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# Studies on an Aged Preparation of Nucleoside Diphosphatase. Kinetics and Reaction Mechanism*

Vern L. Schramm† and J. F. Morrison‡

ABSTRACT: A kinetic study has been made of the reaction catalyzed by a purified preparation of nucleoside diphosphatase which has been subjected to prolonged storage at −10°.

Such treatment has been shown to affect some, but not all, of the properties of the enzyme. Thus both preparations have approximately the same molecular weight and are activated by the allosteric modifier, magnesium adenosine triphosphate ($MgATP^{2-}$). However, whereas the freshly prepared enzyme exhibits nonlinear double-reciprocal plots of velocity as a function of the magnesium inosine diphosphate ($MgIDP^-$) concentration and its maximum velocity is reduced in the presence of the modifier, the aged enzyme gives linear plots of $1/v$ *vs.* $1/MgIDP^-$ and its maximum velocity is unaffected by the addition of modifier. The kinetic data are consistent with a reaction mechanism in which it is proposed that the aged enzyme possesses distinct substrate and modifier sites, that there is random addition of one molecule (or independent combinations of multiple molecules) of each of these reactants to the enzyme under rapid equilibrium conditions and that the modifier facilitates the combination of substrate without influencing the rate of product formation. Values for the various kinetic constants have been determined.

It has been shown previously (Schramm and Morrison, 1968) that, after prolonged storage, the kinetic properties of the allosteric enzyme, nucleoside diphosphatase, are altered in that plots of $1/v$ *vs.* $1/MgIDP^-$ are linear, rather than curvilinear, as obtained with freshly prepared enzyme. Further, it was reported that the aged enzyme is still capable of being activated by the allosteric modifier, $MgATP^{2-}$. These results have prompted an investigation of the mechanism of the reaction as catalyzed by the aged enzyme. It was considered that since the reaction of substrate with this enzyme, both in the absence and presence of modifier, obeys Michaelis–Menten kinetics, the analysis and interpretation of the kinetic data would be less difficult than for those derived from investigations with the freshly prepared enzyme. Apart from their intrinsic interest, it was also of interest to compare the results with those obtained from studies on the freshly prepared enzyme.

The results of the present work lend support to the sug-

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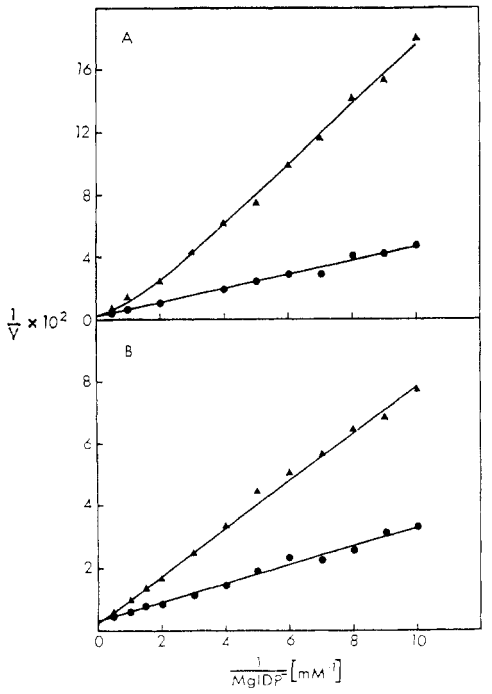



FIGURE 1: Comparison of initial velocity results obtained with the freshly prepared (A) and aged (B) enzymes, both in the absence (▲) and presence (●) of 0.6 mM $MgATP^{2-}$. $IDP^{3-}$ was maintained constant at 0.1 mM. The upper curve of A was fitted by eye. Velocity is expressed as millimicromoles of IMP per minute per microgram of protein.

gestion (Schramm and Morrison, 1969) that the substrate and modifier combine with nucleoside diphosphatase at distinct sites. They also indicate that, in contrast to the ability of $MgATP^{2-}$ to influence the maximum velocity of the reaction catalyzed by freshly prepared enzyme, the modifier does not affect the maximum velocity of the reaction catalyzed by the aged enzyme.

## Materials and Methods

Horseradish peroxidase was supplied by the Sigma Chemical Co.; creatine kinase was prepared and stored as described by Morrison *et al.* (1961). Nucleoside diphosphatase was purified from extracts of rat liver acetone powders by the method of Schramm and Morrison (1968) and stored for 6 months at −10° before use. The source of other materials has been given previously (Schramm and Morrison, 1968).

