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The Inhibition of Pig Kidney Alkaline Phosphatase by Oxidized or Reduced Nicotinamide–Adenine Dinucleotide and Related Compounds

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1. The inhibition of alkaline phosphatase by NAD^+, NADH, adenosine and nicotinamide was studied. 2. All of these substances except NAD^+ act as uncompetitive inhibitors, i.e. double-reciprocal plots are parallel. NAD^+, however, is a 'mixed' inhibitor of alkaline phosphatase and is less potent than NADH. 3. Inhibition studies with pairs of the inhibitors suggest that, in spite of the difference in type of inhibition, NAD^+ and NADH bind to alkaline phosphatase at a common site. Adenosine and nicotinamide also seem to bind at the NAD site and the binding of adenosine is facilitated by nicotinamide, and vice versa. 4. The facilitation may indicate the occurrence of an induced fit for NAD^+ and NADH. Attempts to desensitize alkaline phosphatase to NAD^+ and NADH inhibition by partial denaturation were unsuccessful. 5. The results are discussed in terms of a two-site model in which separate, but interacting, regions exist on the enzyme to accommodate the adenosine and nicotinamide moieties of NAD, and a single-site model in which the adenosine part of the molecule is bound preferentially and this interacts with the nicotinamide fraction. 6. The activity of alkaline phosphatase can be changed fourfold by alteration of the NAD^+/NADH ratio. This sensitivity to the redox state of the coenzyme could be a means of controlling phosphatase activity.

In a study of the effects of a number of biological compounds on mammalian alkaline phosphatases (EC 3.1.3.1) Fishman *et al.* (1962, 1963) discovered that L-phenylalanine was inhibitory and was particularly so towards enzyme preparations obtained from intestine. Subsequently it was shown that placental alkaline phosphatase shares with intestinal enzyme the property of sensitivity towards L-phenylalanine (Ghosh & Fishman, 1966). The nature of this inhibition has been well studied in Fishman's laboratory (see Fishman & Ghosh, 1967, for a review) and the specificity towards intestinal and placental enzymes has been exploited by clinical biochemists to determine the proportion of plasma alkaline phosphatase originating from the intestine or placenta. More recently, L-tryptophan has been shown to be an inhibitor of placental alkaline phosphatase (Lin *et al.*, 1971) and a number of aromatic compounds resembling L-phenylalanine in chemical structure have been found to be inhibitory (Fishman & Sie, 1971).

Ghosh & Fishman (1968) examined the action of L-phenylalanine on alkaline phosphatase for allosteric effects, which might suggest a regulatory function for the amino acid. Co-operative interactions were not found, however, and the nature of the inhibition, which is uncompetitive, seems to be unusual among allosteric inhibitors. Because there is a lack of evidence for a direct link between amino acid absorption and alkaline phosphatase and because the effects of aromatic compounds appear to mimic those of L-phenylalanine to some extent, it was decided to investigate the actions of other biological compounds containing aromatic moieties.

The location of alkaline phosphatase at sites where transport is known to occur, e.g. the brush border of intestine and the proximal convoluted tubules of the kidney, and the adenosine triphosphatase activity of the enzyme (Eaton & Moss, 1967; Fernley & Walker, 1966; Haussler *et al.*, 1970; Norman *et al.*, 1970; Wass & Butterworth, 1971), led us to examine the effects of NAD^+ and NADH on kidney alkaline phosphatase. The $[NAD^+]/[NADH]$ ratio is believed to be linked to the cytoplasmic $[ATP]/[ADP][P_i]$ ratio because the high activities of 3-phosphoglycerate kinase (EC 2.7.2.3) and glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12) may maintain the reactants of the system near equilibrium *in vivo* (Veech *et al.*, 1970; Gumaa *et al.*, 1971).

$$\frac{[ATP]}{[ADP][P_i]} = \frac{[NAD^+]}{[NADH]} \times \frac{K\,[\text{Glyceraldehyde 3-phosphate}]}{[\text{3-Phosphoglycerate}]}$$

Thus changes in the $[NAD^+]/[NADH]$ couple may contribute to the control of the concentration of ATP in the cell and, in turn, may contribute to the control

of transport processes linked to a membrane-bound adenosine triphosphatase.

The present paper describes some experiments in which the effects of NAD^+ and NADH on pig kidney alkaline phosphatase were studied to see if direct control of the enzyme could be mediated by the redox state of the coenzyme. Both forms of NAD were found to be inhibitory, but differences in the nature and potency of the inhibition were noted.

