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STUDIES ON INOSINE MONOPHOSPHATE DEHYDROGENASE. ISOTOPE EXCHANGE AT EQUILIBRIUM

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Summary

Investigations on the mechanism of the IMP dehydrogenase (IMP: NAD^+ oxidoreductase, EC 1.2.1.14) reaction have been made at pH 7.0 by measuring rates of isotope exchange at chemical equilibrium with K^+ maintained at a constant concentration. The results are generally in accord with the conclusions reached on the basis of the steady-state kinetic data obtained previously and confirm that there is random addition of IMP and NAD to the enzyme. The data also indicate clearly that at pH 7.0 catalysis is faster than the rate of IMP and/or XMP release which is rate limiting for the reaction sequence. The binding of IMP to the enzyme at pH 8.1 has been demonstrated to occur in the absence of both K^+ and NAD and is independent of the K^+ concentration.

Introduction

Steady-state kinetic studies on the reaction catalysed by IMP dehydrogenase (IMP: NAD^+ oxidoreductase, EC 1.2.1.14) [1] have indicated that the mechanism is a partly random one in which IMP and K^+ add randomly to the enzyme while NAD reacts only with the enzyme · K and the enzyme · K · NAD complexes. The aim of the present study was to obtain further information about the reaction mechanism using the technique of isotope exchange at equilibrium under conditions where K^+ was present at a relatively high concentration. The investigations were made at pH 7.0 because of the unfavourable equilibrium of the reaction at pH 8.1. The results show that there is random addition of IMP and NAD to the enzyme, that catalysis is faster than reactant release and that exchange is limited by the rate of IMP and/or XMP release.

Materials

[8-^{14}C]IMP (33 Ci/mol) was purchased from Schwarz Bioresearch, and [2-8^{3}H]NAD (3.46 Ci/mmol) from New England Nuclear. [*Adenine*-U-^{14}C]-

NAD (136 Ci/mol) was obtained from the Radiochemical Centre, Amersham. The latter compound was purified as described in Methods. Imidazole came from Fluka AG (purum), acetic anhydride from British Drug Houses (analytical reagent grade), and ammonium bicarbonate and formic acid from Ajax Chemicals Ltd., Sydney, Australia. DEAE cellulose paper was Whatman DE81. The preparation of IMP dehydrogenase and the source of all other chemicals have been described previously [1,2].

Methods

Chromatographic separation of IMP from XMP and of NAD from $NADH_2$ was achieved by ascending chromatography on DEAE-cellulose paper by the method of Morrison [3], using 0.2 M formic acid adjusted to pH 4.4 with NH_4OH and 0.1 M ammonium bicarbonate (pH 8.3), respectively, as the developing solvents.

For the isotope-exchange experiments, reaction mixtures contained, in a total volume of 0.25 ml, 0.1 M imidazole-acetate buffer (pH 7.0), 100 mM KCl, 5 mM dithiothreitol, varying equilibrium concentrations of reactants and [^{14}C]IMP (40 000 cpm) or [^{14}C]NAD (10—20 000 cpm). The reactions, at 37°C, were initiated by the addition of enzyme (4.5 μg) and stopped at various time intervals by delivering a sample of the reaction mixture (35 μl) by syringe onto DEAE-cellulose paper. The spot to which the sample was applied had been soaked just beforehand with 8 M urea (35 μl). This procedure was adopted to obtain immediate inactivation of the enzyme (cf. ref. 3). Inactivation could also be achieved by applying samples to spots previously impregnated with urea and dried or by the addition of urea after application of the samples. The presence of urea affected the development of the chromatogram only in that the size of the pyridine nucleotide spots was increased. The addition of an equal volume of ethanol was not effective in stopping the reaction. Velocities were determined from the slope of a plot of counts appearing in the appropriate product against time and the specific radioactivity of the substrate.