Enzymic activity was determined at 30° in the presence of 0.1 M triethanolamine-HCl buffer containing 0.01 mM EDTA (pH 8.5). The amount of enzyme added corresponded to 0.2–0.8 μg of protein/ml while the total volume of reaction mixtures was either 1.0 or 2.0 ml. $MgIDP^-$ and $MgATP^{2-}$ were used as the variable reactants with the free $IDP^{3-}$ being held constant at 0.1 mM. At this concentration, free $IDP^{3-}$ has only a negligible activating effect on the reaction. The total concentrations of $MgCl_2$ and IDP to give the required concentrations of $MgIDP^-$ at a fixed concentration of free $IDP^{3-}$ were calculated as described by Morrison *et al.* (1961). For this purpose, the stability constant for $MgIDP^-$ was taken to be 4000 $M^{-1}$ (Schramm and Morrison, 1968; O'Sullivan and Perrin, 1964). $MgATP^{2-}$ was formed by the addition of equimolar amounts of $MgCl_2$ and ATP. To ensure that initial velocities were being measured, reactions were run for at least three time periods. After stopping the reaction with acid, the formation of IMP or $P_i$ (as indicated in the legends to the figures) was determined (Schramm and Morrison, 1968). Weighted mean values and the standard error of the means were calculated as described by Morrison and Uhr (1966).

Molecular weight estimations of nucleoside diphosphatase were carried out as follows: A mixture of creatine kinase (50 μg), horseradish peroxidase (5 μg), and nucleoside diphosphatase (20 μg) in a volume of 0.15 ml of Tris-HCl buffer (0.1 M, pH 8.5), containing 0.01 mM EDTA, 0.1 mM dithiothreitol, and 5 mg/ml of sucrose, was layered on a column (1.2 × 9.5 cm) of G-200 Sephadex and eluted with the same buffer from which sucrose was omitted. Fractions of ten drops (0.2880 g ± 0.0003 g) were collected and the enzymes were located by testing for their catalytic activity. Elution volumes of the marker enzymes were plotted as a function of the logarithm of the molecular weight (Andrews, 1964) and the molecular weight of nucleoside diphosphatase was estimated on the basis of its elution volume. The molecular weights of creatine kinase and horseradish peroxidase were taken to be 81,000 (Noda *et al.*, 1954) and 40,000 (Maehly, 1955), respectively. Other experimental methods were as described by Schramm and Morrison (1968).

*Analysis of Data.* The results of kinetic experiments were analyzed by the computer programs of Cleland (1963), except that they were modified to make a weighted fit of the data. A weighting factor of $1/v^2$ was used since it was found that the standard deviation of mean initial velocity values was proportional to the velocity value (Cleland, 1967; Schramm and Morrison, 1969). Data that gave linear plots of $1/v$ *vs.* $1/A$ were fitted to eq 1. Data conforming to linear competitive

$$v = \frac{VA}{K - A} \tag{1}$$

$$v = \frac{VA}{K\left(1 + \frac{I}{K_{is}}\right) + A} \tag{2}$$

$$v = \frac{VA}{K\left(1 + \frac{I}{K_{is}}\right) + A\left(1 + \frac{I}{K_{ii}}\right)} \tag{3}$$

$$v = \frac{VA}{K\left(\frac{1 + \frac{M}{K_N}}{1 + \frac{M}{K_D}}\right) + A} \tag{4}$$

inhibition, to linear noncompetitive inhibition, and to slope hyperbolic, competitive activation patterns were fitted to eq 2, 3, and 4, respectively.

## Results

*Comparison of the Kinetic Properties of the Two Enzyme Preparations.* A comparison of the kinetic properties of

