart homogenate (Colomb *et al.*, 1968), it appeared desirable to verify whether this dual cellular localization accompanies differences in catalytic behavior and molecular parameters. This paper describes some physical and kinetic properties of the NDP-kinase purified from beef heart cytosol (cytosolic NDP-kinase). The cytosolic NDP-kinase and its mitochondrial counterpart are compared and differences in properties are discussed as they relate to the molecular heterogeneity of beef heart NDP-kinase.

Experimental Section

Materials. Nucleotides used in this study (P. L. Biochemicals and Sigma) were checked by chromatography for purity (Krebs and Hems, 1953; Duée, 1968) and accordingly further purified by chromatography according to Hurlbert (1957). [^{32}P]Phosphoric acid was obtained from the Commissariat à l'Energie Atomique (Saclay).

Preparation of [γ-^{32}P]ATP and [β-^{32}P]ADP. [γ^{32}P]ATP was prepared according to Glynn and Chappell (1964). [β-^{32}P]ADP was obtained from [γ-^{32}P]ATP by a coupled reaction involving adenylate kinase in the presence of AMP and purified by exchange chromatography on Dowex 1.

Assays for Monitoring Enzyme Purification. A simple spectrophotometric assay was used to scan for enzyme activity in the course of enzyme purification. The reaction mixture in 3 ml contained 10 μM ATP, 10 μM AOPCP, 0.5 mM phosphoenolpyruvate, 70 mM KCl, 20 mM $MgSO_4$, 33 μM NADH, 0.1 M triethanolamine buffer (pH 7.6), 6 units of pyruvate kinase, 9 units of lactate dehydrogenase, and the NDP-kinase preparation. In this test AOPCP was chosen as nucleoside diphosphate, because, at the concentration used, it reacted quite efficiently with NDP-kinase but not with pyruvate kinase. The reaction was started by the addition of AOPCP and carried out at 25°. The decrease in absorbancy at 340 nm was followed with a Zeiss spectrophotometer.

An isotopic assay, more precise than the spectrophotometric one and referred to as standard isotopic assay was used to measure the activity in fractions where enzyme was detected. It was carried out, as described in a previous paper (Colomb *et al.*, 1969), with the following medium: 6 mM ATP, 12 mM $MgCl_2$, 0.2 mM [^{32}P]ADP, and 0.1 M triethanolamine-HCl buffer (pH 8).

Assay Used in Kinetic Studies. The same isotopic method as that mentioned above was used except that in this case the concentrations of ADP and ATP were varied and that the free Mg^{2+} concentration (Mg) was kept constant. Under these conditions, the ratios of Mg-ADP to free ADP and of Mg-ATP to free ATP also remain constant, which allows a better analysis of the enzyme mechanism. Values of 4000 and 70,000 M^{-1} were taken for the stability constants of $Mg\text{-}ADP^{1-}$ and $Mg\text{-}ATP^{2-}$, respectively, as determined in 0.1 M buffers at pH 8 by O'Sullivan and Perrin (1964). The small interference due to the monovalent cations in the test medium was neglected. The concentrations of total Mg^{2+}, required to maintain (Mg) at a fixed value were calculated from the relationship

$$(\text{total Mg}) = (\text{Mg}) + (\text{total ATP})\frac{K_2(\text{Mg})}{1 + K_2(\text{Mg})} + (\text{total ADP})\frac{K_1(\text{Mg})}{1 + K_1(\text{Mg})}$$

Physical Measurements. Sedimentation in sucrose gradient was performed essentially as described by Martin and Ames (1961). Centrifugation was carried at 4° for 15 hr at 38,000 rpm using a Beckman L2 50B centrifuge and a SW50 rotor. After centrifugation, the contents of the tubes were pumped through a 1-mm flow cell of a LKB Uvicord connected to a Varian recorder to monitor the uv absorbance. Standard proteins of known sedimentation coefficients and molecular weights were used as internal markers: bovine serum albumin (mol wt 67,000, s_{20} = 4.4 S, Hughes and Dintzis, 1964), hexokinase (mol wt 95,000, s_{20} = 5.5 S, Kenkare and Colowick, 1965), rabbit muscle glyceraldehyde phosphate dehydrogenase (mol wt 145,000, s_{20} = 7.3 S, Allison and Kaplan 1964). The NDP-kinase as well as the enzyme markers were located in the gradient by activity measurements on the fractions collected.

Stokes radius of NDP-kinase was determined by filtration on Bio-Gel P300 columns. Bio-Gel P300 was preferred to Sephadex G-200 which was found to retain or inactivate NDP-kinase. Standard proteins with known Stokes radii were used as markers: catalase 52.2 Å, glyceraldehyde phosphate dehydrogenase 43 Å, and serum albumin 36.1 Å (Ackers, 1964). Bio-Gel P300 was suspended in 0.2 M NaCl–0.1 M Tris-HCl buffer (pH 7.5) and allowed to swell at 3° for at least 48 hr. The gel was poured into glass columns of a diameter of 1.3 cm to a total length of 50 cm and allowed to settle under flow. Samples of standard proteins and of NDP-kinase (between 1 and 2 mg), dissolved in 0.1 ml of 0.2 M NaCl–0.1 M Tris buffer (pH 7.5), were then applied to the top of the gel. Elution was carried out at 2° at a flow rate of 2 ml/hr. Protein position was determined by monitoring the absorbancy of the eluate with an Uvicord cell at 280 mμ and by measuring the enzymatic activity in collected fractions. Gel filtration data are presented in terms of V_e/V_0, where V_e is the elution volume of a given molecular species and V_0 the void volume of the column measured with Blue Dextran 2000 (Pharmacia).

Electrofocusing (Vesterberg and Svensson, 1966) was carried out with an ampholine concentration of 1% in a 0–45% discontinuous sucrose gradient in a LKB 8101 column at 4° with 400 V for the first 15 hr and 800 V for the further 25 hr. After elution from the electrophoresis column, the pH of the collected fractions was determined at 20° and the NDP-kinase activity was assayed with the standard isotopic test.

Results

Enzyme Purification. Beef hearts were collected at the slaughterhouse immediately after the death of animals. At each step of the purification process, the adenylate kinase activity was checked by the use of the hexokinase and glucose-6-phosphate dehydrogenase system (Colomb *et al.*, 1969).

