Purification, Properties, and Allosteric Activation of Nucleoside Diphosphatase*

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ABSTRACT: Nucleoside diphosphatase (nucleoside diphosphate phosphohydrolase, EC 3.6.1.6) from rat liver has been purified over 800-fold and used for preliminary kinetic studies of the metal ion activation, substrate specificity, and mechanism of action of the enzyme, as well as of its allosteric activation by nucleoside triphosphates. The essential divalent metal ion requirement was met by Mg^{2+}, Mn^{2+}, or Ca^{2+} and it appears that these ions are involved in the formation of metal–nucleotide complexes that function as substrates for the reaction. At pH 8.5 and a concentration of 0.5 mM, the rate of hydrolysis of the magnesium complexes of various nucleoside diphosphates and thiamine pyrophosphate decreased in the order: uridine diphosphate $\simeq$ guanosine diphosphate $\simeq$ inosine diphosphate $\gg$ cytosine diphosphate $\geqslant$ thiamine pyrophosphate $\gg$ adenosine diphosphate. No hydrolysis of d-thymidine diphosphate was detected. When $MgIDP^-$ was used as the substrate and IDP^{3-} maintained at a concentration of 0.1 mM, double-reciprocal plots of the initial velocity as a function of substrate concentration were curvilinear. Relatively high concentrations of magnesium–nucleoside triphosphates or IDP^{3-} had the effect of increasing the initial reaction velocity at lower substrate concentrations with the result that the double-reciprocal plots became linear. It was concluded that the above results were consistent with the idea that while two molecules of substrate can undergo interdependent reaction with the enzyme, the addition of only one is necessary for reaction to occur. From the data relating to the activating effects of magnesium–nucleoside triphosphates and IDP^{3-}, it has been concluded that these allosteric activators combine with the enzyme at one of the sites at which $MgIDP^-$ can react. Higher concentrations of free Mg^{2+} have been found to inhibit the reaction.

A number of enzymes which are involved in the control of intracellular reactions are characterized by the fact that double-reciprocal plots of initial velocity as a function of substrate concentration are nonlinear or curvilinear, rather than linear (Atkinson, 1966; Stadtman, 1966). Enzymes of this type are also subject to the influence of modifiers, which may or may not be structurally related to the substrate, and have been considered to belong to a special class known as allosteric enzymes (Monod *et al.*, 1963). Although studies have been made on a large number of these enzymes and various hypotheses proposed to account for the results (*cf.* Kowalik and Morrison, 1968), no definitive conclusions of general applicability can be drawn about their reaction mechanisms. Thus there appeared to be good reasons for undertaking a detailed kinetic investigation of the reaction catalyzed by an allosteric enzyme with the object of elucidating the reaction sequence.

Because of the interest in this laboratory in the mechanism of action of enzymes which catalyze phosphoryl group transfer reactions, the enzyme chosen for study was nucleoside diphosphatase. This enzyme was discovered by Yamazaki and Hayaishi (1965) who showed that it was activated by nucleoside triphosphates which did not undergo reaction. An additional advantage in connection with kinetic investigations of this enzyme is that it catalyzes a relatively simple reaction, *viz.*, nucleoside diphosphate $\xrightarrow{M^{2+}}$ nucleoside monophosphate $+ P_i$.

As a prelude to a more detailed quantitative kinetic study of the reaction, attention was directed toward obtaining from rat liver a highly purified preparation of nucleoside diphosphatase which has been used to investigate some of the general and kinetic properties of the enzyme. The results indicate that the enzyme has an essential requirement for the bivalent metal ions, Mg^{2+}, Mn^{2+}, or Ca^{2+}, and a broad substrate specificity in that it hydrolyzes UDP,[1] GDP, IDP, CDP, thiamine pyrophosphate, and ADP. Further, it has been confirmed that double-reciprocal plots of the initial velocity of the reaction as a function of the concentration of $MgIDP^-$ are nonlinear and that the presence of the allosteric activators, $MgATP^{2-}$ or IDP^{3-}, gives rise to linear double-reciprocal plots.

Materials and Methods

Materials. The sodium salts of all nucleotides were purchased from P-L Biochemicals with the exception of dTDP and TPP which were purchased from Calbiochem, and the phosphonate analogs of ATP which were purchased from Miles Laboratories. ATP was re-

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[1] For abbreviations, see *Biochemistry* 5, 1445 (1966).

crystallized twice from ethanol at 2° by the procedure of Berger (1956) and IDP, which contained about 5% IMP, was purified by a modification of the method of Morrison *et al.* (1961). A solution of IDP (1.3 g) was adsorbed onto a column (3.5 × 12 cm) of Dowex 1 (formate, 200–400 mesh) and eluted by using a linear gradient formed from 1 M formic acid and 1 M formic acid containing 2 M ammonium formate. Both mixing and reservoir vessels contained 1 l. of solution. Each of the purified preparations of ATP and IDP showed the presence of only a single spot which absorbed ultraviolet light after chromatography in isobutyric acid–NH_3 specific gravity 0.88–water (66:1:33, v/v). Stock solutions of the nucleotides were adjusted to pH 7.6 with NaOH and stored at −10°. Other nucleoside tri- and diphosphates contained small amounts of the corresponding di- and monophosphates, but were used without further purification. Concentrations of all nucleotides were checked by measurement of their absorption according to Bock *et al.* (1956). $MgCl_2$, $CaCl_2$, and $MnCl_2$ were reagent grade products from E. Merck, AG, Darmstadt, which were treated with dithizone in carbon tetrachloride (Morrison and Uhr, 1966). Solutions were standardized according to the procedure outlined by Morrison *et al.* (1961). EDTA was a laboratory reagent from British Drug Houses. Phenylmethylsulfonyl fluoride and dithiothreitol were obtained from the California Corp. for Biochemical Research. DEAE-Sephadex and Sephadex were supplied by Pharmacia, hydroxylapatite by Bio-Rad Laboratories, and cellulose by Whatman. All other reagents were commercial products of the highest grade available and were used without further purification. [8-^{14}C]ATP-Li_4 (16.8 mCi/mmole) in 50% ethanol was obtained from Schwarz Bioresearch, Inc.

Measurement of Enzyme Activity. All experiments have been carried out at pH 8.5 since at this pH, all nucleoside di- and triphosphates can be considered to exist in solution only in their fully ionized forms of NDP^{3-} and NTP^{4-}, respectively. Thus the only metal-nucleotide complexes that have to be taken into account are $MNDP^-$ and $MNTP^{2-}$ (O'Sullivan and Perrin, 1964). Because metal nucleotide diphosphates function as substrates for and metal nucleoside triphosphates as activators of the reaction (see Results), these complexes were used as the variable reactants while the free NDP^{3-} was held constant. The concentrations of total NDP and metal chloride to give the required concentrations of $MNDP^-$, while maintaining free NDP^{3-} at the desired concentration, were calculated as described by Morrison *et al.* (1961). For this purpose it was assumed that the stability constants for all metal–nucleoside diphosphate complexes were the same as those determined for the various metal complexes of ADP (Walaas, 1958). Thus the stability constants for $MNDP^-$ were taken to be 4000, 25,000, and 2200 M^{-1}, when M was magnesium, manganese, and calcium, respectively (O'Sullivan and Perrin, 1964). As these same authors also showed that the stability constant for $MgATP^{2-}$ has a relatively high value of 73,000 M^{-1}, this complex was formed by the addition of equimolar amounts of $MgCl_2$ and ATP. Other nucleoside triphosphates were assumed to bind magnesium with the same affinity as ATP (Walaas, 1958), and thus the metal complexes were formed by the equimolar addition of nucleotide and $MgCl_2$.

