ACID AND ALKALINE INORGANIC PYROPHOSPHATASE

OF

STREPTOCOCCUS FAECIUM F24

by

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INTRODUCTION

STATEMENT OF THE PROBLEM

Microbial, plant and animal cells contain inorganic pyrophosphatase activity. Mammalian cells have as many as four different types of pyrophosphatase activities which are characterized by their pH optimum and cofactor requirements. Those plant cells examined have both an acid activity that does not require a cofactor and an alkaline, Mg^{++}-dependent pyrophosphatase activity. Most bacteria contain a single activity, which is optimum at alkaline pH and requires Mg^{++}.

Streptococcus faecium F24, however, was shown to have two activities; one at pH 5.6 which required Co^{++}, and another maximally active at pH 8.6 in the presence of Mg^{++}. The acid activity is further enhanced by the addition of L-histidine. The purpose of the research reported in this thesis was to determine the nature of these two activities. The question asked was "Are these two activities expressions of two distinct proteins or are they expressions of a single molecule under different conditions?". Distinct proteins may be associated with different cellular structures, thus, the localization of the two activities was determined. The regulation of pyrophosphatase activities was studied on the assumption that activities on distinct proteins may be regulated separately. The physical characteristics and the inactivation kinetics of the two activities were compared.

Inorganic pyrophosphatase (pyrophosphate phosphohydrolase, E.C. 3.6.1.1.), or PPase[1] activity has been observed in every cell type for which it has been examined (78). In fact, it may be possible to predict that if it is not found, the lack of activity indicates an enzymic instability under the conditions used for the assay. The presence of this enzyme is essential to prevent massive accumulation of PP which is a product of almost every biosynthetic pathway that occurs in the cell, and which is utilized in very few reactions (Table 1). The potential concentration of PP in a growing cell must be quite high. During the biosynthesis of macromolecules, one molecule of PP is released for every amino acid activated, for every subunit polymerized into RNA and DNA, and for every sugar polymerized into polysaccharide. In addition, PP is produced during the synthesis of coenzymes and sugar nucleotides, and from the activation of choline and ethanolamine, in some systems. Table 2 shows some of the many reactions that result in PP release.

PP accumulation is detrimental to the stability of the cell. The molecule is highly charged. It may chelate metal ions, thus inhibiting the activity of enzymes requiring metallic cofactors (42). Enzymes involved in macromolecule biosynthesis, such as aminoacyl t-RNA synthetases (7, 38, 76, 105), and DNA-dependent RNA polymerase (105)

[1] Abbreviations to be used in the thesis: PPase = inorganic pyrophosphatase, PP = inorganic pyrophosphate, P_i = orthophosphate, Co/Mg = ratio of Co^{++}-stimulated PPase activity to that of Mg^{++}-stimulated PPase activity, RNA = ribonucleic acid, DNA = deoxyribonucleic acid, ATP = adenosine triphosphate, ADP = adenosine diphosphate, AMP = adenosine monophosphate, NAD = nicotinamide-adenine dinucleotide, EDTA = (ethylenedinitrilo)-tetraacetic acid, DEAE = diethylaminoethyl, ATPase = adenosine triphosphatase.

TABLE I

Enzymes Utilizing Pyrophosphate as Substrate

EC Number	Systematic Name	Reaction
2.4.2.7.	AMP:pyrophosphate phosphoribosyl-transferase	PP + AMP = adenine + 5-phospho-α-D-ribosyl-pyrophosphate
2.4.2.8.	IMP:pyrophosphate phosphoribosyl-transferase	PP + IMP = hypoxanthine + 5-phospho-α-D-ribosyl-pyrophosphate
2.4.2.9.	UMP:pyrophosphate phosphoribosyl-transferase	PP + UMP = uracil + 5-phospho-α-D-ribosyl-pyrophosphate
2.4.2.10.	Orotidine-5'-phosphate:pyrophosphate phosphoribosyltransferase	PP + orotidine-5'-phosphate = orotate + 5-phospho-α-D-ribosyl-pyrophosphate
2.4.2.11.	Nicotinatenucleotide:pyrophosphate phosphoribosyltransferase	PP + nicotinate ribonucleotide = nicotinate + 5-phospho-α-D-ribosyl-pyrophosphate
2.4.2.12.	Nicotinamidenucleotide:pyrophosphate phosphoribosyltransferase	PP + nicotinamide ribonucleotide = nicotinamide + 5-phospho-α-D-ribosyl-pyrophosphate
2.4.2.14.	Ribosylamine-5-phosphate:pyrophosphate phosphoribosyl-transferase	PP + β-D-ribosylamine 5-phosphate + L-glutamate = L-glutamine + 5-phospho-α-D-ribosyl-pyrophosphate + H_2O
3.6.1.1.	Pyrophosphate phosphohydrolase	PP + H_2O = 2 P_i
4.1.1.38.	Pyrophosphate:oxaloacetate carboxy lyase	PP + oxaloacetate = P_i + phospho-enol-pyruvate + CO_2

TABLE 2

Enzymes Producing Pyrophosphate as a Product

EC Number	Systematic Name	Reaction
2.5.1.1.	Dimethylallylpyrophosphate:isopentenylpyrophosphate dimethylallyltransferase	Dimethylallyl pyrophosphate + isopentenyl pyrophosphate = PP + geranyl pyrophosphate
2.5.1.3.	2-Methyl-4-amino-5-hydroxymethyl-pyrimidine-pyrophosphate:4-methyl-5-(2'-phospho-ethyl)-thiazole 2-methyl-4-amino-pyrimidine-5-methenyltransferase	2-Methyl-4-amino-5-hydroxymethyl-pyrimidine pyrophosphate + 4-methyl-5-(2'-phospho-ethyl)-thiazole = PP + thiamine monophosphate
2.5.1.6.	ATP:L-methionine S-adenosyltransferase	ATP + L-methionine + H_2O = PP + P_i + S-adenosylmethionine
2.7.7.1.	ATP:NMN adenylyltransferase	ATP + nicotinamide ribonucleotide = PP + NAD
2.7.7.2.	ATP:FMN adenylyltransferase	ATP + FMN = PP + FAD
2.7.7.3.	ATP:pantetheine-4'-phosphate adenylyltransferase	ATP + pantetheine-4'-phosphate = PP + dephospho-CoA
2.7.7.4.	ATP:sulphate adenylyltransferase	ATP + sulphate = PP + adenylylsulphate
2.7.7.6.	Nucleosidetriphosphate:RNA nucleotidyltransferase	m NTP + RNA_n = m PP + RNA_{n+m}

continued

TABLE 2 (Cont.)

EC Number	Systematic Name	Reaction
2.7.7.7.	Deoxynucleosidetriphosphate:DNA deoxynucleotidyltransferase	n dNTP + DNA_n = n PP + 2 DNA_n
2.7.7.9.	UTP:α-D-glucose-1-phosphate uridylyltransferase	UTP + α-D-glucose-1-phosphate = PP + UDPglucose
2.7.7.10.	UTP:α-D-galactose-1-phosphate uridylyltransferase	UTP + α-D-galactose-1-phosphate = PP + UDPgalactose
2.7.7.11.	UTP:α-D-xylose-1-phosphate uridylyltransferase	UTP + α-D-xylose-1-phosphate = PP + UDPxylose
2.7.7.13.	GTP:α-D-mannose-1-phosphate guanylyltransferase	GTP + α-D-mannose-1-phosphate = PP + GDPmannose
2.7.7.14.	CTP:ethanolaminephosphate cytidylyltransferase	CTP + ethanolamine phosphate = PP + CDPethanolamine
2.7.7.15.	CTP:cholinephosphate cytidylyl-transferase	CTP + choline phosphate = PP + CDPcholine
2.7.7.18.	ATP:nicotinatemononucleotide adenylyltransferase	ATP + nicotinate ribonucleotide = PP + deamido-NAD
2.7.7.19.	ATP:polynucleotide adenylyl-transferase	ATP + $(adenylate)_n$ = PP + $(adenylate)_{n+1}$
2.7.7.20.	ATP:sRNA adenylyltransferase	ATP + $sRNA_n$ = PP + $sRNA_{n+1}$

continued

TABLE 2 (Cont.)

EC Number	Systematic Name	Reaction
2.7.7.21.	CTP:sRNA cytidylyltransferase	CTP + sRNA = PP + $sRNA_{n+1}$
2.7.7.23.	UTP:2-acetamido-2-deoxy-α-D-glucose-1-phosphate uridylyltransferase	UTP + 2-acetamido-2-deoxy-D-glucose-1-phosphate = PP + UDP-2-aceamido-2-deoxy-D-glucose
2.7.7.24.	deTTP:α-D-glucose-1-phosphate thymidylyltransferase	deTTP + α-D-glucose-1-phosphate = PP + deTDPglucose
2.7.7.25.	Nucleosidetriphosphate:sRNA nucleotidyltransferase	ATP + $sRNA_n$ = PP + $sRNA_{n+1}$
3.6.1.8.	ATP pyrophosphohydrolase	ATP + H_2O = PP + AMP
3.6.1.12.	deCTP nucleotidohydrolase	deCTP + H_2O = PP + deCMP
6.1.1.1.	L-Tyrosine:sRNA ligase	ATP + L-tyrosine + sRNA = PP + AMP + L-tyrosyl-sRNA
6.1.1.2.	L-Tryptophan:sRNA ligase	ATP + L-tryptophan + sRNA = PP + AMP + L-tryptophanyl-sRNA
6.1.1.3.	L-Threonine:sRNA ligase	ATP + L-threonine + sRNA = PP + AMP + L-threonyl-sRNA
6.1.1.4.	L-Leucine:sRNA ligase	ATP + L-leucine + sRNA = PP + AMP + L-leucyl-sRNA

continued

TABLE 2 (Cont.)

EC Number	Systematic Name	Reaction
6.1.1.5.	L-Isoleucine:sRNA ligase	ATP + L-isoleucine + sRNA = PP + AMP + L-leucyl-sRNA
6.1.1.6.	L-Lysine:sRNA ligase	ATP + L-lysine + sRNA = PP + AMP + L-lysyl-sRNA
6.1.1.7.	L-Alanine:sRNA ligase	ATP + L-alanine + sRNA = PP + AMP + L-alanyl-sRNA
6.1.1.9.	L-Valine:sRNA ligase	ATP + L-valine + sRNA = PP + AMP + L-valyl-sRNA
6.1.1.10.	L-Methionine:sRNA ligase	ATP + L-methionine + sRNA = PP + AMP + L-methionyl-sRNA
6.1.1.11.	L-Serine:sRNA Ligase	ATP + L-serine + sRNA = PP + AMP + L-seryl-sRNA
6.1.1.12.	L-Aspartate:sRNA ligase	ATP + L-aspartate + sRNA = PP + AMP + L-aspartyl-sRNA
6.1.1.13.	D-Alanine:polyphosphoribitol ligase	ATP + D-alanine + polyribitolphosphate = PP + AMP + O-D-alanylpolyribitolphosphate
6.2.1.1.	Acetate:CoA ligase	ATP + acetate + CoA = PP + AMP + acetyl-CoA
6.2.1.2.	Acid:CoA ligase	ATP + an acid + CoA = PP + AMP + an acyl-CoA

continued

TABLE 2 (Cont.)

EC Number	Systematic Name	Reaction
6.2.1.7.	Cholate:CoA ligase	ATP + cholate + CoA = PP + AMP + choloyl-CoA
6.3.1.1.	L-Aspartate:ammonia ligase	ATP + L-aspartate + NH_3 = PP + AMP + L-asparagine
6.3.2.1.	L-Pantoate:β-alanine ligase	ATP + L-pantoate + β-alanine = PP + AMP + L-pantothenate
6.3.2.11.	L-Histidine:β-alanine ligase	ATP + L-histidine + β-alanine = PP + AMP + carnosine
6.3.4.1.	Xanthosine-5'-phosphate:ammonia ligase	ATP + xanthosine-5'-phosphate + NH_3 = PP + AMP + GMP
6.3.4.5.	L-Citrulline:L-aspartate ligase	ATP + L-citrulline + L-aspartate = PP + AMP + L-argininosuccinate
6.3.5.1.	Deamido-NAD:L-glutamine amido-ligase	ATP + deamido-NAD + L-glutamine + H_2O = PP + AMP + NAD + L-glutamate
6.3.5.2.	Xanthosine-5'-phosphate:L-glutamine amido-ligase	ATP + xanthosine-5'-phosphate + L-glutamine + H_2O = PP + AMP + GMP + L-glutamate

are inhibited by PP. Succinic dehydrogenase is inhibited by PP. Both PP and malonate have structural similarities and compete with the substrate of this enzyme (80). Accumulation of PP may also result in P_i depletion within the cell. P_i is not only a substrate of several enzymes, but is also an allosteric effector of others (13). In addition, PP itself has been shown to be an allosteric effector (73). Therefore, it is to the advantage of the cell, from considerations of P_i deployment and metabolic stability, to hydrolyze the PP molecule.

In fact, the cell may have been programmed such that all biosynthetic pathways, which are energetically not feasible, may result in PP liberation. The reaction would thus be driven to completion by the mass action law upon hydrolysis of PP by a potent PPase. Such a mechanism would prevent reversal of these important syntheses. In support of this hypothesis, first proposed by Kornberg (74), high levels of PP have been found only in the fat bodies of butterflies (57), in Aspergillus niger mycelia (93), and in yeast, aerated in the presence of lithium salts (83).

However, P_i is not the only form of inorganic phosphate found in cells. Many organisms contain polyphosphate kinase (ATP:polyphosphate phosphotransferase, E.C. 2.7.4.1.) which synthesizes long chain polyphosphates. Organisms as diverse as the bacterial genera Corynebacterium, Aerobacter, Nitrobacter, and Staphylococcus, as diverse as spinach leaves, corn roots, wax moths and rat liver nuclei accumulate polyphosphate (56). Although PP will serve as a substrate for polyphosphate kinase, in bacteria, polyphosphates accumulate only under adverse growth conditions or during stationary phase of growth (55).

Therefore, this enzyme is not active during the period of rapid PP formation and is not the primary means of disposal of this material. Enzymic breakdown of polyphosphate does not result in accumulation of PP (64).

It has recently been suggested that primordial cells hydrolysed PP to obtain energy for biosynthesis and maintainence (18). PP has been shown to function as an energy source for CO_2 fixation (166), for photophosphorylation (19), and for oxidative phosphorylation in mitochondrial systems (20). This suggests that the enzyme was an early one which has been retained by all successful evolutionary branches. A search of the literature shows that the PPase activity has become quite diverse as evolution progressed. The following discussion will include mammalian, insect, plant and microbial PPase activities. Although there is an activity requiring similar optimal conditions in all cases, a comparison of the complement of microbial and mammalian PPase activities suggests that diversification of the enzyme has occured as cells became more highly differentiated.

MAMMALIAN PYROPHOSPHATASE ACTIVITIES

The discovery of PPase was based on an error of identification. Early workers (84), while attempting to isolate and characterize the phosphorus-containing compounds of cells, discovered an easily hydrolyzable compound which could be broken down to P_i by 7 minute exposure to 100 C in 1 N HCl. This was found to be present in many cells and was described as PP. In an attempt to learn about the metabolism of this material, which in some cases constituted as much as

25% of the total phosphorus, cells were examined for an enzyme that would hydrolyze such a compound. Thus, PP was added to cell-free extracts, P_i was formed and PPase was discovered. Subsequently it was shown that the easily hydrolyzable phosphorus-containing compound was not PP at all, but was a mixture of nucleotide phosphates. Since then, high levels of PP have been found in few cells, presumably because of rapid hydrolysis by PPase.

PPase activity was first demonstrated in muscle tissue by Lohman in 1928 (84). Kay (70) then examined tissues of various types and found the enzyme to be optimally active at pH 7.2-7.8 and to be present, in order of decreasing activity, in human kidney cortex and duodenal mucosa, rat bone and lung, and pig kidney. Thereafter, early studies demonstrated the presence of this enzyme in several other animal sources, including bones and cartilage of rabbits (172), tubercles (106) and sputum (63) from tuberculosis patients, rabbit kidney (159), liver (85), placenta and its adnexes (104), blood (148) and brain tissue (53, 144). In addition, the activity has been found in microbes, plants and insects but the characteristics of PPase from these systems will be discussed in later sections.

Early studies to characterize the activity showed that the pH optimum for PPase depended upon the source of the enzyme. Kay's preparations were found to have maximal activity at pH 7.2-7.8 (70), bone at 8.6-9.3 (172), and tubercles at 7.4-7.6 (106). The PPase from rabbit kidney-autolysate (159) had two pH optima, one at 3.8-4.0 and another at 8.6-8.9. Later studies of muscle preparations by Lohman (85) indicated that maximum hydrolysis of PP at alkaline pH required

Mg^{++} at a Mg^{++}/PP ratio of two. The studies of Bamann and Gall (21) were of a clarifying nature. They suggested that PPase existed in three "iso-dynamic" forms which, they predicted, could be demonstrated in all mammalian cells. Such a suggestion came from their examination of the PPase activities of pig liver acetone powder preparations. Upon treatment of the powder with either NH_4OH or acetate, they were able to extract enzyme activity with a pH optimum at either 5.5 or 4.0, respectively. The insoluble powder left after these treatments had associated with it two activities; one with a pH optimum at 4.1 and the other with an optimum at 7.4-8.1. The insoluble alkaline enzyme and the soluble pH 5.5 enzyme were activated by Mg^{++}. The pH 4 enzyme was not affected by the cation in either the soluble or the insoluble form. Since the alkaline activity was unstable to incubation at 37 C, the acid enzyme of the insoluble powder could be examined. This was the first demonstration that PPase activities with different pH optima could be studied separately.

Naganna and Menon (111) found only two enzyme types in human blood. One, with an acid pH optimum, was associated with the plasma and was not stimulated by Mg^{++}, while the other was associated with the erythrocytes, and was stimulated by the presence of Mg^{++} at alkaline pH. The latter enzyme could be measured only with lysed preparations. Inhibition studies showed that the alkaline enzyme was sensitive to iodoacetate and alloxan, suggesting that the activity might depend on the presence of a thiol group. A similar enzyme was isolated from swine brain (53). It was maximally active at pH 6.5-7.4 and required a 30-fold excess of Mg^{++} over PP for optimal activity.

This enzyme was also sensitive to sulfhydryl inhibitors.

The spectrum of PPase activities was increased by Norberg's (116) discovery of a fourth iso-dynamic form of the enzyme in rat liver. Optima were found at pH 4.0, 5.0, 5.8-7.0 and 8.0-8.4. The acid enzymes were unstable and insensitive to Mg^{++} and the neutral and alkaline enzymes were strongly activated by Mg^{++}.

Since there was much confusion concerning the proposed existence of so many different forms of the enzyme activities, the next logical step was to purify each of the "iso-dynamic" forms and study them separately. Even crude separation of rat liver homogenates (158) into soluble and particulate fractions showed that more than 95% of the total Mg^{++}-requiring alkaline PPase was in the soluble fraction. In the microsomes of guinea pig liver Elliot (45) found an enzyme with a pH optimum at 7.4 which was only slightly stimulated by Mg^{++}. Two activities were observed in the supernatant of swine brain homogenates (144). The minor component had optimal activity at pH 5.0 and was not stimulated by Mg^{++}. The other component accounted for 97% of the PPase activity of the supernatant and was maximally active at pH 7.6-7.8. The latter had an absolute requirement for Mg^{++} and could be purified 165-fold with a 37% yield. Inhibition studies suggested thiol involvement. Two PPase activities were found in mouse liver homogenates (132); one with pH optimum at 7.0 and Mg^{++}-dependent was found in the soluble fraction. The other Mg^{++}-independent and optimum at pH 5.3, was found in the mitochondrial fraction. Nordlie and Lardy (117) separated rat liver homogenates into mitochondrial, microsomal and soluble fractions. When each fraction was assayed at pH 7.5

and 5.5 in the presence of Mg^{++}, PPase activity was found in all three fractions. However, the alkaline PPase was located primarily in the soluble fraction, whereas the majority of the acid PPase was in the microsomal fraction. Pynes and Younathan (130) found a similar distribution in pigeon pancreas.

Rafter (133) first demonstrated the existence of a second acceptor for the P_i of PP. He suggested that two routes for the hydrolysis of PP were possible:

$$\text{Reaction 1:} \quad PP + H_2O \rightleftharpoons 2\ P_i$$

$$\text{Reaction 2:} \quad PP + X \rightleftharpoons X\text{-}P + P_i.$$

A mechanism for the formation of glucose-6-phosphate in mouse liver mitochondria that required the presence of both PP and glucose (Reaction 2) was found. Both PP hydrolysis and glucose-6-phosphate formation were optimum at pH 5.2. The two activities were inactivated in a parallel manner, suggesting that they were on the same molecule.

Stetten (154) and Stetten and Taft (155) in a study of rat liver homogenates found that the majority of the alkaline PPase which had optimal activity in the presence of equimolar Mg^{++} and PP was in the soluble fraction. Another PPase, originally isolated from the liver and kidney was found in the microsomes, had a pH optimum at 5.4, and did not require Mg^{++}. The acid PPase activity was inhibited noncompetitively by glucose. After extensive inhibition and activation studies it was shown that both of the activities, as proposed by Rafter, were associated with a single protein. Later, it was found that a glucose-6-phosphatase activity was also a part of this enzyme complex. The three-enzyme complex has been found only in the

microsomes of rat kidney and liver (119, 154) and rabbit kidney, liver and intestinal mucosa (88).

Accumulated evidence suggests that the pH 5.3 PPase activity of microsomes is part of a multi-enzyme complex containing three activities: a PPase, a glucose-6-phosphatase, and a PP-glucose phosphotransferase. Deoxycholate treatment of rat liver microsomes (12) released all activities, which could then be fractionated with ammonium sulfate to a parallel 1.5- to 2.0-fold purification of all three. Different fractions had the same ratios of the three activities. Adsorption by calcium phosphate gels destroyed all three activities concurrently and the thermal inactivation kinetics of all three were similar. Glucose-6-phosphate acted as a competitive inhibitor of PPase activity and conversly PP acted as a competitive inhibitor of glucose-6-phosphatase. In vitro activity of the complex was increased by pretreatment at pH 9.5-9.8 (156). Detergent treatment altered all three activities concurrently; deoxycholate, cholate and Triton X-100 activated the complex, whereas sodium lauryl sulfate and the Tweens were inhibitory (150). All three activities in both liver and kidney were increased in fasted rats (123), and decreased in insulin treated rats (122). Alloxan-diabetic rats and cortisone treated rats had high levels of all three activities (46, 122). Treatment of rats with the steroid, Triamcinolone, in the presence or absence of actinomycin D resulted in increased enzyme levels. This suggested that the effect of the gluco-corticoids on the complex involved an activation of all activities, rather than a regulation of their synthesis (12).

Rat liver mitochondria also have a multiplicity of PPase

activities. Nordlie and Lardy (118) found an acid PPase in addition to a neutral Mg^{++}-requiring PPase activity. The acid activity was inhibited by Mg^{++} and ATP. The enzyme was thought to function in the reaction: $PP + ADP \rightleftharpoons P_i + ATP$, thus conserving the energy of the anhydride bond of PP.

A comparative study was made to determine whether all PPase activities were regulated coordinately. Nordlie and Schultz (120) found that the soluble, Mg^{++}-activated alkaline PPase and the microsome-bound acid PPase were independently regulated. The two activities responded independently toward adrenalectomy and hydrocortisone injections. The alkaline PPase was present at higher activities, but the changes in activity brought about by the alterations in hormonal levels were greater for the acid enzymes.

Alkaline PPase activity has also been found in purified human alkaline phosphatase preparations from liver (36), small intestine (108), HeLa cells (37) and rat kidney (102). The activity of PPase in these preparations does not require Mg^{++}, even though it has a pH optimum at pH 8.9 (43). After fractionation of human liver and small intestine preparations with acetone and ammonium hydroxide precipitation, both the phosphatase and PPase were found in the same peak after elution from Sephadex G-100 and DEAE-cellulose and in the same band after electrophoresis (43). Both had similar heat inactivation kinetics, and in mixed substrate reactions, the hydrolysis of each substrate was inhibited by the presence of the other. L-phenylalanine inhibits the activity of intestinal alkaline phosphatase but not that from other sources. PPase activity in intestinal preparations of

alkaline phosphatase was also inhibited by L-phenylalanine, but neither activity of liver phosphatase responded to the amino acid. Arsenate was a competative inhibitor for both activities from both tissues, and the Ki was the same for both substrates. This suggested that both activities were at a single site on the protein. In addition, organic pyrophosphates were hydrolysed by the preparation (44).

Neither could the alkaline phosphatase and PPase activity from HeLa cell cultures be separated on a 134- to 150-fold purification through butanol extraction and Sephadex G-200 elution (37). Both activities responded similarly to heat inactivation, and were equally inhibited by 0.2 mM L-cysteine. Treatment of HeLa cell cultures with prednisolone induced both activities 5-fold. Inclusion of phenyl phosphate in the medium of BSC-1 cell line resulted in an 8-fold increase in phosphatase and a 10-fold increase in PPase activity. In vivo evidence for this association comes from the observation of Russell (139) that patients with a deficiency of cytoplasmic alkaline phosphatase in certain cells (hypophosphatasia) have pyrophosphaturia, suggesting that the inborn error is associated with an impaired metabolism of PP.

Heppel and Hilmoe (60) isolated a PPase from bull seminal fluid that had a pH optimum of 8.6, but did not require Mg^{++} and, in fact, was inhibited by it. This enzyme may be of the type associated with alkaline phosphatase.

Soluble PPase, active at neutral to alkaline pH and requiring Mg^{++}, has been isolated from many sources. This activity has been shown in most cases to be inhibited by Ca^{++}. Reversal can be effected

by addition of excess Mg^{++} or EDTA treatment. Results for other inhibitors are variable. Soluble PPase isolated from mammalian sources is sensitive to parachloromercuribenzoate (pCMB) and alloxan (53, 62, 111, 131, 144). This characteristic differentiates them from plant and insect PPase which are not sensitive (101, 112). Other divalent cations such as Co^{++}, Fe^{++}, Mn^{++}, Ba^{++}, Hg^{++} and Cu^{++} have been shown to be inhibitory to this activity (62, 111, 144, 151). The soluble PPase does not seem to respond as sharply to changes in endocrine levels, adrenalectomy or fasting, as does the particulate enzyme (120).

Kesselring and Siebert (71) isolated a soluble PPase from rat liver nuclei which, they suggest, functions by driving NAD synthesis. This enzyme had a pH optimum at 7.3 in the presence of Mg^{++}. The enzyme is quite specific for both Mg^{++} and PP, and is inhibited by NAD, ATP and excess PP.

Recently it has been suggested (62) that the PPase of mouse liver cytoplasm belongs to the class of proteins with allosteric properties. It could be shown with a crude supernatant that the enzyme had allosteric properties of a K-system with homotropic and heterotropic effects. Mg^{++} functioned as an activator, $MgPP^{-2}$ as the substrate, and MgADP as an inhibitor. MgATP, AMP, and adenosine had no effect. If the enzyme was preincubated at 40 C, the allosteric cooperativity of the substrate was lost.

Thus several forms of PPase activity have been found in mammalian systems. A microsome bound PPase, active at pH 5.3, seems to be part of a three-enzyme complex. Two activities were found associated with mitochondria. Two soluble enzymes were found, one that requires Mg^{++}

at a pH near neutrality and another active at pH 8.6 that does not require addition of a cationic cofactor. The latter activity may be a special class of alkaline phosphatase activity. Those activites requiring Mg^{++} are inhibited by excess PP. This will be discussed in a later section.

None of the mammalian PPase activities have been purified to a state of homogeneity, and although studies have been performed to determine the K_m and the optimal Mg/PP ratios, they all suffer from having been done with crude preparations. For this reason kinetic parameters were not reported here.

PPASE ACTIVITY OF OTHER ORGANISMS

A. Plant PPases

Both acid, Mg^{++}-independent, and alkaline, Mg^{++}-requiring PPase activities have been found in plants. The acid activity was found in a wide variety of plants (28, 48, 96) and was thought originally to be the only PPase in plants. However, in 1951, Naganna, Raman, Venogopol and Sripathi (112) discovered that fresh potato extract contained a potent Mg^{++}-dependent alkaline PPase as well as the acid PPase. The alkaline enzyme was unaffected by thiol inhibitors, but was inhibited by Zn^{++}, Ca^{++} and fluoride. Subsequently, twenty-three different plant sources were examined and found to contain both acid and alkaline PPase (113).

Alkaline PPase activity has been found in maize leaf chloroplasts when isolated in non-aqueous media (146). Etiolated plants respond to brief periods of illumination or to continuous illumination by

synthesizing increased amounts of PPase. This is not a response to increased photosynthesis since the PPase response occurs before chlorophyll is formed. It was suggested that the PPase functions in driving CO_2 fixation, as shown in the following reactions:

Reaction 3: Pyruvate + ATP + $P_i \rightleftharpoons$ phospho-pyruvate + AMP + PP

Reaction 4: Phospho-pyruvate + $CO_2 \rightleftharpoons$ oxalacetate + P_i.

Basidiomycetes contain two acid PPase activities that are not influenced by Mg^{++} (161); an alkaline PPase has not been reported in this system.

B. Insect PPase

A comprehensive study of PPase from firefly lanterns was carried out by McElroy, Coulombre and Hays (101). Dried lanterns of Photinus pyralis were extracted and fractionated. The enzyme was purified to a 20-fold increase in specific activity with a 3.9% recovery. The purified PPase required Mg^{++} at a ratio of 1:1 with PP at its optimum pH of 8.3. Mg^{++} was the only cation to activate the enzyme and excess PP was found to be inhibitory. The enzyme was specific for PP and could not use as substrate thiamin pyrophosphate, NAD, ATP, ADP, tripolyphosphate, meta- or hexa-metaphosphate, glycerol phosphate or hexose diphosphate. Neither iodoacetate or alloxan was inhibitory. Fluoride, Ca^{++} and Cu^{++} at 0.1 mM inhibited the activity to the extent of 50-60%.

Gilmore and Calaby (52), in an examination of locust thoracic muscle, found a PPase with optimal activity at pH 8.3 which required Mg^{++} at a ratio of 2:1 with PP. This enzyme appeared to be inhibited by excess PP like the firefly enzyme.

