Muscle Pyrophosphatases

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Some of the fundamental properties of pyrophosphatases in rabbit skeletal muscle were studied. pH optima for these enzymes occurred at pH 5.2 and 7.4. Acid pyrophosphatase was found to be associated with muscle particulate components sedimented at 18,000 G, while a neutral one was present in the soluble fraction. Sonic disruption of the particulate fractions inactivated the former and fractionation with organic solvents and DEAE cellulose column chromatography resulted in total destruction of the latter. The neutral pyrophosphatase could be partially purified only by fractionation with ammonium sulfate and is active only in the presence of magnesium ions. Magnesium ions are specifically required not only for activation but for stabilization of the neutral, but not for the acid, pyrophosphatase. Data on the rates of the reaction or inactivation under various conditions (pH, temperature, substrate, and modifiers) for the two enzymes are presented.

Hashimoto *et al.* (1959) pointed out that inorganic pyrophosphate, when incubated with myosin A in 0.5*M* KCl at pH 7.0 and 30° C., was hydrolyzed to orthophosphate more rapidly than was expected when spontaneous reversion of this substance occurred under the same conditions. They interpreted the phenomenon as the result of the action of inorganic pyrophosphatase (PPase) mixed in their myosin A preparations. Inorganic PPase has been known to be widely distributed in living organisms as well as in various organs in tissue fluids (Bauer, 1936; Forti, 1961; Lohman, 1928; Naganna *et al.*, 1955; Rafter, 1958; Schneider, 1948; Swanson, 1952), and some of them were obtained in highly purified form or as crystals (Bailey and Webb, 1944; Kunitz, 1952; Pynes and Younathan, 1967). However, little is known about the PPase in mammalian muscle.

In the meat industry, inorganic polyphosphates are commonly used to promote water-holding or binding properties of cured meat products such as hams and sausages. It is also recognized empirically that the effect of polyphosphates added to meat diminishes with time. Among polyphosphates used in the meat industry, only pyrophosphate has been found to be essential on the basis of its specific interaction with muscle structural proteins (Yasui *et al.*, 1964). It is, therefore, of practical importance to study PPase in muscle and its destructive effect on pyrophosphate in meat.

The objective of this research was to isolate PPase from rabbit skeletal muscle and obtain information on its behavior.

MATERIALS AND METHODS

Skeletal muscle tissue was obtained from rabbit dorsal lumbar and pelvic limb muscle immediately after exsanguination.

Preparation of Subcellular Fraction. The separable fat and connective tissue were removed and the muscle was ground in a chilled meat grinder. The ground tissue was dispersed in 6 volumes of 0.25*M* sucrose and blended in a cold Waring Blendor for 3 minutes. The homogenate was fractionated into nuclei plus cellular debris (800 G), mitochondria (8500 G), submicroscopic particles (18,000 G), microsomes (54,000 G), and soluble fraction by the differential centrifugation method of Schneider (1948) and Hogeboom (1955). The sediments from the latter four fractions were used to measure enzymic activity.

Direct Preparation of Neutral PPase. Since the neutral PPase mostly resides in the water-soluble fraction, it was obtained directly from a water extract of rabbit muscle. The extract was dialyzed against neutralized water with 5 m*M* $MgCl_2$ at 3° C. for 24 hours, and the insoluble precipitates were removed by centrifugation for 20 minutes at 9000 r.p.m. The supernatant fluid was used for experiments as a crude enzyme preparation.

Measurement of Activity. Hydrolysis of pyrophosphate by acid and neutral PPases was measured in the following reaction mixtures: 40 m*M* citrate or acetate buffer (pH 5.3), 0.25*M* sucrose, 1 m*M* pyrophosphate, and 0.5 mg. of protein per ml. for the former, and 25 m*M* tris-maleate buffer (pH 7.0), 5 m*M* $MgCl_2$, 1 m*M* pyrophosphate, and 0.2 mg. of protein per ml. for the latter. The reaction was carried out at 30° and 25° C., respectively, for acid and neutral enzymes. At measured intervals of time, 2 ml. of 10% trichloroacetic acid was pipetted into 2 ml. of reaction mixture in the test tube. The PPase activity in the reaction mixture was determined by measuring the liberated orthophosphate according to the method of Martin and Doty (1949).