STEP 1. PREPARATION OF THE CYTOSOL FRACTION. Heart muscle was cut in small pieces which were extensively washed out in 0.25 M sucrose buffered at pH 7.6 by 10 mM Tris-HCl before homogenization in order to lower blood contamina-

TABLE I: Purification of the Nucleoside Diphosphokinase from Beef Heart Cytosol.

Step	Vol (ml)	Protein (mg)[a]	Total Adenylate Kinase Act. (Units)[b]	Sp NDP-kinase Act. (Units/mg of Protein)[c]	Total NDP-kinase Act. (Units)[c]
1. Crude cytosol fraction	3800	47,900	32,600	1.7	80,000
2. Heat denaturation	3350	16,100	510	4.7	75,000
3. Ammonium sulfate	190	3,880	181	17.8	69,000
4. DEAE-cellulose eluate	1040	230	Not detectable	62.1	14,000
5. Ammonium sulfate	5.5	52.8	Not detectable	222.0	11,770
6. CM-cellulose eluate	14.5	11.6	Not detectable	360.0	4,176

[a] Protein estimation was performed as described by Zak and Cohen (1961). [b] One unit of adenylate kinase catalyzes the conversion of 1 μmole of ADP into ATP per min at 28°. [c] One unit of NDP-kinase catalyzes the exchange of 1 μmole of [^{32}P]ADP with ATP per min at 28°.

tion as much as possible. Two successive centrifugations at 10,000*g* for 15 min and at 30,000*g* for 1 hr, respectively, were performed at 4° to eliminate mitochondria and mitochondrial fragments. A further centrifugation at 100,000*g* for 1 hr failed to sediment NDP-kinase; the 30,000*g* supernatant was routinely used for the purification of the cytosolic NDP-kinase.

STEP 2. HEAT TREATMENT. The 30,000*g* supernatant was divided into fractions of 1.5 l. distributed in 3-l. containers. The temperature of the solution was brought to 65° in about 20 min under constant stirring. The preparation was maintained at the same temperature for another 15 min, and then rapidly cooled to 1–3°, at which temperature all subsequent operations were performed. The mixture was centrifuged at 10,000*g* for 30 min and the clear supernatant was collected. This step resulted in a 3-fold increase of the specific activity without loss of total activity.

STEP 3. AMMONIUM SULFATE PRECIPITATION. Solid ammonium sulfate was added to bring the supernatant solution to 80% saturation. The mixture was kept at 2–3° for 24 hr and then centrifuged at 10,000*g* for 20 min. The precipitate was dissolved in a small volume of distilled water under magnetic stirring for 12 hr. Any insoluble material was discarded by a 20-min centrifugation at 10,000*g*. The supernatant fluid was dialyzed against 20 volumes of 5 mM NaCl–10 mM Tris-HCl (pH 9), for about 12 hr. The completion of the dialysis was checked by resistivity and pH measurements.

STEP 4. DEAE-CELLULOSE COLUMN CHROMATOGRAPHY. Before use, DEAE-cellulose (0.85 mequiv/g) was converted to the basic form. After equilibration with 5 mM NaCl–10 mM Tris-HCl (pH 9), the DEAE-cellulose was packed into a column (8 × 70 cm) on the top of which the dialyzed supernatant (step 3) was applied. The amount of dry DEAE-cellulose used was 20 times the amount of protein. The elution was carried out with a linear NaCl gradient ranging from 5 to 20 mM NaCl in 10 mM Tris-HCl (pH 9). The NDP-kinase was eluted between 10 mM and 18 mM NaCl, essentially free of adenylate kinase.

STEP 5. AMMONIUM SULFATE PRECIPITATION. This step was introduced to concentrate the large volume eluted from DEAE-cellulose. Proteins precipitated by ammonium sulfate at 80% saturation were collected, then dissolved in a small volume of distilled water, and dialyzed against 50 volumes of 70 mM NaCl–10 mM phosphate buffer (pH 5.5) for about 12 hr. Any insoluble material was removed by centrifugation at 10,000*g* for 20 min.

STEP 6. CM-CELLULOSE COLUMN CHROMATOGRAPHY. The soluble, dialyzed material from step 5 was chromatographed on a CM-cellulose column (0.4 × 20 cm) equilibrated with 70 mM NaCl–10 mM phosphate buffer (pH 5.5). The amount of dry CM-cellulose used (0.6 mequiv/g) was from 20 to 30 times the amount of protein. The enzyme was eluted from the column with a linear pH gradient from 5.5 to 7.0 at a constant ionic strength. The final product was a colorless solution.

An illustration of 210-fold purification of the enzyme from beef heart cytosol with an activity yield of about 5% is given in Table I.

STORAGE AND STABILITY OF ENZYME. NDP-kinase solutions were stored with 0.04% of sodium azide and bovine serum albumin at 2 mg/ml for at least 1 month at 0–2° without loss of activity. In dilute solutions the cytosolic NDP-kinase is unstable. For instance, more than half of the activity was lost after an 1-hr incubation at 28° of an enzyme solution at 1.8 μg/ml in 0.1 M phosphate buffer (pH 7). Maximal protection against loss of activity can be afforded by addition of bovine serum albumin at 2 mg/ml or 2 mM Mg-ADP or 5 mM free ATP. By contrast, $MgCl_2$ or free ADP alone are ineffective. Also acidic pH below 5 rapidly inactivates the enzyme. Inactivation by dilution is not prevented by dithiothreitol and must not be mistaken for the inactivation caused by thiol group reagents.

Molecular Size. The comparative distribution of beef heart cytosol NDP-kinase and protein markers in a sucrose gradient showed a peak of NDP-kinase activity corresponding to a sedimentation coefficient of 5.9 ± 0.1 S and a molecular weight of 103,000, a value similar to that found for the enzyme from beef heart mitochondria. A value in close agreement was obtained (108,000) from gel filtration data with Bio-Gel P300 (Figure 1). Molecular weights of the order of 100,000 have also been reported for NDP-kinases extracted from erythrocytes 100,000 (Mourad and Parks, 1966), yeast 102,000 (Yue *et al.*, 1967), beef liver 107,000 (Pedersen, 1968), and *Bacillus subtilis* 100,000 (Sedmak and Ramaley, 1971); however, the pea seed enzyme has a molecular weight of only 70,000 (Edlund, 1971).