Paper electrophoresis of the extract from 1000 boll weevils (Anthonomus grandis Boheman) resulted in the separation of six bands of protein (79). Four of these bands had PPase activity, with 85% found in one band and only 2 to 7% in any one of the other bands. All but the most active band were variable in their appearance depending upon the preparation used. All bands were inhibited by iodoacetate, fluoride, alloxan and Cu^{++} suggesting that thiol groups were required. It was suggested that the bands represent isoenzymes, but it is possible that they are due to population differences.

MICROBIAL PPASES

As in the case of mammalian tissue, PPase in microbial systems was also discovered during a study of acid-labile phosphate, thought to be PP, but later shown to be nucleotide phosphates.

Boyland (29), in a study of yeast phosphate metabolism, was first to show the existence of a microbial PPase, and also first to show a separation of PPase from hexosephosphatase activity. PPase, but not hexosephosphatase, was associated with acetone-dried preparation of a yeast extract. Lüers, Zychlinski and Bengtsson (87) found PPase activity in both malt and yeast; the yeast enzyme had a pH optimum of 6.4-6.8.

PPase activity was found in several bacterial genera, such as Clostridium acetobutylicum Weizmann, Propionibacterium jensenii Van Neil (127), Bacillus subtilis, Alkaligenes faecalis and Aerobacter aerogenes (128). All were similarly active at neutral to alkaline pH and required Mg^{++} for activity.

Crude preparations from mycelia of both Aspergillus niger and Penicillium chrysogenum were found to have PPase activity over a wide range of pH (75). Added Mg^{++} stimulated the Penicillium enzyme at its optimal pH of 6-7, but the Aspergillus enzyme was inhibited by Mg^{++} at its optimal pH of 3-4. It was suggested that there are two types of PPase activity in molds. Thus, the PPase complement from molds is similar to that from plants.

Selective inactivation of extracts of a Stockholm bottom yeast by treatment at high temperature (22), or at pH 4.0 (23), showed that glycerol phosphatase and PPase could be studied in the absence of the activity of the other. In addition, PPase was selectively adsorbed to calcium phosphate gels. The PPase was maximally active at pH 6.0-6.5 and required Mg^{++}. The cation requirement was thought to be not merely to stabilize the enzyme, but to be involved in a "ligatorial mechanism" between the PP, and the enzyme. Bauer (24) proposed that the active complex was a ring structure $\left(\begin{smallmatrix} PP\text{----}Mg \\ enzyme \end{smallmatrix}\right)$. Excess PP was inhibitory. The inhibition by fluoride could be reversed by addition of PP, but not Mg^{++}.

PPase was first extensively purified by Bailey and Webb (16) from a yeast preparation. A 133-fold purification with a 4.6% recovery was obtained. The preparation was not homogeneous, since moving boundry electrophoresis showed a second band after three hours. The preparation was characterized by a pH optimum of 7.2 and an optimum Mg/PP ratio of 5. In excess Mg^{++} the K_m was 0.03 mM. The activity was inhibited by excess PP, Ca^{++} and fluoride. The inhibition by Cu^{++}, iodoacetate, and alloxan was reversed by cysteine, thus implicating

thiol groups. Heppel and Hilmoe (59) also purified a PPase from yeast and obtained a 10-fold higher specific activity than Bailey and Webb. Their enzyme showed maximal activity at pH 8.6 with a Mg^{++}/PP ratio of 1.

Kunitz (77) purified yeast PPase to a 139-fold increase in specific activity with a 12.4% recovery and crystallized the enzyme. The activity of the preparation was similar to that obtained by Bailey and Webb. Optimal conditions were 40 C, pH 7.0, and a Mg^{++}/PP ratio of 1, at which it was possible to measure hydrolysis at a rate of 1000 molecules PP/molecule PPase/sec. Mg^{++} was required for maximal activity, but Co^{++} and Mn^{++} showed slight stimulatory activity alone; in the presence of Mg^{++}, however, both were inhibitory. Ca^{++} inhibited the activity of all three cations. The optimal ratio of Mg^{++}/PP of 1 at 7.2 was shifted to 100 at pH 5.3. Under optimal conditions the kinetics of the reaction were zero order, but in excess Mg^{++} the order deviated after 40% hydrolysis and could not be plotted as a straight line by the method of Lineweaver and Burk. Thus, the order of the reaction depended upon the relative concentration of substrate and cation. High Mg^{++} concentrations shifted the optimal pH from 7.2 toward the acid side. The enzyme was specific for PP; in the presence of Mg^{++}, ATP, ADP or thiamine pyrophosphate were not used as substrates. However, there was slight activity toward tri- or tetrapolyphosphate in the presence of Mn^{++}. This activity was less than 1.0% of that of PP. The enzyme was not heat stable and rapidly lost activity when incubated at temperatures above 40 C. The molecular weight was shown to be 60,000 by calculations from data collected

from ultracentrifugal, electophoretic and viscosity measurements of the crystalline preparation (140).

Crystalline yeast PPase has been used in other studies and was until only recently the only crystalline PPase available. Since that time it has been shown that this preparation has other activities. Schlessinger and Coon (142) showed that it would hydrolyze organic pyrophosphates in the presence of Zn^{++}. Turn-over numbers for PP were calculated to be 37,000 in the presence of Mg^{++} and 33,000 in the presence of Zn^{++}. ADP and ATP were hydrolyzed in the presence of Zn^{++}; their turn-over numbers were found to be 230 and 870, respectively. These are very low values, as compared to PP hydrolysis in the presence of Mg^{++}, but the authors suggest that this is evidence that the substrate range of the enzyme depends upon the cation present. In further extending the range of substrates for crystalline yeast PPase, Avaeva (14, 15) has shown this preparation to hydrolyze serine pyrophosphate with maximum activity at pH 6 and 8 at a Zn^{++}/serine-pyrophosphate ratio of 2. Co^{++} and Mn^{++} were also stimulatory, but Mg^{++} was inactive. With Zn^{++} at pH 6, the serine pyrophosphate hydrolysis was faster than ATP hydrolysis.

Cohn used the Kunitz preparation to study the reversal of the PPase reaction (34). She found that yeast crystalline PPase catalyzes a rapid exchange of 18_O between P_i and water in the absence of PP. This indicates that exchange is not a reversal of the overall reaction, but that hydrolysis is a two-step mechanism:

$$\text{Reaction 5:}\quad HP_2O_7^{-3} + EH \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EPO_3^{-2} + H^+$$

$$\text{Reaction 6:}\quad EPO_3^{-2} + H_2O \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EH + HOPO_3^{-2}$$

The P_i-water exchange is 500 times as fast as the rate of complete reversal. The model extended implicates a phospho-enzyme intermediate in the PPase reaction.

Most bacterial PPases have been found to be active at neutral to alkaline pH range and require Mg^{++} for optimal activity. Escherichia coli enzyme measured after cell lysis with petroleum ether had a pH optimum at 6.7 (31), whereas cells lysed with deoxycholate had a pH optimum at 7.6 (17); both required Mg^{++} for activity. Schito and Pesce (141) studied a PPase preparation from E. coli purified 400-fold and found the pH optimum to be 9.0 and the optimal Mg^{++}/PP ratio to be 1. Thus, the optimum pH of a preparation appears to be related to its state of purity. The K_m of this last preparation was 0.014 mM. It was inhibited by fluoride, Co^{++} and EDTA, but thiol inhibitors had no effect. Josse (67) also purified PPase from E. coli. He obtained a 500-fold purification and crystallized the enzyme. The pH optimum of his preparation was similar to that of the preparation of Schito and Pesce, i.e. 9.0. The optimum Mg^{++}/PP ratio was also 1. Fluoride and guanidine were inhibitory. The preparation was not active on organic pyrophosphates, but did hydrolyze tri-and tetra-polyphosphates at a low rate (0.016 and 0.007 respectively as compared to 1.0 for PP). In the presence of other cations, the pH optimum was observed to shift toward the acid side, but there was no change in the range of substrate specificity as in the case of yeast PPase. Both Zn^{++} and Co^{++} stimulated at pH 7.5, but the activity at this pH in the presence of Co^{++} was still less than that with Mg^{++}. The Zn^{++}-stimulated activity was slightly higher than that of Mg^{++} at this pH. In contrast to

yeast PPase, the E. coli preparation was exceptionally heat stable; in 0.01 M Mg^{++} 95% of the activity remained after incubation at 80 C for 10 min, whereas 15% remained without Mg^{++} addition.

The PPase activity from other organisms has also been shown to be heat stable. Marsh and Militzer (94) found a particulate-bound PPase of a thermophile to be quite heat stable. The enzyme had a pH optimum at 7.5, required a Mg^{++}/PP ratio of 0.5, and was maximally active at 60 C. On heating at high temperature, a long lag was observed before denaturation occurred. Other enzymes from the thermophile exhibited single phase kinetics of denaturation. Interestingly enough, yeast PPase also exhibited a similar lag phenomenon, but was rapidly denatured thereafter (77). In a second study of thermal resistant PPases, it was shown that heat stability of the PPase from Bacillus stearothermophilus increased with the increase of growth temperature, and with the presence of Mg^{++} (30). Protection by Mg^{++} has also been found with PPases from mesophiles other than E. coli. Johnson and Johnson (66) found that the PPase from Azotobacter agilis was resistant to 75 C for 120 min only in the presence of Mg^{++}. Partially purified enzyme was also protected by Mg^{++} against inactivation by protease and by 6 to 9 M urea (65). Thiol inhibitors were without effect for this enzyme. In an examination of extracts of many bacterial strains, Blumenthal, Johnson and Johnson (27) found heat-labile PPases in gram positive sporeforming rods, gram positive cocci such as Staphylococcus and Streptococcus, and in Mycobacterium phlei. Gram negative rods, such as Proteus vulgaris, E. coli, Pseudomonas fluorescens and Serratia indica had PPases which were heat-stable in the presence

of Mg^{++}.

Akagi and Campbell (6) obtained a 136-fold purification of PPase from Desulfovibrio desulfuricans, an obligate anaerobe. This purified preparation was specific for PP and had maximal activity at pH 8.0-8.5, and with a Mg^{++}/PP ratio of 1. Mn^{++} and Co^{++} were slightly stimulatory, but at a much lower efficiency. Inhibition by excess PP could be reversed by addition of Mg^{++}. In excess Mg^{++} the K_m was 1.93 mM. Neither thiol inhibitors nor 1 mM fluoride were inhibitory. The PPase exhibited two phase inactivation kinetics when exposed to temperatures of 40 C and above. An initial rapid phase was followed by a slower second phase.

The PPases of other anaerobic bacteria have not been studied extensively. D'Eustachio, Knight, and Hardy (40) demonstrated that the PPases from several anaerobes were unstable in the absence of reductants. Addition of KBH_4 stimulated the activity by a factor of 2 to 40-fold. PPases from aerobic bacteria were not stimulated by the inclusion of the reductant.

The possibility of multiplicity of PPases in differentiating microorganisms was suggested by the report of Levinson, Sloan and Hyatt (82). They reported that the PPase from spore extracts of Bacillus megaterium QM 1551 was qualitatively and quantitatively different than that from the extract of vegetative cells. Their report presented evidence that crude extracts from spores had optimal PPase activity at pH 6.8-7.0 and required Mn^{++}, while the PPase from crude extracts from vegetative cells was optimally active with Zn^{++}, Co^{++} or Mg^{++}. The optimal ratio of metal ion to PP found in their system

at pH 7 was 0.33. Thus, they suggested that the transition from spore to vegetative cell is accompanied by a diminution of Mn^{++}-stimulated PPase, and an increase in the Co^{++}-activated enzyme. They suggested that the Mn^{++}-stimulated PPase may function in spore germination.

In a re-examination of the question Tono and Kornberg (163) subjected crude extracts of B. megaterium QM 1551 to polyacrylamide electrophoresis. No difference between vegetative cell and spore PPase was found by this method. Electrophoresis of spore and vegetative extracts resulted in a single band with R_f 0.45 (band 1) in both cases. However, when extracts of sporulating cells were examined, two activity bands were observed, in that a second band appeared at R_f 0.54 (band 2). These were separated by preparative polyacrylamide electrophoresis and compared. The two activities responded similarly in their substrate range, pH optimum in the presence of Mn^{++}, and to the range of metal ions that stimulated at pH 7.3. Both were found to be unstable in the absence of Mn^{++}. However, when activity from the band 2 was exposed to Mn^{++} and subjected to electrophoresis, there was a partial conversion to band 1. Both were similarly inhibited by (2-amino-2-hydroxy-methyl)-1, 3-propanediol (Tris) and not affected by fluoride. The molecular weight of both activities was calculated to be 58,000 on the basis of $S_{20,W} = 4.0$.

In confirmation of these findings, it was also shown that the PPases of vegetative cells and spores from B. subtilis were identical (164). Spore and vegetative PPases were purified 390- and 1,230-fold, respectively. Both required Mn^{++} for stability, and both gave a single band after polyacrylamide electrophoresis under three different

sets of conditions. The enzymes had similar amino acid compositions, which were characterized by high levels of glutamate, aspartate, alanine and leucine. The molecular weight of both was approximately 68,000 ($S_{20,W} = 4.4$). Their substrate specificity was identical and the same as that of B. megaterium. In the presence of Mn^{++} the optimal pH was 7.5, but in the presence of Mg^{++} it was pH 9.0. The optimal activity in the presence of Mg^{++} was about 70% higher than the optimum in Mn^{++}. Both enzymes were inhibited by Tris, and not influenced by 1 mM pCMB, 10 mM iodoacetate or 1 mM fluoride. On the basis of these results it was concluded that the PPase from spores is the same as that from vegetative cells, and that the differences observed by Levinson et. al. (82) were the result of the profound changes that Mn^{++} exerts on the activity and structure of these enzymes.

Evidence for multiplicity of PPase activity in non-differentiating cells was found by Meloni, Pesce, and Schito (103). Two peaks of PPase activity could be separated by DEAE-cellulose chromatography of the extracts of P. fluorescens and Staphylococcus aureus. Both peaks from the Pseudomonas extract had the same pH optimum, the same cation requirements and the same size, as shown on Sephadex G-200. Other microorganisms tested showed only one peak of PPase activity.

Two PPase activities are found in E. coli, the constitutive one described before, and another, present in phosphate-deprived cells, or certain mutants, which is identical with the inducible non-specific alkaline phosphatase first described by Horiuchi, Horiuchi and Minuzo (61) and Torriani (165). The activity is present only in cells grown in phosphate-limited medium, and is maximal at pH 8.2. In addition,

Anderson and Nordlie (8) showed that purified E. coli alkaline phosphatase had PP-glucose phosphotransferase activity at pH 7.9. This could be an alkaline bacterial counterpart of the acid microsomal three-enzyme complex of mammalian cells.

PPase from crude preparations of Saccharomyces mellis had two pH optima (168), one at 5.5 that did not require Mg^{++}, and another at pH 8.0 that was Mg^{++}-dependent. The acid PPase appeared to be a complex with acid phosphomonoesterase activity of the yeast, since the level of PPase increased concurrently with induction of the phosphatase when the cells were grown in low levels of P_i. Maximal alkaline activity was observed when the Mg^{++}/PP ratio was 5. Both excess PP and excess Mg^{++} were inhibitory. It was suggested that a Mg^{++}-enzyme complex was the active form.

Another possibility of multiplicity of PPase in bacteria was found in the report of Oginsky and Rumbaugh (124) concerning an acid, Co^{++}-stimulated PPase activity in Streptococcus faecium F24. The organism also contained an alkaline, Mg^{++}-dependent PPase. The acid activity differed from all others studied in that it was 1)dependent upon Co^{++}, 2) enhanced by histidine and 3) present at higher activity than the Mg^{++}-stimulated alkaline activity. The acid PPase had maximal activity at pH 5.3 in the presence of Co^{++}; Zn^{++} and Mn^{++} stimulated only slightly. Zn^{++}, Fe^{++}, Ca^{++}, EDTA and high concentrations of PP were inhibitory. No inhibition was observed by arsenite, arsenate, fluoride, cyanide, semicarbazide, hydroxylamine or azide. NAD, thiamine pyrophosphate, hexose-diphosphate, or glycerol phosphate was not utilized as substrate. ADP and ATP hydrolysis was stimulated by

the addition of Co^{++} and histidine, but occurred at low rates as compared with the hydrolysis of PP. The amino acid enhancement was only relatively specific, with L-histidine giving maximum activity, but cysteine, lysine and histamine stimulating somewhat. A constant ratio of Co^{++}/histidine was not found over a wide range of concentrations, thus indicating that a histidine-Co^{++} complex was not involved. The concentration of histidine required appeared to be more directly related to the amount of enzyme, although a definite ratio of histidine to enzyme was not obtainable experimentally. Several other microorganisms were found to have PPase activity at pH 5.3 that was stimulated by Co^{++}: E. coli, A. aerogenes, P. vulgaris and B. subtilis all responded slightly to Co^{++} at pH 5.3, but were inhibited by addition of histidine. The PPases of Lactobacillus, Streptococcus, Staphylococcus and Leuconostoc strains all responded to Co^{++} and were further enhanced by histidine. Thus, this enzyme may be restricted to the family Lactobacillaceae.

In summary, most bacterial systems contain an alkaline PPase that is maximally stimulated by Mg^{++}. A second PPase activity may be found in alkaline or acid phosphatase. Although higher organisms have a multiplicity of PPase activities, bacteria and yeast appear to have only one that has high activity for PP. No evidence has been presented that would clearly show more than one enzyme for hydrolysis of PP in bacteria.

SUBSTRATE OF PPASE

The identity of the substrate of the alkaline PPase has been a

matter for speculation for many investigators. When Mg^{++} and PP are in solution together, there are many species of $(Mg)_x(P_2O_7)_y$ complexes, depending upon the relative concentration of each compound and the pH of the solution. In addition, when the enzyme test suspension is a crude extract, other proteins are present. Thus, there are many possibilities for binding of ions, to PPase and to other proteins. The early speculation of Bauer, of a "ligatorial" mechanism and an enzyme-PP-Mg^{++} ring complex, has much support from recent studies of alkaline PPase. A different mechanism must be proposed for the acid enzymes that do not require any added cofactor.

Bloch-Frankenthal (26) proposed that $MgPP^{-2}$ is the substrate of alkaline PPase. She suggests that the optimal ratio of Mg^{++}/PP for formation of $MgPP^{-2}$ depends upon the pH of the reaction mixture and that the ratio required increases with decreasing pH. Mg^{++}, in alkaline solution, forms a complex with PP, depending upon the concentration of each component: at near equimolar concentrations $MgPP^{-2}$ is formed. When the complex is formed there is a resultant decrease in the pH of the solution. At constant PP concentration, the Mg^{++} concentration was varied and the change of pH measured in each mixture, to find the ratio where maximum $MgPP^{-2}$ was formed. The activity of erythrocyte alkaline PPase was measured in this series of PP-Mg^{++} mixtures. Both the formation of maximal $MgPP^{-2}$ and the maximal PPase activity of the enzyme occurred at the same Mg^{++}/PP ratio. She concluded from these results that $MgPP^{-2}$ is the substrate. She also suggested that the reason high Mg^{++}/PP ratios were required at low pH (53, 111) is that the substrate complex is not easily formed at

lower pH levels. Thus, more Mg^{++} is required to push the reaction.

Robbins, Stulberg and Boyer (137) also found that $MgPP^{-2}$ was the substrate for rat brain alkaline PPase. All experiments were performed at pH 8.3 and the actual amount of free Mg^{++} in each solution was titrated with EDTA. Thus, an estimate of the concentration of different species present could be made. They observed inhibition by excess PP, but suggested that excess $MgPP^{-2}$ is also an inhibitor.

Josse (68), using a purified preparation of PPase from E. coli, also studied the kinetics of the Mg^{++}-stimulated alkaline PPase. He performed reactions at pH 9.1 at five different PP concentrations with 13 different concentrations of Mg^{++} in each. With the results he obtained he tested five models with the aid of a computer-model fitting procedure. The values fit only two of the five models tested. The model with best fit was one in which $MgPP^{-2}$was the substrate, excess PP an inhibitor, and Mg_2PP bound weakly, if at all. The K_m was calculated to be 0.005mM. It was concluded that the active site of the enzyme carries one or more positive charges at pH 9.1 and is of limited dimensions. He suggested that Mg^{++} binds to PP to form a rigid six-membered ring. The enzyme may then apply "torque" to break the rigid bond.

Several papers (30, 65, 66, 67) have reported that PPase is protected from denaturation by the presence of Mg^{++}. Cohn (35) has shown directly, by nuclear magnetic resonance, that Mg^{++} binds to crystalline yeast PPase. Thus, a complete model to account for the activity of alkaline PPase must account for at least Mg^{++} binding, PP inhibition, and selection of $MgPP^{-2}$ over all other types of Mg-PP complexes

for substrate.

REGULATORY STUDIES

Very few studies have been done on the regulation of PPase. Blumenthal, Johnson, and Johnson (27) reported that both gram positive and gram negative rods, Streptococcus and Mycobacterium have constitutive alkaline PPases, but that Micrococcus lysodeikticus and S. aureus have "inducible" enzymes. That is, no PPase was detected unless PP was added to the medium. No other possible inducers were tested and no studies were performed to determine whether the PP was taken up by the intact cell. The answer may lie in the fact that their methods were not adequate to measure PPase in Staphylococcus citreus or albus, Micrococcus roseus, Sarcina lutea or Neisseria. Yet, PPase had been detected in S. albus in an earlier study by another worker (115). It does not seem that an enzyme of this type would require induction by exogenous sources of substrate since the substrate would be produced endogenously in great amounts during growth.

Josse (67), in his study of the PPase of E. coli K12, was unable to detect alterations in the level of PPase related to phase or rate of growth, composition of the medium, or conditions of phage induction or protein synthesis. The alkaline PPase of E. coli K12 is thus constitutive.

The regulation of alkaline phosphatase on the other hand, has been extensively studied. Its synthesis has been shown to be repressed by high levels of P_i in the medium. Thus, if PPase activity were shown to be an activity of the phosphatase molecule, the two

activities must be regulated coordinately. This was shown to be the case for alkaline phosphatase of rat kidney (102), and E. coli (67), and for acid phosphatase of S. mellis (168). However, in the latter case, coordinate reduction of acid PPase and acid phosphatase in high P_i concentrations resulted in increased levels of the alkaline PPase.

Alterations of levels of the enzyme have been found in E. coli and A. aerogenes (147). Strains that are streptomycin-resistant have been isolated and assayed for PPase. In both cases, the resistant mutants had lower levels of PPase than the parent cells. The levels were lowered additionally when the strains were grown in the presence of the antibiotic.

The second band of PPase activity on electrophoresis arising during sporulation of B. megaterium (163) might be an indication of regulation of activity. The structure of the enzyme may be influenced by the in vivo fluctuations of ion concentration during sporulation. The answer awaits a more thorough study of the PPase.

The soluble alkaline PPase in mammalian cells has not been shown to be regulated, even though treatment such as fasting, or changes in hormone levels has been shown to influence the levels of the microsomal acid PPase (12, 46, 122, 123). Results of examinations of crude preparations of mouse liver alkaline PPase suggest that this enzyme may be an allosteric protein. Its activity is influenced by levels of Mg^{++} and Mg-ADP which act as effectors (62). Confirmation of this hypothesis awaits study of the purified enzyme.

APPROACH TO THE PROBELM

A. Localization Studies

If the acid and alkaline activities of PPase in S. faecium were associated with different proteins it was possible that each was localized in a different cellular component. Bacteria are of relatively simple structure thus it was only possible to examine the periplasmic space, the membrane, the ribosomes and the cytoplasm for the presence of each activity. After disruption of the cell the various components were obtained by differential centrifugation.

B. Regulation of the PPase Activities

It had been reported (Barber, unpublished results) that different levels of PPase activity could be recovered from S. faecium F24 grown in different media, and that increased glucose concentrations in the growth medium resulted in decreased levels of PPase activity. This suggested the possibility of a regulatory phenomenon controlling the PPase levels in S. faecium F24. The phenomenon was examined from the standpoint of a regulatory study as well as a means to determine whether the acid and alkaline PPase activities responded coordinately to different cultural conditions. It seemed that PPase might be required at higher activity during the exponential phase of growth when a great deal of biosynthesis was occurring. Thus cells were harvested at various time during the growth cycle, and the specific activity of both PPase activities was determined. Growth rates were altered by two different methods to observe whether this influenced the rate of synthesis of either activity.

C. Physical Properties of the PPase Activities

If the acid and alkaline PPases were expressions of activity of two separate proteins, the original question could be best answered by purifying the two and comparing the physical and chemical properties of each. However, when one is dealing with activities from a crude preparation, one does not know how many molecular species are being assayed. Several possibilities existed: 1) both activities were associated with a single protein, 2) there are two proteins each with a single activity, 3) there are two proteins, one with both acid and alkaline activity, and another with a single activity , or 4) there were two proteins with both activities. It seemed necessary to analyze the whole crude preparation to differentiate among these possibilities. For this reason the whole crude extract was examined by several methods. Attempts were made to separate activities on the basis of charge density, size, and electrophoretic mobility on starch gel and polyacrylamide.

D. Inactivation Studies

Thermal inactivation studies were performed to determine whether the two activities had similar denaturation kinetics or whether they were equally protected by Mg^{++} or Co^{++}. Both activities were also examined in increasing concentrations of urea to determine whether there was a differential effect.

MATERIALS AND METHODS

GENERAL GROWTH CONDITIONS

A. Media

All media and solutions were sterilized in the autoclave for 15 min at 15 psi and 121 C. All reagents and media were prepared with water passed through a deionizer and then through a Corning glass distillation unit.

1. Trypticase soy medium (TS broth or agar). The broth was prepared by dissolving 30 g of the dry powder, obtained from Baltimore Biological Laboratories, per liter of water. The powder contains in g/liter: 17.0 g trypticase peptone, 3.0 g phytone peptone, 5.0 g NaCl, 2.5 g K_2HPO_4, and 2.5 g glucose. The final pH was 6.8. TS agar was prepared by adding 15 g agar (Difco) per liter to the broth.

2. Tryptone-yeast extract medium (TYE medium). The following components were dissolved in distilled water and the volume made to 900 ml: 5 g K_2HPO_4, 10 g yeast extract (Difco) and 10 g tryptone (Difco). This medium and a solution of 30% (w/v) glucose were sterilized separately. After an appropriate amount of the glucose solution was added to the medium, the volume was brought to one liter with sterile water. The final pH was 6.8.

3. Glycerol oxidase factor medium (GOF medium). This is a modification of the medium used by Claridge and Hendlin (32). To 500 ml of water were added the solids and solutions as shown in

Table 3. The amino acids were each placed in 50 ml water and dissolved by adding concentrated HCl to the cysteine and tyrosine, and 20% (w/v) KOH to the tryptophan before their addition into the medium. The medium volume was made to 900 ml and the pH adjusted to 6.8. The medium and a solution of 30% (w/v) glucose were sterilized separately. The glucose was added just before inoculation along with sterile water to bring the volume to one liter.

B. Cultures

Organisms used in this study were Streptococcus faecium F24, originally obtained from the late Dr. J. M. Sherman, and Escherichia coli Crookes. The culture was confirmed to be S. faecium by its lack of reduction of litmus milk, methylene blue and 2,3,5-triphenyltetrazolium chloride, and its sensitivity to 0.04% tellurite (170). These characteristics distinguished it from Streptococcus faecalis (strain 10C1) which reduced all three of the compounds listed above, and which was not sensitive to 0.04% tellurite.

C. Growth Conditions

Streptococcal strains were maintained as stab cultures in TS agar deeps. One stab culture was maintained as a master culture and was entered only for bimonthly transfers. Others were used for inoculation into growth media. All stab cultures were grown at 37 C for 5 hr and then refrigerated until used. E. coli Crookes was maintained on TS agar slants; other incubation and storage conditions were the same as for streptococci.

Starter cultures were obtained by transferring cells from a stab culture to TS broth and incubating at 37 C for 5 hr. Cells from this

TABLE 3

Components of GOF Medium

Constituents	per liter
Solids	
K_2HPO_4	5.0 gm
Na Acetate	2.0
Na Thioglycollate	0.1
Casein hydrolysate (vitamin free)	10.0
DL-tryptophan	0.2
L-cysteine	0.2
L-tyrosine	0.4
Solutions	
Salts B[1]	10.0 ml
Base mixture[2]	4.0
Vitamin mixture[3]	3.3
Folic acid (0.3 mg/6 ml H_2O	2.0
Biotin (0.03 mg/.6 ml ethanol)	0.2
Final pH - 6.8	

[1]Salts B	Ascorbic acid	0.2 g/100 ml
	$MgSO_4$	4.0
	NaCl	0.2
	$FeSO_4$	0.2
	$MnCl_2$	0.4
[2]Base mixture	Adenine SO_4	0.374
	Guanine HCl	0.340
	Uracil	0.250
[3]Vitamin mixture	Nicotinic acid	0.150
	Riboflavin	0.030
	Ca Pantothenate	0.030
	Pyridoxal HCl	0.060
	Thiamine HCl	0.060

turbid culture were then inoculated into appropriate media. Usually at least one passage was made through the appropriate medium before the cells were used for the experiment. On transfers from TS broth to GOF medium, several (ca. 3-5) passages in the defined medium were required to circumvent a very long lag period before growth started. The inoculum size generally used was 0.1 ml per 10 ml medium. Erlenmeyer flasks were used for all experiments, and the medium was dispensed so that flasks used for standing culture experiments were filled to 9/10 volume, while those for aerated culture were filled to 1/10 volume. Aerated cultures were incubated in a Gyratory shaker (New Brunswick Scientific Co., New Brunswick, N.J.). Standing cultures were used unless otherwise stated. Media were always prewarmed to the appropriate temperature before inoculation.

D. Harvest

Harvest of stationary phase cells was performed after 16-18 hr of incubation at the appropriate temperature. Unless otherwise stated, this was always 37 C. The cultures were poured directly into centrifuge bottles and the cells sedimented at 1300 x g in a Sorvall Model G for 20 min at room temperature. The cells were then washed twice with distilled water and resuspended in buffer or water to the desired optical density at 520 nm. Tubes used for these determinations were always 16 x 150 mm colorimetric tubes, and optical densities for all cell density, P_i, and protein determinations were determined in a Bausch and Lomb Spectronic 20 spectrophotometer. Cultures harvested during exponential phase were poured over crushed ice made from the growth medium. These cells were then sedimented and washed in the

same manner as the stationary cells. When large volumes of exponential phase cultures were required, the cells were harvested with the Szent-Györgyi and Blum continuous flow apparatus for the Sorvall model SS-3, in the cold room. In these cases, the cells were inoculated from 16 hr cultures to an initial optical density of ca. 0.200 at 520 nm. Replicate liter flasks were inoculated at 30 min intervals and 500 ml flasks at 15 min intervals. Each replicate culture was incubated 60 min, poured over crushed medium-ice and then into the continuous flow apparatus. The cells were sedimented at 10,000 x g at a flow rate of 80 ml/min. Faster flow rates resulted in excessive frothing and poor clearance of cells from the medium. After the last culture was harvested, 400 ml of cold water was used to flush out the medium. The apparatus was dissassembled, the cells collected and washed twice with cold water, and then resuspended to the desired optical density, in a buffer appropriate for the experiment to be performed.