Experimental

Materials

Nicotinamide, adenosine, NAD^+ and bovine serum albumin were obtained from the Sigma (London) Chemical Co. Ltd., London S.W.6, U.K., and NADH from P-L Biochemicals Inc., Milwaukee, Wis., U.S.A. The compounds were used without further purification. Disodium *p*-nitrophenyl phosphate and guanidine hydrochloride were obtained from British Drug Houses Ltd., Poole, Dorset, U.K. and A.R.-grade Tris was from Fisons Scientific Apparatus Ltd., Loughborough, Leics., U.K.

Enzyme procedures

Purification of alkaline phosphatase. Alkaline phosphatase was purified from pig kidney (Butterworth, 1968). The specific activity of the preparation was 25 μmol of *p*-nitrophenol released/min per mg. The enzyme, in 0.05M-Tris–HCl buffer, pH7.6, was stored at −15°C and diluted on the day of the experiment with a solution of bovine serum albumin (0.1% in water). The degree of dilution required to bring the enzymic activity within a suitable range for assay varied slightly from batch to batch but was usually in the ratio of one part of enzyme to three or four parts of albumin solution.

Assay of alkaline phosphatase. The increase in E_{400} as *p*-nitrophenol is released from *p*-nitrophenyl phosphate was monitored continuously in a Perkin–Elmer 124 double-beam spectrophotometer fitted with a thermostatically controlled multiple cell-holder and with a pen recorder. All assays were made at 30°C in 0.1M-Tris–HCl buffer, pH9.0, and reaction mixtures were of 3ml volume. Enzymic activity (v) was expressed as μmol of *p*-nitrophenol released/min per ml of diluted enzyme solution.

Effects of inhibitors. Stock solutions of the various inhibitors described in the Results section were freshly prepared in 0.1M-Tris–HCl buffer and the pH of the solution was readjusted to 9.0 if necessary. Suitable portions were included in reaction mixtures to give the concentrations indicated in the Results section. When adenosine was used the stock solution was maintained at 40–50°C to prevent crystallization. Solutions of NAD^+ were kept at 0°C between runs to minimize the temperature-dependent decomposition that occurs at pH9.0. Breakdown during the assays was assumed to be negligible (based on rates of decomposition given by Lowry *et al.*, 1961).

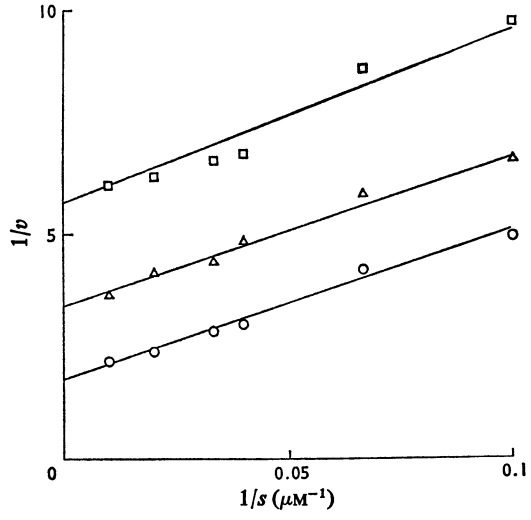


Fig. 1. *Double-reciprocal plots of NADH inhibition*

Concentration of NADH: ○, zero; △, 0.1mM; □, 0.25mM. Details of the conditions under which the inhibition was studied are given in the Experimental section.

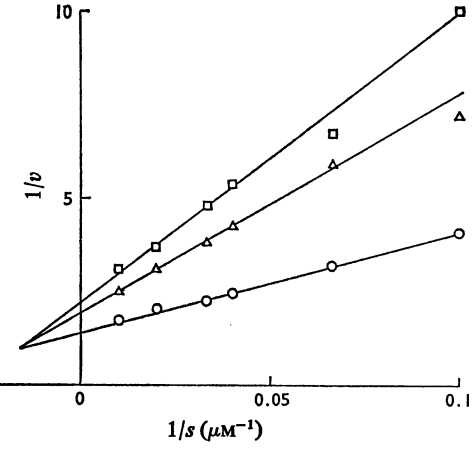


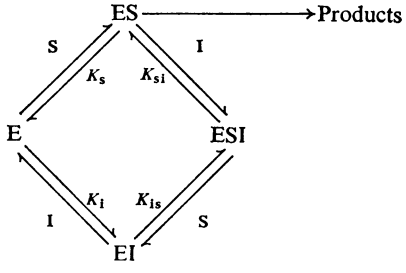
Fig. 2. *Double-reciprocal plots of NAD^+ inhibition*

Concentrations of NAD^+: ○, zero; △, 0.5mM; □, 1.0mM. See the Experimental section for other details.

