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The Action of Cyanate on Human and Pig Kidney Alkaline Phosphatases

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1. At concentrations of cyanate up to 0·2 M there is an apparently reversible combination with alkaline phosphatase (EC 3.1.3.1), but higher concentrations inhibit alkaline phosphatase irreversibly by a process that is time-dependent. 2. The effect of 0·2 M-cyanate on the enzymic reaction velocity depends on the substrate concentration. There is inhibition when the substrate concentration is 1·0 mM or higher, but at lower substrate concentrations cyanate has an activating effect. 3. The pH-dependence of the reversible reaction suggests that cyanate may react with a thiol group at or near the active site of the enzyme, preventing a conformational change that is believed to be important in the mechanism of action of alkaline phosphatase. 4. Prolonged treatment with 0·6 M-cyanate probably carbamoylates all free amino groups in the enzyme molecule and generates a new enzyme with decreased $V_{max.}$ and increased K_m.

Stark, Stein & Moore (1960) found that cyanate inhibited ribonuclease with the formation of ϵ-carbamoyl-lysine and *S*-carbamoylcysteine derivatives. A preliminary investigation showed that cyanate inhibited alkaline phosphatase (EC 3.1.3.1) and the inhibition exhibited some interesting features (Butterworth, 1967). Fishman & Ghosh (1967) obtained evidence that thiol and amino groups are important for the catalytic action of rat intestinal alkaline phosphatase and it appeared that a study of the effects of cyanate on alkaline phosphatase would be worth while, since further information might be gained on the role of amino and thiol groups at or near the active centre of the enzyme.

MATERIALS AND METHODS

Substrates. Disodium *p*-nitrophenyl phosphate was obtained from British Drug Houses Ltd., Poole, Dorset, and disodium α-naphthyl phosphate was purchased from Hopkin and Williams Ltd., Chadwell Heath, Essex.

Other chemicals. The best grade of NaOCN available was obtained from British Drug Houses Ltd. All other chemicals were of analytical grade.

Enzyme preparations. Alkaline phosphatase was prepared from human and pig kidneys as described by Butterworth (1968*a*,*b*).

Method of assay. Reaction mixtures were of 3 ml. volume and consisted of 1 ml. of 9 mM-*p*-nitrophenyl phosphate, 0·1 ml. of 100 mM-$MgCl_2$ and 1·8 ml. of 0·1 M-Na_2CO_3–$NaHCO_3$ buffer, pH 10. The reaction was started by the addition of 0·1 ml. of enzyme. The substrate and $MgCl_2$ solutions were prepared in the Na_2CO_3–$NaHCO_3$ buffer and adjusted to pH 10 if necessary. Phosphatase activity was determined at 37° by following the increase in E_{400}. In some experiments 1 ml. of 18 mM-α-naphthyl phosphate replaced *p*-nitrophenyl phosphate and the activity was then measured by monitoring the increase in E_{335}. Alkaline phosphatase activity was expressed as μmoles of product released/min./ml. of enzyme solution.

Inhibition studies with cyanate. NaOCN, dissolved in 0·1 M-Na_2CO_3–$NaHCO_3$ buffer, was substituted for buffer solution in the reaction mixtures to give the concentrations of cyanate indicated in the Results section. The amount of inhibition was expressed as a percentage of the activity of non-inhibited controls.

Effect of pH *on inhibition by cyanate.* This was studied by using 0·1 M-Na_2CO_3–$NaHCO_3$ buffers covering the pH range 9·2–10·8.

Time-dependence of inhibition by cyanate. Enzyme was preincubated at 37° with 0·6 M-NaOCN in Na_2CO_3–$NaHCO_3$ buffers at pH 10 and pH 10·4. At timed intervals 0·1 ml. portions of the preincubation mixture were assayed for phosphatase activity. Mixtures of enzyme in buffers at pH 10 and pH 10·4 were incubated to serve as controls.