Reaction mixtures contained in a total volume of either 1.0 or 2.0 ml: triethanolamine-HCl buffer, 0.1 M; EDTA, 0.01 mM; and enzyme, as well as the indicated concentrations of substrate (and in some cases activators). After the addition of the components and before the addition of enzyme (0.2–0.8 μg of protein), the tubes were incubated for 3 min at 30°. The enzyme was diluted with 0.1 M Tris-HCl buffer containing 0.01 mM EDTA and 0.1 mM dithiothreitol (pH 8.0) and added to the reaction mixtures in a volume of 1–4 μl. For the addition of enzyme a Hamilton microsyringe was used and it was fitted with a Hamilton repeating dispenser which delivers $^1/_{50}$ of the syringe volume/delivery. All experiments were run for two time periods, between 1 and 60 min, to ensure that initial velocities were being measured, and the reaction was stopped in a manner depending upon which product was to be determined. Extinction measurements were made using either a Shimadzu or Gilford 300 spectrophotometer.

A unit of activity was taken to be the amount of enzyme which releases 1 μmole of IMP or inorganic phosphate per min from 0.5 mM $MgIDP^-$ under the above conditions.

Estimation of P_i. The reaction was stopped by the addition of 0.1 ml of 3.5 or 7.0 N H_2SO_4 depending upon whether the volume of the reaction mixture was 1.0 or 2.0 ml. The tubes were mixed on a Vortex mixer and were then plunged into ice to minimize the acid hydrolysis of nucleotide and warmed to room temperature before the addition of 0.2 ml of 1% (w/v) *p*-methylaminophenol sulfate in 3% (w/v) sodium sulfite to 1.0 ml of reaction mixture or equivalent amounts of the reagents to 2.0 ml of reaction mixture. This was followed by the addition of 0.5 ml of 2.5% (w/v) ammonium molybdate in 1 N H_2SO_4 to 1.0 ml of reaction mixture or 0.5 ml of 5% (w/v) ammonium molybdate in 2 N H_2SO_4 to 2.0 ml of reaction mixture. The solution of acid molybdate was added to the tubes at 12–25-sec intervals and the extinction measurements were made at 650 mμ after 8 min at room temperature.

Estimation of IMP. The reaction was stopped by the addition of 0.1 ml of 3.5 N HCl to 1.0 or 2.0 ml of reaction mixture and the tubes were placed in ice. IMP was determined using a specific IMP dehydrogenase prepared from extracts of *Aerobacter aerogenes*, strain P14, and free of $NADH_2$ oxidase (Magasanik *et al.*, 1957). Before assaying, the contents of the tubes were neutralized by the addition of 0.1 ml of 3.5 N KOH after which was added 0.27 or 0.55 ml of solution (for 1- and 2-ml reaction mixtures, respectively) containing Tris-HCl buffer (0.25 M, pH 7.6), NAD (5.1 mM), dithiothreitol (0.25 mM), and partially purified IMP dehydrogenase (0.1 mg of protein). Incubation was carried out at 30° until the reaction was complete (about 40 min). The formation of $NADH_2$ was determined at 340 mμ in a cell 1- or 2-cm light path. To allow for the presence of small amounts of IMP in the solutions of IDP, blanks contained all components except the nucleoside diphosphatase.

Reaction velocities were determined by measurement of P_i production when nucleotides other than IDP were

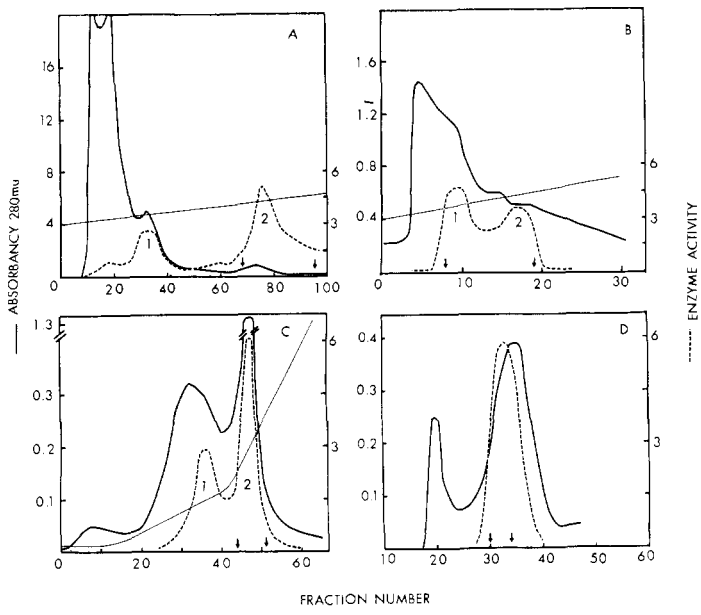


FIGURE 1: Patterns for the elution of protein (—) and enzyme (- - -) from columns of (A) DEAE-Sephadex (first), (B) DEAE-Sephadex (second), (C) hydroxylapatite–cellulose, and (D) G-200 Sephadex. The fine lines represent the rate of increase in the concentration of the eluting gradients which were formed as described in the text. The arrows indicate the enzyme fractions taken for further treatment. Enzyme activity is given in arbitrary units.

added as substrates or when only a narrow range of relatively high substrate concentrations was used. For wider ranges of substrate concentrations which extended down to relatively low values, estimation of IMP proved to be more satisfactory. There was good agreement between the results obtained with the two methods.

Separation of Nucleotides. Nucleotides were separated by chromatography on DEAE-cellulose paper by the method outlined by Morrison and Cleland (1966).

Determination of Protein. This was carried out by the method of Lowry *et al.* (1951) using bovine serum albumin as the standard.

Fractionation with Ammonium Sulfate. The weight (w) of ammonium sulfate required to give the various degrees of saturation was calculated from the formula, $w = 0.515V(S_2 - S_1)/(1.0 - 0.292S_2)$, where V represents the volume in milliliters and w is expressed in grams. S_1 and S_2 represent the initial and desired degrees (0–1.0) of saturation at 0° (Kunitz, 1952; Noltmann *et al.*, 1961).

Results

Purification of Nucleoside Diphosphatase. The enzyme was purified by a modification of an unpublished method which was communicated to the authors by Professor Hayaishi. The introduction of certain modifications had the effect of increasing the yield and stability of the enzyme. The procedure is given below.