The Stokes radius of the cytosolic NDP-kinase was approximated to 39.5 Å by graphical interpolation, using the filtration data on protein markers with known Stokes radii. Calculation from the equation of Ackers (1964) gave an identical value (39.4 ± 0.6 Å).

Isoelectric Point. The isoelectric point of the purified cytosolic NDP-kinase was measured by electrofocusing (*cf.*

TABLE II: Nucleotide Specificity.[a]

System of Nucleotides	Rate of Exchange (%)
ATP–ADP	100
UTP–ADP	32
UTP–UDP	31
GTP–ADP	12
CTP–ADP	21
CTP–CDP	15

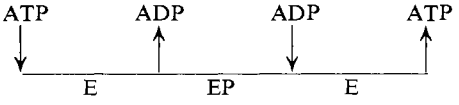
[a] The nucleoside diphosphates and triphosphates were used at a final concentration of 0.02 and 0.2 mM. The free Mg^{2+} concentration was 10 mM.

Methods). As shown in Figure 2, most of the activity focused at pH 8.5; there were two minor peaks at pH 7.7 and 7.2. This differs from behavior of the mitochondrial enzyme, in which the isoelectric point of a predominant electrophoretic form is around 7 (Colomb *et al.*, 1971).

Substrate Specificity. The reaction velocity for various nucleotides is shown in Table II. When measured at noninhibitory concentrations of substrate, the ATP–ADP exchange was three times faster than the UTP–ADP exchange and five to seven times faster than the CTP–ADP and GTP–ADP exchanges. On the other hand, the UTP–ADP exchange proceeded at the same rate as the UTP–UDP exchange. The rates of the CTP–ADP and the CTP–CDP exchanges also were similar. Clearly, the nature of the nucleoside triphosphate, but not that of the nucleoside diphosphate, controls the rate of the overall exchange reaction.

Ping-Pong Kinetics. NDP-kinase from beef heart cytosol displayed a maximal activity over a broad range of pH from 6.0 to 8.5. In this study, all kinetics were carried out at pH 8.0 since at this pH, ATP and ADP are virtually fully ionized, which allows an easier determination of the percentage of the free and Mg-bound nucleotides (see Methods).

The double-reciprocal plot of the ADP–ATP-exchange rate catalyzed at different fixed concentrations of ATP, when ADP was the variable substrate, yielded a family of parallel straight lines (Figure 3A,B). Such kinetics is typical of a Ping-Pong mechanism which in the case of the ADP–ATP exchange is most simply depicted by a two-step reaction



where E is the free enzyme and EP a stable phosphorylated intermediate; EP accumulates upon phosphorylation of E by ATP (first step) and is discharged by ADP to give ATP (second step). The initial rate equation for a Ping-Pong mechanism (Cleland, 1963) applied to the ADP–ATP exchange is

$$\frac{1}{v} = \frac{1}{V_{max}}\left[1 + \frac{K_{ATP}}{(ATP)} + \frac{K_{ADP}}{(ADP)}\right] \quad (1)$$

From data in Figure 3, the Michaelis constant for ADP was 0.05 mM. Michaelis constants for ATP taken from 9 experiments were found to vary from 0.04 to 0.1 mM with an average ratio K_{ATP}/K_{ADP} of the order of 6 (extreme values 3 and 10).

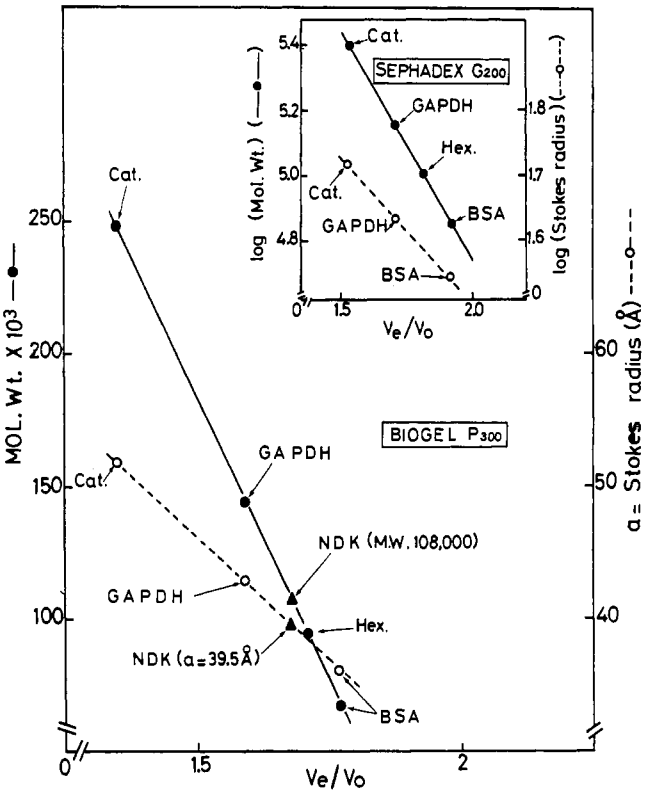


FIGURE 1: Chromatography of NDP-kinase on Bio-Gel P300. Experimental conditions are given in Methods. Note that the plot of log (molecular weight) *vs.* V_e/V_0 is linear with Sephadex G-200, whereas linearity with Bio-Gel P300 is obtained by plotting molecular weight *vs.* V_e/V_0.

Homologous or heterologous exchanges carried out with different couples of nucleotides revealed the same basic Ping-Pong mechanism as for the ADP–ATP exchange. The Michaelis constants for the nucleoside diphosphates were found to

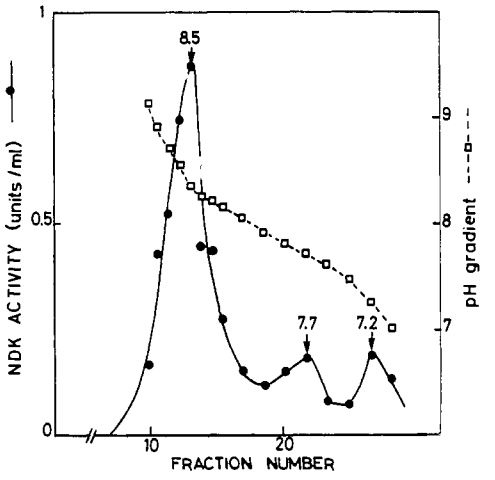


FIGURE 2: Electrofocusing profile of NDP-kinase. A discontinuous 0–45% sucrose gradient containing 1% (w/v) ampholine pH 3–10 was prepared in a LKB 110-ml electrofocusing column thermostated at 4°. Nine units of NDP-kinase in 4.6 ml of a mixture of 1% ampholine–15% sucrose was introduced, during the course of the gradient preparation at the corresponding level of density in the column. The cathode was at the bottom of the column. Fractions of 1.5 ml were collected. Other conditions as described in Methods.