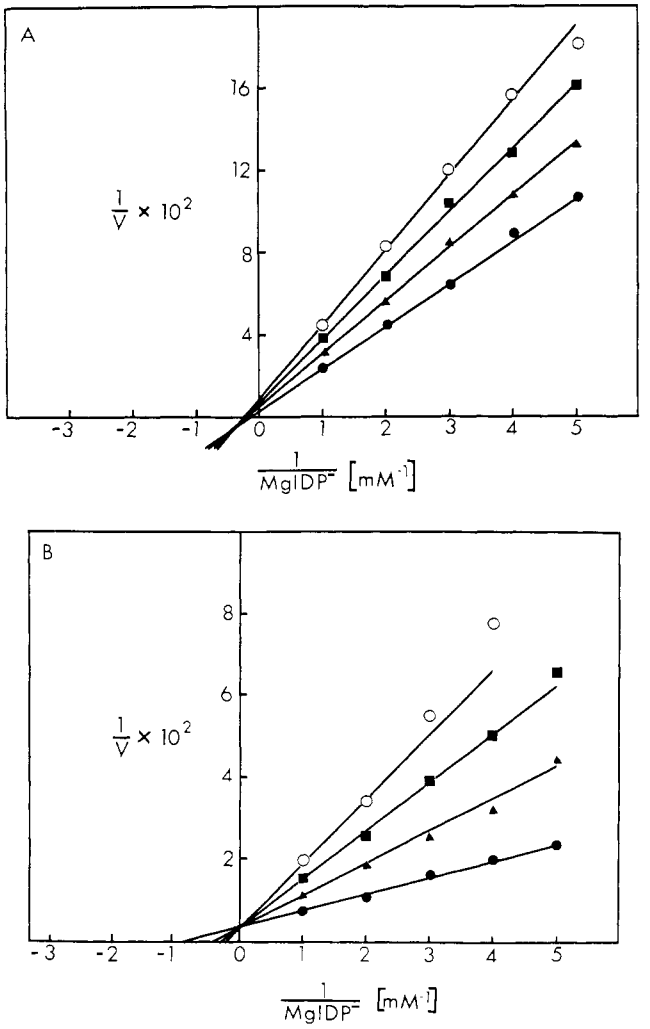



FIGURE 2: Inhibition of the reaction by free $Mg^{2+}$ both in the absence (A) and presence of $MgATP^{2-}$ (B). Concentrations of free $Mg^{2+}$ for (A) were: ●, 5; ▲, 10; ■, 15; and ○, 20 mM and for (B) were: ●, 5; ▲, 20; ■, 35; and ○, 50 mM. In B the ratio of $MgATP^{2-}$:$MgIDP^-$ was 2:1. Lines in A and B were drawn by using the constants obtained by fitting the data to eq 3 and 2, respectively. Initial velocity is expressed as millimicromoles of IMP per minute per microgram of protein.

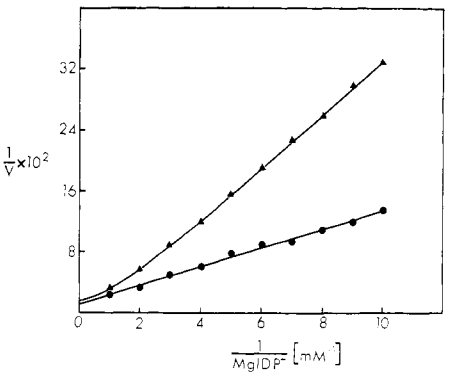



FIGURE 3: Effect of free $Mg^{2+}$ on the kinetics of the reaction. (●) The concentration of free $IDP^{3-}$ was held constant at 0.1 mM so that at any point, the concentration of free $Mg^{2+}$ was 2.5 times that of the substrate. (▲) Free $Mg^{2+}$ was maintained at a fixed concentration of 10 mM with the result that the concentration of free $IDP^{3-}$ varied from 0.025 to 0.0025 mM. The upper curve was fitted by eye. Initial velocities are expressed as millimicromoles of IMP per minute per microgram of protein.

freshly prepared (untreated) and aged preparations of nucleoside diphosphatase is illustrated in Figure 1. In the absence of the allosteric modifier, $MgATP^{2-}$, the untreated enzyme gives a plot of $1/v$ *vs.* $1/MgIDP^-$ which is curvilinear and whose asymptote can be extrapolated to cut the vertical ordinate at a negative value (Figure 1A). On the other hand, a similar plot using the aged enzyme is linear and the intercept of the curve with the vertical ordinate has a positive value (Figure 1B). With both preparations, the addition of $MgATP^{2-}$ causes an increase in the reaction velocities at nonsaturating concentrations of $MgIDP^-$ and double-reciprocal plots of velocity as a function of substrate concentration are linear (Figure 1A,B). Although it would appear that $MgATP^{2-}$ does not influence the maximum velocity of the reaction, as obtained with each enzyme preparation, a definitive conclusion cannot be reached as no allowance has been made for the inhibitory effect of free $Mg^{2+}$ (see below; Schramm and Morrison, 1969).