Chromatography and Gel Filtration. The diethylaminoethyl (DEAE) cellulose (Brown Co., Calif.) and the crosslinked dextran gel (Sephadex G-100, Pharmasia, Upsala, Sweden) were used for column chromatography and gel filtration, respectively. Before use, DEAE cellulose was washed successively on a Büchner funnel with 1*N* NaOH, deionized water, and starting buffer for the elution (50 m*M* tris-maleate buffer at pH 7.0 and 5 m*M* $MgCl_2$) and placed on the column. The

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crosslinked dextran gel was washed 8 to 12 hours before use and equilibrated with the starting buffer (25 m*M* tris-maleate at pH 6.3, 6 m*M* $MgCl_2$, and 10 m*M* KCl). Usually 10 to 15 mg. of protein, equilibrated by dialysis against the starting buffers, was chromatographed by applying stepwise ascending KCl gradient, or was gel-filtered.

The effluent was collected by a Toyo weight system fraction collector SF-200A, and examined at 280 mμ in a Hitachi spectrophotometer EPU-2A. All experiments were carried out at 2° to 4° C. Total protein recovered was calculated to be more than 95%.

Ultracentrifugal Analysis. The ultracentrifugal sedimentation patterns were determined with a Spinco Model E ultracentrifuge at 56,100 r.p.m. and 4° C.

Disruption of Particles. Sonic disruption of the particulate fraction was carried out at 1° C. in the Kubota sonic oscillator (10 kc. per second).

Protein Concentration. The concentration of protein was calculated by multiplying the nitrogen content as determined by a micro-Kjeldahl procedure by a factor of 6.25, or was estimated directly by the biuret reaction.

RESULTS

Localization of Enzymes and Their Activities at Different pH Values. The activities of PPase with each fraction obtained from muscle homogenate were studied at different pH values. Figure 1 shows that there are two kinds of PPases in muscle, one existing in particle fractions and the other in the soluble fraction. The PPase associated with particles shows its optimal activity at pH 5.2 and is richest in the sediments at 18,000 G. The other PPase in the soluble fraction exhibits its pH optimum at about 7.0, and its activity is greatly enhanced by the addition of $MgCl_2$.

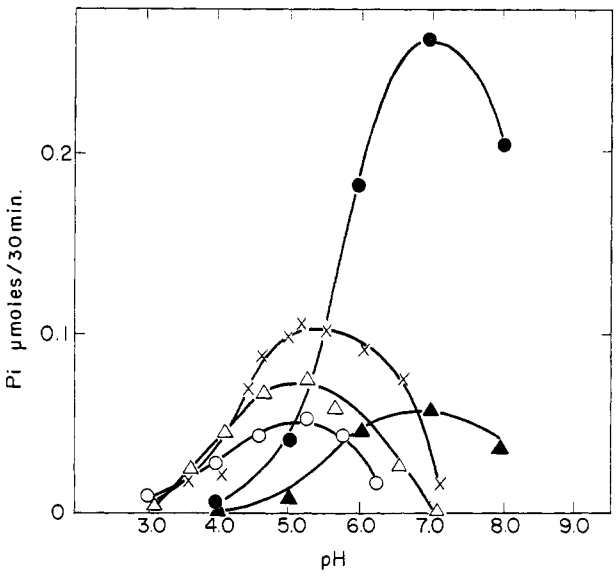

Figure 1. Effect of pH on pyrophosphatase activity at 30° C. of subcellular fractions of muscle

○ Mitochondria fraction
× Submicroscopic particle fraction
△ Microsomal fraction
● Soluble fraction with 2.3 m*M* $MgCl_2$
▲ Soluble fraction without $MgCl_2$

40 m*M* citrate buffer, pH 3.0 to 6.0
40 m*M* tris-maleate buffer, pH 6.0 to 8.0
Incubation period 30 min.