Preparation of Liver Acetone Powder. Livers were obtained from adult white rats and dropped immediately into cold, distilled water. After several minutes, they were removed, placed in redistilled acetone (approximately 10 volumes at −10°), and treated in a Waring Blendor until the suspension appeared uniform (2–4 min). The mixture was then filtered on a Buchner funnel and the pad was treated again with acetone as described above. The pad was broken up and left at room temperature overnight after which it was ground in a mortar. The powder was stored at −10°. Under these conditions, the loss of nucleoside diphosphatase activity was 25% over a period of 10 months. Sixty rat livers yielded 160 g of acetone powder.

Extraction of Acetone Powder. Unless otherwise stated, all operations were carried out at 4°, buffers contained EDTA (0.01 mM) and dithiothreitol (0.1 mM), and centrifugations were done at 13,000g for 20 min. Dialyses were carried out against either two changes of 15 or 100 volumes of buffer solution. Acetone powder (120 g) was extracted by stirring for 1 hr with 720 ml of 0.05 M Tris-HCl buffer containing 0.25 mM phenylmethylsulfonyl fluoride (pH 7.8). The suspension was centrifuged and the precipitate was reextracted with 240 ml of the same buffer solution as described above. The supernatant solutions were combined.

First Ammonium Sulfate Fraction. The extract was brought to 0.3 saturation by the addition of solid ammonium sulfate over a period of 2 hr and after stirring for a further 15 min, the precipitate was removed by centrifugation. Solid ammonium sulfate was then added to the supernatant solution over a period of 1 hr to bring it to 0.7 saturation. After the addition was complete, stirring was continued for an additional 15 min before the precipitate was collected by centrifuging. The precipitate was dissolved in 300 ml of 0.05 M acetate buffer (pH 6.0) and dialyzed for 17 hr against the same buffer.

Fractionation on CM-Sephadex. CM-Sephadex (C-50) which had been equilibrated against acetate buffer (0.05 M, pH 6.0) was added to a coarse, sintered-glass funnel, containing filter paper (Whatman No. 531), so as to give a pad (6 × 6 cm) after the application of gentle suction. The dialyzed solution from the previous step was passed, with gentle suction, through the pad which was then washed with acetate buffer (two 100-ml portions).

Second $(NH_4)_2SO_4$ Fractionation. The filtrate was fractionated with solid $(NH_4)_2SO_4$ in a manner similar to that outlined above and the fraction precipitating between 0.4 and 0.6 saturation was collected by cen-

TABLE I: Summary of Yields and Specific Activities of Fractions Obtained during the Purification of Nucleoside Diphosphatase from Rat Liver Acetone Powder.[a]

Fraction	Vol (ml)	Protein (mg)	Total Units (μmoles/min)	Sp Act. (mμmoles/min per μg)
Extract	985	43,750	3,060	0.07
$(NH_4)_2SO_4$ precipitate (0.3–0.7 saturation)	375	33,750	2,470	0.07
Effluent from CM-Sephadex column	440	20,240	2,630	0.13
$(NH_4)_2SO_4$ precipitate (0.4–0.6 saturation)	104	9,980	1,530	0.15
Eluate from first DEAE-Sephadex column	750	495	1,070	2.16
Eluate from second DEAE-Sephadex column	154	103	1,010	9.8
Eluate from hydroxylapatite column	5.8	64	460	7.2
Eluate from G-200 Sephadex column	4.5	10.3	610	59.2

[a] Weight of rat liver acetone powder was 120 g. Details are given in the text.

trifuging. The precipitate was dissolved in 40 ml of 0.01 M Tris-HCl buffer containing 0.2 M NaCl (pH 8.0) and dialyzed for 17 hr against the same buffer.

First Chromatography on DEAE-Sephadex. A column (4.5 × 22 cm) of DEAE-Sephadex (A-50) was equilibrated against the Tris-HCl buffer referred to above. The dialyzed solution from the previous step was applied to the column and the enzyme was eluted by using a linear gradient formed from 0.05 M Tris-HCl containing 0.2 M NaCl (pH 8.0) and the same buffer containing 0.35 M NaCl. The total volume of the elution buffers was 21. Fractions (25 ml) were collected and those with enzyme of the highest specific activity (68–96 of peak 2, Figure 1A) were pooled.

Second Chromatography on DEAE-Sephadex. The solution of pooled fractions was diluted with Tris-HCl buffer (0.05 M, pH 8.0) so as to reduce the concentration of NaCl to approximately 0.2 M and applied to a second column (2 × 23 cm) of DEAE-Sephadex which had been prepared as described above. The same procedure as in the previous step was used for elution of the enzyme, except that each vessel contained 600 ml of the appropriate buffer solutions. Fractions (14 ml) containing enzyme of the highest specific activities (8–19 of peaks 1 and 2 of Figure 1B) were pooled and dialyzed for 17 hr against 0.005 M phosphate buffer (pH 6.8).

Chromatography on Cellulose–Hydroxylapatite. A column (3.5 × 4 cm) of a 1:1 (w/w) mixture of cellulose and hydroxylapatite was prepared and washed with 500 ml of the above phosphate buffer. The solution from the previous step was applied and the enzyme was eluted with two successive linear gradients of phosphate buffer (pH 6.8) from 0.01 to 0.12 M and from 0.15 to 0.50 M. Both vessels contained 150 ml of buffer at the appropriate concentrations. Fractions (10 ml) containing nucleoside diphosphatase of the highest specific activities (43–51 from peak 2, Figure 1C) were pooled and the solution was concentrated by pressure dialysis from 108 to 6 ml.

Chromatography on G-200 Sephadex. The concentrated solution from the previous step was added to the top of a column (2.5 × 70 cm) of G-200 Sephadex which had been equilibrated with Tris-HCl buffer (0.1 M, pH 8.0) containing 0.2 mM dithiothreitol. Elution was carried out with the same buffer and fractions (7 ml) containing enzyme of the highest specific activities (30–34, Figure 1D) were pooled. The solution was concentrated by pressure dialysis to a volume of 4.5 ml.

A summary of the yields and specific activities of the various fractions obtained during the purification procedure is given in Table I.

Properties of the Purified Enzyme. Storage of the enzyme at concentrations of 1–3 mg/ml at 5° in the presence of Tris-HCl buffer (0.1 M, pH 8.0) containing 0.2 mM dithiothreitol and 0.01 mM EDTA resulted in a variable loss of activity of up to 20%/week. When such solutions were frozen by immersion in Dry Ice–ethanol and stored at −10°, there was no change in specific activity for periods up to 8 months. However, after 3–4 months storage, the enzyme exhibited normal Michaelis–Menten kinetics in contrast to the nonlinear kinetics shown by preparations which had been stored for shorter periods. Repeated freezing and thawing had no effect on the activity of the enzyme.