The [^{14}C]NAD was markedly impure as judged by the fact that on chromatography under the aforementioned conditions, radioactivity was present from the origin up to the spot of NAD which runs ahead of $NADH_2$. The material also contained an inhibitor of the enzyme since the observed velocity decreased as the concentration of labelled NAD was increased. Purification of [^{14}C]NAD was carried out by the same procedure as used to separate NAD and $NADH_2$. After development, the chromatogram was dried in a stream of air at room temperature to remove completely the ammonium bicarbonate, eluted with water and stored at −15°C after the addition of an equal volume of ethanol. Chromatography of samples of the purified product showed that about 10% of the radioactivity was present in the same position as $NADH_2$ and that approx. 10% of the [^{14}C]NAD was not converted to [^{14}C]$NADH_2$ following incubation with excess IMP and IMP dehydrogenase. Allowance was made for the 80% purity of the [^{14}C]NAD in all calculations of the exchange rates. Because the purified [^{14}C]NAD gave rise to inhibition of the NAD-$NADH_2$ exchange, these reactions were run with the lowest satisfactory level of labelled nucleotide. For comparisons of the NAD-$NADH_2$ and IMP-XMP exchange

rates, the former exchange was measured at three different levels of label (and inhibition) and the uninhibited velocity determined by extrapolation of [^{14}C]-NAD to zero concentration. The IMP-XMP exchange rate was independent of the amount of [^{14}C] IMP added.

The occurrence on chromatograms of counts from the [^{14}C]NAD preparation in the $NADH_2$ position may be linked to the observation that although spots containing NAD were ultraviolet-light absorbing, but not fluorescent, one hour after separation, they were fluorescent 24 h later. To minimize the possible occurrence of this effect before separation of NAD and $NADH_2$, chromatograms were developed within 1 h of sample application.

Analysis of data

Velocities were first plotted graphically in double reciprocal form, and then fitted to the appropriate rate equation by using the computer programs of Cleland [4]. The data were fitted to Eqn. 1 if double reciprocal plots were linear or Eq. 2 if substrate inhibition was observed. The parameters so determined have been used to draw the lines in the figures.

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

$$v = \frac{VA}{K + A + A^2/K_i} \tag{2}$$

Binding studies

The binding of IMP to IMP dehydrogenase was measured at pH 8.1 and 20°C in the presence of 0.1 M Tris · HCl buffer and 5 mM dithiothreitol. The enzyme concentration was 180 μg per ml and the total volume was 0.2 ml. The ultrafiltration method of Paulus [5] was used in conjunction with Visking, instead of UM10 Diaflo, membranes [6]. Counting of enzyme-ligand complex deposited on the membranes was performed in a Packard Tri-Carb liquid scintillation spectrometer, using Triton scintillant. Blank values (obtained in the absence of enzyme) did not vary with the concentration of IMP or KCl, and corresponded to less than 1 μl of solution.

Results

Determination of the equilibrium constant for the IMP dehydrogenase reaction

At pH 8.1, the optimum pH for the IMP dehydrogenase reaction and the pH at which steady state kinetic investigations was carried out [1,7], the position of the equilibrium lies so far towards product formation that it is not feasible to determine an equilibrium constant or to study isotope exchanges under equilibrium conditions. But, at pH 7.0, the equilibrium is more favourable for the latter study. The equilibrium constant was determined in reaction mixtures initially containing 0.15 mM NAD, 0.15 mM IMP and either 0.45 mM or 0.90 mM XMP, by measuring the concentration of $NADH_2$ present after the reaction had reached equilibrium. The two estimates of K_{eq} were in agreement, giving a mean value of $14.5 \cdot 10^{-7}$ M.