Partial denaturation. Portions of the enzyme that had been diluted with the albumin solution were kept at 40°C for up to 60min. At various time-intervals the enzyme was assayed for residual activity and sensitivity to inhibition. In a similar experiment the enzyme was treated with 3M-guanidine–HCl for up to 90min before remeasurement of the activity and degree of inhibition.

Determination of kinetic parameters

Kinetic constants and maximum velocities were calculated by a statistical method (Wilkinson, 1961). The results are presented in double-reciprocal plots (Lineweaver & Burk, 1934).



Scheme 1. *Inhibition by a single inhibitor*

The equilibrium constants describing each of the steps are shown in the figure.

Results

Inhibition by NAD$^+$ and NADH

Both NAD$^+$ and NADH inhibit pig kidney alkaline phosphatase at pH9.0. In Figs. 1 and 2 are shown typical double-reciprocal plots. All such plots were linear and thus for calculation of apparent inhibition constants it was assumed that rapid equilibration of all enzyme–ligand complexes occurred (Frieden, 1964). The values of the kinetic constants indicated in Scheme 1 were obtained from

Table 1. *Summary of kinetic data obtained with pig kidney alkaline phosphatase*

Kinetic constants were calculated from experimental results obtained at 30°C and pH9.0, by assuming the mechanism shown in Scheme 1. K_s and K_{si} are respectively the equilibrium constants for the binding of substrate, *p*-nitrophenyl phosphate, with the enzyme and for the combination of inhibitor with the enzyme–substrate complex. K_i is the equilibrium constant for the binding of inhibitor to the free enzyme. The mean values ± S.D. are given and the number of observations is included in parentheses.

Constant	Mean value (mM)
K_s	0.018 ± 0.0042 (11)
NADH inhibition (K_{si})	0.16 ± 0.06 (10)
NAD$^+$ inhibition (K_s)	0.49 ± 0.11 (9)
(K_{si})	1.73 ± 0.41 (6)
Nicotinamide inhibition (K_{si})	17.4 ± 3.4 (12)
Adenosine inhibition (K_{si})	24.8 ± 9.6 (11)

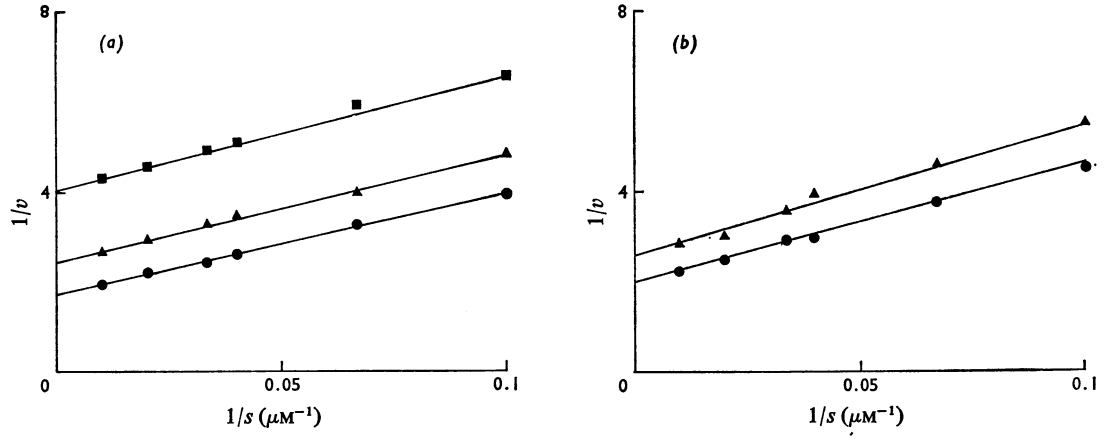


Fig. 3. *Double-reciprocal plots for inhibition by (a) nicotinamide and (b) adenosine*

The inhibitors were present at concentrations of zero (●), 10mM (▲) and 25mM (■). See the Experimental section for details of the conditions under which the studies were made.

the slopes and intercepts of linear plots. Values for the various constants are shown in Table 1, together with those obtained for the other substances that were tested as inhibitors.

