JANET PEASE

PROPERTIES OF ALKALINE PHOSPHATASE FROM *BACILLUS SUBTILIS*

BACHELOR OF SCIENCE WITH HONORS

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by

Janet Pease

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I. INTRODUCTION

An increase in the activity of alkaline phosphatase was observed in an *in vitro* system containing soluble and particulate fractions from *Bacillus subtilis* (Whiteley and Oishi, 1963). In order to determine whether the increase was due to activation of preformed enzyme or enzyme synthesis, a study of the properties of the enzyme was required. Although alkaline phosphatase was found to be an exoenzyme in *B. subtilis* (Cashel and Freese, 1964), little was known about the other properties of the enzyme.

In contrast, the alkaline phosphatase of *Escherichia coli* has been studied extensively. The properties of a highly purified enzyme, mechanism of action, localization of the enzyme in the cell, as well as the genetics and regulation of enzyme synthesis have been investigated. In addition, *in vitro* synthesis of alkaline phosphatase has also been reported (Bishop, *et al.*, 1964; Manson, *et al.*, 1965). Because of the possible influence of these findings on the problem of enzyme activation or synthesis in *B. subtilis*, information concerning the *E. coli* enzyme will be reviewed briefly.

II. REVIEW OF THE LITERATURE

A. Properties and Mechanism of Action of *E. coli* Alkaline Phosphatase

1. Function.

Alkaline phosphatase was thought to provide the cell with inorganic phosphorus by hydrolysis of phosphate esters (Torriani, 1960). In cultures supplied with both organic and inorganic phosphorus, the latter was used preferentially for growth. When the inorganic phosphate was exhausted, alkaline phosphatase synthesis increased and phosphate was released from organic esters. Mutants lacking alkaline phosphatase were unable to grow on a medium containing glycerol phosphate as the only phosphate source but grew normally when inorganic phosphorus was supplied (Levinthal, 1959).

2. Substrate Specificity.

The enzyme was found to be active on a wide range of monoesters tested (Heppel, *et al.*, 1962). Pyrophosphatase and ATPase activities were also observed, but no diesterase activity was found. These reactions had similar Michaelis constants indicating that the enzyme interacted specifically with the phosphate group, and other structural components of the substrate molecule were unimportant.

3. Size and Electrophoretic Species.

Purified alkaline phosphatase was found to be a spherical molecule of 80,000 molecular weight, sedimenting as a single peak in the analytical ultracentrifuge with a sedimentation coefficient of 6.3s (Garen and Levinthal, 1960). The enzyme was composed of two identical monomers (Rothman

and Byrne, 1963), each containing an atom of Zn^{++} which was required for enzymatic activity (Plocke, et al., 1962a,b). The enzyme traveled in four or five bands when subjected to electrophoresis through starch gels (Bach, et al., 1961), suggesting that the peptide chains making up the monomer could exist in several states with respect to net charge (Levinthal, et al., 1962). All bands were thought to be products of the same gene because they were altered to the same extent by a single mutation (Bach, et al., 1961).

4. Stability.

The enzyme was found to have unusual stability with respect to denaturation by heat, trichloroacetic acid, and proteolytic enzymes. In crude extracts, the enzyme remained active after 30 minutes at 85°C while the purified enzyme was unstable at the same temperature unless 0.01M Mg^{++} was present (Garen and Levinthal, 1960). Phosphate also protected the enzyme against heat inactivation while salt (0.1M NaCl) reduced its stability (Heppel, et al., 1962). Both intact enzyme and monomers derived by reduction of the enzyme with urea and thioglycolate were resistant to attack by proteolytic enzymes (Levinthal, et al., 1962). Precipitation of these enzymes with 5% TCA resulted in only a 50% loss of enzyme activity (Schwartz and Lipmann, 1961). Sixty per cent of the activity lost when the enzyme was subjected to pH's between 3.5 and 6.0 was recovered when the pH was increased to 8.0 (Pigretti and Milstein, 1965). Treatment of cells with toluene or storage of cells had no effect on activity (Torriani, 1960).

5. Inhibitors.

In addition to end product inhibition by orthophosphate (Horiuchi, et al., 1959), other inhibitors of enzymatic activity included $HAsO_4^{=}$ (Garen and Levinthal, 1960), CN^-, Co^{++}, Pb^{++}, Cu^{++}, and chelating agents such as EDTA and 1,10-phenylanthroline (Plocke and Vallee, 1962b). The metal ions and chelating agents probably inhibit enzymatic activity by displacing Zn^{++} from the enzyme.

6. Mechanism of the Reaction.

The active center of the enzyme has been identified as a phosphate-accepting serine residue (Schwartz and Lipmann, 1961; Engstrom, 1962), and the amino acid sequence around it has been determined (Schwartz, et al., 1963; Milstein, 1963). Studies on the binding of labeled orthophosphate to this serine residue showed that incorporation was greatest at pH 4.0 and decreased with increasing pH, while the reaction velocity was maximum at high pH (Engstrom, 1962; Schwartz, 1963). However, phosphate inhibited the reaction at pH 8.8 (Torriani, 1960) where binding was minimal (Schwartz, 1963). From this evidence, Schwartz (1963) hypothesized that the enzyme had two sites: an ionic binding site, possibly containing Zn^{++}, and a site of phosphorylation, the hydroxyl group of serine. A phosphate ester or orthophosphate would be attracted to the binding site, then transferred to the site of phosphorylation with the accompanying hydrolysis of the substrate (or removal of the H^+ ion), leaving the phosphate attached to the serine. The final step would be the dissociation of the phosphate from the enzyme.

To explain the variation in the incorporation of P^{32} with changing pH, it was suggested that the rate of transfer of the phosphate from the binding site to the phosphorylation site was not favored at high pH due to the negative charge on the phosphate inhibiting the nucleophilic attack by serine (Schwartz, 1963). Thus, at pH 9.0 phosphate could still bind ionically to the enzyme and block the attachment of the substrate but little phosphorylation could occur. The fact that more than one mole of phosphate is bound by the enzyme suggests two active sites per molecule (Pigretti and Milstein, 1965).

B. Genetic Regulation of Alkaline Phosphatase Synthesis in E. coli

1. Structural Genes.

Synthesis of alkaline phosphatase depended on the presence of the requisite structural gene (P) (Levinthal, 1959; Garen, et al., 1961). Mutations in this gene resulted in the synthesis of an altered enzyme, detected by changes in the fingerprint patterns of tryptic digests of the enzyme (Garen, et al., 1961). In some instances, mutant heterozygotes (P-/P-) could produce active enzyme, presumably by intracistronic complementation (Garen and Garen, 1963b). In vitro complementation was also demonstrated with mutant monomeres produced by treatment of CRM with urea and thioglycolate or acid (Levinthal, et al., 1962; Schlesinger and Levinthal, 1963; Schlesinger, et al., 1963). Since the combining of the monomeres was found to be dependent upon the presence of Zn^{++} (Schlesinger and Levinthal, 1963), and its release occurred when dimers were reduced to monomeric form (Schlesinger, et al., 1963), it was proposed that the dimer was held together by ionic bonds stabilized by Zn^{++} (Schlesinger and Levinthal, 1963). Intergeneric complementation was demonstrated

between the monomers of S. marcescens and E. coli, suggesting that although the amino acid composition of the enzymes of the two organisms differed, they had similar secondary structures and active sites (Levinthal, et al., 1962).

2. Regulatory Genes.

Repression of alkaline phosphatase synthesis by inorganic phosphate was first described by Horiuchi and workers (1959). The synthesis of the enzyme was under control of two regulator genes, R1 and R2, since mutations were found to map in two different regions (Echols, et al., 1961). Because these constitutive mutations were recessive in both the cis and the trans positions, it was postulated that both genes acted through a cytoplasmic product. The nature of the product of the R1 gene was suggested by the fact that most R1 mutants produced much lower levels of enzyme than the wild type or R2 mutants (Garen and Echols, 1962a). It was proposed that the R1 gene caused the production of an inducer which was converted by the product of the R2 gene in the presence of high phosphate into a repressor. The theory was further supported by evidence showing that phosphatase negative mutations mapped in the R1 region and complemented with P^- mutations (Garen and Echols, 1962b).

The chemical nature of the repressor has been studied from several aspects. In a genetic approach, the regulator genes were said to direct the formation of proteins because some of the constitutive mutations could be counteracted by a suppressor mutation mapping at a distant site (Garen and Garen, 1963a). It was suggested that the suppressor mutation had changed a nonsense amino acid code word to sense through a change in the S-RNA. Thus, the suppressor mutation would only be effective if the product of the repressor gene were protein.

Studies on the effects of inhibitors also supported the hypothesis that the repressor is a protein (Gallant and Stapleton, 1964). Inhibiting DNA synthesis in thymineless cells or using 5-fluorouracil to inhibit RNA synthesis had no effect on the repression of alkaline phosphatase, whereas chloramphenicol apparently prevented the synthesis of the repressor.

Direct chemical evidence that the R2a gene specifies a protein was obtained by Garen and Otsuji (1964). A specific "R2a protein" produced in cells grown in low phosphate medium was isolated. Cells having mutations in the R2a locus produced altered R2a protein. Since the regulation of the protein was similar to that of alkaline phosphatase (produced in low but not high phosphate medium), R2a protein could not be the repressor itself but could be a precursor to the repressor or participate in some other way in its formation.

C. Localization of Alkaline Phosphatase

1. Comparison of Enzyme Localization in E. coli and B. subtilis.

The E. coli enzyme was found to be primarily soluble (Bishop, et al., 1964) with only 5-10% associated with the ribosomes (Yearbook Carnegie Inst., 1960). The enzyme was localized between the cell wall and the plasma membrane, and was released into the medium upon spheroplast formation using EDTA and lysozyme (Malamy and Horecker, 1961, 1964) or EDTA alone (Neu and Heppel, 1964a). However, the enzyme was not released when spheroplasts resulting from penicillin treatment were formed (Malamy and Horecker, 1964). Thus, the release of alkaline phosphatase was dependent on the action of EDTA which was reported to cause a change in the permeability of the cell wall (Leive, 1965).

In contrast to the soluble E. coli enzyme, the B. subtilis enzyme was associated mainly with the particulate fraction (Whiteley and Oishi, 1963). However, a large percentage of the total enzyme was reported to be excreted into the medium during the logarithmic growth phase of B. subtilis (Cashel and Freese, 1964).

2. Exoenzymes in B. subtilis.

Numerous degradative enzymes besides alkaline phosphatase have been reported to be exoenzymes in B. subtilis: amylase (for a review see Pollock, 1962), penicillinase (Pollock, 1961), RNAase (Nishimura, 1959), proteolytic enzymes (Hagihara, et al., 1958; Rappaport, et al., 1965), and lysozyme (Richmond, 1959). The mechanism of liberation of these exoenzymes is unknown (reviewed by Pollock, 1962). Some (e.g., penicillinase; Kushner and Pollock, 1961) may be synthesized at the cell membrane or be released from the cell wall (e.g., lysozyme; Young and Spizizen, 1963). Amylase appears to be activated just prior to its release (Yoshida, et al., 1960a,b), with only small amounts of active enzyme being trapped inside the cell by sudden chilling (Oishi, et al., 1963). The role of the B. subtilis lysozyme and proteolytic enzymes in the excretion of enzymes and other macromolecules such as RNA (Demain, et al., 1964, 1965) is not known (Nomura, et al., 1958; Pollock, 1962).

III. METHODS AND MATERIALS

A. Strains

B. subtilis strain 6, a partial constitutive, was obtained from the Department of Genetics, Institute of Applied Microbiology, University of Tokyo. B. subtilis strain K, wild type, came from the Department of Enzymology, Institute of Applied Microbiology, University of Tokyo.

B. Conditions of Cultivation and Derepression

Cultures were maintained on TSY slants. Derepression of strain 6 was optimal in nutrient broth, whereas strain K was derepressed maximally in lactate medium (NaCl 3.0 g, Na lactate, 2.0 g, Bacto peptone 10.0 g, Tris 3.6 g/l, pH 6.8). All cultures were incubated with shaking at 37°C. Cells were harvested, washed once with distilled H_2O and frozen.

C. Preparation of Extracts

1. Sonic Oscillation.

For experiments with strain K, cell-free extracts were prepared by subjecting cell suspensions to sonic oscillation at 10 kc in a Raytheon sonic oscillator for 50 minutes followed by centrifugation at 27,000 xg for 30 minutes to remove debris and intact cells. The resulting supernatant fraction was used as the source of enzyme. When small samples were to be assayed (for example, in experiments on the derepression of strain 6), 2 mls of a cell suspension were subjected to 10 kc in a MSE oscillator for 3 minutes.

2. Toluene Treatment and Freezing and Thawing.

Cells were treated with toluene (0.1 ml toluene/1.0 ml cell suspension) and incubated for the indicated length of time at 37°C before assaying or subjected to repeated rapid freezing at 10°C and thawing at 37°C.

3. French Pressure Cell.

Cells were usually suspended in TMK buffer (0.01M Tris, 0.1M KCl, 0.01M Mg^{++} Acetate; Schaechter, 1963) and disrupted by passage once through a French pressure cell. The resulting suspension was centrifuged at 27,000 xg for 30 minutes and the supernatant fraction ("crude extract") was removed. For one experiment, TSM buffer (0.01M Tris, 0.004M succinic acid, 10^{-4}M Mg Acetate; Aronson and McCarthy, 1961) was used instead of TMK.

D. Preparation of Ribosomes

A crude extract was centrifuged at 27,000 xg for one hour and the supernatant fraction was centrifuged further at 100,000 xg for 3 hours. The supernatant from the second centrifugation, S_{100}, was usually dialyzed against 100 volumes distilled water for 4 hours. The sediment from the second centrifugation, P_{100}, was resuspended in TMK buffer and usually dialyzed against TMK as indicated under the appropriate figures. For some experiments, P_{100} fraction was resuspended in TMK buffer and sedimented at 100,000 xg for 3 hours. The sediment was referred to as P_{100-2}, the supernatant fraction as S_{100-2}.

E. Localization

1. Cellular Distribution.

An extract, prepared by using the French pressure cell, was centrifuged at 5,000 xg to remove intact cells, and then centrifuged at 27,000 xg for 30 minutes to sediment "debris." The supernatant fraction was recentrifuged as described under preparation of ribosomes except centrifugation at 27,000 xg for 1 hour was omitted.

2. Protoplast Formation.

Cells from cultures in the logarithmic growth phase were washed once in stabilizing solution (15% lactose, 0.01M $MgCl_2$, 0.1M NaCl, 0.01M Tris, pH 7.3) and then resuspended in the stabilizing solution at a concentration of 0.2 g wet weight cells per ml solution. This suspension was warmed to 40°C and an equal volume of stabilizing solution containing 800 u per ml lysozyme (also at 40°C) was added. After 40 minutes of incubation with occasional stirring, remaining intact cells and protoplasts were removed by centrifugation at 5,000 xg for 20 minutes and resuspended in stabilizing solution lacking lysozyme. The suspension was divided into two parts and the detergent lubrol (Imperical Chemical Industries, London) was added to both to make a final concentration of 0.10%. After 10 minutes of contact with lubrol, the cells were lysed and both fractions were centrifuged, one at 27,000 xg for 20 minutes to sediment intact cells and protoplast debris, the other at 3,000 xg for 20 minutes to sediment intact cells only.

F. Sephadex Gel Filtration

An attempt was made to fractionate the ribosomes present in a crude extract by gel filtration using Sephadex G-50 (Pharmacia, Co., Uppsala,

Sweden). A column 20 cm long and 1.5 cm in diameter was loaded with 2 ml of crude extract and eluted with TMK buffer. The protein-containing fraction was divided into two parts: that which was eluted in the first 3.5 mls following the void volume, "sephadex A," and that which was eluted in the following 4.0 mls, "sephadex B." The fractions and the crude extract were then centrifuged at 100,000 xg for 3 hours, the sediment resuspended in TMK buffer, and analyzed by sucrose density gradient centrifugation.

G. Sucrose Gradients

If the ribosomal preparation had been frozen, it was first centrifuged at 27,000 xg for 15 minutes to remove aggregated materials. One-tenth ml of the preparation was then layered on a linear 5 to 20% sucrose gradient containing TMK buffer (the Mg^{++} concentration depended on the experiment) and centrifuged at 115,000 xg usually for 2.5 hours. The tubes were punctured at the bottom and 2 drops collected in each fraction. The fractions containing 10^{-2}M Mg^{++} were diluted with 1.0 ml of 0.01M Tris, pH 7.4, and those containing 10^{-4}M Mg^{++} were diluted with 1.0 ml of 0.01M Tris, pH 7.4, and 10^{-4}M Mg^{++}. The optical density of the diluted fractions at 260 mu was measured and the alkaline phosphatase activity determined.

H. Determination of Sedimentation Coefficients by Analytical Ultracentrifugation

Ribosomal preparations or extracts were centrifuged at 50,740 xg at 20°C in a Spinco Model E analytical ultracentrifuge equipped with Schlieren optics. Both single vector and wedge window cells were used. The observed sedimentation coefficients were calculated by the moving boundary method

using the formula $S = \frac{1}{W^2} \frac{d \ln \bar{x}}{dt}$ (Schachman, 1959).