*Inhibition of the Reaction by Free $Mg^{2+}$.* The aged preparation of nucleoside diphosphatase is similar to the untreated enzyme in that it is activated by $IDP^{3-}$ and inhibited by free $Mg^{2+}$ (Schramm and Morrison, 1968). Thus, the correct interpretation of the kinetic data requires that these effects be taken into account. The problem of the activation of the reaction by $IDP^{3-}$ was circumvented by holding this nucleotide species at a fixed, relatively low concentration of 0.1 mM where it causes negligible activation. The condition was achieved as a result of varying the concentrations of free $Mg^{2+}$ and $MgIDP^-$ in a constant ratio of 2.5:1 so that the concentration of free $Mg^{2+}$ increased concomitantly with the substrate. It was necessary, therefore, to undertake a detailed kinetic study of the inhibition of the aged enzyme by free $Mg^{2+}$, in the absence and presence of $MgATP^{2-}$, so as to obtain values for the inhibition constants which could be used in calculating uninhibited initial velocities.[1]

The results of the inhibition of the reaction by free $Mg^{2+}$ are shown in Figure 2. These indicate that in the absence of $MgATP^{2-}$ (Figure 2A), free $Mg^{2+}$ causes linear, noncompetitive inhibition, while in the presence of $MgATP^{2-}$ (Figure 2B), linear competitive inhibition is obtained. It is also apparent from the results of Figure 2 that the measured initial velocities over the range of substrate concentrations used for maximum velocity determinations (*cf.* Figure 4) would differ significantly from the uninhibited values. The data of Figure 2A,B were fitted to eq 3 and 2, respectively, to give the values for the apparent inhibition constants that are recorded in Table I.

Experiments were also carried out over a wider range of substrate concentrations with free $Mg^{2+}$ at a fixed concentration of 10 mM. When the resulting data were plotted in double-reciprocal form (Figure 3), the plots appeared to exhibit slight nonlinearity in contrast to that of Figure 2A.

[1] Velocities obtained by correcting the measured initial velocities for the inhibitory effects of free $Mg^{2+}$, as described in Table I, are referred to as uninhibited initial velocities.

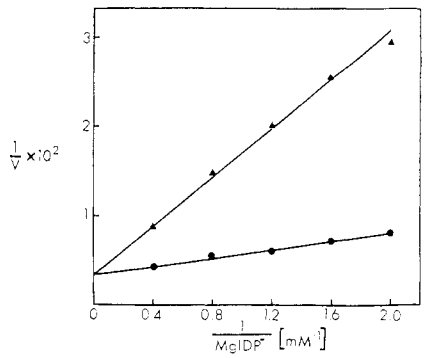



FIGURE 4: Effect of a saturating concentration of $MgATP^{2-}$ on the maximum velocity of the reaction. (▲) No $MgATP^{2-}$; (●) the concentrations of $MgATP^{2-}$ and $MgIDP^-$ were varied in a constant ratio of 2:1. Initial velocity is expressed as millimicromoles of $P_i$ per minute per microgram of protein.

This finding suggests that free $Mg^{2+}$ combines with the enzyme in such a way as to alter its kinetic properties and to cause the interdependent reaction of more than one molecule of substrate. In view of the results of Figure 3, the values of the inhibition constants for $Mg^{2+}$ (Table I) must be regarded only as reasonable estimates of the true values.

*Kinetics of the Reaction in the Presence and Absence of $MgATP^{2-}$.* The maximum velocity of the reaction in the absence of $MgATP^{2-}$ was determined by measuring initial velocities over a range of higher substrate concentrations from 0.5 to 2.5 mM while the maximum velocity of the reaction in the presence of a saturating concentration of the modifier was determined by using the same range of substrate concentrations and varying the concentrations of substrate and modifier in constant ratio. The resulting initial rates were then corrected, as previously elaborated (Schramm and Morrison, 1969), by multiplying by factors that varied from 1.03 to 1.33 (*cf.* Table I). The data so obtained are plotted in double-reciprocal form in Figure 4, which not only illustrates the activation by $MgATP^{2-}$ over the whole range of substrate concentrations, but also shows that the modifier has no significant effect on the maximum velocity. This result is in contrast to that which was obtained previously with the untreated enzyme and which showed that the maximum velocity is reduced considerably in the presence of a saturating concentration of $MgATP^{2-}$ (Schramm and Morrison, 1969).[2]

