Biochemistry

Volume 12, Number 9 April 24, 1973

Yeast Inorganic Pyrophosphatase. I. New Methods of Purification, Assay, and Crystallization†

Barry S. Cooperman,* Ning Yu Chiu, Robert H. Bruckmann, Gerald J. Bunick, and George P. McKenna

ABSTRACT: New methods are described for the purification, assay, and crystallization of yeast inorganic pyrophosphatase. The purification method employs two chromatographic steps and is suitable for large-scale use. A 450-mg (>95% pure) preparation is described. The assay utilizes a pH-Stat and is continuous. The equation linking proton production to phosphate production is presented. Advantages and limitations of the method are discussed. Crystals, large enough for X-ray diffraction studies, are shown and the conditions for growth are described.

Phosphoryl transfer is among the most widespread classes of biochemical reactions, figuring importantly in biosynthesis, regulation of enzymatic activity, and overall energy utilization and conservation. Despite this central position, only recently have model systems for phosphoryl transfer began to yield plausible mechanistic routes for enzymatic catalysis (Benkovic and Sampson, 1971; Benkovic and Dunikoski, 1971; Kirby and Younas, 1970; Lloyd and Cooperman, 1971). Studies on phosphoryl transfer enzymes have yielded excellent information on the structure of enzyme–substrate complexes (Mildvan, 1970), although in no case have the details of the catalytic process been elucidated at the same level as, say, ribonuclease A (Richards and Wyckoff, 1971).

In this and the two accompanying papers we present the first part of a continuing study of the mechanism of action of yeast inorganic pyrophosphatase (EC 3.6.1.1 pyrophosphate phosphohydrolase) which has several desirable properties as a paradigm for phosphoryl transfer enzymes. It is relatively small (estimated mol wt 63,000–71,000) (Ridlington *et al.*, 1972; Schachman, 1952), stable, plentiful (about 1% of total soluble protein in yeast), is typical in requiring divalent metal ion for activity, and catalyzes a simple, one substrate, reaction. A review of the properties of the enzyme has appeared recently (Butler, 1971).

This paper describes new methods for purification, crystallization, and assay of the enzyme. A new purification scheme was required because of our inability to consistently reproduce earlier methods (Kunitz, 1952, 1961). The new crystallization procedure yields crystals of large enough size to be suitable for X-ray diffraction studies. The new, continuous, pH-Stat assay is, at least for some purposes, much more convenient and rapid than previous methods.

While this work was in progress, two new procedures for inorganic pyrophosphatase purification, one similar to our own, have appeared (Negi and Irie, 1971; Ridlington *et al.*, 1972).

Experimental Section

Materials

2-Methyl-2,4-pentanediol (MPD)[1] (Aldrich) was freshly redistilled before use. All other chemicals were reagent grade and were used without further purification. Cakes (1 lb) of compressed Red Star baker's yeast were obtained from the Universal Food Co.

Water for stock solutions was glass distilled and passed through a Barnstead standard mixed-bed deionizing column. Solutions of Tris, inorganic pyrophosphate, and NaCl used to make buffers were passed over Chelex-100 (Bio-rad).

Methods

Enzyme Assays. pH-Stat assays were performed with an ABU1C autoburet equipped with a 2.5-ml buret, a PHM 28 pH meter, and a TTT II titrator, all Radiometer equipment. NaOH solutions (0.01 N) were prepared with vigorously boiled distilled water, stored in a reservoir protected with ascarite, and standardized daily against standard HCl solution (Harleco). Assays were performed under a N_2 atmosphere. A con-

† From the Department of Chemistry, University of Pennsylvania, Philadelphia, Pennsylvania 19104. *Received August 28, 1972.* This work was supported by Research Grant AM-13202 from the National Institutes of Health.

[1] Abbreviations used are: MES, 2-(*N*-morpholinoethanesulfonic acid); MPD, 2-methyl-2,4-pentanediol; P, PO_4^{3-}; HP, HPO_4^{2-}; H_2P, $H_2PO_4^-$; PP, $P_2O_7^{4-}$; HPP, $HP_2O_7^{3-}$; H_2PP, $H_2P_2O_7^{2-}$.