I. Fractionation of Extracts

All manipulations were performed in the cold. Protamine sulfate (0.5 ml of a 2% solution per 10 ml of extract) was gradually added to a crude extract with stirring and the resulting precipitate removed by centrifugation at 27,000 xg for 20 minutes. The sediment was resuspended in 2.0 mls of distilled water and combined with a crude extract that had been treated with 20 γ/ml of both RNAase and DNAase for 20 minutes. Fractionation of this mixture with powdered ammonium sulfate gave fractions of 0-34%, 34-44%, 44-47%, 47-50%, 50-55%, 55-62%, 62-75%. Fractions between 34-47% saturation, containing 65% of the enzymatic activity, were combined and reprecipitated with saturated $(NH_4)_2SO_4$ solution, pH 8.0. Approximately a 3-fold increase in specific activity was obtained through the procedure.

J. Electrophoresis

S_{100} preparations from strains 6 and K, partially purified strain 6 enzyme (prepared as described under fractionation of extracts) and partially purified E. coli enzyme (Worthington Biochemical Corp. BAP-SF 6145) were subjected to electrophoresis through acrylamide gels using either 11% or 7.5% gel (prepared according to a pamphlet from Canal Industrial Corp., 4935 Cordell Ave., Bethesda, Maryland). All enzyme preparations were layered as solutions without polymerization on the large pore upper gel and were subjected to a field of 4 amps per gel at room temperature. The gels were stained for enzyme activity using the method of Allen and Hyncik (1963), substituting Fast Blue for Nuclear Red, and fixed according to Levinthal (1962).

K. Assay

The enzyme was measured by determining the rate of hydrolysis of p-nitrophenylphosphate (NPP), in the presence of 0.6M Tris buffer, pH 9.4, and either 2.0 or 1.2 x 10^{-3}M NPP, in a total volume of 1.0 ml. In the case of the strain 6 enzyme, 10^{-4}M Mg^{++} was also included in the reaction mixture. After incubation at 37°C for the desired length of time, the reaction was stopped by adding 1.0 ml of either 1.0N NaOH or 2.0M K_2HPO_4, pH 8.5. For some experiments (noted under the appropriate figures) slight modifications of these conditions were used. A unit is defined as the amount of enzyme preparation which gave a change in optical density of 0.1 per minute. Protein determinations were made using the method of Lowry and workers (1951).

L. Abbreviations

The following abbreviations are used in this paper: EDTA, ethylenediaminetetraacetic acid; Tris, (hydroxymethyl) amino-methane; PVS, polyvinylsulfate; NPP, p-nitrophenylphosphate; CRM, cross-reacting material.

IV. RESULTS

A. Properties of the Reaction

1. pH Optima, Buffer Concentration and K_m Values.

The hydrolysis of NPP by crude extracts obtained from derepressed strain K (wild type) was compared with that of an S_{100} preparation (see Methods) from strain 6 (partial constitutive). The reaction for each preparation was found to be linear with time and proportional to the amount of enzyme present. For the strain K enzyme, the concentration of Tris buffer necessary for maximum activity was 1.0M (figure 1). In contrast, the strain 6 enzyme showed maximum activity when the Tris buffer concentration was 0.8M. A pH of 9.4 was optimal for the strain K enzyme while the strain 6 enzyme showed maximum activity at pH 10.0 (figure 2). The optimum substrate concentration for the strain K enzyme was approximately 2×10^{-3}M and the K_m, 1.9×10^{-4} (figure 3). In contrast, the strain 6 enzyme had a much larger K_m of 2.3×10^{-3} and a biphasic curve was obtained with increasing substrate concentration (figure 4).

2. Effect of NaCl, EDTA, and Mercaptoethanol.

In contrast to the E. coli enzyme (Wilson, et al., 1964), the strain 6 enzyme was inhibited 50% by 0.1M NaCl. However, like the E. coli enzyme (Garen and Levinthal, 1960; Plocke, et al., 1962a), the strain 6 enzyme was inhibited by EDTA, a concentration of 10^{-4}M producing 65% inhibition of activity in a crude preparation.

Since mercaptoethanol was assumed to promote the formation of the most stable configuration of the enzyme by increasing the ease with which

stimulated by supernatant factors. At 10^{-4}M Mg^{++}, when increasing amounts of supernatant were added to a constant amount of a particulate preparation during assay, an increase in activity was observed only after the supernatant concentration in the reaction mixture reached a certain level (figure 6). In contrast, at 10^{-2}M Mg^{++}, activity increased when the supernatant concentration in the reaction mixture was much lower (figure 7). As seen from Table 2, the activities per ml of enzyme increased more in the presence of 10^{-2}M Mg^{++} (73%) than in the presence of 10^{-4}M Mg (30%).

B. Electrophoresis

For comparative purposes, the electrophoretic species of alkaline phosphatase from B. subtilis strains 6 and K and E. coli were examined using disc gel electrophoresis. The E. coli enzyme migrated in 4 bands, 3 close together as reported by Levinthal (1962) and one closer to the top of the gel. Three to four multiple bands were seen with both soluble preparations of strains 6 and K enzymes and the partially purified strain 6 enzyme. However, an accurate determination of the number of bands was not possible due to the difficulty in obtaining good separation of bands, possibly because of interference from other proteins since very concentrated preparations were used. It may be tentatively concluded, however, that the patterns of migration of the two B. subtilis strains were not similar and that these patterns also differed from that obtained with the E. coli enzyme.

C. Localization

1. Extracellular.

It was reported that B. subtilis excreted alkaline phosphatase quantitatively into the medium during the logarithmic growth phase. (Cashel

and Freese, 1964). The possibility of such excretion in strain 6 was examined by studying the appearance of the enzyme intracellularly and in the medium (figure 8). Derepression began after approximately four hours of incubation. At this time, most of the enzyme was present in the medium. After the sixth hour, the amount of enzyme in the cells increased, but the amount in the medium decreased. This loss of activity could have been due to dilution or to the action of extracellular proteases (Hagihara, et al., 1958; Rappaport, et al., 1965). By the eighth hour, the amount of enzyme retained in the cells exceeded that found in the medium. After 25 hours of growth, a specific activity of 240 was attained; 71% of the activity was found in the cells.

2. Cellular.

a. Permeability barrier. Both strains 6 and K appear to have a permeability barrier to the substrate (figure 9). The permeability of strain K cells changed after 2 min of exposure to toluene, the enzymatic activity rising from 15 units/ml to 20.6 units/ml. However, the released enzyme was partially inactivated during the subsequent incubation with toluene. A slight rise in activity followed by a larger increase after 45 minutes of toluene treatment was observed with strain 6 cells. Figure 10 shows the increase in activity of strain 6 cells upon repeated freezing and thawing. Cells frozen and thawed 8 times and then treated with toluene showed a decrease in activity.

b. Localization between the cell wall and cell membrane. An attempt was made to determine whether alkaline phosphatase was localized between the cell wall and cell membrane as in E. coli (Malamy and Horecker, 1961). Cells were treated with lysozyme and the resulting protoplasts were lysed when 1% lubrol was added (figure 11). The enzymatic activity of the

protoplast debris was corrected for activity due to the presence of intact cells. Whether the enzyme was localized between the cell wall and the cell membrane could not be determined because most of the enzymatic activity was lost during separation of the protoplasts from the supernatant fraction containing lysozyme. Released proteases or Mg^{++} present during protoplast formation could have caused the inactivation. The remaining activity was divided between the particulate and supernatant fractions, suggesting that at least part of the enzyme was membrane bound.

3. Particulate Nature of Alkaline Phosphatase.

a. Intracellular distribution of enzyme. Previous work indicated that much of the cellular alkaline phosphatase in strain 6 was associated with the particulate fraction (Whiteley and Oishi, 1963). Following this line of inquiry, a study of the cellular distribution of the enzyme revealed that 80% was particulate, either bound to the ribosomes (P_{100} fraction) (44%) or associated with the debris (36%) (Table 3). The alkaline phosphatase associated with the ribosomes was strongly bound; when the ribosomes were washed (P_{100-2}), specific activity increased, showing that other proteins were more easily removed than alkaline phosphatase.

b. Ribosomes of B. subtilis. A study of the ribosomes of B. subtilis was undertaken when it was observed that much of the cellular alkaline phosphatase was associated with the P_{100} fraction. The sedimentation patterns of various ribosomal preparations are shown in figures 12 and 13. The observed sedimentation coefficients of the ribsomes in the crude extract were smaller than those in the P_{100-2} preparation due to the difference in viscosity of these two preparations. When the observed sedimentation coefficients of ribosomes in the crude extract were multiplied by a partial correction factor for viscosity, $\frac{n}{n_o}$, (Schachman, 1959), the 54S coefficient

was raised to approximately 70S, the 41S to 50S and the 79S to 100S. No correction factor was made for viscosity in the P_{100-2} preparations.

As observed with E. coli ribosomes (Tissieres, 1959), at 10^{-2}M Mg^{++}, 70S and heavier forms were predominant especially in the crude extract while at 10^{-4}M Mg^{++}, 50S and 30S were the main species present. However, in contrast to E. coli, the ribosomes of B. subtilis appear to be fairly unstable. Summarizing the results of approximately 20 sucrose gradient centrifugations, degradation of B. subtilis ribosomes occurred in crude extracts prepared with either TSM or TMK buffer (0.01M KCl) at 10^{-2}M Mg^{++}. In contrast, E. coli extracts prepared in TSM buffer were stable for at least 20 hours (Bolton, 1958). At a higher salt concentration (0.1M KCl), and 10^{-2}M Mg^{++}, 70S and 50S B. subtilis ribosomes were more stable whereas the 30S ribosome was degraded under these conditions (no 30S ribosomes appeared in the P_{100-2} preparation at 10^{-2}M Mg^{++}). At 10^{-4}M Mg^{++}, 30S and 50S units were more stable in low salt (0.01M KCl in TMK buffer) or in TSM buffer.

c. Enzyme distribution on the ribosomes. When a crude extract containing 10^{-4}M Mg^{++} was centrifuged in a sucrose gradient, the pattern shown in figure 14 was obtained. By comparing this pattern of sedimentation with that obtained with preparations analyzed both by centrifugation in the analytical ultracentrifuge and in sucrose gradients, an estimate could be made of the sedimentation coefficients of the UV absorbing peaks shown in this figure. The enzyme distribution tended to follow that of the ribosomes, with peaks occurring in the 70S, 50S and 30S fractions. Most of the enzyme was found associated with the 30S ribosome.

When ribosomes at 10^{-2}M Mg^{++} were centrifuged in a sucrose gradient, again most of the enzyme was found in the 30S fractions with lesser

amounts in the 50S and 70S fractions (figure 15). The multiple enzyme peaks in the 30S region may indicate breakdown of the ribosomes resulting in the formation of components with coefficients in the range of 25S and 21S as well as 30S. The largest peak of enzyme ribosomal bound enzyme activity coincided with the lightest breakdown product, suggesting that the enzyme might become more active as the ribosomes disintegrated.

d. Activation of ribosomal bound enzyme by changes in Mg^{++} concentration. The activity of the enzyme on ribosomes prepared at 10^{-2}M Mg^{++} (but assayed at 10^{-4}M Mg^{++}) was compared with the same preparation dialyzed to a Mg^{++} concentration of 10^{-4}M. When equal amounts of the two preparations were analyzed by density gradient centrifugation, activation of the enzyme was observed in the 10^{-4}M Mg^{++} preparation in all fractions except those containing soluble RNA (figures 16 and 17). If the activated preparation was redialyzed against 10^{-2}M Mg^{++}, most of the activity disappeared except for that in the 30S fractions (figure 18). These effects of Mg^{++} concentration on the activities of different ribosomal fractions are summarized in Table 4. The striking increase in activity when the Mg^{++} concentration was changed from 10^{-2}M to 2.5×10^{-4}M was most clearly seen with the 80S-100S fractions (a 9-fold increase). Increases in activity were also noted with the 70S (a 4-fold increase) and 50S fractions (a 3-fold increase). The activity of 30S ribosomes was least affected by changes in Mg^{++} concentration, perhaps because most of the enzyme was already fully activated. The slight activation that did occur was in the heavier portion of the 30S region suggesting that more latent enzyme was present on intact 30S ribosomes than on partially degraded ones.

The amount of soluble enzyme also increased in the 10^{-4}M Mg^{++} preparation but was reduced again when the preparation was dialyzed at 10^{-2}M Mg.$^{++}$

An attempt was made to repeat this effect by assaying a P_{100-2} fraction for enzymatic activity after it had been dialyzed at 10^{-2}M Mg^{++} and then at 10^{-4}M Mg.$^{++}$ As seen from Table 5, dialysis at 10^{-4}M Mg^{++} and incubation at that Mg^{++} concentration had little effect on activity of the particulate enzyme. The 10% increase in the activity of the soluble enzyme upon dialysis at 2.5×10^{-4}M Mg^{++} would not account for the large activation in the previous experiment.

e. Activation of ribosomal bound enzyme by PVS. Preliminary evidence indicated that activation may occur when ribosomes prepared in TSM buffer at 10^{-4}M Mg^{++} are dialyzed in that buffer in the presence of polyvinyl-sulfate (PVS) which was added to inhibit RNAase (figures 14, 19, 20). Since the crude extract and the ribosomes dialyzed without PVS had nearly the same activities, the effect was not due to inactivation of the preparation without PVS during dialysis. Thus, interaction between the anion and the basic protein may have caused some activation, suggesting again that even at 10^{-4}M Mg^{++} some enzyme on the 30S ribosome may be in latent form. An alternative explanation for this phenomenon would be that PVS facilitated the removal of enzyme associated with the 50S ribosome and its transfer to the 30S units since the 50S fractions had less activity than the controls.

f. Fractionation of enzyme bearing ribosomes. A preliminary study has indicated that the 30S ribosomes bearing enzyme tended to be separated from other 30S ribosomes during sephadex gel filtration. Sedimentation analysis of fractions in sucrose gradients indicated that ribosomes with enzyme attached were filtered more rapidly through the gel, i.e., were excluded more completely from the gel (figure 21). The first fraction (A)

had 1.7 times more activity associated with the 30S units than the second(B) fraction. Little fractionation should occur because of the high molecular weight of the ribosome; bound protein would not be expected to influence filtration. This effect remains unexplained and will be explored in future experiments.

g. Nonspecific binding of alkaline phosphatase by ribosomes. Preliminary evidence indicated that the enzyme binds nonspecifically to the ribosomes. Strain K ribosomes (P_{100} preparation) from repressed cells (fully repressed strain 6 ribosomes could not be obtained since strain 6 was a partial constitutive) and strain 6 ribosomes (P_{100} fraction) from derepressed cells were incubated with approximately 340 units soluble strain 6 enzyme (S_{100} preparation) in TMK buffer containing either 10^{-2}M or 10^{-4}M Mg^{++}. Since the enzyme in the S_{100} preparation was known to be stable in TMK buffer, the amount of binding of the enzyme to the P_{100} fraction was estimated by measuring the decrease in activity of the supernatant fraction after the particulate fraction had been removed by centrifugation. Repressed strain K preparations bound more enzyme than the derepressed strain 6 P_{100} fractions (Table 6). The washed particulate preparations (P_{100-2}) retained most of the enzyme bound especially at high Mg^{++} concentrations.

V. DISCUSSION

A. Comparison of *E. coli* and *B. subtilis* (strains 6 and K) Alkaline Phosphatases

A comparison of some of the properties of *E. coli* and *B. subtilis* alkaline phosphatases is made below (Table 7).

TABLE 7

Comparison of *E. coli* and *B. subtilis* Alkaline Phosphatase

Property	*E. coli*	*B. subtilis* strain K	*B. subtilis* strain 6
Effect of:			
0.01 M Mg^{++}	none+	stimulation	inhibition
0.1 M NaCl	stimulation**	--	inhibition
EDTA	inhibitory+	--	inhibitory
toluene	none*	inhibitory	inhibitory
Opt. conc. Tris	1.6 M**	1.0 M	0.8 M
Opt. pH	8.0-11.0**	9.4	10.0
K_m	1.2×10^{-5}+ (dilute NPP)	1.90×10^{-4}	2.36×10^{-3}
Localization	soluble+	particulate	particulate
Permeability barrier	none*	present	present

* strain W
\+ strain K12
** Worthington Biochem. Corp.

Perhaps the greatest difference between these enzymes was their localization; in the particulate fraction in *B. subtilis*, in the soluble

fraction in E. coli. Another major difference was the effect of Mg^{++} on these enzymes. The E. coli enzyme only required Mg^{++} if the Tris buffer concentration was below 0.01M. For B. subtilis strain K, Mg^{++} was required for optimal alkaline phosphatase activity, and a Tris buffer concentration of 0.6M would not eliminate this requirement. In contrast, Mg^{++} inhibited the B. subtilis strain 6 enzyme in 0.6M Tris buffer.

Differences in the conditions for optimal activity with respect to pH and Tris buffer concentration were also noted with the different enzymes. A pH optimum of 9.4 was observed with B. subtilis strain K enzyme while 10.0 was the optimal for the strain 6 enzyme. Alkaline phosphatase from E. coli exhibited maximum activity in the pH range of 8.0 - 11.0 (Wilson, et al., 1964). A high concentration of Tris buffer was necessary for maximum activity of the E. coli enzyme. This requirement was explained as due to the participation of the buffer as a phosphate acceptor in a transphosphorylation reaction (Wilson, et al., 1964). Possibly this mechanism also occurred in the B. subtilis enzyme catalyzed reaction since high levels of Tris buffer were necessary for optimum activity of both B. subtilis enzymes.