Isotope exchange rates under various conditions

The effect on the rates of the IMP-XMP and NAD-$NADH_2$ exchanges of raising in constant ratio the equilibrium concentration of IMP and XMP is shown in Fig. 1. It is apparent that the IMP-XMP exchange initially increases and then decreases as the concentration of IMP/XMP is raised. By contrast there is no clearly defined inhibition of NAD-$NADH_2$ exchange at higher levels of IMP/XMP. When the equilibrium concentrations of NAD/$NADH_2$ are raised in constant ratio (Fig. 2) again there is a fall off in the rate of the IMP-XMP exchange while no inhibition of the NAD-$NADH_2$ exchange is observed. The maximum rate of the latter exchange is considerably greater than that of the IMP-XMP exchange (Table I). When the equilibrium concentrations of IMP, XMP, NAD and $NADH_2$ are raised in constant ratio, measurement of the IMP-XMP exchange indicates that there is no decrease in the rate at relatively high concentrations of the reactants (Fig. 3). Measurement of both exchanges under three sets of equilibrium conditions (Table II) shows that, at the lowest reactant concentrations, the rate of the two exchanges is of a similar magnitude. However, the NAD-$NADH_2$ exchange increases much more rapidly than does the IMP-XMP exchange as the concentration of reactants is increased.

The effect on the IMP-XMP exchange of raising the concentrations of unlike substrate-product pairs (IMP and $NADH_2$ or XMP and NAD) is illustrated in Figs. 4 and 5. Both sets of data give good fits to Equation (2) and thus it may be concluded that the dead-end complexes enzyme · (K) · IMP · $NADH_2$ and enzyme · (K) · XMP · NAD are formed.

Steady state velocity pattern at pH 7.0

To determine if the steady state velocity pattern at pH 7.0 was equilibrium

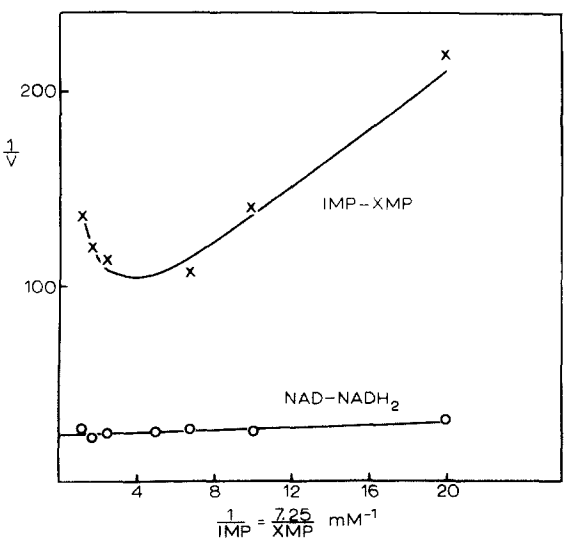

Fig. 1. Effect of increasing the concentration of the IMP/XMP pair on the initial velocity of the IMP-XMP (X - - - - - - X) and NAD-$NADH_2$ (○ - - - - - - ○) exchanges. The fixed concentrations of NAD and $NADH_2$ were 0.5 mM and 1.0 mM, respectively. The data for the NAD-$NADH_2$ and IMP-XMP exchanges were fitted to Eqns. 1 and 2, respectively. Velocity is expressed as μmol of XMP or $NADH_2$ formed per min per mg of enzyme.

