Nucleoside Diphosphokinase from Beef Heart Mitochondria. Purification and Properties*

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ABSTRACT: A nucleoside diphosphokinase has been partially purified from beef heart mitochondria. The purified enzyme has a molecular weight of 103,000 ± 3,000 and an isoelectric point of the order of 9.5. The optimum pH depends upon the adenosine diphosphate concentration; it is 7.4 at low concentrations of adenosine diphosphate and ranges from 6 to 8 at high concentrations of adenosine diphosphate. Inactivation of the enzyme by an SH reagent such as *p*-mercuribenzoate is released by incubation with dithiothreitol. The adenosine diphosphate–adenosine triphosphate exchange catalyzed by the purified enzyme is twice as fast as the uridine diphosphate–uridine triphosphate, adenosine diphosphate–uridine triphosphate, or adenosine diphosphate–deoxyadenosine triphosphate exchanges and four to five times faster than the adenosine diphosphate–cytidine triphosphate, cytidine diphosphate–cytidine triphosphate, or guanidine diphosphate–guanidine triphosphate exchanges. The phosphonic analog of adenosine triphosphate, namely, AOPOPCP, is totally inactive as phosphate donor. The reaction mechanism obeys the Ping-Pong kinetics with Michaelis constants of 0.10 mM for adenosine diphosphate and 1.4 mM for adenosine triphosphate at a pH of 7.4. A study of the distribution pattern of the free and magnesium-bound forms of adenosine diphosphate and adenosine triphosphate shows that free adenosine diphosphate is preferred to magnesium adenosine diphosphate as phosphate acceptor, and that magnesium adenosine triphosphate is the only phosphate donor. High concentrations of magnesium adenosine diphosphate are inhibitory. Free adenosine triphosphate competes with free adenosine diphosphate for the phosphorylated form of nucleoside diphosphokinase. AMP is a noncompetitive inhibitor with respect to adenosine diphosphate. In contrast with nucleoside diphosphokinase from liver mitochondria, nucleoside diphosphokinase prepared from beef heart mitochondria does not exhibit allosteric properties under the experimental conditions reported in this paper.

Nucleoside diphosphokinase (nucleoside triphosphate:nucleoside diphosphate transphosphorylase, EC 2.7.4.6), an enzyme first detected in muscle and intestinal mucosa by Krebs and Hems (1953) and in yeast by Berg and Joklik (1953), has since been found in a variety of animal and plant tissues. Later Mourad and Parks (1965) and Norman *et al.* (1965) reported the phosphorylation by ATP of nucleoside diphosphokinase isolated from erythrocytes and from artichoke mitochondria. Considerable interest focused upon the finding of Norman *et al.* as suggesting a means whereby the mitochondrial nucleoside diphosphokinase could be involved in oxidative phosphorylation.

In a preliminary report from this laboratory (Colomb *et al.*, 1966) it was shown that the transphosphorylation reaction between ADP and ATP catalyzed by beef heart mitochondria nucleoside diphosphokinase follows a Ping-Pong mechanism. The evidence was based on kinetic data and isolation of a phosphorylated form of the enzyme. A similar conclusion concerning nucleoside diphosphokinase isolated from other sources than heart has been reported from other laboratories (Mourad and Parks, 1965, 1966a,b; Goffeau *et al.*, 1967; Pedersen, 1968). Although this common feature allows a description of a general basic mechanism for the nucleoside diphosphokinase reaction, a critical evaluation of physical and catalytic properties of the various nucleoside diphosphokinase preparations reported in the literature shows substantial differences and suggests that several types of nucleoside diphosphokinase may exist and may be distinguished according to their sources.

On the other hand, as shown by a study now in progress in our laboratory, nucleoside diphosphokinase is localized not only in mitochondria but also in cytosol of mammalian cells. Based on the fact that mitochondrial nucleoside diphosphokinase could act as a coupling factor in oxidative phosphorylation (Wadkins and Lehninger, 1963; Zalkin *et al.*, 1965; Bygrave and Lehninger, 1966; Glaze and Wadkins, 1967), the above finding prompted us to compare the properties of mitochondrial and cytoplasmic enzymes in order to delineate possible differences between them.

The present paper deals with the purification, the properties and the kinetics of nucleoside diphosphokinase isolated from beef heart mitochondria. Particular attention has been given to the role played by free and Mg-bound ADP or ATP in the kinetics of the ADP–

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ATP exchange reaction. A further communication will describe the physical and kinetic properties of the cytoplasmic nucleoside diphosphokinase from beef heart.

Experimental Procedures

Materials. Analytical grade sucrose was obtained from Mallinckrodt. ATP, ADP, DEAE-cellulose, CM-cellulose, and cristalline bovine serum albumin were purchased from Sigma. UDP, UTP, CDP, CTP, GDP, and dATP, purchased from Pabst, were purified before use by chromatography according to Hurlbert (1957). Reducing the contamination of nucleoside triphosphate by nucleoside diphosphate was absolutely necessary because of the inhibitory effect of nucleoside diphosphate when it is present in an amount above a critical level (*cf.* Results). In any experiment, the amount of the residual contamination of nucleoside triphosphate by nucleoside diphosphate was carefully evaluated and taken into account. AOPOPCP was obtained from Miles and Co., Elkart. Hexokinase, glucose 6-phosphate dehydrogenase, phosphoglycerate kinase, glyceraldehyde phosphate dehydrogenase, myokinase, alcohol dehydrogenase, and catalase were obtained from C. F. Boehringer and Soehne. Dithiothreitol (Cleland's reagent) was purchased from Calbiochem. γ-Globulins (average molecular weight 160,000) were obtained from Mann. Bio-Gel was purchased from Bio-Rad. [^{32}P]-Phosphoric acid was a generous gift from the Commissariat à l'Energie Atomique. [^{32}P]ADP was prepared according to a modified technique of Glynn and Chappell (1964) as described by Nachbaur (1968). [^{14}C]-Nucleotides were obtained from Schwarz Laboratories. All other reagents were of the highest quality commercially available.

Beef hearts were collected at the slaughterhouse immediately after the death of animals. Heart muscle was cut in small pieces which were extensively washed out in 0.25 M sucrose before homogenization in order to lower as much as possible blood contamination. After homogenization of the tissue, mitochondria were prepared by a modified technique of Crane, as described by Smith (1967) but with only one final washing. Integrity of the mitochondrial structure was verified by electromicrography (courtesy of Professor J. André): usually the preparations were devoid of significant contamination by other subcellular particles. The lack of contaminating erythrocytes was checked by spectrophotometry and was ascertained by the absence of the typical oxyhemoglobin or hemoglobin spectrum.

Protein estimation was performed with a modified Folin reagent (Zak and Cohen, 1961); for the calibration curve bovine serum albumin was used. For the fractions containing ammonium sulfate, all traces of this salt had to be eliminated by dialysis before protein estimation.

Assay of the Nucleoside Diphosphokinase Activity. The medium used for the determination of the nucleoside diphosphokinase activity was made of 6 μmoles of ATP, 12 μmoles of $MgCl_2$, 0.2 μmole of [^{32}P]ADP (70,000 cpm), and 100 μmoles of triethanolamine-HCl buffer (pH 7.4) in a final volume of 1 ml, which also included the volume of the incubated enzymic fraction. The incubation was initiated with the nucleoside diphosphokinase and proceeded at 28° for 5 min. The reaction was stopped by adding 0.1 ml of 30% trichloroacetic acid. The nucleotide separation was achieved by paper chromatography according to Krebs and Hems (1953) or according to Duée (1968).

After location under ultraviolet light of the areas corresponding to ADP and ATP, the respective sections were cut out from the paper sheets.

After radioactivity counting, the amount of incorporation in ATP was calculated as described by Boyer *et al.* (1959). The amount of enzyme was always chosen to give an exchange by transphosphorylation between ADP and ATP of about 30% of the maximum possible exchange.

The enzymic activity was expressed in units: we define one unit as that amount of nucleoside diphosphokinase which catalyzes the exchange of 1 μmole of [^{32}P]-ADP/min.

Assay of the Adenylate Kinase Activity. A detection test of adenylate kinase based on the use of the hexokinase and glucose 6-phosphate dehydrogenase system was performed at each step of the purification process (Oliver, 1955). The fractions were incubated for 30 min at room temperature with dithiothreitol (final concentration 10^{-3} M) in order to reactivate any denatured adenylate kinase.

In order to estimate nucleoside diphosphokinase activity in fractions containing adenylate kinase, a study of the distribution of $^{32}P_i$ in the P_β and the P_γ of ATP was carried out as described previously (Laturaze and Vignais, 1964) and the activity relevant to the specific catalysis of nucleoside diphosphokinase was calculated according to the formula, $[(\beta/\gamma) - 1]/[(\beta/\gamma) + 1]$, where β and γ represent the labeling of each of the two terminal phosphate groups in ATP.