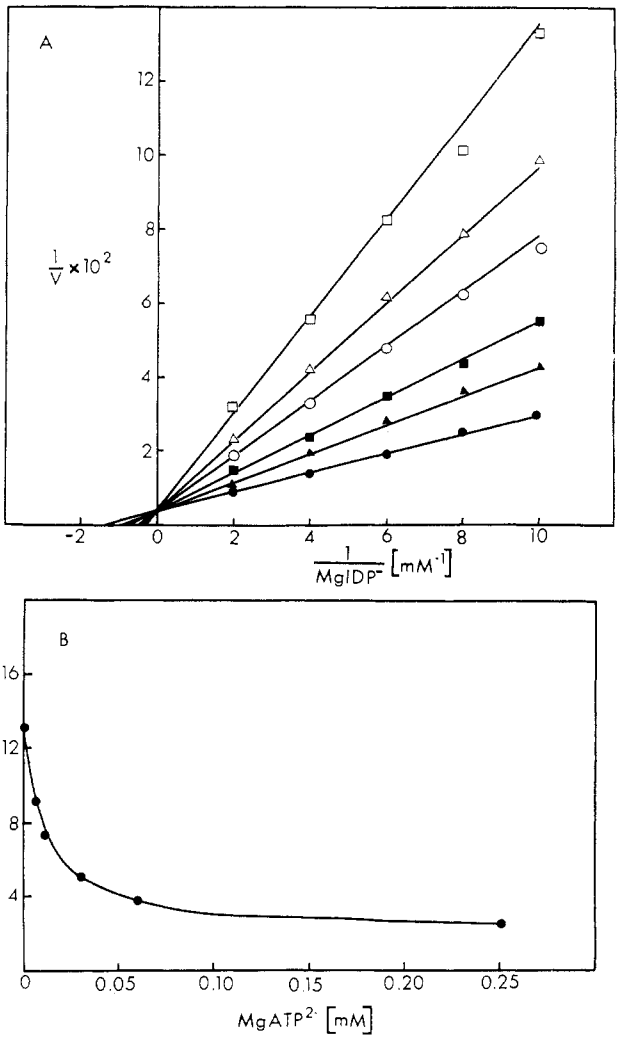



FIGURE 5: $MgATP^{2-}$ studies. (A) Effect of $MgATP^{2-}$ on the initial velocity of the reaction. Concentrations of $MgATP^{2-}$ were: □, 0; △, 0.006; ○, 0.012; ■, 0.03; ▲, 0.06; and ●, 0.25 mM. Lines were drawn by using the constants obtained by fitting the data to eq 4. (B) Replot of the slopes of the lines of A against the concentration of $MgATP^{2-}$ to illustrate that such a plot is nonlinear. Velocity is expressed as millimicromoles of IMP per minute per microgram of protein.

TABLE I: Kinetic Constants[a] for the Inhibition of the Reaction by Free $Mg^{2+}$ in the Absence and Presence of $MgATP^{2-}$.

| Kinetic Constant | $-MgATP^{2-}$ | $+MgATP^{2-}$ |
|---|---|---|
| $K$ | 3.86 ± 1.17 | 0.60 ± 0.02 |
| $K_{is}$ | 14.9 ± 1.38 | 10.2 ± 0.48 |
| $K_{ii}$ | 6.02 ± 2.58 | |

[a] Weighted mean values from three sets of data including that of Figure 2. These were used to calculate uninhibited initial velocities from the apparent values as obtained from plots of product formation against time. The relationship used was

$$\text{unhibited velocity} = \text{apparent velocity} \times \frac{K\left(1 + \frac{I}{K_{is}}\right) + A\left(1 + \frac{I}{K_{ii}}\right)}{K + A}$$

where $I$ and $A$ represent the concentrations of free $Mg^{2+}$ and $MgIDP^-$, respectively.

To gain further information about the function of the

[2] From Table III of this paper, it is apparent that free $Mg^{2+}$ is more inhibitory in the absence of $MgATP^{2-}$ than in its presence. Thus, correction for the inhibition would result in separation of the lines in such a way that a higher maximum velocity would be obtained in the absence of $MgATP^{2-}$.