For NADH, parallel straight lines indicated an uncompetitive type of inhibition as seen when L-phenylalanine acts on some alkaline phosphatases (Fishman & Ghosh, 1967). The convergent double-reciprocal plots for NAD^+ were indicative of a 'mixed' pattern of inhibition, however. This difference in the manner of inhibition is surprising in view of the close structural similarity of oxidized and reduced forms of the coenzyme. A comparison of the kinetic constants reveals that NADH is more firmly bound to alkaline phosphatase than is NAD^+.

Inhibition by nicotinamide and adenosine

In a study of the binding of NAD^+ to dogfish lactate dehydrogenase (EC 1.1.1.27) McPherson (1970) found that AMP was a competitive inhibitor. In addition, X-ray-crystallographic studies showed that AMP causes a conformational change in lactate dehydrogenase, allowing NMN to bind to the enzyme–AMP complex. As shown in Fig. 3, both nicotinamide and adenosine inhibit alkaline phosphatase so that an obligatory ordered binding of part of the NAD^+ molecule to facilitate the binding of the remainder, comparable with the actions of the dehydrogenase, seems unlikely at first sight. Nicotinamide and adenosine were both found to inhibit uncompetitively, but with some adenosine plots there was evidence of slight convergence. The pattern of inhibition was judged to be mainly uncompetitive, however, and other kinds of inhibition were not considered further. Thus it seems likely that the mode of binding of adenosine and nicotinamide to kidney alkaline phosphatase is similar to that which occurs with NADH. The inclusion of small amounts of nicotinamide in reaction mixtures enhanced the inhibitory effect of adenosine and vice versa. For example, a mixture of nicotinamide and adenosine which would have been expected to cause a 50% inhibition of alkaline phosphatase, was found to inhibit about 80% of the enzymic activity. This potentiation was studied further in the interaction experiments described below.

Interactions between molecules of different inhibitors

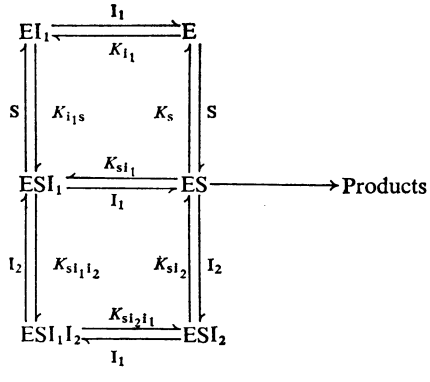
Because of the similarities in structure of NAD^+ and NADH it seemed reasonable to assume that they bind to alkaline phosphatase in a similar manner. The difference in the type of inhibition produced by these coenzyme forms, however, led us to an examination of the effects on the inhibition of the enzyme by one form in the presence of the other.

Scheme 2 shows a mechanism for the reaction of an enzyme with two inhibitors, I_1 and I_2, which are assumed to give mixed and uncompetitive behaviour respectively. Assuming a rapid equilibrium mechanism the rate equation is given by:

$$1/v = \frac{(1+K_s/s+[I_2]/K_{si_2})}{V} + \frac{[I_1](K_s/K_{i_1}s+1/K_{si_1}+[I_2]/K_{si_1}K_{si_1i_2})}{V}$$

where s is the substrate concentration and V is the maximum velocity.

Plots of $1/v$ against $[I_1]$ in the presence of I_2 are linear. At a constant substrate concentration, results obtained at various concentrations of I_2 intersect at a point where $[I_1]$ equals $-K_{si_1}K_{si_112}/K_{si_2}$, i.e. $-K_{si_2i_1}$. A comparison of the magnitude of $K_{si_2i_1}$ with K_{si_1} indicates whether the presence of I_2 facilitates or antagonizes the binding of I_1. Where an enzyme–substrate–I_1I_2 complex is unable to form, i.e. there is exclusive binding of one of the inhibitors, plots of $1/v$ against $[I_1]$ in the presence of various concentrations of I_2 are parallel. Equivalent experiments in which $1/v$ is plotted against $[I_2]$ with or without I_1 present give estimates of $K_{si_1i_2}$ which may then be compared with K_{si_2}.