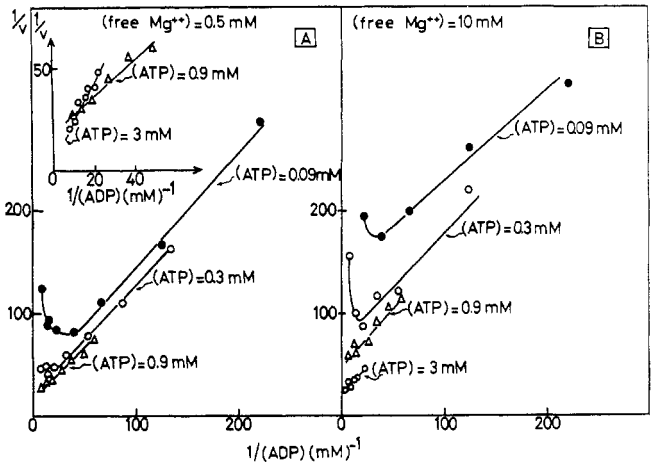


FIGURE 3: Initial velocity pattern for the ADP–ATP-exchange reaction. The concentrations given in the figure are for total ADP, ATP, and $MgCl_2$. The rate of exchange, v, is given in micromoles of [^{32}P]ADP exchanged per minute.

be of the same order of magnitude, 0.04–0.06 mM, in the following exchanges: ATP–ADP, UTP–ADP, UTP–UDP, GTP–ADP, CTP–ADP, and CTP–CDP. On the other hand, K_{UTP} was similar in UTP–ADP and UTP–UDP exchanges, and two to three times as high as K_{ATP} in the ATP–ADP exchange.

Inhibition by Excess ADP. The range of ADP, ATP, and $MgCl_2$ concentrations required for the Ping-Pong kinetics to be exhibited was rather limited due mainly to inhibition by excess substrate. This was especially noticeable for ADP. For instance, 0.04 mM ADP was found to be inhibitory when the concentrations of total ATP and free Mg^{2+} were held at 0.09 and 0.5 mM, respectively (Figure 3A); however, inhibition by excess ADP could be released by increasing the ATP concentration. Inhibition by excess ATP also occurred, but at much higher concentrations than in the case of ADP (above 0.9 mM in Figure 3A, insert). Initial velocity studies with different couples of nucleotides showed a significant difference between ADP and other nucleoside diphosphates as to the inhibitory effect displayed at high concentration of nucleoside diphosphate. This is illustrated in Figure 4. A maximal velocity of the ADP–ATP exchange was obtained at 0.02 mM ADP and an inhibition of 50% (with respect to the maximal activity) at 0.078 mM ADP. In comparison the rate of the UDP–UTP and ADP–UTP exchanges reached a maximum at 0.02 mM UDP and no inhibition occurred at higher UDP or ADP concentrations. One explanation for the inhibitory effect of excess ADP (I_1) and excess ATP (I_2) in the ADP–ATP exchange is that I_1 and ATP compete for the free enzyme E, on one hand, and I_2 and ADP for the phosphorylated enzyme EP, on the other. To take account of these postulated competitive interactions, the "inhibition terms" $K_{ATP}/V_{max} \times (ADP)/K_{i_{ADP}}$ and $K_{ADP}/V_{max} \times (ATP)/K_{i_{ATP}}$ where $K_{i_{ADP}}$ and $K_{i_{ATP}}$ are the dissociation constants for $E \cdot I_1$ and $EP \cdot I_2$ complexes, were included in eq 1

$$\frac{1}{v} = \frac{1}{V_{max}} \times \left[1 + \frac{K_{ATP}}{(ATP)}\left(1 + \frac{(ADP)}{K_{i_{ADP}}}\right) + \frac{K_{ADP}}{(ADP)}\left(1 + \frac{(ATP)}{K_{i_{ATP}}}\right)\right] \quad (2)$$

Provided the ATP to ADP ratio (α ratio) be kept constant when the ADP and ATP concentrations are varied, eq 2 can be rearranged as

$$\frac{1}{v} = \frac{1}{V_{max}} \times \left[1 + \frac{K_{ATP}}{K_{i_{ADP}}}\frac{1}{\alpha} + \frac{K_{ADP}}{K_{i_{ATP}}}\alpha + \left(\frac{K_{ATP}}{\alpha} + K_{ADP}\right)\frac{1}{(ADP)}\right] \quad (3)$$

At a given α ratio, a plot of $1/v$ *vs.* $1/(ADP)$ will be linear, with a vertical intercept

$$\frac{1}{V_{max}}\left(1 + \frac{K_{ADP}}{K_{i_{ADP}}}\frac{1}{\alpha} + \frac{K_{ADP}}{K_{i_{ATP}}}\alpha\right)$$

including all the inhibition terms and with a slope $(K_{ATP}/\alpha + K_{ADP})$, from which K_{ATP} and K_{ADP} can be derived. Likewise, a plot of $1/v$ *vs.* $1/(ATP)$ will yield a straight line which corresponds to

$$\frac{1}{v} = \frac{1}{V_{max}} \times \left[1 + \frac{K_{ATP}}{K_{i_{ADP}}}\frac{1}{\alpha} + \frac{K_{ADP}}{K_{i_{ATP}}}\alpha + (K_{ATP} + \alpha K_{ADP})\frac{1}{(ATP)}\right] \quad (4)$$

and which differs from the preceding one only by its slope value $(K_{ATP} + \alpha K_{ADP})$.

