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CHARACTERIZATION OF ENZYMATIC HYDROLYSIS OF INORGANIC POLYPHOSPHATES BY FLOW INJECTION ANALYSIS AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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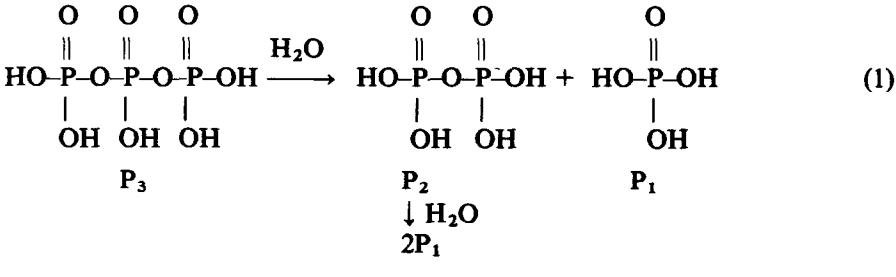
SUMMARY

A flow injection system using a molybdenum(VI) reagent and a high-performance liquid chromatographic system using a molybdenum(V)–molybdenum(VI) reagent were designed to characterize the substrate specificity and the metal ion specificity in the hydrolysis of polyphosphates by inorganic pyrophosphatase (EC 3.6.1.1). The rapid reaction with a half-life of the order of 2 min was easily measured. The enzyme was quite specific for inorganic pyrophosphate of the ten polyphosphates tested at pH 7.2 and 30°C in the presence of magnesium ion. Magnesium ion activated the enzymatic reaction, while calcium and strontium ions inhibited the effect of magnesium ion.

INTRODUCTION

Orthophosphate (P_1), pyrophosphate (diphosphate, P_2) and tripolyphosphate (triphosphate, P_3) have been extensively used in various industrial fields[1,2]. These inorganic compounds also play an important rôle in biological metabolisms associated with nucleotides[3,4]. Kinetic researches on both the chemical hydrolysis and the enzymatic hydrolysis of P_2 and P_3 are required to help to understand their stability and reactivity in environmental waters[5] and biological cycles[1–4].

P_3 and P_2 are hydrolyzed slowly in the absence of a catalyst, with approximate half-lives of 1 and 3 years, respectively, in a neutral medium at 30°C[6]. The final product is P_1. At least one hydroxyl group per phosphate moiety in all phosphate species is considered to be unprotonated at pH 7.



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Inorganic pyrophosphatase (P_2ase or PP_iase, EC 3.6.1.1) is distributed widely in almost all living cells and is likely to be indispensable to living systems[7–22]. The P_2ase isolated from baker's yeast has been well characterized by X-ray structural analysis[8] and amino acid sequence analysis[9]. The enzyme is a peanut-shaped dimer with the overall dimensions 100 × 50 × 50 Å. Each monomer peptide chain in the P_2ase dimer contains 285 amino acid residues, corresponding to a molecular weight of 32042. The approximate molecular weight of the P_2ase dimer with two identical subunits is 64000, and this value will be used in this paper to calculate enzyme concentrations.

P_2ase catalyzes the hydrolysis of pyrophosphate in a neutral medium as follows:

$$P_2 \xrightarrow{P_2\text{ase, } H_2O} 2\,P_1 \qquad (2)$$

Much attention has been focused on the reaction mechanisms and the substrate specificity of P_2ase in the presence of various metal ions[7–32]. Two or three metal ions are likely to participate in the enzymatic reaction. The interesting possibility of using P_2ase as a marker enzyme in cancer research[14] has also been suggested. One of our current projects is to monitor the activities of free enzyme and P_2ase-carriers in environmental waters, with a view to determining the kinetic factors that may control the lifetimes of polyphosphates and the process of eutrophication.

