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STUDIES ON INOSINE MONOPHOSPHATE DEHYDROGENASE. STEADY STATE KINETICS

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Summary

The reaction catalyzed by IMP dehydrogenase (IMP: NAD^+ oxidoreductase EC 1.2.1.14) from *Aerobacter aerogenes* has been investigated kinetically at pH 8.1 as a three reactant system by means of steady-state velocity studies in the absence of products, as well as by inhibition studies using products and substrate analogues. The mechanism appears to be a partially random one in which IMP and K^+ can bind randomly to the free enzyme while NAD does not react unless K^+ or both K^+ and IMP are present on the enzyme. While the steady-state velocity data can be analysed adequately on the basis that rapid equilibrium conditions apply, this is only an approximate description of the mechanism since product inhibition studies indicate that there is a significant concentration of an enzyme · XMP (enzyme · K · XMP) complex in the steady-state.

Introduction

IMP dehydrogenase (IMP: NAD^+ oxidoreductase, EC 1.2.1.14) catalyses the reaction

$$\text{IMP} + \text{NAD} \xrightarrow{M^+} \text{XMP} + \text{NADH}_2$$

where M^+ represents an essential monovalent cation which may be K^+ or NH_4^+ [1]. In addition, the enzyme from *Aerobacter aerogenes* requires a reducing agent for its activity [1]. Some of the kinetic properties of the enzyme from this source have been reported by Hampton and his co-workers [2]. They maintained a relatively high, constant concentration of K^+ and considered that the reaction involved only IMP and NAD. From qualitative product inhibition studies it was concluded that the reaction conformed to an ordered mechanism with IMP adding to the enzyme before NAD. [2].

In the present investigations on the mechanism of the IMP dehydrogenase reaction, consideration has been given also to the role of the essential monovalent cation. Since preparations of the enzyme from *Aerobacter aerogenes* consist of a mixture of molecular weight isomers [3,4], it was necessary to avoid the complications that would arise by using a mixture of enzyme forms with different kinetic parameters. Therefore, the kinetic experiments were performed under conditions where the enzyme exists essentially as one molecular species [4]. The investigation has been made at the optimum pH for the reaction (pH 8.1), at which the reaction is essentially irreversible.

The results of steady-state velocity, product inhibition and dead-end inhibition studies suggest that the mechanism is a partly random one in which there is random addition of K^+ and IMP to the free enzyme while NAD reacts only with the E · K and E · K · IMP forms. Further it appears that the rate of chemical interconversion of the central complexes is sufficiently slow compared with all other steps of the reactant addition sequence that rapid equilibrium conditions are approximated.

Materials and Methods

IMP dehydrogenase was prepared from *A. aerogenes*, strain P-14, by the procedure of Brox and Hampton [3] and exhibited the properties described previously [3]. $NADH_2$, GMP and XMP were purchased from P-L Biochemicals Inc., nicotinic acid from Fluka AG (purum) and lithium chloride from British Drug Houses Ltd. All other reagents were as described earlier [4].

Measurement of enzyme activity

Assays were performed by continuously recording the increase in absorbance at 340 nm due to the formation of $NADH_2$, using cells of 1 cm light path in a Cary 14 spectrophotometer. Assay mixtures contained, in a final volume of 3.0 ml: 0.1 M Tris · HCl buffer (pH 8.1) containing dithiothreitol (5 mM), and concentrations of reactants as indicated in the figures. The assay mixtures were preincubated for 5 min at 37°C in the thermostatted cell compartment of the spectrophotometer, and the reaction was then initiated by the addition of 20—40 μg of enzyme.

As $NADH_2$ proved to be a poor product inhibitor it was necessary to use relatively high concentrations of this compound to demonstrate inhibition. To avoid the artifactual results that can be obtained under these conditions and which are attributable to stray light effects associated with wide slit openings [5,6,7], studies with $NADH_2$ were made by using cells of 5 mm light path and by increasing the dynode voltage.

Analysis of data

Data were first plotted graphically to check the linearity of double reciprocal plots and to determine the patterns given by families of such plots. An overall fit of each set of data was then made to the appropriate rate equation by using one of the computer programs of Cleland [8] in conjunction with a Univac 1108 computer. This method yields the best estimates of the kinetic parameters which were used in connection with the drawing of the lines illustrated in the figures.