Studies on enzyme kinetics also revealed differences between the three enzymes. For the E. coli enzyme, a plot of 1/V vs. 1/S yielded a biphasic curve, with dilute substrate concentrations (below 10^{-3}M) giving K_m values of approximately 10^{-5}, while substrate concentrations above 10^{-3}M gave higher K_m values (approximately 10^{-3}; Heppel, et al., 1962). Higher K_m values were obtained in dilute substrate concentration and biphasic reciprocal plots were not found with the B. subtilis enzymes.

B. Extracellular Enzymes in B. subtilis

The increase in enzymatic activity observed in the medium during late log phase could be explained by lysis although there was no noticeable decrease in the optical density of the culture at this point. Cashel and Freese (1964) noted similar results with B. subtilis under conditions in which little lysis occurred. This suggests that a change in permeability may occur during late log phase; amylase and lysozyme were also excreted at this time (Nomura, et al., 1958).

C. Enzymes Bound to Ribosomes

1. Possible Explanations for the Presence of Enzymes on the Ribosomes.

All proteins are associated with the ribosomes during their synthesis and are usually released as soon as completed (McQuillen, et al., 1959). However, certain degradative enzymes; RNAase, DNAase (Elson, 1959), B-galactosidase, alkaline phosphatase, D-serine deaminase, acid phosphatase, L-threonine deaminase (Aronson, et al., 1960), B-glucosidase (Kihara, et al., 1961), and leucine aminopepidase (Bolton, et al., 1959) are associated with E. coli ribosomes and, like B. subtilis alkaline phosphatase, they resist separation from these particles.

The presence of these enzymes on the ribosomes could be explained in several ways. Some of the enzymes are probably nascent protein that has remained tightly bound to the enzyme. The small percentage (5-10%) of the cellular alkaline and acid phosphatases, D-serine deaminase, and L-threonine deaminase bound to the ribosomes suggests that these enzymes are nascent protein (Aronson, et al., 1960). A fraction of the ribosomal

bound B-galactosidase has been shown to meet the kinetic requirements of nascent protein (Zipser, 1963), whereas ribosomal bound B-glucosidase in yeast was shown to be a direct precursor to the soluble enzyme (Kihara, et al., 1961). Because of the large percentage of alkaline phosphatase associated with the ribosomes (44%) in B. subtilis, it seems unlikely that the bound enzyme is nascent protein.

Some degradative enzymes may have a function associated with their localization on the ribosomes. Regulation of B-glucosidase was shown to be dependent on the controlled release of nascent enzyme from the ribosome; during repression the amount of ribosomal bound enzyme increased (Hauge, et al., 1961). At 10^{-2}M Mg^{++} a cysteinlyglycinase was found specifically associated with the heavier ribosomal aggregates, suggesting that it had a function in protein synthesis (McCorquodale, 1963). Triphosphatases were also found to be specifically associated with the polysome fractions and were released to 50S, 30S and soluble RNA fractions (Raacke and Fiala, 1964). Thus it was suggested that the polysome could bind proteins that could not attach to the separated 70S ribosomes.

GTPase activity has been found to coincide with the ribosomal fractions where maximum amino acid incorporation occurred, suggesting a GTP split was connected with protein synthesis (Conway and Lipmann, 1964). GTP was shown to be a requirement for the release of nascent protein from reticulocyte ribosomes (Morris, 1963). The reversible dissociation of DNAase from the E. coli 70S ribosome also suggested that the enzyme may have a function in protein synthesis (Tal and Elson, 1963).

Some of the associations of enzymes with ribosomes probably result from methods used in the preparation of extracts. The quantity of

B-galactosidase bound to the ribosomes depended on how the cells were broken (Boezi, et al., 1961). The association of all of the cellular RNAase with the 30S ribosome could have occurred during extract formation due to nonspecific binding of the basic protein to the ribosome, a phenomenon which has been observed in vitro (Neu and Heppel, 1964c). The enzyme was reported to bind very tightly to the nucleoprotein, remaining attached to it during electrophoresis and DEAE cellulose chromatography (Elson, 1959) but could be removed by incubation of the ribosomes in 0.5M NH_4Cl (Spirin, et al., 1963). An RNAase with different chromatographic properties than the ribosomal bound enzyme was released from the cell within a few minutes after treatment of the cells with EDTA and lysozyme (Neu and Heppel, 1964a). Further study showed that there were two RNAases, one associated with the ribosomes and the other with the debris (Anrahu and Mizuno, 1965). These enzymes differed not only in elution position on DEAE cellulose but in rates of reaction and activity against different substrates. However, the debris RNAase still could bind to the ribosomes in vitro suggesting that its presence in the debris depended on how the extract was made.

Alkaline phosphatase in B. subtilis resembled E. coli RNAase in its tendency to nonspecifically bind to the 30S ribosome. In contrast to the RNAase, B. subtilis alkaline phosphatase also was bound in small amounts to other ribosomes. Thus, it will be pertinent to determine whether the soluble and particulate alkaline phosphatases are identical in B. subtilis and whether the amount of binding depends on the method of preparation.

2. Activation.

Evidence was found for the existence of a partially latent alkaline phosphatase associated with the B. subtilis ribosomes. Increases in activity were observed when the P_{100} fraction was incubated in the presence of the S_{100} fraction or when the P_{100-2} fraction was dialyzed from 10^{-2}M Mg^{++} to 2.5×10^{-4}M Mg^{++}.

Other ribosomal bound enzymes have been detected in latent form in E. coli. An aminopeptidase was activated by chymotrypsin, trypsin or RNAase (Matheson, 1963). Similarly, ribosomal bound RNAase was revealed only upon ribosomal degradation (Neu and Heppel, 1964c). B-galactosidase was activated while attached to ribosomes by the addition of specific antisera (Cowie, et al., 1961). An increase in tryptophan synthetase activity was observed when low molecular weight ribosomes were incubated in the presence of the supernatant fraction (Marushige, et al., 1964). The latter was interpreted as an activation of preformed enzyme on the ribosome rather than protein synthesis, since the process was insensitive to DNAase, chloramphenicol, and streptomycin. In vitro complementation between mutant subunits of B-galactosidase occurred faster if one subunit was still attached to the ribosome (Zipser and Perrin, 1963).

The explanation for the activation of ribosomes which had been dialysed from 10^{-2}M Mg^{++} to 10^{-4}M Mg^{++} prior to sucrose gradient analysis is not clear since the increase in activity could not be noted by directly assaying P_{100-2} fractions for enzymatic activity. 10^{-3}M Mg^{++} slightly inhibited the particulate enzyme. This observation procludes the possibility that the differences in activities of the two preparations could be due to changes in the amount of Mg^{++} present as a result of the 10^{-2}M

preparation binding more Mg^{++} than the 10^{-4}M preparation. Perhaps soluble components activated the latent enzyme in the 10^{-2}M Mg^{++} P_{100-2} preparation. Soluble factors were not present in the individual ribosome fractions from the sucrose gradient. Possibly the ribosomes in the activated 10^{-4}M Mg^{++} preparation were partially degraded before the assay and therefore had higher activity than the ribosomes of the P_{100-2} fraction at 10^{-4}M Mg^{++} which were intact as shown by sucrose gradient analysis.

The observed activation of the particulate fraction by the soluble fraction in B. subtilis could be the result of release of the ribosomal bound enzyme by supernatant factors, as in the case of tryptophan synthetase (Marushige, et al., 1964), or could be caused by the combination of soluble monomers with ribosomal bound monomers, as with B-galactosidase (Zipser and Perrin, 1963). The difference in the activation at 10^{-2}M Mg^{++} and 10^{-4}M Mg^{++} suggested that Mg^{++} may stimulate activation as was observed with the enzyme catalyzed release of hemoglobin from reticulocyte ribosomes (Morris, 1961). The possible relationship between this activation and the previously reported in vitro increase in enzymatic activity in B. subtilis (Whiteley and Oishi, 1963) or in E. coli (Bishop, et al., 1964; Manson, et al., 1965) will be investigated in future experiments.

BIBLIOGRAPHY

Allen, J. and G. Hyncik. 1963. Localization of Alkaline Phosphatase in Gel Matrices following Electrophoresis. J. Histochem. Cytochem., 11, 169.

Anraku, Y. and D. Mizuno. 1965. A Ribonuclease from the Debris of Escherichia coli. Biochem. Biophys. Res. Comm., 18, 462.

Aronson, A. I., E. T. Bolton, R. J. Britton, D. B. Cowie, J. D. Duerksen, B. J. McCarthy, K. McQuillen, R. B. Roberts. 1960. Enzymic Activities of Ribosomal Particles. Yearbook Carnegie Inst., 59, 238.

Aronson, A. and B. J. McCarthy. 1961. Studies of Escherichia coli Ribosomal RNA and Its Degradation Products. Biophys. J., 1, 215.

Bach, M. L., E. R. Singer, C. Levinthal and I. W. Sizer. 1961. The Electrophoretic Patterns of Alkaline Phosphatase from Various Escherichia coli Mutants. Fed. Proc., 20, 255.

Bishop, D. H., C. Roche, and B. Nisman. 1964. Induction of Alkaline Phosphatase in a Subcellular Preparation from Escherichia coli. Biochem. J., 90, 378.

Boezi, J. A., E. T. Bolton, R. J. Britten, D. B. Cowie, B. J. McCarthy, J. E. Midgley and R. B. Roberts. 1961. Ribosomal Enzymes. Yearbook Carnegie Inst., 60, 322.

Bolton, E. T. 1958. Stability of Ribonucleoprotein Particles of Escherichia coli. in Microsomal Particles and Protein Synthesis. Pergamon Press, New York, p. 18.

Bolton, E. T., R. J. Britten, D. B. Cowie, B. J. McCarthy, K. McQuillen and R. B. Roberts. 1959. Enzyme Content of Ribosomes. Yearbook Carnegie Inst., 58, 275.

Cashel, M. and Freese, E. 1964. Excretion of Alkaline Phosphatase by Bacillus subtilis. Biochem. Biophys. Res. Comm., 16, 541.

Conway, T. W. and F. Lipmann. 1964. Characteristics of a Ribosomal-linked Guanosine Triphosphatase in Escherichia coli Extracts. Proc. Natl. Acad. Sci. U.S., 52, 1462.

Cowie, D. B., S. Spiegelman, R. B. Roberts, and J. D. Duerksen. 1961. Ribosomal Bound B-Galactosidase. Proc. Natl. Acad. Sci., 47, 114.

Dayan, J. and I. B. Wilson. 1964. The Phosphorylation of Tris by Alkaline Phosphatase. Biochim. Biophys. Acta., 81, 620.

Demain, A. L., R. W. Burg, and D. Hendlin. 1965. Excretion and Degradation of Ribonucleic Acid by Bacillus subtilis. J. Bact., 89, 640.

Demain, A. L., I. M. Miller, and D. Hendlin. 1964. Production of Extracellular Guanosine 5' Monophosphate by Bacillus subtilis. J. Bact., 88, 991.

Echols, H., A. Garen, S. Garen and A. Torriani. 1961. Genetic Control of Repression of Alkaline Phosphatase in Escherichia coli. J. Mol. Biol., 3, 425.

Engstrom, L. 1962. Incorporation of Inorganic Phosphate into Alkaline Phosphatase from Escherichia coli. Biochim. Biophys. Acta., 56, 606.

Elson, D. 1959. Preparation and Properties of a Ribonucleoprotein isolated from Escherichia coli. Biochim. Biophys. Acta., 36, 362.

Elson, D. 1961. A Ribonucleic Acid Particle Released from Ribosomes by Salt. Biochim. Biophys. Acta., 53, 232.

Gallant, J. and Stapleton, R. 1964. Physiological Evidence on the Nature of the Repressor of Alkaline Phosphatase Synthesis in Escherichia coli. J. Mol. Biol., 8, 431.

Garen, A. and H. Echols. 1962a. Properties of Two Regulatory Genes for Alkaline Phosphatase. J. Bact., 83, 297.

Garen, A. and H. Echols. 1962b. Genetic Control of Alkaline Phosphatase Synthesis in Escherichia coli. Proc. Natl. Acad. Sci., 48, 1398.

Garen, A. and S. Garen. 1963a. Genetic Evidence on the Nature of the Repressor for Alkaline Phosphatase in Escherichia coli. J. Mol. Biol., 6, 433.

Garen, A. and S. Garen. 1963b. Complementation In Vivo between Structural Mutants of Alkaline Phosphatase from Escherichia coli. J. Mol. Biol., 7, 13.

Garen, A. and C. Levinthal. 1960. A Fine Structure Genetic and Chemical Study of the Enzyme Alkaline Phosphatase of Escherichia coli. Biochim. Biophys. Acta., 38, 470.

Garen, A., C. Levinthal and F. Rothman. 1961. Alterations in Alkaline Phosphatase Induced by Mutations. J. Chim. Phys., 58, 1068; Chem. Abstracts, 56, 13349i.

Garen, A., and N. Otsuji. 1964. Isolation of a Protein Specified by a Regulator Gene. J. Mol. Biol., 8, 841.

Hagihara, G., H. Matsubara, M. Nakai and K. Okunuki. 1958. Crystalline Bacterial Proteinase. J. Biochem. (Tokyo), 45, 185.

Hauge, J. G., A. M. MacQuillan, A. L. Cline and H. O. Halvorson. 1961. The Effect of Glucose Repression on the Level of Ribosomal Bound B-Glucosidase. Biochem. Biophys. Res. Comm., 5, 267.

Heppel, Leon A., D. R. Harkness, and R. J. Hilmoe. 1962. A Study of the Substrate Specificity and Other Properties of Alkaline Phosphatase of Escherichia coli. J. Biol. Chem., 237, 841.

Horiuchi, T., S. Horiuchi, and D. Mizuno. 1959. Possible Negative Feedback Phenomenon Controlling the Formation of Alkaline Phosphomonoesterase in Escherichia coli. Nature, 183, 1529.

Kihara, H. D., A. S. L. Hu, and H. O. Halvorson. 1961. The Identification of a Ribosomal Bound B-glucosidase. Proc. Natl. Acad. Sci., 47, 489.

Kushner, D. J. and M. R. Pollock. 1961. Location of Cell Bound Penicillinase in Bacillus subtilis. J. Gen. Micro., 26, 255.

Leive, L. 1965. A Nonspecific Increase in Permeability in Escherichia coli Produced by EDTA. Proc. Natl. Acad. Sci., 53, 745.

Levinthal, C. 1959. Genetic and Chemical Studies with Alkaline Phosphatase of Escherichia coli. Brookhaven Symposia in Biology, 12, 76.

Levinthal, C., E. R. Signer and K. Fetherolf. 1962. Reactivation and Hybridization of Reduced Alkaline Phosphatase. Proc. Natl. Acad. Sci., 48, 1230.

Lowry, O. H., N. J. Rosenbrough, A. L. Farr, and R. S. Randall. 1951. Protein Measurement with Folin Phenol Reagent. J. Biol. Chem., 193, 265.

Malamy, M. and B. Horecker. 1961. Localization of Alkaline Phosphatase in Escherichia coli K12. Biochem. Biophys. Res. Comm., 5, 104.

Malamy, M. H. and B. L. Horecker. 1964. Release of Alkaline Phosphatase from Cells of Escherichia coli upon Lysozyme Spheroplast Formation. Biochemistry, 3, 1889.

Manson, L. A., J. Pelmont, A. Yapo, C. Roche, and B. Nisman. 1965. The Biosynthesis of Alkaline Phosphatase with a Particulate Fraction of Escherichia coli. Biochem. J., 95, 215.

Marushige, K., T. Yura, and M. Imai. 1964. Role of Ribosomes in the Cell Free Formation of Tryptophan Synthetase in Escherichia coli. Biochim. Biophys. Acta., 87, 90.

Matheson, A. T. 1963. The Localization and Properties of an Aminopeptidase in Escherichia coli B. Canad. J. Biochem. Phys., 41, 9.

McCorquodale, D. J. 1963. Some Properties of Ribosomal Cysteinlyglycinase in Escherichia coli B. J. Biol. Chem., 238, 3914.

McQuillen, K., R. B. Roberts and R. J. Britten. 1959. Synthesis of Nascent Protein by Ribosomes in Escherichia coli. Proc. Natl. Acad. Sci., 45, 1437.

Milstein, C. 1963. The Amino Acid Sequence around the Reactive Serine in Alkaline Phosphatase. Biochim. Biophys. Acta., 67, 171.

Morris, A. J. 1963. The Release of Protein from Reticulocyte Ribosomes. Biochem. Biophys. Res. Comm., 11, 201.

Morris, A. and R. S. Schweet. 1961. Release of Soluble Protein from Reticulocyte Ribosomes. Biochim. Biophys. Acta., 47, 415.

Neu, H. C. and L. A. Heppel. 1964a. The Release of Ribonuclease into the Medium when Escherichia coli Cells Are Converted to Spheroplasts. Biochem. Biophys. Res. Comm., 14, 109.

Neu, H. C. and L. A. Heppel. 1964b. On Surface Localization of Enzymes in Escherichia coli. Biochem. Biophys. Res. Comm., 17, 215.

Neu, H. C. and L. A. Heppel. 1964c. Some Observations on the Latent Ribonuclease of Escherichia coli. Proc. Natl. Acad. Sci., 51, 1267.