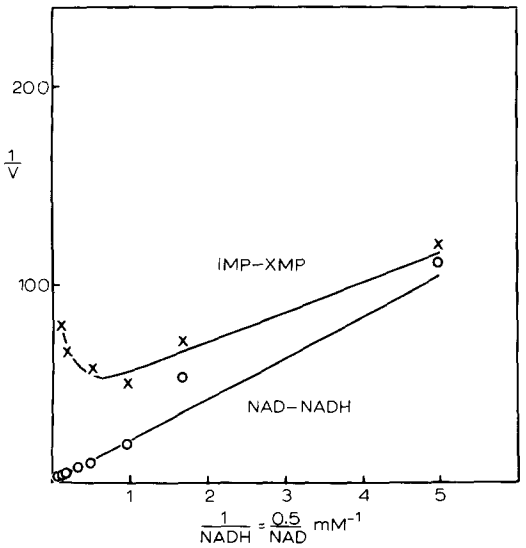

Fig. 2. Effect of increasing the concentration of the NAD/$NADH_2$ pair on the initial velocity of the IMP-XMP (X - - - - - - X) and NAD-$NADH_2$ (○ - - - - - - ○) exchanges. The fixed concentrations of IMP and XMP were 0.6 mM and 4.35 mM, respectively. The data for the NAD-$NADH_2$ and IMP-XMP exhanges were fitted to Eqns. 1 and 2 respectively. Velocity is expressed as μmol of XMP or $NADH_2$ formed per min per mg of enzyme.

ordered, as it is at pH 8.1 (1), the velocity was determined as a function of the concentrations of K^+ and NAD. The results (Fig. 6) show that a common vertical intercept is not obtained, in contrast to the result at pH 8.1 (1).

Binding studies

IMP dehydrogenase was retained completely by Visking membranes and was

TABLE I

COMPARISON OF THE APPARENT KINETIC PARAMETERS FOR THE IMP DEHYDROGENASE REACTION

The values were determined by fitting the data of Figs. 1 and 2 to either Eqn. 1 or 2 according to whether the plot was linear or non-linear. V_m represents the maximum exchange velocity that would be obtained at infinite concentrations of the variable reactants in the absence of substrate inhibition and is expressed as μmol per min per mg of protein. K values are Michaelis constants and expressed as mM.

Variable reactants	Concentrations of fixed reactants (mM)	Kinetic parameter	Value of kinetic parameter	
			Exchange reaction	
			NAD-$NADH_2$	IMP-XMP
NAD/$NADH_2$	IMP, 0.6 XMP, 4.35	V_m	0.85 ± 0.14	0.026 ± 0.006
		K_{NAD}	8.9 ± 2.5	0.41 ± 0.22
		K_{NADH_2}	17.8 ± 5.0	0.82 ± 0.44
IMP/XMP	NAD, 0.5 $NADH_2$, 1.0	V_m	0.041 ± 0.001	0.021 ± 0.007
		K_{IMP}	0.012 ± 0.006	0.17 ± 0.08
		K_{XMP}	0.087 ± 0.043	1.23 ± 0.58

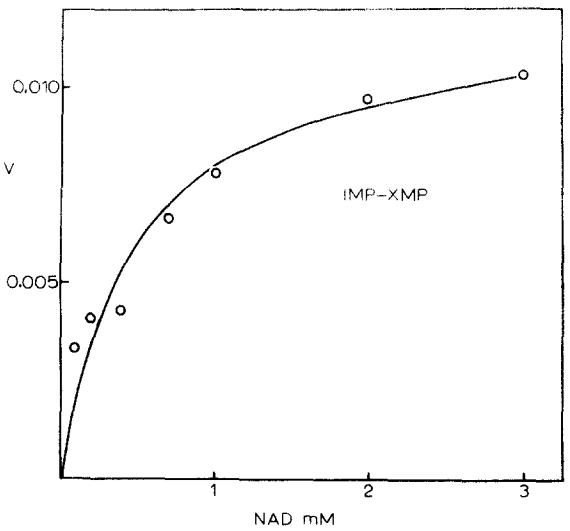

Fig. 3. Effect on the initial velocity of the IMP-XMP exchange of increasing in constant ratio the equilibrium concentrations of IMP, XMP, NAD and $NADH_2$. [NAD = IMP] : [$NADH_2$ = XMP] = 3.8. The data were fitted to Eqn. 1. Velocity is expressed as μmol of XMP formed per min per mg of enzyme.

not inactivated by standing at room temperature (20°C) for the duration of the experiment or on being subjected to a pressure of 30 lb/inch^2. The amount of IMP bound was proportional to the enzyme concentration up to a concentration twice that used in the binding experiments. However, at higher levels this relationship did not hold. The binding of IMP to the enzyme at 20°C in the absence and presence of K^+ is illustrated in Fig. 7. It will be noted that the binding of IMP to the enzyme is not affected significantly by K^+. At a fixed concentration of IMP (0.12 mM) the amount bound to the enzyme was not significantly different at 20 and 37°C.