At each step of the purification run, adenylate kinase activity was expressed in units: 1 unit of enzyme catalyzes the conversion of 1 μmole of ADP into ATP per min.

Column Chromatography. DEAE-cellulose (0.85 mequiv/g) and CM-cellulose (0.6 mequiv/g) were transformed, respectively, to the basic and acidic form before the final decantation which eliminated fines. Medium cellulose fiber sizes were used for a good rate of elution. All experiments were carried out with the same batch of cellulose.

The gradients were achieved with a special nine-chamber device as described by Peterson and Sober (1960). The eluates were monitored with an Uvicord cell at 253.7 or 280 mμ and collected with a LKB fraction collector. A second detection method based on a conductivity assay was also used and proved very useful for the purified fractions of low protein content which showed no appreciable ultraviolet absorption. These tests were made with a continuous-flow cell (LKB 5312 B) connected to a LKB conductolyzer type 5300 B.

Zonal Centrifugation. Density gradient centrifugations, performed essentially as described by Martin and Ames (1961), were carried out in the SW39 rotor (Spinco L2 65B centrifuge) at 2° for 15 hr at 38,000

rpm. The sucrose gradients (5–20%, w/v) contained 0.05 M Tris-HCl (pH 7.5). The markers used were bovine serum albumin (molecular weight 67,000, s_{20} = 4.4 S; Hughes and Dintzis, 1964), hexokinase (molecular weight 95,000, s_{20} = 5.5 S; Kenkare and Colowick, 1965), rabbit muscle glyceraldehyde phosphate dehydrogenase (molecular weight 145,000, s_{20} = 7.3 S; Allison and Kaplan, 1964), γ-globulins (molecular weight 160,000, s_{20} = 7.1 S; Putnam, 1965), and catalase (molecular weight 250,000, s_{20} = 11.4 S; Martin and Ames, 1961). The sedimentation was followed by elution of the fractions with a special device that included continuous ultraviolet control. The nucleoside diphosphokinase was located in the gradient by activity measurements on the fractions collected. For all the proteins tested except γ-globulins, the expression given by Svedberg and Pedersen (1940) relating the molecular weight to the sedimentation coefficient was verified.

Electrophoretic Mobility. The isoelectric point of the enzyme was measured by electrophoresis on cellulose acetate (Sepraphore III, Gelman Instrument Co., Ann Arbor). Aliquots (5 μl) of the nucleoside diphosphokinase preparation were used from each run. After 70-min electrophoresis under a stabilized potential of 10 V/cm, the strips were cut transversly into bands of 3 mm width. Each band was placed in a centrifuge tube containing 300 μl of 2% bovine serum albumin. After 15-min incubation at 0°, the ADP–ATP exchange reaction was started by addition of 200 μl of the standard incubation medium used for the assay of nucleoside diphosphokinase activity, at 28°, as described above.

Calculation of the Concentration of Free and Mg-Bound Adenine Nucleotides. To assess the significance of some kinetic data, it was necessary to estimate the concentration of the Mg–nucleotides complexes and of the free nucleotides present in the incubation medium. At pH 7.4, which is optimal for nucleoside diphosphokinase activity and which therefore has been used throughout this study, the predominant species of ATP and ADP are ATP^{4-}, $HATP^{3-}$, ADP^{3-}, $HADP^{2-}$, as well as the corresponding Mg complexes. Taking 6.97 and 4.88 as pK values for the equilibria $HATP^{3-} \rightleftarrows ATP^{4-}$ and $Mg\text{–}HATP^{-} \rightleftarrows Mg\text{–}ATP^{2-}$ (O'Sullivan and Perrin, 1964), the ratios ATP^{4-}:$HATP^{3-}$ and $Mg\text{–}ATP^{2-}$:$Mg\text{–}HATP^{1-}$ were found equal to 2.7 and 330, respectively. On the other hand, values of 75,000 and 500 M^{-1} were taken as stability constants for $Mg\text{–}ATP^{2-}$ and $Mg\text{–}HATP^{-}$ (O'Sullivan and Perrin, 1964). When substituting $Mg\text{–}HATP^{-}$ by $Mg\text{-}ATP^{2-}/330$ in the expression $Mg\text{–}HATP^{-} = 500(Mg^{2+})(HATP^{3-})$, we found a value of 49,000 M^{-1} for K_1, the "theoretical" stability constant of $Mg\text{-}ATP^{2-}$ at pH 7.4 ($K_1 = Mg\text{–}ATP^{2-}/(ATP^{4-} + HATP^{3-})(Mg^{2+})$). This value may be compared with 20,000 M^{-1} which is the stability constant of Mg–ATP experimentally determined according to Watanabe *et al.* (1963) using the same conditions (ionic strength and pH) as those currently used for the kinetic studies described in this paper.

The same type of calculation was applied to ADP. A pK of 6.65 for $HADP^{2-} \rightleftarrows ADP^{3-}$ and a pK of 4.70 for $Mg\text{–}HADP \rightleftarrows Mg\text{–}ADP^{-}$ were taken from O'Sullivan and Perrin (1964), likewise the stability constant of 4000 M^{-1} for $Mg\text{–}ADP^{-}$. For Mg–HADP the stability constant calculated from the data of Taqui Khan and Martell (1962) was found equal to 40 M^{-1}. By following the same rationale as for ATP we obtained a value of 3100 M^{-1} for the "theoretical" stability constant K_2 ($K_2 = Mg\text{–}ADP^{-}/(ADP^{3-} + HADP^{2-})(Mg^{2+})$) at pH 7.4. This value may be compared with 3000 M^{-1} found for the experimental stability constant determined, at pH 7.4, according to Watanabe *et al.* (1963).

From the above data it is obvious that at pH 7.4 in a medium containing ATP, ADP, and $MgCl_2$, the species $Mg\text{–}HATP^{1-}$ and Mg–HADP are present in negligible amount and that therefore the distribution of the predominant species is: $(ATP)_T = (ATP^{4-}) + (HATP^{3-}) + (Mg\text{–}ATP^{2-})$, $(ADP)_T = (ADP^{3-}) + (HADP^{2-}) + (Mg\text{–}ADP^{-})$, and $(Mg)_T = (Mg) + (Mg\text{–}ATP^{2-}) + (Mg\text{–}ADP^{-})$.

In this expression the subscript T refers to the total concentrations of ATP, ADP, and Mg. For convenience, subsequently in this paper we shall use the terms "free nucleotides" or "Mg–nucleotides" to designate the above-mentioned species which are predominant at pH 7.4.

From the above equations and from the relationships which relate K_1 and K_2 to free and complexed ATP and ADP

$$(Mg)^3(K_1K_2) + (Mg)^2[(K_1K_2)(ADP)_T + (ATP)_T + (Mg)_T + K_1 + K_2] + Mg[K_2(ADP)_T + K_1(ATP)_T - (K_1 + K_2)(Mg)_T + 1] - (Mg)_T = 0$$

This equation has three roots: two negative and one positive. The latter one corresponds unambiguously to the real value of Mg. The equation was solved with an IBM 7044 digital computer using an ALGOL program[1] (Chéruy, 1968). From the calculated value of (Mg), it was possible to derive the respective values of (ATP), (ADP), (Mg–ATP), and (Mg–ADP).

Treatment of Kinetic Data. A kinetic analysis has been made by fitting the experimental data to

$$v = \frac{x}{Ax + Bx^2 + C}$$

where x is (ADP) and A, B, and C are factors depending upon rate constants and upon ATP and Mg concentrations. Their dimension is as follows: A ($M^{-1}t$), B ($M^{-2}t$), and C (t), where M is a concentration and t a time. The experimental data were fitted to this equation in terms of x/v as a function of $Ax + Bx^2 + C$ by means of an iterative procedure; this procedure, based on Tchebitcheff's approximation, gave those values of A, B, and C that minimized the term

$$\max_x \left| \frac{\frac{x}{v} - (Ax + Bx^2 + C)}{x/v^2} \right|$$

[1] The ALGOL and FORTRAN programs are available from Laboratoire d'Automatique, Faculté des Sciences, 38-Grenoble, France.

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

Step	Vol (ml)	Protein (mg)	Total Adenylate Kinase Act. (units)[a]	Total Nucleoside Diphospho-kinase Act. (units)	Specific Nucleoside Diphosphokinase (units/mg)
I. After sonication	210	2550	380	572	0.225
II. Before DEAE-cellulose column	180	595	180	195	0.328
III. DEAE-cellulose eluate	325	29.2	0	76	2.600
IV. CM-cellulose eluate	7.5	1.3	0	36	26.700

[a] Units are defined under Experimental Procedures.

The calculations were carried out on a Bull Γ60 digital computer using a FORTRAN program (Chéruy, 1968).