Nishimura, S. and M. Nomura. 1959. Ribonuclease of Bacillus subtilis. J. Biochem. (Tokyo), 46, 161.

Nomura, M., J. Hosoda and H. Yoshihawa. 1958. Studies on Amylase Formation by Bacillus subtilis. J. Biochem. (Tokyo), 45, 737.

Oishi, M., H. Takahasi, and B. Maruo. 1963. Intracellular Amylase in Bacillus subtilis. J. Bact., 85, 246.

Pigretti, M. and C. Milstein. 1965. Acid Inactivation of and Incorporation of Phosphate into Alkaline Phosphatase from Escherichia coli. Biochem. J., 94, 106.

Plocke, D. J., C. Levinthal and B. L. Vallee. 1962a. Alkaline Phosphatase of Escherichia coli: A Zinc Metalloenzyme. Biochem., 1, 373.

Plocke, D. J. and B. L. Vallee. 1962b. Interaction of Alkaline Phosphatase of Escherichia coli with Metal Ions and Chelating Agents. Biochem., 1, 1039.

Pollock, M. 1961. Measurement of Liberation of Penicillinase from Bacillus subtilis. J. Gen. Micro., 26, 239.

Pollock, M. R. 1962. Exoenzymes. Gunsalus, I. C. and R. Y. Stanier. The Bacteria, vol. 4, Academic Press, Inc., New York, p. 121.

Raache, I. D. and J. Fiala. 1964. Polyribosome-bound Nucleotide Triphosphatases in Escherichia coli. Proc. Natl. Acad. Sci., 51, 323.

Rappaport, H. P., W. S. Riggsby and D. A. Holden. 1965. A Bacillus subtilis Proteinase. J. Biol. Chem., 240, 78.

Richmond, M. H. 1959. Formation of a Lytic Enzyme by a Strain of Bacillus subtilis. Biochim. Biophys. Acta, 33, 78.

Rothman, F. and R. Byrne. 1963. Fingerprint Analysis of Alkaline Phosphatase of Escherichia coli K12. J. Mol. Biol., 6, 330.

Schachman, H. K. 1959. Ultracentrifugation in Biochemistry. Academic Press, New York, pp. 75-103.

Schaechter, M. 1963. Bacterial Polyribosomes and Their Participation in Protein Synthesis In Vivo. J. Mol. Biol., 7, 561.

Schlesinger, M. and Levinthal, C. 1963. Hybrid Protein Formation of Escherichia coli Alkaline Phosphatase Leading to In Vitro Complementation. J. Mol. Biol., 7, 1.

Schlesinger, M., A. Torriani and C. Levinthal. 1963. In Vitro Formation of Enzymatically Active Hybrid Protein from Escherichia coli Alkaline Phosphatase. Cold Spr. Harb. Symp. Quant. Biol., 28, 539.

Schwartz, J. 1963. Phosphorylation of Alkaline Phosphatase. Proc. Natl. Acad. Sci., 49, 871.

Schwartz, J. H., A. M. Crestfield and F. Lipmann. 1963. The Amino Acid Sequence of a Tetradecapeptide Containing the Reactive Serine in Escherichia coli Alkaline Phosphatase. Proc. Natl. Acad. Sci., 49, 722.

Schwartz, J. H. and F. Lipmann. 1961. Phosphate Incorporation into Alkaline Phosphatase of Escherichia coli. Proc. Natl. Acad. Sci., 47, 1996.

Spirin, A. S., N. A. Kiselev, R. S. Shakulov and A. A. Bogdanov. 1963. Study of the Structure of the Ribosome: Reversible Unfolding of the Ribosome Particles in Ribonucleoprotein Strands and a Model of the Packing. Biokhimiya, 28, 920.

Tal, M. and D. Elson. 1963. Reversible Release of Deoxyribonuclease, Protein and RNA from Ribosomes. Biochim. Biophys. Acta, 72, 439.

Tissieres, A., J. D. Watson, D. Schlessinger and B. R. Hollingworth. 1959. Ribonucleoprotein Particles from Escherichia coli. J. Mol. Biol., 1, 221.

Torriani, A. 1960. Influence of Inorganic Phosphate in the Formation of Phosphatase by Escherichia coli. Biochim. Biophys. Acta, 38, 460.

Whiteley, H. R. and M. Oishi. 1963. An Increase in Alkaline Phosphatase in an In Vitro System Derived from Bacillus subtilis. Biochem. Biophys. Res. Comm., 13, 6.

Wilson, I., J. Dayan, and C. Cyr. 1964. Some Properties of Alkaline Phosphatase from Escherichia coli. J. Biol. Chem., 239, 4182.

Yoshida, A. and T. Tobita. 1960a. Studies on the Mechanism of Protein Synthesis. Biochim. Biophys. Acta, 37, 513.

Yoshida, A., T. Tobita and J. Koyama. 1960b. Biological and Chemical Properties of Amylase Precursor. Biochim. Biophys. Acta, 44, 388.

Young, F. E. and J. Spizizen. 1963. Biochemical Aspects of Competence in a Bacillus subtilis Transformation System. II. Autolytic Enzyme Activity in Cell Walls. J. Biol. Chem., 238, 3126.

Zipser, D. 1963. Studies on the Ribosomal-bound B-Galactosidase of Escherichia coli. J. Mol. Biol., 7, 739.

Zipser, D. and D. Perrin. 1963. Complementation on Ribosomes. Cold Spr. Harb. Symp. Quant. Biol., 28, 533.

TABLE 1

The Effect of Mg^{++} on the Soluble and Particulate Enzymes of Strain 6

Mg^{++} concentration	% inhibition soluble enzyme	% inhibition particulate enzyme
10^{-2} M	67%	86%
5×10^{-3} M	57%	68%
10^{-3} M	21%	11%
10^{-4} M	0%	0%
10^{-5} M	16%	0%
0	26%	-

Soluble enzyme (S_{100} fraction) was prepared as indicated in Methods except that dialysis was against 100 volumes of distilled H_2O for 4 hours with hourly changes of H_2O. The particulate enzyme (P_{100} fraction) was dialyzed against 200 volumes of TMK buffer (10^{-4} M Mg^{++}).

TABLE 2

The Effect of the Supernatant on the Activity of the Particulate Fraction at Different Mg^{++} Concentrations

Mg^{++} concentration	amount supernatant	activity in units/ml
10^{-4} M	0.1 ml	2.1
	0.2	1.9
	0.3	2.0
	0.4	2.4
	0.5	2.6
10^{-2} M	0.1	1.1
	0.2	1.6
	0.3	1.5
	0.4	1.8
	0.5	1.9

Conditions as described in figures 6 and 7.

TABLE 3

Localization of Cellular Alkaline Phosphatase in Strain 6

fraction	total units	% recovery	sp. act.
whole extract	21,000	100	34.8
supernatant	14,500	69	38.2
debris	7,500	36	39.4
S_{100}	3,140	22	29.6
P_{100}	9,200	63	261
S_{100-2}	94	1	5.3
P_{100-2}	6,400	70	867

Fractions were prepared and assayed as described in Methods.

TABLE 4

Relationship between Mg Concentration and Activity on Different Ribosomes

figure	Mg	activity (units/ml/ 80-100s	70s	50s	30s
16	10^{-2}	.21	.20	.17	.79
17	2.5×10^{-4}	1.8	.77	.51	.98
18	10^{-2}	.18	.08	.09	.90

Conditions as described under figures 16, 17 and 18.

TABLE 5

The Effect of Dialysis and Incubation at Different Mg^{++} Concentrations on the Activity of the Enzyme

Preparation	Mg^{++} concentration of dialysis	time incubated at 10^{-2}M Mg^{++}	time incubated at 10^{-4}M Mg^{++}	Activity
P_{100-2}	10^{-2}	-	-	10.90
"	"	3	-	10.72
"	"	-	3	10.10
"	"	18	-	10.45
"	"	-	18	10.38
"	10^{-4}	-	-	10.90
"	"	-	15	10.38
S_{100}	10^{-2}	-	-	8.66
"	"	3	-	7.98
"	"	-	3	8.16
"	"	18	-	7.88
"	"	-	18	7.88
"	10^{-4}	-	-	9.84
"	"	-	15	8.90

The P_{100-2} and S_{100} fractions were prepared as described in Methods. The fractions were dialyzed against 200 volumes TMK buffer containing the indicated amount of Mg^{++} for 4 hours.

TABLE 6

Binding of Alkaline Phosphatase by Particulate Preparations from Strains 6 and K

Strain	Preparation	Mg^{++} conc.	% Enzyme removed from S_{100} frac.	% Enzyme retained by P_{100} fraction after washing
K	P_{100}	10^{-2}	59%	88%
"	P_{100}	10^{-4}	69%	83%
6	P_{100}	10^{-3}	46%	79%
"	P_{100}	10^{-4}	42%	75%

P_{100} fractions were prepared as described in Methods and dialyzed against 200 volumes TMK buffer at the indicated Mg^{++} concentration for 4 hours. The fractions were incubated with an S_{100} preparation for 1 hour in the cold and then centrifuged at 100,000 xg for 3 hours. The sediment was washed by resuspension in TMK buffer at the indicated Mg^{++} concentration and recentrifugation at 100,000 xg for 3 hours.

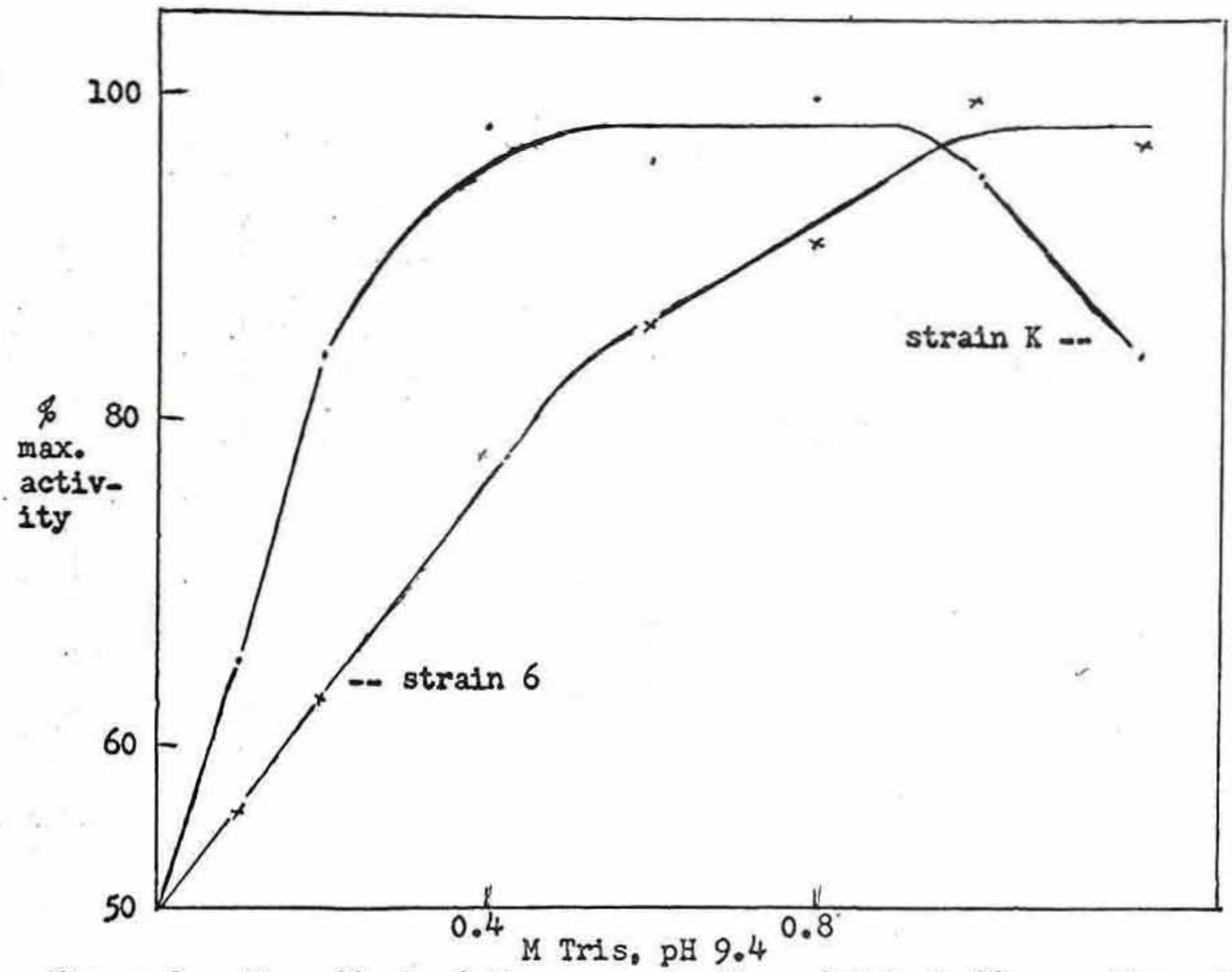

Figure 1. The effect of the concentration of Tris buffer on the activities of the enzymes of strains K and 6. A strain K extract and a strain 6 S_{100} fraction were prepared and assayed as described in Methods.

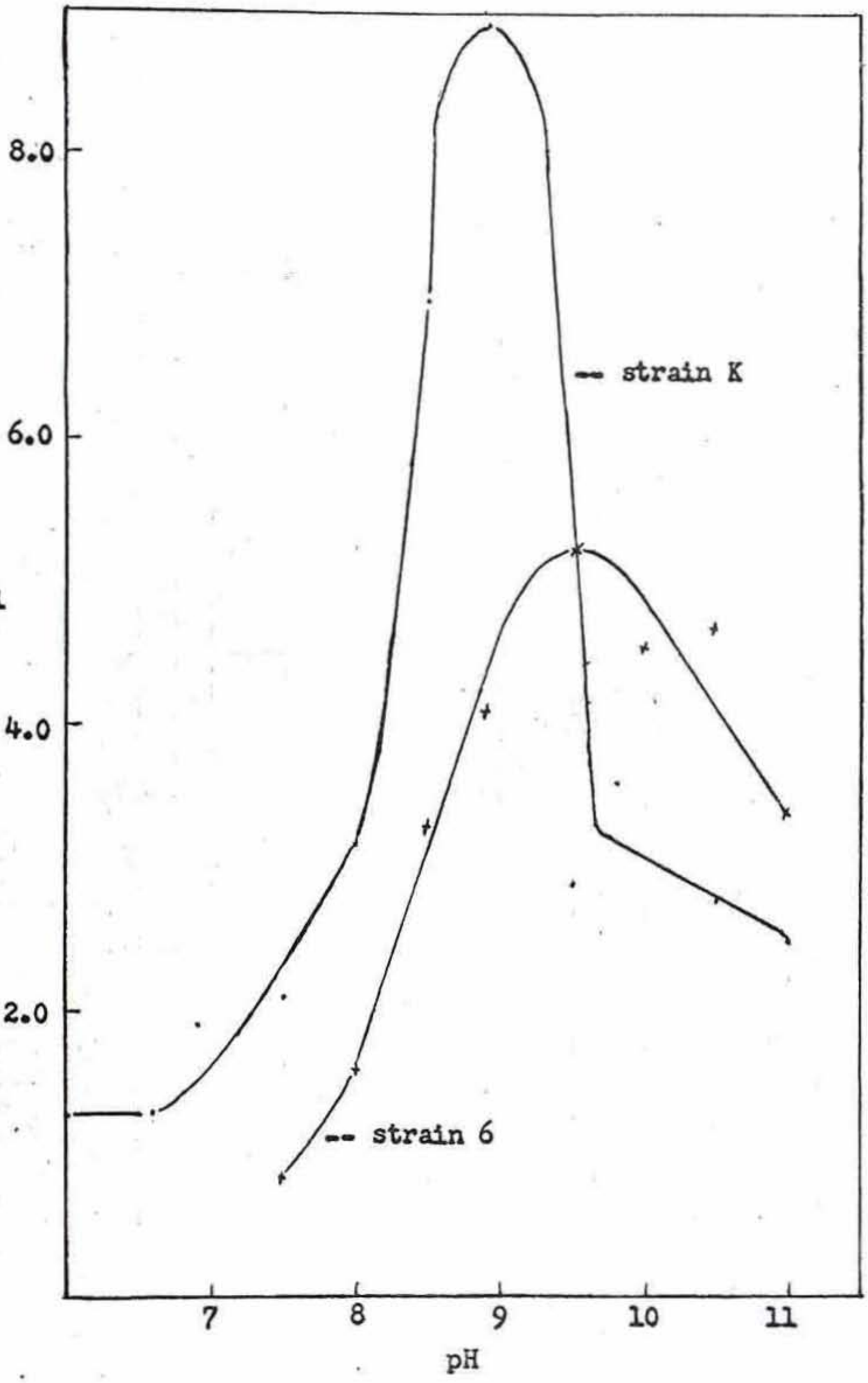

Figure 2. The effect of pH on the activities of the enzymes of strains 6 and K. A strain K extract and a strain 6 S_{100} fraction were prepared as described in Methods.