Results

Steady-state velocity studies in the absence of inhibitors

Steady-state velocity studies were performed by varying the concentrations

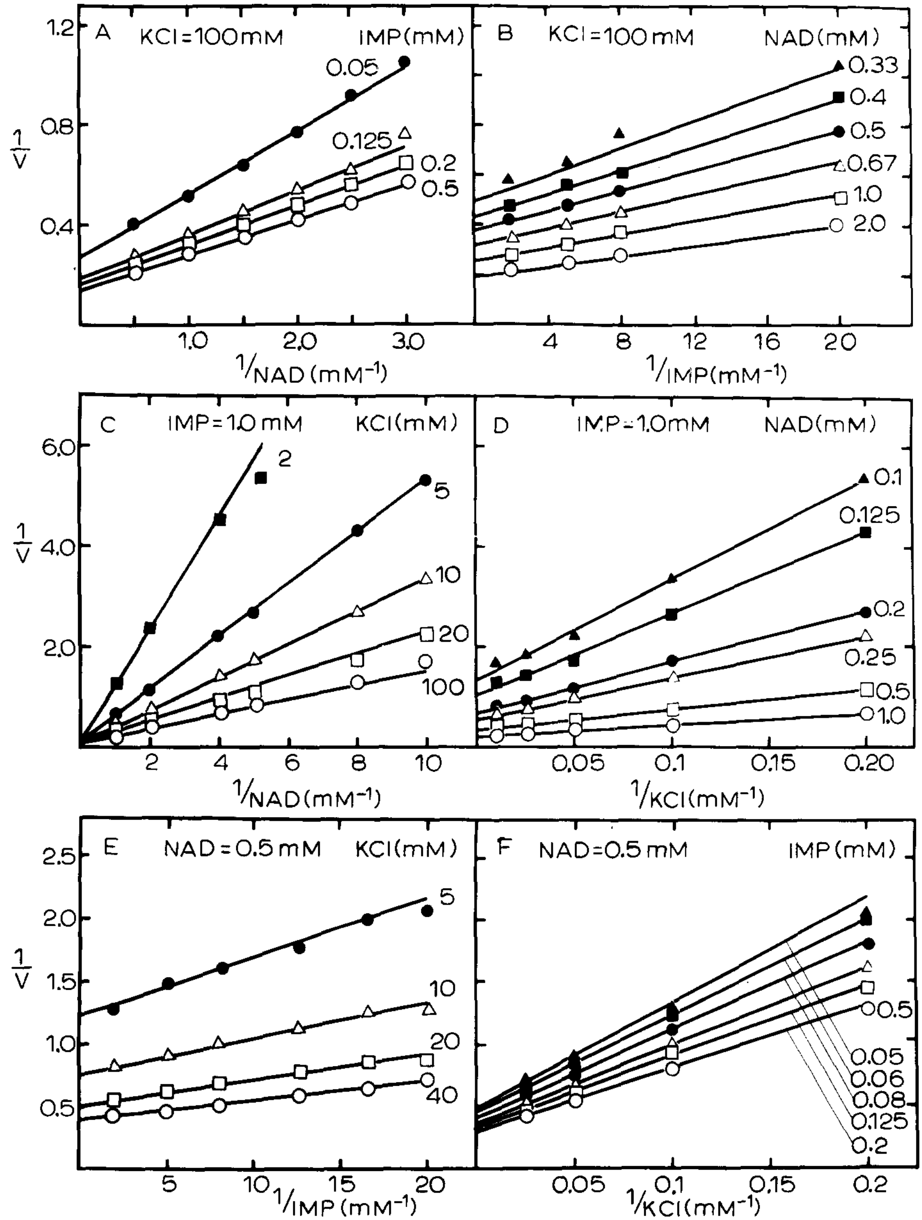
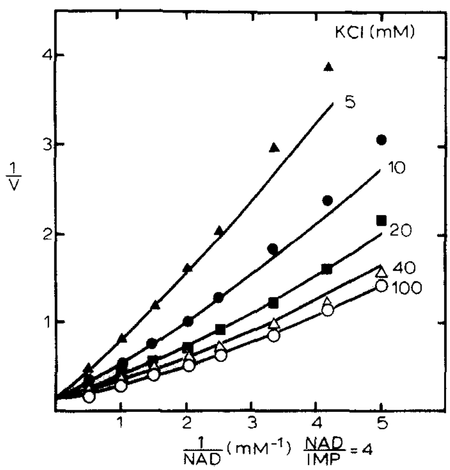


Fig. 1. Effect of varying different reactant pairs on the velocity of the reaction. Velocities are expressed as μmol of $NADH_2$ formed per min per mg of enzyme. The data of A, B, E and F were fitted to Eqn. 7 of ref. 8. The data of C and D were fitted to the equation $v = VAB/(K_{ia}K_b + K_bA + AB)$.

of a pair of reactants while holding the third reactant at a fixed concentration. Each of the double reciprocal plots thus obtained showed an intersecting pattern (Fig. 1), but it should be noted that with NAD as the variable substrate at different fixed concentrations of K^+, the intersection point is on the vertical ordinate (Fig. 1c).

When NAD and IMP were varied in constant ratio at different fixed concentrations of K^+, a series of curved double reciprocal plots with a common intersection point on the vertical ordinate is obtained (Fig. 2). Thus the maximum



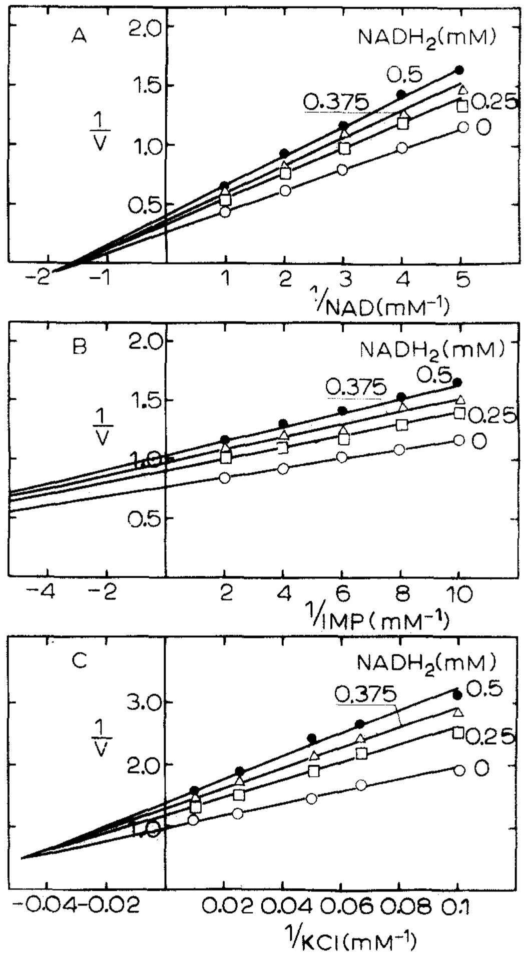


Fig. 2. Effect of K^+ concentration on the velocity of the reaction when IMP and NAD are varied in constant ratio. The data were fitted to the equation

$$y = (Vx^2)/a\left[\left(1 + \frac{K_{im}}{M}\right) + b\left(1 + \frac{K_m}{M}\right) + x^2\right]$$

Velocities are expressed as μmol of $NADH_2$ formed per min per mg of enzyme.

Fig. 3. Product inhibition by $NADH_2$. Velocities are expressed as μmol of $NADH_2$ formed per min per mg enzyme. The concentrations of fixed reactants were KCl 100 mM, IMP 0.2 mM and NAD 0.25 mM in B and 0.5 mM in C. The data were fitted to Eqn. 10 of ref. 8.