Results

Purification of the Nucleoside Diphosphokinase from Beef Heart Mitochondria. A typical purification run on the mitochondrial enzyme is given in Table I. All operations were performed at 0–3°.

Beef heart mitochondria, suspended in Tris-HCl (0.01 M)–NaCl (0.005 M, pH 9) to give a protein concentration of 10–20 mg/ml, were disrupted by exposure to sonic oscillations in a Branson sonifier for three periods of 40 sec at 9 kc (step I). The suspension was dialyzed for 12 hr against 20 volumes of 0.005 M NaCl–0.01 M Tris-HCl (pH 9) for equilibration. The particles were removed by centrifugation at 30,000 rpm for 40 min in a Spinco centrifuge with a rotor 30. After measuring the protein concentration, pH, and ionic strength (step II) of the colored, limpid supernatant, it was then charged to a column of DEAE-cellulose previously equilibrated at pH 9 with 0.005 M NaCl and 0.01 M Tris-HCl buffer.

The amount of DEAE-cellulose used was at least 50 times the amount of protein. The elution was performed with a linear saline gradient ranging from 0.005 to 0.018 M NaCl in 0.01 M Tris-HCl (pH 9).

The nucleoside diphosphokinase was eluted between 0.012 and 0.015 M NaCl (step III). Apart from the nucleoside diphosphokinase activity recovered from DEAE-cellulose at pH 9, which accounts for most of the enzyme activity present in the mitochondrial extract, some nucleoside diphosphokinase activity was found in fractions eluted at a more acidic pH. For instance, it has been reported that a fraction containing nucleoside diphosphokinase associated with cytochrome *c* and structural protein is eluted at pH 7.5 with 0.1 M Tris-HCl (Laturaze and Vignais, 1964). This result suggests that nucleoside diphosphokinase may be isolated in a free or in a combined form.

The fraction eluted at pH 9 between 0.012 and 0.015 M NaCl was adjusted to pH 5.5 with dilute phosphoric acid. The ionic strength was kept low enough to allow the fixation of nucleoside diphosphokinase by CM-cellulose in the subsequent chromatography. CM-cellulose, previously equilibrated with 0.07 M NaCl and 0.01 M phosphate buffer (pH 5.5), was used in a 20:1 ratio to the protein fraction obtained from the preceding step. The protein fraction was poured on the column; at pH 5.5 the nucleoside diphosphokinase was fixed. Elution with NaCl and phosphate buffer was carried out with a continuous gradient ranging from pH 5.5 to 7, the ionic strength being kept constant. The enzyme was eluted at pH 6 (step IV). At this stage, in most preparations, no adenylate kinase was detected. It must be recalled that the contaminating adenylate kinase activity was always assayed after preincubation with dithiothreitol, as described in Methods, in order to reactivate any denatured adenylate kinase activity. If any contaminating adenylate kinase remained in the preparation of nucleoside diphosphokinase, a further step of purification was carried out. The eluate was concentrated by precipitation with ammonium sulfate (final concentration 531 g/l.) overnight at 0–3°. The precipitate was collected by centrifugation for 10 min at 10,000*g* and then dissolved in distilled water. Any insoluble material was centrifuged. The concentrated fraction was equilibrated against 100 volumes of 0.01 M Tris-HCl (pH 9) with two changes of buffer. The dialyzed fraction was poured on a small column of DEAE-cellulose equilibrated with 0.01 M Tris-HCl (pH 9) containing NaCl (0.005 M). Elution carried out with the same buffer led to purification of nucleoside diphosphokinase from the last traces of adenylate kinase.

Molecular Weight. The molecular weight and the sedimentation coefficient of nucleoside diphosphokinase from beef heart mitochondria were estimated by sucrose gradient centrifugation (*cf.* Methods). They were determined by following the peak of enzymatic activity since the ultraviolet absorption was found to be not always reliable, due to protein contaminants of close molecular weight. Calculation made by using catalase,

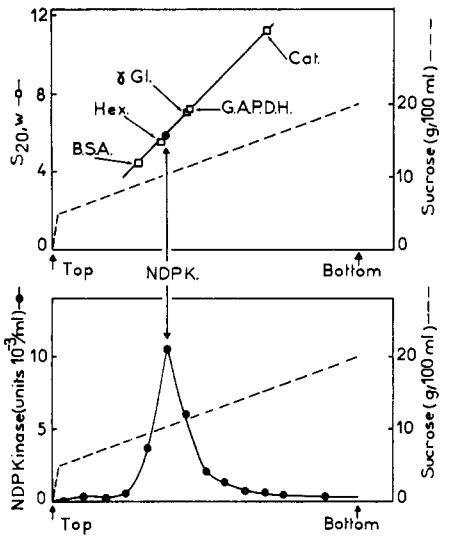


FIGURE 1: Sucrose density gradient centrifugation. The samples were diluted in Tris-HCl (0.05 M, pH 7) to a concentration of 5 mg/ml. They were layered (0.1 ml) on a 4.7-ml sucrose gradient of 5–20% sucrose in 0.05 M Tris-HCl (pH 7). After centrifugation as described in Methods, the tube was punctured and the elution was performed from bottom to top by pushing a solution of 25% sucrose in the tube with a constant speed driven syringe. Fractions (200 μl) were collected and their enzymic activity was tested. For other details, see Methods.

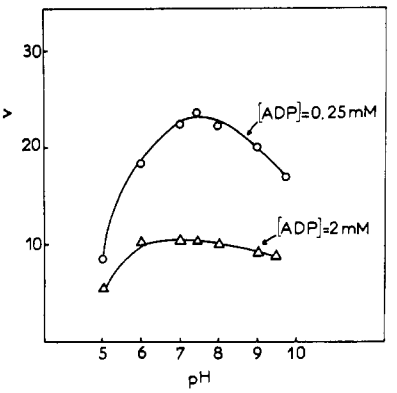


FIGURE 2: Effect of pH. Enzyme concentration was 23 munits/tube. Other conditions of incubation as indicated under methods. Below pH 7 ADP and ATP ensured the buffering of the medium and were adjusted to the desired pH in the presence of Mg^{2+} before addition to the medium. The pH of the different media was checked and eventually adjusted before incubation. The rate v is the amount of [^{32}P]ADP (millimicromoles) exchanged per minute.

glyceraldehyde phosphate dehydrogenase, γ-globulin, hexokinase, and bovine serum albumin as protein standards (*cf.* Methods) gave values of 5.9 ± 0.1 S and 103,000 ± 3,000 for the sedimentation coefficient and the molecular weight, respectively (Figure 1). It is noteworthy that the sedimentation coefficient of the nucleoside diphosphokinase activity from a crude extract of beef heart mitochondria has the same value as that of the purified enzyme. The value of molecular weight given here is different from that of 124,000 reported by Beyer (1968a) for an enzyme which may be similar or closely related. It may be recalled that the molecular weight of nucleoside diphosphokinase from various sources differs markedly: 30,000 for a basic molecular weight unit of the bovine liver enzyme (Wadkins and Glaze, 1965), 102,000 for the nucleoside diphosphokinase from yeast (Yue *et al.*, 1967), and 107,000 for the bovine liver enzyme studied by Goffeau *et al.* (1968) and Pedersen (1968).

Isoelectric Point. The purified enzyme was subjected to electrophoresis on cellulose acetate strips in glycine–NaOH buffers at different pH values ranging from 9.0 to 10.8 and in triethanolamine-HCl buffers at different pH values from 8 to 9. The molarity of the buffers was adjusted in order to maintain the resistivity at a constant value (700 ohms cm). The enzyme activity was located on the strips as described in experimental procedures. This method gave an estimated isoelectric point of 9.5. It is interesting to note that the electrophoretic mobility of the nucleoside diphosphokinase activity in a crude extract of beef heart mitochondria or in a purified enzymatic preparation is the same.

Stability Effect of SH Reacting Reagents. Purified preparations of nucleoside diphosphokinase from step IV of Table I can be stored at −20° for periods up to 2 weeks and thawed with no detectable loss of activity. After 1 month the enzyme is progressively denaturated with the formation of an inactive precipitate.

The sulfhydryl reagent, *p*-mercuribenzoate, at 10^{-4} M inhibits about 50% of the enzyme activity. Addition of dithiothreitol at 10^{-8} M reverses totally the inhibition.

pH Optimum. As shown in Figure 2, the activity of the mitochondrial nucleoside diphosphokinase is maximum at a pH of 7.4 when ADP concentration is maintained at 0.25 mM, a concentration which is optimum under the test conditions used. In contrast, a broad pH optimum from 6 to 8 is obtained at 2 mM ADP, a concentration which is inhibitory as will be described in detail later. Since the ionization state of ADP and ATP, and consequently the distribution pattern of the different Mg–nucleotides complexes, are pH dependent, the differences between the pH curves obtained at 0.25 mM ADP and at 2 mM ADP are probably explained by the ionization state of complexes formed between the enzyme and nucleotides present themselves as free or Mg complexes.