Table I. Effect of Sonic Oscillation on Acid PPase

	Control	Sonicated 5 min.	Sonicated 10 min.	Sonicated 30 min.
Activity[a] with MgCl (2.5 m*M*)	—	0.001	0.002	—
Activity[a] without $MgCl_2$	0.008	0.001	0.002	0.002

[a] Activity=μmoles orthophosphate/minute/mg. protein.

Table II. Ammonium Sulfate Fractionation of Crude Neutral PPase Preparation[a]

	Activity[b]	Index
Crude prep.	0.053	100
Sediments at 0.45 sat.	0.065	122
Sediments at 0.65 sat.	0.236	445
Supernatant at 0.65 sat.	0.021	40

[a] 5 m*M* $MgCl_2$ always present in system.
[b] Activity same as in Table I.

Purification of PPases. To purify the acid PPase, it is presumably necessary to isolate the enzyme from particles. An attempt was made to disrupt particles by applying sonic oscillation. As shown in Table I, this attempt failed to extract the enzyme. Contrary to the case with liver PPase (Nordlie and Lardy, 1961), neither increase in activity nor activation by $MgCl_2$ could be observed, but, on the contrary, activities decreased through this treatment. Therefore, well-washed submicroscopic particles were used for acid PPase assay throughout this study.

Figure 1 indicates that neutral PPase residues were in the soluble fraction. It seems much more convenient for further purification if this PPase can be extracted directly from muscle homogenate. Another preparation method was then developed as described. The crude enzyme thus prepared possessed activity two times higher than that of its mother water extract, only after dialysis against water for 24 hours (0.03 to 0.05 μmole of orthophosphate per minute per mg. of protein).

The crude enzyme was purified by an ammonium sulfate fractionation method in the presence of 5 m*M* $MgCl_2$ (Table II). The specific activity of the crude enzyme was taken as 100. Protein precipitated within 0.45 to 0.65 saturation of ammonium sulfate indicated the highest activity (455), resulting in a 4.5-fold increase in purity of the enzyme. The enzyme is stable only in the presence of Mg ions. Procedures performed without Mg ion inevitably brought about inactivation. Experiments using organic solvents, ethanol, isobutyl alcohol, acetone, and ethyl Cellosolve to replace ammonium sulfate were unsuccessful. All the solvents brought about deactivation or failed to obtain invariable results, even when the procedures were carried out at −5° C. and in the presence of 5 m*M* $MgCl_2$.

The fraction precipitated between 0.40 and 0.65 saturation of ammonium sulfate, however, revealed at least three components in its ultracentrifugal pattern. In order to fractionate further, it was chromatographed or gel-filtered through DEAE or Sephadex G-100 col-

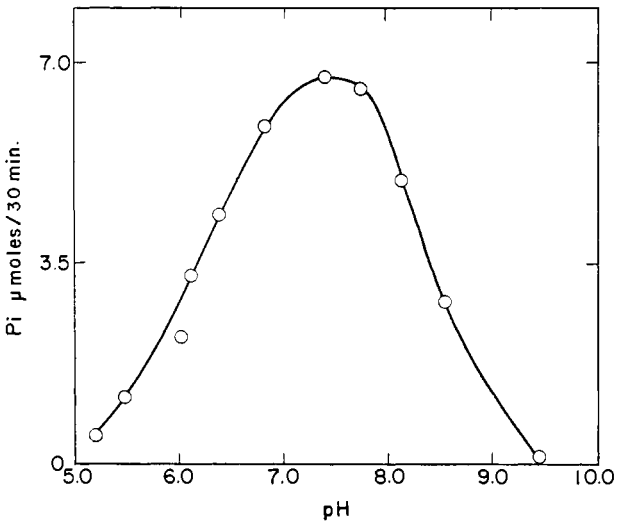

Figure 2. Effect of pH on partially purified neutral PPase at 25° C.