Scheme 2. *Inhibition by two inhibitors*

In this mechanism it is assumed that the enzyme binds two inhibitors, I_1 and I_2. I_1 combines with both free enzyme and the enzyme–substrate complex, but I_2 is limited to combination with the complex only. When K_{i_1} is infinity both I_1 and I_2 are uncompetitive inhibitors.

When I_1 and I_2 bind only to the enzyme–substrate complex, i.e. both act as uncompetitive inhibitors, the appropriate rate equation is given by:

$$1/v = \frac{K_s}{V_s} + \frac{(1+[I_2]/K_{si_2})}{V} + \frac{[I_1](1/K_{si_1} + [I_2]/K_{si_1}K_{si_1i_2})}{V}$$

Thus exclusive binding is indicated by parallel plots of $1/v$ against $[I_1]$ and convergent plots intersect at $-K_{si_2i_1}$.

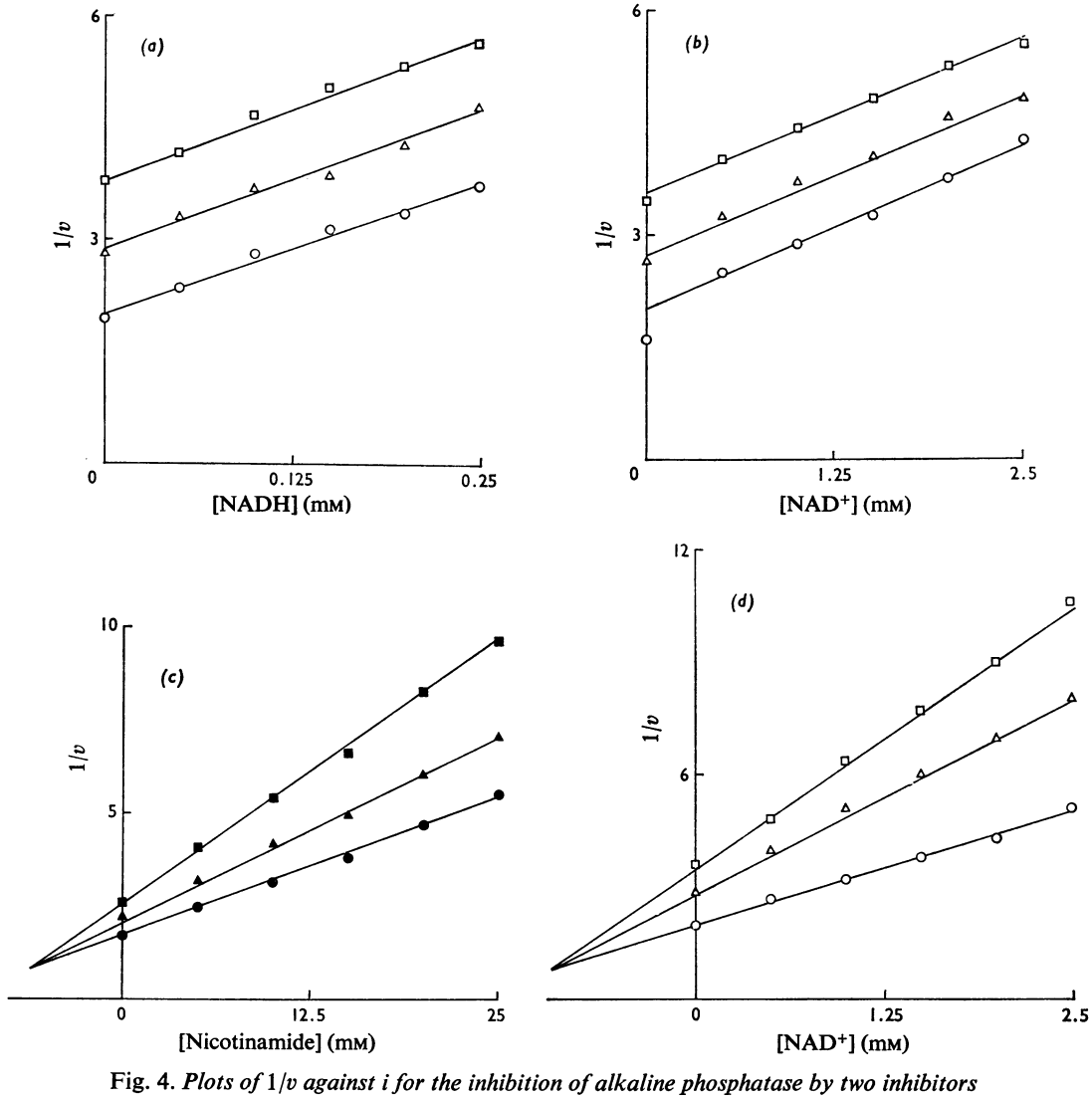


Fig. 4. *Plots of* $1/v$ *against* i *for the inhibition of alkaline phosphatase by two inhibitors*