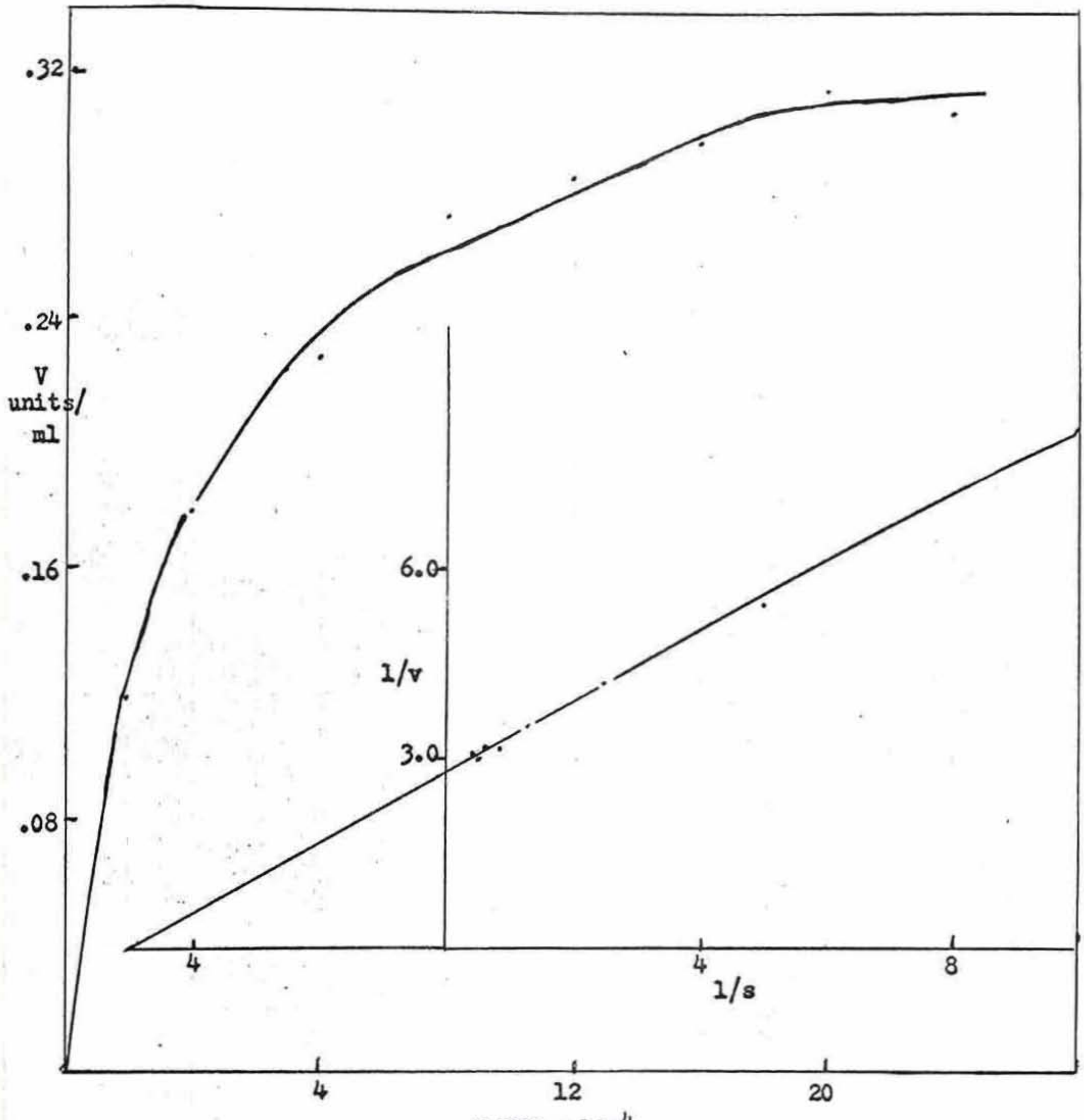

Figure 3. The relationship between substrate concentration and reaction velocity for strain K enzyme. An extract was prepared and assayed according to the Methods section.

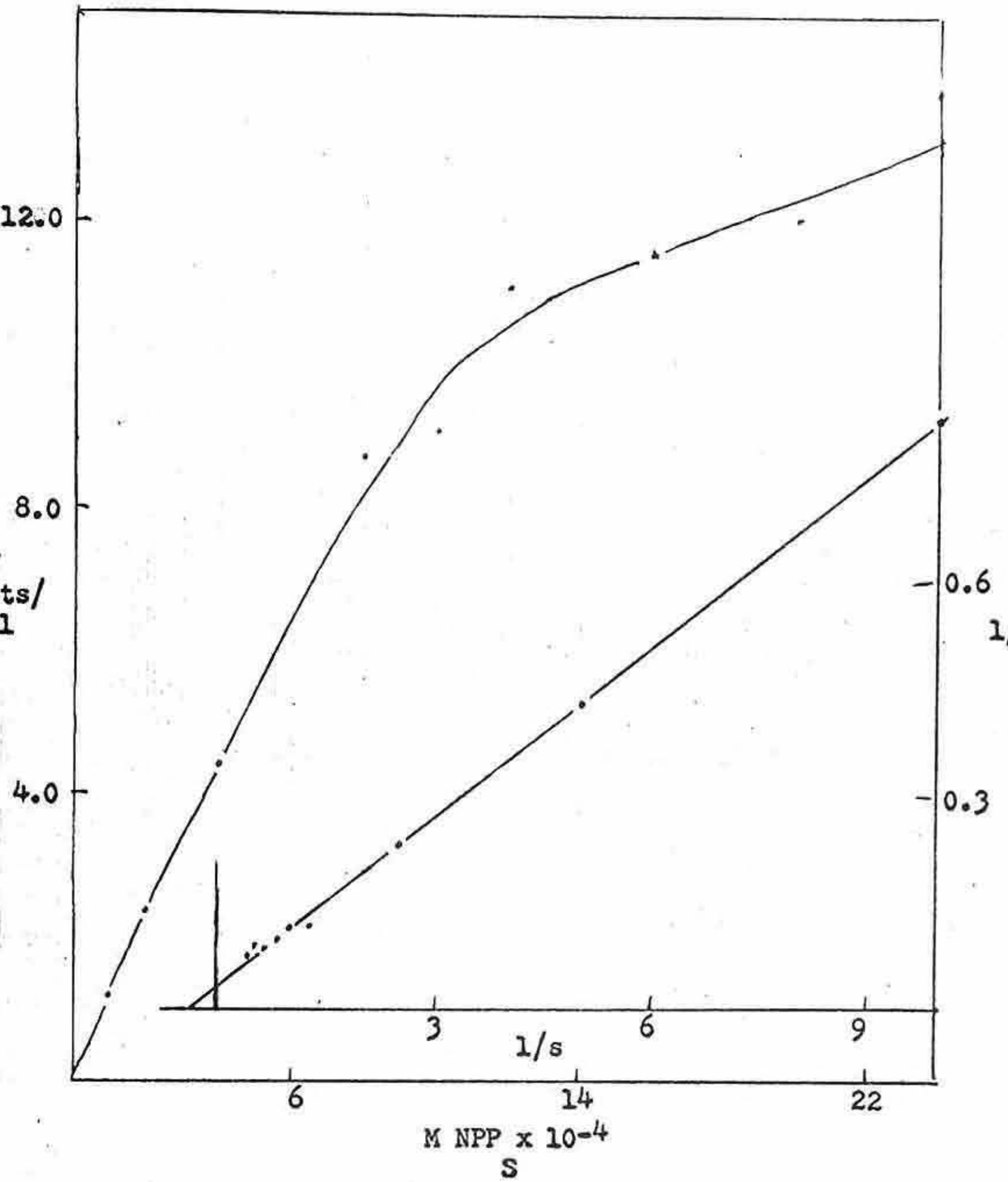

Figure 4. The relationship between substrate concentration and reaction velocity for strain 6 enzyme. Soluble strain 6 enzyme (S_{100} fraction) was prepared and assayed as described in Methods.

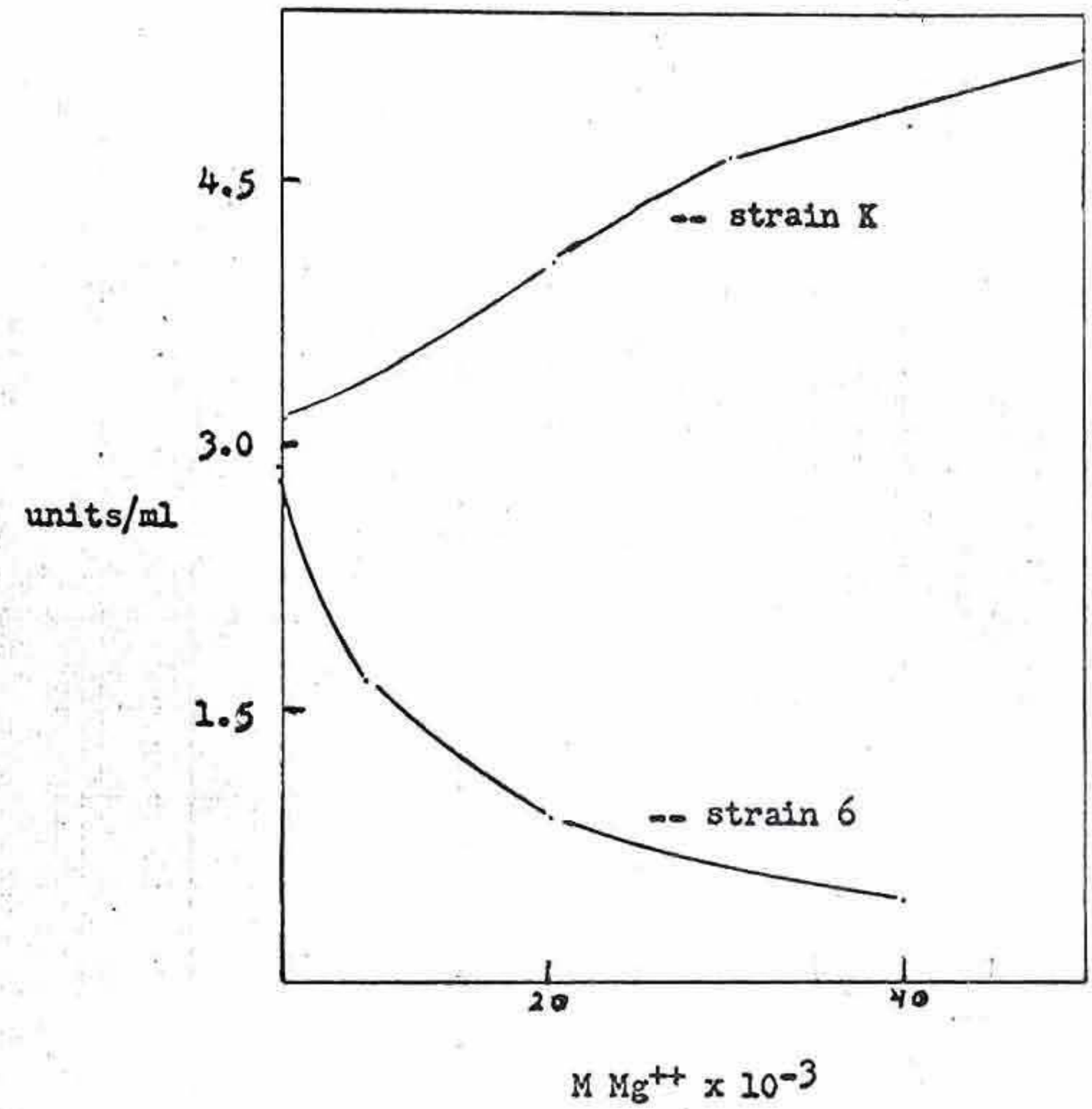

Figure 5. The effect of Mg^{++} on the activities of strains K and 6 enzymes. Extracts were prepared from strains K and 6 cells using sonic oscillations and assayed as described in the Methods section.

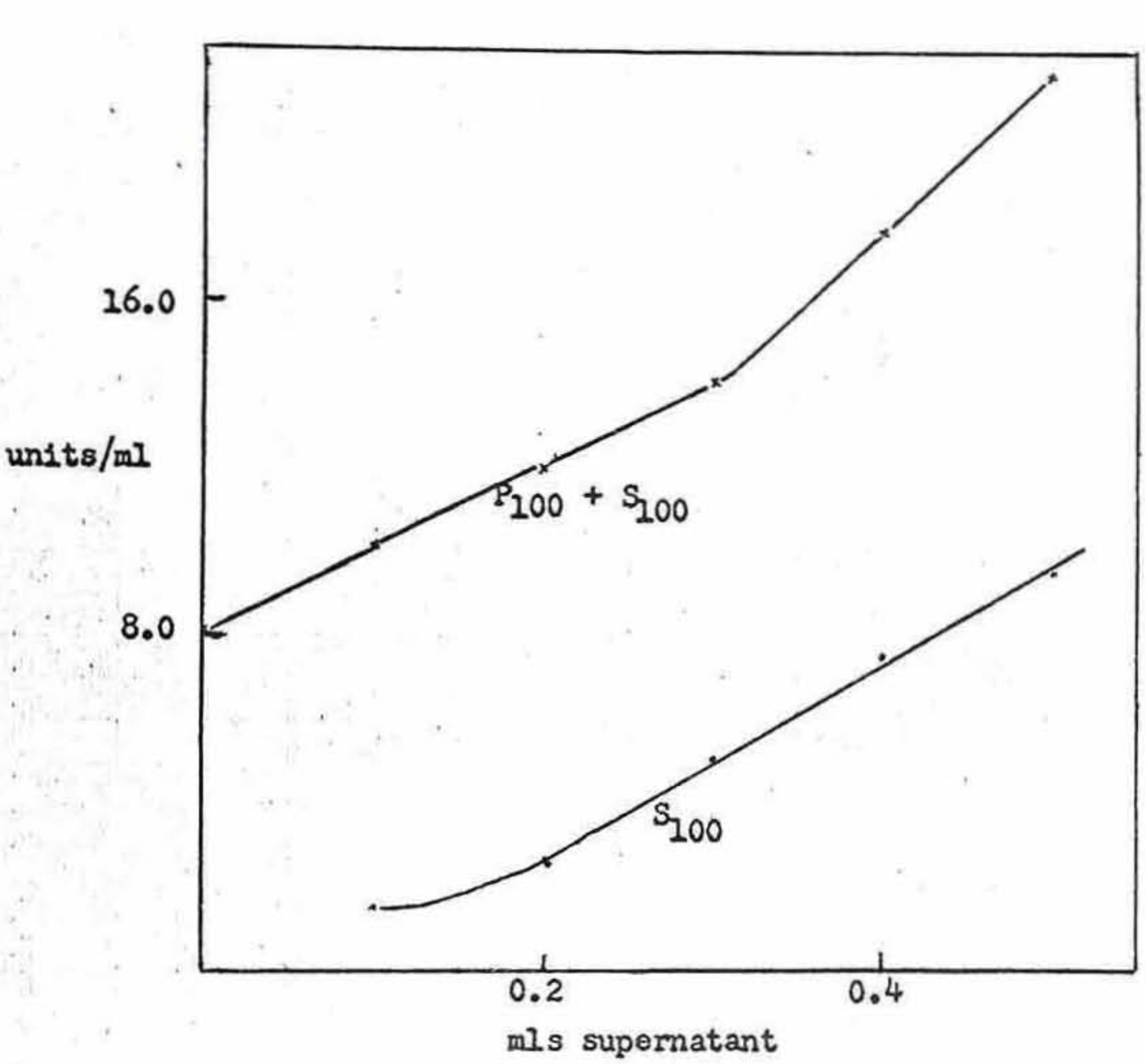

Figure 6. The effect of the supernatant fraction on the activity of a particulate fraction at 10^{-4}M Mg^{++}. The soluble enzyme (S_{100} fraction) was prepared as described in Methods. The particulate enzyme (P_{100}) was prepared from an extract containing 10^{-4}M Mg^{++} TMK. The extract was centrifuged at 27,000g for 30 minutes, the supernatant removed and recentrifuged at 100,000g for 3 hours. The sediment was resuspended in 10^{-4}M Mg^{++} TMK and dialyzed against 200 volumes of that buffer for 3 hours. The enzyme preparations were assayed as described in the Methods section except the reaction mixture contained 10^{-2}M mercaptoethanol.