TABLE I

APPARENT KINETIC CONSTANTS ASSOCIATED WITH THE REACTION OF K^+ (M), IMP (A) AND NAD (B) WITH IMP DEHYDROGENASE

Values for the apparent kinetic constants were obtained by fitting at least two sets of data for each reactant pair, including those illustrated in Fig. 1, to the appropriate rate equation and taking the weighted mean of the values for each apparent constant. When A and M or A and B were varied, the data were fitted to Eq. 7 of ref. 8. For experiments when M and B were varied the data were fitted to the equation $v = VAB/(K_{ia}K_b + K_bA + AB)$

Variable substrates	Fixed substrate (mM)	Apparent kinetic constant (mM)			
		K_{ia}	K_a	K_{im}	K_m
A, M	B; 1.0	0.036 ± 0.011	0.049 ± 0.005	7.6 ± 2.6	10.3 ± 2.7
	0.5	0.038 ± 0.004	0.039 ± 0.003	16.4 ± 2.2	16.9 ± 0.8
		K_{ia}	K_a	K_{ib}	K_b
A, B	M; 100	0.030 ± 0.002	0.062 ± 0.004	0.61 ± 0.06	1.08 ± 0.04
	20	0.036 ± 0.004	0.066 ± 0.011	1.08 ± 0.20	2.00 ± 0.17
	5	0.040 ± 0.004	0.090 ± 0.026	1.89 ± 0.47	4.25 ± 0.64
		K_{im}	K_b		
M, B	A; 1.0	25.3 ± 1.7	0.69 ± 0.05		
	0.5	23.9 ± 2.2	0.76 ± 0.07		
	0.1	28.6 ± 3.3	0.62 ± 0.07		

TABLE II

KINETIC CONSTANTS FOR PRODUCT INHIBITION OF THE IMP DEHYDROGENASE REACTION

The concentrations of fixed substrates are shown in Figs. 3 and 4. The data, including those illustrated in Figs. 3 and 4, were analyzed according to Eqs. 10 and 8 of Reference 8, for non-competitive and competitive inhibition, respectively. Where more than one experiment of each type was performed, the weighted mean value of the apparent constants is recorded. Values for true K_i for XMP were calculated from apparent K_i values assuming that XMP functions as an analogue of IMP and using the kinetic constants from Table IV.

Inhibitor	Variable substrate	Apparent K_i (mM)		True K_i (mM)	
		$K_{i_{slope}}$	$K_{i_{intercept}}$	K_i [a]	K_I [a]
$NADH_2$	NAD	0.79 ± 0.08	0.53 ± 0.04		
	IMP	1.11 ± 0.25	1.41 ± 0.11		
	KCl	0.50 ± 0.05	1.10 ± 0.07		
XMP	NAD	1.46 ± 0.06	2.35 ± 0.02	0.21 ± 0.02 [a]	0.54 ± 0.05 [a]
	IMP	0.21 ± 0.01 (0.28 ± 0.03) [b]			
	KCl	1.10 ± 0.18	1.87 ± 0.16 (1.67 ± 0.18) [b]	0.16 ± 0.03	

[a] It was assumed that the dissociation constants for the reaction of the inhibitor XMP with the E and EM complexes were the same (K_i) but different from the dissociation constant for its reaction with EMB (K_I).

[b] When calculation of the value for a true inhibition constant was precluded because the expression for the apparent inhibition constant contained both K_i and K_I, a comparison was made between the experimental and calculated values for the apparent inhibition constant. The latter values, which are given in brackets below the experimental values, were calculated by substituting values for the various kinetic constants into the appropriate relationship.

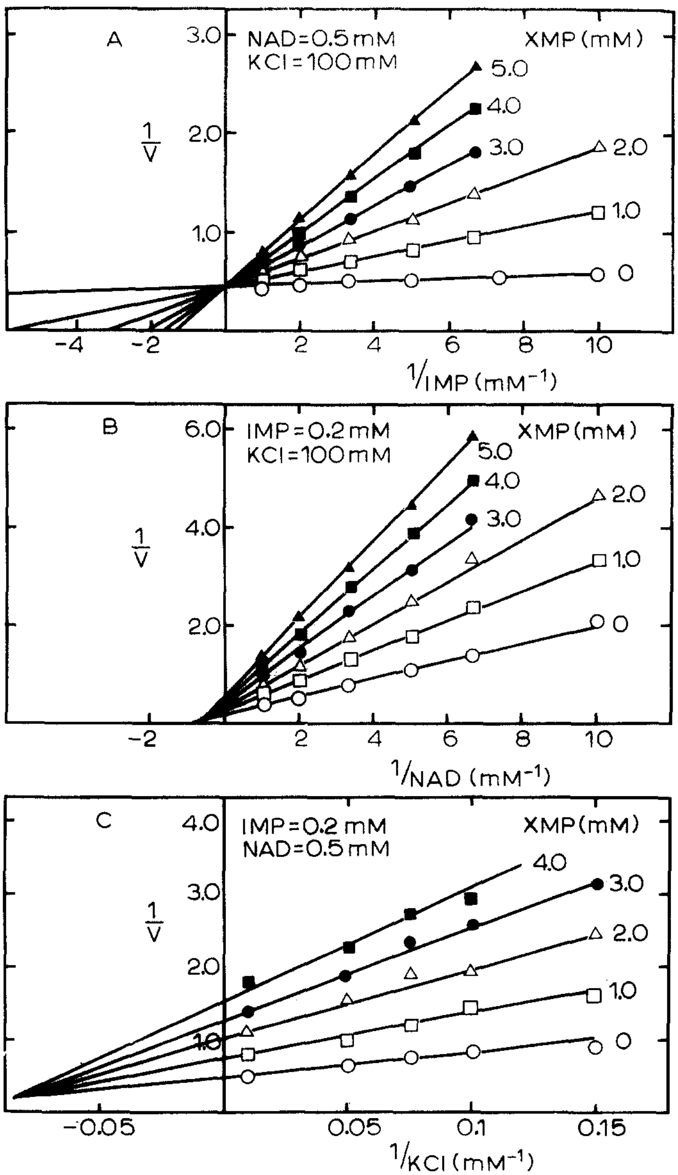


Fig. 4. Product inhibition by XMP. Velocities are expressed as μmol of $NADH_2$ formed per min per mg enzyme. The concentrations of fixed reactants were KCl 100 mM, IMP 0.2 mM and NAD 0.5 mM. The data of A were fitted to Eqn. 8 and those of B and C to Eqn. 10 of ref. 8.