(*a*) NAD^+ concentrations were fixed at zero (○), 1.0mM (△) and 2.0mM (□) and the concentration of NADH was varied. (*b*) NADH concentrations were fixed at zero (○), 0.1mM (△) and 0.2mM (□) and the concentration of NAD^+ was varied. (*c*) NAD^+ concentrations were fixed at zero (●), 0.5mM (▲) and 1.0mM (■) and the concentration of nicotinamide was varied. (*d*) Nicotinamide concentrations were fixed at zero (○), 5mM (△) and 10mM (□) and the concentration of NAD^+ was varied. The substrate concentration was 1mM throughout. Other conditions were as indicated in the Experimental section.

The results of experiments with two inhibitors present are shown in Figs. 4 and 5 and the results are summarized in Table 2. It seems that NADH and NAD^+ can exclude each other from binding to the enzyme–substrate complex, although presumably NADH does not interfere with the reaction between NAD^+ and free enzyme. Nicotinamide antagonizes NADH to a small extent but mildly facilitates the binding of NAD^+. Adenosine tightens the binding of nicotinamide considerably and vice versa, but antagonizes binding of NAD^+ and NADH. The potentiation of adenosine binding by nicotinamide may be indicative of a conformational event accompanying the binding of these compounds. Such a change would presumably be associated also with the binding of NAD^+ and NADH.

Desensitization

The linearity of all inhibition plots seems to eliminate the existence of co-operative allosteric effects, but since the binding of nicotinamide and adenosine show some kind of interaction, attempts were made to hinder the interaction by subjecting alkaline

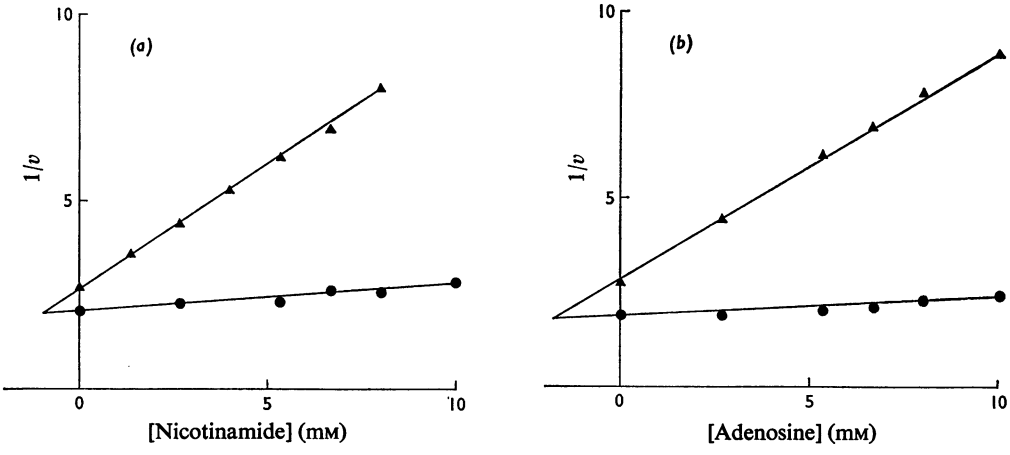


Fig. 5. *Plots of* $1/v$ *against* i *for inhibition by two inhibitors*

(*a*) Adenosine concentrations were fixed at zero (●) and 10mM (▲). (*b*) Nicotinamide concentrations were fixed at zero (●) and 10mM (▲). In all plots the substrate concentration was 100μM. Other conditions were as indicated in the Experimental section.

Table 2. *Interaction between pairs of inhibitors*

Values for K_{si_1} should be compared with $K_{si_2i_1}$, and K_{si_2} compared with $K_{si_1i_2}$. A smaller value for the constant obtained with both inhibitors signifies facilitation of binding. All data refer to pH9.0 and 30°C. $K_{si_1} \cdot K_{si_1i_2}$ should equal $K_{si_2} \cdot K_{si_2i_1}$. It can be seen from the values that reasonable agreement is obtained.

