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Simplified Preparation of a Phosphatase Inhibitor and Further Studies of its Action

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1-Pyrrolidinecarbothioic acid (2-pyridylmethylene)hydrazide chelates Zn^{2+} but not Mg^{2+} . This compound is about twice as effective as EDTA for inhibiting alkaline phosphatase from calf mucosa, and approx. 1000-fold more effective than EDTA for inhibiting acid phosphatase from wheat germ. The compound did not inhibit pyridoxine kinase activity in human leucocytes at the highest concentration tested ($33\ \mu\text{M}$). Therefore it may be a useful tool for either examining or eliminating the effects of phosphatases in complex enzyme systems.

In connection with studies of anti-tumour agents, Agrawal *et al.* (1974) have developed a series of inhibitors of alkaline phosphatase. Lee *et al.* (1973) demonstrated that some of these compounds chelate Zn^{2+} but not Mg^{2+} . This suggested to us that such inhibitors might be useful for either examining or eliminating the effects of alkaline phosphatase in systems that also contained Mg^{2+} -requiring enzymes such as kinases.

The most effective inhibitor prepared by Agrawal *et al.* (1974) was 1-pyrrolidinecarbothioic acid [(5-hydroxy-2-pyridyl)methylene]hydrazide. 5-Hydroxy-pyridine-2-carbaldehyde, the starting material for the synthesis of that compound, is not commercially available and involves a multistep synthesis (Blanz *et al.*, 1970). However, pyridine-2-carbaldehyde is readily available. Since previous reports had indicated that the 5-hydroxy group improved anti-tumour activity (Agrawal *et al.*, 1973), but did not greatly increase alkaline phosphatase inhibition (Lee *et al.*, 1973), we decided to examine the properties of the simpler analogue 1-pyrrolidinecarbothioic acid (2-pyridylmethylene)hydrazide:

Furthermore, since Saleem & Alikhan (1974) and Masuda & Hagiwara (1972) have demonstrated that, like alkaline phosphatase, at least some acid phosphatases are inhibited by EDTA, thus suggesting that they require the presence of bivalent metal ions, the effect of this compound on an acid phosphatase from wheat germ was studied.

Materials and Methods

Methyl dithiocarbamate was prepared by the method of Audrieth *et al.* (1954), starting with 0.11 mol of 85% hydrazine hydrate, 0.11 mol of

carbon disulphide and 0.11 mol of methyl iodide, giving a final yield of 58.2%. 1-Pyrrolidinecarbothioic acid hydrazide was prepared by the method of Agrawal *et al.* (1974) with 8 mmol of methyl dithiocarbamate and 24 mmol of pyrrolidine as the amine, giving a final yield of 50.4%. 1-Pyrrolidinecarbothioic acid (2-pyridylmethylene)hydrazide was prepared by a method similar to that of Agrawal *et al.* (1973). Pyridine-2-carbaldehyde (214 mg; 2 mmol) was added to 290 mg (2 mmol) of 1-pyrrolidinecarbothioic acid hydrazide in 10 ml of 50% (v/v) ethanol and the mixture was warmed to dissolve. After the reaction mixture had cooled, a few drops of dilute acetic acid were added and the solution was heated at 60°C for 5–10 min. A white precipitate formed when the mixture was cooled. The precipitate was filtered and recrystallized from 50% ethanol, giving crystals with m.p. $190\text{--}191^\circ\text{C}$ [yield 303 mg (64.7%)] (Found: C, 56.55; H, 6.18; N, 24.23. $\text{C}_{11}\text{H}_{14}\text{N}_4\text{S}$ requires C, 56.41; H, 5.98; N, 23.93%).

The effect of the inhibitor was measured with alkaline phosphatase from calf mucosa (Sigma Chemical Co., St. Louis, MO, U.S.A.) by using three substrates: β -naphthyl phosphate and glycerol 2-phosphate were used by the methods of McCoy *et al.* (1965) and Oser (1965) respectively; *p*-nitrophenyl phosphate was used in a reaction mixture with the following final composition in 3 ml: 1.0 M-2-amino-2-methylpropane-1,3-diol buffer, pH 10, at 37°C ; 0.2 mM- MgCl_2 ; enzyme 0.83 μg to 0.168 mg of protein/ml; 2.5 mM substrate. After the incubation, 0.1 ml of 18 M-NaOH was added to stop the reaction. NaOH (0.1 ml; 18 M) was added before the incubation to control tubes. A_{410} of the *p*-nitrophenol was measured and compared with that of standard solutions.