TABLE II: Summary of Values for the Kinetic Parameters Obtained by Analysis of Data from Studies on Aged Nucleoside Diphosphatase.

| Kinetic Parameter | Values[a] as Obtained from the Data of | | |
|---|---|---|---|
| | Figure 2[b] | Figure 4 | Figure 5 |
| $V$ (mμmoles/min μg) | 246 ± 64[c]<br>279 ± 10[d] | 297 ± 6 | 258 ± 9 |
| $K_a$ (mM) | 3.9 ± 1.2 | 3.4 ± 0.01 | 3.0 ± 0.01 |
| $K_A$ (mM) | 0.60 ± 0.02 | 0.67 ± 0.04<br>(0.69 ± 0.03)[f] | 0.62 ± 0.05 |
| $K_{im1}$ | | | 0.071 ± 0.002<br>(0.039 ± 0.010)[f] |
| $K_{im2}$ | | | 0.0112 ± 0.0003 |

[a] Values were obtained as described in the legends to Figures 2, 3, and 4. [b] Weighted means of three experiments. [c] No $MgATP^{2-}$. [d] $MgATP^{2-}$ was present at a saturating concentration. [e] Calculated from the relationship $K_A = K_a K_{im2}/K_{im1}$. [f] The figures in brackets represent values for the corresponding kinetic constants as obtained by Schramm and Morrison (1969) with freshly prepared enzyme.

modifier in enhancing the catalytic activity of the enzyme at nonsaturating concentrations of substrate, investigations were made of the initial velocities of the reaction over a range of lower $MgIDP^-$ concentrations, at different fixed concentrations of $MgATP^{2-}$. After correction of the velocities for the inhibition by $Mg^{2+}$, using factors ranging from 1.01 to 1.10 (*cf.* Table I), the data were fitted to eq 4. The results illustrated in Figure 5 show that the experimental points fit well to the theoretical curves (Figure 5A) and that the slopes of the lines vary as a hyperbolic function of the reciprocal of the modifier concentration (Figure 5B). Values for the kinetic constants are listed in Table II.

*Determination of the Molecular Weights of Untreated and Aged Preparations of Nucleoside Diphosphatase.* As the kinetic characteristics of nucleoside diphosphatase were altered by its storage at −10°, it was possible that this change could arise from dissociation of the enzyme into smaller subunits. To test this possibility, the molecular weights of the untreated and aged enzyme preparations were determined by chromatographing catalytic quantities of each on a column of Sephadex G-200 as described under Methods. Two such determinations for each preparation gave average values of 95,000 for the untreated and 103,000 for the aged enzyme. Thus it appears that the changes in the kinetic properties of nucleoside diphosphatase cannot be ascribed to the slow dissociation or association of the enzyme under the conditions of storage. It is of interest to note that Yamazaki and Hayaishi (1968) have estimated the molecular weight of nucleoside diphosphatase from bovine liver to be 100,000.

## Discussion

The kinetic data obtained from initial velocity studies of the reaction catalyzed by an aged preparation of nucleoside diphosphatase appear to be consistent with the mechanism illustrated in Scheme I, where E, A, M, and P represent enzyme, substrate ($MgIDP^-$), allosteric modifier ($MgATP^{2-}$), and products ($Mg^{2+}$, IMP, and $P_i$), respectively. In this Scheme, it is considered that the enzyme possesses distinct sites for the combination of A and M so that they can add in a random manner and both can be present on the enzyme at the same time. Further, it is assumed that the presence of M on the enzyme enhances the combination of A, but does not affect the maximum velocity of the reaction. That is, both the EA and MEA complexes give rise to products at the same rate ($k_1$). It follows, therefore, that the activation of the reaction by M is due to the fact that, at any particular nonsaturating concentration of A, a greater proportion of the total enzyme ($E_t$) is saturated with substrate than is the case in the absence of M.