TABLE I: Purification of Inorganic Pyrophosphatase (Based on 50 lb of Compressed Yeast).

Step No.	Vol (ml)	Protein[a] (mg)	Total Act. (Kunitz Units)	Sp Act.[a] (Kunitz Units)	Recov (%)	Purification[a]
1	29×10^3	1.4×10^5	9.3×10^4	0.66	100	1.0
2						
70% pad	3.5×10^3	5.2×10^4	6.8×10^4	1.3	73	2.0
75% pad	1.3×10^3	2.0×10^4	1.3×10^4	0.67	14	1.0
3	950	2.7×10^4	5.2×10^4	1.95	56	3.0
4	480	3.1×10^3	2.4×10^4	7.8	26	11.8
5						
251–291	690	441 (475)	2.2×10^4	49 (45)	24	74 (68)
244–250 and 292–294	150	59 (65)	2.3×10^3	39 (35)	2	59 (53)

[a] Protein measured by the biuret method. In step 5, values in parentheses were calculated using $A^{1\%}_{280\ nm} = 14.5$.

stant temperature of 30° was maintained by a circulating thermostatted water bath (Porta-Temp, Precision Scientific). The standard assay solution of 10.0 ml was 3.0 mM in tetrasodium pyrophosphate, 2.0 mM in magnesium chloride, and was run at a fixed pH of 8.20 ± 0.05.

Measurement of enzyme activity by phosphate analysis was performed as described previously (Cooperman and Mark, 1971).

Activities are reported in Kunitz units (Kunitz, 1952).

Crystallization. All operations are at 4°.

Concentrated enzyme solutions were made up by desalting the ammonium sulfate pads (specific activity 35–45) on a G-25 (water) column, lyophilizing the enzyme solution, dissolving it in the appropriate amount of water, and centrifuging it to remove small amounts of insoluble material.

METHOD 1. Enzyme solution and a 0.5 M MES–NaOH buffer (pH 6.0) were combined in a thoroughly cleaned and siliconized microscope well slide and MPD was added in small drops around the outside and carefully blended into the enzyme–buffer solution, which at the end contained 0.4 mg of enzyme, 16% MPD, and 0.03 M MES buffer in a total volume of 0.032 ml. The slides were sealed in plastic boxes containing a 20-ml reservoir solution, 0.03 M MES (pH 6.0), in 25% MPD. Crystals began to appear in about 1 week, when the MPD concentration in the well slide reached 20%, and then continued for an additional 4–6 weeks. Larger crystals resulted when samples were seeded with small crystals. In this way, crystals as large as 1.8 × 0.5 × 0.1 mm have been obtained.

METHOD 2. In some cases small amounts of material (lacking inorganic pyrophosphatase activity) precipitated out of solution at an MPD level of 10%. This material was removed by centrifugation, 20–30 μl of supernatant were placed in microscope well slides, and the slides were sealed as before, with a reservoir containing buffer and 16% MPD. Crystals began to appear in about 1 week which were apparently isomorphous with those grown by method 1. The reason for the variation in recrystallization conditions from one preparation to another are not now understood.

Other Methods. Protein concentration was measured by the biuret method (Layne, 1957), or, on highly purified samples, by $A_{280\ nm}$ measurement, assuming an $A^{0.1\%}_{280\ nm}$ of 1.45 (Kunitz, 1952).

Polyacrylamide gel electrophoresis at pH 9.5 were performed according to the methods of Ornstein (1964) and Davis (1964), using a Canalco Model 66 apparatus. Protein was visualized by Amido Black staining. $A_{650\ nm}$ scans were obtained in the laboratory of Dr. Neville Kallenbach, using a Gilford gel scanning spectrophotometer.

Results

Purification. All operations following the toluene plasmolysis step were performed in a cold room at 4°. pH values were measured between 0 and 5°.

STEP 1: TOLUENE PLASMOLYSIS AND WATER EXTRACTION were as described by Kunitz (1952).

STEP 2: AMMONIUM SULFATE PRECIPITATION AND AUTOLYSIS were as described by Kunitz (1952), except that after discarding the 50% saturated $(NH_4)_2SO_4$ pad, pads from 70 to 75% saturated $(NH_4)_2SO_4$ were collected and worked up separately.