Polyacrylamide gel electrophoresis of the purified enzyme showed the presence of five to six bands (Figure 2). It is possible that more than one band possesses enzyme activity, especially since the elution patterns from columns of DEAE-Sephadex and hydroxylapatite (Figure 1) suggest that the enzyme may undergo association–dissociation reactions. But this point has yet to be investigated further. However, a comparison of the kinetic properties of nucleoside diphosphatase, as present in the two fractions obtained by elution of the sec-

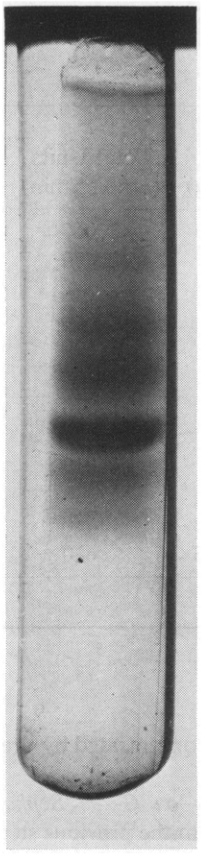


FIGURE 2: Separation of the protein components of the purified nucleoside diphosphatase (50 μg) on polyacrylamide gel. The unpublished procedure of Orr and Blakley was used.

ond DEAE-Sephadex column (Figure 1B), has shown that no differences could be detected. All subsequent kinetic studies have been made with the most highly purified preparation of the enzyme (Table I).

In view of the possibility that the enzyme could undergo association–dissociation reactions, it was of importance to determine if the initial velocity of the reaction was a linear function of enzyme concentration over a range which exceeded that used in the kinetic investigations. The fact that such a relationship does hold is

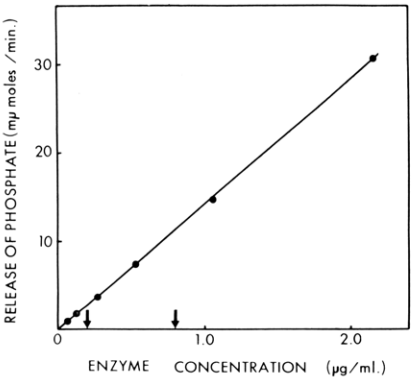


FIGURE 3: Initial velocity of the reaction as a function of the enzyme concentration at a $MgIDP^-$ concentration of 2.5 mM. The arrows indicate the range of enzyme concentrations used in most experiments.

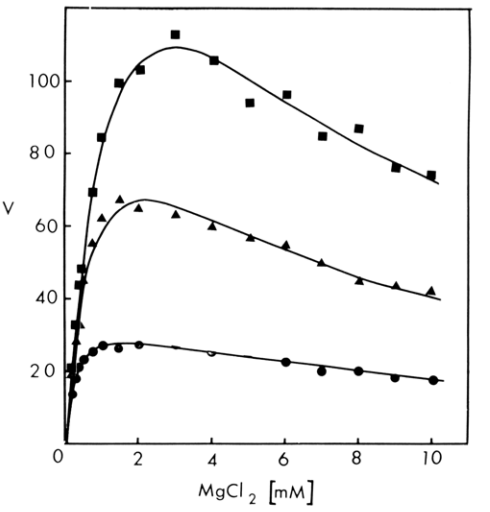


FIGURE 4: Effect of the total magnesium concentration on the initial velocity of the reaction at different fixed concentrations of IDP. The concentrations of total IDP were 0.5 mM (●), 1.0 mM (▲), and 2.0 mM (■). Velocity is expressed as millimicromoles of IMP per minute per microgram of protein.

illustrated in Figure 3. With the exception of those experiments carried out with what can be regarded as poor substrates, the concentrations of enzyme fell within the range covered by the arrows in Figure 3.

Activation of the Enzyme by Mg^{2+}. In the absence of added metal ions, there was a slow enzymatic reaction when unpurified samples of IDP were used as substrate. However, it would appear that this activity was due to the presence, in commercial preparations of IDP, of one or more bivalent metal ions which could activate the enzyme. Thus there was no hydrolysis of purified IDP unless Mg^{2+}, Ca^{2+}, or Mn^{2+} was present in the reaction mixture.

To gain preliminary information about the function of metal ions, the initial velocity of the reaction was studied as a function of the concentrations of total magnesium and total IDP. The results (Figure 4) indicate that the concentrations of $MgCl_2$ required to obtain an apparent maximum velocity increase as the concentration of total IDP is increased and that each apparent maximum velocity is attained under conditions where the ratio of magnesium: total IDP is greater than 1. These results are generally in accord with those expected if $MgIDP^-$ were acting as the substrate. Because the stability constant for $MgIDP^-$ is likely to be similar to the low value for $MgADP^-$, *viz.*, 4000 M^{-1} (Walaas, 1958; O'Sullivan and Perrin, 1964), a considerable excess of $MgCl_2$ would be required to completely convert IDP into its metal complex. But at magnesium concentrations which are well in excess of the total IDP concentration, the initial velocity falls. This could be interpreted to indicate that, at higher concentrations, free Mg^{2+} acts as an inhibitor of the reaction. In subsequent experiments, $MgIDP^-$ was used as the variable substrate and further investigations were made of the effects of free IDP^{3-} and free Mg^{2+} on the initial velocity of the reaction.

Effects of $MgIDP^-$ Concentration on the Initial Ve-

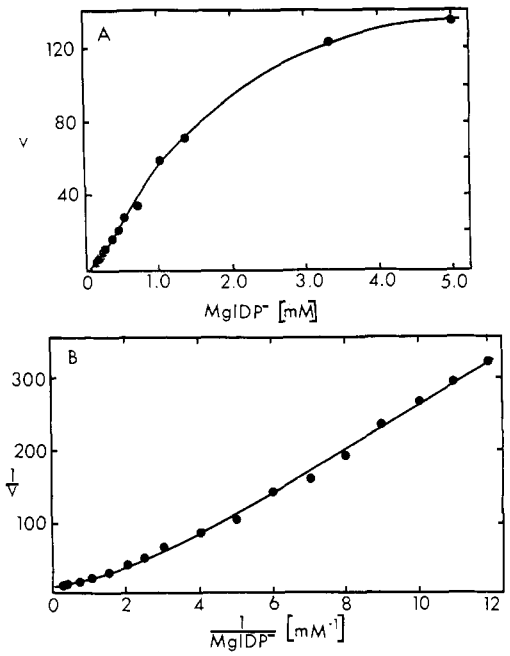
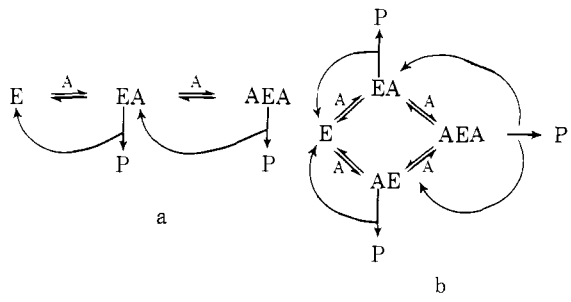


FIGURE 5: Effect of $MgIDP^-$ concentration on the initial velocity of the reaction. The data are plotted as velocity against substrate concentration (A) and in double-reciprocal form (B). Velocity is expressed as millimicromoles (A) or micromoles (B) of IMP per minute per microgram of protein.

locity of the Reaction. When free IDP^{3-} was maintained constant at a concentration of 0.1 mM, a plot of initial velocity against the concentration of $MgIDP^-$ gave a curve which might be considered as being sigmoidal (Figure 5A), but it is not possible to reach any definite conclusion about its shape. However, when the same data are plotted in double-reciprocal form, it becomes clear that the kinetics of the nucleoside diphosphatase reaction are not of the Michaelis–Menten type since the plot is not linear (Figure 5B). Indeed, the curve has the shape of a nonrectangular hyperbola. Further, it will be noted that extrapolation of the linear portion of the curve, which appears to be an asymptote, gives a negative intercept on the ordinate. A comparison of the two types of plot shown in Figure 5 indicates that the plotting of data in double-reciprocal form is a far more satisfactory method of revealing nonlinear reaction kinetics.