Acid phosphatase from wheat germ (Sigma Chemical Co.) was dissolved in deionized water and used at a concentration of 5.5 μg of protein/ml in the final reaction mixture. For the acid phosphatase reaction, two tubes were prepared in duplicate containing the following reagents: 2.65 ml of sodium

acetate buffer (0.15 M, pH 5.0); 0.5 ml of water; 0.2 ml of enzyme. The tubes were preincubated for 3 min and 0.3 ml of 0.01 M- β -naphthyl acid phosphate was added to each tube. To one tube 0.2 ml of 2 M-NaOH was added immediately and a stop-watch was started. After 10 min of incubation at 37°C, 0.2 ml of 2 M-NaOH was added to the second tube. The difference in fluorescence (excitation 350 nm, emission 420 nm) of the duplicate tubes was used to determine the reaction rate. NaOH (0.2 ml; 2 M) was added to the standard-curve tubes before the fluorescence was read, and the volume of water was adjusted to maintain the same final volume in all tubes. For the inhibition studies, the inhibitor was prepared at a concentration of 1 mg/ml in ethanol and diluted 1:100 with water to give a concentration of 43 μ M. EDTA was prepared at a concentration of 0.2 mM. For 15 min before addition of the substrate, portions of these solutions were incubated with the buffer appropriate for the particular substrate being tested, water and enzyme to obtain a range of concentrations. The volume of water was adjusted accordingly to compensate for inhibitor volumes.

For re-activation studies, Zn^{2+} was added to the assay mixture as $ZnCl_2$ in concentrations ranging from 0.33 to 16.6 μ M. In one experiment, Zn^{2+} was added with the inhibitor and preincubated for 5 min before the enzyme was added. In a second experiment, the enzyme was added with the inhibitor and preincubated for 15 min before Zn^{2+} was added. In all cases, substrate was the final substance added to the assay mixture.

Pyridoxine kinase activity in homogenates of human leucocytes was determined by the method of Chern & Beutler (1975a,b).

Results and Discussion

Chelation of Zn^{2+} was indicated by progressive changes in the u.v. spectrum with increasing Zn^{2+} concentrations (Fig. 1). Although it seems likely that the stoichiometric ratio of Zn^{2+} to inhibitor is 1:2, the u.v. spectra obtained in the present paper and by Lee *et al.* (1973) for an analogue show little difference between a 1:2.7 ratio and a 1:2.0 ratio. We obtained similar results at pH 7.4 and 5.0. No change in the u.v. spectrum of our inhibitor was observed in the presence of Mg^{2+} cations at concentrations of 13.3, 26.6 and 53.3 μ M at pH 9.3, 7.4 or 5.0. This is in agreement with the results of Lee *et al.* (1973) and suggests that our compound retained the ability to selectively chelate Zn^{2+} .

A final inhibitor concentration of 5 μ M produced about 50% inhibition of alkaline phosphatase over a 200-fold range of enzyme concentration (0.83 μ g to 0.168 mg of protein/ml) and with all three substrates. Typical results are shown in Fig. 2. The 5 μ M value is comparable with the 4 μ M concentration of the 5-

Fig. 1. Effect of Zn^{2+} on the spectrum of 1-pyrrolidine-carbothioic acid (2-pyridylmethylene)hydrazide

All samples were at pH 9.3 in 0.05 M-bicarbonate buffer and contained inhibitor at a concentration of 53.8 μ M. Zn^{2+} concentrations (added as $ZnCl_2$) from top to bottom were 0, 3.3, 6.6, 13.3, 19.9 and 26.6 μ M.

hydroxy analogue required for 50% inhibition (Agrawal *et al.*, 1974). With EDTA, a final concentration of 13.3 μ M was needed to produce 50% inhibition when Mg^{2+} was omitted from the reaction mixture. If Mg^{2+} was present, it was necessary to increase the concentration of EDTA accordingly, since EDTA chelates both Zn^{2+} and Mg^{2+} .

Preincubation of the inhibitor with alkaline phosphatase for at least 15 min was necessary to obtain maximum inhibition. Inhibition could be prevented if the inhibitor was preincubated with Zn^{2+} for 5 min before addition of the enzyme (Fig. 3). However, if the inhibitor was incubated with the enzyme for 15 min, subsequent addition of Zn^{2+} had little effect in restoring activity (Fig. 3). Mg^{2+} concentrations up to 4.1 mM had no effect. The fact that enzyme concentration had little effect on the inhibition curves in Fig. 2 suggests competitive inhibition. However, the data in Fig. 3 show that the inhibition is only partly reversible. In an attempt to resolve this paradox, we measured the Zn^{2+} content of the reaction mixture by aspiration into the air/ H_2 flame of a Jarrell-Ash model 82-516 atomic-absorption spectrophotometer. The reaction mixtures for both acid and alkaline phosphatases contained Zn^{2+} at a concentration of approx. 3 μ M. This was independent of the enzyme concentration. The data in Fig. 2 are compatible with the hypothesis that most of the inhibitor was bound to extraneous Zn^{2+} and that the fraction actually bound to the enzyme was so small that the effect of changes in enzyme concentration was not detectable.