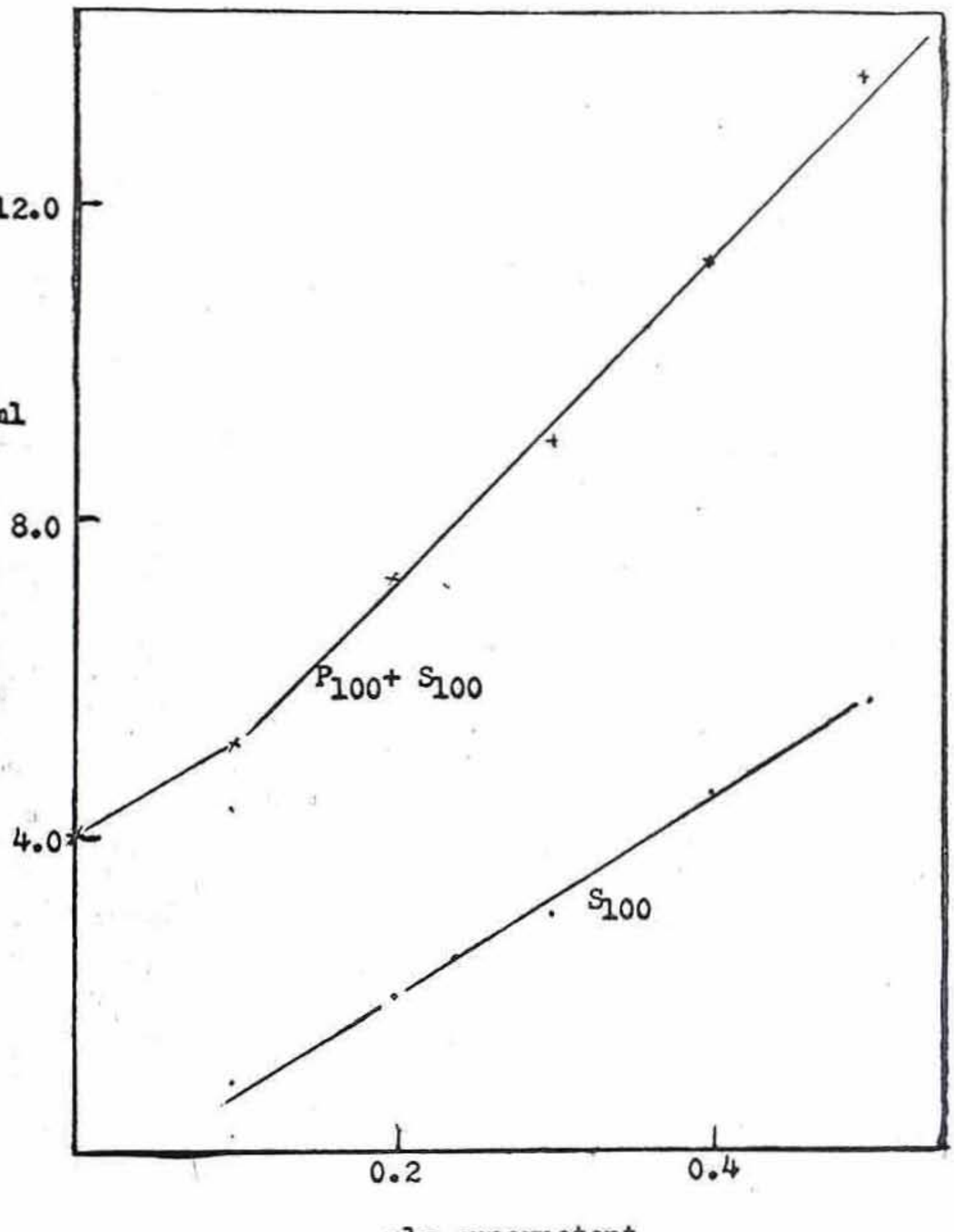

Figure 7. The effect of supernatant fraction (S_{100}) on the activity of a particulate fraction (P_{100}) at 10^{-2}M Mg^{++}. Conditions were the same as in Figure 6 except the particulate fraction was prepared at 10^{-2}M Mg^{++} TMK and dialyzed against that buffer.

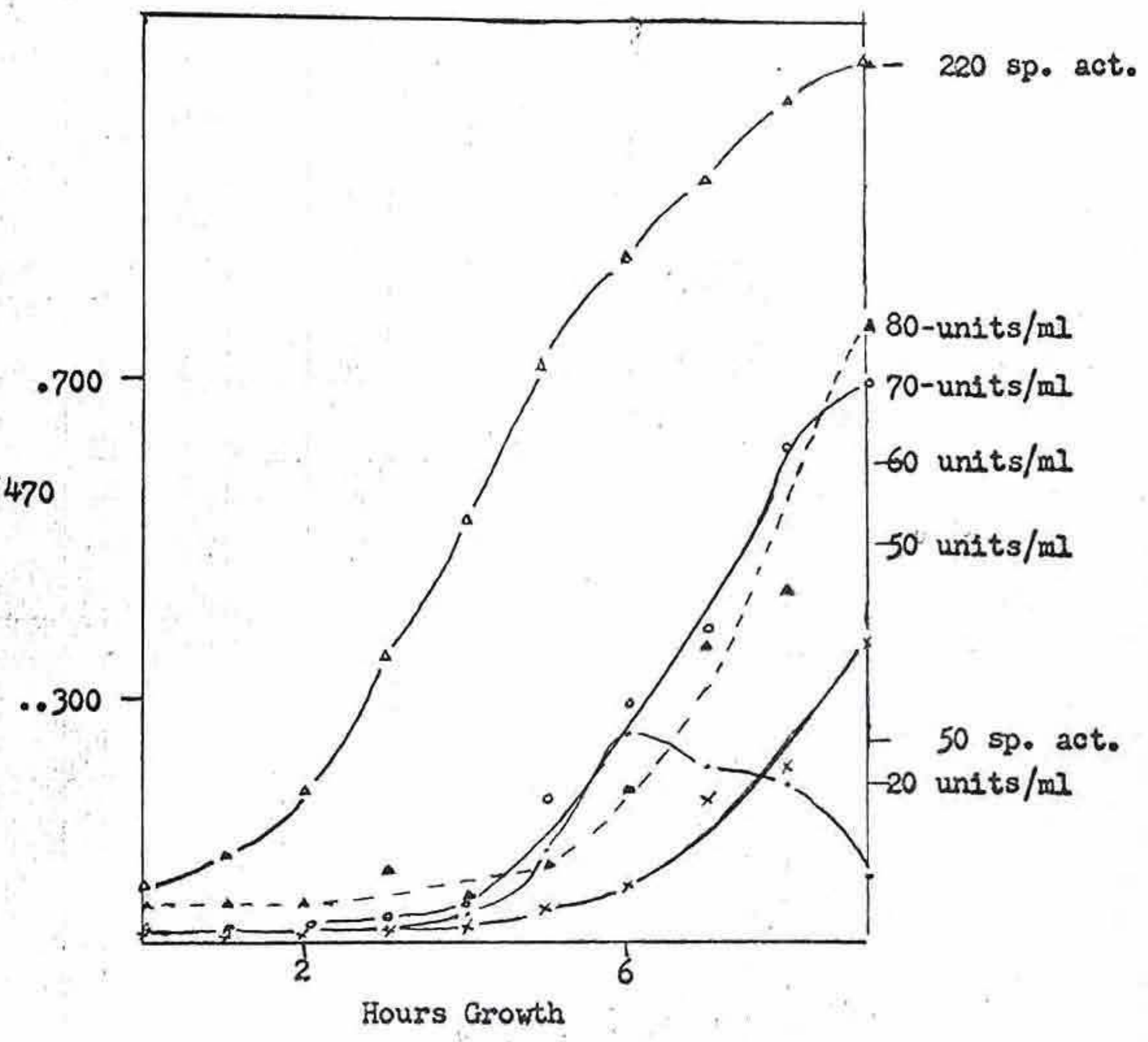

Figure 8. Derepression of strain 6. Samples were collected and assayed as described in Methods. △ — △ O.D.$_{470\ mu}$, ▲ - ▲ specific acitvity of cells, ○ — ○ activity of cells in medium, · — · activity of medium, × — × activity of cells.

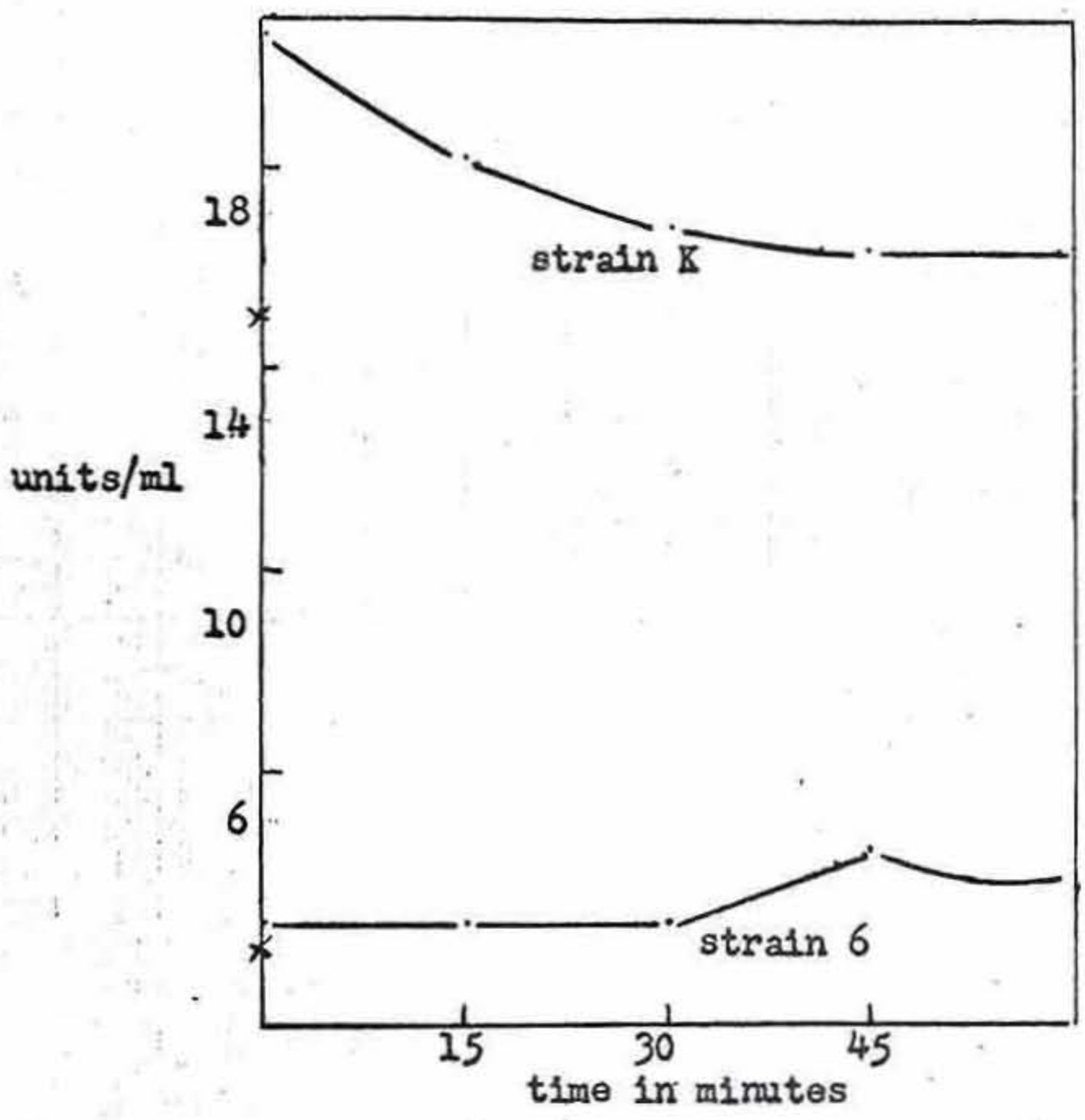

Figure 9. The effect of incubation with toluene on activities of strain 6 and K cells. Cells were treated with toluene and assayed as in Methods. x = activity before treatment.

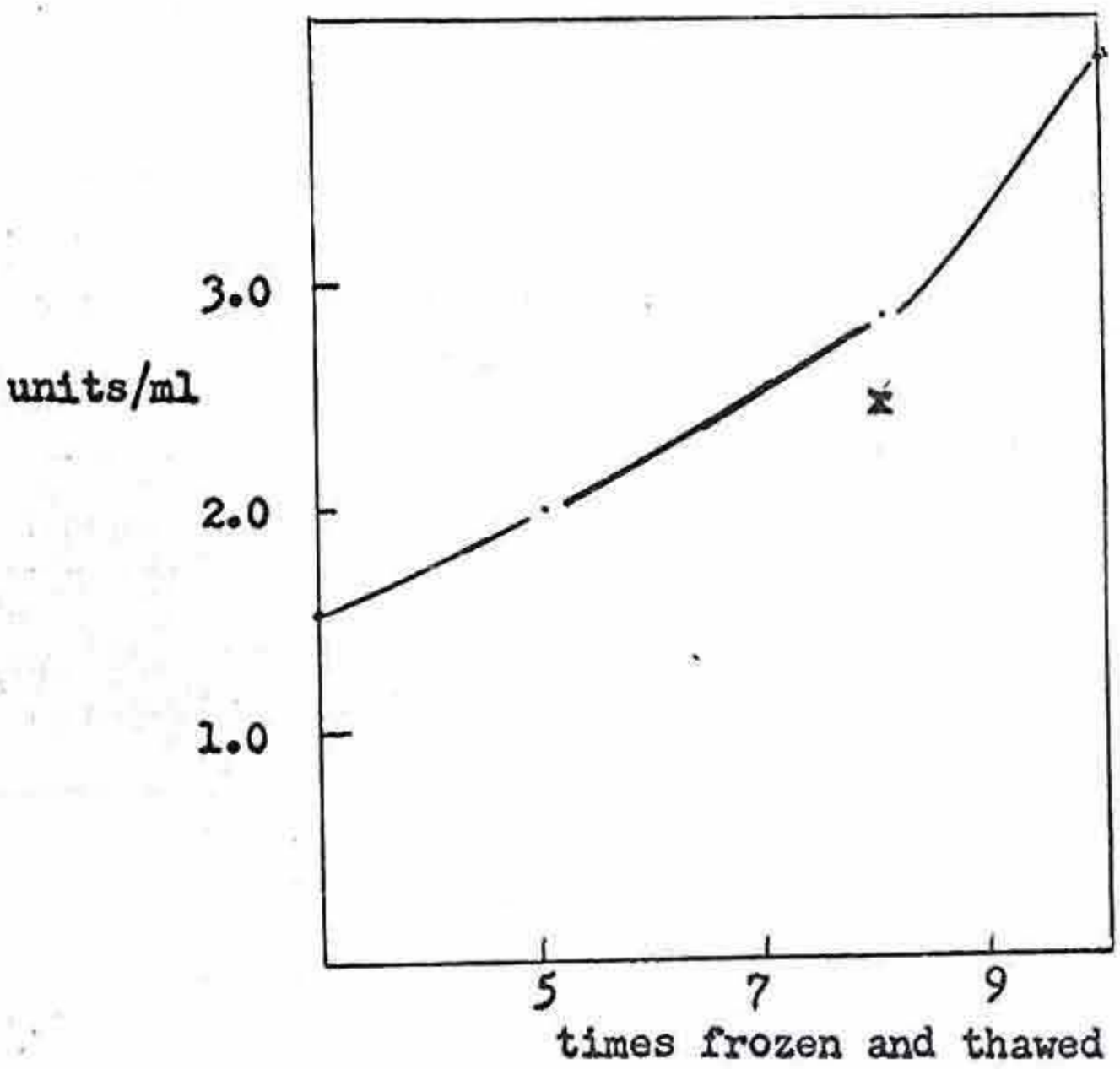

Figure 10. The effect of freezing and thawing of cells on their activity. Cells were frozen, thawed and assayed as described in Methods. After the eighth time of freezing and thawing, a portion was treated with toluene (45 mins. incubation) and its activity indicated by x.

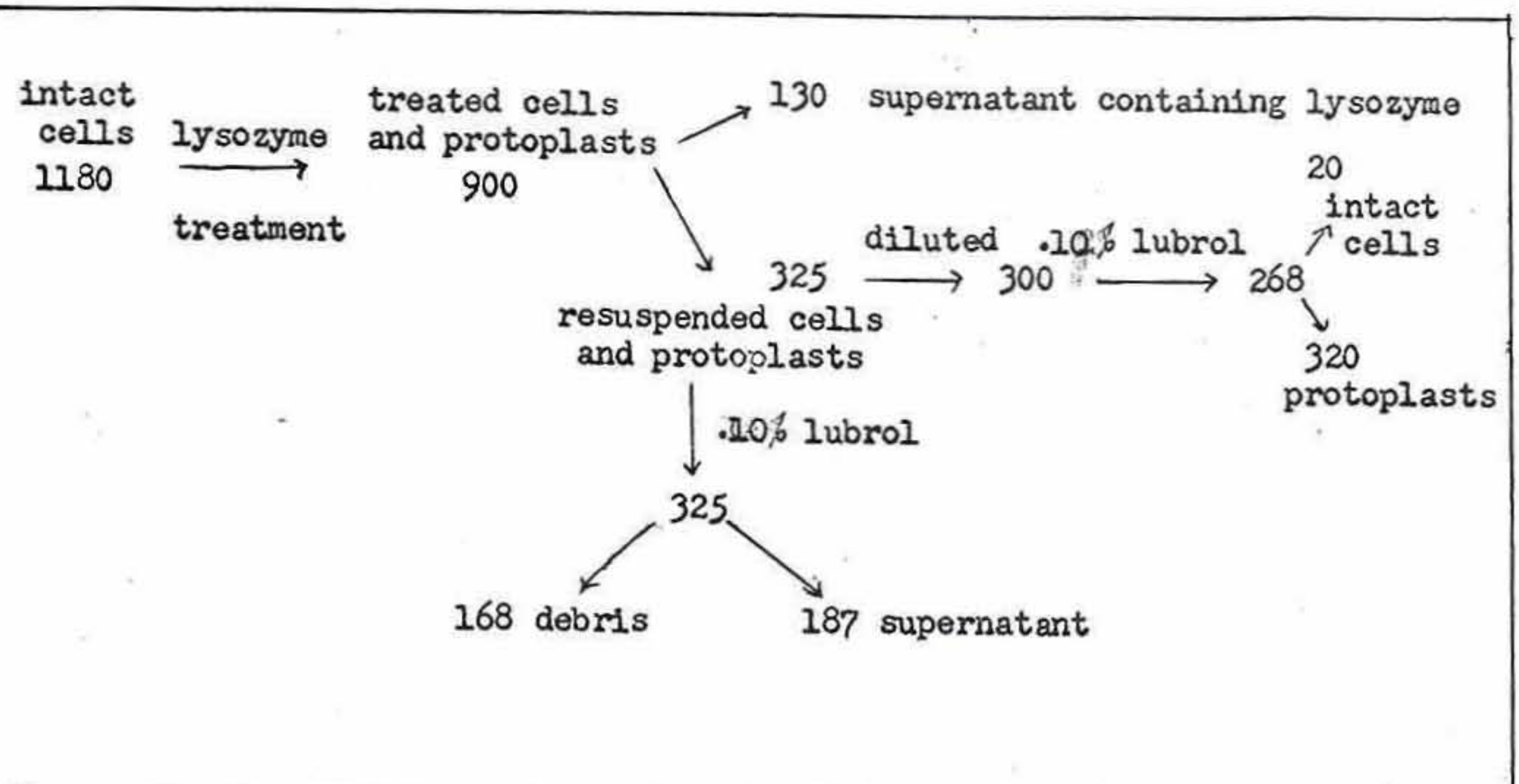

Figure 11. Localization of alkaline phosphatase. Activity given in units/ml. Conditions as described in Methods and Materials.