Starch-gel electrophoresis. This was carried out by the method of Smithies (1955) with the discontinuous buffer system of Poulik (1957) at pH 8·6.

RESULTS

Four fractions of human kidney alkaline phosphatase, designated A, B, C and D, are obtained by the preparation method used (Butterworth, 1968*a*). Fig. 1 shows the effect of cyanate on the hydrolysis of *p*-nitrophenyl phosphate by fraction A, which constitutes the main enzyme fraction. The results for cyanate inhibition obtained with fractions B, C and D were identical with those of fraction A. In a similar experiment in which the substrate was

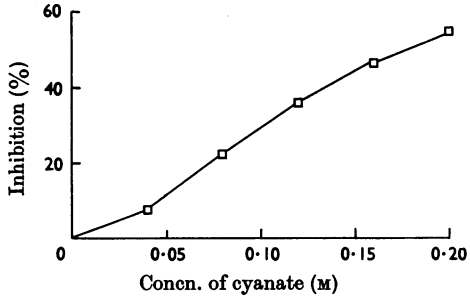

Fig. 1. Inhibition of human kidney alkaline phosphatase by cyanate. The substrate was 3mM-*p*-nitrophenyl phosphate.

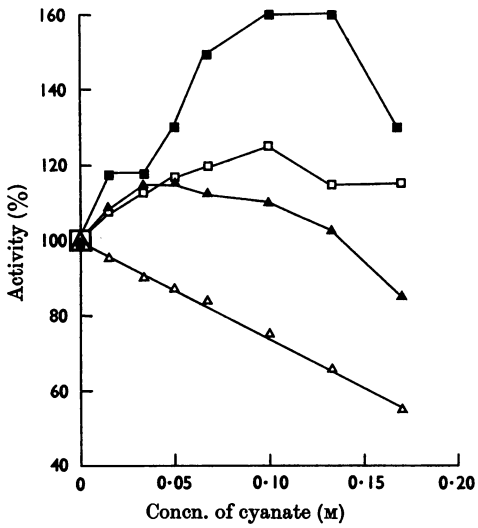

Fig. 2. Effect of cyanate on the activity of pig kidney alkaline phosphatase. Determinations were made at the following concentrations of *p*-nitrophenyl phosphate: △, 1·0mM; ▲, 0·5mM; □, 0·2mM; ■, 0·1mM.

6mM-α-naphthyl phosphate an identical inhibition pattern was seen. The inhibition of pig kidney alkaline phosphatase by cyanate resembled that of human enzyme very closely.

With both human and pig kidney enzymes the inhibition behaviour depended on the substrate concentration in the reaction mixture. Fig. 2 shows some results obtained with pig enzyme at different substrate concentrations. When the concentration of *p*-nitrophenyl phosphate was 1·0mM or higher cyanate was inhibitory, but as the concentration of substrate was decreased cyanate was found to have an activating effect. A similar result was seen when α-naphthyl phosphate was used as substrate.

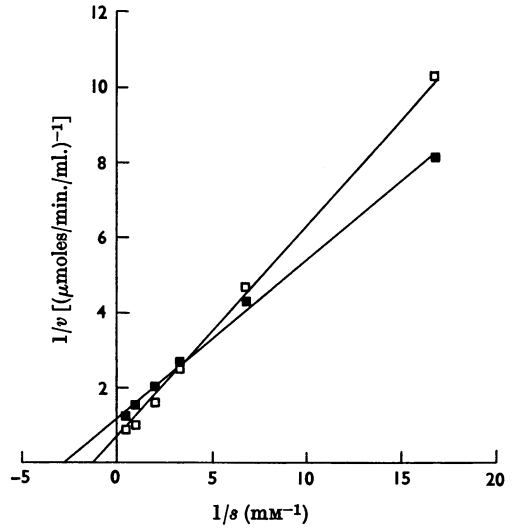

Fig. 3. Plots of $1/v$ against $1/s$ obtained for the hydrolysis of *p*-nitrophenyl phosphate by human kidney alkaline phosphatase in the absence (□) and presence (■) of 0·2M-cyanate.