The above results are consistent with one or other of the hypotheses which are illustrated diagrammatically in Scheme I. In these it is supposed that two molecules

SCHEME I



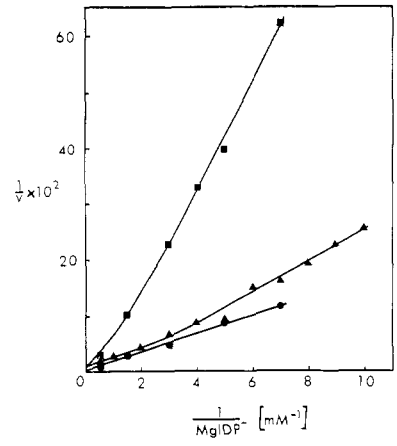


FIGURE 6: Inhibition of the reaction by free Mg^{2+} at a concentration of 20 mM (■) and activation by free IDP^{3-} at a concentration of 1.0 mM (●). For control experiments (▲), the concentration of IDP^{3-} was held constant at 0.1 mM. Velocity is expressed as millimicromoles per minute per microgram of protein.

of substrate (A) react interdependently with the enzyme (E) in either an ordered (a) or random (b) manner and that the resulting enzyme forms, containing one (EA, AE) and two (AEA) molecules of substrate, break down to yield products (P). These types of reaction mechanism have also been discussed by Cleland (1967). The experimental data are not in agreement with the breakdown of only AEA, for in this case, a parabolic double-reciprocal plot would be obtained. The curve shown in Figure 5B differs from the parabola drawn by Yamazaki and Hayaishi (1965) using data obtained by varying the concentration of total IDP in the presence of a fixed concentration of $MgCl_2$. It should be pointed out that these authors could also have drawn through their experimental points at lower substrate concentrations, a straight line which, on extrapolation, would have given a negative intercept on the ordinate. Because of the differences in the approach used in the present work and that of Yamazaki and Hayaishi (1965), no quantitative comparison can be made of the two sets of results.

Effects of Free Mg^{2+} and IDP^{3-} on the Initial Velocity of the Reaction. If the concentration of free Mg^{2+} were held constant at a concentration of 20 mM and the free IDP^{3-} concentration allowed to vary, double-reciprocal plots of velocity against $MgIDP^-$ concentration (Figure 6) still maintained their nonlinear form, but as judged from the increased slope of the asymptote, marked inhibition occurs. Although no conclusion could be drawn from these data about the type of inhibition, it was subsequently shown to be of the noncompetitive type (V. Schramm and J. F. Morrison, unpublished data). Because of the noncompetitive inhibition of the reaction by free Mg^{2+}, it is unlikely that the intersection point on the ordinate of Figure 5B represents the reciprocal of the true maximum velocity. Under conditions where the concentration of $MgIDP^-$ is increased while the concentration of free IDP^{3-} is held constant, there will be a simultaneous increase in the concentration of free Mg^{2+} to levels which cause significant inhibition. Indeed, it may be calculated, using a stability constant of

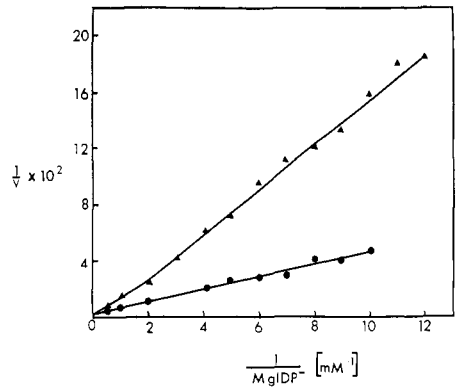


FIGURE 7: Activation of the reaction by $MgATP^{2-}$. The concentrations of $MgATP^{2-}$ were (▲) none and (●) 0.6 mM. IDP^{3-} was held constant at a fixed concentration of 0.1 mM. Velocity is expressed as millimicromoles of IMP per minute per microgram of protein.

4000 M^{-1} for $MgIDP^-$, that the concentration of free Mg^{2+} will be 2.5 times that of $MgIDP^-$. It follows then that part of the curvature of the plot shown in Figure 5B will be due to the inhibition by free Mg^{2+}.

Effect of Allosteric Activators on the Reaction Kinetics. In contrast to the nonlinear double-reciprocal plots obtained in the absence of activators and in the presence of a low fixed concentration of free IDP^{3-} (Figure 5B), the addition of relatively high concentrations of $MgATP^{2-}$ or an increase in the concentration of free IDP^{3-} gave rise to linear double-reciprocal plots (Figures 6 and 7). One possible explanation for this finding is that under these conditions only one molecule of substrate reacts with the enzyme.[2] In both cases it will be noted that the degree of activation decreases as the concentration of $MgIDP^-$ increases. Further, it is clear that the maximum velocity of the reaction is greater in the presence of 1.0 mM free IDP^{3-} than in the presence of 0.1 mM free IDP^{3-} (Figure 6). This finding can be explained as being due to the reduction in the inhibitory effect of free Mg^{2+} whose concentration at higher substrate concentrations must fall as a result of increasing the fixed IDP^{3-} concentration.

The activating effect of $MgATP^{2-}$ was not dependent upon it undergoing chemical change, for there was no hydrolysis of ATP either in the absence of substrate or during the course of the reaction. Thus when labeled ATP was used and the nucleotides were separated by chromatography on DEAE-cellulose paper, the number of counts per minute in the ATP spot was equivalent to the number added.