The purpose of this study was to design analytical systems based on flow injection analysis (FIA)[23–28] and high-performance liquid chromatography (HPLC)[27,28] which could be employed for the rapid and automatic measurement of P_2ase activity. The FIA system employing a molybdenum(VI) reagent was useful for the selective determination of the product (P_1) in the presence of the substrate (P_2) in eqn. 2. On the other hand, the HPLC system using a molybdenum(V)–molybdenum(VI) reagent for the post-column reaction was employed for the simultaneous determination of P_1, P_2, P_3 and other oligophosphates[1,6] as substrates. A marked effect of alkaline earth metal ions on the P_2ase activity at pH 7.2 and 30°C was observed. Only magnesium ion activated the enzymatic hydrolysis of P_2, and calcium and strontium ions inhibited its effect. Substrate specificity was also investigated in the presence of various substrates, *i.e.*, P_2, P_3 and ten other oligophosphates. The P_2ase was found to be quite specific for P_2.

EXPERIMENTAL

Samples and reagents

Orthophosphate, KH_2PO_4, pyrophosphate, $Na_4P_2O_7 \cdot 10H_2O$ and tripolyphosphate, $Na_5P_3O_{10}$, were commercially available (Wako, Osaka). Only tripolyphosphate was purified by repeated crystallization, yielding $Na_5P_3O_{10} \cdot 6H_2O$. An equilibrium mixture of oligophosphates was prepared according to the literature[29].

Inorganic pyrophosphatase (EC 3.6.1.1) isolated from baker's yeast was commercially available from Sigma (St. Louis, MO, U.S.A.). The specific activity of lyophilized enzyme according to Sigma was 500–600 U per mg protein at pH 7.2 and 25°C. The P_2ase activity measurements in this work were made at pH 7.2 (5 m*M* Tris–HCl buffer) and at 30°C.

The molybdenum(VI) reagent for FIA experiments was prepared by dissolving

5.3 g of ammonium molybdate, $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, in 1 l of 0.3 *M* sulphuric acid. The molybdenum(VI) concentration was about 0.03 *M*.

The molybdenum(V)–molybdenum(VI) reagent for HPLC experiments was prepared as follows. About 5.3 g of ammonium molybdate were dissolved in 1 l of 2.0 *M* sulphuric acid to prepare a 0.03 *M* molybdenum(VI) solution. (Subsequently, 1.8 *M* sulphuric acid was found to be better than 2.0 *M* sulphuric acid for rapid colour development.) To the molybdenum(VI) solution, 0.65 g of metallic zinc (sandy, free from As) were added. A part of the Mo(VI) was reduced to Mo(V) during the complete dissolution of the zinc with magnetic stirring. The resultant molybdenum(V)–molybdenum(VI) reagent was stable at least for a month and ready for the determination of orthophosphate and polymeric phosphates by the so-called heteropoly blue method.

Apparatus and procedures

The apparatus for the FIA and HPLC systems was the same as that used previously[23,27,30–32]. The main components were reciprocating pumps with two plungers (Seishin PSU-3.2W and Kyowa KHU-W-52), a spectrophotometer with a 8-μl flow cell (JASCO, UVIDEC 100-IIW) and an HPLC system (Hitachi 635). An anion-exchange separation column (25 cm × 4 mm I.D., TSK-Gel SAX, 10 μm) was employed.

Eluent I for the isocratic elution of phosphates was 0.20 *M* potassium chloride–1 m*M* ethylenediaminetetraacetate disodium salt (Na_2EDTA)–10 m*M* ammonia. For the gradient elution, eluents I and II (0.50 *M* KCl–1 m*M* Na_2EDTA–10 m*M* NH_3) were used. The gradient characteristics (convex, 180 min) were determined by the chloride concentration, *C* (*M*), at time *t* (min); $C = (4.7 \cdot 10^{-3} + 5.3 \cdot 10^{-4}\, t)^{0.3}$. All HPLC experiments, whether isocratic or gradient elutions, were made at the flow-rate of 1 ml/min.

RESULTS AND DISCUSSION

Determination of P_2ase activity by FIA

The FIA system in Fig. 1 was designed so that the enzymatic reaction in eqn. 2 could be stopped instantaneously and the product P_1 could be detected selectively in the presence of the substrate P_2. Each flow-rate of water and molybdenum(VI) reagent was adjusted to 1.0 ml/min by using a reciprocating pump with two plungers. A sample solution (S) was injected into the water stream via a loop-valve injector (100 μl). The molybdenum(VI) reagent was acidic enough to stop the enzymatic