ENZYME STUDIES

A. Preparation of Crude Enzyme

Cell suspensions, made to a desired optical density, were placed in a 25 x 65 mm lusteroid tube maintained in an ice bath and sonicated with the standard size probe of a Bronwill Biosonik Sonicator (Rochester, New York). Sample size was never more than 5 ml, and if the sonication time was longer than 3 min, the preparation was cooled in an ice bath for 30 sec, at that time. The intensity was set at "50" for all preparations. Sonication was carried out for 3 to 6 min,

depending upon the concentration of cells.

Particulate material was sedimented at 20,000 x g for 20 min in an International refrigerated centrifuge model PR-2. If the sonicate was from a heavy cell suspension, both the supernatant and the light portion of the sediment were collected and recentrifuged in the same force field. Only the supernatant was recovered from the second centrifugation. This material was used without further purification in most experiments and will be referred to as "crude sonicate".

B. Partial Purification

Nucleic acids were precipitated from the sonicate by the streptomycin method of Tono and Kornberg (163). One ml of a 5% (v/v) streptomycin sulfate (or 1% (w/v) protamine sulfate) solution was added per 5 ml crude sonicate. The precipitating agent was added dropwise to the sonicate with constant stirring in an ice bath. The mixture was then held at 0 C for 15 min without stirring, after which it was centrifuged at 20,000 x g for 20 min. The supernatant was recovered.

Ammonium sulfate precipitation was used as a means of obtaining a partial purification. The supernatant of the streptomycin-treated crude sonicate was maintained in an ice bath and then with constant stirring, an appropriate amount of saturated ammonium sulfate was added dropwise to bring the suspension to 50% saturation. This was allowed to stand without agitation for 20 min and was then centrifuged for 20 min at 20,000 x g. The procedure was repeated to obtain fractionation at higher concentrations of ammonium sulfate. The supernatant was assayed at low saturation levels. The precipitate from the final fraction was dissolved in buffer and both supernatant and

precipitate were assayed.

C. Enzyme Assay

1. PPase. Acid PPase activity was assayed in a volume of 0.85 ml, containing 0.028 M acetate buffer at pH 5.6, 5.8 m moles $CoCl_2$, 2.9 m moles L-histidine and 1.4 m moles $Na_4P_2O_7$. Final pH after all additions was 5.4. The alkaline enzyme reaction mixture contained 0.014 M borate-KCl buffer at pH 8.6, 2.64 m moles $MgCl_2 \cdot 6 H_2O$ and 1.4 m moles $Na_4P_2O_7$ in 0.85 ml. The final pH, after all additions, was 8.0. The reaction mixtures, containing the extract, buffer, and cation were prewarmed to 37 C for 1 min. The reactions were started by the addition of 0.15 ml 0.01 M PP, incubated at 37 C for 10 or 15 min and stopped by addition of 0.15 ml 10% (w/v) trichloroacetic acid. No visible precipitation of protein occurred under these conditions. In both cases, a zero time control was included for each sample analysed. Each analysis was done at least in duplicate. No increase in P_i on incubation was detected with enzyme without PP, or PP without enzyme.

2. Alkaline Pase. Alkaline phosphatase was determined by the method of Torriani (165). The reaction mixture contained 0.033 mg ρ-nitrophenyl phosphate, disodium 5 1/2 H_2O (A grade, Calbiochem, Los Angeles, Calif.) in 0.5 M Tris buffer at pH 8.0. Enzyme was added to start the reaction. Total volume was 3 ml. The reaction was followed at room temperature in the Beckman DU spectrophotometer at 420 nm. ρ-Nitrophenol was used as standard.

3. PP-glucose phosphotransferase. This activity was tested by the method of Stetten (154). Crude sonicate from a stationary

phase culture grown in TS-broth was assayed for the hydrolysis of PP, and the release of P_i. Duplicate acid reaction mixtures, as described in section I above were prepared containing Co^{++}, Co^{++} and histidine, or histidine, in the same concentrations as before. To one set was added glucose, to a final concentration of 0.4 M and to the other set was added the same volume of water. Enzyme was added to start the reaction and incubation was carried out at 37 C for 15 min. The reaction was stopped by addition of 10% trichloroacetic acid , and P_i was assayed.

D. Phosphorus Determination

A modification of the colorimetric Fiske-SubbaRow method (47) was used to determine the amount of P_i released by the enzyme. To the 1.0 ml reaction mixture were added 0.5 ml 4% (w/v) ammonium molybdate, 0.5 ml 10 N H_2SO_4 and 1 ml of a mixture containing 1% (w/v) p-methylaminophenol sulfate (Elon; Eastman Organic Chemicals, Rochester, New York) and 3% (w/v) $NaHSO_3$. The volume was brought to 10 ml with water, and the tube contents mixed thoroughly. The color was allowed to develop at room temperature for 15 min, before the optical density was read at 670 nm. Product P_i was determined from a standard curve, which was linear over the range from 5 to 45 μg P_i. To verify the concentration, standards containing 10 and 20 μg P_i were assayed with each determination.

E. Protein Determination

Proteins were determined by the Reiner and Cheung (135) modification of the Folin-Ciocalteau procedure. Crystalline bovine serum albumin (A grade, Calbiochem, Los Angeles, Calif.) was used as a protein

standard. The standard curve at 625 nm was linear from 25-200 μg protein. 50 and 75 μg bovine serum albumin standards were assayed with each determination.

F. Bacterial N Determination

The mg bacterial N content of each cell suspension was calculated from the optical density at 520 nm of 0.1 ml whole cell preparation diluted in 9.9 ml water. Previous analysis in this laboratory using micro-Kjeldahl determinations of suspensions with varied optical density indicated that for S. faecium, the optical density multiplied by a factor of 265 results in μg bacterial N/0.1 ml. The factor for E. coli is 750.

LOCALIZATION OF PPASE ACTIVITY

A. In Lysozyme Treated Cells

Stationary phase cells, grown 17 hr at 37 C in TYE medium, supplemented with 0.5% glucose (w/v), were harvested, washed in H_2O and suspended to 2.5 mg bacterial N/ml in buffer. They were incubated with 200 μg/ml lysozyme (General Biochemicals, Chagrin Falls, Ohio), 0.66 M sucrose and 0.016 M Tris buffer at pH 7.2 for 1 hr at 37 C. That protoplasts were formed by this treatment was shown by progressive development of osmotic fragility. Cells were diluted (1:5) in water at intervals after exposure to lysozyme and optical density at 520 nm was determined. After 60 min the optical density was only 20% of that of the original cell suspension. The protoplasts were centrifuged at 12,000 x g for 15 min and both the supernatant and the particulate material recovered. The protoplasts were then diluted in an

equal volume of distilled water to cause lysis. This viscous suspension was treated with 1.0 µg/ml ribonuclease and 1.0 µg/ml deoxyribonuclease (Sigma, St. Louis, Mo.) in the presence of 0.1 M Mg^{++} for 30 min at 37 C and then centrifuged at 30,000 x g for 20 min. Both the supernatant and the pellet were recovered. Bibb and Straughn (25) identified this pellet, in their studies of S. faecium F24, as the membrane fraction, using as criteria, the absence of cell wall rhamnose and characteristic membrane forms in electron micrographs. Both the supernatant and the membrane fraction were assayed.

B. In Sonicates

To ascertain whether any activity was associated with the ribosomal fraction (162), cells were harvested from exponential phase of growth in TS broth. Half of the cells were suspended in 1 mM Tris at pH 7.9, and the other half diluted in 1 mM Tris + 3 mM Mg^{++} at pH 7.8. Both contained 5.8 mg bacterial N/ml. Each was sonicated for 4 min and then centrifuged at 20,000 x g in the PR-2 refrigerated centrifuge. The resulting supernatant was recentrifuged in an LKB ultracentrifuge at 104,000 x g for 1 hr. Both the pellet and the supernatant were assayed for acid and alkaline PPase activity.

REGULATORY STUDIES

A. Effects of Cultural Conditions

1. Glucose concentration in standing culture. A five-hour starter culture, grown in TS broth or GOF medium, was inoculated into 500 ml flasks containing 400 ml GOF, TS broth or TYE medium supplemented with increasing concentrations of glucose. Those tested were

from 0 to 1.0% (w/v) as described in the Results section for each experiment. These were incubated at 37 C for 17-24 hr, harvested and washed as described before. Cells were suspended in H_2O to a common optical density at 520 nm for each experiment. Whole cell samples were removed before sonication. Each suspension was sonicated for 5 min. Both whole cells and sonicates were assayed for acid and alkaline PPase. The protein content of the sonicates was determined. Yields were calculated on the basis of total bacterial N per 100 ml medium.

For comparative purposes, the effect of glucose on the stationary phase levels of PPase in E. coli Crookes was also examined. Cells were inoculated into medium containing the following components in g/l: 1.0 g KH_2PO_4, 1.0 g K_2HPO_4, 1.0 g NaCl, 0.7 g $MgSO_4 \cdot 7H_2O$, 4.0 g ammonium sulfate, 0.5 g Na Citrate$\cdot 2H_2O$, and 5.0 g vitamin free casein hydrolysate, (final pH - 6.9) and incubated 18 hr at 37 C. The glucose concentration was varied in this medium from 0.1 to 1.0%. Cells were harvested, washed and suspended in water to a common optical density at 520 nm. Crude sonicates were assayed for acid and alkaline PPase and protein concentration. In addition, analysis was made of sonicates of cells harvested from the same medium, but without the casein hydrolysate, in which glucose was the only carbon source. Stationary phase cells were harvested at 18 hr and treated as above.

2. Glucose concentration in aerobic culture with lipoic acid. Starter cultures in TS broth were inoculated into 250 ml flasks containing 25 ml GOF medium supplemented with 0.5% (w/v) glucose. The concentration of α-lipoic acid was varied from 0 to 2.5 μg/ml.

Flasks were incubated on the shaker for 18 hr at 37 C. Cells were harvested, washed and sonicated as previously described. Acid PPase and yields were calculated. Thereafter 1.25 µg/ml lipoic acid was included in GOF medium for aerobic growth of S. faecium. The effect of glucose on PPase levels of cells grown aerobically was tested by inoculating GOF medium supplemented with varying glucose concentrations. These cultures were placed on the shaker and incubated 18 hr at 37 C. The cells were harvested, and sonicates prepared and assayed.

3. P_i concentration. GOF medium was made without P_i by using Tris-maleate to buffer the medium at a final pH of 6.8. Additions from sterile 0.2 M phosphate buffer at pH 7.0 were made to bring the final medium concentration to 8 and 20 mM P_i. Complete GOF medium, containing 28 mM P_i, was also included in the experiment. A starter culture grown 5 hr in TS broth was inoculated into these media and incubated 15 hr at 37 C. Cells were harvested, washed, sonicated and assayed for acid and alkaline PPase as described before.

4. PP concentration. A 5-hr starter culture grown at 30 C was inoculated into TS broth and incubated overnight at 30 C. After 16 hr of growth, this was inoculated, to an initial optical density of 0.200 at 520 nm, into fresh TS broth containing 0, 1.0 and 10 mM PP. Growth was stopped by pouring over ice and harvested at 1 hr and 2 hr intervals. Crude sonicates were made from cell suspensions, which were all at a uniform concentration of bacterial N/ml.

B. Variations of PPase During Growth

1. In TS broth at 37 C. Five-hr starter cultures were inoculated into 500 ml flasks containing 400 ml fresh TS broth and

incubated 16 hr at 37 C. These stationary phase cells were diluted into fresh, prewarmed TS broth to an initial optical density at 520 nm of ca. 0.200. Inoculations were made at intervals, and all cultures were stopped by pouring over TS-ice and harvested at the same time. All samples were made to a common optical density in water, and crude sonicates prepared. Determinations made for each experiment included acid and alkaline PPase, protein, growth rate and mg bacterial N/ml.

2. In GOF and TYE medium at 37 C. The procedure for TS broth was repeated using these two media. Starter cultures were prepared in the same medium to be used for the experiment. All media were prewarmed to 37 C.

3. In TS broth at 30 C. The procedure for 37 C was repeated except that all growth, including that of the starter culture, occurred at 30 C. All media were prewarmed to 30 C before inoculation.

4. In GOF-200 and GOF-1 medium. The levels of PPase were determined at intervals during growth in GOF and GOF limited in tryptophan. A preliminary experiment to show which of several components of GOF could be used most efficiently to control the rate of growth was performed. GOF medium was made at 2-fold concentration without uracil, cysteine, tyrosine, tryptophan or folic acid. Each of the above constituents was dissolved separately at 10-fold final medium concentration. The components and the medium were sterilized separately. A series of deficient GOF media was then made by adding all but one of the components back to the medium. All other constituents were at the same final concentration as shown in Table 3. These media

were dispensed into sterilized 16 x 125 mm screw capped tubes and inoculated from a 16 hr culture in GOF complete medium. This culture had been passed through GOF medium 5 times prior to its use as inoculum. Transfers of 0.01 ml were made daily through each of the deficient media until no more growth occurred. The most critical component appeared to be tryptophan, since no growth occurred beyond the third passage, whereas there was growth in all other deficient media to at least the seventh passage. Growth in uracil- and folic acid-deficient media continued through the tenth passage.

Complete GOF medium contained 200 μg/ml tryptophan (GOF-200). Limited medium was made to contain only 1 μg/ml tryptophan (GOF-1). These two media were used in an experiment to determine whether the growth rate influenced the levels of PPase during exponential phase. Starter cultures were prepared in GOF-1 or GOF-200. These were inoculated into the same medium and incubated 16 hr at 37 C. Stationary phase cells were diluted into prewarmed medium to an initial optical density of ca 0.100 at 520 nm. Samples were taken at intervals, and poured over crushed ice. Crude sonicates were prepared and assayed for acid and alkaline PPase and protein. Growth rate of the cells in each medium was determined.

C. Calculation of Growth Rates

Growth rates, reported as doubling time, were estimated from plots on semi-log paper of optical density (520 nm) of the culture vs time of incubation.

SOME PHYSICAL PROPERTIES OF PPASE ACTIVITIES

A. DEAE-cellulose Chromatography

DEAE-cellulose (Selectacel type 40, Schleicher and Schuell, Keene, N. H.) was equilibrated to constant pH in 0.01 M Tris buffer at pH 8.0, degassed with a water vacuum and poured into a 1.5 x 15 cm column, at room temperature. The column was equilibrated to 4 C overnight. Two ml of a 65-75% ammonium sulfate precipitated preparation containing 15 mg protein/ml was allowed to absorb to the cellulose and the proteins then eluted with a stepwise gradient of 100 ml each of 0.05, 0.15, 0.5, and 1 M NaCl. Ten ml fractions were collected with a Buchler circular fraction collector (Buchler Instrument Co., Fort Lee, N.J.). Fractions were assayed for both PPase activities and for protein content.

B. Gel-filtration in Sephadex G-100

Gel-filtration was used as a method to attempt to separate the two PPase activities, as well as a means to estimate the molecular weight (9). Sephadex G-100 beads (Pharmacia, Piscataway, N. J.) were hydrated by heating with H_2O in a boiling water bath for 5 hr. The slurry was cooled to room temperature and the fines poured off. After the desired buffer was then added, mixed and the gel allowed to settle, the fines were again decanted. Buffer addition was repeated 2 times. Finally, the gel was suspended in enough buffer so that when it was stirred, incorporated air bubbles could escape rapidly to the surface. This mixture was then degassed with a water vacuum, and allowed to equilibrate to 4 C. Part of the mixture was then poured into a 2.5 x 45 cm column, already partly filled with the appropriate buffer.

After a 5 to 6 cm layer of Sephadex had settled to the bottom, the outlet was opened. The pressure head was then lowered slowly to 15 cm, where it was maintained during the rest of the pouring of the column and thereafter by the use of a Mariotta flask. As the bed volume increased, the clear buffer on top was removed and more slurry was added, until the bed reached a height of about 35 cm. A sample applicator was placed on top, to prevent disturbance of the gel when the sample was applied. Each column was equilibrated with 500 ml buffer before use. Samples of 1 or 3 ml were placed on the gel so that the first tube was under the column at the time that half of the sample had entered the gel. Samples of 60 drops, equivalent to 3 ml, were measured with a drop counting device and were collected on a Gilson linear fraction collector. The elution volume of each protein was located by graphic estimation of the midpoint. The void volume of each column was determined with 0.2% Blue Dextran (Pharmacia, Piscataway, N. J.) at the beginning of each experiment. Non-enzymatic marker proteins were assayed by adsorption at specified wave lengths (Table 4) and both Mg^{++}- and Co^{++}-activated PPase were assayed enzymatically as before. During the standardization of the column, proteins were applied sequentially; a second protein sample was placed on the column as soon as there was no danger of overlap with the first. Elution volume is not influenced by this procedure (9).

C. Electrophoresis

1. In starch gel. A 13% (w/v) starch gel (Connaught Laboratories, Toronto, Ont.) was prepared in 0.025 M boric acid + 0.01 M NaOH at pH 8.6. The bridge buffer was 0.3 M boric acid + 0.06 M NaOH

TABLE 4

Proteins Used to Standardize Sephadex G-100 Columns for Molecular Weight Estimation of PPase

Protein[1]	mg Protein Applied to Column	λ for Estimation (nm)	Molecular[2] Weight
Myoglobin	2	410	17,800
Chymotrypsinogen A 3x recrystallized	4	280	25,000
Ovalbumin 5x recrystallized	8	280	45,000
Bovine serum albumin fraction 5	8	280	67,000[3]

[1]from Mann Research Laboratories, N.Y., N.Y.
[2]from Ref. 10.
[3]Dimer.

at pH 8.6. Samples were inserted as filter paper squares which had been soaked in the enzyme preparation or in hemoglobin (obtained from Dr. D. Rigas, University of Oregon Medical School), which was used as a marker. These were placed between halves of the vertically sliced gel. The halves were then rejoined and the gel placed in an E.C. electrophoresis apparatus (Weston Instruments, Philadelphia, Pa.). Eight volts/cm were applied for 4 hr with water cooling. After 4 hr, the papers were removed, and the gel was sliced horizontally. Half was stained for proteins by Amido Schwartz (125), and the other half assayed for enzyme activity. Assays were carried out by incubating the gels for 20 min at room temperature in reaction mixtures at pH 5.6 containing Co^{++} and L-histidine, at pH 8.0 containing Mg^{++}, and at pH 7.0 containing Co^{++}, Mg^{++} and L-histidine to detect any intermediate bands. The gel was then immersed in the triethylamine-molybdate reagent of Sugino and Miyoshi (157). This method is specific for P_i and a sharp, discrete zone of yellow precipitate appears within seconds where P_i has been released.

2. <u>In polyacrylamide</u>. Crude sonicate, containing 2 mg/ml protein, was applied to a standard 7% (w/v) polyacrylamide gel at pH 9.5, prepared by the method of Ornstein and Davis (126). Five milliamps per tube was applied for 45 min at 4 C in a Canalco (Canalco, Bethesda, Md.) apparatus. Columns were then washed with distilled water and placed in reaction mixtures described on page 44. Columns were incubated for 20 min at room temperature, washed and assayed for P_i by the Sugino and Miyoshi method (157). Protein zones were defined in a duplicate column by staining with Amido Schwartz (125).

D. Sucrose Gradient Centrifugation

Solutions containing 5 and 20% (w/v) sucrose were made in 2 mM acetate buffer at pH 5.6. Three 5-20% linear sucrose gradients, 4.0 ml each, were dispensed with a single outlet of a Buchler Polystaltic pump (Buchler Instrument Co., Fort Lee, N.J.) into 4.0 ml polyallimer centrifugation tubes. These were allowed to equilibrate for 4 hr in the cold room. A 0.1 ml sample of a 50-70% ammonium sulfate precipitated fraction, containing 2 mg/ml protein was applied to 2 of the gradients, 0.1 ml ovalbumin (0.8 mg/ml) was placed on the third gradient. This procedure was repeated for a 5-20% sucrose gradient made up in 3 mM borate buffer at pH 8.6. All six tubes were placed in a 6-place SW-56 rotor and spun in an LKB preparative ultracentrifuge at 157,000 x g (ave) for 16 hr. A series of 0.2 ml samples were collected from each tube by punching a hole in the bottom and forcing the samples out with positive pressure. Each sample was assayed for protein content by absorbance at 280 nm. Every third sample was assayed for both acid and alkaline PPase activity to determine the range of samples with enzymic activity, and then every tube within this range was assayed for both activities.

INACTIVATION STUDIES

A. Thermal Inactivation

To determine whether the two PPase activities had different rates of thermal denaturation, a crude sonicate preparation was subjected to different temperatures. Crude sonicate, prepared in 0.2 M veronal acetate (VA) buffer at pH 7.0 and containing 0.5 mg protein/ml

was pipetted at room remperature into a tube preheated in a water bath. The solution was rimmed around the tube so that the temperature of the preparation reached bath temperature as quickly as possible. Samples were withdrawn at intervals over 15 min and placed in an ice bath until the heating period was concluded. Each sample was then assayed for both acid and alkaline PPase activity. Enzyme preparations in the same buffer, but containing either 3.5 μg/ml Co^{++}, or 3.5 μg/ml Mg^{++} were also treated in the same manner, to determine whether addition of a cation altered the rate of inactivation.

B. In Urea

To the complete enzyme reaction mixtures were added various concentrations of urea. The crude sonicate prepared in 3 mM VA buffer at pH 5.6 and containing 20 mg/ml protein was diluted and appropriate amounts added. The mixture was allowed to equilibrate to 37 C for 2 min. The reaction was started by the addition of PP. After 10 min incubation, the reaction was stopped by addition of 10% TCA, and P_i was measured as before. Urea added to standards containing 20 μg P_i did not influence the Fiske-SubbaRow determination.

RESULTS

CHARACTERISTICS OF CRUDE SONICATE FROM S. FAECIUM F24

A. pH Dependence of PPase Activities

A crude sonicate, prepared from cells in exponential phase of growth in TS broth was assayed over a range of pH values from 4.5 to 9.0. Additions to the reaction mixtures were Co^{++}, Co^{++} and L-histidine, Mg^{++}, or Mg^{++}, Co^{++} and L-histidine. Crude sonicate was added to start the reaction since the pH determination was made after all other components had been added. Activity was measured after a 10 min incubation period. Figure 1 shows that in the presence of Co^{++} a peak occurred at pH 5.4. Addition of L-histidine increased that activity about 30%, but did not shift the pH optimum. In the presence of Mg^{++}, a broad maximum occurrred from pH 7.5 to 8.5. When both cations were included in the reaction mixture, the profile was altered considerably; no sharp peak occurred in the acid or alkaline range, and the activity in the neutral range was higher. Mg^{++} inhibited the acid PPase activity and Co^{++} and L-histidine inhibited the alkaline activity. No P_i was formed in the absence of cation.

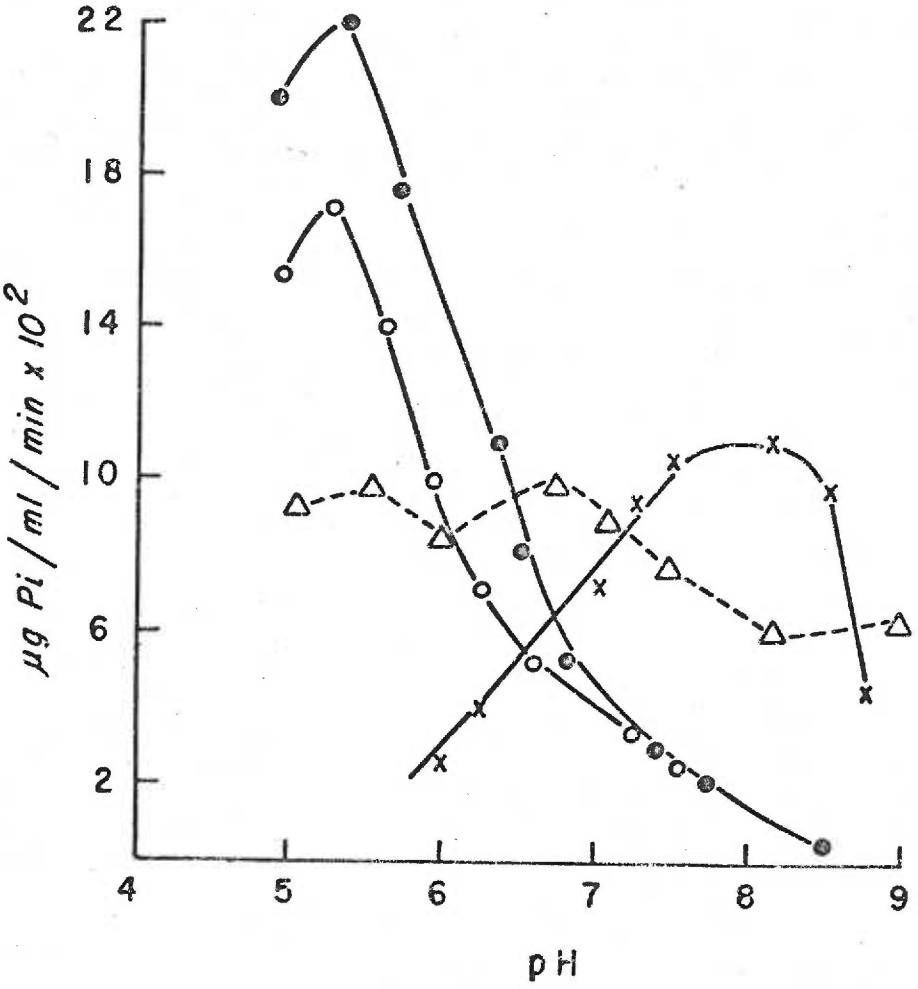
B. Histidine Enhancement

Where several amino acids had been tested for their ability to enhance the acid PPase reaction in the presence of Co^{++}, L-histidine had been found to have maximal activity (124). A study was made in the course of this research to determine whether the reaction was specific for L-histidine, or if it could be satisfied by D-histidine,

FIGURE 1

Effect of pH on the Co^{++}- and Mg^{++}-dependent PPase Activities

A 3 mM VA buffer was used at all pH values tested. Each reaction mixture contained 1.4 m moles $Na_4P_2O_7$. pH was measured after all additions but the crude sonicate had been made. Reaction mixtures contained; (O) 5.8 m moles $CoCl_2$; (●) 5.8 m moles $CoCl_2$ and 2.9 m moles L-histidine; (X) 2.6 m moles $MgCl_2$; (△) 5.8 m moles $CoCl_2$, 2.9 m moles L-histidine and 2.6 m moles $MgCl_2$.


22
18
14
10
6
2
μg Pi / ml / min x 10²
4
5
6
7
8
9
pH

or imidazole itself. Table 5 shows that the stimulation by D- or L-histidine was equal and amounted to 30%. Imidazole did not increase the level of Co^{++}-stimulated activity. In the absence of Co^{++} no activity was observed. All reaction mixtures thereafter contained L-histidine.

C. Other Enzyme Activities

1. Alkaline phosphatase. A crude sonicate from S. faecium F24, grown to stationary phase in TS broth at 37 C, was assayed for alkaline phosphatase. No hydrolysis of ρ-nitrophenyl phosphate at pH 8.5 was detected after 60 min incubation at room temperature.

2. PP-glucose phosphotransferase. Stetten had shown that a glucose-dependent hydrolysis of PP occurred at acid pH, using liver or kidney microsomal preparations as the source of enzyme (154). E. coli alkaline phosphatase also had PP-glucose phosphotransferase activity (8). To determine whether S. faecium had this type of activity, a crude sonicate was prepared from stationary phase cells in TS broth. This preparation was assayed at acid pH with and without 0.4 M glucose, by the standard method described in the Material and Methods section. No activity was observed in the absence of Co^{++}, and no significant differences were observed between the assays that contained 0.4 M glucose and those that did not (Table 6).

PPASE ACTIVITY OF OTHER ORGANISMS

A. Streptococcus faecalis 10C1

Since S. faecalis is closely related to S. faecium, it was of interest to observe whether they both had a similar spectrum of PPase

TABLE 5

Specificity of Histidine Enhancement of Co^{++}-dependent PPase

Additions	μg P_i/min/ml
Co^{++}	200
Co^{++} + L-histidine	263
Co^{++} + D-histidine	260
Co^{++} + D-, L-histidine*	263
Co^{++} + Imidazole	195
None	0
Mg^{++} (pH 8.4)	115

Concentrations used: $CoCl_2$, 5.8 m moles; $MgCl_2$, 2.6 m moles; D-, L-histidine, and Imidazole, 2.9 m moles.
*D- and L-histidine, 1.45 m moles each.

TABLE 6

PP-glucose Phosphotransferase
Activity of S. faecium F24

Additions	µg P_i/ml/min	
	without glucose	with 0.4 M glucose
Co^{++}	168	116
Co^{++}, histidine	264	300
histidine	15	6
None	8	7

TABLE 7

PPase Activities of
S. faecalis 10C1 and S. faecium F24

Organism	µg P_i/mg bacterial N/min		Co/Mg
	acid PPase	alkaline PPase	
S. faecalis 10C1	193	141	1.4
S. faecium F24	261	87	3.1

activities. Crude sonicates were prepared from stationary phase cells of both species and assayed for acid and alkaline PPase. Table 7 shows that S. faecalis does have a Co^{++}-stimulated acid PPase, as well as the alkaline activity. The Co/Mg ratio is not as large as that observed in S. faecium.

B. E. coli Crookes

E. coli has been shown to have a Mg^{++}-stimulated alkaline PPase activity (17, 31, 67, 141). It was of interest to see if such an unrelated organism would have a Co^{++}-stimulated activity, such as that of S. faecium. E. coli Crookes was grown in a minimal salts, casein hydrolysate medium with 0.1% (w/v) glucose for 16 hr. The cells were harvested, washed, sonicated, and assayed for both acid and alkaline PPase. No activity was observed at pH 5.6 in the presence or absence of Co^{++} and histidine. At pH 8.0, however, a Mg^{++}-stimulated PPase was detected. The specific activity was 150 μg P_i/mg bacterial N/min.