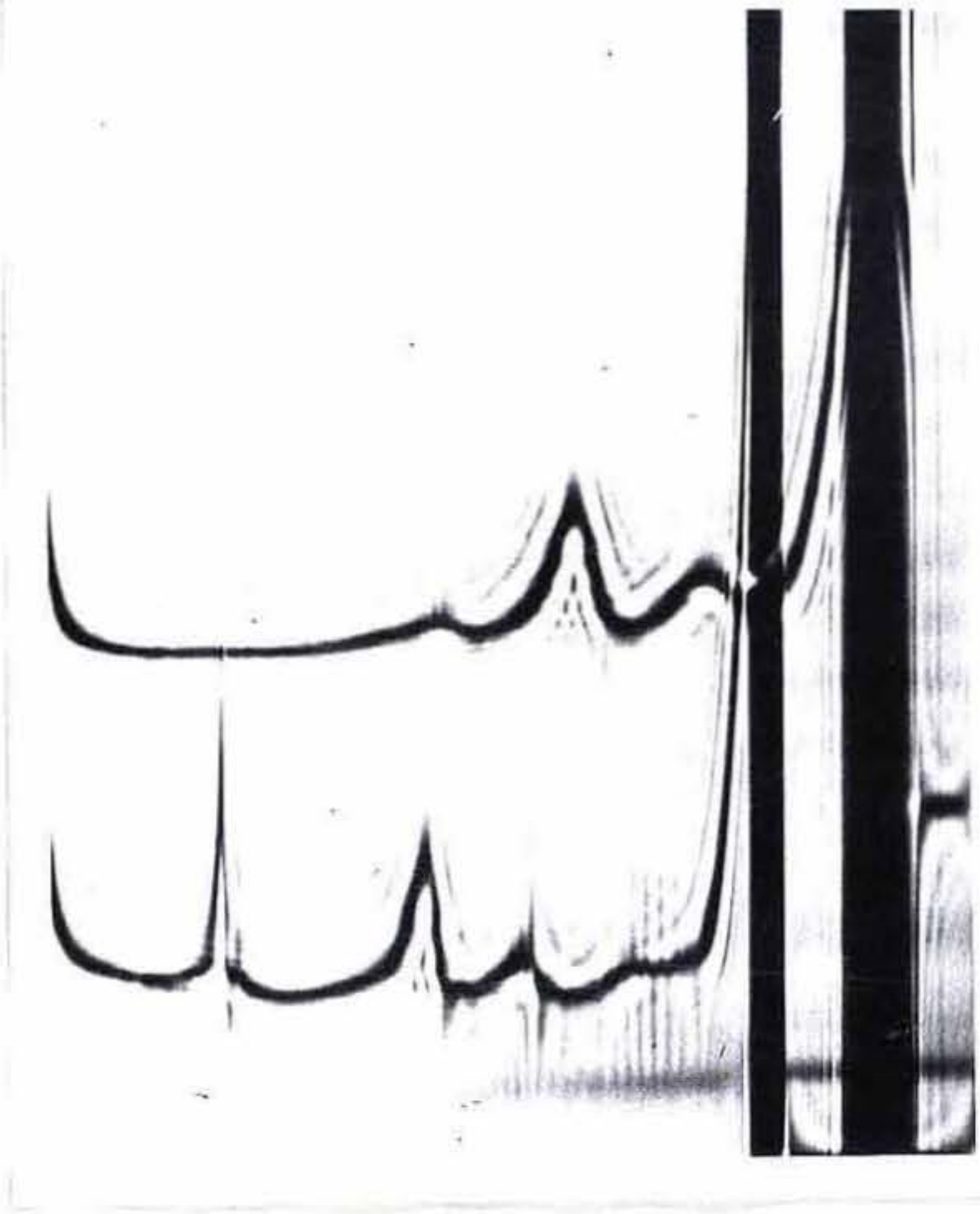

Figure 12. Schlieren patterns obtained with extracts at 10^{-4} (top) and 10^{-2} (bottom) M Mg^{++}. Extracts were prepared in TMK containing 0.5 mg/ml bentonite. Observed sedimentation coefficients (top, left to right): 54S, 41S, 27S; Bottom: 79S, 54S, 41S, 25S.

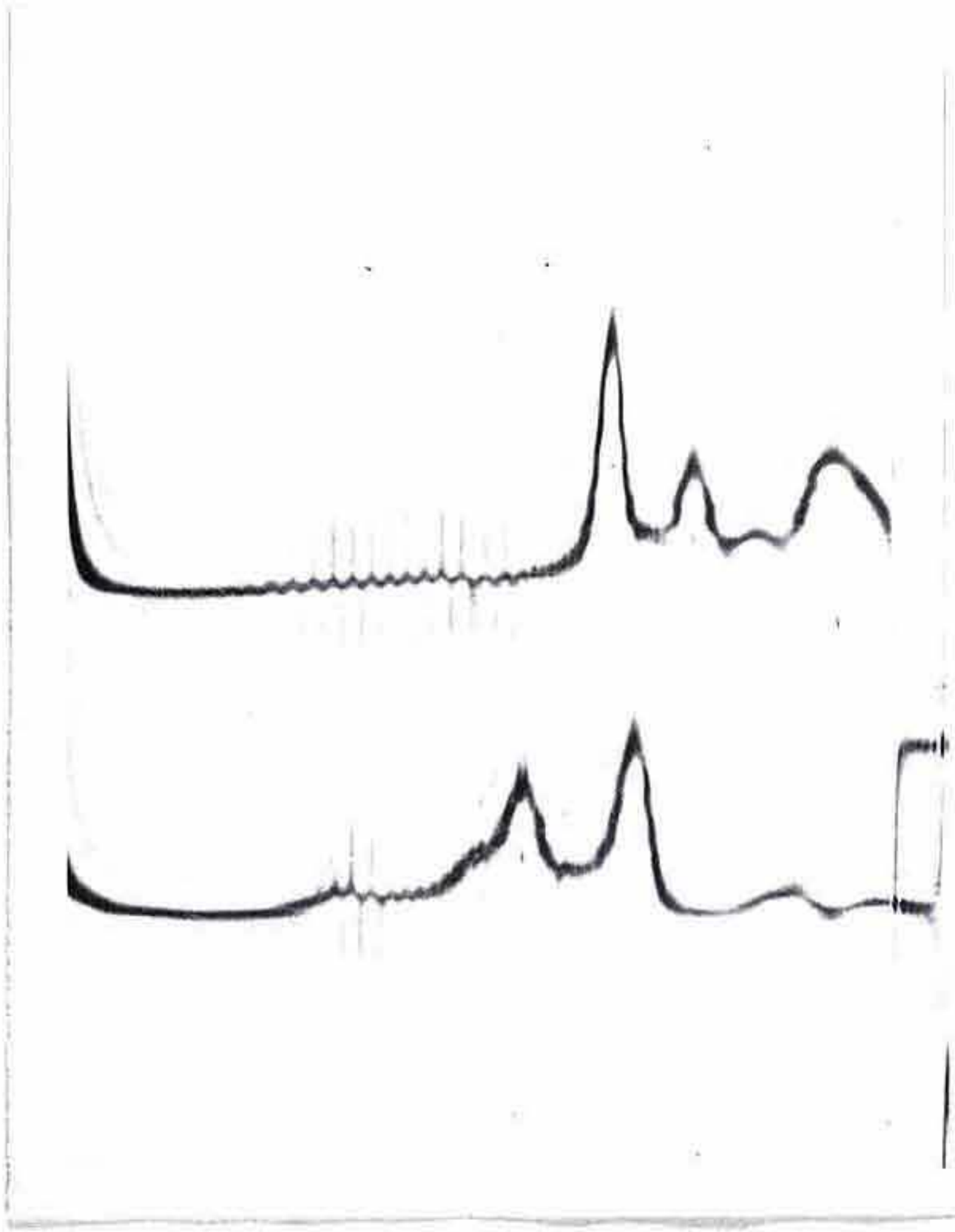

Figure 13. Schlieren patterns obtained with a P_{100-2} preparation at 10^{-2} and 10^{-4}M Mg^{++}. The P_{100-2} fraction had been prepared from an extract made with 10^{-4}M Mg^{++} TMK. After washing, the preparation was diyided in half, with one part dialyzed against 500 volumes TMK at 10^{-4} (top) and the other part against 500 volumes TMK at 10^{-2}M Mg^{++}. Observed sedimentation coefficients (Top, left to right): 48S, 30S, 22S; (Bottom): 82S, 65S, 48S, 22S.

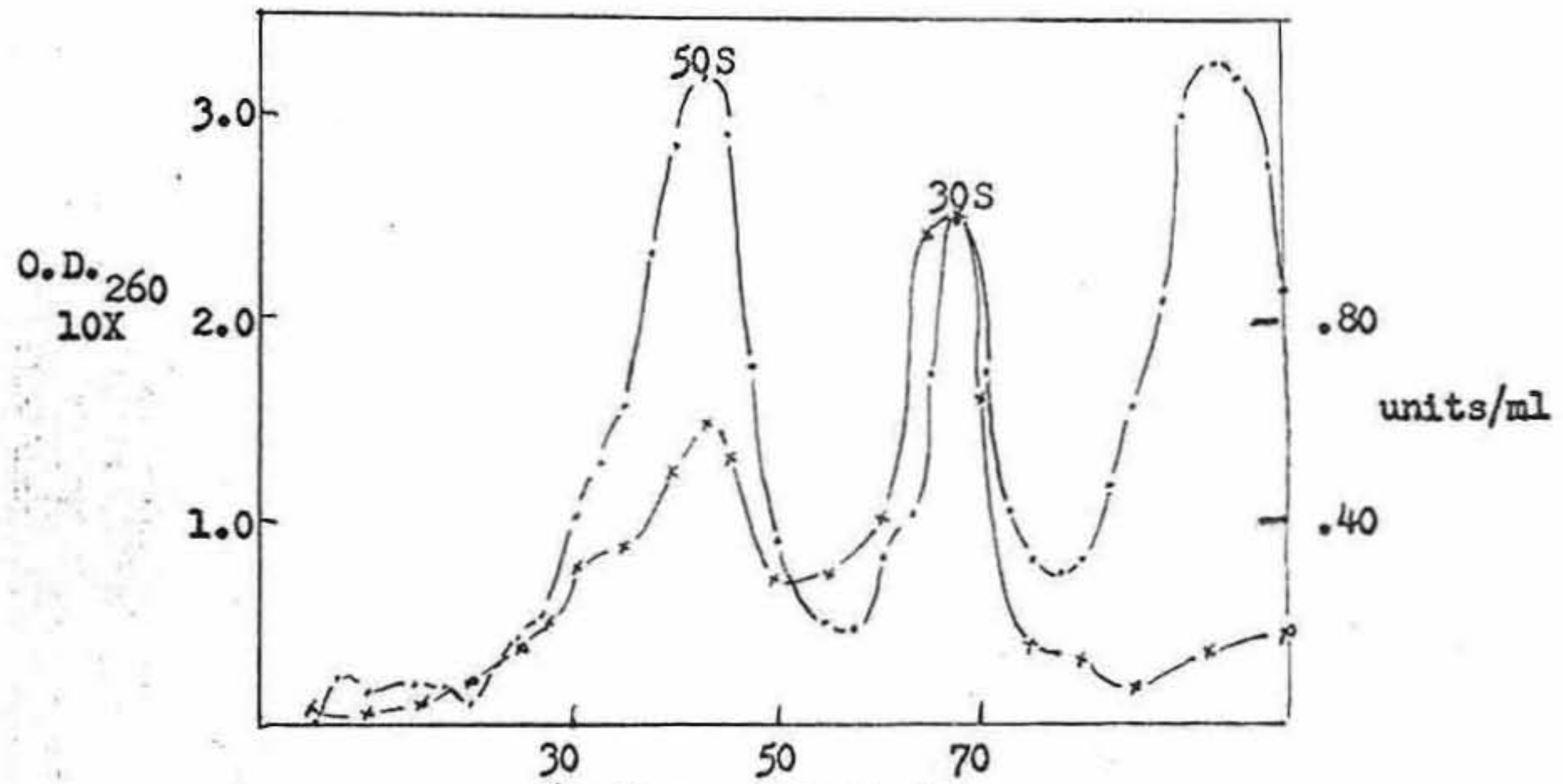

Figure 14. Ribosomal pattern and enzyme distribution at 10^{-4}M Mg^{++} in a sucrose gradient. A crude extract was prepared in TSM buffer (10^{-4}M Mg^{++}), dialyzed against 200 volumes of that buffer for 3 hours, and subjected to sucrose gradient analysis as described under Methods.
×—× enzyme ·—· $O.D._{260}^{10X}$ mu

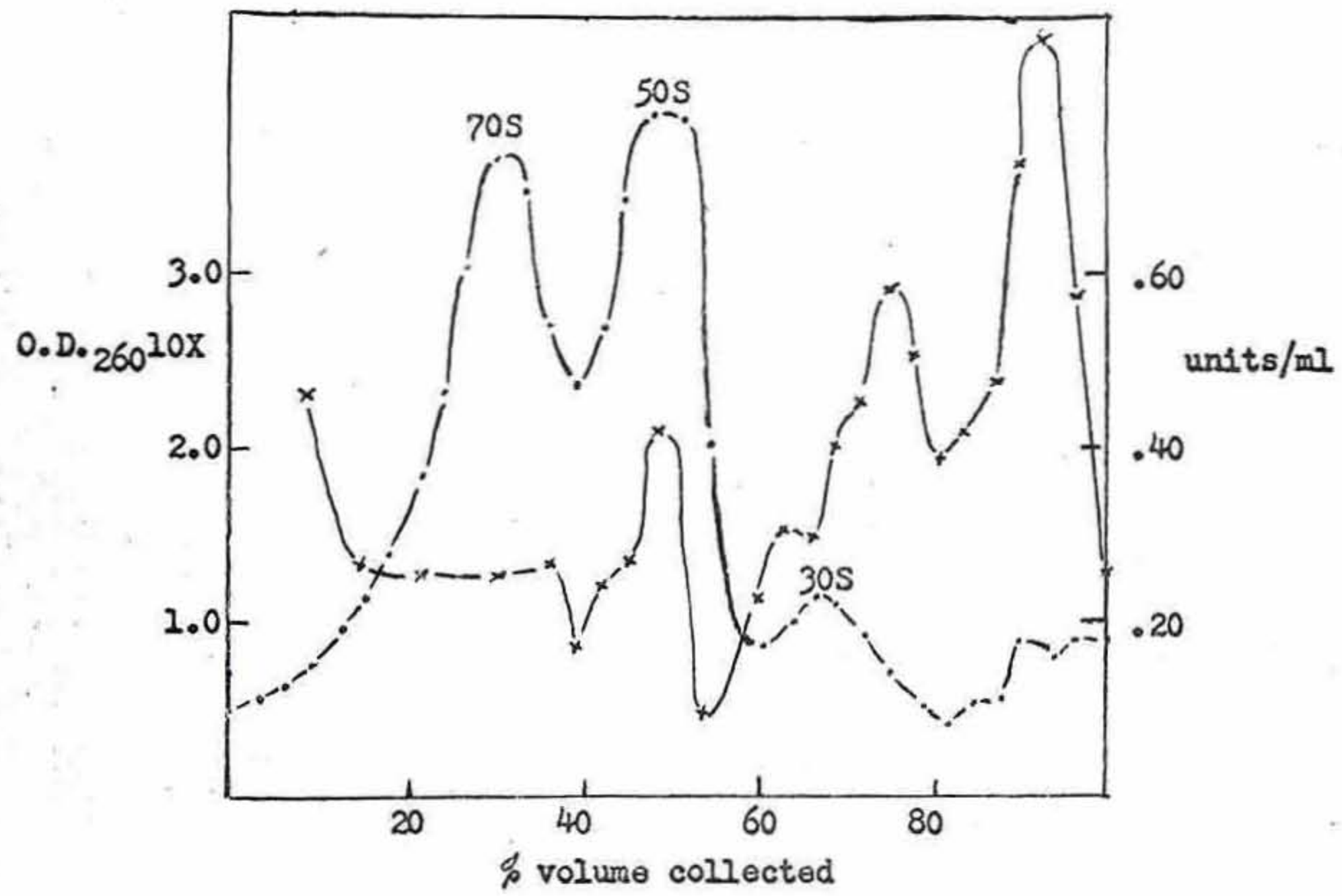

Figure 15. Ribosomal pattern and enzyme distribution at 10^{-2}M Mg^{++} in a sucrose gradient. A P_{100} fraction was prepared in TMK buffer containing 10^{-2}M Mg^{++} as described in Methods except it was not dialyzed. The time of centrifugation in the gradient was 90 minutes. ·—· O.D.$_{260}$ 10X mu ×—× enzyme