Specificity. The ADP–ATP exchange catalyzed by the mitochondrial NDP kinase is about twice as fast as the UDP–UTP, ADP–UTP, or ADP–dATP exchanges and four to five times faster than the ADP–CTP, CDP–CTP, and GDP–GTP exchanges (Table II). The rate of exchange depends much more upon the NTP than on the NDP species. For instance, when UTP is the phosphate donor, the ADP–UTP and UDP–UTP exchanges show comparable rates. In the same manner, the ADP–CTP and CDP–CTP exchanges proceed at the same rate. These observations suggest that the over-all process of exchange is rate limited by the step of phosphorylation of the free enzyme by NTP. No transphosphorylation between ADP and the phosphonic analog of ATP, namely, AOPOPCP, can be detected. The range of specificities found for low and noninhibitory concentrations of NDP holds as well for a high

TABLE II: Nucleotide Specificity.[a]

Expt	System of Nucleotides	Rate of Exchange (%)
1	[^{14}C]ADP and ATP	100
	[^{14}C]ADP and UTP	49
	[^{14}C]UDP and UTP	47
	[^{14}C]ADP and CTP	20
	[^{14}C]CDP and CTP	23
	[^{14}C]GDP and GTP	25
2	[^{32}P]ADP and ATP	100
	[^{32}P]ADP and dATP	50
	[^{32}P]ADP and AOPOPCP	<1

[a] The reaction mixture contained 0.25 mM [^{14}C]-nucleoside diphosphate, 3 mM nucleoside triphosphate, 6 mM Mg^{2+}, and 50 mM triethanolamine buffer (pH 7.4, expt 1). In expt 2 the same medium was used except that [^{14}C]XDP (X meaning nucleoside) was replaced by [^{32}P]ADP.

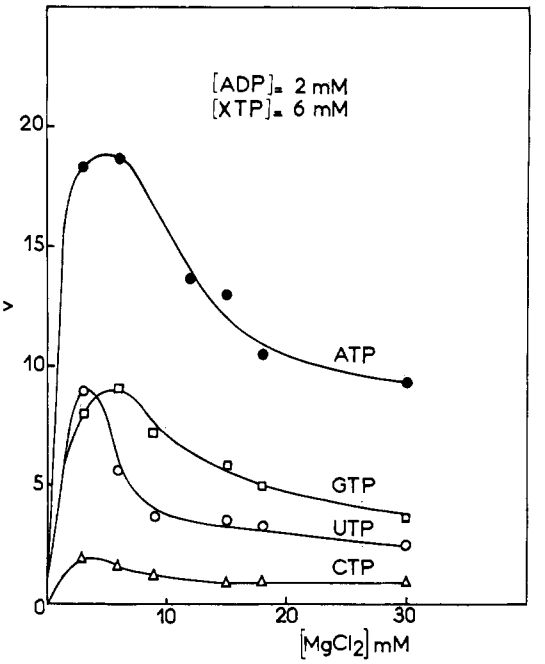


FIGURE 3: Specificity of nucleoside diphosphokinase at high concentration of ADP. Enzyme (64 munits) was added per tube. Conditions of incubation are similar to those used for the routine test with ATP and ADP. The concentrations given in the figure are for total XTP, ADP, and $MgCl_2$. The rate of exchange is given in millimicromoles of [^{32}P]-ADP exchanged per minute.

and inhibitory concentration of ADP (2 mM) (Figure 3). It is noteworthy that the nucleotide specificity of nucleoside diphosphokinase from beef heart mitochondria reported in this paper is markedly different from that of the beef heart phosphoryltransferase described by Beyer (1964a–c) and of other nucleoside diphosphokinases (Chiga and Plaut, 1959; Ratliff *et al.*, 1964; Nakamura and Sugino, 1966; Glaze and Wadkins, 1967; Goffeau *et al.*, 1967; Groot and Van Den Bergh, 1968).

Effects of ADP and ATP Concentrations at Different $MgCl_2$:ATP Ratios. Preliminary experiments showed that at low and noninhibitory concentrations of ADP the rate of the ADP–ATP exchange was maximum for a $MgCl_2$:ATP ratio equal to 2. In Figure 4a,b is shown the double-reciprocal plots of the exchange rate at different fixed concentrations of ATP when ADP is the variable substrate and when the $MgCl_2$:ATP ratio is maintained at a fixed value of 2. At low concentrations of ADP a series of parallel straight lines is obtained which is typical of a Ping-Pong mechanism as described by Cleland (1963). The apparent Michaelis constant for ADP calculated from the slopes of these parallels, as well as the apparent V_{max}, increases to a limit value when the concentration of ATP increases. From the data reported in Figure 4a, a Michaelis constant for ADP of 0.10 mM at an infinite ATP concentration can be calculated. Conversely, the K_m for ATP at an infinite ADP concentration calculated by extrapolation from a range of noninhibitory concentrations of ADP is 1.4 mM. The K_m values for ADP and ATP found by Mourad and Parks (1966a) in the case of the nucleoside diphosphokinase from human erythrocytes were 0.04 and 0.4–0.8 mM, respectively. In spite of these differences between beef heart and erythrocytic nucleoside diphosphokinases, in both cases the K_m for ATP is from 10 to 20 times greater than the K_m for ADP.

An increase in the concentration of ADP beyond the optimal concentration causes an inhibition of the nucleoside diphosphokinase reaction. The concentration of ADP above which a decrease of the rate of exchange is observed depends upon the concentration of ATP: for instance 0.17 mM ADP is not yet inhibitory at 6 mM ATP whereas 0.07 mM ADP is already inhibitory at 0.09 mM ATP.

Evidence for an allosteric behavior of the beef liver nucleoside diphosphokinase based on cooperative effects of Mg–ATP has been recently provided (Goffeau *et al.*, 1967). The plot of log $v/(V_{max} - v)$ against log (ATP) at two different concentrations of ADP, noninhibitory (inset Figure 4a) and inhibitory (inset Figure 4b), gives a Hill interaction coefficient of about 1 for the beef heart nucleoside diphosphokinase, a result which contrasts with that obtained for the liver enzyme.

As will be discussed in details below, increasing the $MgCl_2$:ATP ratio above 2 within the range of low and noninhibitory concentrations of ADP does not alter the rate of exchange (*cf.* Figure 7b). On the contrary, decreasing the concentration of $MgCl_2$ below that of ATP results in a striking inhibition of the reaction (Figure 5). When the rate of exchange is determined at different fixed concentrations of ATP, with ADP as variable substrate, and when the $MgCl_2$:ATP ratio is maintained at 0.5 (Figure 6), a family of converging lines in the Lineweaver and Burk graphical representation is obtained. Curiously enough the intersection of these