velocity appears to be independent of the concentration of K^+ (cf. Fig. 1c). The curvature of these plots and the intersecting patterns of Fig. 1 indicate that all three reactants combine with the enzyme before either product is released. The apparent kinetic constants obtained from analysis of the data of Fig. 1 are recorded in Table I. When NH_4^+ was used as the activating cation instead of K^+, the initial velocity patterns were similar to those illustrated in Fig. 1.

Product inhibition

The inhibition by $NADH_2$ was linear non-competitive with respect to each of the three reactants (Fig. 3). The linearity of the slope replot of the data obtained with NAD as the variable substrate (Fig. 3a) contrasts with the parabolic slope replot reported by Brox and Hampton [2] which may have been an artifactual result caused by stray light effects with $NADH_2$ at relatively high concentrations [5,6,7].

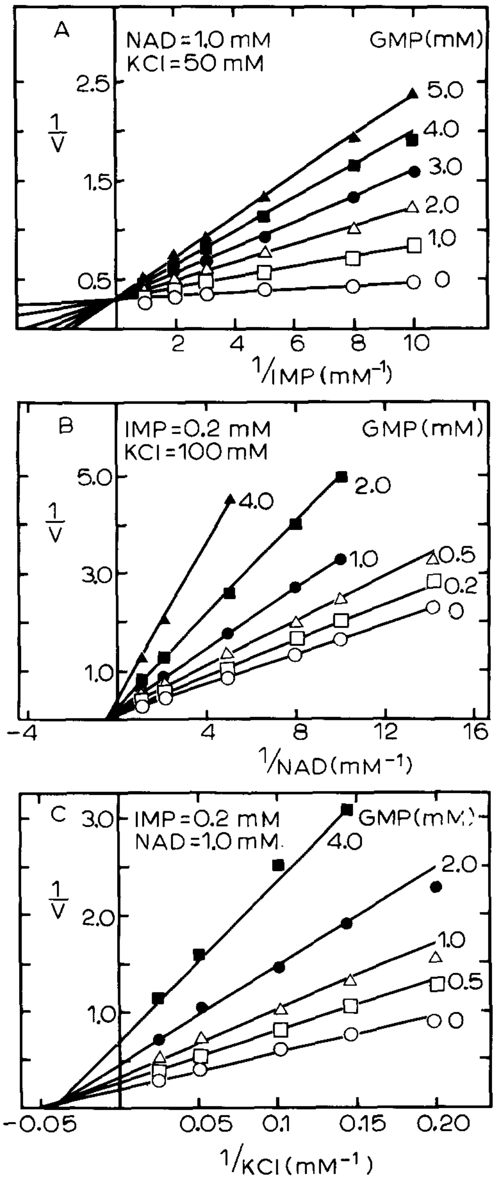


Fig. 5. Inhibition by GMP. Velocities are expressed as μmol of $NADH_2$ formed per min per mg enzyme. The concentrations of fixed reactants were: (A) NAD 1.0 mM and KCl 50 mM, (B) IMP 0.2 mM and KCl 100 mM, (C) IMP 0.2 mM and NAD 1.0 mM. The data of A were fitted to Eqn. 8 and those of B and C to Eqn. 10 of ref. 8.

XMP acts as a linear competitive inhibitor with respect to IMP and as a linear non-competitive inhibitor with respect to both NAD and K^+ (Fig. 4). The kinetic constants derived from the product inhibition data are recorded in Table II.

Inhibition by substrate analogues

GMP functioned as an inhibitory analogue of IMP giving rise to inhibitions

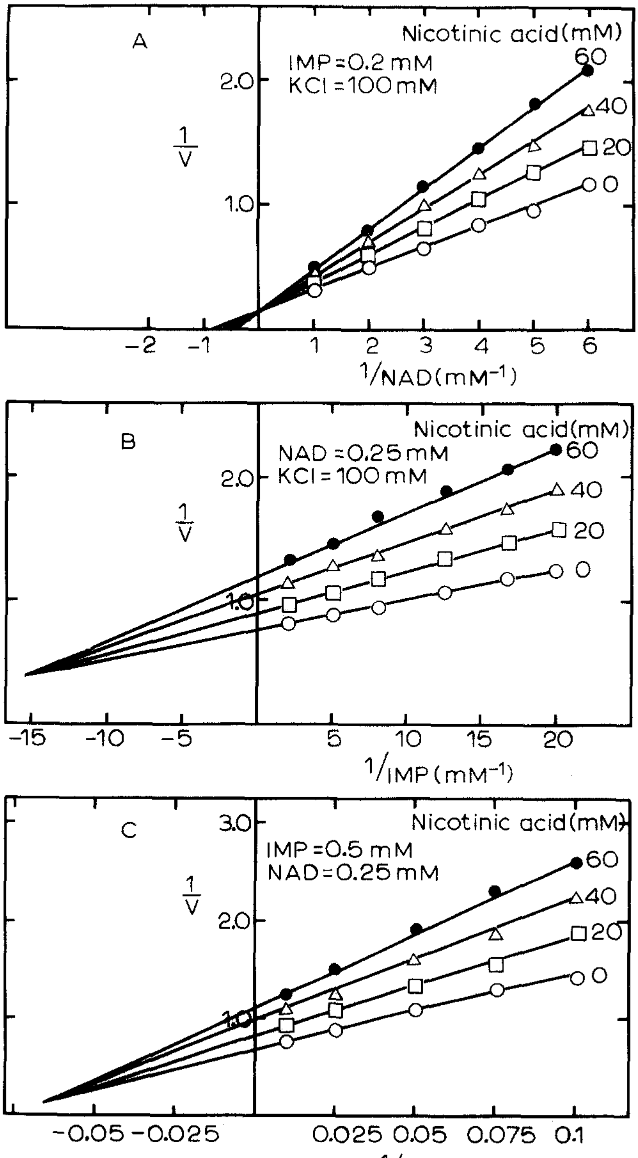
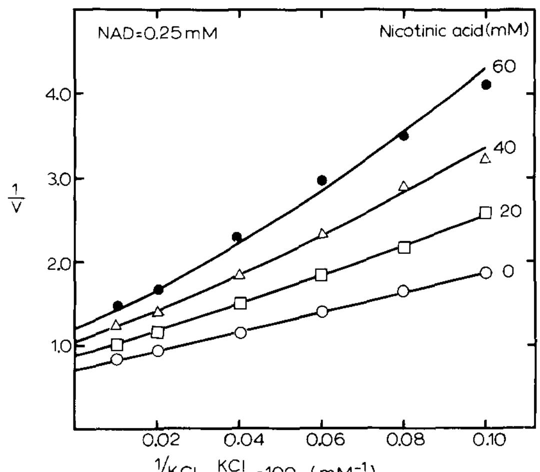