STEP 3: REFRACTIONATION WITH AMMONIUM SULFATE. First 30 g of $(NH_4)_2SO_4$/100 ml of autolysate was added. Then small increments of $(NH_4)_2SO_4$ were added, and the pads were collected and assayed. Those pads with the highest specific activity were pooled, dissolved in sufficient buffer I (0.03 M Tris-HCl (pH 7.5), 1 mM $MgCl_2$) so that the $A_{280\ nm}$ did not exceed 240 (solution viscosity at $A_{280\ nm} = 240$ is 1.9), and desalted on a G-25 (medium) column equilibrated with buffer I. For the preparation described in Table I, a volume of 650 ml was applied to a 51 × 9 cm column (bed volume 3250 ml, void volume 1400 ml).

STEP 4: FIRST DEAE-SEPHADEX *A*50 COLUMN. A DEAE-Sephadex A50 column (53 × 9 cm; bed volume, 3.4 l.; void volume, 1 l.) was equilibrated with buffer I. The desalted solution from step 3 (950 ml) was applied, and a linear NaCl gradient was used to elute protein from the column. The mixing vessel and reservoir initially contained 6.7 l. of buffer I, and 6.7 l. of buffer I which was 1 N in NaCl, respectively. The flow rate was 2.5 ml/min. The first 950 ml of effuent was discarded and thereafter 12-ml fractions were collected. The elution profile is shown in Figure 1. Fractions 297–318 were pooled and desalted on a G-25 medium column (51 × 9 cm) which had been preequilibrated with buffer II (0.05 M Tris-HCl–5.0 mM pyrophosphate, pH 7.05).

STEP 5: SECOND DEAE-SEPHADEX A50 COLUMN. A DEAE-Sephadex A50 column (47 × 9 cm) was equilibrated with buffer II. The desalted solution from step 4 (480 ml) was applied, and a double (NaCl, pyrophosphate) linear gradient was used to elute protein from the column. The mixing vessel

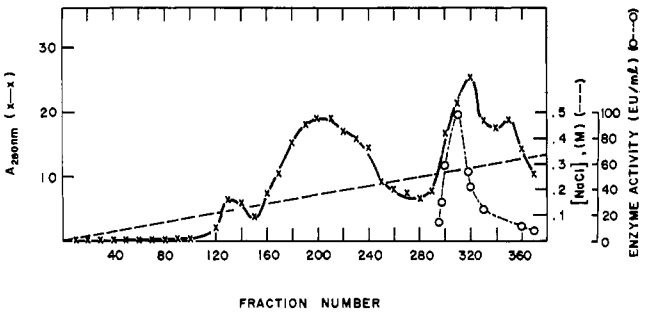

FIGURE 1: Elution profile of first DEAE-Sephadex A50 column (step 4). Dashed line (- - -) represents NaCl gradient.

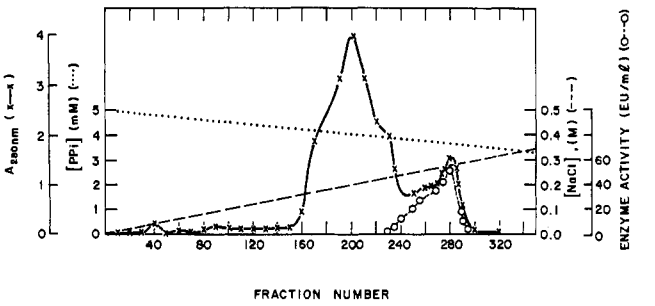

FIGURE 2: Elution profile of second DEAE-Sephadex A50 column (step 5). Dashed line (- - -) represents NaCl gradient. Dotted line (· · ·) represents pyrophosphate gradient.

and reservior initially contained 6 l. of buffer II and 6 l. of buffer III (0.05 M Tris-HCl–1.0 N NaCl, pH 7.05), respectively. The first 500 ml of effuent was discarded. The flow rate and fraction volumes were as in step 5. The elution profile is shown in Figure 2. Fractions 251–291, having almost constant specific activity equal to 44 ± 4, were pooled. Fractions 244–250 and 292–294, having an average specific activity of 35, were pooled separately. Enzyme was precipitated by bringing both pools to 90% $(NH_4)_2SO_4$ saturation. The pads were stored at −20°, and in this form were stable indefinitely. On occasion the enzyme peak was insufficiently resolved from the main protein peak in Figure 2 to give a good purification, and it was necessary to repeat step 5.