Another possible explanation for inhibition by excess ADP(I_1) or excess ATP(I_2) is the accumulation of ternary complexes of the type $E<^{ATP}_{I_1}$ or $EP<^{ADP}_{I_2}$, typical of noncompetitive inhibition. In the case for instance of inhibition by excess ADP, the term $(1/V_{max})[(K_{ATP}/ATP)(ADP/K_{i_{ADP}})]$ in eq 2 would have to be replaced by $(1/V_{max})[(K_{ATP}/ATP) \times (K_{i_{ATP}}/ADP)]$. The resulting equation after introduction of the α ratio

$$\frac{1}{v} = \frac{1}{V_{max}}\left[\left(\frac{K_{ATP} \cdot K_{i_{ADP}}}{\alpha}\right)\frac{1}{(ADP)^2} + \left(\frac{K_{ADP}}{K_{i_{ATP}}} + \frac{K_{ATP}}{\alpha} + K_{ADP}\right)\frac{1}{(ADP)}\right] \quad (5)$$

shows that in noncompetitive inhibition a plot of $1/v$ *vs.* $1/$ ADP is not linear but parabolic.

Experimental data presented in Figure 5 allow a choice between the two above-mentioned alternatives, namely competitive and noncompetitive inhibitions by excess substrate. Plots of $1/v$ *vs.* $1/$ADP at three different α ratios were strictly linear, a result in complete agreement with the hypothesis of competitive inhibition caused by excess ADP.

The vertical intercepts in Figure 5 have been replotted *vs.* $1/\alpha$ (insert of Figure 5). The points so obtained can be joined by a straight line which corresponds to

$$\frac{1}{v} = \frac{1}{V_{max}}\left[1 + \frac{K_{ATP}}{K_{i_{ADP}}}\frac{1}{\alpha} + \frac{K_{ADP}}{K_{i_{ATP}}}\alpha\right] \quad (6)$$

Since, in the present experiment, there was no inhibition by excess ATP, the term $K_{ADP}/K_{i_{ATP}}$ in eq 6 could be left out, which simplifies eq 6 as follows

$$\frac{1}{v_{int}} = \frac{1}{V_{max}}\left[1 + \frac{K_{ATP}}{K_{i_{ADP}}}\frac{1}{\alpha}\right] \quad (7)$$

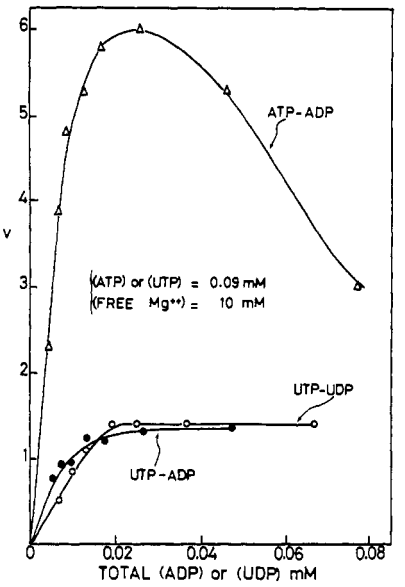


FIGURE 4: Effect of high concentrations of nucleoside diphosphate on the enzyme activity. The concentrations are for total ADP, UDP, ATP, and UTP and for free Mg^{2+}. The rate of exchange, v, is given in micromoles of [^{32}P]ADP or [^{14}C]UDP exchanged per minute.

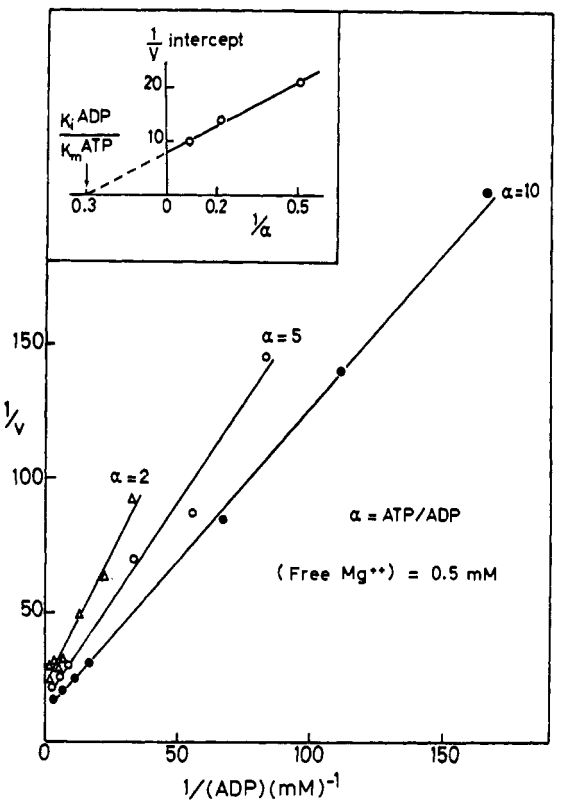


FIGURE 5: Double-reciprocal plot of $1/v$ *vs.* 1/ADP at several fixed ATP to ADP ratios (α ratios). The concentrations are for total ADP and total ATP. The free Mg^{2+} concentration was kept constant (0.5 mM). The rate of exchange, v, is given in micromoles of [^{32}P]ADP exchanged per minute.

A plot of $1/v_{int}$ *vs.* $1/\alpha$ is linear and cuts the horizontal axis at a point giving K_{iADP}/K_{ATP}. The ratio K_{ATP}/K_{iADP} was approximated to 3.3, a value rather close to that of K_{ATP}/K_{ADP} found experimentally of the order of 6.

Effect of Mg^{2+} on Kinetics. The Ping-Pong kinetics was demonstrated for a larger range of ADP and ATP concentrations when free Mg^{2+} was held at 10 mM (Figure 3B) instead of 0.5 mM (Figure 3A). This finding points to the dependence of the kinetics on $MgCl_2$. To study the effect of the free Mg^{2+} concentration (Mg) on the rate of the ADP–ATP exchange, an original method was developed.