Studies on the binding of [*adenine*-^{14}C]NAD to the enzyme showed that in the presence of 0.1 M Tris · HCl (pH 8.1), 5 mM dithiothreitol and 0.2 mM NAD, 0.8 μmol of NAD per g of IMP dehydrogenase was bound. The observed binding was not affected by K^+, although it was increased slightly in the presence of IMP. The binding of label from [2,8-^{3}H]NAD was found to correspond to 2.6 μmol per g of enzyme, apparently three times as much as with [*adenine*-U-^{14}C]NAD. The difference between results using the two samples

TABLE II

EQUILIBRIUM EXCHANGE RATES FOR THE IMP DEHYDROGENASE REACTION

Concentration of reactants (mM)				Exchange rate (μmol/min/mg enzyme)	
[IMP]	[XMP]	[NAD]	[$NADH_2$]	IMP-XMP	NAD-$NADH_2$
0.1	0.38	0.1	0.38	0.008	0.014
0.2	1.45	0.5	1.0	0.011	0.042
2.0	7.6	2.0	7.6	0.015	0.193

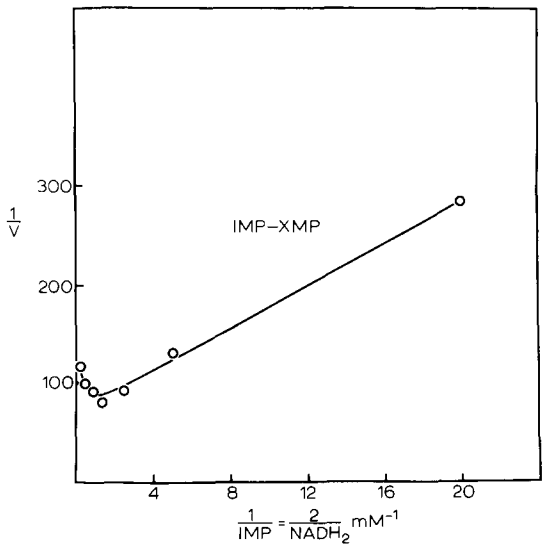

Fig. 4. Effect of increasing the concentration of the IMP/$NADH_2$ pair on the initial velocity of the IMP-XMP exchange. The concentrations of NAD and XMP were fixed at 0.2 mM and 1.45 mM, respectively. The data were fitted to Eqn. 3. Velocity is expressed as μmol of XMP formed per min per mg of enzyme.

of labelled NAD indicates that labelled contaminants could be responsible for the observed binding of label to the enzyme especially as the preparation of [*adenine*-U-^{14}C]NAD contains an inhibitor of the enzyme (see Methods). The weak binding of NAD to the enzyme precluded studies over a range of nucleotide concentrations and the use of other binding techniques such as that of Colowick and Womack [8].