A sample of inorganic pyrophosphatase (specific activity 45), when subjected to polyacrylamide gel electrophoresis, was resolved into two bands, with an intensity ratio of 36:1.

Crystallization. Figure 3 is a photograph of crystals of the enzyme prepared from a mixed MPD–water solution (see Methods) the largest one shown having dimensions 0.66 × 0.17 × 0.06 mm. These crystals are considerably larger than those previously obtained from mixed ethanol–water and 65–70% saturated ammonium sulfate solutions (Kunitz, 1961; Ridlington *et al.*, 1972). A separate communication on the unit cell and space group of these crystals will appear shortly.

Comments on the Purification. It has been suggested (Moe and Butler, 1972; Rapaport *et al.*, 1972; Baykov *et al.*, 1972), and demonstrated by us in an accompanying paper (Cooperman and Chiu, 1973), that free pyrophosphate binds to the enzyme. The point of the second DEAE chromatography was to utilize this binding to increase the negative charge on the enzyme, and thus specifically retard it on the anion-exchange column. Judging from Figure 2, this strategy appears to have succeeded. Rigorously deionized buffer had to be used to ensure that divalent metal ion in trace amounts would not serve as a cofactor for the enzyme and lead to hydrolysis of pyrophosphate.

The specific activity of our most purified samples (40–45 Kunitz units/mg) is similar to that reported by previous workers (Kunitz, 1952; Negi and Irie, 1971; Ridlington *et al.*, 1972). In addition, the specific activity is virtually constant across the enzyme peak in step 5. From the gel electrophoresis scan we can estimate our preparation to be about 97% pure.

pH-Stat Assay. a. THEORY. Under the assay conditions, there are four dominant species in solution MgPP, HPP, Mg^{2+}, and HP and they are linked by eq 1 and 2. Although

$$Mg^{2+} + HPP^{3-} \rightleftharpoons MgPP^{2-} + H^+ \quad (1)$$

$$H_2O + MgPP^{2-} \xrightarrow[\text{pyrophosphatase}]{\text{inorganic}} 2HP^{2-} + Mg^{2+} \quad (2)$$

there is no net uptake or release of protons directly associated with hydrolysis step 2, Mg^{2+} released in this step will complex with excess pyrophosphate with concommitant release of a proton into solution, thus coupling proton release to pyrophosphate hydrolysis. When pyrophosphate concentration falls to the level of Mg^{2+} concentration, proton release will come to a virtual halt, although hydrolysis will proceed to completion.

A complete treatment of the assay system requires consideration of four additional species present in minor amounts: PP, MgHPP, MgHP, and H_2P.[2] The total of eight species are linked by five equilibrium expressions and three conservation equations (3–5): $K_1 = (H)(PP)/(HPP)$; $K_2 = (MgPP)/[(Mg)(PP)]$; $K_3 = (H)(HP)/(H_2P)$; $K_4 = (MgHP)/[(HP)(Mg)]$; $K_{12} = (MgHPP)/[(Mg)(HPP)]$.

$$(PP)_T = (PP)_t^* + \frac{(P)^*}{2}t \quad (3)$$

where $(PP)_t^* = (PP)_t + (HPP)_t + (MgPP)_t + (MgHPP)_t$ and $(P)_t^* = (HP)_t + (H_2P)_t + (MgHP)_t$.

$$(Mg^{2+})_T = (Mg^{2+})_t + (MgPP)_t + (MgHPP)_t + (MgHP)_t \quad (4)$$

$$(\Delta H)_t = (MgHPP)_0 + (HPP)_0 + 2(PP)_T - [(MgHPP)_t + (HPP)_t + 2(PP)_t^*] - [(HP)_t + (MgHP)_t + 2(H_2P)_t] \quad (5)$$

Equation 5 expresses the conservation of acidic protons. $(\Delta H)_t$ refers to the concentration of protons released into solution, as measured by the amount of NaOH solution added to maintain constant pH,[3] and the subscripts 0 to t refer to zero time and to time equal to t, respectively. The first group of three terms gives the total concentration of acidic protons in reagents at zero time. The term $2(PP)_T$ is included because two acidic protons from water are introduced for every pyrophosphate molecule hydrolyzed. The second group of three terms gives the total concentration of acidic protons in unreacted starting material at time t. The third group of three terms gives the total concentration of acidic protons in products at time t.