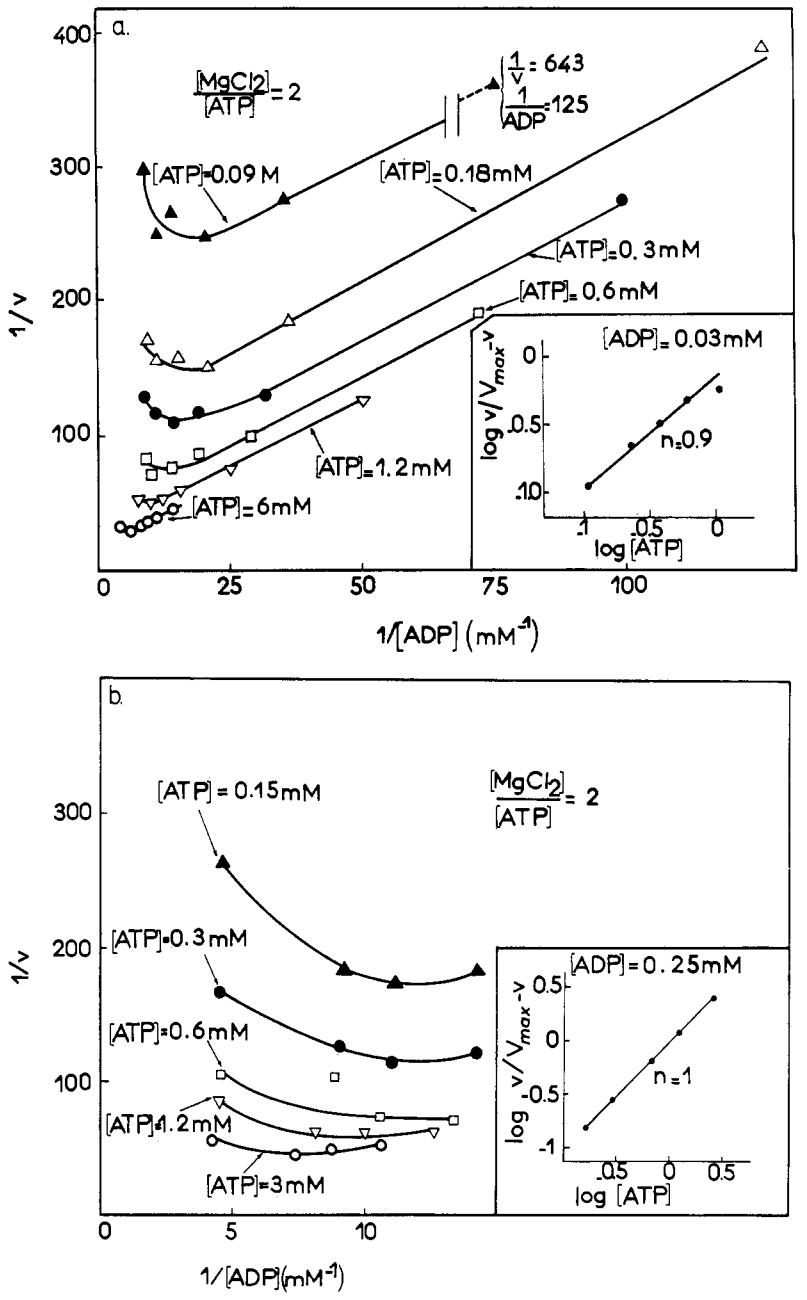


FIGURE 4: Double-reciprocal plots of $1/v$ *vs.* 1/ADP at several fixed concentrations of ATP and of $MgCl_2$. Nucleoside diphosphokinase (30 munits) was added per tube in part a and 50 munits in part b. Other conditions of incubation are indicated under Methods. The concentrations given in the figure are for total $MgCl_2$, ADP, and ATP. The rate of exchange, v, is given in micromoles of [^{32}P]ADP exchanged per minute.

lines is characterized by positive coordinates ($1/v$ and $1/ADP > 0$). This anomalous feature in terms of enzymatic inhibition is accounted for by the following changes of V_{max} and K_m (for ADP) as the ATP concentration is raised: the ratio of the apparent K_m for ADP to the apparent V_{max} increases whereas the value of $1/V_{max}$ decreases; in the classical Ping-Pong mechanism (Cleland, 1963) the ratio K_m^{ADP}/V_{max} would remain constant. In other words, the apparent K_m for ADP increases with increasing concentrations of ATP, a result which points to a competition between ADP and ATP. From the distribution of nucleotide species (Table III) it appears that for a $MgCl_2$:ATP ratio equal to 0.5, 54% of the ATP is free ATP and that less than 6% of ADP is Mg–ADP. It is obvious that under these conditions, the deviation from the Ping-Pong kinetics (see Figure 6) is due to the presence of an excess of free ATP in the medium. As will be discussed later, the anomalous intersection at $1/v > 0$ and $1/ADP > 0$ results from the superimposed effects of free ATP acting as inhibitor in the second step of the nucleoside diphosphokinase reaction and of Mg–ATP acting as substrate in the first step of the reaction.

Effect of Free and Mg-Bound Species of ADP and ATP.

TABLE III: Distribution of Free and Mg-Bound ADP and ATP.[a]

Total ADP (mM)	Total ATP (mM)	Total Mg (mM)	Mg–ADP (mM)	Free ADP (mM)	Mg–ATP (mM)	Free ATP (mM)
0.0130	0.600	0.300	0.0007	0.0123	0.281	0.319
0.0330	0.600	0.300	0.0017	0.0313	0.280	0.320
0.0730	0.600	0.300	0.0038	0.0692	0.279	0.321
0.1130	0.600	0.300	0.0058	0.1072	0.227	0.323

[a] This distribution pattern refers to Figure 6. As described in detail in Methods, Mg–ADP is essentially represented by $Mg–ADP^{2-}$, Mg–ATP by $Mg–ATP^{2-}$. Free ADP and free ATP are represented by ADP^{3-} and $HADP^{2-}$, on the one hand, and by ATP^{4-} and $HATP^{3-}$, on the other, in the ratios mentioned in Methods.

To relate the inhibitory effects of high concentrations of ADP to either Mg–ADP or free ADP, we carried out two parallel experiments in which the total ATP concentration was maintained at a fixed value of 0.6 mM. In the first experiment (Figure 7a), the total ADP concentration was held at 0.43 mM, a concentration which is inhibitory as already stated, and the $MgCl_2$ concentration was varied from 0 to 6 mM. In Figure 7a, the rate of exchange and the concentration of Mg–ADP have been plotted together with respect to the $MgCl_2$ concentration. A maximum rate is observed at 0.6 mM $MgCl_2$, *i.e.*, when the $MgCl_2$:ATP ratio reaches a value of 1. Under these conditions, 0.06 mM ADP, *i.e.*, 15% of the initial amount of the ATP, is complexed with Mg^{2+}. Increasing the $MgCl_2$ concentration above 0.6 mM results both in an abrupt increase in the concentration of the Mg–ADP complex and in a marked inhibition of the rate of exchange.

In the second experiment (Figure 7b) the total ADP concentration was maintained at 0.03 mM, a concentration which is not inhibitory. In this case, the rate of exchange is maximal when the $MgCl_2$:ATP ratio is equal to 2 (Figure 7b) and when 0.024 mM ADP (representing 70% of the total ADP) is complexed with Mg^{2+}. The decrease of the exchange rate above the optimal $MgCl_2$:ATP ratio is much less abrupt at low ADP concentration (Figure 7b) than at high ADP concentration (Figure 7a). These results reveal the inhibitory effect of the Mg–ADP species and indicate that the inhibition due to Mg–ADP is critically related to the total ADP concentration. They show that Mg–ADP behaves as an inhibitor at high concentrations of ADP and that Mg–ADP may be used as a phosphate acceptor at low concentrations of ADP although free ADP is preferred to Mg–ADP under these conditions.

The role played by free ATP in the inhibition of the ADP–ATP exchange reaction above the optimal $MgCl_2$ concentration seems to be negligible due to the low percentage of free ATP present under these conditions (Figure 7a,b).

To determine the effect of free ATP and Mg–ATP with respect to either Mg–ADP or free ADP, the following experiment was carried out. The total ATP concentration was kept constant at 0.9 mM and the total ADP concentration was varied. Three different concentrations of $MgCl_2$ were used (0.6 and 3 mM); for each concentration of $MgCl_2$ the calculated ratio of free ATP to Mg–ATP did not vary. Reciprocal plots of the data (Figure 8a,b) where the variable substrate is the fraction of free ADP (Figure 8a) or Mg–ADP (Figure 8b), yield a family of straight and converging lines which meet at $1/v$ intercept. The inhibition pattern in Figure 8a clearly indicates that free ATP even at relatively low concentrations behaves as a competitive inhibitor with respect to free ADP, a result which strongly suggests that Mg–ATP is the true phosphate donor in the ADP–ATP exchange reaction. Conversely, as shown in Figure 8b, the rate of exchange at a given concentration of Mg–ADP unexpectedly decreases when the concentration of Mg–ATP is raised by increasing the concentration of $MgCl_2$. This inhibition, although apparently paradoxical with respect to the above results (Figure 8a), is easily understood if it is related to the decrease in free ADP concentration when the Mg concentration is raised. It confirms that free ADP is preferred to Mg–ADP in the ADP–ATP exchange reaction and that Mg–ADP competes with free ADP for nucleoside diphosphokinase. The same experiment carried out at 6 mM ATP (actually

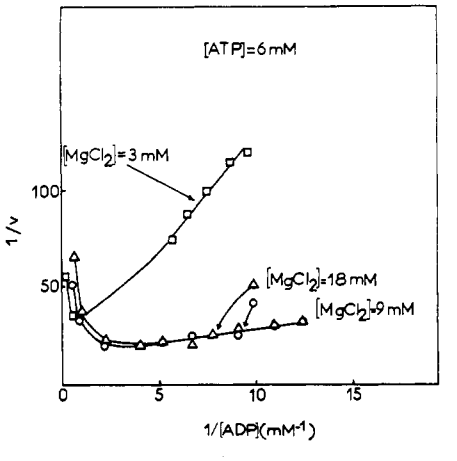


FIGURE 5: Effect of $MgCl_2$ concentrations on reaction velocities. Enzyme (50 munits) was added per tube. Other conditions are described under Methods. The concentrations given in the figure are for total $MgCl_2$, ADP, and ATP. The rate of exchange, v, is given in micromoles of [^{32}P]ADP exchanged per minute.