LEVELS OF PPASE ACTIVITY DETECTABLE WITH WHOLE CELLS

Assays of fresh whole cell suspension, harvested at stationary phase, resulted in positive Fiske-SubbaRow tests regardless of whether PP, Co^{++} or histidine were included in the reaction mixture, suggesting that whole cell assays may, in fact, measure only P_i leakage (Table 8). However, if the cells were allowed to remain overnight or longer in water or buffer in the refrigerator, increasing levels of PPase activity were observed, for example, day 1: no detectable levels of enzyme; day 3: 35 μg P_i/ml/min; day 5: 158 μg P_i/ml/min. Variable levels of PPase were observed in aged cells from different

TABLE 8

Acid PPase Assay of Whole Cells

Additions	μg P_i/mg bacterial N/min	
	1	2
Complete	2.2	3.3
$-Co^{++}$, -histidine	3.0	---
-PP	---	3.2
zero time	2.6	3.4

experiments, which depend upon the medium used for growth, and the age of the culture harvested. These, no doubt, reflect the state of the integrity of the cell wall or membrane, or may suggest that the cell experiences a change in permeability upon storage. Sonication resulted in crude extracts with specific activities that were 100-fold or more greater than those of whole cell assays.

LOCALIZATION OF PPASE ACTIVITIES

Since whole cell preparations of S. faecium F24 were impermeable to PP, it was of interest to determine where in the cell the enzyme activities could be found. To make such a determination, a method was required which would separate the cell wall from the cell membrane. Lysozyme treatment of gram positive cells results in destruction of the cell wall (25), and thus allows one to study the membrane in the absence of the wall. In addition, treatment of gram negative cells with lysozyme and EDTA in sucrose has been shown to release enzymes that are localized between the membrane and the wall. These have been called "periplasmic" enzymes (92).

A. In Lysozyme Treated Cells

The location of the enzyme activities was determined by forming protoplasts of S. faecium F24, lysing them and examining the resultant supernatant and particulate fractions after differential centrifugation.

The optimal concentration of lysozyme used for formation of protoplasts was found by treating, with varying concentrations of lysozyme, cells harvested at the stationary phase of growth in TYE medium

supplemented with 0.5% (w/v) glucose. The reaction mixture contained 0.66 M sucrose, which acted as an osmotic buffer to stabilize the protoplasts formed. Lysozyme treatment proceeded for a total of 120 min at 37 C. The rate of formation of protoplasts was followed by observing the decrease in optical density at 520 nm in samples taken at intervals and diluted 1 to 5 with water. Figure 2 shows that in concentrations greater than 40 µg lysozyme/ml, lysis is rapid, with a 50% decrease in optical density within 30 min, and 75% decrease within 60 min. Treatment with 200 µg/ml for 60 min at 37 C was used in the following experiments.

In a preliminary experiment, whole cells were treated with lysozyme in a sucrose-stablilized reaction mixture. After 60 min, the sample was centrifuged at 12,000 x g. The total reaction mixture, the supernatant and the protoplasts, recovered and lysed by dilution in water, were assayed for acid PPase. Data in Table 9 show that only about 5% of the potential enzyme was released into the supernatant by lysozyme treatment. Thus PPase is not a "periplasmic" enzyme. Some of the protoplasts may have lysed during centrifugation or other manipulations, and this may account for the low levels observed in the supernatant.

In another experiment, it could be shown (Table 10) that if the protoplasts were assayed in 0.66 M sucrose little of the potential enzyme was detectable. Thus the enzyme does not seem to be bound to the outside of the membrane. Total PPase was available from lysed protoplasts only. The amount of enzyme measurable in stabilized protoplasts varied with the amount of pipetting and handling to which they

FIGURE 2

Effect of Lysozyme on Optical Density of S. faecium F24

A cell suspension, containing 2.3 mg bacterial N/ml, was made from cells at stationary phase of growth in TYE medium. Cells were incubated in 0.1 M Tris buffer at pH 7.2, 0.66 M sucrose and varying concentrations of lysozyme at 37 C. Samples were removed at intervals and diluted 1:5 in H_2O. The optical density at 520 nm was determined. Lysozyme concentrations used were: (●) control, (○) 40 µg/ml, (×) 120 µg/ml, (▲) 200 µg/ml, and (△) 400 µg/ml.

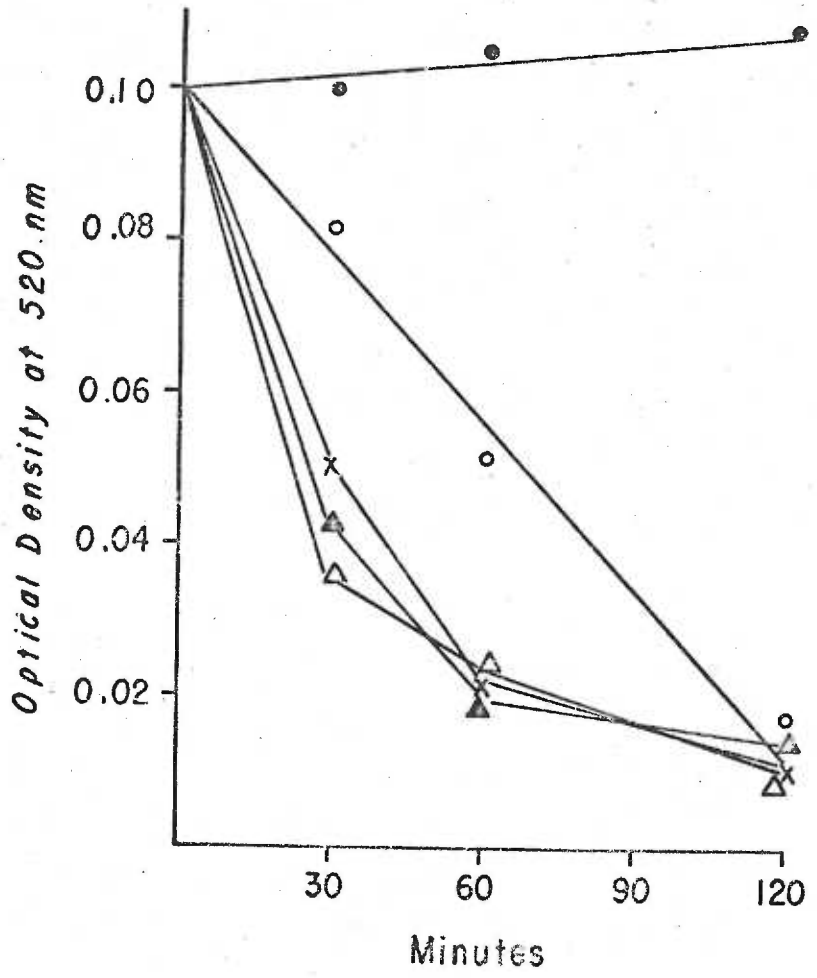

0.10
0.08
0.06
0.04
0.02
Optical Density at 520 nm
30
60
90
120
Minutes

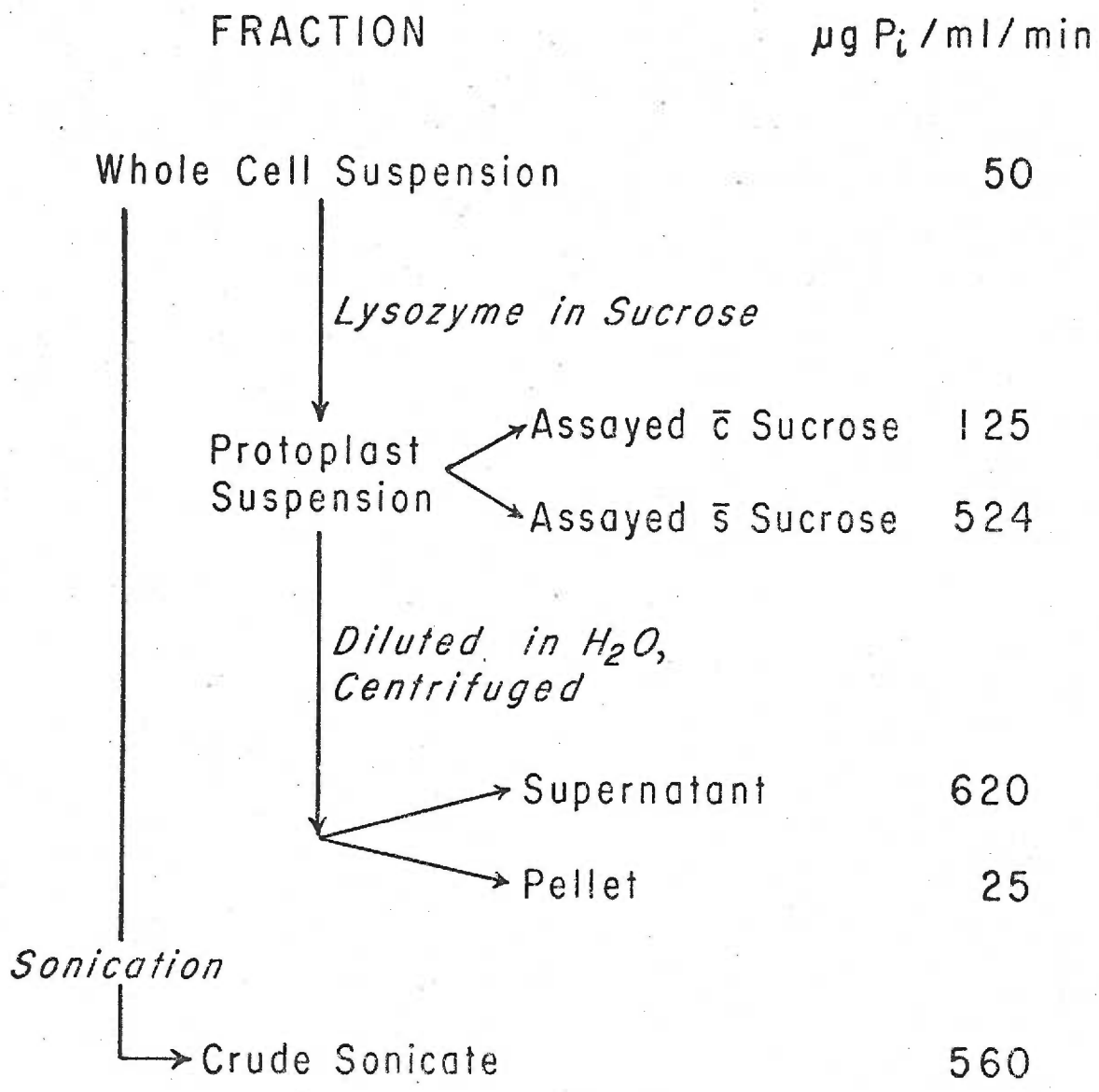
TABLE 9

Acid PPase Activity Released into the Medium by Sucrose Stabilized, Lysozyme Treated Cells

Fraction Assayed	μg P_i/ml/min
Total reaction mixture diluted in H_2O	317
Lysed protoplasts	301
Supernatant	17
Untreated whole cells	14

TABLE 10

Acid PPase Activity of Stablilized and Lysed Protoplasts


FRACTION
µg Pi / ml / min
Whole Cell Suspension
50
Lysozyme in Sucrose
Protoplast Suspension
Assayed c̄ Sucrose
125
Assayed s̄ Sucrose
524
Diluted in H2O, Centrifuged
Supernatant
620
Pellet
25
Sonication
Crude Sonicate
560

had been subjected. They are very fragile and lyse readily upon exposure to shear forces, or prolonged storage. The pellet fraction from the lysed protoplasts contained less than 5% of the total potential activity. As noted in the Discussion, the pellet is most likely membrane.

One problem encountered was that when the protoplasts lysed, nucleic acids were released, causing an increase in viscosity. The solution became difficult to pipette accurately, and the pellet did not sediment well. Therefore, after lysis of the protoplasts, deoxyribonuclease and ribonuclease were added, and the lysate subjected to a second incubation at 37 C for 30 min. The supernatant fraction of cells treated in this manner were shown to contain 88.5% of the total activity. Only 10% was found in the pellet (Figure 3).

Once the techniques had been established for the localization of the acid PPase activity, the procedure was repeated to show whether the alkaline PPase activity was similarly located. Table 11 shows that both activities are predominantly inhabitants of the cytoplasm, and not bound to the particulate fraction.

B. In Sonicated Cells

Sonication and lysozyme treatment released similar amounts of PPase activity (Table 10); since it could be shown that sonication of lysates did not destroy the activity of the lysates, sonicates were used as the source of enzyme for the rest of the experiments.

An experiment was designed to determine whether the activities were associated with the ribosomes in the cytoplasm. Crude sonicates were prepared from cells in the exponential phase of growth in TS

FIGURE 3

Localization of Acid PPase Activity in Lysozyme and Nuclease Treated Cells

A cell suspension of stationary phase cells, grown in TYE medium, was made to contain 0.4 µg bacterial N/ml in 0.1 M Tris buffer at pH 7.6, containing 0.66 M sucrose and 200 µg/ml lysozyme. The mixture was incubated 60 min at 37 C, and then centrifuged at 10,000 x g. The protoplast pellet was resuspended in buffer containing 1 µg ribonuclease and 1 µg/ml deoxyribonuclease and 0.01 M $MgSO_4$ and incubated 30 min at 37 C. This suspension was centrifuged at 20,000 x g for 30 min. The pellet was resuspended. The protoplast was assayed in 0.66 M sucrose, the crude lysate is the lysozyme treated cell suspension before centrifugation.

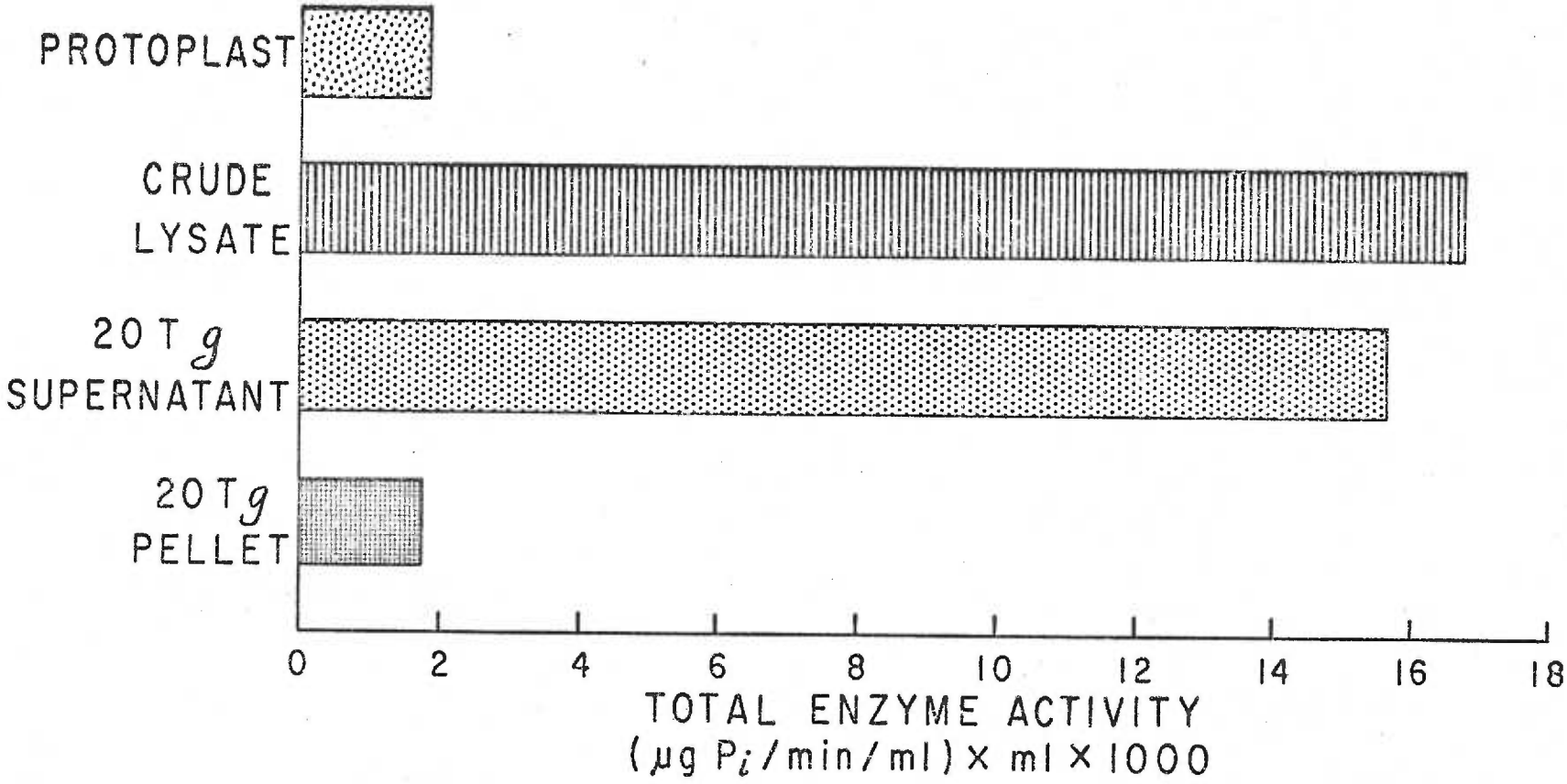

PROTOPLAST
CRUDE LYSATE
20 T g SUPERNATANT
20 T g PELLET
0
2
4
6
8
10
12
14
16
18
TOTAL ENZYME ACTIVITY
(μg Pi/min/ml) x ml x 1000

TABLE II

Localization of Acid and Alkaline PPase Activities in Lysozyme Treated Cells

Fraction	μg P_i/ml/min		Co/Mg
	Acid PPase	Alkaline PPase	
Total lysate	360	150	2.5
Lysate medium	16	7	2.2
Protoplast cytoplasm	243	90	2.7
Protoplast membrane	3.8	1.1	3.7

broth. The cells were sonicated in 1 mM Tris buffer at pH 8.0 with or without 3 mM Mg^{++}. After removal of the wall and debris, by centrifugation at 20,000 x g, the resultant supernatant was centrifuged at 104,000 x g for 60 min. The results presented in Figure 4 show that neither the acid nor the alkaline activities sedimented during this treatment. Only about 3% of either activity was detected in the particulate fraction; this may have been trapped during sedimentation.

REGULATORY STUDIES

A. Influence of Cultural Conditions

1. Glucose concentration in standing culture. Barber (unpublished results) made the observation in this laboratory that increased concentrations of glucose in TYE medium resulted in decreased concentrations of PPase, as assayed with whole cells. She had obtained the results shown in Table 12. Since it had been shown that little of the potential activity was expressed by whole cells it was of interest to determine whether the results observed by Barber were, in fact, alterations in level of enzyme, or an expression of the stability of the cell. Thus, an experiment was performed to differentiate these two possibilities. Crude sonicates, from cells grown 18 hr at 37 C in GOF medium supplemented with increasing concentrations of glucose, were prepared and assayed. Table 13 shows that, indeed, under these conditions the levels of acid PPase did decrease with growth in increasing concentrations of glucose. The cell yield, calculated as mg bacterial N/100 ml medium, increased with increasing concentration of glucose, as would be expected. The experiment was

FIGURE 4

Localization of Acid and Alkaline PPase Activities in Sonicates of S. faecium F24

Cells from exponential phase of growth in TS broth were made to 5.8 mg bacterial N/ml in 1 mM Tris buffer at pH 8.0, and with or without 3 mM Mg^{++}. The suspension was sonicated 4 min, and centrifuged at 20,000 x g for 20 min. The supernatant was recovered, assayed and recentrifuged at 104,000 x g for 1 hr. The pellet was suspended in buffer to the original volume.

Hatched bars are acid PPase values, and open bars are values for alkaline PPase activity.

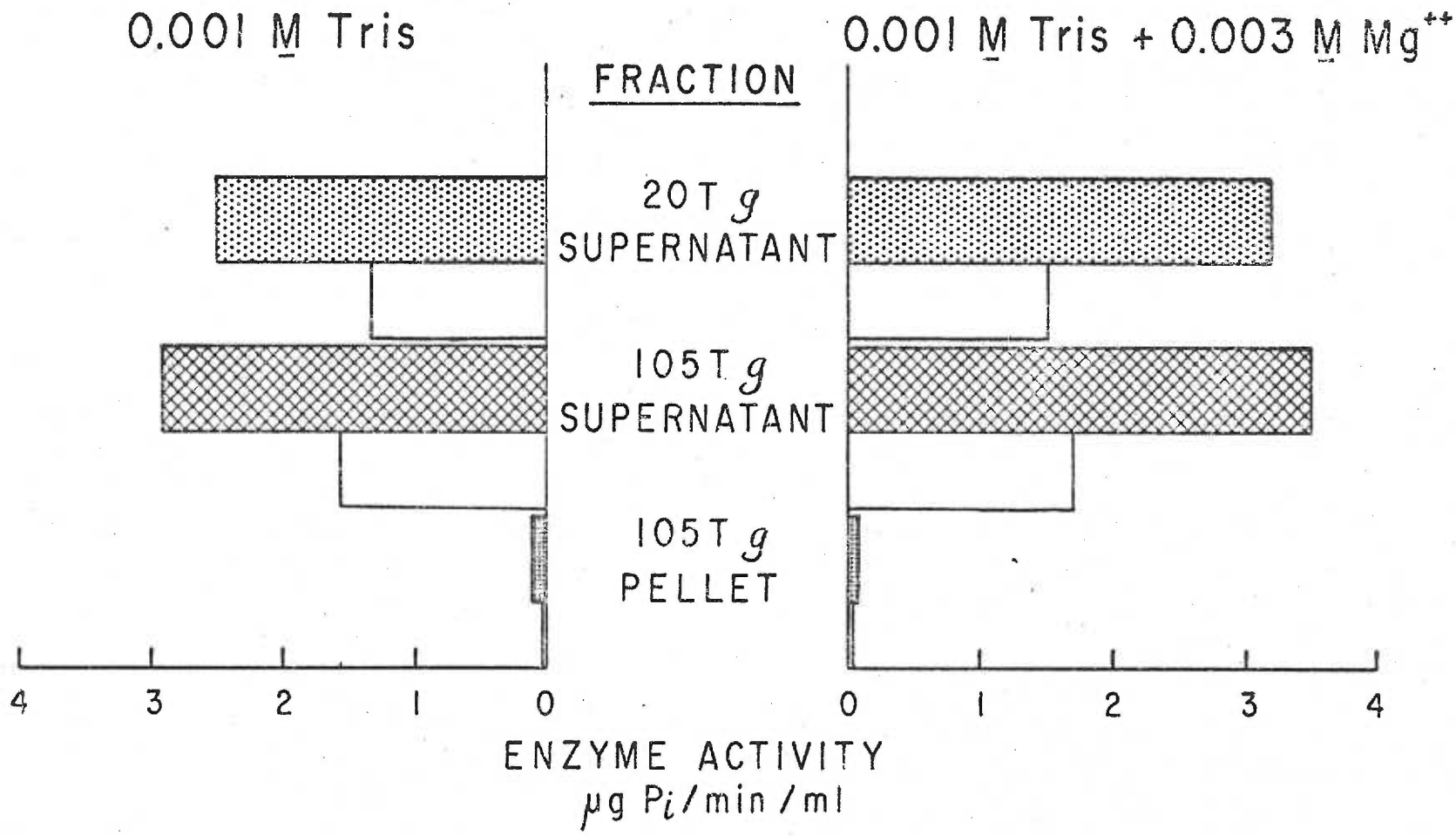

0.001 M Tris
0.001 M Tris + 0.003 M Mg++
FRACTION
20T g SUPERNATANT
105T g SUPERNATANT
105T g PELLET
4
3
2
1
0
0
1
2
3
4
ENZYME ACTIVITY
µg Pi / min / ml

TABLE 12

Effect of Glucose Concentration in TYE Medium on Stationary Phase Levels of PPase Activities of Whole Cells[1]

% glucose	μg P_i/mg bacterial N/min		Co/Mg
	Acid PPase	Alkaline PPase	
0.0	14	5.9	2.4
0.1	7.4	3.0	2.5
0.5	5.6	3.5	1.6

[1]Barber, unpublished results

TABLE 13

Effect of Glucose Concentration in GOF Medium on Stationary Phase Levels of Acid PPase of Whole Cells and Crude Sonicates

% glucose	Yield[1]	μg P_i/mg bacterial N/min	
		Whole cells	Sonicates
0.01	1.0	3.0	270
0.1	2.8	3.0	218
0.5	7.1	13.0	132

[1]mg bacterial N/100 ml medium

repeated, using more closely spaced glucose concentrations in GOF medium, and a 24 hr incubation period to assure that cells had reached stationary phase. Results, as shown in Figure 5A, were calculated as μg P_i/mg protein/min, but if these values are recalculated in terms of μg P_i/mg bacterial N/min a similar curve can be drawn. The yield of cells increased up to 0.5% glucose and then remained stable, showing a mirror image of the acid PPase activity.

To determine whether this response was specific to GOF medium, and to observe whether both PPase levels responded similarly, the experiment was repeated using GOF and TYE medium. Both activities were assayed. Table 14 shows that both acid and alkaline activities decreased coordinately with increasing glucose concentration in each medium. The specific activities of the activities were much smaller in TYE medium. Yield data were not obtained for this experiment.

That the response was observed in all media used in these experiments was shown in the results of assay of crude sonicates from cells grown to stationary phase in TS broth supplemented with increasing concentrations of glucose (Figure 5B). TS broth contains 0.25% glucose without further addition. Both the acid and alkaline PPase activities decrease with increasing glucose concentration.

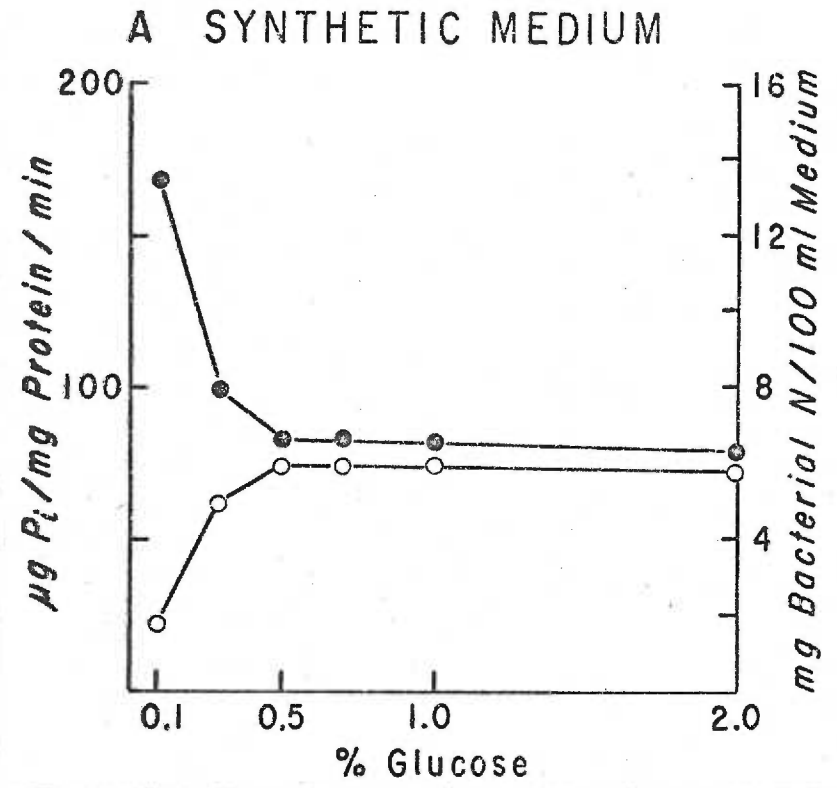
2. Glucose concentration in aerobic culture with lipoic acid. Adequate growth under aerobic conditions does not occur in S. faecium in the absence of lipoic acid (39). Thus, lipoic acid was included, in increasing concentrations, in GOF medium containing 0.5% glucose. Cells were incubated in these media for 16 hr with shaking. Table 15 shows that the yield of cells was greatly increased by

FIGURE 5

Effect of Glucose Concentration on PPase Activities of Stationary Phase Cells

A. Cells were grown to stationary phase in GOF medium supplemented with increasing concentrations of glucose. (●) acid PPase activity, (O) cell yield in mg bacterial N/100 ml medium.

B. Cells were grown to stationary phase in TS broth supplemented with increasing concentrations of glucose. (●) acid PPase, (O) alkaline PPase, (X) cell yield in mg bacterial N/100 ml medium.


A SYNTHETIC MEDIUM
200
100
16
12
8
4
μg P_i/mg Protein/min
mg Bacterial N/100 ml Medium
0.1
0.5
1.0
2.0
% Glucose

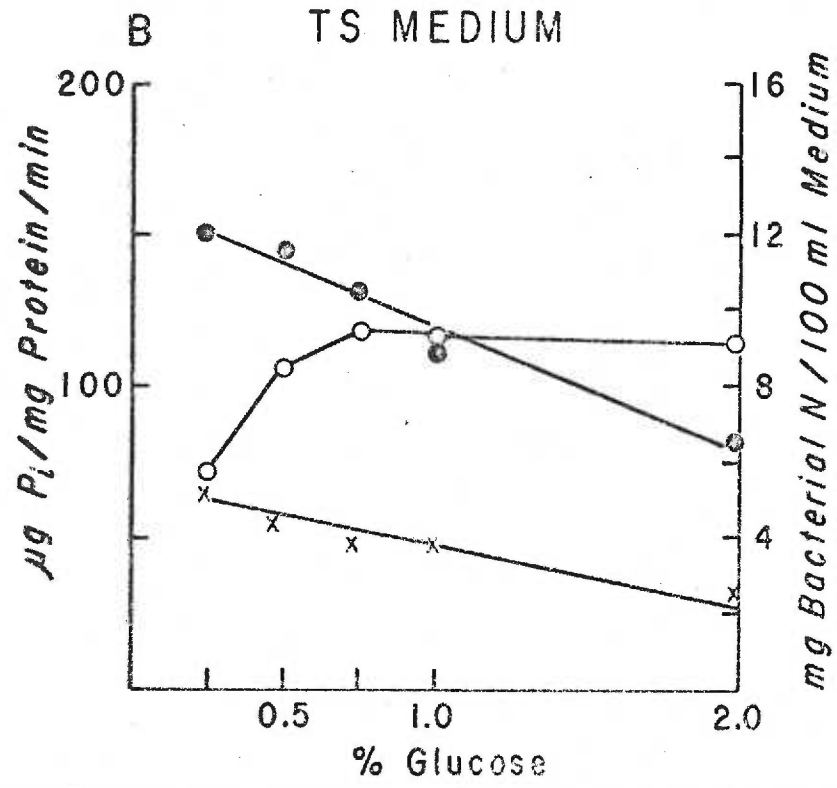

B TS MEDIUM
200
100
16
12
8
4
μg P_i/mg Protein/min
mg Bacterial N/100 ml Medium
0.5
1.0
2.0
% Glucose

TABLE 14

Effect of Glucose Concentration in GOF Medium and TYE Medium on Stationary Phase Levels of Acid and Alkaline PPase Activities in Crude Sonicates

% glucose	GOF Medium			TYE Medium		
	μg P_i/mg bacterial N/min		Co/Mg	μg P_i/mg bacterial N/min		Co/Mg
	Acid PPase	Alkaline PPase		Acid PPase	Alkaline PPase	
0.1	166	41	4.0	80	25	3.2
0.5	101	26	3.0	18	6	3.0
1.0	92	22	4.2	16	6	2.7

TABLE 15

Effect of Lipoic Acid on the Levels of Acid PPase Activity of Cells Grown Aerobically in GOF Medium

Lipoic acid concentration (µg/ml)	Cell yield	µg P_i/mg bacterial N/min
0.0	1.2	142
0.5	5.2	130
1.25	6.3	80
2.0	6.1	70
2.5	6.1	60

addition of 0.5 µg/ml lipoic acid, and that the levels of acid PPase activities decreased. Addition of 1.24 µg/ml lipoic acid to the assay mixture did not influence the P_i determination.