Activation at low substrate concentrations coupled with inhibition at higher concentrations led to 'crossed' Lineweaver & Burk (1934) double-reciprocal plots obtained for human kidney alkaline phosphatase in the presence and absence of cyanate (Fig. 3).

Fig. 4 shows the pH-dependence of the inhibition by 0·2M-cyanate determined at a substrate concentration of 3mM. The amount of inhibition was fairly constant as the pH was increased from 9·0 to 10·0, but above pH 10·1 there was a sharp decrease in the inhibition caused by cyanate.

In all the experiments described so far the rates of the enzyme-catalysed reactions remained constant throughout the assay periods, both in the presence and absence of cyanate, indicating a very rapid reaction between cyanate and the enzyme. The duration of the assay period was never longer than 10min. Preincubation of human kidney enzyme with 0·6M-cyanate at 37° led, however, to a progressive fall in enzymic activity, showing that higher concentrations of cyanate inhibited alkaline phosphatase by a process that was time-dependent (Fig. 5). At pH 10·0 the rate of inactivation by 0·6M-cyanate was approx. 1·6 times the rate at pH 10·4. When preincubated enzyme was assayed in the presence of 0·2M-cyanate no further activation or inhibition could be demonstrated. An investigation of K_m and $V_{max.}$ of human kidney alkaline phosphatase showed that both parameters were affected by pretreatment with 0·6M-cyanate.

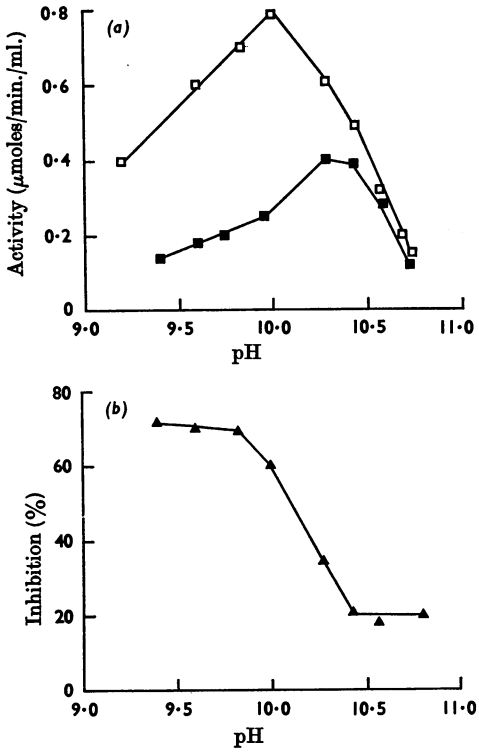

Fig. 4. (*a*) Effect of pH on the activity of human kidney alkaline phosphatase in the presence (■) and absence (□) of 0·2M-cyanate. (*b*) Effect of pH on the amount of inhibition calculated from the curves shown in (*a*).

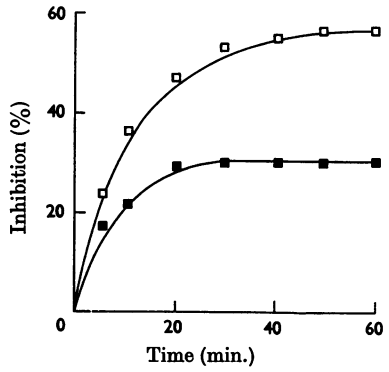

Fig. 5. Time-dependence of the inhibition of human kidney alkaline phosphatase at pH10 (□) and pH10·4 (■) by 0·6M-cyanate.

K_m was increased and $V_{max.}$ was decreased. Dialysis against water or 0·04M-L-lysine for 18hr. at 20° of enzyme that had been treated with cyanate did not restore enzymic activity lost as a result of the preincubation.

The electrophoretic mobility of alkaline phosphatase on starch gel was increased approx. 50% after preincubation with cyanate.