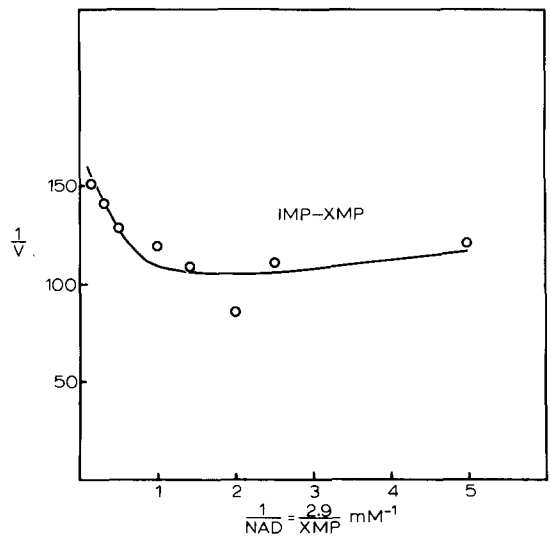

Fig. 5. Effect of increasing the concentration of the XMP/NAD pair on the initial velocity of the IMP-XMP exchange. The concentrations of IMP and $NADH_2$ were fixed at 0.2 mM and 1.0 mM, respectively. The data were fitted to Eqn. 2. Velocity is expressed as μmol of XMP formed per min per mg of protein.

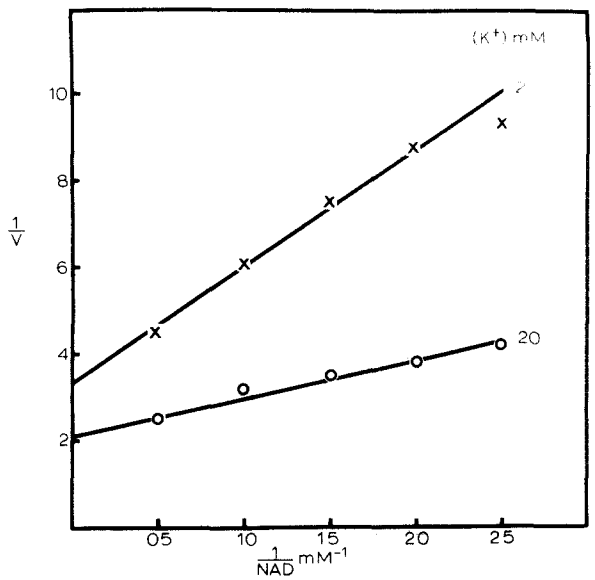

Fig. 6. Steady state velocity pattern at pH 7.0. The concentration of IMP was fixed at 1 mM, and the data were fitted to Eqn. 7 of ref. 4. Velocities were measured as described in ref. 1, except that the buffer was imidazole/acetate at pH 7.0 instead of Tris · HCl at pH 8.1.

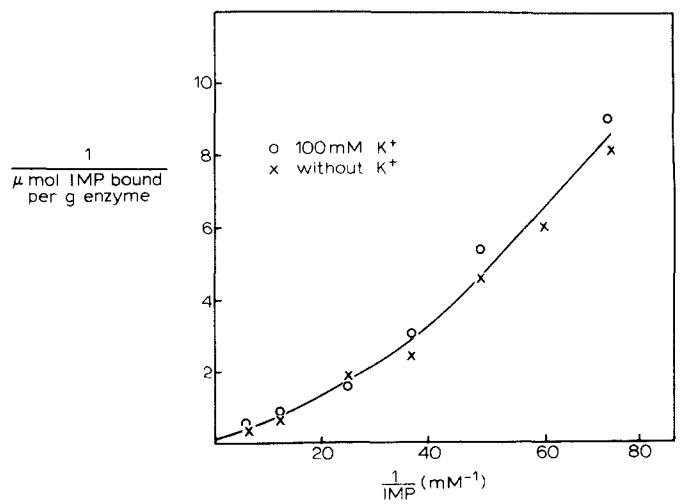

Fig. 7. Effect of K^+ on the binding of IMP to IMP dehydrogenase at pH 8.1. The concentration of K^+ was zero, X ------ X; 100 mM ○ ------ ○.