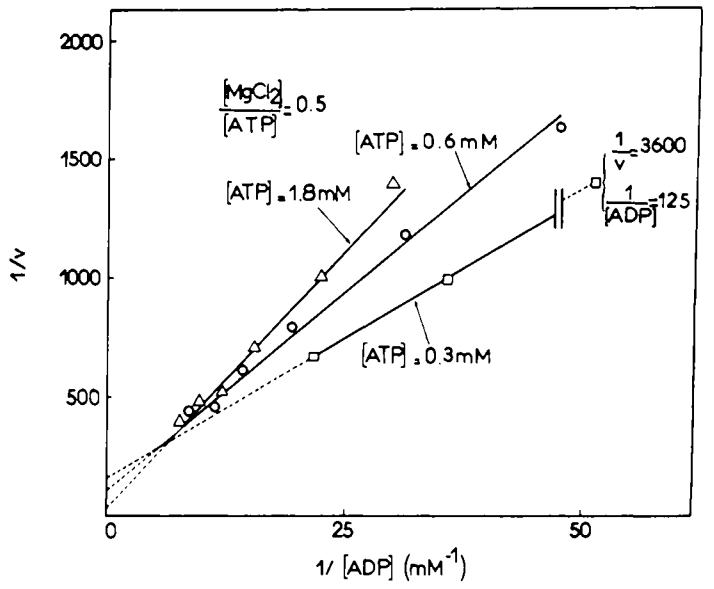


FIGURE 6: Double-reciprocal plots of $1/v$ *vs.* 1/ADP at several fixed concentrations of ATP and for a $MgCl_2$/ATP ratio of 0.5. Nucleoside diphosphokinase (20 munits) was added per tube. Other conditions are described under Methods. The concentrations given in the figure are for total $MgCl_2$, ADP, and ATP. The rate of exchange v is given in micromoles of [^{32}P]ADP exchanged per minute.

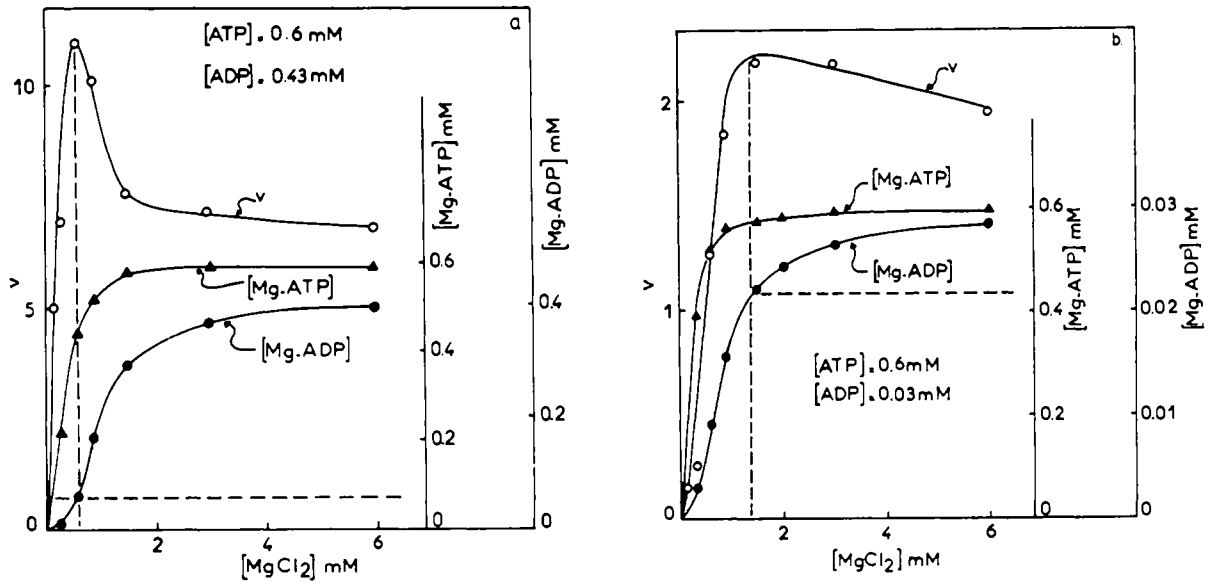


FIGURE 7: Relationship between the accumulation of Mg-ADP and the inhibition of the nucleoside diphosphokinase activity at high ADP concentration (a) and at low concentration (b). Amount of enzyme incubated per tube was 60 munits in part a and 10 munits in part b. Other conditions are described under Methods. The total ATP concentration was 0.6 mM in both parts. The total ADP concentration was 0.43 mM in part a and 0.03 mM in part b. The concentrations of Mg-ADP were calculated as a function of the concentrations of total ATP, ADP, and $MgCl_2$, as described in Methods. The rate of exchange, v, is given in millimicromoles of [^{32}P]ADP exchanged per minute.

the concentration of ATP used in the routine test) gives an inhibition pattern similar to that found with 0.9 mM ATP.

In the experiment to be described now, not only the free ATP:Mg-ATP ratio, but also the free ADP:Mg-ADP ratio was kept constant by maintaining the concentration of free Mg^{2+} at a constant level (Table IV and Figure 9). The rate of exchange was measured under these conditions at three different fixed concentrations of free Mg^{2+}: 0.32, 1.1, and 12.0 mM; it was found maximal at 1.1 mM free Mg^{2+}. The distribution pattern of free and Mg-bound ADP and ATP in this experiment (Table IV) indicates that the decrease of activity at 0.32 mM free Mg^{2+} is paralleled by an increase of free ATP, whereas the decrease of activity observed at 12 mM free Mg^{2+} is paralleled by an increased concentration of Mg-ADP. These data point again to the inhibitory effect of free ATP and of Mg-ADP on the rate of exchange.

Effect of AMP. Mourad and Parks (1966a) have observed that GMP inhibits the exchange catalyzed by erythrocytic nucleoside diphosphokinase, a finding which is at variance with the insensitivity of the bovine liver nucleoside diphosphokinase to AMP (Goffeau *et al.*, 1967; Glaze and Wadkins, 1967). In the case of beef heart nucleoside diphosphokinase, AMP behaves as a noncompetitive inhibitor with respect to ADP; with the low concentration of ATP used in this experi-

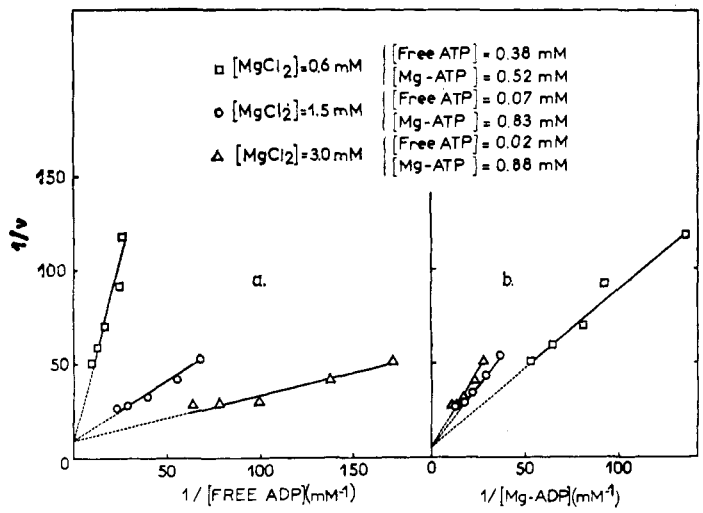


FIGURE 8: Effect of free ADP and Mg–ADP at different fixed concentrations of free ATP and Mg–ATP on the rate of exchange. Amount of nucleoside diphosphokinase incubated per tube was 60 munits. For other conditions, see Methods. Three different concentrations of total $MgCl_2$ were used: 0.6, 1.5, and 3.0 mM. The concentrations of Mg–ADP, Mg–ATP, free ADP, and free ATP were calculated as described in Methods. The rate of exchange v is given in micromoles of [^{32}P]ADP exchanged per minute. The same concentration of Mg–ATP or of free ATP plotted for three different fixed concentrations of $MgCl_2$ actually corresponds to different concentrations of total ADP.

TABLE IV: Distribution of Free and Chelated Species of ADP and ATP at Constant Excess of Free Mg.[a]

Total ADP (mM)	Total ATP (mM)	Total Mg (mM)	Mg–ADP (mM)	Free ADP (mM)	Mg–ATP (mM)	Free ATP (mM)	Free Mg (mM)
0.116	6.00	6.00	0.057	0.059	5.63	0.37	0.31
0.224	6.00	6.08	0.112	0.112	5.64	0.36	0.32
0.440	6.00	6.20	0.222	0.218	5.65	0.35	0.33
0.800	6.00	6.40	0.408	0.392	5.66	0.34	0.32
0.116	6.00	7.00	0.088	0.028	5.88	0.12	1.03
0.224	6.00	7.15	0.174	0.105	5.89	0.11	1.09
0.440	6.00	7.35	0.341	0.099	5.89	0.11	1.12
0.800	6.00	7.70	0.628	0.172	5.90	0.10	1.17
0.116	6.00	18.00	0.112	0.003	5.99	0.01	11.9
0.224	6.00	18.20	0.218	0.006	5.99	0.01	12.0
0.440	6.00	18.45	0.428	0.012	5.99	0.01	12.0
0.800	6.00	18.90	0.779	0.021	5.99	0.01	12.1

[a] The distribution pattern of Table IV refers to Figure 9. For convenience, the free Mg concentrations reported in Figure 9 were the mean of the values given in Table IV. For nucleotide species definition, see Table III.

ment, the inhibitory effect of AMP could be more clearly demonstrated (Figure 10). The calculated K_i value for AMP is about 4 mM.