Pairs of inhibitors	Constants (mM) K_{si_1}	K_{si_2}	$K_{si_2i_1}$	$K_{si_1i_2}$	Comments on binding
NADH and NAD^+			∞	∞	Exclusive
Nicotinamide and adenosine	17.4	24.8	0.9	1.85	Facilitated
NADH and nicotinamide	0.16	17.4	0.3	42.0	Antagonistic
NADH and adenosine			∞	∞	Exclusive
NAD^+ and nicotinamide	1.73	17.4	0.9	11.0	Facilitated
NAD^+ and adenosine	1.73	24.8	4.9	70.5	Antagonistic

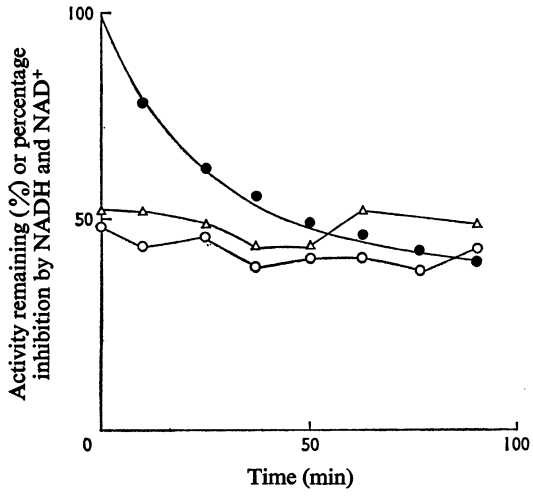


Fig. 6. *Effect of partial denaturation by guanidine on the inhibition of alkaline phosphatase by NADH and NAD+*

The change in activity and degree of inhibition is shown as a function of time of treatment with 3M-guanidine. ●, Phosphatase activity; △, NADH inhibition; ○, NAD^+ inhibition. See the Experimental section for details of the denaturation procedure and assay method.

phosphatase to partial denaturation. Denaturation was brought about either by keeping at 40°C or by treating with 3M-guanidine–HCl at 0°C. Fig. 6 shows the percentage inhibition produced by 1mM-NAD^+ and 0.1mM-NADH at various stages of the denaturation by guanidine. Within 1h the alkaline phosphatase activity fell by 50–60%, but there was no significant change in the degree of inhibition caused by NAD^+ and NADH. Similar experiments showed that the interaction between nicotinamide and adenosine was unaffected by partial denaturation.

Inhibition at different [NAD+]/[NADH] ratios

Fig. 7 shows the variation in alkaline phosphatase activity with changes in the ratio of the two forms of the coenzyme. The activity was determined with a substrate concentration of 0.1mM so that measured velocities were close to $V_{max.}$. Changing the ratio from zero to infinity brought about an approximately fourfold increase in the velocity. The presence of 5mM-Mg^{2+} did not change the overall shape of the inhibition plot.

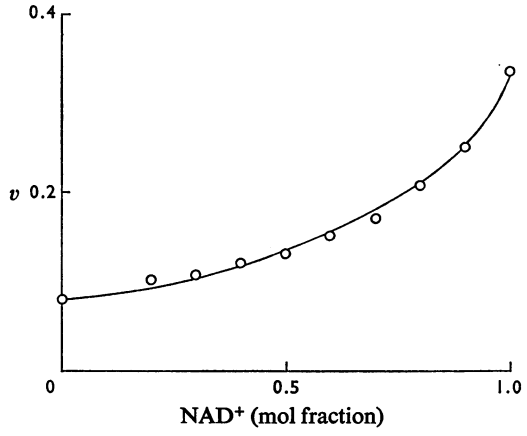


Fig. 7. *Variation in alkaline phosphatase activity with changes in the [NAD+]/[NADH] ratio*

The total coenzyme concentration was 1mM. Thus 1.0 on the horizontal axis represents 1mM-NAD^+ and zero NADH. Measurements of activity were made as indicated in the Experimental section.