The results obtained in the presence and absence of activators are in general agreement with either of the reaction mechanisms (a and b) that are illustrated in Scheme II and in which it is supposed that only one molecule of the activator (M) combines at one of the catalytic sites on the enzyme at which substrate can react and that because of the structural similarities of the activator and substrate molecules, their combinations at this site are mutually exclusive (*cf.* Atkinson *et al.*, 1965). Thus when M is present at sufficiently high concentrations, essentially all the enzyme would be present in the ME form with which only one molecule of sub-

SCHEME II

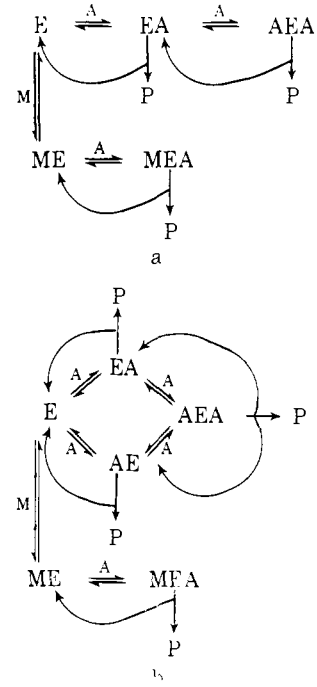


TABLE II: Effect of Magnesium Complexes of Nucleotides and Polyphosphates on the Rate of Hydrolysis of $MgIDP^-$.[a]

Additions	Initial Velocity (mμmoles/min per μg)
None	18.5
$MgATP^{2-}$	46.8
$MgITP^{2-}$	54.7
$MgGTP^{2-}$	52.3
$MgUTP^{2-}$	37.7
$MgCTP^{2-}$	28.8
β,γ-Phosphonate analog of $MgATP^{2-}$ [b]	17.8
α,β-Phosphonate analog of $MgATP^{2-}$ [c]	20.1
Magnesium pyrophosphate	13.9
Magnesium tripolyphosphate	19.5
Magnesium trimetaphosphate	19.0

[a] Both $MgIDP^-$ and the test compounds were present at a concentration of 0.143 mM. The concentration of free IDP^{3-} was 0.1 mM. [b,c] The oxygen atom linking the β- and γ- or α- and β-phosphoryl groups was replaced by a methylene group.

[2] The enzyme is regarded as a catalytic unit containing two sites at which substrate can combine. This assumption does not preclude the possibility that an enzyme molecule consists of more than one catalytic unit, but if this were so it would be further assumed that the catalytic units are kinetically equivalent.

TABLE III: Effect of $MgATP^{2-}$ on the Rate of Hydrolysis of Various Magnesium Nucleoside Diphosphate Complexes and Magnesium Thiamine Pyrophosphate.[a]

Substrate	Substrate Concentration			
	0.5 mM		0.143 mM	
	$-MgATP^{2-}$	$+MgATP^{2-}$	$-MgATP^{2-}$	$+MgATP^{2-}$
$MgIDP^-$	65.5	108.0	18.1	40.2
$MgUDP^-$	64.1	92.0	25.8	39.0
$MgGDP^-$	63.0	98.5	21.4	41.2
$MgCDP^-$	3.3	20.4	0.88	4.2
$MgADP^-$	0.23	0.60	0.04	0.15
$MgdTDP^-$	<0.01	<0.03	<0.002	<0.002
Mg-thiamine pyrophosphate	2.0	12.9	0.29	3.06

[a] Substrates and $MgATP^{2-}$ were present at equimolar concentrations. Initial velocity in millimicromoles per minute per microgram.

strate would react. Additional support for the above scheme comes from kinetic investigations which showed that only one molecule of activator reacts with each catalytic unit of the enzyme and that saturation with activator does not cause inhibition of the reaction (Yamazaki and Hayaishi, 1965; V. Schramm and J. F. Morrison, unpublished data).

The increase in reaction velocity, at a low substrate concentration, as a result of the addition of a fixed concentration of various magnesium–nucleoside triphosphate complexes, is shown in Table II. The same table also indicates that magnesium phosphonate analogs of ATP, which contained a methylene group rather than an oxygen atom between the α- and β- or β- and γ-phosphoryl groups, were inactive as were the magnesium complexes of tripolyphosphate and trimetaphosphate. Magnesium pyrophosphate, on the other hand, caused inhibition of the reaction.

Substrate Specificity of the Enzyme. To determine the relative rates of hydrolysis of the magnesium complexes of various nucleoside diphosphate complexes, their reaction velocities were measured at two different concentrations both in the presence and absence of the allosteric activator, $MgATP^{2-}$. From the results illustrated in Table III, it may be concluded that the nucleotides fall into three classes according to their rates of hydrolysis. The magnesium complexes of IDP, UDP, and GDP can be considered as good substrates since they undergo reaction at much greater rates than the magnesium complexes of CDP and ADP which hence can be regarded as poor substrates. The evidence suggests that $MgdTDP^-$ is not a substrate for nucleoside diphosphatase, although the enzyme is capable of hydrolyzing thiamine pyrophosphate.

At a substrate concentration of 0.5 mM, the good substrates, $MgIDP^-$, $MgUDP^-$, and $MgGDP^-$, underwent reaction at comparable rates, but at the lower substrate concentration of 0.143 mM, the relative rates of hydrolysis decreased in the order: $MgUDP^- > MgGDP^- > MgIDP^-$. The rates of hydrolysis of all substrates at both concentrations were increased considerably on the addition of an equimolar concentration of $MgATP^{2-}$. The relative increase in velocity ranged from 1.5 to 10.6 with $MgUDP^-$ and magnesium thiamine pyrophosphate, respectively, as substrates. In so far as the results with IDP, UDP, GDP, and thiamine pyrophosphate are concerned, they are similar to those reported by Yamazaki and Hayaishi (1965).

A more detailed study of the kinetics of the hydrolysis of the good substrates showed that, with free NDP^{3-} at a fixed concentration of 0.1 mM, $MgGDP^-$ is similar to $MgIDP^-$ in giving nonlinear double-reciprocal plots while $MgUDP^-$ differs in that it gives a linear plot (Fig-

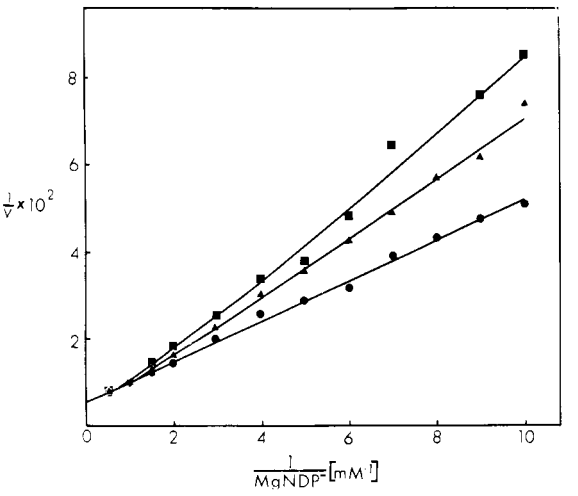


FIGURE 8: Effect of the concentration of various magnesium nucleoside diphosphate complexes ($MgNDP^-$) on the initial velocity of the reaction. (●) $MgUDP^-$, (▲) $MgGDP^-$, and (■) $MgIDP^-$. The free concentration of each nucleoside diphosphate was maintained constant at 0.1 mM. Velocity is expressed as millimicromoles of inorganic phosphate per minute per microgram of protein.