[2] H_2PP, P, MgP, and Mg_2PP are present in only negligible quantities.

[3] The derivation presented neglects the small dilution resulting from NaOH addition.

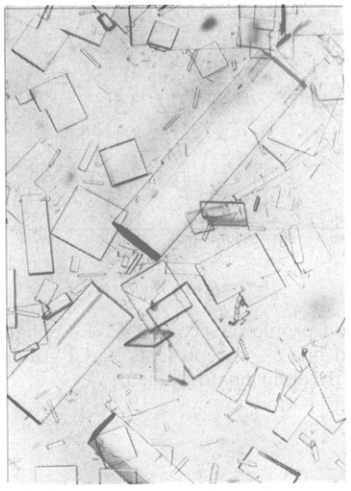

FIGURE 3: Crystals of inorganic pyrophosphatase grown from mixed MPD–water solution.

Using the equilibrium constants defined above, we can solve for $(\Delta H)_t$ as a function of $(P)_t^*$ and $(Mg^{2+})_t$

$$(\Delta H)_t = \frac{(P)_t^*}{2} \times \left[\frac{1}{1 + \frac{K_1}{(H^+)}L_t} - \frac{2}{1 + \frac{K_3}{(H^+)}[1 + K_4(Mg^{2+})_t]}\right] + (PP)_T \frac{\frac{K_1}{(H^+)}(L_t - L_0)}{\left[1 + \frac{K_1}{(H^+)}L_0\right]\left[1 + \frac{K_1}{(H^+)}L_t\right]} \quad (6)$$

where $L_{0,t} = [1 + K_2(Mg^{2+})_{0,t}]/[1 + K_{12}(Mg^{2+})_{0,t}]$. Since $(Mg^{2+})_t$ is itself a function of $(P)_t^*$ (see Appendix), specifying $(P)_t^*$ allows a calculation of $(\Delta H)_t$. The results of such a calculation are shown in Figure 4, using the values for the equilibrium constants summarized in Table II. The important feature of this plot is that in the initial phase of hydrolysis, when $(PP)_t^* > (Mg^{2+})_T$, $(\Delta H)_t$ is proportional to $(P)_t^*$, so that in this region the rate of hydrogen ion release is a valid measure of inorganic pyrophosphatase activity.

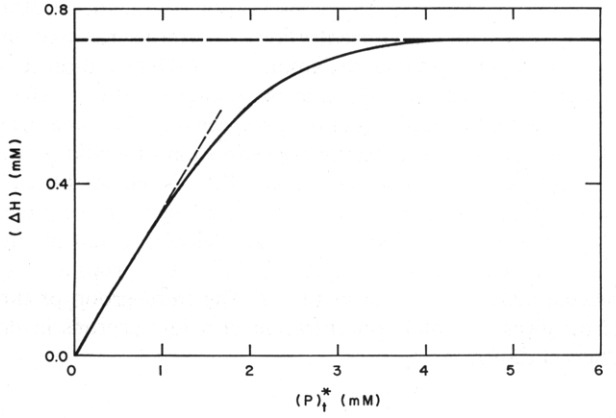

FIGURE 4: Theoretical curve of $(\Delta H)_t$ *vs.* $(P)_t^*$ for standard assay conditions eq 6. Dashed line represents continuation of initial slope. Plateau $(\Delta H)_t$ value: 0.73×10^{-3} M.

TABLE II: Equilibrium Constants for pH-Stat Assay.