25 m*M* tris-maleate buffer, pH 5.0 to 8.0
25 m*M* glycine-NaOH buffer, pH above 8.0
Incubation period 30 min.

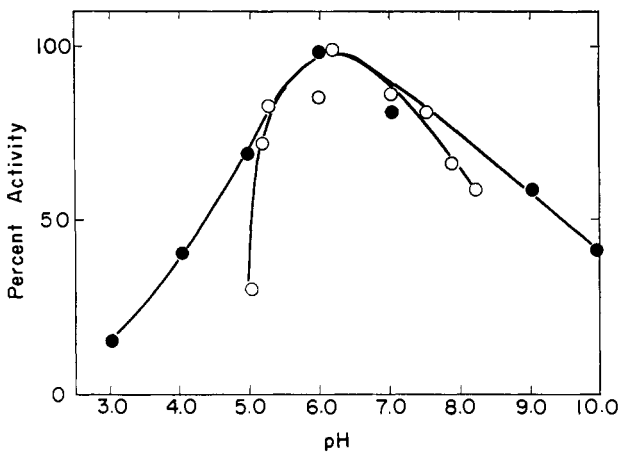

Figure 3. Stability of acid and neutral PPases at different pH values and 1° C.

● Acid PPase after storage for 72 hours
○ Neutral PPase (crude enzyme) with 5 m*M* $MgCl_2$ after storage for 60 hours

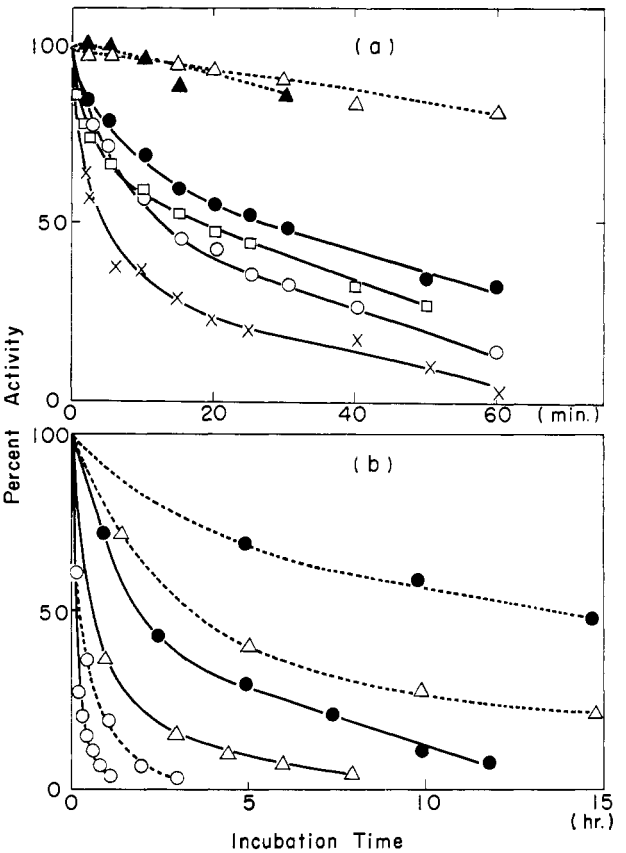

Figure 4. Stability of PPases at different temperatures

a. Acid PPase at pH 5.2, pH adjusted by:

● 40 m*M* citrate buffer
×, △ 40 m*M* acetate buffer
□ 40 m*M* EDTA buffer
○, ▲ Without buffer

—— 50° C.
– – – 30° C.

b. Crude neutral enzyme at pH 7.0

● 30° C.
△ 40° C.
○ 50° C.