Fig. 6. Inhibition by nicotinic acid. Velocities are expressed as μmol of $NADH_2$ formed per min per mg enzyme. The concentrations of fixed reactants were: (A) IMP 0.2 mM and KCl 100 mM, (B) NAD 0.25 mM and KCl 100 mM, (C) IMP 0.5 mM and NAD 0.25 mM. The data of A were fitted to Eqn. 8 and those of B and C to Eqn. 10 of ref. 8.

which are linear competitive with respect to IMP and linear non-competitive in relation to both NAD and K$^+$ (Fig. 5). Nicotinic acid acted as an inhibitory analogue of NAD, causing linear competitive inhibition with respect to this substrate and linear non-competitive inhibition with respect to IMP and K$^+$



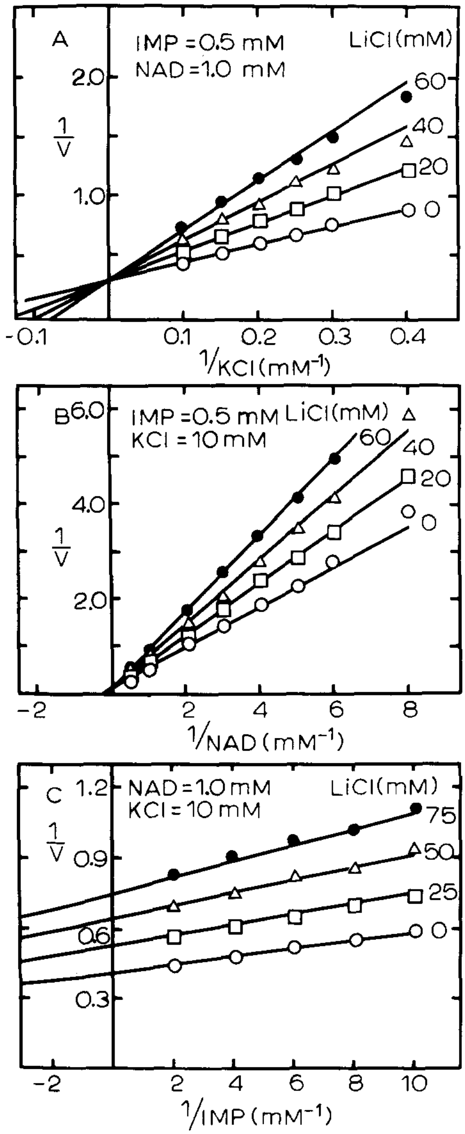


Fig. 7. Inhibition by nicotinic acid with respect to IMP and K$^+$, simultaneously varied. Velocities are expressed as μmol of $NADH_2$ formed per min per mg of enzyme. The concentration of NAD was 0.25 mM. The data were fitted to the equation

$$y = \frac{Vx^2}{b + cI + dI^2 + ex + gxI + x^2 + ra^2 I}$$

Fig. 8. Inhibition by Li$^+$. Velocities are expressed as μmol of NADH formed per min per mg of enzyme. The concentrations of fixed reactants were: (A) IMP 0.5 mM and NAD 1.0 mM, (B) IMP 0.5 mM and KCl 10 mM, (C) NAD 100 mM and KCl 10 mM. The data of A and B were fitted to Eqn. 8 and those of C to Eqn. 10 of ref. 8.

(Fig. 6). The inhibition by nicotinic acid was also investigated by varying the concentrations of IMP and K^+ in constant ratio, at a fixed concentration of NAD. The result (Fig. 7) shows that the ordinate intercept varies as a linear function of the concentration of nicotinic acid. A number of other potential analogues of NAD which did not cause significant inhibition included ADP-ribose, ADPribose plus nicotinamide, desaminoNAD, isonicotinic hydrazide, tetrahydronicotinamide, AMP and ADP. Three other analogues, NMN, NADP and thioNAD, functioned as very poor substrates.

TABLE III

KINETIC CONSTANTS FOR INHIBITION OF THE IMP DEHYDROGENASE REACTION BY SUBSTRATE ANALOGUES

The data, including those illustrated in Figs. 5, 6 and 8, were analyzed according to Eqns. 10 and 8 of ref. 8, for non-competitive (NC) and competitive (C) inhibition, respectively. Where more than one experiment of each type was performed, the weighted mean value of the apparent kinetic constants is recorded. True K_i values were calculated from the apparent K_i values using the appropriate relationships together with the concentrations of fixed substrates which are shown in the figures and the values of the kinetic constants given in Table IV.