When cells were aerated in GOF medium, supplemented with 1.25 µg/ml lipoic acid and containing increasing amounts of glucose, assay of crude sonicates resulted in the data shown in Table 16. The acid PPase activity increased in 0.1% glucose and then decreased with increasing glucose concentrations thereafter. Addition of lipoic acid did not alter the glucose effect observed in anaerobically grown cells. Assay of crude sonicates prepared from cells grown without aeration for 18 hrs in lipoic acid supplemented GOF medium showed the results presented in Table 17.

3. Effect of P_i concentration. The synthesis of alkaline phosphatase is repressed in high concentration of its product, P_i. Experiments were designed to show whether PPase levels were influenced by the concentration of P_i in the medium. GOF medium was prepared using Tris-maleate buffer instead of K_2HPO_4, and made to a final pH of 6.8. P_i was added in varying amounts from sterile solution of 0.2 M phosphate buffer at pH 7.0. Crude sonicates were prepared after 18 hr incubation at 37 C, and assayed for acid and alkaline PPase. In addition, each medium was assayed for P_i. The constituents of the medium contained enough phosphate to support the growth of S. faecium F24, without addition of supplemental amounts. However, addition of P_i did not radically change the levels of PPase found in the cells (Table 18). There does seem to be a slight shift from large Co/Mg ratios usually observed in fully supplemented GOF medium (3.2), to

TABLE 16

Effect of Glucose Concentration on Acid PPase Activity of Cells Grown Aerobically in GOF Medium Supplemented with Lipoic Acid

% glucose	µg P_i/mg bacterial N/min	Cell yield
0.01	150	0.5
0.10	250	1.9
0.50	155	5.5
1.00	100	5.4

TABLE 17

Effect of Glucose Concentration on PPase Activities of Cells Grown Anaerobically in GOF Medium Supplemented with Lipoic Acid

% glucose	µg P_i/mg bacterial N/min		Co/Mg
	Acid PPase	Alkaline PPase	
0.05	195	66	3.0
0.5	164	50	3.3
1.0	155	39	4.0
5.0	88	35	2.6

TABLE 18

Effect of P_i Concentration on PPase Activities of Cells Grown in GOF Medium

Molarity of added P_i	μg P_i/mg bacterial N/min		Co/Mg	μg P_i/ml measured
	Acid PPase	Alkaline PPase		
0	160	115	1.9	46
8	130	75	1.7	370
20	165	92	1.8	950
28	135	42	3.2	1400

TABLE 19

Effect of PP Concentration on Acid PPase Activity of Cells Grown in TS Broth at 30 C

Molarity of added PP	Time hrs	Yield	μg P_i/mg bacterial N/min
0	1	.83	74
	2	1.23	73
0.001	1	.85	74
	2	1.46	77
0.010	1	.82	--
	2	1.36	63

smaller ones in low concentration of P_i (1.7).

4. Effect of PP concentration. The presence of substrate in the medium induces the synthesis of some enzymes, the classical example being β-galactosidase. To test the possibility that PPase was such an inducible enzyme, cells were grown at 30 C in TS broth for 18 hr. They were then diluted into fresh TS broth, containing varying levels of PP, to an initial optical density of 0.100. These cultures were incubated for 1 or 2 hr at 30 C, poured over ice, harvested and sonicated. There appeared to be no difference in the rate of increase in cell yield, and no significant induction of PPase activity by the inclusion of PP in the medium (Table 19). Most probably the cells are not permeable to PP as was seen in experiments using whole cells.

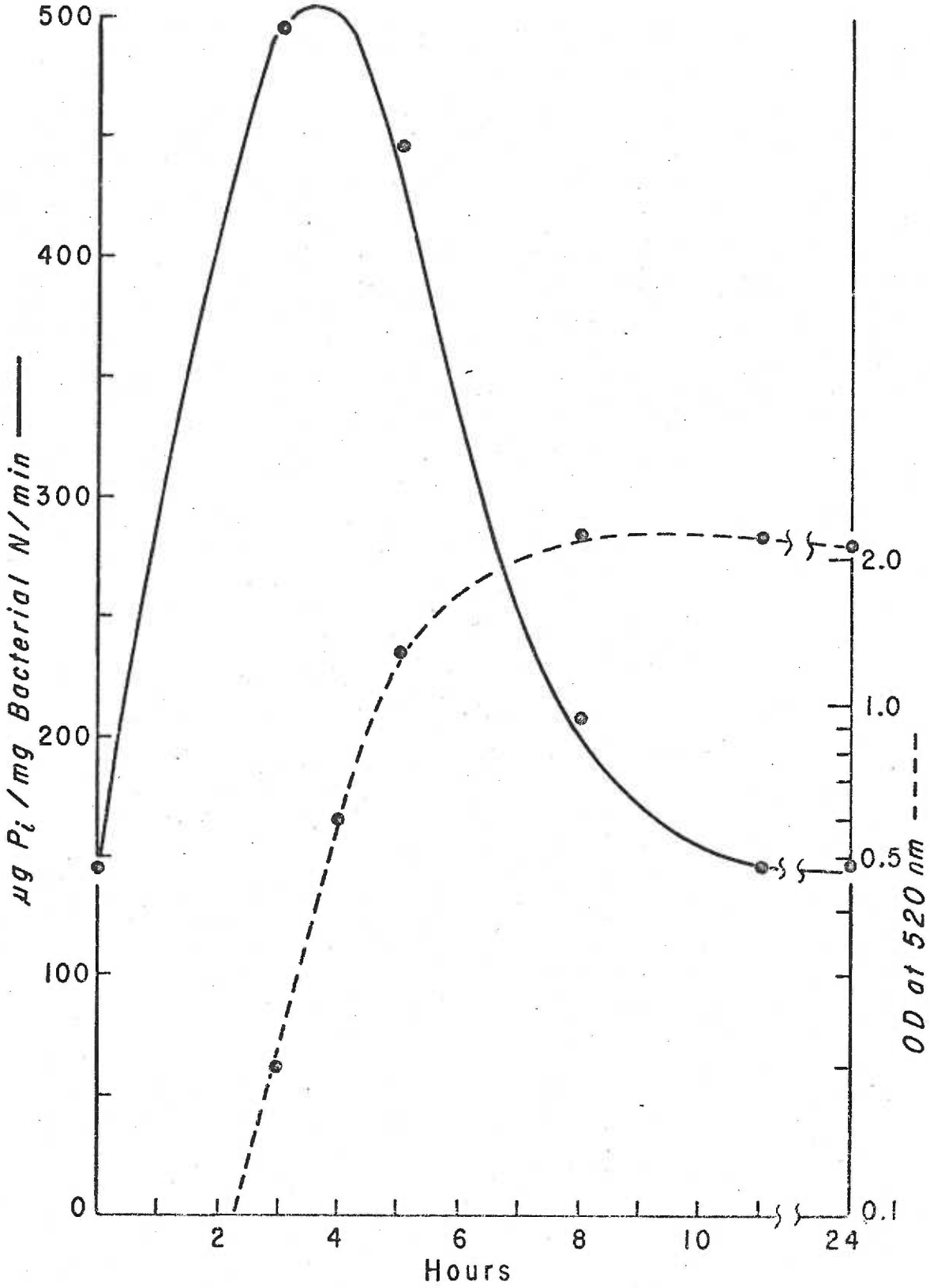
B. Levels of PPase During Growth

1. In TS broth at 37 C. It had been found earlier using whole cell assays, (Barber, unpublished results) that enzyme levels in cells grown to stationary phase in TS broth were quite low. To investigate this further, TS broth was inoculated and incubated at 37 C. Growth was monitored and cells were harvested at 0, 3, 5, 8, and 11 hr. Crude sonicates of cells harvested at each time were prepared and assayed for acid activity. Figure 6 shows the specific activity of the stationary phase culture to be 145 μg P_i/mg bacterial N/min. After 3 hr of growth, a 3.5-fold increase in specific activity was detected. The level decreased as the culture approached stationary phase. A larger inoculum was used in the following experiments so that this increase in specific activity could be followed more closely. Thus, the initial optical density of prewarmed TS broth was

FIGURE 6

Specific Activity of Acid PPase During Growth from Small Inoculum in TS Broth

Cells were inoculated into flasks of TS broth and allowed to incubate at 37 C. Inoculum size was 0.1 ml per 100 ml medium. Samples were withdrawn at intervals for optical density determination at 520 nm (- - -), and for harvest. Cells were washed and sonicated. Crude sonicates were assayed for acid PPase activity (——).


500
400
300
200
100
0
μg P_i / mg Bacterial N / min
2.0
1.0
0.5
0.1
OD at 520 nm
2
4
6
8
10
24
Hours

made to 0.250 at 520 nm with cells from a 16 hr TS broth culture. Samples were taken at hourly intervals and crude sonicates prepared. A 4.5 fold increase in specific activity of sonicates occurred in the first hour of growth (Figure 7). Moreover, if the experiment was repeated and samples taken at 15 min intervals, it could be shown that the increase in acid PPase activity was detectable within 15 min and continued for 60 min. The total enzyme activity of the cultures climbed, came to a steady level and did not decrease as rapidly as did the specific activity. To observe whether both acid and alkaline enzymes responded in the same manner, the experiment was repeated with the exception that the initial optical density was increased to 0.300. Figure 8 shows that both specific activities increased two-fold during the first hour and then began to decrease. The only variable to account for the smaller increase in specific activity observed in this experiment appeared to be the initial optical density. It was later observed that if the initial optical density of a culture was made to 0.500, no increase in the specific activity was measurable at all.

Since E. coli has a Mg^{++}-activated PPase, it was of interest to determine whether that enzyme varied in specific activity during growth, as did the alkaline PPase of S. faecium. Thus, E. coli Crookes was inoculated to an initial optical density of 0.200 in TS broth at 37 C and assayed at hourly intervals for alkaline PPase activity. No increase was noted when tested at hourly intervals. It was proposed that the increase might be faster than that in S. faecium, since the growth rate of E. coli is also faster. Therefore the experiment was repeated, assaying samples taken at 20 min intervals. No

FIGURE 7

Specific Activity of Acid PPase During Growth from Large Inoculum in TS Broth

Inoculum size was 1 ml per 10 ml medium. The conditions of incubation and assay were the same as described for Figure 6. The inset shows total enzyme units as $\mu g\ P_i$/min in the culture.

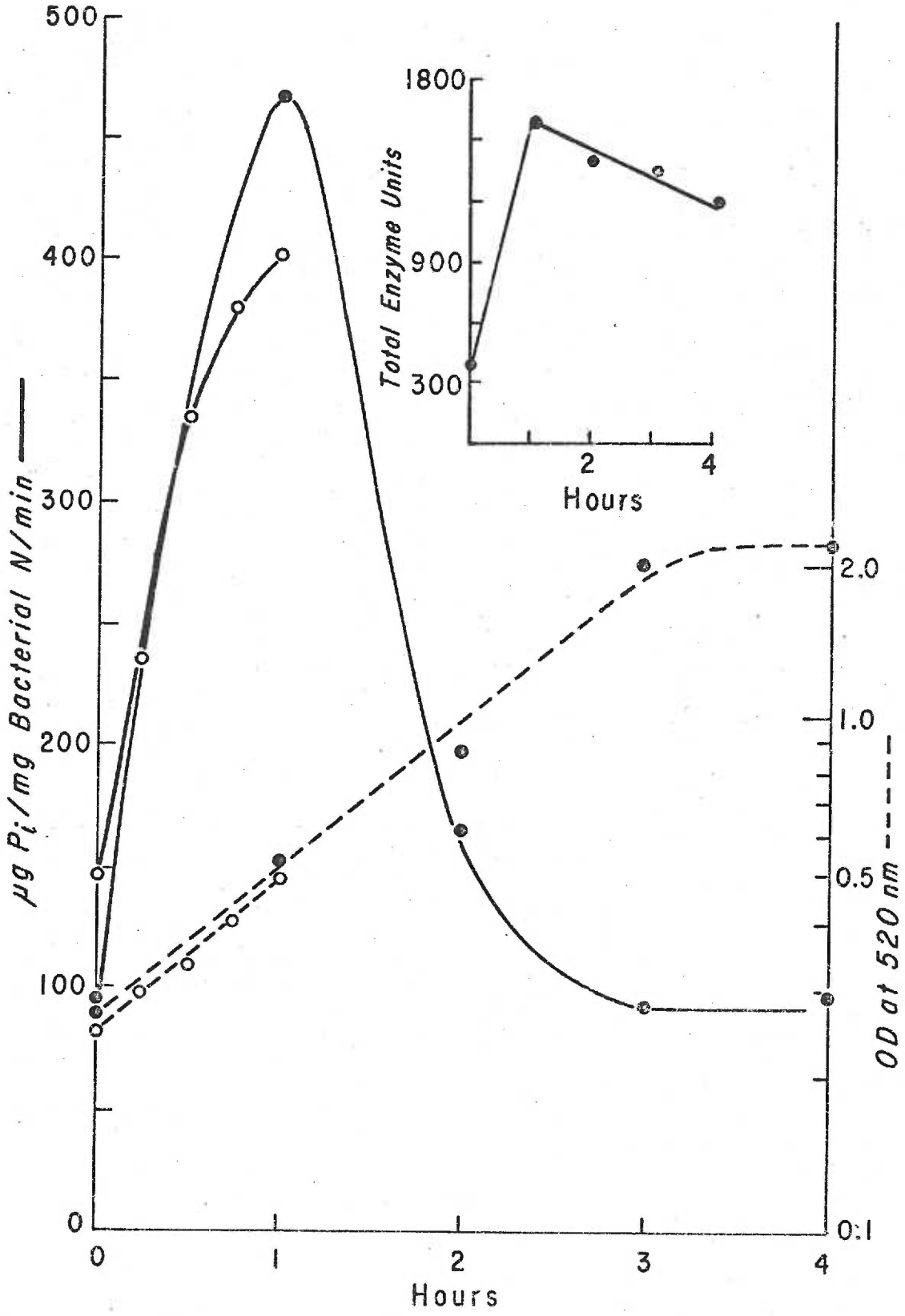
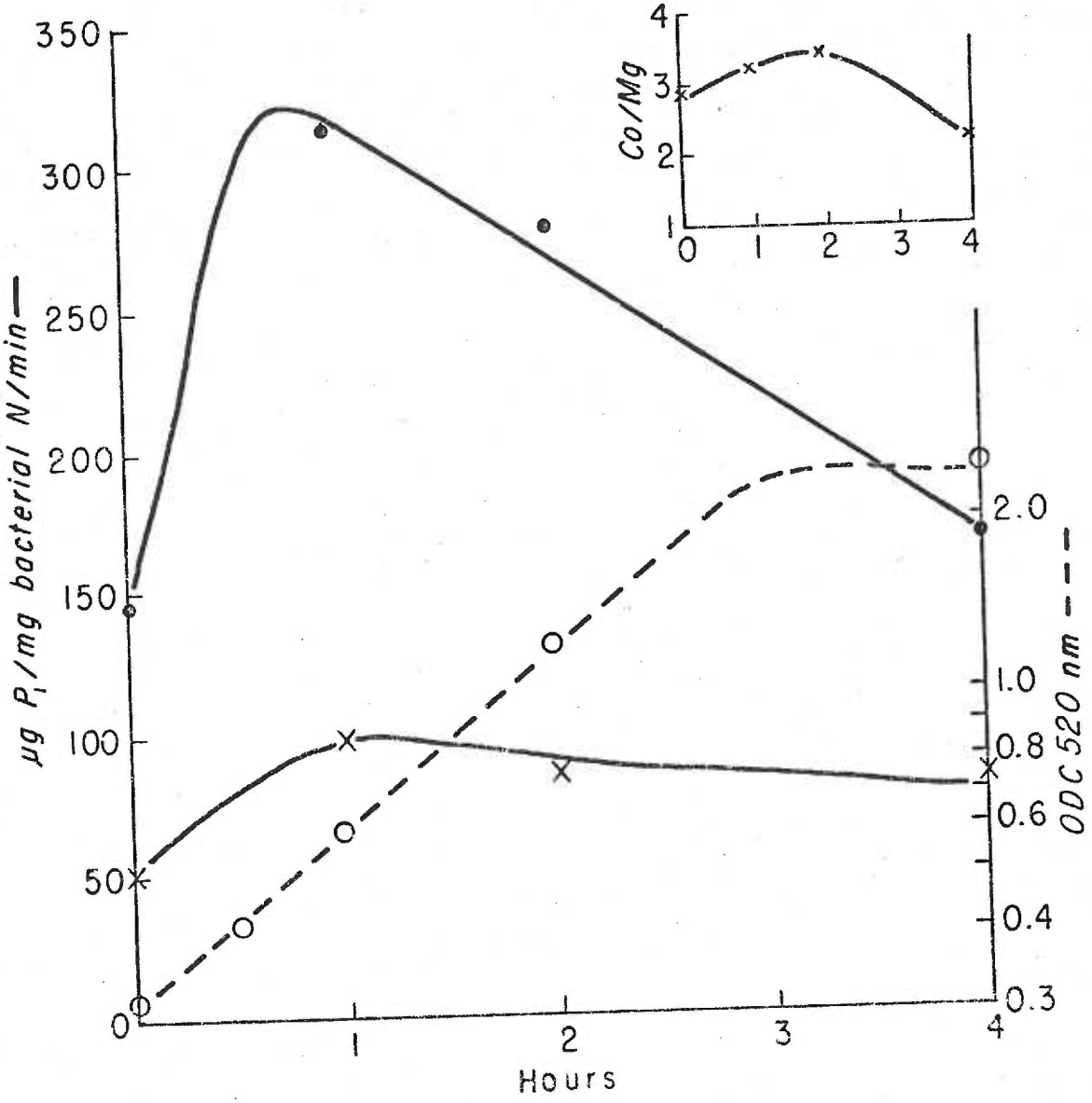

500
400
300
200
100
0
μg Pi/mg Bacterial N/min
0
1
2
3
4
Hours
2.0
1.0
0.5
0.1
OD at 520 nm
1800
900
300
Total Enzyme Units
2
4
Hours

FIGURE 8

Specific Activities of Acid and Alkaline PPase During Growth from Large Inoculum in TS Broth

Inoculation size was 1.5 ml/10ml medium. The conditions of incubation and assay were the same as described in Figure 6. (●) acid PPase activity, (X) alkaline PPase activity, (---) growth curve.


350
300
250
200
150
100
50
0
μg P_i/mg bacterial N/min
1
2
3
4
Hours
2.0
1.0
0.8
0.6
0.4
0.3
ODC 520 nm
Co/Mg
4
3
2
1
0
1
2
3
4

change in the specific activity was observed (zero time = 222, 20 min = 246, 40 min = 245, and 60 min = 234 μg P_i/mg bacterial N/min).

The possibility of enzyme activation by a substance present in exponential phase cells, but not in stationary phase cells, or enzyme inhibition by a substance present in stationary phase cells, but not in exponential phase cells was examined. Crude sonicates from S. faecium grown for 1 hr in TS broth and crude sonicates from cells grown 16 hr in TS broth were mixed and assayed. Only an additive effect was observed; no stimulation or inhibition that would amount to a 4-fold increase in activity was detected. Neither did 15 min incubation of the two preparations prior to assay result in a significant alteration of the additive effect.

2. In TS broth at 30 C. The cause of this rapid increase of activity was investigated. It was first proposed that the increase might be a response to rapid growth, and thus might not occur at a lower growth rate. In the first experiment, the growth rate was decreased by lowering the incubation temperature from 37 C to 30 C. The initial optical density was made to 0.300 in TS broth and the enzyme activity assayed hourly. Figure 9 shows that no burst of synthesis occurred at 30 C as it had at 37 C. Doubling times were determined to be 48 and 80 min at 37 C and 30 C, respectively (figure 10). If the activities, as μg P_i/min/100 ml medium, of both PPases are plotted against mg protein/100 ml medium, a differential graph can be constructed (figure 11). Only values obtained during the increase in specific activity were plotted. It can be seen that a real difference existed between differential rates of enzyme synthesis at 37 and at

FIGURE 9

Specific Activities of Acid and Alkaline PPase During Growth from Large Inoculum in TS Broth at 30 C

Inoculum size was 1.5 ml/10ml medium. The conditions of incubation and assay were the same as described for Figure 6, with the exception that the incubation temperature was 30 C. (●) acid PPase activity, (O) alkaline PPase activity, (---) growth curve.

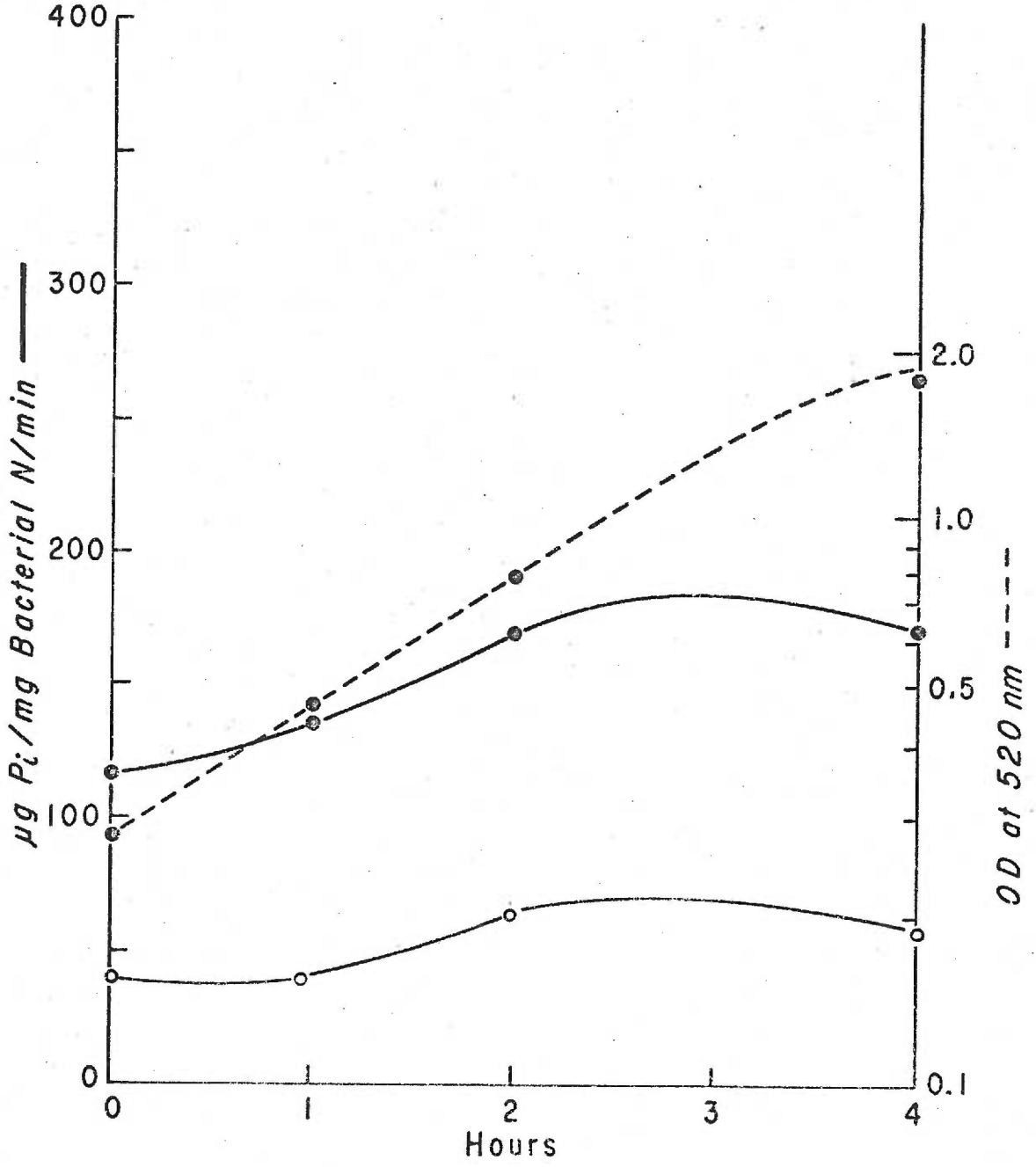

400
300
200
100
0
μg Pi/mg Bacterial N/min
2.0
1.0
0.5
0.1
OD at 520 nm
0
1
2
3
4
Hours

FIGURE 10

Growth Curves of S. faecium F24 in

TS Broth at 37 C and 30 C

(0) growth at 37 C; (X) growth at 30 C.

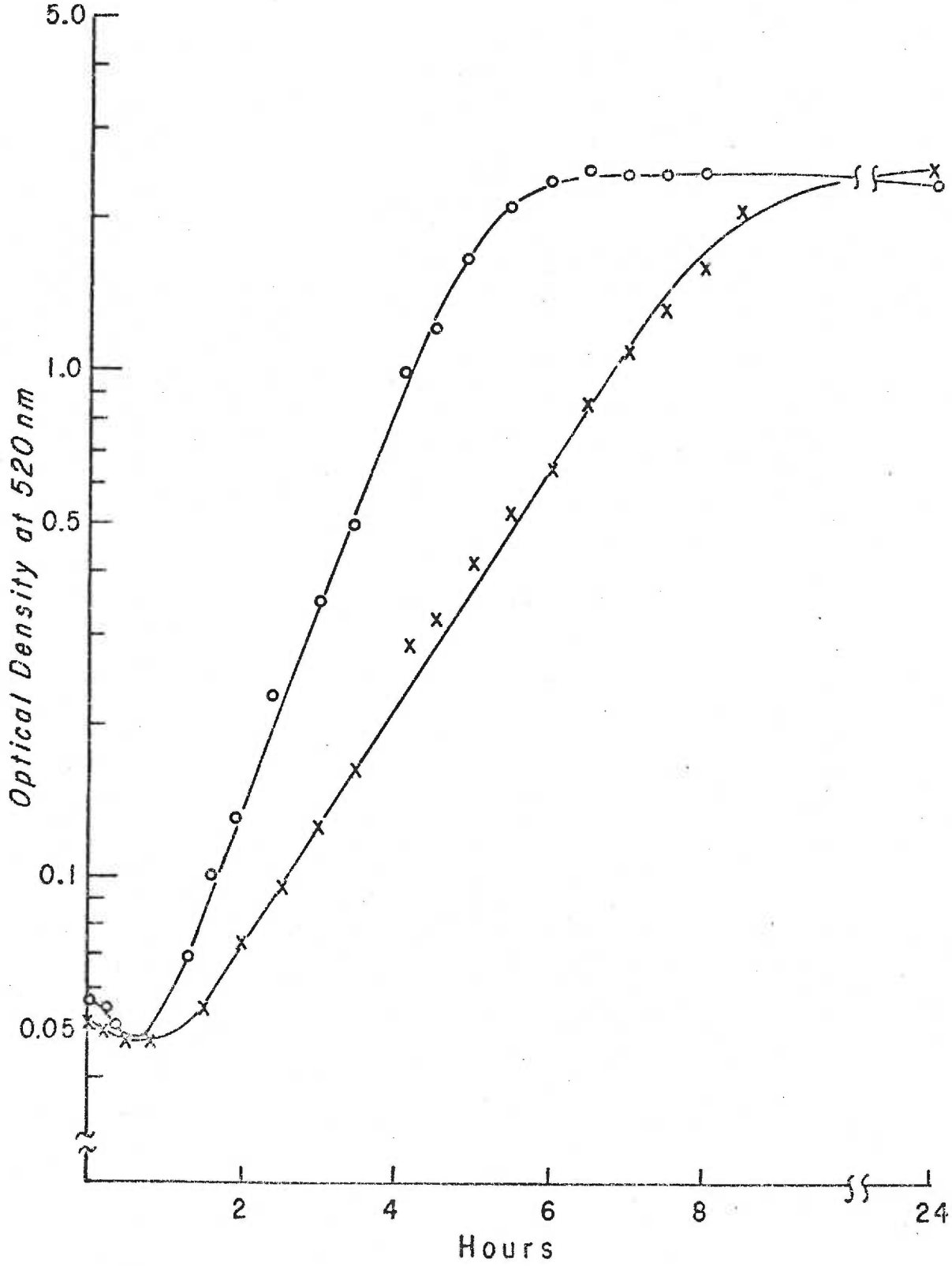
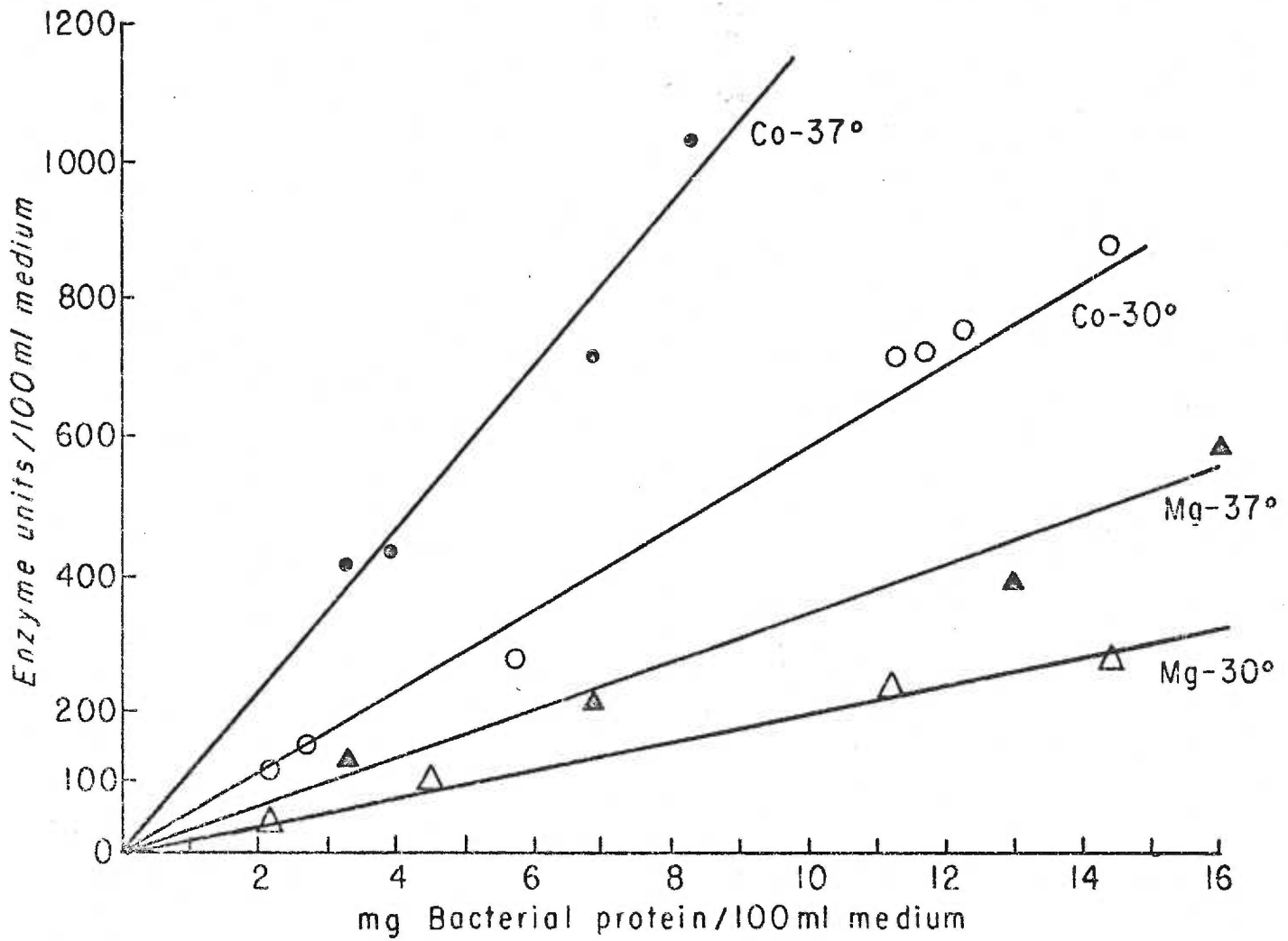

5.0
1.0
0.5
0.1
0.05
Optical Density at 520 nm
2
4
6
8
24
Hours

FIGURE 11

A Differential Plot of Increase of Acid and Alkaline PPase Activities of Cells Growing at 30 C and 37 C

Enzyme units are μg P_i/min.