Discussion

The steady-state kinetic investigations of the IMP dehydrogenase reaction at pH 8.1 indicated that IMP binds to the enzyme in the absence of K^+ and NAD and that the presence of K^+ on the enzyme does not affect the binding of IMP [1]. These conclusions are confirmed by the binding data which were obtained at pH 8.1 and which show that the binding curves for IMP are not significantly different in the absence and presence of K^+. In contrast to the linear double

reciprocal plots obtained with IMP as a variable substrate [1], the plot for the binding of IMP to the enzyme (Fig. 6) is not linear but appears to be a non-rectangular hyperbola. This might suggest that there is interdependent combination of multiple molecules of IMP with the enzyme. However, in view of the kinetic data [1], it seems more likely that the results (Fig. 6) arise as a consequence of the use of the Paulus ultrafiltration technique with an enzyme that undergoes polymerization [2,9]. For the binding experiments it was necessary to use IMP dehydrogenase at an initial concentration which is 10-fold greater than that for the kinetic studies and as filtration proceeds the enzyme concentration would increase further. Thus the higher polymeric forms would be favoured, and if the various enzyme species with different molecular weights were to exhibit different binding parameters, a curve with a form similar to that shown in Fig. 6 could be obtained. The non-linearity of the binding curve precluded determination of the dissociation constant for the enzyme · IMP complex.

For the isotope exchange studies the reaction was simplified by considering that, in the presence of a fixed concentration of KCl, it could be treated as one involving two substrates and two products. A comparison of the IMP-XMP and NAD-$NADH_2$ exchanges (Table II) shows that at lower concentrations of the reactants, the rates are similar. However, the NAD-$NADH_2$ exchange rate increases much more markedly than the IMP-XMP exchange rate as a function of the equilibrium concentrations of the reactants so that the two rates can differ by an order of magnitude. The inequality of the exchange rates and the fall off in the IMP-XMP exchange at higher concentrations of IMP/XMP (Fig. 1) or NAD/$NADH_2$ (Fig. 2) indicates that, at pH 7.0, the reaction does not conform to a rapid equilibrium, random mechanism. The possibility that the reaction has an ordered or Theorell-Chance mechanism is not consistent with the results of Fig. 2. The fitting of the data for the NAD-$NADH_2$ and IMP-XMP exchanges to Eqns. 1 and 2, respectively yields theoretical curves whose asymptotes will intersect when extrapolated to lower concentrations of NAD/$NADH_2$. In an ordered mechanism the exchange rate between the outer reactant pair can never become greater than that between the inner reactant pair. The failure to obtain inhibition of the IMP-XMP exchange as the concentrations of all reactants are raised in constant ratio (Fig. 3) is also inconsistent with the reaction proceeding via an ordered mechanism [10,11].

The results of Fig. 2 suggest that the NAD-$NADH_2$ exchange could be slower than the IMP-XMP exchange at sufficiently low concentrations of NAD/$NADH_2$ and hence that the reaction conforms to a random mechanism in which the rate of reactant release is slower than catalysis [12]. This suggestion is supported by the analytical data (Table I) which indicate that the apparent K_m values for NAD and $NADH_2$ (IMP and XMP) are higher when the NAD-$NADH_2$ (IMP-XMP) exchange rate is measured than when the IMP-XMP (NAD-$NADH_2$) exchange is determined. These relationships can be considered in a manner similar to that described by Cleland for data on NADP-isocitrate dehydrogenase [12]. Thus, for the NAD-$NADH_2$ exchange where these reactants are fixed (Fig. 1) only enough IMP and XMP need be present to keep catalysis faster than reactant release, and the apparent K_m values for IMP and XMP are below the levels that would convert half of the enzyme into central complexes.

On the other hand, for the IMP-XMP exchange (Fig. 1) it is necessary to keep the level of complexes containing IMP and XMP high enough to support rapid exchange rates and thus a higher apparent K_m value is observed. It will be noted (Table I) that the NAD-$NADH_2$ exchange is markedly faster than the IMP-XMP exchange at concentrations of IMP and XMP which are probably high relative to their K_m values. (Values have been determined only at pH 8.1 [11]). Thus it appears that the exchange velocities are limited by the rate of IMP-XMP release. At fixed concentrations of NAD/$NADH_2$, the NAD-$NADH_2$ exchange is only twice that of IMP-XMP (Fig. 1) and this result suggests that the concentrations of the pyridine nucleotides are relatively low so that catalysis and IMP/XMP release occur at comparable rates.