Using stability constants of 70 mM^{-1} for Mg-ATP^{2-} and 4 mM^{-1} for Mg-ADP^{1-} (see Methods), a number of "Mg terms" were calculated and listed in Table III. These Mg terms essentially depend on the postulated substrates (free or Mg-bound nucleotides). As detailed below, the Mg terms are proportional to the slopes in the reciprocal plots of $1/v$ *vs.* 1/nucleotide when the ATP/ADP ratio is kept constant; varying (Mg) may alter the Mg terms and thereby the slope values. Assuming for instance that Mg-ATP (and not free ATP) is substrate, 1/(ATP) in eq 4 will be replaced by 1/(Mg-ATP) or by (1/(total ATP))[(1 + 70(Mg))/70(Mg)], where (1 + 70(Mg))/70(Mg) is the Mg term related to Mg-ATP (Table III, line 4) and 70 stands for the stability constant (mM^{-1}) of Mg-ATP^{-2}. Plotting $1/v$ *vs.* 1/(total ATP) will yield a straight line with a slope proportional to the Mg term, (1 + 70(Mg))/70(Mg), and therefore increasing as (Mg) decreases. An opposite variation of the slope value would be expected to occur if free ATP (instead of Mg-ATP) were substrate. The same reasoning applies if $1/v$ is plotted *vs.* 1/(total ADP) (Table III, line 1). It is therefore possible to predict, according to the slope variation as a function of (Mg), which nucleotide species (free or Mg bound) is the substrate of NDP-kinase.

Using this rationale, the data of two kinetic experiments differing essentially by the α ratio (30 and 5) will now be examined (Figure 6 and Table IV). In both cases, the slopes of the straight lines representing $1/v$ *vs.* 1/total ADP or 1/total ATP decreased when Mg was raised from 0.1 to 5 mM. Referring to the tabulated Mg terms (Table III, line 1 or 4), this result indicates that Mg-bound ADP and ATP are preferred as substrates to free ADP and ATP.

To assess the degree of preference of Mg-ADP over free ADP, the data obtained with the α ratio of 30 were chosen (Figure 4) because at high α ratio the Mg term related to ATP is negligible as compared to the one related to ADP. In this case the slope variations as a function of (Mg) essentially reflect the effect of ADP and allow an easy discrimination between Mg ADP and free ADP as substrates. As predicted in Table III (line 3) increasing (Mg) will result in an increase of the Mg terms and of the corresponding slopes if free ADP is substrate. Conversely, there will be no change in Mg term and thereby no change in the slope value if Mg-ADP is substrate. Figure 4B shows the data of Figure 4A plotted as $1/v$ *vs.* 1/Mg-ADP. The identical slopes obtained at three different (Mg) values obviously fit Mg-ADP as substrate and allow us to calculate a K_M value of 0.07 mM for Mg-ADP. It is noteworthy that the affinity of beef heart cytosol NDP-kinase for Mg-ADP differs from that of its mitochondrial counterpart for which free ADP is preferred to Mg-ADP as substrate (Colomb *et al.*, 1969).

Finally to delineate the relative participation of Mg-ATP as substrate with respect to free ATP, slopes obtained by plotting $1/v$ *vs.* 1/total ADP or ATP at low and high α ratios (5 and 30) were compared (Table IV). At a given (Mg), the values of slopes were significantly lower at $\alpha_2 = 5$ than at $\alpha_1 = 30$ and the slope difference, which is proportional to the Mg term related to ATP, decreased as (Mg) increased.

TABLE III: Mg Terms[a] as a Function of (Mg)[b] and α[c] for Four Possible Substrates of the ATP $\rightleftarrows$ ADP Exchange.

Abscissa Parameter in Reciprocal Plot	Mg Terms for Mg-ADP	Mg Terms for Free ADP	Mg Terms for Mg-ATP	Mg Terms for Free ATP
1/Total ADP	$\frac{1 + 4(Mg)}{4(Mg)}$	$1 + 4(Mg)$	$\frac{1 + 70(Mg)}{\alpha 70(Mg)}$	$\frac{1 + 70(Mg)}{\alpha}$
1/Free ADP	$\frac{1}{4(Mg)}$	1	$\frac{1 + 70(Mg)}{\alpha[1 + 4(Mg)]70(Mg)}$	$\frac{1 + 70(Mg)}{\alpha[1 + 4(Mg)]}$
1/Mg-ADP	1	$4(Mg)$	$\frac{1 + 70(Mg)}{\alpha[1 + 4(Mg)]}$	$\frac{[1 + 70(Mg)]4(Mg)}{\alpha[1 + 4(Mg)]}$
1/Total ATP	$\frac{\alpha[1 + 4(Mg)]}{4(Mg)}$	$\alpha[1 + 4(Mg)]$	$\frac{1 + 70(Mg)}{70(Mg)}$	$1 + 70(Mg)$
1/Free ATP	$\frac{\alpha[1 + 4(Mg)]}{4(Mg)[1 + 70(Mg)]}$	$\frac{\alpha[1 + 4(Mg)]}{1 + 70(Mg)}$	$\frac{1}{70(Mg)}$	1
1/Mg-ATP	$\frac{\alpha[1 + 4(Mg)]}{1 + 70(Mg)}$	$\frac{\alpha[1 + 4(Mg)]70(Mg)}{1 + 70(Mg)}$	1	$70(Mg)$

[a] The Mg terms are used to evaluate the influence of the free Mg concentration (Mg) on the slope values in the Lineweaver–Burk representations shown in Figures 5 and 6. The experimental slope change, positive or negative, obtained by varying (Mg) is compared to the predicted slope change calculated from the Mg terms for any of the four possible substrates (Mg-ADP, free ADP, Mg-ATP, free ATP). A postulated substrate is considered as an active component of the enzyme system when the predicted and experimental data fit together. [b] (Mg) = free Mg^{2+} concentration. [c] α = ATP/ADP.

Such a variation is consistent only with Mg-ATP acting as substrate; opposite variation would occur if free ATP were substrate (see Table III, line 1).

It was shown above that excess ADP inhibits NDP-kinase activity. One may wonder whether this inhibition is due to Mg-ADP or free ADP. This may be answered by considering the variation of the $1/v$ intercept as a function of (Mg) (Table IV, last column). The vertical intercepts can be calculated as

$$\frac{1}{V_{max}}\left(1 + \frac{K_{Mg\text{-}ATP}}{K_{i_{free\ ADP}}}\right)\frac{1 + 70(Mg)}{[1 + 4(Mg)]70(Mg)}\frac{1}{\alpha}$$

$$\frac{1}{V_{max}}\left(1 + \frac{K_{Mg\text{-}ATP}}{K_{i_{Mg\text{-}ADP}}}\right)\frac{1 + 70(Mg)}{1 + 4(Mg)}\frac{4}{70}\frac{1}{\alpha}$$

according as the inhibition by excess ADP is caused by free ADP or Mg-ADP. These relationships predict that an increase of (Mg) will result in a decrease of the $1/v$ intercept if excess free ADP is inhibitory; the reverse would hold if the inhibition was due to excess Mg-ADP. As shown in Table IV the $1/v$ intercept increased with increasing (Mg). This result leads us to conclude that the form of excess ADP which inhibits the NDP-kinase activity is Mg-ADP. In other words Mg-ADP, and not free ADP, behaves either as the substrate (at low concentrations) or as the inhibitor (at excess concentrations).