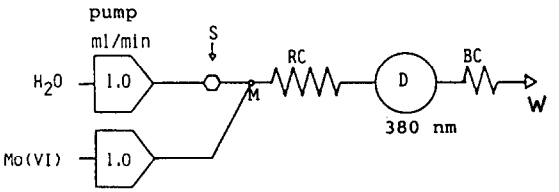

Fig. 1. FIA system for the selective determination of orthophosphate in the presence of polyphosphates. D = Spectrophotometric detector; S = sample injector; M = T-joint; RC = reaction coil (5 m × 0.5 mm I.D. PTFE, 30°C); BC = back-pressure coil (1 m × 0.25 mm I.D. PTFE); W = waste.

reaction by acidifying the sample zone at the confluence point M. Of P_3, P_2 and P_1 tested, only P_1 reacted with the molybdenum(VI) reagent in the reaction coil (RC) to form molybdophosphoric acid. The absorbance of this yellow complex was monitored at 380 nm with a spectrophotometer (D). A back-pressure coil (BC) was located at the exit of the cell.

The molybdenum(VI) reagent used in this work has scarcely been used for the determination of phosphates in a manual batch method. The main reasons for this are that the molybdenum(VI) reagent shows strong UV absorption over a wide wavelength range, 200–400 nm, and the molar absorptivity of the yellow molybdophosphate complex changes steeply with wavelength, even at 380 nm as employed in this work. Some difficulty due to the high background level of the reagent could not be avoided when a sample was injected into a carrier stream of the molybdenum(VI) reagent; a negative peak, associated with the dilution of the streaming reagent by the sample, was observed at low sample concentration.

On the other hand, this inherent difficulty could be overcome by employing the two-line FIA system shown in Fig. 1; a sample was injected into the carrier stream of water, and subsequently mixed with the flowing molybdenum(VI) reagent. With this system, very stable and even background levels could be maintained, which permitted the reproducible detection of P_1 without the appearance of a negative peak even at zero sample concentration. Compared to the heteropoly blue method employed in the following section, this method was less sensitive, but had the advantage that the colour development of P_1 was so rapid at normal temperature as to enable the selective detection of P_1 without the chemical hydrolysis of polyphosphates.

Metal ion specificity

P_2ase was allowed to react with P_2 at 30°C in a buffered medium (pH 7.2, 5 m*M* Tris–HCl buffer) containing Mg^{2+}, Ca^{2+} and/or Sr^{2+}. Aliquots of the incubated reaction mixture (each 100 μl) were successively injected at 1-min intervals into the manifold (Fig. 1) to monitor the progressive formation of P_1 in the incubated solution.

Fig. 2 shows kinetic FIA profiles for the enzymatic hydrolysis of P_2 at constant substrate and enzyme concentrations but varying types of metal ions. As shown in Fig. 2a, with magnesium ion the peak height or the amount of P_1 in the FIA profile increased linearily with time and then tended to become constant toward the end of the enzymatic reaction. The slope of the kinetic FIA profile corresponds to the rate of enzymatic hydrolysis. In contrast to the rôle of magnesium ion as an activator, calcium ion is a very poor activator as shown in Fig. 2d where the slope is almost zero. It is also evident from Fig. 2b and c that calcium ion depressed the activation effect of magnesium ion. Strontium ion also inhibited the effect of magnesium ion, though not as strongly as Ca^{2+}. P_2ase activities in the presence of Mg^{2+}, Ca^{2+} and/or Sr^{2+} are shown in Table I. Each value is relative to the maximum activity (100%) with Mg^{2+} and negligibly low values are indicated as 0%. No experiment with Ba^{2+} was made because the incubated solution gradually became turbid under the same conditions.