—— Without $MgCl_2$
– – – With 5 m*M* $MgCl_2$

umns. Although the fraction was separated into three main peaks by stepwise KCl gradient from 0 to 1.5*M*, all peaks showed little activity. The results of gel filtration by Sephadex G-100 were less effective than by chromatography; it did not separate the fraction, and the activity decreased. Then, the partially purified neutral PPase by ammonium sulfate fractionation served for this experiment. pH dependence of the enzymic activity of this preparation in the presence of $MgCl_2$ is presented in Figure 2 and its optimum pH is shown to be 7.4.

Stability of PPases. Stability of acid and neutral PPases at different pH values and temperatures is shown in Figures 3 and 4. The acid enzyme is stable at pH 6.0 (Figure 3) after 60 hours' storage at 1° C., and the neutral enzyme is stable at pH 6.3 in the presence of 5 m*M* $MgCl_2$. Both enzymes are inactivated slowly at 30° when preincubated at pH values of their optimal activities (Figure 4, *a* and *b*). The inactivation rate of the neutral enzyme was markedly retarded by the presence of $MgCl_2$ (Figure 4, *b*), while that of the acid enzyme appeared to be affected by the kind of buffers used (Figure 4, *a*), acetate buffer being promotive and citrate inhibitory. However, the inactivation rate at 30° C. of acid PPase was so slow that the effect of the buffer used failed to be apparent within 30 to 60 minutes. Times required for 50% inactivation of acid PPase at 50° C. were 12.0 minutes for control, 4 minutes for the acetate buffer, 18.5 minutes for the ethylene(dinitrole)tetraacetate (EDTA) buffer, and 26.0 minutes for the citrate buffer (Figure 4, *a*). When the crude enzyme preparation of the neutral PPase was incubated without $MgCl_2$ at 50°, 40°, and 30° C., 5, 40, and 120 minutes, respectively, were required for 50% deactivation, but in the presence of 5 m*M*, $MgCl_2$ 20, 210, and 810 minutes were needed, respectively (Figure 4, *b*).

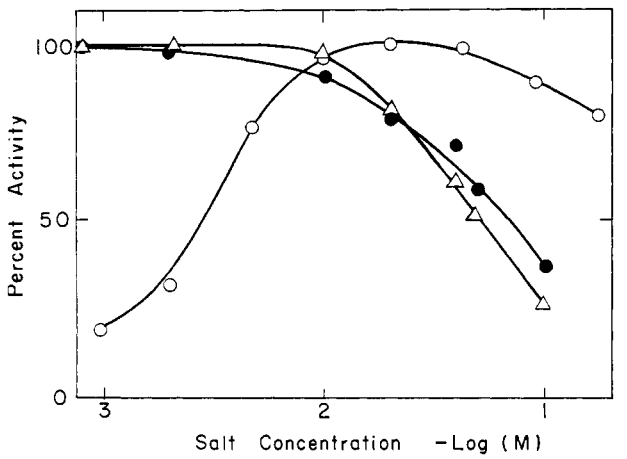

Figure 5. Effect of $MgCl_2$ and $CaCl_2$ on muscle PPases

○ Magnesium effect on neutral PPase at pH 7.0 and 25° C.
● Magnesium effect on acid PPase at pH 5.2 and 30° C.
△ Calcium effect on acid PPase at pH 5.2 and 30° C.

Effect of Divalent Metal Ions on Acid and Neutral PPases. The inhibition of the acid PPase and the activation of the neutral PPase by $MgCl_2$ are shown in Figure 5. $MgCl_2$ in concentration up to 1 m*M* exerts no inhibitory effect on the acid PPase. There is about 10% inhibition at 5 m*M* $MgCl_2$ and 30% at 20 m*M* $MgCl_2$, while higher concentrations of $MgCl_2$ produced greater inhibition. At such higher concentrations the magnesium-activated neutral enzyme also was inhibited.

The neutral enyzme is activated to the maximum extent with 10 m*M* $MgCl_2$. Substitution of $MgSO_4$ or $(CH_3COO)_2Mg$ for $MgCl_2$ showed the same results, suggesting that only Mg^{2+} is necessary for activation or stabilization of the enzyme.