1200
1000
800
600
400
200
100
0
Enzyme units/100 ml medium
Co-37°
Co-30°
Mg-37°
Mg-30°
2
4
6
8
10
12
14
16
mg Bacterial protein/100 ml medium

30 C. At 37 C the acid activity increased at a rate of 115 PPase units/mg protein, and the alkaline activity at 43 units/mg protein. At 30 C the values were only 64 and 20, respectively.

3. In GOF-200 and GOF-1 medium. To determine whether the above results were related to growth rate or to a temperature effect, the growth rate was regulated in another way. The concentration of tryptophan was decreased from the normal 200 µg/ml in GOF medium (GOF-200) to 1 µg/ml (GOF-1). The culture was started in GOF-200 and then transferred to GOF-1. Five 24 hr passages were made through the appropriate medium before the cells were used in the experiment. Cells from stationary phase were diluted into fresh medium to an initial optical density of 0.100. Samples were taken at intervals and crude sonicates prepared. These were assayed for acid and alkaline PPase activities and protein. A two fold decrease in growth rate was observed in GOF-1 medium below that with normal tryptophan concentration (Figure 12). The doubling time in GOF-200 was 60 min and that in GOF-1 was 132 min. Increases in specific activity of both PPase activities during the first hour of growth were observed in both media (Figure 13). Thus, the response in GOF-200 and GOF-1 is similar to that observed in TS broth. If a differential plot is made of these values, it was observed (Figure 14) that the rates in both cases were the same. The rate of increase of acid activity was 102 enzyme units/mg protein and that for the alkaline activity was 33 units/mg protein, in either medium.

FIGURE 12

Growth Curves of S. faecium F24

in GOF-200 and GOF-1 Medium

(O) growth in GOF-200; (X) growth in GOF-1.

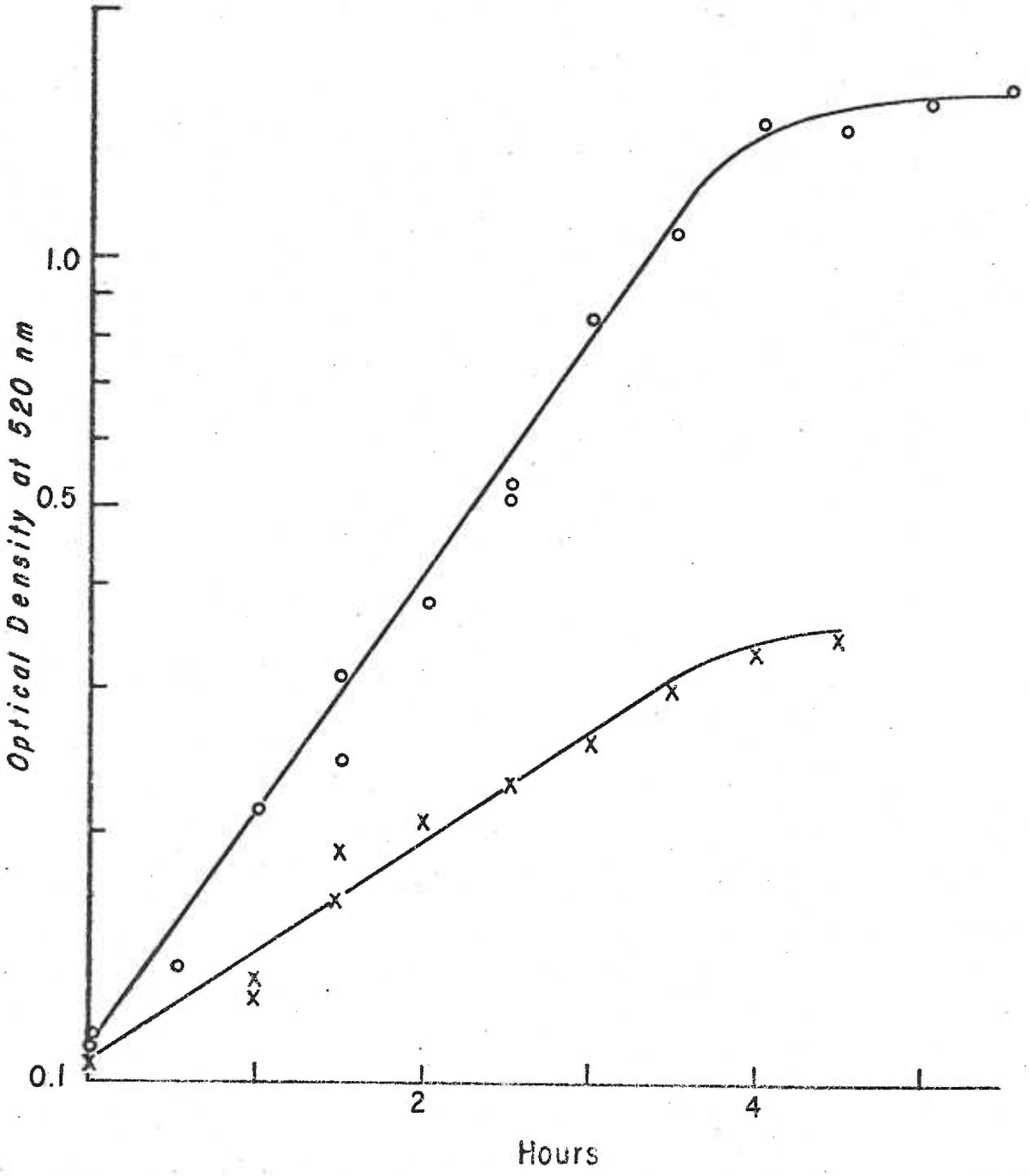

Optical Density at 520 nm
1.0
0.5
0.1
2
4
Hours

FIGURE 13

Specific Activities of Acid and Alkaline PPase During Growth from Large Inoculum in GOF-200 and GOF-1 Medium

Cells were inoculated to an initial optical density at 520 nm of 0.100. The cultures were incubated at 37 C for varying intervals, harvested and the resultant cell suspensions sonicated. These sonicates were assayed for enzyme activity.

Open figures designate acid (O), and alkaline (□) PPase activities of cells grown in GOF-1. Closed figures designate acid (●), and alkaline (■) PPase activities of cells grown in GOF-200.

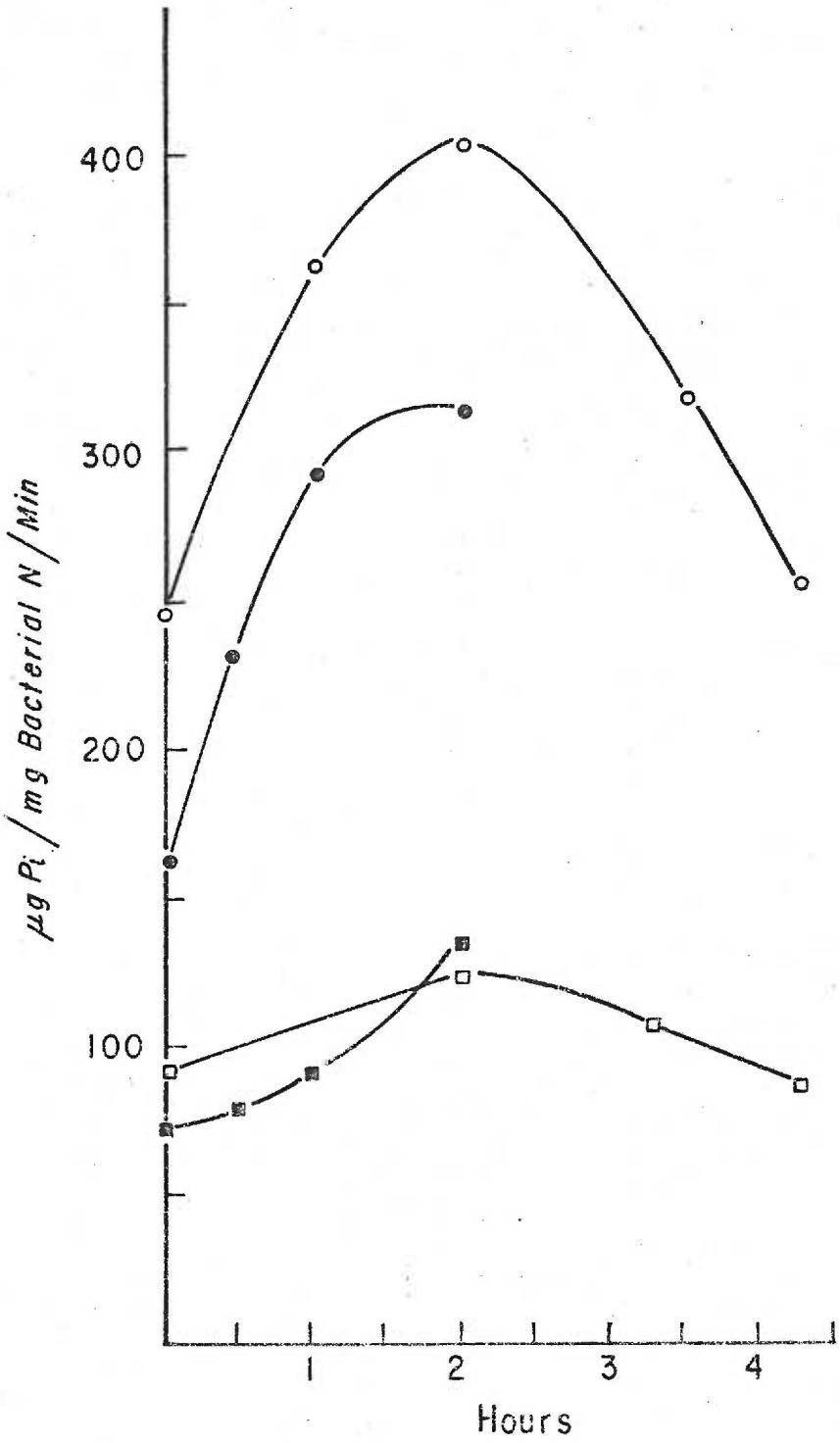

400
300
200
100
μg Pi / mg Bacterial N / Min
1
2
3
4
Hours

FIGURE 14

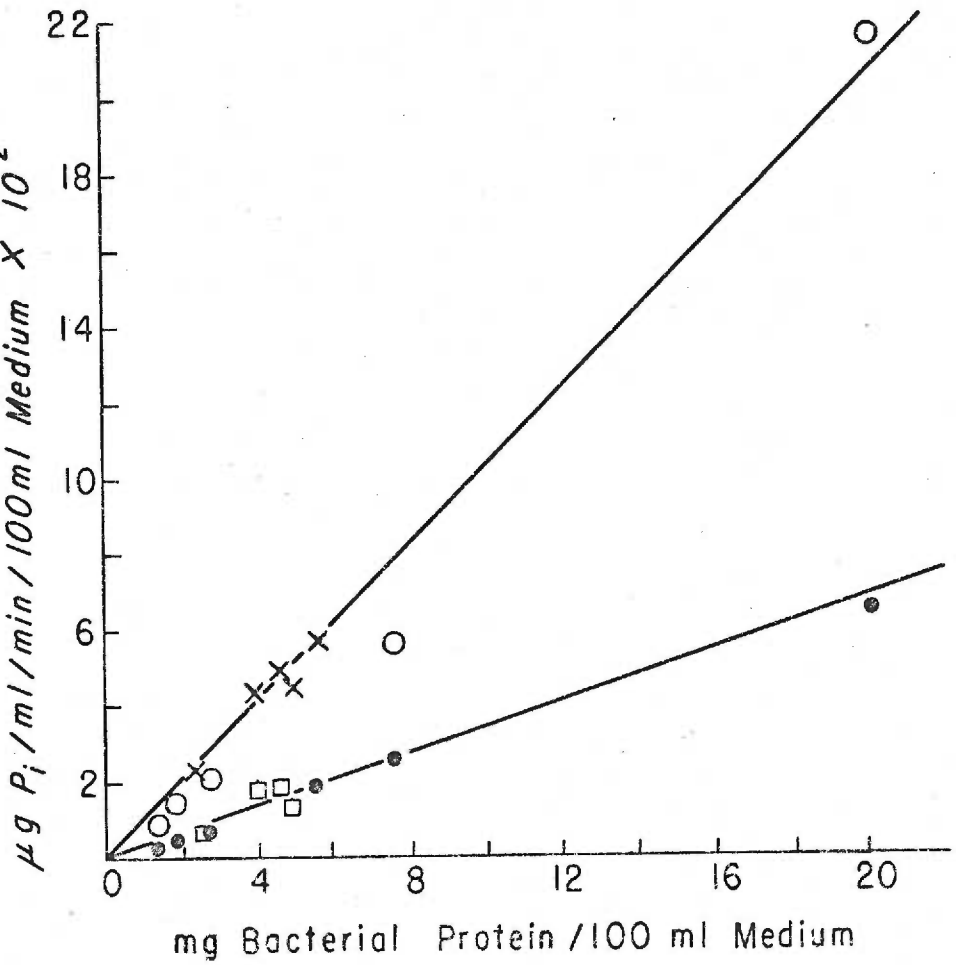
A Differential Plot of Increase in Acid and Alkaline PPase Activity of Cells Growing in GOF-200 and GOF-1 Medium

(O), acid PPase from GOF-200;

(X), acid PPase from GOF-1;

(●), alkaline PPase from GOF-200;

(□), alkaline PPase from GOF-1.


22
18
14
10
6
2
0
4
8
12
16
20
μg P_i / ml / min / 100ml Medium X 10^2
mg Bacterial Protein / 100 ml Medium

AMMONIUM SULFATE FRACTIONATION

Preliminary attempts were made to effect a partial purification of the PPase activities observed in exponential phase of growth. Since cells had maximal activity after growth for 1 hr in TS broth, crude sonicates were prepared from these cells for the purpose of ammonium sulfate fractionation. Prior to treatment with ammonium sulfate each preparation was treated with streptomycin to remove nucleic acids. The results of a typical ammonium sulfate fractionation are presented in Table 20. The characteristic features of ammonium sulfate fractionations were:

1. Only about half of each PPase activity precipitated in 65-75% saturated ammonium sulfate.
2. The PPase activities were only purified by a factor of 5- to 7-fold.
3. The Co/Mg ratio did not vary significantly in any fraction.

Other experiments using different concentrations of ammonium sulfate show that even precipitates above 65-75% saturation contain only small fractions of the activity. Thus, the PPase activities precipitated over a wide range of ammonium sulfate concentrations, and no significant purification was effected. For this reason, crude preparations were used thereafter unless stated otherwise.

PHYSICAL CHARACTERISTICS OF PPASE ACTIVITIES

A. DEAE-cellulose Chromatography

The fraction precipitating in 65-75% saturated ammonium sulfate was dissolved in 0.01 M Tris buffer at pH 8.0 and applied to a column

TABLE 20

Ammonium Sulfate Fractionation of PPase Activities

Fraction	mg protein / ml	Volume	Total mg protein	μg P_i/mg protein / min Co	Mg	Total activity Co	Mg	% recovered Co	Mg	Co/Mg
Crude	9.58	6.3	61.5	196	66	11,800	3,970	100	100	3.0
Protamine sulfate supernatant	5.96	6.0	35.8	279	67	9,950	2,470	85	61	4.1
0-50% ammonium sulfate supernatant	2.28	11.9	27.2	340	99	9,210	2,700	78	68	3.4
50-65% ammonium sulfate supernatant	0.53	14.4	7.6	936	286	7,100	2,180	60	55	3.3
65-75% ammonium sulfate precipitate	1.54	3.0	4.6	1000	466	4,500	2,060	38	52	2.1

equilibrated with the same buffer. The sample was eluted with step gradients of 100 ml each 0.05, 0.15, 0.5, and 1.0 M NaCl in the same Tris buffer. Figure 15 shows that both activities were eluted in the same fraction. The inset shows little variation in the Co/Mg ratio across the main part of the peak. The material applied to the column had a specific activity of 1,000 μg P_i/mg protein/min and that from the peak tube was only 1,685, thus only a 1.7-fold increase in specific activity was observed. However, only 30% of the activity was recovered. Tris buffer was later found to be inhibitory. When a crude sonicate was diluted in water and assayed, the values for acid and alkaline PPases were 3.7 and 2.6 μg P_i/ml/min, respectively. These values were decreased to 2.6 and 1.5 when 0.01 M Tris buffer was used as a diluant. This most likely accounts for the lack of greater increase in specific activity, when it is obvious from the graph that considerable protein has been separated from the PPase peak.

B. Gel-filtration on Sephadex G-100

1. Partial purification. When crude sonicate samples were placed on a Sephadex G-100 column previously equilibrated with 3 mM VA buffer at pH 7.0 and eluted with the same buffer, both activities were found in the same single peak (Figure 16). Maximum activity of each PPase activity was found in the same fraction. Recovery by this method is ca. 85%, and the purification factor is about 8.

2. Molecular weight estimation. Sephadex G-100 was also used to estimate the molecular weight of the PPase activities, by the method of Andrews (9). Figure 17 shows the positions of 4 marker proteins eluted sequentially from G-100. When the molecular weight of

FIGURE 15

Fractionation on a DEAE-cellulose Column of a Sample Precipitated by 65-75% Saturated Ammonium Sulfate

A 2.0 ml sample of 65-75% saturated ammonium sulfate precipitated protein dissolved in 0.01 M Tris buffer at pH 8.0 was applied to a 1.5 x 15 cm column of DEAE-cellulose equilibrated overnight at 4 C in the same buffer. The sample contained 1.5 mg protein/ml and a specific activity of 1000 μg P_i/mg protein/min for acid PPase and 466 for alkaline PPase activity. The sample was eluted with step gradients of NaCl. (X) acid PPase, (O) alkaline PPase, (——) optical density at 254 nm.

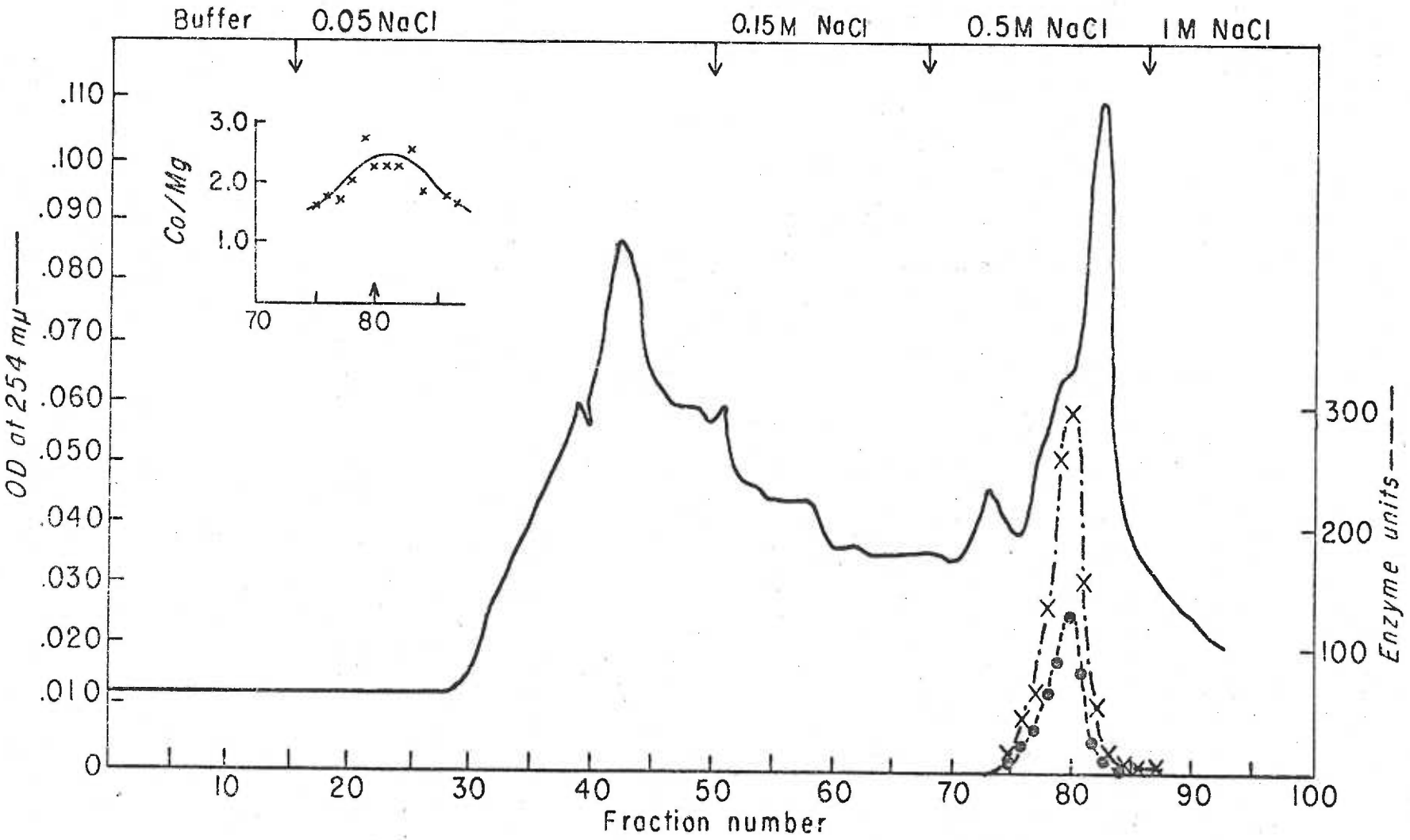

Buffer
0.05 NaCl
0.15 M NaCl
0.5 M NaCl
1 M NaCl
OD at 254 mμ
.110
.100
.090
.080
.070
.060
.050
.040
.030
.020
.010
0
Co/Mg
3.0
2.0
1.0
70
80
10
20
30
40
50
60
70
80
90
100
Fraction number
300
200
100
Enzyme units

FIGURE 16

Sephadex G-100 Gel-filtration of PPase Activities from Crude Sonicate

A 3 ml sample of crude sonicate, containing 1.4 mg protein/ml with specific activity of 113 and 41 µg P_i/mg protein/min for acid and alkaline activity respectively, was applied to a 2.5 x 37 cm column of Sephadex G-100. The proteins were eluted with 3 mM VA buffer at pH 7.0. Three ml samples were collected and assayed; (O) acid PPase, (●) alkaline PPase. The arrow indicates the void volume as determined with 0.2% Blue Dextran.

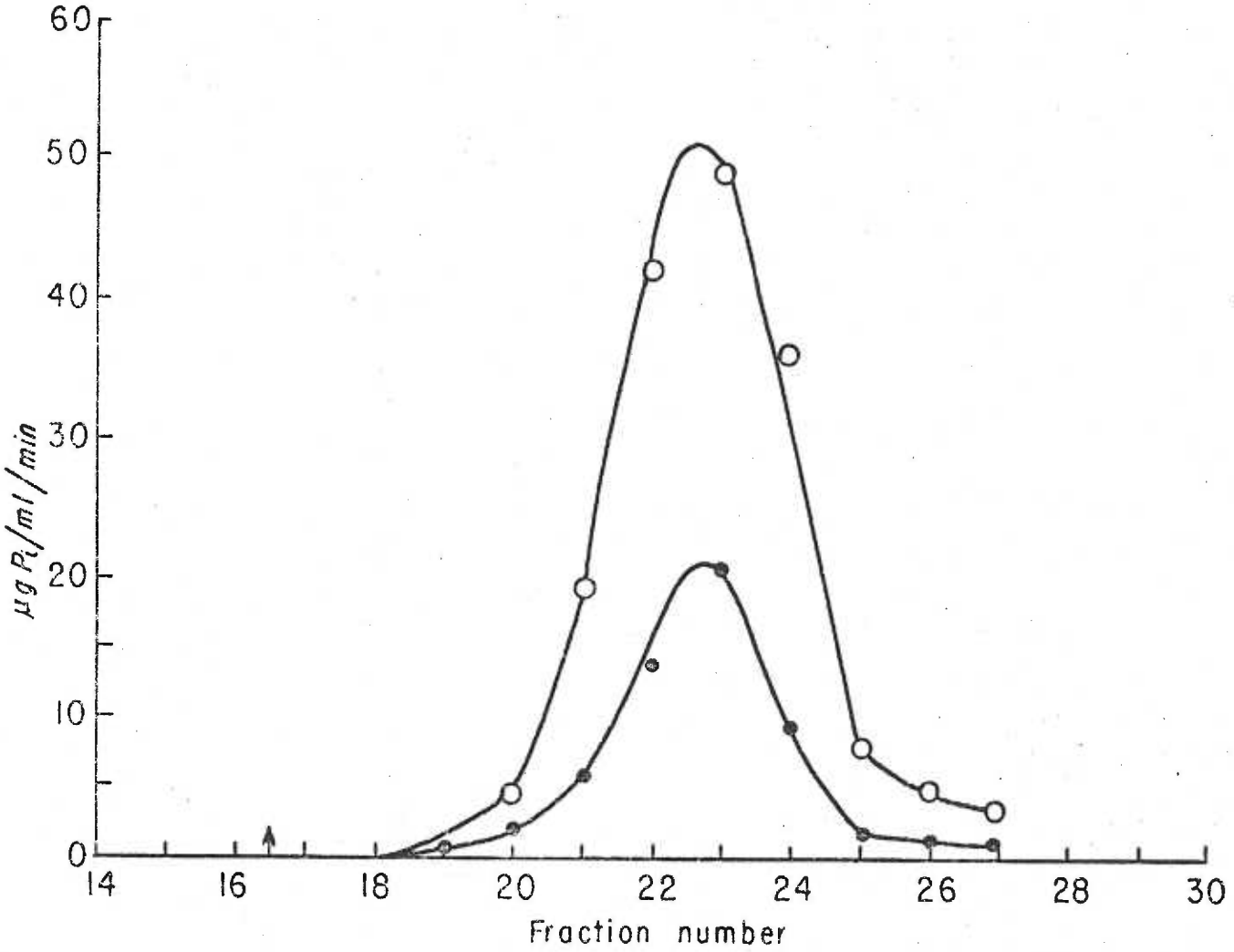

60
50
40
30
20
10
0
μg Pi/ml/min
14
16
18
20
22
24
26
28
30
Fraction number

FIGURE 17

Sephadex G-100 Gel-filtration of Marker Proteins

Proteins were dissolved individually in 3 mM VA buffer at pH 7.0. Three ml samples at concentrations shown in Table 4 were applied to a 2.5 x 38 cm column of Sephadex G-100 equilibrated to the same buffer. Three ml samples were collected and assayed.