As mentioned previously[23], the slopes of the kinetic FIA profiles or the reaction rates were proportional to the enzyme concentrations and independent of the substrate concentrations over the range 0.1–1 m*M* P_2. The Michaelis constant, K_m,

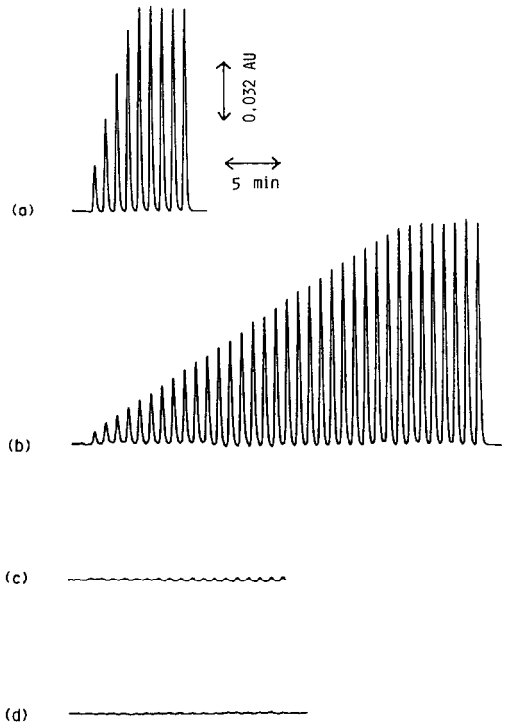

Fig. 2. Kinetic FIA profiles for the enzymatic hydrolysis of pyrophosphate. (a) $[Mg^{2+}] = 0.4$ mM; (b) $[Mg^{2+}] = 0.4$ mM, $[Ca^{2+}] = 0.04$ mM; (c) $[Mg^{2+}] = 0.4$ mM, $[Ca^{2+}] = 0.4$ mM; (d) $[Ca^{2+}] = 0.4$ mM. In all experiments, $[P_2] = 0.2$ mM and $[P_2ase] = 3 \cdot 10^{-9}$ M.

for P_2ase has been shown to be as low as 0.005 mM[7–23]. Hence the observed reaction rate, V, can be regarded as the maximum reaction rate, V_{max}, in the well known Michaelis–Menten expression; at $[P_2] \gg K_m$:

$$V = \frac{V_{max}[P_2]}{[P_2] + K_m} = V_{max} \tag{3}$$

The enzymatic hydrolysis of P_2 with a half-life of a few minutes can be measured by FIA as shown in Fig. 2a. A further attempt to design a more efficient FIA system is in progress to enable the measurement of enzymatic reactions with half-lives shorter than 1 min and to determine kinetic parameters such as the Michaelis constant by means of FIA experiments at lower substrate concentrations. The specific activity of P_2ase at 30°C was estimated from the kinetic profiles to be 730 U/mg, *i.e.*, one mg protein liberated 730 μmol P_1 per minute. This value is somewhat higher than the value at 25°C, 500–600 U/mg, according to Sigma, but is in agreement with 600–750 U/mg given in the literature[7–23]. It is stressed that the amounts and the concentrations of P_2ase in this study are based on the protein content according to Sigma and the molecular weight (64 000) in the literature[8,9].

TABLE I

EFFECT OF METAL IONS ON THE P_2ase ACTIVITY UNDER THE CONDITIONS INDICATED IN FIG. 2

Metal ions		*Concentrations (mM)*		*Relative activity (%)*
Mg^{2+}	–	0.4	–	100
–	Ca^{2+}	–	0.4	0
–	Sr^{2+}	–	0.4	0
Mg^{2+}	Ca^{2+}	0.4	0.04	27
Mg^{2+}	Ca^{2+}	0.4	0.2	3
Mg^{2+}	Ca^{2+}	0.4	0.4	0
Mg^{2+}	Sr^{2+}	0.4	0.04	95
Mg^{2+}	Sr^{2+}	0.4	0.2	61
Mg^{2+}	Sr^{2+}	0.4	0.4	44

Determination of P_2ase activity by HPLC

The activity measurement by FIA is based on the selective detection of either the substrate or the product. If one wanted to know the total mass balance of chemical species in a enzymatic reaction it would be necessary to monitor the amounts of the individual substrates, products and, if present, intermediates. This is important in cases where more than one substrate, such as P_2 and P_3, are involved in an incubated solution. The HPLC system in Fig. 3 was designed for the simultaneous determination of orthophosphate and polymeric phosphates. It is based on an anion-exchange separation using a TSK-GEL SAX (10 μm) and post-column detection using a molybdenum(V)–molybdenum(VI) reagent. The post-column reaction system can be interchanged with the high-pressure FIA system with three pumping lines, which has been extensively used for FIA experiments on phosphorus compounds[27–32]. The pumping line P_b is installed for multi-purpose use and is not always necessary. The molybdenum(V)–molybdenum(VI) reagent was prepared by a more simple method than that described previously[30–32]. It was stable for at least a month and reacted with orthophosphate to form a heteropoly blue complex which could be sensitively detected at its absorption maximum, 820–830 nm. The hydrolysis of po-