Fig. 2. Effect of 1-pyrrolidinecarbothioic acid (2-pyridylmethylene)hydrazide on alkaline phosphatase activity. Enzyme concentrations were 0.83 (○), 8.3 (●), 16.8 (□) and 168 (■) μg of protein/ml. The substrate was *p*-nitrophenyl phosphate.

Fig. 3. Re-activation of phosphatases after exposure to 1-pyrrolidinecarbothioic acid (2-pyridylmethylene)hydrazide. For alkaline phosphatase, the final inhibitor concentration was $7.5 \mu\text{M}$. For the top two curves, enzyme [final concentration 0.83 (▲) or $8.3 \mu\text{g}$ of protein/ml (○)] was added after the inhibitor was preincubated with Zn^{2+} for 5 min. For the bottom two curves, enzyme [0.83 (●) or $8.3 \mu\text{g}$ of protein/ml (Δ)] was incubated with the inhibitor for 15 min before the Zn^{2+} was added. The substrate was *p*-nitrophenyl phosphate. For acid phosphatase (the two middle curves), the final concentrations of inhibitor and enzyme were $41.1 \mu\text{M}$ and $11 \mu\text{g}$ of protein/ml respectively. In one case (■) the enzyme was incubated with the inhibitor for 15 min before Zn^{2+} was added. In the other case (□) the inhibitor was incubated with Zn^{2+} for 10 min before the enzyme was added. The substrate was β -naphthyl phosphate.

Higher concentrations ($20\text{--}30 \mu\text{M}$) of inhibitor were required to produce 50% inhibition in acid phosphatase. However, these were still much lower than the concentrations ($20\text{--}30 \text{mM}$) of EDTA required to produce 50% inhibition, even in the absence of Mg^{2+} . With acid phosphatase, the order of mixing the inhibitor, Zn^{2+} and enzyme had no consistent effect on the results. Zn^{2+} re-activated the enzyme whether added to the inhibitor before or after the enzyme (Fig. 3).

Inhibitor concentrations up to $33 \mu\text{M}$ (the highest concentration tested) had no effect on the activity of pyridoxine kinase in leucocyte homogenates. The ability to inhibit phosphatase activity without affecting kinases should provide a useful tool for evaluating the role of phosphatases in the turnover of various phosphate esters. It could also be used to provide increased confidence that measurements of kinase activity represent total activity and not net gain over losses due to phosphatases. The inhibitor

might also be of use with other Zn^{2+} -containing enzymes.

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References

- Agrawal, K. C., Booth, B. A. & Sartorelli, A. C. (1973) *J. Med. Chem.* **16**, 715–717
- Agrawal, K. C., Lee, M. H., Booth, B. A., Moore, E. C. & Sartorelli, A. C. (1974) *J. Med. Chem.* **17**, 934–938
- Audrieth, L. F., Scott, E. S. & Kippur, P. S. (1954) *J. Org. Chem.* **19**, 733–741
- Blanz, E. J., French, F. A., DoAmaral, J. R. & French, D. A. (1970) *J. Med. Chem.* **13**, 1124–1130
- Chern, C. J. & Beutler, E. (1975a) *Anal. Biochem.* **67**, 97–109
- Chern, C. J. & Beutler, E. (1975b) *Clin. Chim. Acta* **61**, 353–365
- Lee, M. H., Sznycer-Pochner, E., Agrawal, K. C., Wolpert, M. K. & Sartorelli, A. C. (1973) *Biochem. Pharmacol.* **22**, 1477–1485
- Masuda, T. & Hagiwara, K. (1972) *Nippon Nogei Kagaku Kaishi* **46**, 437–441
- McCoy, E. E., Park, J. & England, J. (1965) *Clin. Chim. Acta* **12**, 453–461
- Oser, B. L. (ed.) (1965) *Hawk's Physiological Chemistry*, 14th edn., pp. 1119–1121, McGraw-Hill, New York
- Saleem, M. & Alikhan, M. A. (1974) *Comp. Biochem. Physiol. B* **49**, 129–137