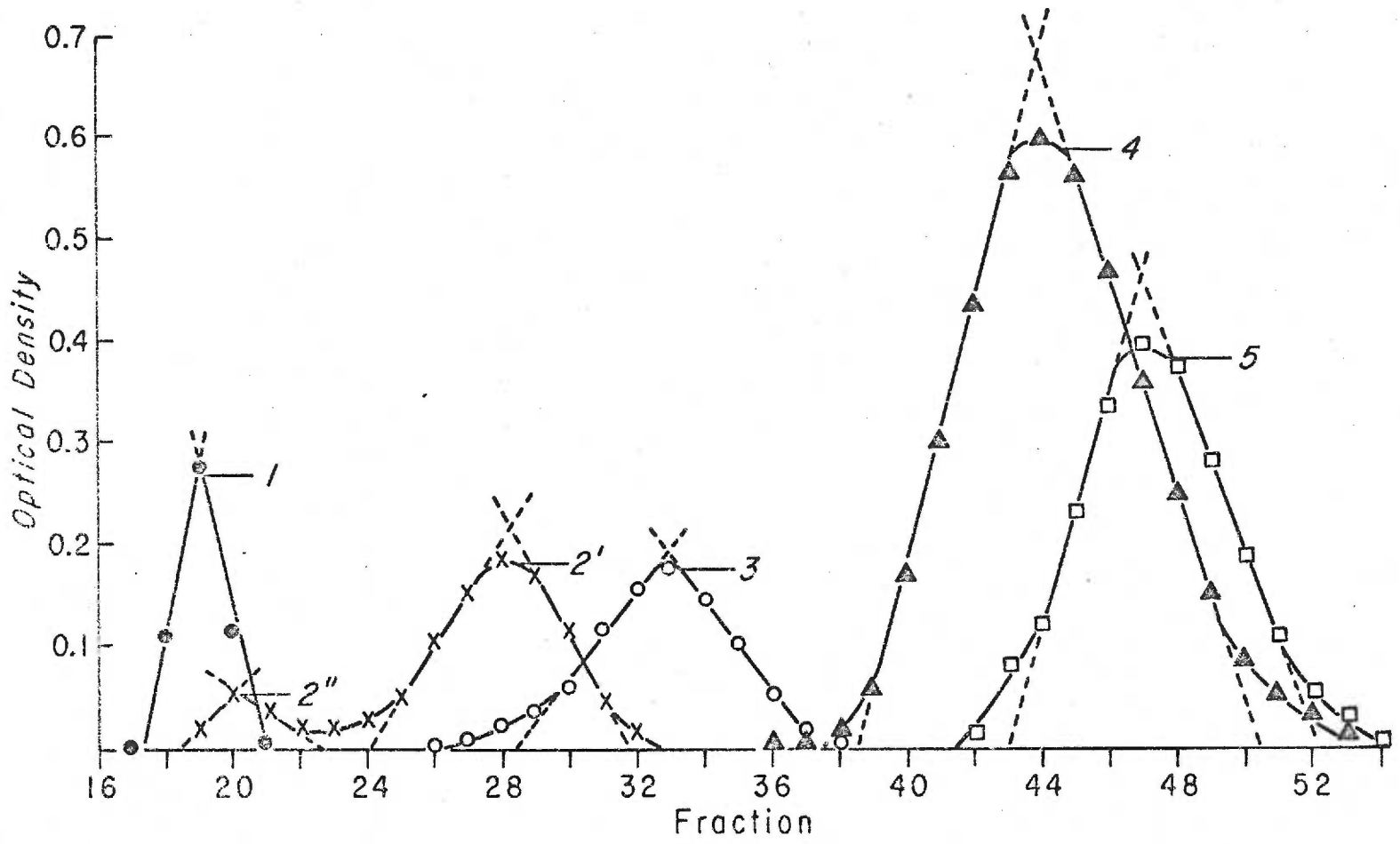
Peak 1, Blue Dextran measured at 520 nm.

Peak 2' & 2'', Bovine serum albumin (monomer and dimer) measured at 280 nm.

Peak 3, Ovalbumin measured at 280 nm.

Peak 4, Chymotrypsinogen A measured at 280 nm.

Peak 5, Myoglobin measured at 410 nm.


0.7
0.6
0.5
0.4
0.3
0.2
0.1
Optical Density
1
2"
2'
3
4
5
16
20
24
28
32
36
40
44
48
52
Fraction

each marker was plotted against the ratio of elution volume (V_e) of the protein to the void volume (V_o) of the column on semi-log paper, a straight line relationship was observed (Figure 18). When the V_e/V_o of PPase is plotted on this curve a resultant molecular weight of 65,000 to 70,000 can be estimated.

3. Nature of the PPase activities. Since other methods to separate the two activities had failed, it was hypothesized that the enzyme existed as a pH-dependent polymer; at acid pH it was in one form and at alkaline pH it was in another polymeric state. If these states involved changes in size, one should be able to detect them by gel-filtration. Thus, the Sephadex G-100 gel was equilibrated first with 3 mM acetate buffer at pH 5.3. The sonicate was applied to the gel and eluted, as were standard marker proteins. This procedure was repeated with 2 mM borate buffer at pH 8.6. Results are presented in Table 21. It can be seen that there was a drastic change in the V_e/V_o of PPase activity at pH 8.6. It appeared that at alkaline pH the protein with PPase activity increased in size, and thus was eluted from the gel sooner. However, a drastic change in the elution volume of the marker proteins also occurred. The possibility existed that a buffer effect rather than a pH effect was being observed. Therefore, the procedure was repeated using 3 mM VA buffer at pH 5.3, and 8.6. Table 22 shows that there was no effect of pH on the position of PPase or the marker proteins under these conditions. Even though the elution volume was changed by the use of different buffers, the two activities were always found in the same fractions.

FIGURE 18

Standard Curve for Estimation of Molecular Weight by Gel-filtration

The dotted area covers the range of V_e/V_o observed for PPase after 4 different gel-filtration experiments. All were performed using 3 mM VA buffer at pH 7.0.

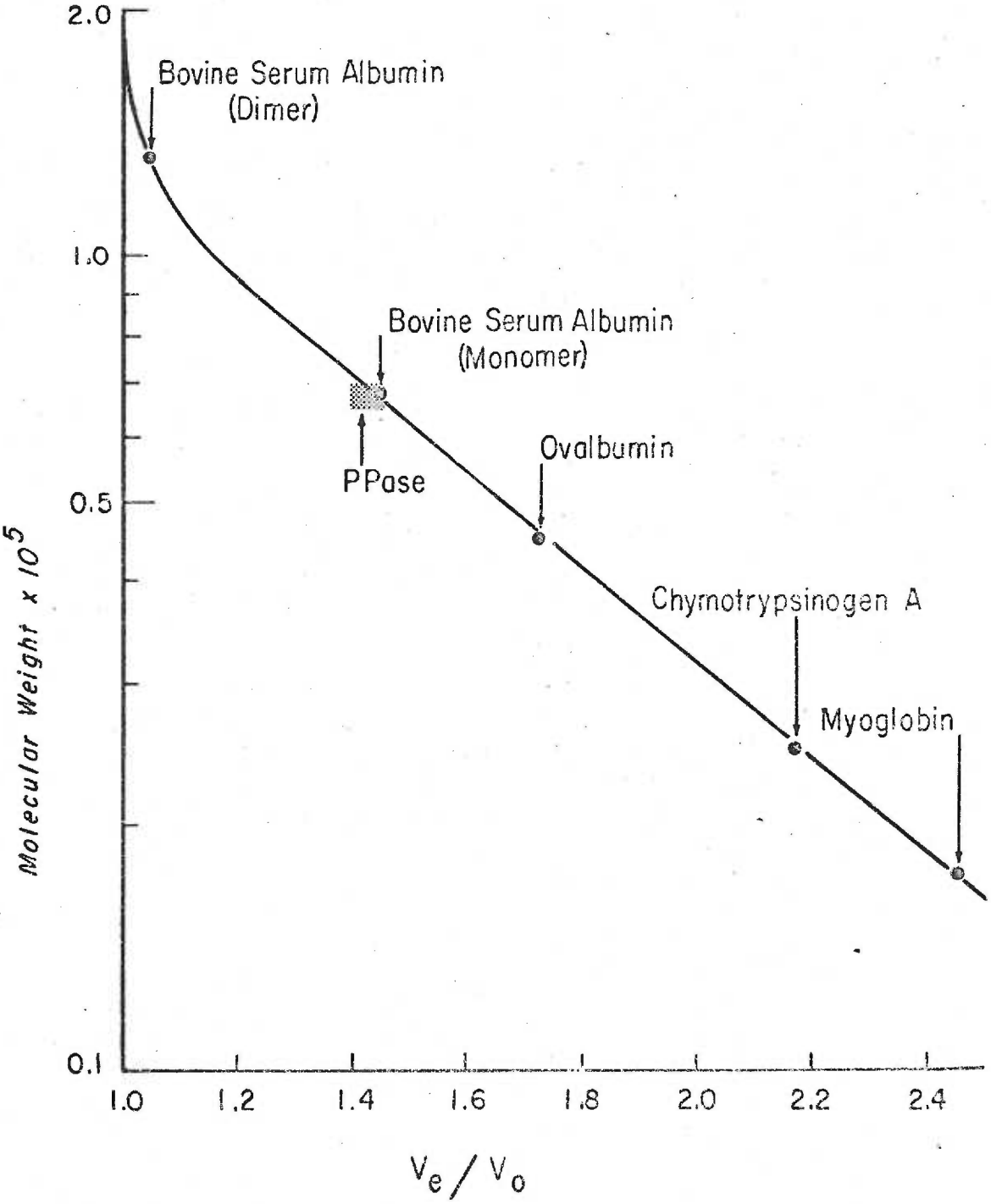

2.0
1.0
0.5
0.1
Molecular Weight x 10^5
Bovine Serum Albumin
(Dimer)
Bovine Serum Albumin
(Monomer)
PPase
Ovalbumin
Chymotrypsinogen A
Myoglobin
1.0
1.2
1.4
1.6
1.8
2.0
2.2
2.4
V_e / V_o

TABLE 21

V_e/V_o Ratio of Proteins Eluted from Sephadex G-100 with VA, Acetate and Borate Buffers

Protein	V_e/V_o VA pH 7.0	Acetate pH 5.3	Borate pH 8.6
PPase	1.45	1.35	1.15
Bovine serum albumin	1.47	1.57	
Ovalbumin	1.73	1.54	1.39
Chymotrypsinogen A	2.18	2.88	2.63
Myoglobin	2.46	3.47	2.50

TABLE 22

V_e/V_o Ratios of Proteins Eluted from Sephadex G-100 with 3 mM VA Buffer at pH 7.0, 5.3 and 8.6

Protein	V_e/V_o pH 7.0	pH 5.3	pH 8.6
PPase	1.45	1.45	1.45
Ovalbumin	1.73	1.80	1.75
Chymotrypsinogen A	2.18	2.33	2.31
Myoglobin	2.46	2.46	

C. Electrophoresis

1. In starch gel. Crude sonicates were prepared from exponential phase cells, in 3 mM VA buffer at pH 7.0. Replicate filter papers were dipped into the crude supernatant and then placed against the vertically sliced starch gel. The gel was rejoined, and current applied for 4 hr. At the end of that time, the gel was cut into strips; one was stained with Amido Schwartz for proteins, and the others were assayed for alkaline or acid PPase activity. As shown by the graphic representation in Figure 19, all assays for PPase resulted in a single band of PP hydrolytic activity. Both acid and alkaline PPase activities were in that band.

2. In polyacrylamide. Crude sonicates from both stationary and exponential phases of growth in TS broth were applied to polyacrylamide gels and allowed to migrate in an electrical field. Results were the same for both preparations. Many protein bands were visualized after staining with Amido Schwartz, as expected. Assay for acid PPase activity resulted in a single narrow band 5 mm from the salt front. Assay at pH 7.0 with both Mg^{++} and Co^{++} also resulted in a band 5 mm behind the salt front, but also 2 slower moving wide bands. When assayed at alkaline pH with Mg^{++}, maximum activity was shown to be in the fast moving band, but some activity was associated with the broader, slow moving bands. No P_i was precipitated in the absence of PP, Co^{++} or Mg^{++} in the reaction mixtures.

D. Sucrose Gradient Centrifugation

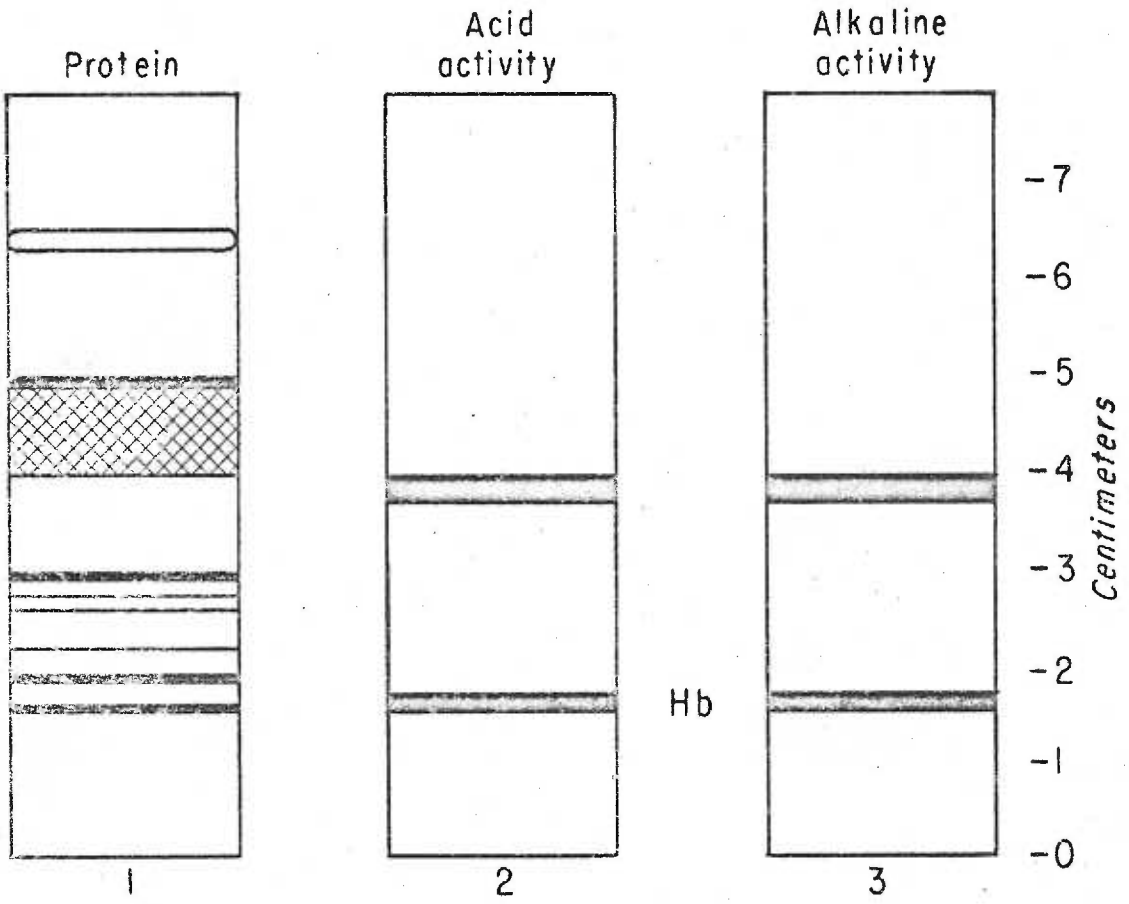
PPase activity, precipitated by 50-70% saturated ammonium sulfate, was dissolved in 3 mM VA buffer at pH 7.0 and 0.1 ml placed on

FIGURE 19

Starch Gel Electrophoresis of PPase Activities from Crude Sonicate

Crude sonicates were applied to a 13% starch gel on filter paper strips. Eight volts/cm were appled for 4 hr. Proteins were stained with Amido Schwartz. Acid and alkaline PPase activities were assayed. Lower line in column 2 and 3 represents the position of the hemoglobin marker.

STARCH GEL ELECTROPHORESIS



duplicate 5-20% sucrose gradients, made in either borate buffer at pH 8.6 or acetate buffer at pH 5.6. A 0.1 ml sample of a 2 mg/ml solution of ovalbumin was also applied to one each of the two sucrose gradient types. These 6 tubes were centrifuged for 16 hr at 106,000 x g (ave). Much of the protein was sedimented to the bottom of the tube (Figure 20). The PPase moved slowly in this force field, but both activities sedimented at the same rate, in either buffer. The distance of migration was similar to that of ovalbumin.

INACTIVATION STUDIES

Enzymatic activities found on separate proteins are subject to different rates of inactivation. Conversly, if the two PPase activities were on the same protein, they should have the same kinetics of denaturation. Two methods were used to determine the relative rates of inactivation; treatment at elevated temperature and at high concentrations of urea.

A. Thermal Inactivation

Crude sonicates were prepared from cells in the exponential phase of growth in TS broth. These were treated at different temperatures to determine the stability of the PPase activities. Table 23 shows that after 15 min at 60 C, little inactivation had occurred, whereas at 80 C, 95% of both activities were destroyed within 5 min. Therefore 70 C was selected for the rest of these studies. The rate of inactivation of both acid and alkaline PPase in 0.3 M VA buffer at pH 7.0 was then followed more closely. The Co^{++}-stimulated activity was inactivated in 2 phases, an initial rapid phase and a later slow one

FIGURE 20

Sucrose Gradient Centrifugation of Crude Sonicates

Applied to a 5-20% sucrose gradient was 0.1 ml of a sample precipitated in 50-70% saturated ammonium sulfate, containing 2.0 mg protein/ml with specific activity of 120 and 45 μg P_i/mg protein/min for acid and alkaline PPase, respectively. After centrifugation for 16 hr at 157,000 x g (ave.) 0.2 ml samples were collected and assayed; (●) acid PPase, (○) alkaline PPase, (---) optical density at 520 nm.

A. 5-20% sucrose gradient made in 3 m<u>M</u> acetate buffer at pH 5.6.

B. 5-20% sucrose gradient made in 2 m<u>M</u> borate buffer at pH 8.6.

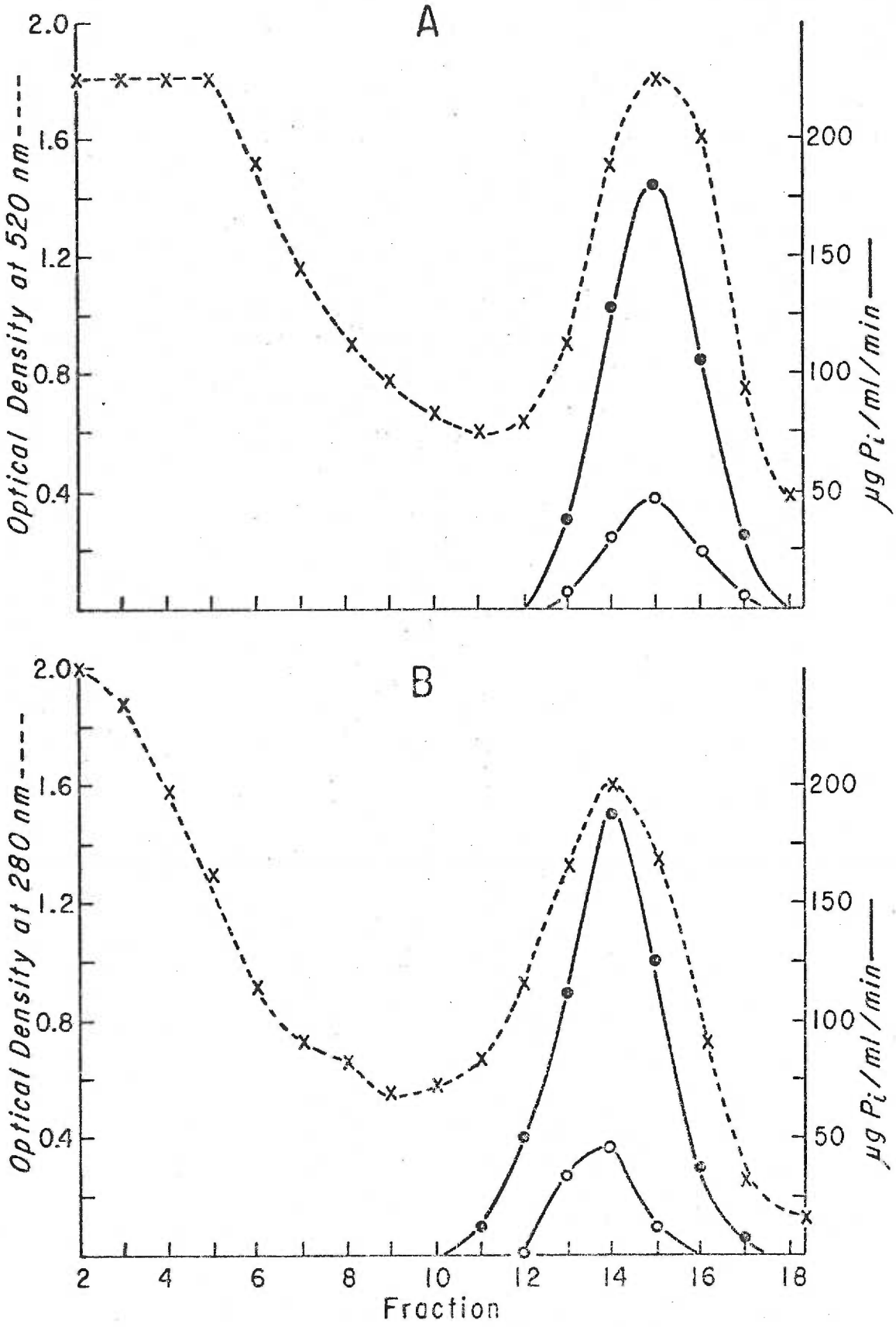

A
B
Optical Density at 520 nm
Optical Density at 280 nm
μg Pi/ml/min
Fraction
2.0
1.6
1.2
0.8
0.4
200
150
100
50
2
4
6
8
10
12
14
16
18

TABLE 23

Thermal Inactivation of PPase Activities at 60, 70, and 80 C

Time heated	% Unheated Activity					
	60 C		70 C		80 C	
	Acid PPase	Alkaline PPase	Acid PPase	Alkaline PPase	Acid PPase	Alkaline PPase
0	100	100	100	100	100	100
5	86	73	57	57	5	<5
10	87	74	40	36	2	<5
15	92	95	15	13	1	<5

(Figure 21A). The alkaline enzyme was inactivated linearly at a rate of 4%/min.

To determine whether Mg^{++} or Co^{++} protected against denaturation, a crude supernatant was buffered in 0.3 M VA buffer at pH 7.0 and then heated in the presence of either Co^{++} or Mg^{++}. Both stationary phase and exponential phase preparations were tested, but since their responses were similar, only the data for the exponential phase activities are presented (Figure 21B). The presence of Co^{++} during heating resulted in a 30% increase in the alkaline PPase activity, thereafter the activity decreased at a rate similar to that in buffer alone (4%/min). Addition of Mg^{++} also altered the rate and kinetics of inactivation of the alkaline PPase. Two phases were observed under these conditions; an initial rapid rate of inactivation and a subsequent rate similar to that in buffer alone. The rate of inactivation of the acid PPase activity was not altered by either Mg^{++} of Co^{++}; the kinetics of inactivation were the same as in buffer alone.

B. In Urea

Crude supernatant was prepared from exponential phase cells in 3 mM acetate buffer at pH 5.6. Both alkaline and acid enzyme assays were carried out in varying concentrations of urea. Figure 22 shows that low concentrations of urea stimulated the acid PPase activity. As the levels of urea increased beyond 1.5 M, the activity decreased rapidly. The alkaline enzyme was sensitive to low concentrations of urea and was rapidly inactivated. Addition of urea did not alter the pH of the reaction mixtures, nor did it influence the P_i assay. It is not known why the alkaline PPase assay resulted in higher activity

FIGURE 21

Thermal Inactivation of Acid and Alkaline PPase Activities

A crude sonicate containing 0.57 mg protein/ml was prepared in 0.2 M VA buffer at pH 7.0. The activity of the preparation was 159.0 and 38.1 µg P_i/ml/min for acid and alkaline PPase, respectively. The preparation was heated at 70 C, samples were withdrawn at intervals and diluted in the same buffer, cooled in an ice bath, and assayed.

A. Thermal inactivation in 0.2 M VA buffer. Values represented by 100% are 159 µg P_i/ml/min for acid PPase (△, ▲) and 38 ug P_i/ml/min for alkaline PPase (●, ○). (Results of 2 separate experiments.)

B. Thermal inactivation in 0.3 mM Co^{++} or Mg^{++}. Values represented by 100% are 156 µg P_i/ml/min for acid PPase in Co^{++} (●), 43 for alkaline PPase in Co^{++} (▲), 141 for acid PPase in Mg^{++} (○), and 34 for alkaline PPase in Mg^{++} (△).

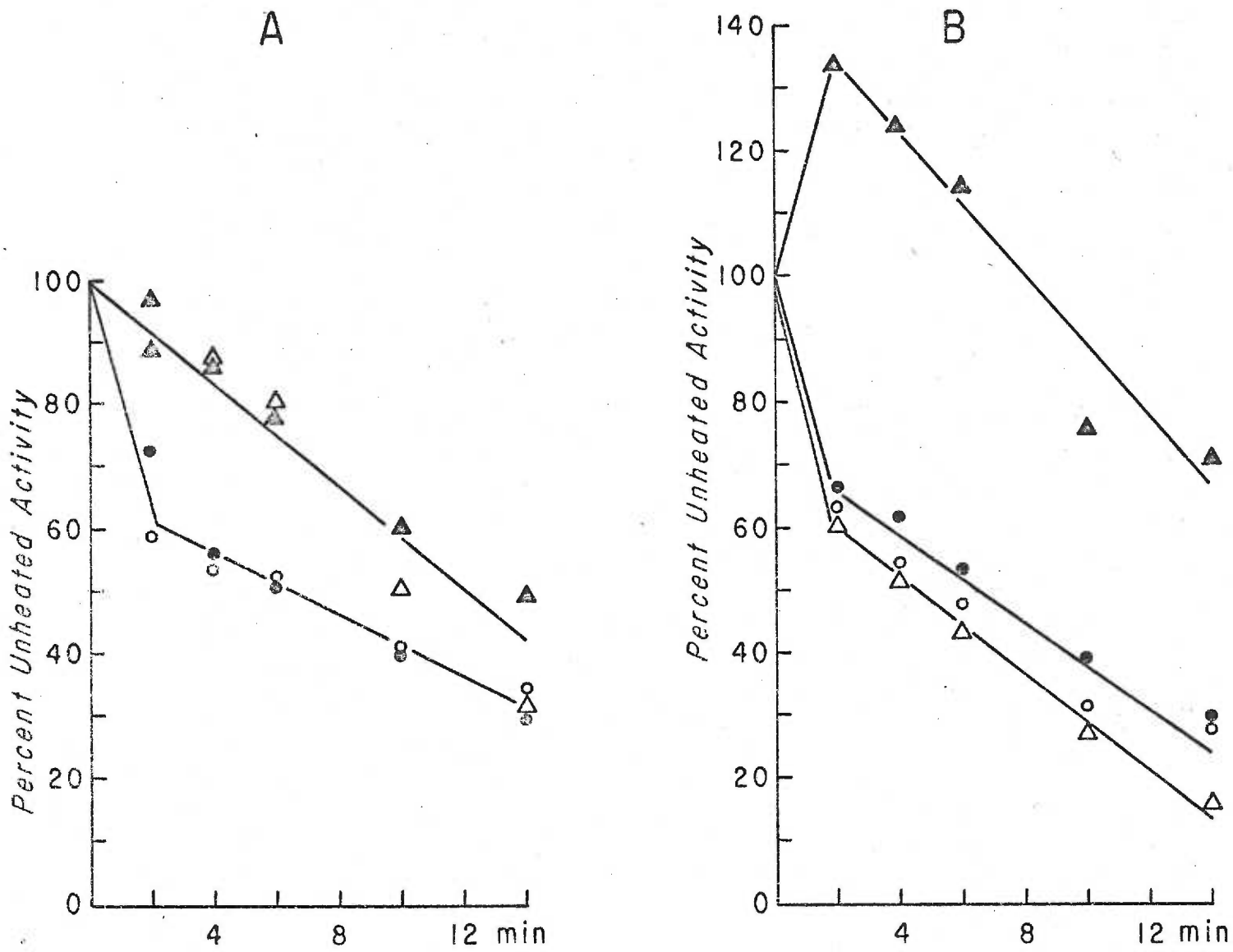
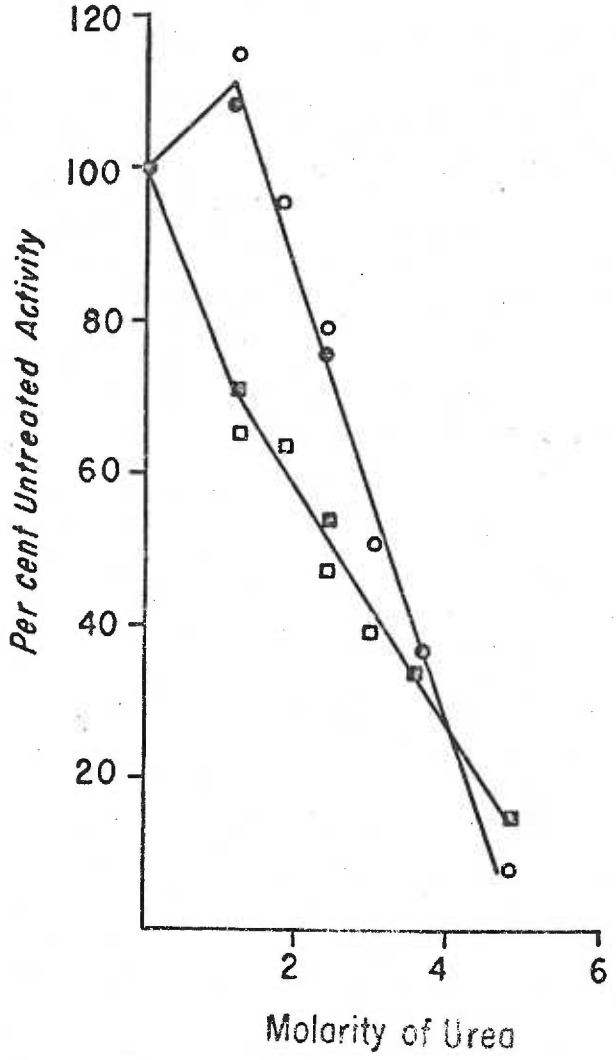

A
B
Percent Unheated Activity
100
80
60
40
20
0
140
120
4
8
12 min

FIGURE 22

Inhibition of Acid and Alkaline PPase Activities by Urea

A crude sonicate was prepared in 3 m$\underline{M}$ VA buffer at pH 5.6 containing 20 mg protein/ml. Appropriate dilutions were made into reaction mixtures for assay of acid or alkaline PPase, containing urea. Substrate was added to start the reaction. After 10 min incubation the reaction was stopped and assayed. Values represented by 100% activity were 6200 μg P_i/ml/min for acid PPase (●, ○) and 7300 μg P_i/ml/min for alkaline PPase (■, □). (Results of duplicate experiments.)