$K_1 = 3 \times 10^{-10}$ M	Wolhoff and Overbeck (1959)
$K_2 = 5 \times 10^5$ M^{-1}	Vasil'ev (1957)
$K_{12} = 2.2 \times 10^3$ M^{-1}	Vasil'ev (1957) and Lambert and Watters (1957)
$K_3 = 6.3 \times 10^{-8}$ M	Grzybowski (1958)
$K_4 = 2 \times 10^2$ M^{-1}	Greenwald *et al.* (1940) and Smith and Alberty (1956)

General eq 6 is sufficiently complex that it may not be entirely clear why $(\Delta H)_t$ is proportional to $(P)_t^*$. This can be understood as follows. In the initial phase of hydrolysis, $(PP)_t^*$ is both greater than $(Mg^{2+})_T$ and much greater than the apparent dissociation constant for MgPP complex formation (23 μM, from Table II) so that it is a reasonable approximation that during this phase all Mg^{2+} is bound to pyrophosphate. With this approximation, the only species whose concentrations change in this phase are HPP, PP, HP, and H_2P. Using a derivation similar to that presented above for the general case, one can show that for this approximation $(\Delta H)_t$ is given by

$$(\Delta H)_t = \frac{(P)_t^*}{2}\left[\frac{1}{1 + \frac{K_1}{(H^+)}} - \frac{2}{1 + \frac{K_3}{(H^+)}}\right] \quad (7)$$

The proportionality constant predicted by eq 7, 0.386, is very close to the initial slope obtained in Figure 4, 0.345, from general eq 6.

b. RESULTS. The standard assay conditions are: 3 mM PP_i, 2 mM Mg^{2+}, at pH 8.2. pH 8.2 was chosen because falling almost midway between pK_1 and pK_3, it gives a maximal value of $(\Delta H)_t/(P)_t^*$, is not too far off the pH optimum for hydrolysis, and leads to no problems of enzyme instability.

Since substrate concentration is very high compared to K_m (Moe and Butler, 1972), a pseudo-zero-order proton release is obtained when $(\Delta H)_t$ is proportional to $(P)_t^*$; *i.e.*, for the first 0.4 ml of 0.01 N NaOH added.[4] The pseudo-zero-order rate measured in this way is strictly proportional to enzyme concentration. For reasons we do not fully understand, there were occasionally days when a plot of volume change *vs.* enzyme concentration did not extrapolate to zero, *i.e.*, there was a small but definite background volume change. Careful use of this assay thus requires that this background be determined each day and subtracted from the observed rates.

In order to compare activity measured in this manner with that measured by phosphate analysis under the standard Kunitz assay (Kunitz 1952), identical enzyme aliquots were assayed by both methods. It was found that one Kunitz unit corresponds to a volume change of 0.73 ml of 0.01 N NaOH/min. This conversion factor was used to express measured pH-Stat activities as Kunitz activities throughout this paper.

The measured conversion factor agrees well with the values calculated from eq 6. One Kunitz unit corresponds to the formation of 32 μmol of phosphate/min at pH 7.2, 30°. The volume change corresponds to 7.3 μmol of H^+ formed, or 21.2 μmol of phosphate formed per min at pH 8.2, 30° (Figure 4). The ratio of the activity at pH 8.2 to that at pH 7.2 is thus

[4] The autoburet is accurate to at least 0.002 ml.

0.66, which is consistent with previous results (Kunitz, 1952; Moe and Butler, 1972; Rapoport *et al.*, 1972). The measured final $(\Delta H)_t$ at pH 8.2, 0.80 ml, is also in reasonably good accord with the predicted value, 0.73 ml, from eq 6.

The advantages of the pH-Stat assay of inorganic pyrophosphatase activity over the traditional method of analyzing aliquots for phosphate are that it is rapid, easy to do (no stock reagent solutions are needed) and, as with any continuous assay, yields more data, permitting more accurate determination of rates. As such, it has been especially useful in situations where a standard assay can be used: *e.g.*, monitoring activity coming off a column or, as described in an accompanying paper, measuring the effect of various electrophilic reagents on enzymatic activity. A severe limitation on its usefulness for such studies as pH or inhibitor effects is that the initial $(\Delta H)_t/(P)_t^*$ ratio will vary, for example, as inhibitor concentration changes, and this ratio must of course be known before the pH-Stat assay can be used to measure enzyme activity. Although it is possible in principle to calculate this ratio, as we have done in this paper for a very favorable case (high $(\Delta H)_t/(P)_t^*$ and relative insensitivity of the calculation to the values of all but two constants, K_1 and K_3), in general, given the uncertainties in published equilibrium constants, such a calculation will yield only approximate results. Accurate work would require a direct experimental determination which certainly limits the appeal of the pH-Stat method.