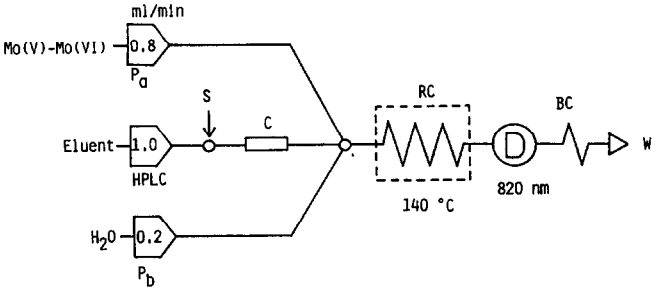

Fig. 3. HPLC system for the determination of orthophosphate and polyphosphates. P_a and P_b = pumps; HPLC = HPLC pumping system; C = separation column; RC = reaction coil (20 m × 0.5 mm I.D. PTFE, 140°C); BC = back-pressure coil (1 m × 0.25 mm I.D. PTFE); other notations in Fig. 1.

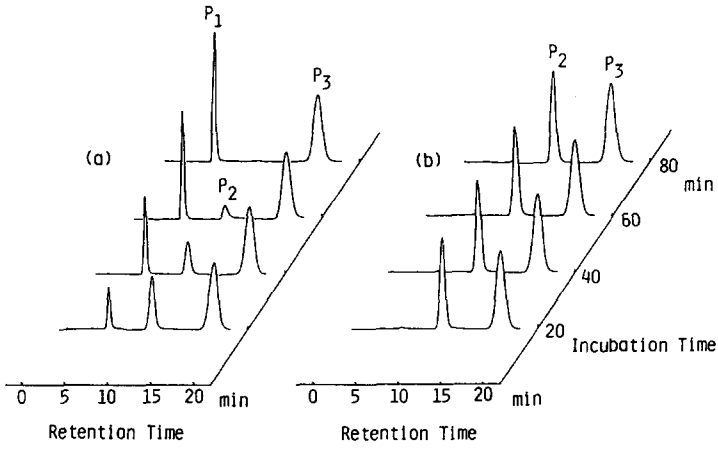

Fig. 4. Kinetic HPLC profiles for the enzymatic hydrolyses of pyrophosphate and tripolyphosphate. (a) With enzyme, [P_2ase] = $1 \cdot 10^{-10}$ *M*; (b) without enzyme. In both experiments [P_2] = 0.1 m*M*, [P_3] = 0.1 m*M* and [Mg^{2+}] = 0.4 m*M*.

lyphosphate to orthophosphate and the colour reaction of the resultant orthophosphate with the reagent proceeded simultaneously in the reaction coil maintained at 140°C.

The isocratic elutions in Fig. 4 were made with eluent I containing 0.20 *M* potassium chloride to monitor not only P_1, P_2 and P_3, but also intermediate species. No positive evidence for the formation of intermediates was observed. The use of an eluent with higher chloride concentration up to 0.23 *M* is recommended to shorten the separation time[33] when only P_1, P_2 and P_3 are to be separated.

Substrate specificity

To examine the substrate specificity of P_2ase in the presence of magnesium ion an equimolar mixture of two substrates, P_2 and P_3, incubated at pH 7.2 and 30°C with or without the presence of P_2ase was analyzed by HPLC. For convenience, the P_2ase concentration was lowered from $3 \cdot 10^{-9}$ *M* (Fig. 2) to $1 \cdot 10^{-10}$ *M*. Aliquots (100 μl) of each incubated sample solution were injected at 20-min intervals and eluted with eluient I as described in the Experimental section. In the absence of P_2ase, both P_2 and P_3 remained unchanged for at least 80 min (Fig. 4b). In the presence of P_2ase (Fig. 4a) the P_2 peak decreased with time and the P_1 peak increased correspondingly, while the P_3 peak remained unchanged. The fact that P_1 was produced by the hydrolysis of P_2 was confirmed by peak area measurements for P_1, P_2 and P_3 with a Chromatopac (Shimadzu). It was also confirmed that P_2ase did not catalyze the hydrolysis of cyclic triphosphate (trimetaphosphate).