Inhibition of AMP. High concentrations of AMP inhibit the ADP–ATP exchange (Figure 7A). To determine the type of inhibition due to AMP, a kinetic study was carried out

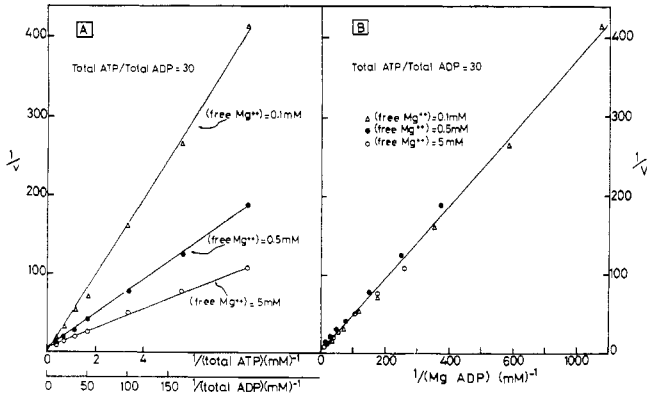


FIGURE 6: Double-reciprocal plot of $1/v$ *vs.* 1/ADP and 1/ATP at a fixed α ratio (30) and at several free $MgCl_2$ concentrations. The concentrations are for total ADP or total ADP (A), or Mg-ADP (B) and free Mg^{2+} concentrations. The rate of exchange, v, is given in micromoles of [^{32}P]ADP exchanged per minute.

TABLE IV: Effect of the Free Mg Concentration (Mg) on the Experimental Slope and Vertical Intercept Values in a Double-Reciprocal Plot ($1/v$ *vs.* 1/Total ADP or ATP).

(Mg) mM	% Mg-ADP[a]	% Mg-ATP	Slope[b] for α_1	Slope[b] for α_2	ΔSlope[c] ($\alpha_2 - \alpha_1$)	$1/v_{int}$[d] for α_2
0.1	28	87	2.00	1.62	0.38	8.0
0.5	66	97	1.00	0.72	0.28	10.0
5.0	95	99	0.70	0.44	0.26	11.8

[a] The percentages of Mg-ATP or of Mg-ADP at a given (Mg) value are the same for α ratios of 30 and 5; at different (Mg) values, they show a much larger variation for Mg-ADP than for Mg-ATP. [b] Slopes are expressed in time units (one slope unit is about 6 min). α_1 and α_2 refer to ATP/ADP ratios of 30 and 5, respectively. [c] The Δslope ($\alpha_1 - \alpha_2$) refers to the difference between the slopes obtained at a given (Mg) value when α ratios are held at 30 and 5. [d] $1/v_{int}$ refers to vertical intercepts; v is expressed in exchange rate units.

at constant ATP/ADP and AMP/ADP ratios. For convenience, these ratios are designated as α and β, respectively. According to the rate equation

$$\frac{1}{v} = \frac{1}{V}\left[1 + \frac{K_{ATP}}{K_{i_{ADP}}}\frac{1}{\alpha} + \frac{K_{ADP}}{K_{i_{2,AMP}}}\beta + \frac{K_{ATP}}{K_{i_{1,AMP}}}\frac{\beta}{\alpha} + \left(K_{ADP} + \frac{K_{ATP}}{\alpha}\right)\frac{1}{ADP}\right] \quad (8)$$

(where $K_{i_{1,AMP}}$ and $K_{i_{2,AMP}}$ are the dissociation constants for E-AMP and EP-AMP, respectively), if AMP competes with ADP and ATP, a plot of $1/v$ *vs.* 1/ADP will be a straight line, which in fact is the case; AMP can therefore be considered as a competitive inhibitor both with respect to ADP and ATP (Figure 6B). By studying the variations of the slope and the $1/v$ intercept values as a function of α and β, the following kinetic constants were derived: $K_{ATP} = 0.5$ mM, $K_{ADP} = 0.07$ mM, $K_{ATP}/K_{i_{1,AMP}} = 0.4$, and $K_{ADP}/K_{i_{2,AMP}} = 0.01$.

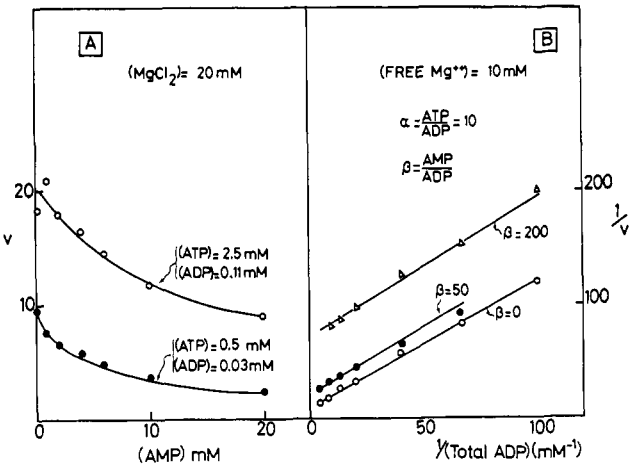


FIGURE 7: Inhibition of AMP of the ADP–ATP exchange. The concentrations are for total ATP and ADP. The rate of exchange is given in micromoles of [^{32}P]ADP exchanged per minute.