Discussion

As shown previously (Colomb *et al.*, 1966) beef heart nucleoside diphosphokinase reacts with [^{32}P]ATP to form a phosphorylated enzyme intermediate which is itself capable of transferring its [^{32}P]phosphate group to ADP to form [^{32}P]ATP. This point has been confirmed recently by Beyer (1968b) and is substantiated by similar findings with nucleoside diphosphokinase extracted from other sources (Norman *et al.*, 1965; Mourad and Parks, 1965; Pedersen, 1968). The isolation of a phosphorylated derivative of nucleoside phosphokinase together with the kinetic data reported in this paper are consistent with a Ping-Pong mechanism (Cleland, 1963), which proceeds according to the following sequence: ATP adds first to nucleoside diphosphokinase; ADP is released and the enzyme is phosphorylated; a second molecule of ADP reacts with the phosphorylated enzyme to form ATP while the free form of the enzyme is regenerated (Scheme I).

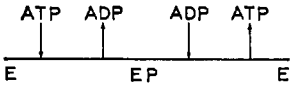
The initial velocity equation for a Ping-Pong mechanism (Cleland, 1963) applied to the ADP–ATP exchange is

$$v = \frac{V_{max}(\text{ADP})(\text{ATP})}{K_{ATP}(\text{ADP}) + K_{ADP}(\text{ATP}) + (\text{ADP})(\text{ATP})} \quad (1)$$

where K_{ADP} and K_{ATP} are the dissociation constants for EP–ADP and E–ATP complexes. However, it is obvious from different data given in this paper that eq 1 applied to the nucleoside diphosphokinase kinetics is

$$v = \frac{V_{max}(\text{Mg–ATP})(\text{ADP})}{K_{\text{Mg–ATP}}\left(1 + \frac{(\text{Mg–ATP})}{K_{i_{\text{Mg–ADP}}}}\right)(\text{ADP}) + K_{\text{ADP}}\left(1 + \frac{(\text{ATP})}{K_{i_{\text{ATP}}}}\right)(\text{Mg–ATP}) + (\text{Mg–ATP})(\text{ADP})} \quad (2)$$

SCHEME I



valid only in a limited range of concentrations of ADP, ATP, and $MgCl_2$ and that the kinetic evidence for a Ping-Pong mechanism depends upon the relative concentrations of the Mg-bound and free forms of ADP and ATP.

Actually, Scheme I does not take into account the occurrence of two abortive enzyme complexes. E–Mg–ADP and EP–ATP which are likely to occur as indicated by data presented in Figures 7 and 8. As shown in Figure 7a,b, increasing the $MgCl_2$ concentration above a critical level, especially at high concentrations of ADP results in a striking inhibition of the rate of exchange. This Mg-dependent inhibition of ADP is best explained by the accumulation of the abortive complex E–Mg–ADP. On the other hand, data in Figure 8a unambiguously demonstrate a competition between free ATP acting as inhibitor and free ADP acting as substrate for the phosphorylated form of nucleoside diphosphokinase, EP. This competition is assumed to lead to the formation of the abortive complex EP–ATP by reaction of free ATP with EP.

These conclusions are accounted for in Scheme II which can be detailed as follows (for convenience free ADP and free ATP have been written ADP and ATP):

$$\text{E} + \text{Mg–ATP} \underset{k_{-1}}{\overset{k_{+1}}{\rightleftarrows}} \begin{cases} \text{E,Mg–ATP} \overset{k_{+2}}{\underset{k_{-2}}{\rightleftarrows}} \text{EP} + \text{Mg} + \text{ADP} \\ \text{EP,Mg–ADP} \end{cases}$$

$$\text{E} + \text{Mg–ADP} \underset{k_{-3}}{\overset{k_{+3}}{\rightleftarrows}} \text{E,Mg–ADP (abortive complex)}$$

$$\text{EP} + \text{ATP} \underset{k_{-4}}{\overset{k_{+4}}{\rightleftarrows}} \text{EP,ATP (abortive complex)}$$

and the rate is given by eq 2, where $K_{\text{Mg–ATP}}$, $K_{i_{\text{Mg–ADP}}}$, K_{ADP}, and $K_{i_{\text{ATP}}}$ are the dissociation constants of E–Mg–ATP, E–Mg–ADP, EP–ADP, and EP–ATP, respectively.

To test the plausibility of this reaction mechanism and also to more easily study the distribution of the different forms of nucleoside diphosphokinase we have carried out, as already described in Figure 9, experiments in which the free Mg^{2+} was maintained in a constant excess amount. Under these conditions the ratio of Mg–nucleotide complexes to the free nucleotides remains constant and rate eq 2 can then be expressed in a form more easily available for mathematical treatment (eq 3). For this purpose, the concentrations of Mg–nucleotides and free nucleotides are expressed as functions of the concentrations of total nucleotides and free Mg^{2+}. As an example, Mg–ATP and free ATP concentrations are given by

$$(\text{Mg–ATP}) = \frac{K_2(\text{free Mg})}{1 + K_2(\text{free Mg})}(\text{ATP})_T$$

$$(\text{free ATP}) = \frac{(\text{ATP})_T}{1 + K_2(\text{free Mg})}$$

where K_2 is the binding constant for ATP and $(\text{ATP})_T$ is the concentration of total ATP (sum of free ATP and Mg–ATP).

The rate eq 3 is given by

$$v = \frac{V_{max}(\text{ADP})_T(\text{ATP})_T}{K'_{\text{ATP}}\left(1 + \frac{(\text{ADP})_T}{K''_{\text{ADP}}}\right)(\text{ADP})_T + K'_{\text{ADP}} \times \left(1 + \frac{(\text{ATP})_T}{K''_{\text{ATP}}}\right)(\text{ATP})_T + (\text{ADP})_T(\text{ATP})_T} \quad (3)$$

where

$$K'_{\text{ATP}} = K_{\text{Mg–ATP}}\frac{[1 + K_2(\text{free Mg})]}{K_2(\text{free Mg})}$$

$$K'_{\text{ADP}} = K_{\text{ADP}}[1 + K_1(\text{free Mg})]$$

$$K''_{\text{ADP}} = K_{i_{\text{Mg–ADP}}}\frac{[1 + K_1(\text{free Mg})]}{K_1(\text{free Mg})}$$

$$K''_{\text{ATP}} = K_{i_{\text{ATP}}}[1 + K_2(\text{free Mg})]$$

K_2 and K_1 are the binding constants for ATP and ADP. In this special case, where the $(\text{ATP})_T$ concentration is fixed, v may be derived from eq 3 as a function of $(\text{ADP})_T$.

$$v = \frac{1}{A + B(\text{ADP})_T + \frac{C}{(\text{ADP})_T}} \quad (4)$$

where

$$A = \frac{1}{V_{max}}\left[1 + \frac{K'_{\text{ATP}}}{(\text{ATP})_T}\right]$$

$$B = \frac{1}{V_{max}}\left[\frac{K'_{\text{ATP}}}{K''_{\text{ADP}}(\text{ATP})_T}\right]$$

$$C = \frac{K'_{\text{ADP}}}{V_{max}}\left[1 + \frac{(\text{ATP})_T}{K''_{\text{ATP}}}\right]$$

According to the method of analyzing the kinetic data (see Methods) and as reported in detail elsewhere (Chéruy, 1968), the fitting of experimental data to eq 4

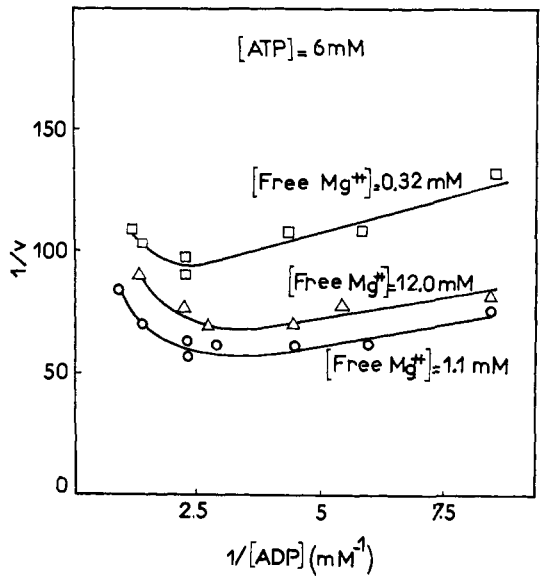


FIGURE 9: Effect of ADP in the presence of a constant excess of free Mg^{2+} on the rate of exchange. Amount of enzyme incubated per tube was 15 munits. Other conditions are described under Methods. The concentrations given in the figure are for total ATP, total ADP, and free Mg^{2+}. The rate of exchange, v, is given in micromoles of [^{32}P]ADP exchanged per minute.