The effect of $CaCl_2$ on the activity of acid PPase was almost the same as that of $MgCl_2$ (Figure 5). Similar observations were made with other animal tissues (Seal and Binkley, 1957) as well as vegetable tissues (Naganna *et al.*, 1955). On the other hand, the neutral PPase is extremely specific for magnesium ion and does not allow replacement by other metals tested—e.g., Ca^{2+}, Mn^{2+}, Co^{2+}, and Ba^{2+}. As far as this experiment is concerned, Mg^{2+} is the only specific metal ion for the activation and stabilization of the enzyme.

Effect of Monovalent Metals and EDTA. Monovalent metals such as Na^+ or K^+ inhibit both enzymes (Figure 6, *a* and *b*). Na^+ and K^+ showed almost the same effect on the acid PPase, whereas the neutral PPase was inhibited more strongly by K^+ than by Na^+, although the ultimate inhibition attained by the two ions was 60%.

Figure 7 illustrates the inhibitory effect of EDTA. The result relating to the neutral PPase clearly shows that the inhibition was merely due to the magnesium-chelating action of EDTA. The acid PPase, on the other hand, was inhibited slightly at 10 m*M* EDTA. Further addition of EDTA gives rise to higher inhibition, as seen in the inhibition by divalent metals. The activating effect of EDTA reported on a PPase from other sources (Swanson, 1952) could not be observed in this experiment.

Substrate Specificity. The action of acid and neutral enzymes on various polyphosphates was measured to determine their ability to split other substrates. Figure 8, *a* and *b*, summarizes the results of these measurements on ATP, hexametaphosphate, and β-glycerophosphate. The data show that the partially purified neutral PPase preparation possessed a high specificity to pyrophosphate. Other organic and inorganic phosphates were slightly hydrolyzed into orthophosphates (Figure 8, *b*). The acid PPase preparation was also fairly specific for pyrophosphate. However, it hydrolyzed ATP and hexametaphosphate at lower rates.

Substrate Effect on Neutral PPase. Chebuliez and Bretagna (1949) pointed out that, in the case of PPases whose activity is highly magnesium-dependent, the true substrate of the enzyme appears to be neither Na (or K)$_4P_2O_7$ nor $(HP_2O_7)^{3-}$ but the complex anion, $(MgP_2O_7)^{2-}$. To ascertain whether the substrate of the muscle neutral PPase is the complex anion, the effects of substrate concentrations were studied (Figure 9). An