We should point out that although we have chosen to use rather high concentrations of pyrophosphate in the standard pH-Stat assay, it should be possible to use much lower concentrations (down to 3×10^{-5} M) by substituting a 0.25-ml buret (which can be read to 0.0001 ml) for the 2.5 ml we have used, and by using more dilute NaOH.

Acknowledgment

We thank Mrs. Maryann Appleby for technical assistance in the early stages of the purification work.

Appendix

Exact Equation for $(Mg^{2+})_t$. Using eq 3 and 4 and the equilibrium constants yields a cubic equation (eq 9) for (Mg^{2+}) where the coefficients

$$A(Mg^{2+})^3 + B(Mg^{2+})^2 + C(Mg^{2+}) - D = 0 \qquad (9)$$

have the following values: $A = 1$, $B = W + X + (PP)_T - (Mg)_T + 0.5(P)_t^*$, $C = XW + X[(PP)_T - (Mg)_T] - W(Mg)_T - [(X/2) - W](P)_t^*$, $D = XW(Mg)_T$, and $X = [K_3 + (H^+)]/K_3K_4$ and $W = [K_1 + (H^+)]/[K_{12}K_2 + K_{12}(H^+)]$.

References

Baykov, A. A., Braga, E. A., and Avaeva, S. M. (1972), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 21*, 80.

Benkovic, S. J., and Dunikoski, L. K. (1971), *J. Amer. Chem. Soc. 93*, 1526.

Benkovic, S. J., and Sampson, E. J. (1971), *J. Amer. Chem. Soc. 93*, 4883.

Butler, L. G. (1971), *Enzymes 4*, 529.

Cooperman, B. S., and Chiu, N. Y. (1973), *Biochemistry 12*, 1670.

Cooperman, B. S., and Mark, D. H. (1971), *Biochim. Biophys. Acta 252*, 221.

Davis, B. J. (1964), *Ann. N. Y. Acad. Sci. 121*, 404.

Greenwald, I., Redish, J., and Kibrick, A. C. (1940), *J. Biol. Chem. 135*, 65.

Grzbyowski, A. K. (1958), *J. Phys. Chem. 62*, 555.

Kirby, A. J., and Younas, M. (1970), *J. Chem. Soc. B*, 1165.

Kunitz, M. (1952), *J. Gen. Physiol. 35*, 423.

Kunitz, M. (1961), *Arch. Biochem. Biophys. 92*, 270.

Lambert, S. I., and Watters, J. I. (1957), *J. Amer. Chem. Soc. 79*, 5606.

Layne, E. (1957), *Methods Enzymol. 3*, 450.

Lloyd, G. J., and Cooperman, B. S. (1971), *J. Amer. Chem. Soc. 93*, 4883.

Mildvan, A. S. (1970), *Enzymes 2*, 466.

Moe, O. A., and Butler, L. G. (1972), *J. Biol. Chem. 247*, 7308.

Negi, T., and Irie, M. (1971), *J. Biochem. (Tokyo) 70*, 165.

Ornstein, L. (1964), *Ann. N. Y. Acad. Sci. 121*, 321.

Rapaport, T. A., Höhne, W. E., Reich, J. G., Heitmann, P. and Rapaport, S. M. (1972), *Eur. J. Biochem. 27*, 237.

Richards, F. M., and Wyckoff, H. W. (1971), *Enzymes 4*, 647.

Ridlington, J. W., Yang, Y., and Butler, L. G. (1972), *Arch. Biochem. Biophys. 153*, 714.

Schachman, H. (1952), *J. Gen. Physiol. 35*, 451.

Smith, R. M., and Alberty, R. A. (1956), *J. Amer. Chem. Soc. 78*, 2376.

Vasilev, V. P. (1957), *Zh. Fiz. Khim. 31*, 692.

Wolhoff, J. A., and Overbeck, M. Th. G. (1959), *Recl. Trav. Chim. Pays-Bas 78*, 759.