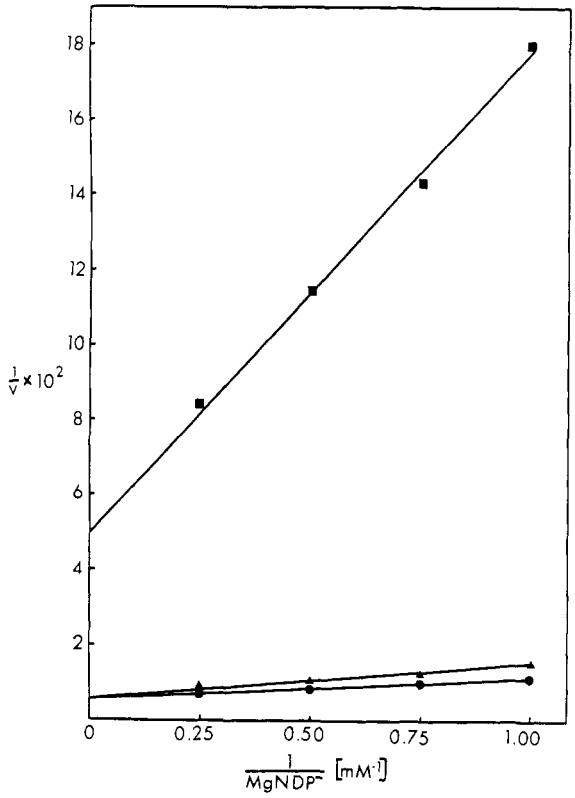


FIGURE 9: Determination of the maximum velocity of the reaction with MgUDP$^-$ (●), MgIDP$^-$ (▲), and MgCDP$^-$ (■) as substrates. The free nucleotide concentration was 0.1 mM. Velocity is expressed as millimicromoles of inorganic phosphate per minute per microgram of protein.

ure 8). The latter result is not inconsistent with the idea that two molecules of each substrate react with the enzyme. If the binding of the first molecule of MgUDP$^-$ were considerably stronger than that of the second, then it is possible that over the substrate concentration range used, only the reaction of the second molecule would be detected. Another feasible explanation is that UDP^{3-} is a much better activator than either IDP^{3-} or GDP^{3-}. It would appear from Figure 8 that the same maximum velocity is obtained with these three substrates. Provided that the inhibitory effect of free Mg^{2+} does not vary with the substrate, this result suggests that a common intermediate reaction involving the release of a phosphoryl group from an enzyme–phosphate complex could be the rate-limiting step of the reaction sequence. If this hypothesis were generally true, a poor substrate such as MgCDP$^-$ should also give the same maximum velocity. However, from Figure 9 it is apparent that the maximum velocity of the reaction with MgIDP$^-$ and MgUDP$^-$ of 170 mμmoles/min per μg of enzyme is very much higher than the value of 20 mμmoles/min per μg of enzyme obtained with MgCDP$^-$. While the above results indicate that the maximum velocity of the reactions with all substrates is not limited by the same slow step, they do not preclude the conclusion that the rate-limiting step with good substrates is the hydrolysis of an enzyme–phosphate complex. It is possible that certain metal–nucleotide complexes are poor substrates because the reaction velocity is limited by the even

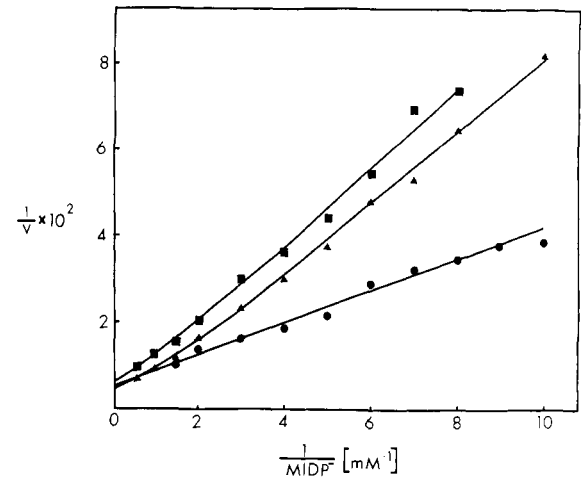


FIGURE 10: Effect of the concentration of various metal–IDP complexes (MIDP$^-$) on the initial velocity of the reaction. (●) MnIDP$^-$, (▲) MgIDP$^-$, (■) CaIDP$^-$. The concentration of IDP^{3-} was held constant at 0.1 mM. Velocity is expressed as millimicromoles of IMP per minute per microgram of protein.

slower rate of release of the nucleoside monophosphate moiety. The apparent linearity of the plots with MgIDP$^-$ and MgCDP$^-$ as the variable substrates, as shown in Figure 9, is due to the use of a higher range of substrate concentrations over which double-reciprocal plots approximate to a straight line.

Initial Velocity Studies with Mg^{2+}, Ca^{2+}, and Mn^{2+} as Activating Metal Ions. Experiments similar to those described above and for which Mg^{2+} was used as the activating metal ion have been carried out with both Ca^{2+} and Mn^{2+}. The initial velocity of the reaction was studied as a function of the concentrations of MgIDP$^-$, CaIDP$^-$, and MnIDP$^-$ at a fixed free IDP^{3-} concentration of 0.1 mM. The results illustrated in Figure 10 in the form of double-reciprocal plots indicate that all three metal–nucleotide complexes function as substrates. Further, they show that while nonlinear plots are obtained with MgIDP$^-$ and CaIDP$^-$, variation of the concentration of MnIDP$^-$ gives rise to a linear plot. Such results are in accord with the hypothesis that two molecules of each substrate react with the enzyme, although it is necessary to postulate that the first molecule of MnIDP$^-$ combines more strongly than the second. This explanation is similar to that given above in connection with the hydrolysis of MgUDP$^-$.

It is difficult to reach any firm conclusion about the relative magnitudes of the maximum velocities with each of the three metal–IDP substrates. They certainly do not appear to differ greatly, but any comparison must relate to the true maximum velocities. To do this, it is essential to take into account the variation in the concentrations of each free metal ion species, which will occur because of the differences in the stability constant values for each complex, and their possible inhibitory effects. With each substrate at a concentration of 2 mM and with free IDP^{3-} fixed at 0.1 mM, the concentration of free Mg^{2+}, Ca^{2+}, and Mn^{2+} would be 5.0, 9.1, and 0.8 mM, respectively.

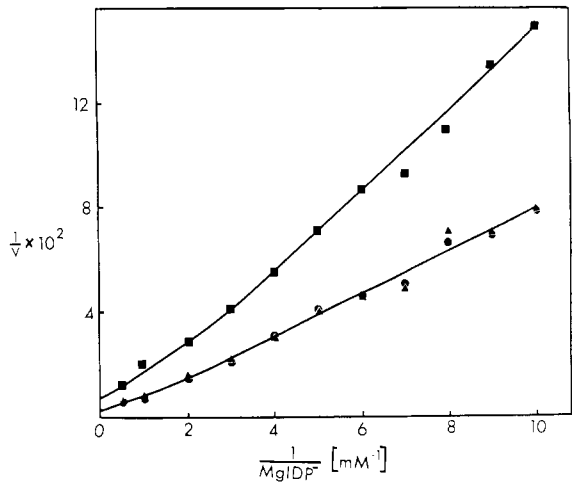


FIGURE 11: Effect of ionic strength and KCl on the initial velocity of the reaction. The concentrations of triethanolamine-HCl buffer (pH 8.5) were 0.1 M (●) and 0.2 M (▲). KCl (■) was added at a concentration of 0.1 M to the reaction mixture containing 0.1 M triethanolamine-HCl buffer. Velocity is expressed as millimicromoles of IMP per minute per microgram of protein.