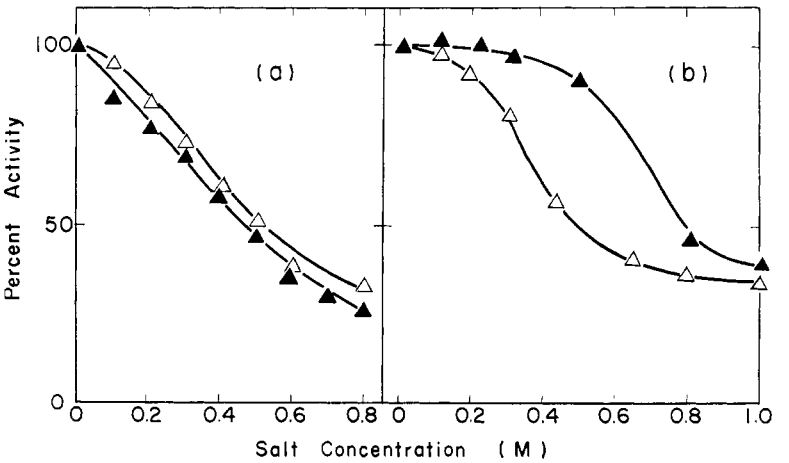

Figure 6. Effect of KCl and NaCl on acid (*a*) and neutral (*b*) PPase

△ KCl
▲ NaCl

5 m*M* $MgCl_2$ present in system for neutral PPase

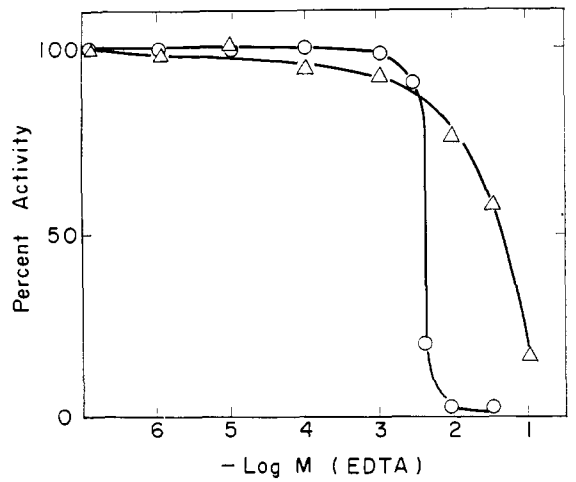

Figure 7. Effect of EDTA on muscle PPases

△ Acid PPase
○ Neutral PPase

Conditions as in Figures 5 and 6

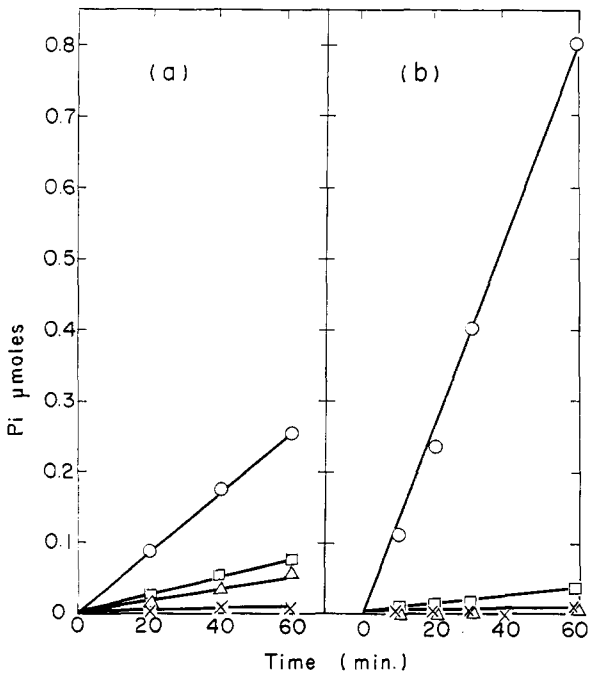

Figure 8. Hydrolysis of different phosphates by acid (*a*) and neutral (*b*) PPases

○ Pyrophosphate
△ ATP
□ Hexametaphosphate
× β-Glycerophosphate

Conditions as in Figures 5 and 6

increase of pyrophosphate concentration in the reaction mixture at pH 7.0 and in the presence of 5 m*M* $MgCl_2$ accelerated the rate of its enzymic hydrolysis as long as $MgCl_2$ was present in excess ($[Mg^{2+}]/[P_2O_7^{4-}] > 5$). When, however, $MgCl_2$ was present in fivefold molar concentration or less in relation to pyrophosphate, the addition of more pyrophosphate inhibited the reaction, as with EDTA (Figure 9). In a second experiment designed to study the effect of initial substrate concentration, the $MgCl_2$ and pyrophosphate concentration