The fall-off at higher concentrations of NAD/$NADH_2$ of the rate of the IMP-XMP, but not of the NAD-$NADH_2$ exchange (Fig. 2), is in accord with the results expected if NAD/$NADH_2$ were the inner reactant pair in an ordered mechanism. However, for the reasons outlined earlier, this simple explanation cannot apply. Data for the IMP-XMP exchanges (Figs. 1 and 2) gave good fits to Equation 2 and thus it might be concluded that higher concentrations of IMP/XMP cause substrate inhibition by displacing NAD/$NADH_2$ from their binding site (Fig. 1) and vice versa (Fig. 2). (In this connection it should be mentioned that this type of inhibition would not be observed when all reactants are raised in constant ratio (cf. Fig. 3)). If inhibition occurred by this mechanism there should also be a fall-off in the NAD-$NADH_2$ exchange rates. Such inhibitions are not obvious and this could be due to the relatively low degree of inhibition and the relatively high magnitude of the NAD-$NADH_2$ exchange. A possible alternative explanation for the results of Fig. 2 is that the fit to Eqn. 2 is fortuitous and the curves for IMP/XMP exchange should cut the vertical ordinate at a finite, rather than at an infinite, value. Such a result would mean that, at infinite concentrations of NAD/$NADH_2$, IMP/XMP are released only from the ternary complexes and at a limiting maximum rate which is lower than their maximum rate of release from binary complexes.

The data of Figs. 4 and 5 could be taken as confirming that the dead-end enzyme complexes which form at pH 8.1 [1] and which involve NAD and XMP, as well as $NADH_2$ and IMP, are also formed at pH 7.0. However, because the IMP-XMP exchange decreases as the concentrations of IMP/XMP or NAD/$NADH_2$ are increased (Figs. 1 and 2) the result cannot be regarded as definitive.

There is broad agreement between the conclusions which may be reached about the mechanism of the IMP dehydrogenase reaction from data obtained by steady-state kinetic investigations at pH 8.1 [1] and isotope exchange studies at pH 7.0. Both sets of results indicate that the reaction conforms to a random mechanism. When the steady-state and dead-end inhibition data obtained at pH 8.1 were analysed on the basis that the IMP dehydrogenase reaction has a rapid equilibrium, random mechanism, the results [1] indicated that the reaction could be described as conforming to this mechanism. However, the product inhibition pattern revealed that the concentration of the enzyme · (K) · XMP complex is significant and hence that the release of XMP is also a slow step. The precise quantitative relationship between the rates of catalysis and XMP release is not known. At pH 7.0, however, it is clear that product release

is slow relative to catalysis. In theory, a reaction having a random mechanism should exhibit non-linear double reciprocal plots of the variation of initial velocity as a function of substrate concentration. Such non-linearity was not obvious with data obtained at pH 7.0 by measuring the steady-state velocity as a function of NAD concentration at different fixed concentrations of K^+. (Fig. 6). Further, the data gave a symmetrical intersecting steady-state velocity pattern (Fig. 6) in contrast to the asymmetric (equilibrium-ordered) pattern obtained at pH 8.1 (Fig. 1C of ref. 1) and the K_m for K^+ was reduced from 25 mM at pH 8.1 to an apparent value of 1.3 mM at pH 7.0 in the presence of 1.0 mM IMP. The symmetrical intersecting pattern could be due either to the random addition of K^+ and NAD to the enzyme or to an ordered addition in which the reaction of K^+ is no longer in thermodynamic equilibrium and in which it dissociates during each catalytic cycle. The variation with pH of the detailed mechanism for the IMP dehydrogenase reaction and the similarity of the kinetic data obtained with this enzyme and isocitrate dehydrogenase [12] are two points of particular interest.

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