The P_2ase activities for P_2 and P_3 in the presence of Ca^{2+} were also examined by the same procedure. P_3 as well as P_2 were not hydrolyzed by P_2ase, as expected from Fig. 2d for P_2. The HPLC results lead to the conclusion that the total mass balance of P_1, P_2 and P_3 in the presence of Mg^{2+} and Ca^{2+} can be quantitatively displayed on the HPLC profiles, and P_2ase catalyzes the hydrolysis of only P_2 in the presence of Mg^{2+}.

The HPLC measurement of P_2ase activities was also carried out for higher

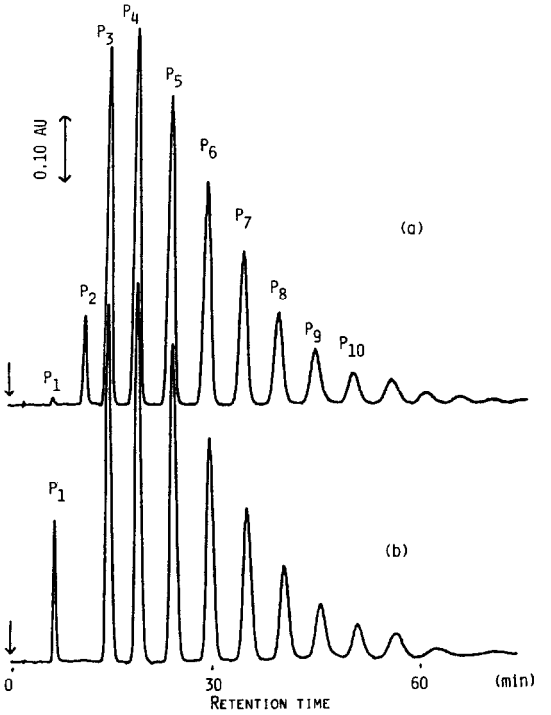

Fig. 5. Kinetic HPLC profiles for the enzymatic hydrolyses of a mixture of oligophosphates obtained by a gradient elution. (a) Without enzyme; (b) with enzyme, [P_2ase] = $3 \cdot 10^{-9}$ *M*. In both experiments the sample concentration is 0.5 m*M* (as P) and the magnesium ion concentration is 1 m*M*.

polymeric phosphates in the presence of Mg^{2+}. All the experimental conditions for the enzymatic reaction and the HPLC procedure were the same as in Figs. 3 and 4, except that a gradient technique was employed using both eluents I and II. The substrate was an equilibrium mixture of polyphosphates whose average degree of polymerization was 5. It contained not only P_1, P_2 and P_3, but also P_4 (tetraphosphate), P_5 (pentaphosphate), P_6 (hexaphosphate), etc., as can be seen in Fig. 5a. These polyphosphates when incubated in the absence of P_2ase were stable for at least an hour to give an HPLC profile identical to that in Fig. 5a. On the other hand, P_2 could not be detected in the HPLC profile of the same substrate incubated for an hour with P_2ase, in agreement with the results in Figs. 2 and 4. An important conclusion is that P_2ase is still specific for P_2 in the presence of Mg^{2+}.

The leading rôle of inorganic pyrophosphate as a substrate, not as a cofactor, in the enzymatic reaction may appear remarkable. Special efforts, therefore, have been made in this paper to demonstrate visually the progress of enzymatic hydrolysis for those who may be unable to find such "inorganic enzyme chemistry" in their textbooks of modern inorganic chemistry. Thus all kinetic profiles in Figs. 2, 4 and 5 were reproduced by direct photocopy, without redrawing, from the original recorder charts with blue background lines. This will allow students to understand the function of P_2ase in facilitating control of the P_2 concentration in a reaction such as ATP → AMP + P_2, which is essential in biological metabolism[4,34].