The results are also in accord with the idea that the reaction occurs under rapid equilibrium conditions so the initial velocity equation can be expressed as

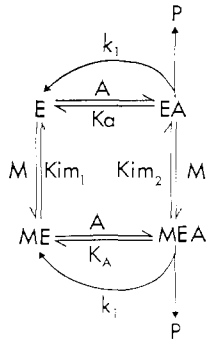
$$v = \frac{VA}{K_a\left[\dfrac{1 + \dfrac{M}{K_{im1}}}{1 + \dfrac{M}{K_{im2}}}\right] + A} \tag{5}$$

where $V = k_1E_t$; $K_a$ and $K_A$ are dissociation constants for the reaction of A with E and ME, respectively; $K_{im1}$ and $K_{im2}$ represent dissociation constants for the combination of M with E and EA, respectively. Because of the rapid equilibrium condition, the relationship $K_aK_{im2} = K_AK_{im1}$ must hold. Equation 5 reduces to eq 6 when $M = 0$ and to eq 7 when $M \rightarrow \infty$. Equation 5 predicts that plots of $1/v$ *vs.* $1/A$ would

$$v = \frac{VA}{K_a + A} \tag{6}$$

$$v = \frac{VA}{K_A + A} \tag{7}$$

SCHEME I




be linear while the slopes of the lines of such a plot would vary as a hyperbolic function of the concentration of M and both these predictions are in accord with the results of Figure 5. The experimental data are also in agreement with the predictions of eq 6 and 7 because of the linearity of the double-reciprocal plots in the absence of M and in the presence of relatively high concentrations of this modifier (Figure 4). Further support for the proposed mechanism comes from the finding that approximately the same maximum velocity is obtained, irrespective of the concentration of M (Figure 4, Table II).

If the reaction illustrated in Scheme I were to occur under steady-state conditions, the initial rate equation would contain terms in $A^2$ and $M^2$. Thus it could be expected that plots of $1/v$ *vs.* $1/A$ would yield nonlinear curves and that the activation of the reaction by M would be a complex function of its concentration. The results illustrated in Figures 1B and 5B show that these predictions are not realized. However, they cannot be used as an argument against the reactions taking place under steady-state conditions because of the distinct possibility that steady-state and rapid equilibrium models cannot be differentiated. The kinetic data of Figures 1B, 4, and 5 are certainly consistent with the idea that rapid equilibrium conditions prevail and this conclusion is supported by the fact that the value for $K_A$, as determined indirectly from the results of Figure 5, is in good agreement with that obtained directly from the analysis of the data of Figure 4 (Table II). Consequently the data have been interpreted in terms of a rapid equilibrium model.

Quantitative analysis of the kinetic data for the reaction gave the values for the kinetic constants that are summarized in Table II. Those for $K_a$ and $K_A$, representing dissociation constants for the reaction of A with E and ME, respectively, are in accord with the proposal that the presence of M on the enzyme facilitates the combination of A. Indeed, it will be noted that the binding of A to the ME complex is sixfold stronger than to the free enzyme (E). A comparison of the values for the kinetic constants, as obtained with freshly prepared and aged nucleoside diphosphatase (Table II), indicates that, with these two enzyme forms, there are no marked differences in the binding of M to E or of A to ME.

The conclusion from the results of the present work with an aged preparation of nucleoside diphosphatase that substrate and modifier combine at distinct, specific sites on the enzyme is similar to that reached from studies on an untreated preparation of the same enzyme (Schramm and Morrison, 1969). In each of these investigations, the kinetic data were consistent with the reaction of one molecule of modifier per mole of enzyme, but the possibility of multiple combinations occurring in an independent manner cannot be excluded since kinetic methods are incapable of distinguishing between these two possibilities. The situation is different with respect to the reaction of substrate with the two enzyme preparations. The evidence suggests that two molecules of substrate react with the untreated (freshly prepared) enzyme in an interdependent fashion while the reaction catalyzed by the aged enzyme involves either the binding of one molecule of substrate or the independent combination of multiple molecules of substrate. As indicated above, the latter two possibilities cannot be differentiated by kinetic techniques and thus the mechanism presented in Scheme I is proposed on the simpler basis of the reaction of a single substrate molecule with the aged enzyme. When the kinetic data for the aged enzyme are considered in relation to the facts that aging of a particular preparation does not alter significantly the molecular weight of nucleoside diphosphatase or its maximum velocity, then it appears that two conclusions are tenable. Firstly, two molecules of substrate do, in effect, react with the aged enzyme and secondly, the aging process causes a change in enzyme conformation which results in the elimination of the effect of one substrate on the combination of the other. It would seem that these conclusions are confirmed by the results of Figure 3 which can be interpreted to indicate that $Mg^{2+}$ combines at the modifier site(s) in such a way as to allow substrate to induce an interaction between the catalytic sites on the enzyme. In this connection, it should be mentioned that the results of studies of the inhibition by $Mg^{2+}$ suggest that this metal ion reacts at both substrate and modifier sites when $MgATP^{2-}$ is absent (Figure 2A and Table I) and only at the substrate site when $MgATP^{2-}$ is present at saturating concentrations (Figure 2B and Table I). Since both the noncompetitive and competitive inhibition patterns are of the linear type, it must be concluded that an enzyme-$(Mg)_2$ complex is not formed in kinetically significant amounts.