120
100
80
60
40
20
Per cent Untreated Activity
2
4
6
Molarity of Urea

than the acid PPase assay in this isolated case.

DISCUSSION

The results will be discussed in terms of the original question asked at the outset of the thesis: "Are the two PPase activities separable?". The research followed four lines of inquiry: 1) Are the two activities located in different areas of the cellular structure?, 2) Are there conditions of growth that cause the alteration of levels of PPase activity and if so do the two activities respond coordinately?, 3) Are the molecular characteristics of the two activities such that they can be separated by physical means?, and 4) Can the two activities be differentiated by their sensitivity to various inactivating agents?.

Properties of the organism used for the study. Since the research involved the enzyme from just one organism, it is of interest to briefly discuss some of the physiological features of S. faecium, which is one of the enterococci. The enterococci are separable into two species on the basis of their response to various agents (39). S. faecalis is insensitive to 0.04% tellurite, reduces tetrazolium salts and methylene blue, and has the greater metabolic diversity, in that it can use as substrate several different sugars and pyruvate. Glucose or ribose may be used by both species as a carbon source, but only S. faecalis can use pyruvate, and only S. faecium can use arabinose. S. faecium has neither tetrazolium nor methylene blue reducing enzymes, and is sensitive to 0.04% tellurite. The two are identifiable by their different Group D antigens (11) which are reflections

of the structural differences of the teichoic acid complements of their membranes. In an analysis of the peptidoglycans of 75 strains of enterococci, it was found that S. faecalis strains contain a murein with an interpeptide chain consisting of 3 alanines, whereas S. faecium strains contain a single aspartic acid as the cross-linking amino acid (69). In addition to the wall and membrane differences, there are structural differences in some internal enzymes. Glucose-6-phosphate dehydrogenase and glycerol-3-phosphate dehydrogenase of S. faecium have a lower electrophoretic mobility in starch gel than do those of S. faecalis (171). Neither have cytochrome pigments, nor a tricarboxylic acid cycle, but S. faecalis does have a fumarate reductase. S. faecalis requires lipoic acid for both aerobic and anaerobic utilization of pyruvate, but not of glucose. S. faecium requires lipoic acid for aerobic utilization of glucose and ribose, but does not require it for anaerobic metabolism (39). S. faecium was selected for these studies because it has higher acid PPase activity than S. faecalis (124). (Both S. faecalis F24 and R have been reclassified as S. faecium.) In addition, this study shows S. faecium F24 to have a greater Co/Mg ratio than S. faecalis 10C1. Whether this is a general characteristic of the two species is not known. Classification experiments done in this laboratory did not unequivocally identify the strain of F24 used as a classical S. faecium, since it would grow in 0.04% tellurite, although poorly. The strain did fit all the other criteria for S. faecium; it did not reduce tetrazolium or methylene blue, and it did require lipoic acid for aerobic growth on glucose. It might be noted that the S. faecium F24 culture used in this study

was originally a transfer obtained from the laboratory where it was first isolated, and has been maintained in stock culture in this laboratory for 20 years.

Properties of the enzyme. The acid enzyme is a unique one in bacterial systems, since all other PPases require Mg^{++} or Mn^{++} for maximal activity. PPase, although found in all organisms, falls into a unique class of enzymes among the heterotrophic bacteria. Only a limited number of enzymes have strictly inorganic substrates, such as PPase, polyphosphatase, carbonic anhydrase, catalase, nitrate reductase, hydrogenases, and nitrogen-fixing enzymes, to name a few. Autotrophic bacteria have a wide variety of enzymes utilizing inorganic substrates.

One of the most striking characteristics of enzymes is their strict specificity. Generally, an enzyme will attack one substrate maximally and others only weakly. These activities are usually maximal at the same pH. Metal requirements may also be satisfied by more than one metal with varying degrees of efficiency, but the pH optimum usually remains the same or is shifted only a small amount in the presence of different metal activators. Thus, it would be of interest to find a single enzyme with two widely separated sharp pH optima, each requiring different cations. The PPase activity of S. faecium F24 appears to be such an enzyme. The activities differ only by a factor of 3 and each pH optimum is well separated from the other. Other supporting data will be discussed later. The enzyme of E. coli does not have the same type of activity spectrum. Josse (67), in his examination of E. coli K12 PPase, did find greater activity with Co^{++}

and Zn^{++} at pH 7.5 over those with these cations at pH 9.1, which was optimum for the Mg^{++} activity. But at the lower pH the Mg^{++}-stimulated activity was still quite high. Studies at pH values lower than 7.5 were not reported. However, experiments in this laboratory show that E. coli Crookes did not contain an acid PPase similar to that of S. faecium F24. Although this is not the same strain that Josse used, one might suppose that the results would be similar. When B. subtilis spore extracts were tested at several pH values with different cations, no Co^{++}-stimulated acid PPase was found (81). Streptococcal PPase activities appear to be unique among bacterial PPases.

The amino acid enhancement can be demonstrated equally well with D- or L-histidine. The carboxyl group appears to be required since neither imidazole nor histamine will enhance. No correlation was found between PP and histidine concentrations; the histidine levels seemed to be more proportional to the enzyme concentration (124).

No alkaline phosphatase activity was observed when S. faecium F24 was grown in TS broth. However, in experiments where cells were grown in GOF with limited P_i, an increase in the level of alkaline PPase was observed. Alkaline phosphatase of E. coli has been shown to be derepressed in low P_i concentrations (61,165) and to have alkaline PPase activity (67). Thus, the increase I observed may be a reflection of increased levels of that alkaline PPase activity of alkaline phosphatase. This speculation could be tested by comparing the starch gel bands resulting from electrophoresis of cytoplasmic material from cells grown in P_i-limited GOF and complete GOF medium. Electrophoresis of crude sonicate from cells grown in the limited

medium should result in one band of alkaline phosphatase with alkaline PPase activity and another band with both Co^{++}- and Mg^{++}-stimulated PPase activity. Whereas electrophoresis of crude sonicates of cells grown in the complete GOF medium should result in only the band with both acid and alkaline PPase activity.

Localization studies. Whole cell assays reflect only about 3 to 4% of the potential PPase activity, as shown by lysozyme lysates. The suspected acid PPase activity, however, is not dependent upon PP, Co^{++} or histidine, suggesting that at a low pH, cells leak P_i into the medium.

Enzymes are located in specific sites in bacteria as well as in higher organisms. Most bacterial cells are of relatively simple structure. They are bounded by a cell membrane and a rigid outer wall and have little internal structure. Enzymes may be found in various relationships to these structures. It was of interest to determine whether the two PPase activities were in the same cell fraction.

Bacterial enzymes released into the medium and active there are called exoenzymes (129), and can be found in the medium after removal of the cells. Because gram negative cell walls fit loosely over the cell membrane, there exists a "periplasmic" space between the two structures which may be the site for various enzymes (92). Treatment of E. coli with lysozyme and EDTA in an osmotically buffered medium, or exposure to rapid osmotic shock after EDTA treatment alone (114), releases the "periplasmic" enzymes into the medium. Alkaline phosphatase activity is released into the medium by such treatment (91, 92), even though the integrity of the spheroplast is maintained (58).

For enzymes so located, the activity can be measured with whole cells even though the cell is impermeable to the substrate. Histochemical methods show exo- and "periplasmic"-enzyme activity to be extramembranous. Few gram positive cells have such a site for enzyme binding. Enzymes found in the "periplasmic" space of gram negative cells may be expressed as exoenzymes in gram positive cells. An example is the exoenzymic nature of alkaline phosphatase of B. subtilis (129), as compared with its "periplasmic" nature in E. coli. An exception, however is the penicillinase of B. subtilis; after treatment of these cells with lysozyme, this enzyme may be measured in the intact protoplast. It is not, however, released into the medium, and appears to be bound to the surface of the membrane. A similar finding was reported for the proteinase of Micrococcus freudenreichii (100). The finding that in S. faecium F24, the PPase was not measurable in whole cells, supernatant after lysozyme treatment in osmotic buffer, nor in intact protoplasts shows that the enzyme is not extramembranous. This could have been predicted from a consideration of its function, and by analogy, since it was found to be an internal enzyme in E. coli.

Several enzymes and proteins have been found to be associated with bacterial membranes; transport proteins (49), structural proteins (134), NADH dehydrogenase (1), polynucleotide phosphorylase (3) and ATPase (2). ATPase was firmly bound to the membrane of S. faecalis (ATCC 9790) and released in an all-or-none fashion only after several successive washings in the absence of Mg^{++} (1). ATPase-free membranes and membrane-free ATPase reassociated in the presence of Mg^{++}. Membranes appeared to have a specific number of sites for

ATPase, and when these were saturated, no more enzyme was bound. Binding was specific for the membranes, in that no membrane-derived ATPase was bound to ribosomes or their subunits. The nature of the binding is not clear, but was supposed to be hydrophobic, since the rate of binding was increased in the presence of Mg^{++} (4). In my experiments with S. faecium F24, PPase did not bind to the membrane, even in the presence of Mg^{++}, nor in the presence or absence of RNase and DNase. However, the enzyme might be associated with the membrane under hypertonic conditions. Some proteins are found in the membrane under these conditions, but are released upon dilution of the membrane with water. This possiblity was not explored.

Ribosomes have been found to bind certain enzymes such as poly A polymerase (173), RNase I and II (152), DNase (160), polynucleotide phosphorylase (54), acid phosphatase (152) and aminopeptidase (166). None have been shown to be an integral part of the ribosome according to the criteria established by Hardy and Kurland (54). Ribosomes, and their subunits, of S. faecium F24 were tested for association of either PPase activity; neither activity was found to be so associated, under the conditions used. Thus the PPase activities of S. faecium F24 were found to be intracellular and soluble.

Regulatory studies. Few studies on the regulation of PPase have been reported. In mammalian cells certain PPase levels appear to be a function of hormone balance or state of nutrition (12, 46, 122, 123). It is not clear whether increased levels are reflections of increased rates of synthesis or of increased activation. Etiolated plant cells are irreversibly stimulated to produce higher levels of alkaline

PPase by a short period of illumination. The mechanism of the increase is not known (146). No extensive regulatory studies have been carried out in bacterial cells. Similar levels of alkaline PPase were recovered from E. coli K12 regardless of the stage of growth, substrate used, or P_i concentration of the medium (67). The finding that both PPase activities of S. faecium F24 vary with the state of growth and the medium used is a unique one. That the level of alkaline PPase of E. coli Crookes was the same throughout the growth stages and in various media confirms that the regulation of PPase in these two organisms differs.

Increased concentration of glucose has been shown to inhibit the formation of many catabolic enzymes. The phenomenon was originally termed a glucose repression, but glucose was found later to be only one of a series of inhibitors. The more general term, catabolite repression, is now used (89). The basis of the phenomenon appears to be that the cell can form catabolites more rapidly from glucose or other efficient substrates, than it can by inducing the synthesis of specific catabolic enzymes. Thus, the intermediates accumulate and repress the formation of various catabolic enzymes. It would seem difficult to justify a catabolite repression of PPase activity a priori, yet analysis of cells grown in a variety of media all had less PPase activity after growth in increased concentrations of glucose. However, the levels varied only by a factor of two, whereas catabolite repression generally represses many fold (89). The effect was observed in all media used for anaerobic and aerobic culture. Addition of increasing concentrations of lipoic acid to aerobic culture in

GOF medium also caused decreased enzyme levels. Lipoic acid, which is required for aerobic growth of S. faecium F24 (39), is a cofactor for oxidative keto-acid decarboxylation. Since S. faecium is unable to initiate aerobic growth in the absence of lipoic acid, the effect of increasing lipoic acid is an increase in cell yield. This is the same effect that glucose has on a culture.

If the activity of an enzyme varies during the growth cycle in response to changing conditions of the medium, its final level in stationary phase will depend upon the physiological age of the cells when growth ceased. For instance, an enzyme may be derepressed by adverse pH caused by metabolism of the substrate when the cells reach a critical concentration. However, if the substrate concentration is limited so that the cell number remains below the critical concentration, the pH will not become adverse. Cells harvested at stationary phase from this culture will not have high levels of the enzyme. Thus, it was important to assay the PPase activities during the growth cycle. In S. faecium F24, the activity was found to fluctuate over a 4- to 5-fold range depending upon the conditions used. Regardless of the medium used for growth, the specific activity of the PPase increased during the exponential phase and declined as stationary phase was approached. Variation of enzyme levels at different stages of growth is not an unusual phenomenon; it has been shown to occur for different enzymes and in different organisms. The level of tryptophan synthetase increases in E. coli after metabolism of glucose. The increased synthesis of enzyme reflects a release from catabolite repression (50). Asparagine dehydrogenase/unit bacterial mass

increases during the end of the growth cycle of Aerobacter aerogenes, and decreases again upon dilution of the organisms into fresh medium. The increased levels are thought to be a response to adverse pH (97). β-galactosidase levels also change during growth of A. aerogenes. There is a progressive decline in activity, a steady level for 3 or 4 generations, a rapid rise at the end of log phase, and a second decline during stationary phase. The rapid rise at the end of log phase is thought to be a response to decreased oxygen tension (136). High levels of catalase observed in late stationary phase of A. aerogenes growth decreased to low values during exponential phase. Since these fluctuations were not observed if the medium was kept at a neutral pH, this, also, was thought to be a response to adverse pH (98). Acid phosphatase levels of A. aerogenes were very high during exponential phase and then fell before stationary phase (99). It was suggested that the loss in specific activity was ascribable to dilution by cell growth after rapid synthesis stopped. In addition, the smaller the inoculum, the higher the maximal activity obtained, as though there were a certain enzyme requirement/unit volume of the medium (99). The results observed for acid phosphatase parallel those observed for the PPase activities for S. faecium F24.

Increased glucose concentrations do not alter the growth rate in the range studied, but they do increase the yield of cells. If the enzyme is synthesized rapidly during growth, to some critical cell concentration, and then is synthesized at a slower rate, the specific activity of the enzyme would be inversely proportional to the cell yield. The following assumptions could account for the results

observed. 1) The medium contains a metabolite or pro-metabolite that, in excess, results in derepression of the PPase activities. 2) The metabolite is used up by the cells during growth. 3) As the concentration of the metabolite in the medium becomes limited, growth can continue but PPase synthesis is progressively repressed. Thus, the specific activity of PPase achieved at stationary phase would be a result of 1) the initial cell concentration, in that the amount of metabolite/cell would be higher with small inoculum, and 2) the final cell yield. This was observed. The identity of the metabolite is unknown. The identity of the derepressor is unknown. PP is the most likely derepressor since other enzymes are known to be induced in the presence of their substrates. The cell is not permeable to PP and thus it is not likely that PP is the metabolite. The metabolite could be amino acids or nucleosides in the medium, or a combination of the two. The metabolites could saturate the polymerization machinery and the activating enzymes so that large concentrations of PP are released. This endogenous PP could then be the derepressor.

The lack of a rapid increase of specific activity at 30 C was thought to be a response to slower growth rate. However, when growth rate was limited by decreasing the concentration of tryptophan in the medium, no change in the rate of increase of PPase was observed. Table 24 shows that there was no correlation between growth rate and the differential rate of increase of PPase activities. In addition, it can be seen that even when the differential rate of enzyme synthesis was halved, both acid and alkaline PPase activities responded coordinately. Thus, the increase in enzyme levels appears to be

TABLE 24

Lack of Correlation Between Growth Rate
and Differential Rate of PPase Synthesis

Growth conditions	Doubling time (min)	Total µg Pi/min / total mg bacterial protein		Co/Mg
		acid PPase	alkaline PPase	
TS broth, 37 C	48	115	43	3.2
TS broth, 30 C	80	64	20	2.8
GOF-200 medium	60	102	34	3.2
GOF-1 medium	115	103	32	3.0

temperature-dependent and not linked to the mechanisms that control rate of growth. It might be hypothesized that the repressor-effector interaction is enhanced at higher temperature. Another possibility is that the polymerizing machinery and activating enzymes do not release PP at as fast a rate at 30 C as at 37 C.

In summary, then, this portion of the research shows that the level of PPases vary coordinately throughout the growth cycle. There is a coordinate change in the rate of increase in both specific activities by growth at low temperature. Stationary phase levels of PPase appear to be functions of enzyme levels attained during growth.

Physical characteristics. The ideal method to show the molecular distinctness of these activities would be to separate them and show that one protein had acid activity and another protein had alkaline activity. However, when one is dealing with a crude sonicate, one cannot be sure how many enzymes are being measured. It could be possible that the Co^{++}- and the Mg^{++}-activated PPases are distinct. It could also be possible that a new set of enzymes were synthesized during the increase in activity observed during exponential phase of growth. When one fractionates an enzyme preparation, to obtain a pure protein, 5 to 20% of the activity is generally discarded in the fraction with lower specific activity. Thus, I could not, at the beginning be certain that I was not discarding a whole species of PPase activity. Primarily for this reason, crude sonicates of cells in exponential phase were examined for multiplicity of PPase in most cases.

Ammonium sulfate fractionation was not a satisfactory method of fractionating PPase from the crude sonicate of exponential phase cells

of S. faecium F24. Josse (67) heated the crude supernatant from E. coli K12 to 85 C for 6 min before fractionating with ammonium sulfate. Thereafter 75% of the enzyme precipitated in 60-80% saturated ammonium sulfate, with a 25-fold purification. Schito and Pesce (141) also heat-treated the crude sonicate from E. coli. The PPase, in this case, precipitated in a 0-75% saturated ammonium sulfate fraction with a 40-fold increase in specific activity. Heat treatment was not feasible for the PPase activity from S. faecium F24, since the activity was heat labile. While half of the PPase activities precipitated in the 60-75% saturated fraction, the purification was only 5-fold. Attempts to precipitate more enzyme from a 75-80% saturated fraction recovered little additional activity. One feature of this experiment was that all fractions contained acid and alkaline PPase activities in similar ratio, indicating that ammonium sulfate fractionation was not separating the two activities. This is not surprising, for the purification factor was low, indicating that not many proteins were separated.

The 60-75% ammonium sulfate precipitated fraction was diluted in Tris buffer, applied to a DEAE-cellulose column and eluted with step gradients of NaCl between 0.01 and 1.0 M in Tris buffer. Others have obtained separation of PPases within this range of NaCl. The two similar PPase activities from P. aeruginosa were separable on DEAE-cellulose, with one eluted at 0.1 M Nacl, and the other at 0.2 M NaCl (103). One peak of PPase activity from Staphylococcus albus was eluted at 0.2 M, and another at 0.4 M NaCl (103). The E. coli alkaline PPase was eluted in a single peak at 0.2 M KCl (67). The steps

made on my column may have been too wide to detect any separation. The two activities were eluted together under these conditions. Complete recovery of activity applied was not obtained. It was later found that PPase activities were inhibited by Tris buffer. A similar inhibition had been noted for E. coli PPase (67).

Gel-filtration has been shown to be a useful method of separating molecules on the basis of their size (9, 10, 153, 169). When the crude extract of S. faecium F24 was passed through Sephadex G-100 equilibrated with 3 mM VA buffer at pH 7.0, a single peak of PPase activity was obtained, with nearly 90% recovery. This peak contained both acid and alkaline activity. Gel-filtration can also be used to estimate the molecular weight of molecules. Andrews (9) and Whitaker (169) have shown that for a homogeneous group of molecules, size is proportional to molecular weight. Large molecules are excluded from the gels and elute early, while smaller proteins are retained in the gel according to their size. Thus, one can correlate the elution volume from Sephadex, of certain proteins, to their molecular weights. Molecular weights of proteins in crude extracts can be estimated by this method, since the elution volume is independent of the state of purity of the preparation (41, 145). When the column was standardized with proteins of known molecular weights and PPase compared, a value of 65,000 to 70,000 was observed. Yeast PPase had a molecular weight of 60,000 when determined by ultracentrifugation methods (140). The molecular weights of B. subtilis and B. megaterium PPases were 68,000 and 58,000 respectively, by density gradient centrifugation (163, 164). Thus, PPase from S. faecium F24 has a molecular weight similar to

other microbial PPases. This gel-filtration method would be a useful technique to apply to PPases from different sources as an index of divergence in size through evolution. Such a study has been performed with malate dehydrogenase (110).

Gel-filtration gives an empirical estimation of the molecular weight. More recently Ackers (5) and Seigal and Monty (145) have shown the method to be one that separates by molecular size. They found that proteins that were not spherical, such as fibrinogen, did not elute according to their molecular weight. It was suggested that the elution volume of a protein is a function of its molecular size, expressed as Stokes (molecular) radius. To use the method for the determination of the molecular weight of an unknown protein, the minimum assumptions are that the protein is globular and spherical. Certain other variations between elution volume and molecular weight have been observed for other proteins, and must be considered when interpreting data from unknown proteins (138). The dextran molecule of Sephadex may act as a substrate thus retarding elution of such proteins as lysozyme. Proteins with associated carbohydrates, such as ovomucoid, also interact with the gel and are not eluted as a function of their molecular weight or size. Proteins which dissociate into subunits may do so on the column, depending upon the concentration of the protein applied, pH or ionic strength. For example, chymotrypsinogen undergoes a concentration-dependent aggregation, and glutamate dehydrogenase dissociates at low concentration on Sephadex G-200 and is inactivated (138).

An observation pertinent to this study, made by Andrews (10), is

that alkaline phosphatase of E. coli has a molecular weight of 74,000 as determined by gel-filtration. Ultracentrifugation data show it to be 75,000 to 80,000 (51), thus the gel-filtration and ultracentrifugation data correlate for this enzyme. Since PPase is of similar molecular weight and also cleaves PP, it seems possible that the gel-filtration data might correlate with ultracentrifugal data in this case as well.

It has been shown for several proteins that pH of the buffer does not alter the elution volume (86). Thus, when it was observed that the apparent molecular weight of PPase was increased when eluted at pH 8.5 in 3 mM borate buffer, it was thought that a pH-dependent dimerization was being detected. Many proteins, including alkaline phosphatase (143) and malate dehydrogenase (110), have been shown to dissociate at low pH and to reassociate at higher pH. Was the alkaline activity then a polymeric expression of the monomeric activity observed at pH 5.6? Tests run in various buffers showed that 2 mM borate buffer and 3 mM acetate buffer caused anomolous behaviour of all the standard proteins on the gel as well as PPase. The standard curve of log molecular weight vs V_e/V_o was not linear in these buffers. However, when all proteins were eluted with 3 mM VA buffer at different pH values the relationship was linear at all pH values tested. Thus, when one is performing such a pH-dependent study, it is important to use the same buffer over the entire pH range.

The apparent pH-dependent molecular weight changes were also examined by sucrose density gradient centrifugation. The PPase activity sedimented at equal rates in both borate and acetate sucrose gradients.

Thus, the phenomenon observed in Sephadex with borate and acetate buffers was probably one of interaction of the protein with the gel. However, the distance migrated through the sucrose gradient was so small that differences might not be obvious.

One of the most powerful methods available to show enzyme multiplicity is starch gel electrophoresis. This method separates molecules on the basis of their size and charge, and has been used to resolve proteins unseparable by other methods. This is the classical method used to separate isozymes. Electrophoresis of crude sonicates of S. faecium F24 resulted in a single band containing both the Co^{++}- and Mg^{++}-dependent PPase activity. Hemoglobin, which was used as a marker protein, has a molecular weight of about 67,000. If the molecular weight of PPase, as determined by gel-filtration, is accurate then PPase has a greater charge than hemoglobin since it migrated faster, yet is of similar molecular weight. In addition, since gel-filtration failed to separate the PPase activities on the basis of size, the results of starch gel electrophoresis show them also to have the same charge at pH 8.6.

Polyacrylamide disc electrophoresis at pH 9.0 also showed a single fast moving band to have both Mg^{++}- and Co^{++}-dependent PPase activity. However, there are additional bands, which move more slowly through the gel, that have only alkaline activity.

Inactivation studies. Thermal inactivation studies showed that treatment at 80 C for 5 min resulted in 95% loss of both activities. Thus, the PPase of S. faecium F24 is not of the heat stable type found in E. coli, which retains 10% of its activity after 8 min at 90 C (67),

nor of the Azotobacter type which is also quite heat stable (66). Both activities of S. faecium are stable to 20 min incubation at 60 C and are denatured at 80 C; however, the two PPase activities have somewhat different inactivation kinetics at 70 C. The acid PPase was denatured in two-phase kinetics regardless of whether Co^{++} or Mg^{++} was present. The alkaline PPase activity was inactivated linearly in buffer, had two-phase kinetics in the presence of Mg^{++}, and was protected and enhanced in the presence of Co^{++}.

The alkaline activity was inactivated linearly by increasing concentrations of urea at pH 8.4. When treated with urea at pH 5.6, the acid activity was stable, and even slightly enhanced, by urea concentrations that inactivated 30% of the alkaline activity.

Both urea and high temperature are thought to inactivate by causing unfolding of the protein. In addition, both cause subunits to separate. If the two PPase activities are associated with the same protein, one could suppose: 1) that the active sites are in different areas of the molecule, or 2) that the active sites are the same, but that at pH 5.6 they are less sensitive to denaturation, ie, the acid environment may cause additional conformational changes which require higher concentrations of urea to unfold.

Molecular distinctness of the two PPases could not be resolved by simple, routine methods used for separation of proteins in crude mixtures. It is not known, of course, whether such resolution could have been obtained by more extensive fractionation procedures. However, one might assume that proteins with similar function would have similar structures and properties. This problem is intensified in

this case by the fact that changes in the pH may affect changes in the state of ionization of 1) the free enzyme, 2) the enzyme-substrate complex, and 3) the substrate. For instance at pH 6.3, PP bears a net negative charge of 2.4 (1.6 of 4 possible sites are protonated), while at pH 8.0, it has a net charge of -3.0 (76).

The metal ions may not be acting in the same manner at the two pH levels. Mg^{++} has been shown to be a part of the substrate at alkaline pH (26, 68, 137); others have shown that it also binds to the enzyme directly (35). The function of Co^{++} in this reaction is unknown. It could function to bind and remove an inhibitor, or it could be involved in a substrate complex, as is Mg^{++} at alkaline pH. Mg^{++} inhibits the acid reaction in the presence of Co^{++}. Mg^{++} has been shown to be involved in many reactions involving organic phosphates, as have Mn^{++} and Zn^{++}. This is not the case for Co^{++}. There is no known Co^{++} requirement for growth of S. faecium, or most other bacteria. Co^{++} exists in most media but at very low levels. Rhizobia require Co^{++} to fix N_2. Yeast, algae, bacteria and other microorganisms take up Co^{++} avidly from culture media but most grow equally well on Co^{++}-free medium. It is probable that many organisms require Co^{++} for conversion into vitamin B_{12} (149). B_{12} does not, however, substitute for the Co^{++} requirement for in vitro activity of the acid PPase activity of S. faecium F24 (124). Arginase of jack-bean is activated by Co^{++}, with a shift in pH optimum from 9.0 to 7.7; it is therefore assumed that Co^{++} attaches to the enzyme molecule (149). Other bacterial enzymes activated by Co^{++} are ornithine peptidase in Bacillus brevis, aspartase in E. coli, citritase, enolase and alkaline

phosphatase (89).

In view of the fact that mammalian cells and higher plants have a multiplicity of PPase activities but that all other microorganisms studied, including those that differentiate, have only a single PPase, it would be unusual to find two distinct enzymes in the Lactobacillaceae. In addition, it is not known what the physiological function of a Co^{++}-dependent activity might be, since there is no known Co^{++} requirement for growth of S. faecium F24. None is included in the medium used, although it is probably present in trace amounts.

The finding that the enzyme is homogeneous when examined by size, charge and charge density suggests that both activities are on the same molecule. In addition, the fact that they are regulated coordinately adds supportive evidence for their identity. The danger of such a conclusion, however, is apparent by observation of the work of Munkres. He concluded from genetic studies, column chromatography, immunochemistry and amino acid analysis that Neurospora malate dehydrogenase and aspartic amino transferase activities were on the same protein (108,109). These two activities were later resolved (72). However, certain enzymes involved in phosphate metabolism have been shown to be homogeneous and yet have more than one activity. Alkaline phosphatase, which has been extensively studied and shown to be homogeneous by several techniques, has a PPase activity and an organic pyrophosphate hydrolytic activity. Yeast PPase has more than one activity depending upon the cation and the pH used. Thus, the PPase of S. faecium F24 could easily fall into such a class of catalytic proteins.

Other non-phosphate metabolizing enzymes have been shown to be members of a class recently named "polycephalic" enzymes. The most extensively studied of these is the complex with aspartokinase and homoserine dehydrogenase activities in E. coli (33). These appear to be associated with the same protein on the basis of genetic, inactivation and purification studies. Thus, there do seem to be protein molecules with more than one activity, each quite different than the other.

CONCLUSION

Experiments to determine the localization of the inorganic PPase activities showed them not to be periplasmic, membrane-associated or ribosomal-bound. Both acid and alkaline PPase activities were soluble. The PPase of S. faecium F24 is a cryptic enzyme, and assays require cell disintegration.

Alterations of cultural conditions influenced the levels of acid and alkaline PPase observed in stationary phase, concurrently in most cases. Increased glucose or lipoic acid concentrations caused a decreased level of the two activities. Low P_i concentration in the medium did not alter the level of acid PPase, but appeared to increase the level of alkaline PPase. This is thought to be due to derepression of alkaline phosphatase, which also has alkaline PPase activity in some other systems.

Levels of PPase were variable during growth. A rapid 4- to 5-fold increase was observed during the exponential phase of growth at 37 C. The rapid increase was not observed during the exponential phase of growth at 30 C. Rates of increase in activity were similar at different growth rates controlled by tryptophan limitation at 37 C.

Acid and alkaline PPase activities could not be distinguished from each other on the basis of charge density, molecular size, sedimentation rate and electrophoretic mobility in starch gel. Differences were observed in polyacrylamide where a rapidly moving band contained both acid and alkaline PPase activity, but slower moving

bands had alkaline activity only. The molecular weight was estimated to be 65,000 to 70,000.

Inactivation studies showed that both activities were denatured rapidly at 80 C, and were stable to 60 C for 20 min. Different inactivation kinetics of the two activities were obtained on heating at 70 C. Treatment with urea also resulted in somewhat dissimilar inactivation kinetics for the two activities.

The two activities were thus shown to be regulated concurrently, and to be inseparable by a variety of methods. However, they have somewhat dissimilar inactivation kinetics. No evidence was obtained to disprove the hypothesis that both PPase activities are functions of the same protein under different ionic conditions.

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