Inhibitor	Variable substrate	Type of inhibition	Apparent K_i (mM)		True K_i (mM)	
			$K_{i\,slope}$	$K_{i\,intercept}$	K_i [a]	K_I [a]
GMP	NAD	NC	0.96 ± 0.04	1.31 ± 0.12	0.14 ± 0.01	0.30 ± 0.03
	K^+	NC	1.15 ± 0.21	1.80 ± 0.35	0.17 ± 0.03	
				(1.15 ± 0.10) [b]		
	IMP	C	0.17 ± 0.01 [c]			
			(0.19 ± 0.02)			
		C	0.31 ± 0.01			
			(0.21 ± 0.02)			
Li^+	NAD	C	62.8 ± 1.7		45 ± 3	
	K^+	C	33.5 ± 1.4		34 ± 1	
		C	42.5 ± 2.6 [d]		43 ± 3	
	IMP	NC	81 ± 18		36 ± 8	
				90 ± 5	48 ± 3	
Nicotinic Acid	NAD	C	59 ± 2			
			(71 ± 5)			
	K^+	NC	70 ± 10		70 ± 10 (K_{i1})	
				91 ± 6		
				(106 ± 8)		
	IMP	NC	54 ± 7		34 ± 5 (K_{i3})	
				104 ± 7	88 ± 8 (K_{i4})	

[a] (i) The dissociation constant for the interaction of GMP with free E or the EM complex is represented by K_i, while that for the interaction with the EMB complex is represented by K_I. (ii) The dissociation constant for the interaction of Li^+ with E and EA is represented by K_i. (iii) Nicotonic acid has been assumed to interact with the E and EA complexes equally well (dissociation constant K_{i1}), and also with the EM and EMA complexes (dissociation constants K_{i3} and K_{i4} respectively).

[b] When calculation of the value for a true inhibition constant was precluded because the expression for the apparent inhibition constant contained two different true inhibition constants, a comparison was made between the experimental and calculated values for the apparent inhibition constant. The latter values, which are given in brackets below the experimental values, were calculated by substituting values for the various kinetic constants into the appropriate relationship.

[c] Concns. fixed reactants: 100 mM KCl, 0.5 mM NAD.

[d] Concns. fixed reactants: 0.5 mM IMP and 0.5 mM NAD.

Li^+ acted as an inhibitory analogue of K^+ in that it caused linear competition with respect to K^+. The inhibition was also linear competitive with respect to NAD while linear non-competitive in relation to IMP (Fig. 8). Analysis of the data of Fig. 5, 6 and 8 gave the results which are recorded in Table III.

Discussion

From the intersecting steady-state velocity patterns obtained with the three reactants (Fig. 1) it follows that the IMP dehydrogenase reaction must proceed via a sequential mechanism which can be ordered, random or partly ordered — partly random. Of the six possible ordered mechanisms, only those for which reactants add in the order: (a) K^+, NAD, IMP, or (b) IMP, K^+, NAD could yield the equilibrium ordered pattern (Fig. 1c, 1d and the common intercept in Fig. 2). However, the competitive inhibition by XMP with respect to IMP (Fig. 4A) rules out (a) while the failure to obtain any uncompetitive inhibitions with inhibitory analogues of the reactants (Fig. 5, 8) eliminates both (a) and (b). In considering possible random mechanisms it is necessary to note that random mechanisms where the rate-limiting step is not solely the interconversion of two central complexes can resemble rapid equilibrium random mechanisms [9]. Nevertheless, none of the double reciprocal plots exhibited non-linearity and it will be considered that any random addition of reactants occurs under what approximates to rapid equilibrium conditions. A completely random combination of K^+, IMP and NAD with the enzyme is not in accord with the equilibrium ordered pattern (Fig. 1c and d). Partly random mechanisms involving the compulsory addition of one reactant, followed by random addition of the other two or the random combination of two reactants followed by the ordered addition of the third can also be eliminated. When rapid equilibrium conditions apply, the latter mechanism requires that equilibrium ordered patterns be obtained with two pairs of reactants while no equilibrium ordered patterns would be obtained with the former mechanism. All six possible sequences for these two basic mechanisms would yield dead-end inhibition patterns at variance with those obtained.

The simplest mechanism that can best account for the results in both qualitative and quantitative terms is that illustrated in Scheme I where M, A and B represent the activating metal ion (K^+ or NH_4^+), IMP and NAD, respectively. It is envisaged that on the addition side of the reaction sequence K^+ and IMP can add randomly to the free enzyme and that NAD does not react unless K^+ or both K^+ and IMP are present on the enzyme. Thus, K^+ induces a conformational change permitting the binding of NAD which then locks K^+ onto the enzyme. It is proposed initially that the interconversion of the central quaternary complexes is sufficiently slow compared with the steps on the addition side of the reaction sequence that rapid equilibrium conditions are approximated. It is assumed now and justified later that combinations of M and A are independent, but that the combination of B with EM differs from that with EMA.

The initial rate equation for the proposed mechanism can be expressed as:

$$v = \frac{V_{\mathrm{MAB}}}{K_{\mathrm{im}}K_{\mathrm{ia}}K_{\mathrm{b}} + K_{\mathrm{ia}}K_{\mathrm{b}}\mathrm{M} + K_{\mathrm{im}}K_{\mathrm{b}}\mathrm{A} + K_{\mathrm{b}}\mathrm{MA} + K_{\mathrm{a}}\mathrm{MB} + \mathrm{MAB}} \quad (1)$$

Each of the kinetic parameters represents a dissociation constant for a reaction denoted in scheme 1. The denominator terms represent respectively the pro-

SCHEME 1

$$\begin{tikzcd}
E \arrow[r, leftrightarrow, "M", "K_{im}"'] \arrow[d, leftrightarrow, "A"', "K_{ia}"] & EM \arrow[r, leftrightarrow, "B", "K_{ib}"'] \arrow[d, leftrightarrow, "A"', "K_{ia}"] & EMB \arrow[d, leftrightarrow, "A"', "K_{a}"] & \\
EA \arrow[r, leftrightarrow, "M", "K_{im}"'] & EMA \arrow[r, leftrightarrow, "B", "K_{b}"'] & EMAB \arrow[r] & \text{Products}
\end{tikzcd}$$

portion of total enzyme present as free enzyme (E), EM, EA, EMA, EMB and EMAB. The rate equation may be rearranged according to which pair of substrates is varied to give Eqns. 2, 3 and 4. M and A varied, B fixed:

$$v = \frac{V\left(\frac{B}{K_b + B}\right)MA}{K_{im}K_{ia}\left(\frac{K_b}{K_b + B}\right) + K_a\left(\frac{K_{ib} + B}{K_b + B}\right)M + K_{im}\left(\frac{K_b}{K_b + B}\right)A + AB} \quad (2)$$