DISCUSSION

Cyanate is able to carbamoylate $-NH_3^+$ groups of amino acids:

$$R{-}NH_3^+ + {}^-OCN \longrightarrow R{-}NH{-}C(=O){-}NH_2$$

Stark *et al.* (1960) demonstrated that the enzymic activity of ribonuclease was lost after cyanate treatment and that acid hydrolysates of the treated enzyme contained homocitrulline. This amino acid is not normally present in ribonuclease, but had been formed by carbamoylation of lysine. The time-dependent reaction of alkaline phosphatase with 0·6M-cyanate and its non-reversal by dialysis suggests that cyanate may react with constituent groups in the enzyme molecule. The lower rate of inactivation at pH10·4 may implicate ϵ-NH_3^+ groups of lysine in the reaction since this pH is close to the pK value of the ϵ-NH_3^+ group and it is the protonated form of amino groups that reacts with cyanate (Warner, 1942; Stark & Smyth, 1963). It seems unlikely, however, that other free amino groups would not react with cyanate under the conditions of the experiments, and the change in electrophoretic mobility at pH8·6 probably results from a decrease in the net positive charge of alkaline phosphatase molecules produced by carbamoylation of all free $-NH_3^+$ groups.

The effects on the activity of alkaline phosphatase produced by the inclusion of relatively lower concentrations of cyanate in the assay mixture suggests that either a reversible combination occurs between the enzyme and modifier or there is a fast irreversible reaction that generates an enzyme of changed characteristics. The steadily increasing inhibition with increasing cyanate concentration is probably indicative of a reversible combination. The reaction of cyanate with amino groups occurs fairly slowly; Stark & Smyth (1963) found the half-life of amino groups to be about 10min. at 50° in 1M-cyanate at pH7. Thus the rapid and reversible effects of cyanate on the initial reaction velocity probably cannot be accounted for by carbamoylation of amino groups. Stark *et al.* (1960) demonstrated that cyanate reacts more rapidly with thiol groups:

$$R{-}S^- + HOCN \rightleftharpoons R{-}S{-}C(=O){-}NH_2 + OH^-$$

Stark (1964) reported that the reacting ionic species are most likely to be those shown in the equation and gave a value of approx. $7 \times 10^4 \text{M}^{-1}\text{min.}^{-1}$ for the rate constant of the reverse action. At pH 10, the concentration of OH^- being 10^{-4}M, the pseudo-unimolecular rate constant for the reverse reaction would be about 7min.^{-1}, giving a half-life of 6sec. This reaction might be fast enough to explain the apparently reversible effects of cyanate.

The rate of carbamoylation of thiol groups decreases above pH 9, the approx. pK value of the –SH group (Stark, 1964), because a change of 1 pH unit will not markedly increase the concentration of R–S^- but will decrease the concentration of cyanic acid tenfold. This decrease in the rate of carbamoylation may account for the decrease in the inhibitory effect of cyanate above pH 10.

The substrate-concentration-dependent action of cyanate is an unusual finding, but a similar phenomenon has been described for the effect of L-lysine on the activity of an alkaline phosphatase preparation (Zittle & Della Monica, 1950). It is analogous to the shift in the pH optimum of alkaline phosphatase to more acid values as the substrate concentration is decreased. Fernley & Walker (1965) have postulated the existence of two conformational states of alkaline phosphatase, the equilibrium between them being pH-dependent, to account for the pH-optimum shift. One conformer is stated to react preferentially with substrate and the other reacts to release P_i. A reaction between cyanate and a thiol group at or near the active site of alkaline phosphatase may confine the molecule to a single conformational state. Such a reaction could perhaps best explain the anomalous effects of cyanate at high and low substrate concentrations.

Prolonged treatment with cyanate such as occurs in preincubation experiments probably leads to reactions between the inhibitor and many other groups present in the enzyme as well as those at the active site. The irreversibly carbamoylated enzyme is not totally inactive, but $V_{max.}$ is decreased and K_m increased.

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