Effects of KCl and Ionic Strength on the Kinetics of the Reaction. The activity of some allosteric enzymes, such as AMP deaminase (Setlow and Lowenstein, 1967; Smiley and Suelter, 1967), can be increased not only by allosteric activators, but also by higher concentrations of monovalent cations and/or an increase in ionic strength. Thus it was of interest to determine if such factors could cause activation of nucleoside diphosphatase. From the results of Figure 11, it is apparent that, at a concentration of 0.1 M, KCl acts as an inhibitor rather than an activator, of the enzyme and does not cause any change in the general shape of the double-reciprocal plot. The inhibition by KCl is not simply due to an increase in ionic strength for a twofold increase in the concentration of triethanolamine-HCl buffer from 0.1 to 0.2 M is without effect. The latter result makes it appear likely that K^+ and not Cl^- is the effective agent, but it should be borne in mind that an increase of 0.1 M in the buffer concentration raises the Cl^- concentration by only 0.024 M. The addition of $MgATP^{2-}$ to the reaction mixture in the presence of KCl resulted in the activation of the enzyme so that double-reciprocal plots were linear (*cf.* Figure 7).

Effect of Ageing on the Kinetic Properties of the Enzyme. After storage of the enzyme for 4 months at −10°, there was an alteration of its kinetic properties as evidenced by the fact that plots of $1/v$ against $1/MgIDP^-$ were linear (Figure 12). However, the enzyme was still capable of being activated by $MgATP^{2-}$ and there was no change in its specific activity. This change in reaction mechanism, which might well be associated with an alteration of the physical properties of the enzyme, will be the subject of further investigations.

Discussion

A study of the reaction catalyzed by a highly purified preparation of nucleoside diphosphatase has indicated quite clearly that bivalent metal ions are required for activity and the function of these ions would appear to be in the formation of metal–nucleotide complexes which act as substrates. It has also become apparent that in using these complexes as substrates, it is of importance to take into account the variation in the concentrations of the free ionic species, Mg^{2+} and NDP^{3-}, which cause inhibition and activation of the reaction, respectively. Thus the nature of the kinetic results obtained can vary according to the equilibrium relationship that exists between the concentrations of the free and complex species.

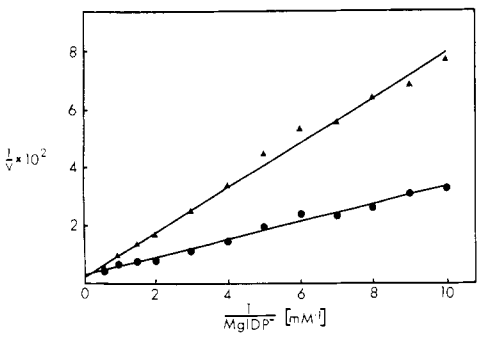


FIGURE 12: Effect of the concentration of $MgIDP^-$ on the initial velocity of the reaction catalyzed by an aged enzyme preparation in the absence (▲) and presence (●) of $MgATP^{2-}$. The $MgATP^{2-}$ concentration was varied in a constant ratio of 1:1 with that of MgIDP. IDP^{3-} was maintained constant at 0.1 mM. Velocity is expressed as millimicromoles of IMP per minute per microgram of protein.

Under the conditions in which the concentration of IDP^{3-} is held constant at a low concentration, plots of $1/v$ against $1/MgIDP^-$ are nonlinear and have the form of a nonrectangular hyperbola which is concave up. Such a result has been shown to be in qualitative agreement with the interdependent reaction of two molecules of substrate with the enzyme to form E–MgIDP and E–$(MgIDP)_2$, both of which can breakdown to yield the products, IMP and P_i. Such an interdependent reaction could come about as a result of a conformational change in the enzyme following the addition of the first substrate molecule. In the presence of compounds that can be considered as allosteric activators, double-reciprocal plots of initial velocity as a function of substrate concentration are linear and it has been suggested that this is due to the reaction of one molecule of activator at one of the two substrate sites. A more rigid test of whether or not the proposals are tenable lies in demonstrating that the initial velocity equation derived on the basis of the mechanism can adequately account for the kinetic data. The results of this approach will be reported later. It should be noted that no comments have been made concerning the structure of the various enzyme forms. While this is of importance with respect to an understanding of the over-all reaction mechanism, no conclusions about the structure of intermediate species of enzyme can be drawn from kinetic data.

In contrast to the nonlinear double-reciprocal plots obtained using $MgIDP^-$ as the substrate, a study of the reaction velocity as a function of the concentrations of $MgUDP^-$ and $MnIDP^-$ gave rise to plots which were

linear. These latter results could be interpreted to indicate that only one molecule of substrate can combine with the enzyme. However, they are not necessarily in disagreement with the reaction of two molecules of substrate, for if the binding of the first were strong, then over the range of concentrations used the results could approximate to those expected if only one molecule of substrate underwent reaction. Further, it is possible that free UDP^{3-} might be a better activator than other free NDP^{3-} species. A change in the relative binding of the two molecules of $MgIDP^{-}$ as a result of ageing the enzyme would also account for the linear kinetics obtained with this preparation. It is of interest to note that under the conditions which yield linear double-reciprocal plots, there is still activation of the enzyme by $MgATP^{2-}$.

The ability of Mg^{2+}, Mn^{2+}, or Ca^{2+} to function as the essential bivalent metal ion for the nucleoside diphosphatase reaction is reminiscent of the results obtained with other phosphotransferases such as creatine kinase (Morrison and Uhr, 1966; O'Sullivan and Cohn, 1966) and in contrast to the finding that Ca^{2+} strongly inhibits pyruvate kinase (Kachmar and Boyer, 1953). On the basis of the hypothesis proposed by Cohn (1963), it would be concluded that nucleoside diphosphatase does not form an enzyme–metal complex. This conclusion is in accord with the postulate that the metal–nucleotide complexes act as substrates.

Although the substrate specificity of the enzyme is broad, only the magnesium complexes of IDP, UDP, and GDP can be considered as good substrates. From a comparison of the reaction velocities and the structure of the nucleotide moiety of substrate, it appears that the 6-oxypurine group, which these three nucleotides have in common, is necessary for full catalytic activity. However, it remains to be determined if this structural feature is of importance with respect to the binding of the first substrate molecule or its ability, in the form of an enzyme–substrate complex, to enhance the reaction of the second molecule of substrate, to the binding of the second substrate molecule or the activation step of the reaction. Irrespective of whether or not a nucleotide is a good substrate, the rate at which it undergoes reaction is increased in the presence of $MgATP^{2-}$. Further, it would seem that the magnesium complexes of the naturally occurring nucleoside triphosphates which function as allosteric activators do not show any specificity for the purine or pyrimidine moiety (*cf.* Yamazaki and Hayaishi, 1965) although phosphonate analogs of ATP and inorganic triphosphates are ineffective as activators of the reactions.

Acknowledgments

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