Discussion

Although the NDP-kinases extracted from a variety of sources (human erythrocytes, Mourad and Parks (1966); calf thymus, Nakamura and Sugino (1966); beef heart mitochondria, Colomb *et al.* (1966, 1969); beef liver mitochondria, Goffeau *et al.* (1967); yeast, Garces and Cleland (1969); pea seeds, Edlund (1971); *Bacillus subtilis*, Sedmak and Ramaley (1971)) are characterized by a same basic Ping-Pong mechanism, they may be distinguished by significant differences in kinetic and physical properties. For instance, mitochondrial NDP-kinases from beef heart and beef liver differ in that AMP inhibits only the heart enzyme and that an allosteric behavior is encountered only with the liver enzyme. NDP-kinase heterogeneity even within a same type of cell has been reported; this is the case for human erythrocytes where multiple forms of NDP-kinase have been separated by electrofocusing, agarose electrophoresis, phosphocellulose chromatography and characterized by molecular weight, affinity for ATP, and effect of temperature on the velocity of the reaction (Cheng *et al.*, 1971). Molecular heterogeneity has also been suggested for NDP-kinases extracted from calf thymus (Nakamura and Sugino, 1966) and from beef liver (Glaze and Wadkins, 1967). While NDP-kinases extracted from beef heart cytosol (this paper) and beef heart mitochondria (Colomb *et al.*, 1969) appear similar in size and shape as shown by density gradient sedimentation and molecular sieve chromatography, they apparently differ in their electrical charge, the isoelectric point of the predominant form of the mitochondrial enzyme being around pH 7 as compared to pH 8.3–8.5 for its cytosolic counterpart and also in some kinetic properties, for instance in that Mg-ADP is preferred as substrate to free ADP by the cytosolic enzyme whereas the reverse holds for the mitochondrial enzyme.

Based on kinetic data, the physiological significance of NDP-kinase may be expressed in terms of unidirectional transphosphorylation [ATP + XDP → ADP + XTP] between ATP and a nucleoside diphosphate XDP other than ADP, for replenishing the cellular cytosol with UTP, CTP, and GTP. That this reaction is mainly controlled both by the ADP concentration and the ratio of ATP to ADP concentrations is based on the following observations. (1) Excess ADP competes with ATP. Since the K_i for excess ADP is roughly twice the average K_M for ADP, it appears that ADP is a true substrate only at concentrations which are in the same range as, or lower than the K_M value. (2) The K_M values for UDP, CDP, and GDP are roughly the same as the K_M for ADP. In other words, considering the reaction ATP + XDP → ADP + XTP, the product ADP will inhibit the enzyme activity, not only by competition with ATP when present in excess concentration as mentioned above, but also at lower concentration by competition with XDP. In the latter case, instead of producing XTP, the reaction will deliver ATP, *i.e.*, the same product as the substrate, an obviously gratuitous catalysis. (3) Inhibition by excess ADP is apparently peculiar to ADP and not shared by other nucleoside diphosphates, such as UDP, CDP, GDP. Similarly to excess ADP, although to a lesser extent, AMP at high concentrations competes with ADP and ATP for binding to the enzyme. (4) The enzyme exhibits a higher affinity for ATP than for other nucleoside triphosphates.

A survey of data related to NDP-kinases listed above shows that the K_M for nucleoside triphosphates is from four to ten times as high as the K_M for nucleoside diphosphates. Furthermore, the K_i for excess ADP in the case of NDP-kinases, from yeast (Garces and Cleland, 1969) and from beef heart cytosol (this paper) was twice as much as the K_M for ADP. There is therefore a good agreement as to the regulatory constraints imposed especially by ADP to the functioning of various NDP-kinases for the purpose of UTP, CTP, and GTP synthesis. A prerequisite for an efficient synthesis of these nucleotides is that the ADP concentration be maintained at the lowest possible level; based on these premises, glycolytic and oxidative phosphorylations appear to be the most likely candidates for a control of the NDP-kinase activity in cell. In this vein, Thompson and Atkinson (1971) have recently postulated that the cellular NDP-kinase activity is regulated by the adenylate energy charge (0.5ADP + ATP)/(AMP + ADP + ATP). In fact, in liver, where GTP is required for phosphoenolpyruvate synthesis, the flow of phosphate from ATP to GDP indirectly controls the production of phosphoenolpyruvate (Garber and Ballard, 1970). In heart, as shown in this paper, the rate of production of UTP, a nucleotide involved in glycogen synthesis, appears to be subjected to a similar regulation.

Acknowledgments

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Nucleoside Diphosphokinase from Beef Heart Cytosol. II. Characterization of the Phosphorylated Intermediate†

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ABSTRACT: Beef heart cytosol NDP-kinase was phosphorylated by [^{32}P]ATP and the bound [^{32}P]phosphate could be discharged on ADP. The phosphorylation and dephosphorylation steps depended on Mg^{2+}, as assessed in rapid-flow experiments. These findings are in good agreement with kinetic results presented in the preceding paper. Phospho-NDP-kinase exhibited the same behavior as the free enzyme on sucrose gradient sedimentation and gel filtration, however, it could be differentiated by its more acid isoelectric point (5.9 instead of 8.5). Coelectrophoresis and cochromatography of alkaline hydrolysates of phospho-NDP-kinase, with reference to standard phosphoamino acids, allowed the identification of 3-phosphohistidine and traces of 1-phosphohistidine in the degradation products. A study of the acid lability of the phosphate linkage in the phosphoenzyme indicated that in native phospho-NDP-kinase, phosphate was bound to the N_1 of the imidazole ring of histidine and that 1-phosphohistidine could be isomerized into 3-phosphohistidine in an alkaline medium. Consistent with the phosphorylation of histidine and its presumed localization at the active site of the enzyme, was the finding that diethyl pyrocarbonate inhibited the exchange reaction and that inhibition was released by hydroxylamine. The enzyme was also inactivated by photooxidation in the presence of Rose Bengal; protection against photooxidation was afforded by ADP or ATP but not by 5′-adenylylmethylenediphosphonate, a methylene analog of ATP which is not a substrate.

In the preceding paper (Colomb *et al.*, 1972), we describe the purification of a nucleoside diphophokinase[1] isolated from beef heart cytosol, its molecular characteristics, and kinetic properties. Here we report physical and chemical studies on the phosphorylated intermediate of this enzyme.

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[1] Abbreviations used are: NDP-kinase and phospho-NDP-kinase, nucleoside diphophokinase and its phosphorylated derivative; AOPCP, adenosine 5′-methylenediphosphonate; AOPOPCP, 5′-adenylylmethylenediphosphonate.