Discussion

The demonstration of the uncompetitive inhibition of alkaline phosphatase by NADH, nicotinamide and adenosine provides further examples of biological compounds that inhibit the enzyme. NAD^+, NADH and adenosine could be regarded as possible phosphate acceptors in a phosphotransferase action catalysed by alkaline phosphatase (Morton, 1958). The existence of phosphate transfer would be shown by a decrease in the rate of release of P_i, and this apparent inhibition would take the form of uncompetitive, non-competitive or 'mixed', depending on the relative rates of transfer of phosphate to water (i.e. hydrolysis) and the alternative acceptor (Hass & Byrne, 1960; Arion & Nordlie, 1964). A decrease in the rate of release of *p*-nitrophenol from the substrate would not be expected, and with alkaline phosphatase it is usually found that the presence of a suitable phosphate acceptor, e.g. diethanolamine, accelerates the release of the alcoholic moiety of the substrate during catalysis (Hausamen *et al.*, 1967). Thus phosphate transfer does not account for the inhibition of alkaline phosphatase by NAD^+, NADH and adenosine.

Fishman & Ghosh (1967) and Lin *et al.* (1971) assumed that L-phenylalanine combined with the enzyme–substrate complex, but the studies of Fernley & Walker (1970) indicate that the uncompetitive inhibition of alkaline phosphatase results from the combination of the inhibitor with a phosphoryl-enzyme intermediate. It seems probable that NADH, nicotinamide and adenosine inhibit by a similar

mechanism. Substrate inhibition occurs when alkaline phosphatase acts on ATP (Wass & Butterworth, 1971). Because of the similarities in structure, it is possible that at least part of the mechanism whereby ATP forms inhibitory complexes with the enzyme resembles that which occurs with NAD^+, NADH and adenosine.

The 'mixed' inhibition caused by NAD^+ was surprising considering the chemical similarity of all the inhibitors tested. There are some changes in molecular structure when NADH is converted into NAD^+. Apart from the extra positive charge, which may be a factor in binding NAD^+ to alkaline phosphatase, a change occurs in the conformation of the ribose attached to the nicotinamide moiety when oxidation of NADH occurs. The alteration results in a change in the distance separating the nicotinamide ring and the side chain linked through C-4 of the ribose. The change in the ribose may also be transmitted to the pyrophosphate backbone (Sarma & Kaplan, 1970*b*). Also, the positively charged N atom may interact electrostatically with the pyrophosphate backbone (Blumenstein & Raftery, 1972). The combined effects of the structural changes presumably account for the differences in the inhibition by NAD^+ and NADH.

The studies of the interaction between adenosine and nicotinamide do not distinguish between the two possibilities of either (*a*) separate but adjacent and interacting sites for adenosine and nicotinamide or (*b*) a single site, which is sufficiently non-specific to allow binding of either nicotinamide or adenosine and allows the bound component to interact favourably with its partner. In solution, NADH appears to take up a conformation in which the nicotinamide and adenine bases are stacked on top of each other (Sarma & Kaplan, 1970*a*), and the situation described in (*b*) would be analogous to a stacked condition. The antagonism of adenosine towards the binding of NAD^+ and NADH also favours, perhaps, the existence of a single site and that the main anchorage is provided by the adenosine moiety. The facilitation of nicotinamide binding by NAD^+ suggests that NAD^+ may bind in a more open rather than a stacked form, so that interactions between the adenosine moiety of NAD^+ and free nicotinamide can occur. The two-site model described in (*a*) would require a mechanism of a transmitted induced fit. Further studies of the inhibition of alkaline phosphatase by NAD^+ and related compounds may furnish information about the ways in which they bind to the enzyme and may give an insight into the geometry of the region on the enzyme where they become located.

The actions of NAD^+ and NADH on alkaline phosphatase may be utilized for controlling the activity of the enzyme *in vivo* and regulate the rate of utilization of ATP, assuming that ATP is the physiological substrate of the enzyme (Wass & Butterworth, 1971). If the regulation of activity results from competition between NAD^+ and NADH for a single regulatory site, alkaline phosphatase seems to provide an example of control by ligand exclusion discussed by Fisher *et al.* (1970). The belief that effector molecules probably only bind to the phosphorylated enzyme may be an important clue to the true function of the enzyme. Membrane transport processes seem to involve phosphorylated intermediates (Kimmich, 1970) and it could be that alkaline phosphatase acts as a phosphorylated carrier or is important in a cycle of phosphorylation–dephosphorylation reactions occurring during transport. Further experiments are needed to test the hypothesis and to determine whether amino acids such as L-phenylalanine bind to alkaline phosphatase at the same site as NAD^+ and NADH.

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