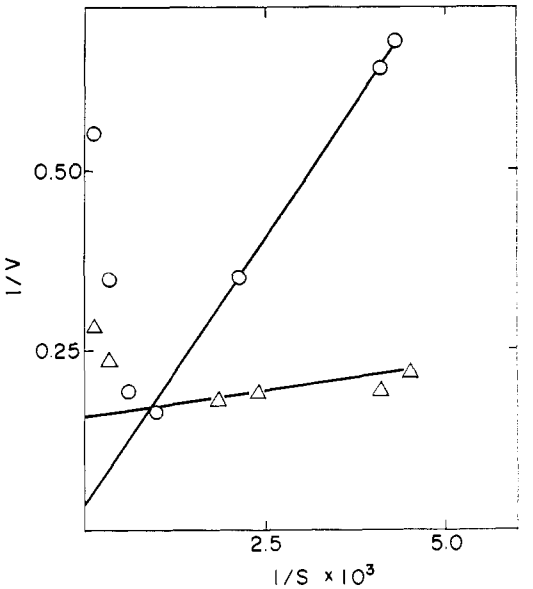

Figure 9. Effect of substrate concentrations on neutral PPase at 25° C. and pH 7.0

△ $[Mg^{2+}]/[(P_2O_7)^{4-}] = 5$
○ $[Mg^{2+}] = 5$ m*M*

v. μmoles orthophosphate/minute/mg. protein
s. Pyrophosphate, mg./liter

was varied in order to maintain a nearly optimum activation at the constant $[Mg^{2+}]/[P_2O_7^{4-}]$ ratio of 5.0 (Figure 9). There was a gradual increase in the rate of hydrolysis as the initial substrate concentration was raised to 20 m*M* and then gradually decreased. Since Figure 9 shows the plot of $1/v$ against $1/s$ in accordance with the linear equation of Lineweaver and Burk (1934):

$$\frac{1}{v} = \frac{Km}{V} \cdot \frac{1}{s} + \frac{1}{V} \qquad (1)$$

where v = initial rate of hydrolysis, V = maximum rate of hydrolysis, s = initial concentration of substrate, and Km = Michaelis constant. Using linear portions of the two curves in Figure 9, we obtain $Km = 1.17 \times 10^{-4}$ M/L when $[Mg^{2+}]/[P_2O_7^{4-}] = 5$ and 5×10^{-3} M L when $[Mg^{2+}]$ is fixed at 5 m*M*. Clearly, the affinity of the substrate for the enzyme is relatively very large in the former case, but retarded in the latter. The results agreed well with those on other magnesium-dependent PPases (Swanson, 1952) and favor the complex anion-substrate theory.

DISCUSSION

There are at least two types of PPases in muscle tissues—acid and neutral. The neutral PPase can easily be characterized by its absolute dependence on magnesium ion, pH dependency of its activity, and reaction kinetics. Perhaps its most important property is its very high affinity to the substrate in the presence of magnesium (Figure 9), although the enzyme partly resembles neutral or alkaline PPases already reported (Balocco, 1962; Gordon, 1950; Schneider, 1948; Seal and Binkley, 1957; Swanson, 1952). It is, in addition, of importance that magnesium ions act not only as activators but also as stabilizers (Figure 4, *b*). The reason that this stabilizing effect of magnesium disap-

pears at some stage of purification is unknown and merits future study in relation to the purification procedures.

In marked contrast to the neutral PPase the acid enzyme shows no requirement for added magnesium ions, and divalent metals in high concentrations partially inhibit its activity (Figure 5). The phosphatase activity tested with various phosphates (Figure 8, *a*) indicates that the activity is probably the same one described by several investigators in rat liver homogenates (Nordlie and Lardy, 1961; Rafter, 1958; Swanson, 1952). The nonrequirement of metal ions probably results from metal ions bound to the particles.

The mechanism associated with the destruction of pyrophosphate added to meat may now be fully explained by the results presented in this paper. The pH value of muscle *post mortem* varies, ranging from ~ 5.0 to ~ 7.0 (Briskey and Wismer-Pedersen, 1961). At any pH values the PPase activity of either the acid or the neutral enzyme or the activities of both may hydrolyze pyrophosphate into orthophosphate even in the presence of high concentrations of neutral salt (Figure 6, *a* and *b*) usually used for meat curing. Once hydrolyzed to pyrophosphate, inorganic tripolyphosphate (Yasui *et al.*, 1964), which is commonly used in ham and sausage manufacture, may also be affected by these two types of pyrophosphatases in a similar way.

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