A and B varied, M fixed:

$$v = \frac{VAB}{K_{ia}K_b\left(1 + \frac{K_{im}}{M}\right) + K_b\left(1 + \frac{K_{im}}{M}\right)A + K_aB + AB} \quad (3)$$

M and B varied, A fixed:

$$v = \frac{V\left(\frac{A}{K_a + A}\right)MB}{K_{im}K_b\left(\frac{K_{ia} + A}{K_a + A}\right) + K_b\left(\frac{K_{ia} + A}{K_a + A}\right)M + MB} \quad (4)$$

It follows from Eqns. 2 and 3 that when A and B or M and A are varied, symmetrical steady-state velocity patterns would be expected and such patterns are obtained (cf. Fig. 1a, b, e and f). Eqn 4. predicts that when M and B are varied the steady-state velocity pattern would be asymmetric, and this pattern is also observed (Fig. 1c and d). The data of Fig. 2 also provide evidence for the equilibrium ordered addition of K^+ and NAD.

The relationships between apparent and true kinetic constants may be derived from Eqns. 2, 3 and 4, and these have been used to calculate the true values for the parameters associated with the reaction (Table IV). The good agreement between the various values for K_{ia}, K_a, K_{ib} and K_b lends quantitative support for the conclusion reached about the reaction mechanism. On the basis that the reaction mechanism is independent of the identity of the activating cation, the values for K_{ia}, representing the dissociation constant for the reaction of IMP with free enzyme, should be the same in the presence of

TABLE IV

KINETIC CONSTANTS FOR THE INTERACTION OF REACTANTS WITH IMP DEHYDROGENASE

Substrate	Kinetic constant	Apparent value (mM)	True value (mM)	Weighted mean (mM)
K^+ (M)	K_{im}	25.3 ± 1.3 [a]	25.3 ± 1.3	25.3 ± 1.3
IMP (A)	K_{ia}	0.038 ± 0.004 [b]	0.038 ± 0.004	0.034 ± 0.002
		0.033 ± 0.002 [b]	0.033 ± 0.002	
	K_a	0.063 ± 0.004 [c]	0.063 ± 0.004	0.060 ± 0.003
		0.049 ± 0.005 [d]	0.063 ± 0.007	
		0.039 ± 0.003 [d]	0.055 ± 0.005	
NAD (B)	K_{ib}	0.61 ± 0.06 [e]	0.49 ± 0.05	0.45 ± 0.04
		1.08 ± 0.20 [e]	0.48 ± 0.09	
		1.89 ± 0.47 [e]	0.31 ± 0.08	
	K_b	1.08 ± 0.04 [e]	0.86 ± 0.03	0.81 ± 0.02
		2.00 ± 0.17 [e]	0.88 ± 0.08	
		4.25 ± 0.64 [e]	0.70 ± 0.11	
		0.69 ± 0.05 [e]	0.71 ± 0.05	
		0.76 ± 0.07 [e]	0.80 ± 0.07	
		0.62 ± 0.07 [e]	0.75 ± 0.09	

[a] Weighted mean of K_{im} values given in Table I.
[b] Each value represents the weighted mean of those obtained at different fixed concentrations of B or M (Table I).
[c] Weighted mean of the three values for K_a obtained at different fixed concentrations of M (Table I).
[d] Values calculated using the relationship: K_a = app. $K_a[K_b + B]/[K_{ib} + B]$ as well as K_b and K_{ib} values of 0.85 ± 0.03 mM and 0.45 ± 0.04 mM, respectively. The latter two values were determined using the relationships: K_b = app $K_b \cdot M/(K_{im} + M)$ and K_{ib} = app $K_{ib} \cdot M/(K_{im} + M)$.
[e] Values taken from Table I.

K^+, or NH_4^+. The values obtained for the kinetic parameters in the presence of NH_4^+ were as follows: K_a = 0.080 ± 0.008 mM, K_{ia} = 0.025 ± 0.004 mM, K_b = 1.08 ± 0.07 mM, K_{ib} = 0.33 ± 0.08 mM, K_{im} = 13.6 ± 0.8 mM. Comparison of these values with those given in Table IV indicates that the nature of the monovalent cation has little effect on the kinetic constants associated with the substrates. However, NH_4^+ binds to the enzyme more strongly than does K^+.

The inhibition patterns expected for analogues of each of the three reactants in the mechanism represented by Scheme 1 are shown in Table V. The results illustrated in Figs. 5, 6 and 8 are qualitatively in accord with the predicted patterns with one exception, i.e., the inhibition by nicotonic acid with respect to K^+ is observed to be non-competitive rather than uncompetitive (Fig. 6c). The latter result could be accounted for on the assumption that nicotinic acid can react not only with the E · K and E · K · IMP complexes but also with E and/or the E · IMP complex. Quantitatively the results are more consistent with the additional combination of nicotonic acid with E · IMP or E and E · IMP than with E alone. The dissociation constant for the interaction of nicotinic acid with the enzyme · K · IMP complex comes directly from the K_i intercept of Fig. 7. This value of 89 ± 11 is in good agreement with that of 88 ± 8 mM calculated for the same constant from the data of Fig. 6 (cf. Table III). In this connection it should be noted that NAD causes substrate inhibition at higher concentrations, probably by combining with the E and/or EA complexes. The finding that Li^+ acts as a competitive inhibitor with respect to both K^+ and

TABLE V

INHIBITION PATTERNS FOR THE MECHANISM IN SCHEME I

C, NC and UC denote competitive, noncompetitive and uncompetitive inhibition, respectively.