Although the aging of nucleoside diphosphatase gives rise to changes in the kinetic properties of the enzyme, as far as its reaction with substrate is concerned, the treatment has little influence on the ability of $MgATP^{2-}$ to act as an allosteric activator. However, it does apparently influence the mechanism of the activation. With the aged enzyme, the modifier appears to function by enhancing substrate combination, while having no effect on the rate of product formation, whereas with the untreated enzyme, it seems that the modifier acts largely by increasing the rate of product formation at lower concentrations of substrate (Schramm and Morrison, 1969).

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# Alteration of the Conformative Response and Inhibition of Xanthosine 5'-Phosphate Aminase by Adenine Glycosides*

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ABSTRACT: Modification of the conformative response of xanthosine 5'-phosphate aminase to its substrate, xanthosine 5'-phosphate, by inorganic pyrophosphate has been found to be essential for its sensitivity to inhibition by adenine glycosides. The millimolar concentration of xanthosine 5'-phosphate which induces a half-maximal conformative response, the conformative response constant or $K_{cr}$, is 0.1. In the presence of inorganic pyrophosphate this value is reduced 33-fold. Such modification can be eliminated by chemical treatment or genetic alteration with the further consequences of loss or diminution of sensitivity to irreversible inhibition by the adenine glycoside antibiotics, psicofuranine and decoyinine, as well as diminution of sensitivity to reversible inhibition by adenosine. Catalytic activity however is not appreciably affected by elimination of the modifying action of inorganic pyrophosphate.

Previous studies (Udaka and Moyed, 1963; Fukuyama and Moyed, 1964) have shown that the adenine glycoside antibiotic psicofuranine (6-amino-9-D-psicofuranosylpurine) causes an irreversible inhibition of XMP aminase (xanthosine 5'-phosphate:ammonia ligase (AMP), EC 6.3.4.1). The inhibition was found to depend upon the presence of the substrates, XMP, and on one of the products, $PP_i$, of the catalytic reaction. A subsequent report (Zyk *et al.*, 1969) presented evidence that exposure of this enzyme to XMP results in a conformative response, a reversible change in the conformation of the enzyme associated with the binding of the substrate, which is greatly enhanced by $PP_i$ and magnesium ions. Enhanced conformative response was found to be essential for the reversible interaction of the enzyme with adenosine. In the present communication we examine the role of the conformative response in the reversible and irreversible binding of adenine glycosides. The results to be presented indicate that the susceptibility of XMP aminase to inhibition by adenine glycosides is determined by the extent of response of the enzyme to the modifying effect of $PP_i$. Loss of responsiveness to $PP_i$ resulting from environmental or genetic changes confers resistance to adenine glycosides.

## Materials and Methods

*Chemicals.* Psicofuranine and decoyinine were gifts of the Upjohn Co. The other adenine glycosides, XMP, trypsin, and pronase were commercial preparations.

*Purification of XMP Aminases.* The previously published purification procedure was employed (Fukuyama and Moyed, 1964). There were modifications in the strains of bacteria and in the method of cultivation. The bacterial strains were *Escherichia coli* B-96, a purine-requiring mutant blocked in inosinicase, and its derivatives B-96-7, B-96-17, and B-96-24, which were selected by Mr. Kerry Donovan for ability to grow in the presence of bacteriostatic concentrations of psicofuranine. Derepressed synthesis of XMP aminase was achieved in all strains by growth for 8 hr in a mineral salts–glucose medium supplemented with 2 mg/ml of Bacto Difco Casamino Acids (vitamin free) and 40 $\mu$g/ml of AMP.

*XMP Aminase Assay.* The procedure was as previously described (Zyk *et al.*, 1969).

*Heat Treatment, Pronase Treatment, and Trypsin Treatment.* The procedures were as previously described (Zyk *et al.*, 1969).

## Results

*Interaction of XMP Aminase with Adenine Glycosides.* In a previous report (Zyk *et al.*, 1969) we described the XMP-

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