REFERENCES

1 D. E. C. Corbridge, *Phosphorus; An Outline of Its Chemistry, Biochemistry and Technology*, Elsevier, Amsterdam, 1978.
2 J. Emsley and D. Hall, *The Chemistry of Phosphorus*, Harper & Row, London, 1976.
3 G. L. Eichhorn (Editor), *Inorganic Biochemistry*, Elsevier, Amsterdam, 1973.
4 R. W. McGilvery and G. W. Goldstein, *Biochemistry; A Functional Approach*, Saunders, New York, 1983.
5 E. J. Griffith, A. Beeton, J. M. Spencer and D. T. Mitchell (Editors), *Environmental Phosphorus Handbook*, Wiley-Interscience, New York, 1973.
6 E. J. Griffith and R. L. Buxton, *J. Am. Chem. Soc.*, 89 (1967) 2884.
7 B. S. Cooperman, *Methods Enzymol.*, 87 (1982) 526.
8 G. Bunick, G. P. McKenna, F. E. Scarbrough, E. C. Uberbacher and D. Voet, *Acta Crystallogr., Sect. B*, 34 (1978) 3210.
9 S. A. Cohen, R. Sterner, P. S. Kein and R. L. Heinricson,*J. Biol. Chem.*, 253 (1978) 889.
10 K. M. Welsh and B. S. Cooperman, *Biochemistry*, 23 (1984) 4947.
11 S.-J. Ting and D. Dunaway-Mariano, *FEBS Lett.*, 165 (1984)251.
12 W. B. Knight, S.-J. Ting, S. Chuang, D. Dunaway-Mariano, T. Haromy and M. Sundaralingam, *Arch. Biochem. Biophys.*, 227 (1983) 302.
13 J. B. Shatton, C. Ward, A. Williams and S. Weinhouse, *Anal. Biochem.*, 130 (1983) 114.
14 J. B. Shatton, A. Williams and S. Weinhouse, *Cancer Res.*, 43 (1983) 3742.
15 S. E. Volk, A. A. Baykov, E. B. Kostenko and S. M. Avaeva, *Biochim. Biophys. Acta*, 744 (1983) 127.
16 W. A. Dick and M. A. Tabatakai, *Soil Sci.*, 137 (1984) 7.
17 I. Laikko, *Caries Res.*, 18 (1984) 258.
18 R. Lahti, *Microbiol. Rev.*, 47 (1983) 169.
19 J. W. Ridlington, Y. Yang and L. Butler, *Arch. Biochem. Biophys.*, 153 (1972) 714.
20 Y. A. Shakkov and P. Nyren, *Acta Chem Scand., Ser. B*, 36 (1982) 689.
21 J. J. Mukherjee and P. R. Pal, *Agr. Biol. Chem.*, 46 (1982) 2735.
22 D. D. Hackney, *J. Biol. Chem.*, 255 (1980) 5320.
23 N. Yoza, H. Hirano, M. Okamura, S. Ohashi, Y. Hirai and K. Tomokuni, *Chem. Lett.*, (1983) 1433.
24 J. Ruzicka and E. H. Hansen, *Flow Injection Analysis*, Wiley, New York, 1981.
25 K. K. Stewart, *Anal. Chem.*, 55 (1983) 931A.
26 J. Ruzicka, *Anal. Chem.*, 55 (1983) 1040A.
27 Y. Baba, N. Yoza and S. Ohashi, *J. Chromatogr.*, 295 (1984) 153.
28 N. Yoza, T. Shuto, Y. Baba, A. Tanaka and S. Ohashi, *J. Chromatogr.*, 298 (1984) 419.
29 T. Miyajima, K. Yamauchi and S. Ohashi, *J. Liquid Chromatogr.*, 4 (1981) 1891.
30 Y. Hirai, N. Yoza and S. Ohashi, *Anal. Chim. Acta*, 115 (1980) 269.
31 Y. Hirai, N. Yoza and S. Ohashi, *J. Chromatogr.*, 206 (1981) 501.
32 N. Yoza, K. Ishibashi and S. Ohashi, *J. Chromatogr.*, 134 (1977) 497.
33 N. Yoza, K. Ito, Y. Hirai and S. Ohashi, *J. Chromatogr.*, 196 (1980) 471.
34 L. Stryer, *Biochemistry*, Freeman, San Francisco, CA, 1975.