Inhibitor	Substrate for which inhibitor is an analogue	Type of inhibitor		
		Varied substrate		
		K^+	IMP	NAD
GMP	IMP	NC	C	NC
Li^+	K^+	C	NC	C
Nicotonic acid	NAD	UC	NC	C

NAD offers good support for the idea that the combination of K^+ and NAD conforms to a rapid equilibrium ordered sequence. The competitive inhibition by Li^+ with respect to NAD also shows that NAD does not combine with an enzyme · Li complex as it does with an enzyme · K complex. The true K_i values associated with the inhibition of the reaction by GMP and Li^+ could be calculated from different sets of data. The quantitative consistency demonstrated throughout Table VI supports the conclusions concerning the reaction mechanism.

For a truly rapid equilibrium random mechanism, inhibition by products occurs only because they act as inhibitory analogues of the substrate and thus give rise to dead-end complexes. The observed inhibition patterns with XMP (Fig. 4) and the results obtained by analysis of the inhibition data (Table II) are in accord with the idea that XMP (Q), like GMP, acts as an inhibitory analogue of IMP to form dead-end E · XMP (EQ), E · K · XMP (EMQ) and E · K · NAD · XMP (EMBQ) complexes and that only the presence of NAD (B) on the enzyme affects its combination. The data obtained with $NADH_2$ were not as expected for a rapid equilibrium random mechanism because the inhibition patterns were all linear noncompetitive, rather than linear competitive with respect to NAD and linear uncompetitive with respect to K^+. Because of the structure similarity between the substrates, it might be considered that the noncompetitive inhibitions could be due to dead-end combinations of $NADH_2$ with the E · K · NAD complex at the IMP site and the E · IMP complex at the NAD site. However, this explanation is ruled out since intercept variation is observed with a double reciprocal plot of data obtained by varying IMP and NAD in constant ratio in the presence of different fixed concentrations of $NADH_2$. Such a finding might be due to the presence of a noncompetitive inhibitor in the preparation of the pyridine nucleotide and in this connection it should be noted that the concentrations of $NADH_2$ required for the inhibition of IMP dehydrogenase are considerably higher than those required to inhibit other dehydrogenases [10,11]. However, the results obtained with $NADH_2$ are also consistent with the postulate that there is a rapid release of $NADH_2$ from the quaternary enzyme · K · XMP · $NADH_2$ complex and that the dissociation of XMP from the resulting ternary complex is not rapid compared with the interconversion of the quaternary complexes. Thus there would be a significant steady-state concentration of E · K · XMP. $NADH_2$ would then give rise to non-competitive inhibition with respect to each reactant by virtue of its ability to combine with the E · K · XMP complex and to reverse steps in the reaction sequence. The poor inhibition by this nucleotide would suggest that the $NADH_2$ is bound weakly to the enzyme · K · XMP complex or that the steady-state concentration of the complex is low.

The results of the analysis of the steady-state velocity and dead-end inhibition data certainly offer good support for the idea that, in the absence of added $NADH_2$, the steady-state concentrations of enzyme-product complexes are sufficiently low that, for all practical purposes, the interconversions of central complexes can be regarded as the rate limiting step. The competitive inhibition by XMP with respect to IMP indicates that, in the event of random release of products, the steady-state concentration of enzyme · K · $NADH_2$ must be negligible as otherwise the inhibition would be noncompetitive. The relative magnitude of rates for the interconversion of central complexes and product release steps will have a marked effect on the rates of isotope exchange at chemical equilibrium. The results of such studies on this system are reported in the following paper [12].

From Scheme 1 it is apparent that at concentrations of K^+ which are high relative to its K_{im} value, the mechanism reduces to one involving the random addition of IMP and NAD, rather than to an ordered mechanism with IMP adding before NAD and XMP dissociating after $NADH_2$ (cf. 2). When the inhibition data obtained with XMP and $NADH_2$, in the presence of 100 mM K^+, were analysed on the assumption that the reaction conformed to an ordered mechanism, discrepancies were found for the inhibition constants associated with XMP but not for those associated with $NADH_2$. The latter result may be simply fortuitous in view of the other evidence which indicates that there is a random addition of IMP and NAD.

Most dehydrogenases have an ordered reaction mechanism with NAD as the first substrate to add and $NADH_2$ as the last product to be released. But IMP and isocitrate [13] dehydrogenases are different in that they combine with their reactants in a random manner. IMP dehydrogenase is also not typical in having a relatively high K_m for NAD. The requirement of IMP dehydrogenase for a monovalent cation is again unusual, although glycerol dehydrogenase from *A. aerogenes* [14] also has the requirement.

This is an unusual dehydrogenase reaction in that it involves the addition of oxygen at carbon atom 2 of XMP. The source of the oxygen atom must be water, because in the present work it has been observed that the rate of the reaction is the same whether it is carried out aerobically or under nitrogen in solutions which have been deoxygenated by passing oxygen-free nitrogen through them. The role of water as a reactant is usually not considered by kineticists because of the difficulty in controlling its concentration in order to elucidate its function. Nevertheless, Brox and Hampton [2] have commented on some possible kinetic effects of the addition of an oxygen source in their proposed mechanism, and Hampton [15] has proposed a chemical mechanism for the reaction in which water would react after IMP. Some further comments can be made in relation to the mechanism in Scheme 1, assuming rapid equilibrium conditions. Thus, if water forms a Michaelis-type complex with the enzyme and is effectively saturating it must add before K^+ and NAD; if it added between them the steady state velocity plots in Fig. 1 (c,d) would be parallel, and if it added after them the steady state velocity would not be a function of the concentrations of K^+ and NAD. Even if at unsaturating concentrations water and IMP could add randomly, when water is saturating it will add before IMP. On the other hand, if water is non-saturating it could be adding at any point in the reaction sequence. A third possibility is that water reacts from solution, without affecting the form of Eq. 1.

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