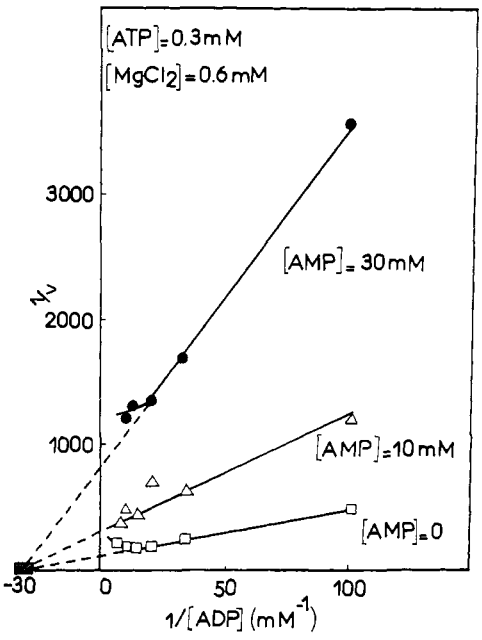


FIGURE 10: Inhibition of nucleoside diphosphokinase by AMP. The amount of enzyme incubated was 15 munits/tube. Other conditions are given under Methods. The concentrations given in the figure are for total ATP, ADP, AMP, and $MgCl_2$. The rate of exchange, v, is given in micromoles of [^{32}P]ADP exchanged per minute.

allows the determination of the values of A, B, and C under the above specified condition. A satisfactory fitting (Figure 11a) has been verified at different fixed concentrations of free Mg^{2+} and of $(ATP)_T$, a result which corroborates the plausibility of the reaction mechanism expressed by Scheme II.

A set of distribution equations for the steady-state conditions has been derived from eq 3.

$$\frac{[E]}{[E_0]} = \frac{K'_{ATP}}{V_{max}(ATP)_T} \times v$$

$$\frac{\text{E-Mg-ATP} + \text{EP-Mg-ADP}}{E_0} = \frac{[E_1] + [E_2]}{[E_0]} = \frac{1}{V_{max}} \times v$$

$$\frac{\text{E-Mg-ADP}}{[E_0]} = \frac{[E_3]}{[E_0]} = \frac{1}{V_{max}(ATP)_T} \frac{K'_{ATP}}{K''_{ADP}} (ADP)_T \times v$$

$$\frac{\text{EP-ATP}}{[E_0]} = \frac{[E_4]}{[E_0]} = \frac{1}{V_{max}(ADP)_T} \frac{K'_{ADP}}{K''_{ATP}} (ATP)_T \times v$$

$$\frac{[EP]}{[E_0]} = \frac{1}{V_{max}(ADP)_T} \frac{K'_{ADP}}{} \times v$$

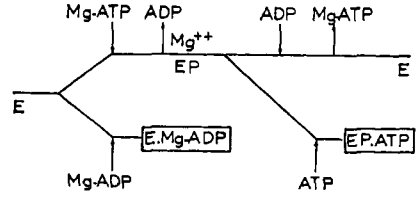
$$[E_0] = [E_1] + [E_2] + [E_3] + [E_4] + [E] + [EP]$$

Data in Figure 11b illustrate the correlation between the distribution pattern of the different possible forms of nucleoside diphosphokinase, calculated from the above equations and the rate of exchange under typical conditions of inhibition by excess free ATP and by excess Mg–ADP. These data provide direct evidence that excess Mg–ADP leads to an accumulation of EP–ATP.

It has been mentioned in this paper that the kinetic behavior of the nucleoside diphosphokinase from beef heart mitochondria is similar in some aspects to that of the nucleoside diphosphokinase isolated from beef liver mitochondria (Goffeau *et al.*, 1967), although marked differences exist which bear on the effects of AMP, allosteric properties, and nucleotide specificities. With respect to the lack of allosteric properties, a possible alteration of the structure of nucleoside diphosphokinase during its purification from beef heart mitochondria, eventually by dissociation into subunits, can be eliminated on the basis of ultracentrifugation and electrophoresis criteria since the nucleoside diphosphokinase activity from crude extracts of mitochondria or from purified preparations exhibit similar characters of sedimentation and of electrophoretic mobility.

In beef heart, nucleoside diphosphokinase is located not only in mitochondria but also in the soluble supernatant fluid obtained after centrifugation of heart homogenates at 100,000g for 1 hr. We have found

SCHEME II



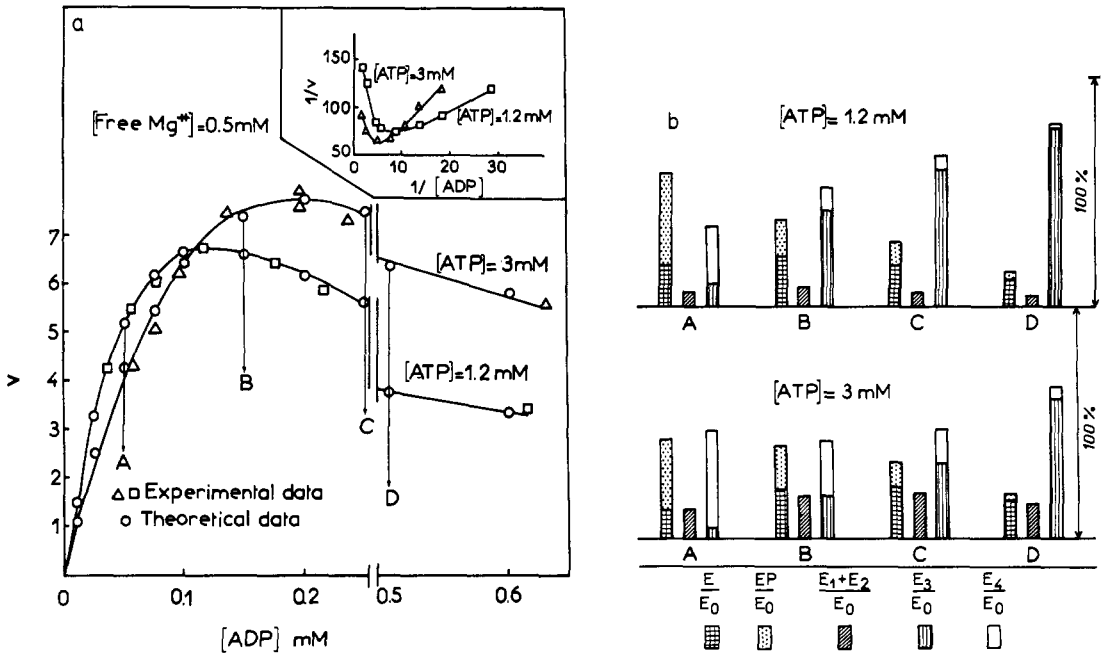


FIGURE 11: Data comparisons and distribution patterns. (a) Comparison of experimental and theoretical data. Two total ATP concentrations were studied with constant free Mg excess and variable total ADP concentrations. The amount of enzyme incubated was 15 munits/tube. The rate of exchange, v, is given in millimicromoles of [^{32}P]ADP exchanged per minute. In the inset, v in $1/v$ is given in micromoles of [^{32}P]ADP exchanged per minute. (b) Distribution pattern of the different possible forms of nucleoside diphosphokinase. This distribution is calculated for four total ADP concentrations considered in part a. E stands for free nucleoside diphosphokinase, E_1 for E-Mg-ATP, E_2 for EP-Mg-ADP, E_3 for E-Mg-ADP, E_4 for EP-ATP, and EP for the phosphorylated nucleoside diphosphokinase.

(unpublished experiments) that the nucleoside diphosphokinase prepared from beef heart mitochondria and the corresponding enzyme prepared from the supernatant fraction of heart homogenates are characterized by a number of similar physical and kinetic properties (molecular weight, isoelectric point, pH optimum, and Ping-Pong kinetics). However, the two enzymes seem to differ by their stability; in contrast with the mitochondrial enzyme, the nucleoside diphosphokinase obtained from the soluble supernatant fraction of beef heart becomes unstable when it is diluted for the activity assay and is totally inactivated by 10^{-4} M *p*-mercuribenzoate. These data suggest that several forms of nucleoside diphosphokinase, which may be isoenzymes, are present in mammalian tissues.

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