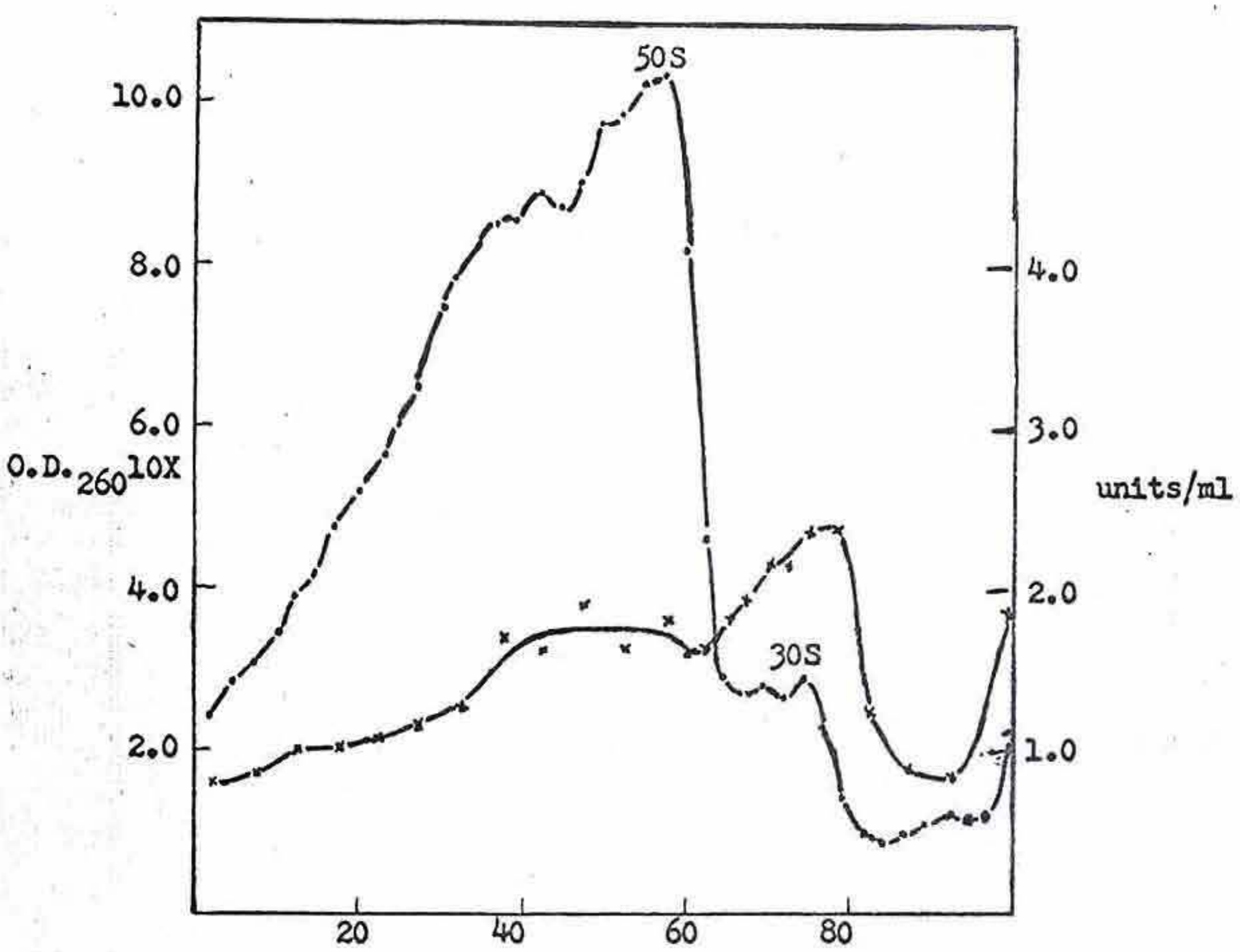

Figure 16. The effect of preparation at 10^{-2}M Mg^{++} on enzymatic activity of ribosomes in a sucrose gradient. A P_{100-2} preparation was dialyzed against 200 volumes TMK buffer containing 10^{-2}M Mg^{++} and 0.1 ml layered on a sucrose gradient and centrifuged for 90 minutes. ·—· $O.D._{260}$, ×—× enzyme

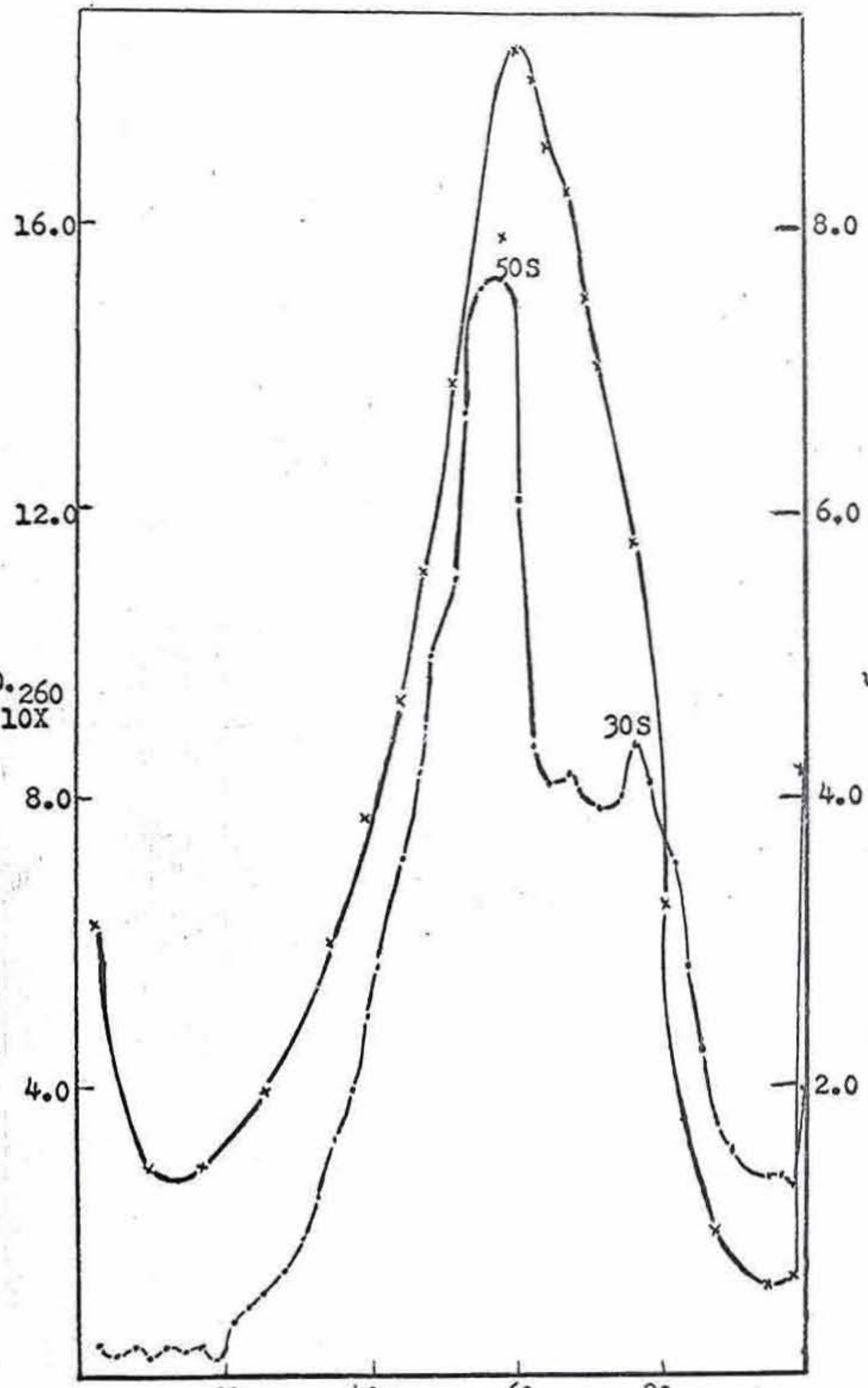

Figure 17. The effect of dialysis from 10^{-2}M Mg^{++} to 2.5×10^{-4}M Mg^{++} on ribosomal bound enzyme activity in a sucrose gradient. The ribosomal preparation from Figure 15 was dialyzed against 300 volumes of 2.5×10^{-4}M Mg^{++} TMK for 4 hours and 0.1 ml layered on a sucrose gradient and centrifuged for 90 minutes. •—• $O.D._{260}$ 10X ×—× enzyme

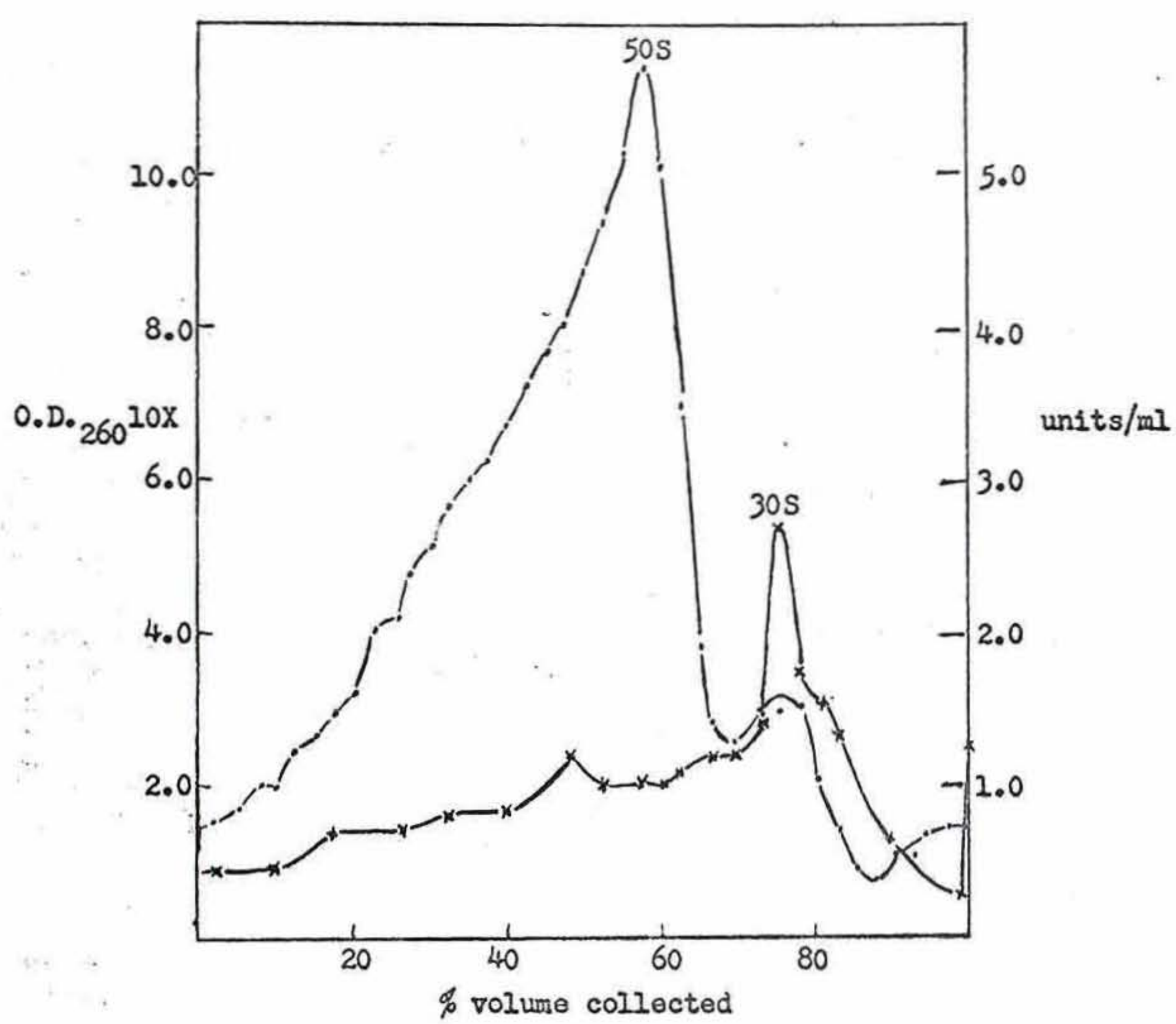

Figure 18. The effect of dialysis from 2.5×10^{-4}M Mg^{++} to 10^{-2}M Mg^{++} on ribosomal bound enzyme activity in a sucrose gradient. The ribosomal preparation from figure 20 was dialyzed against 750 volumes TMK buffer (10^{-2}M Mg^{++}) for 4 hours and 0.1 ml centrifuged in a sucrose gradient for 90 minutes. ×—× enzyme ·—· $O.D._{260}$ 10X

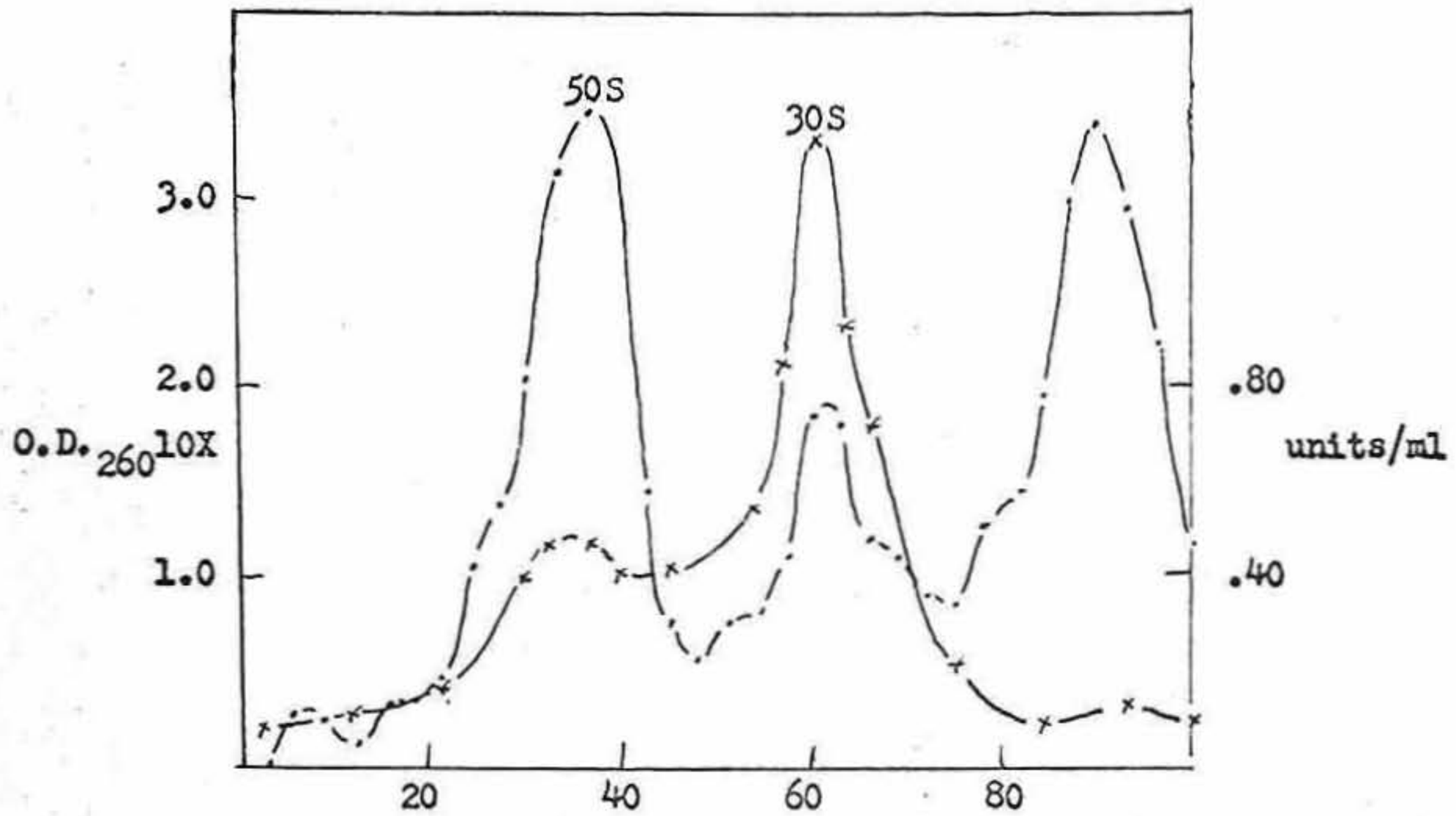

Figure 19. Sucrose gradient analysis of an extract dialyzed at 10^{-4}M Mg^{++} in the presence of PVS. The extract to which 100γ/ml PVS had been added was dialyzed against 200 volumes TSM buffer containing 100γ/ml PVS for 3 hours. ·—· $O.D._{260}$ 10x x—x enzyme

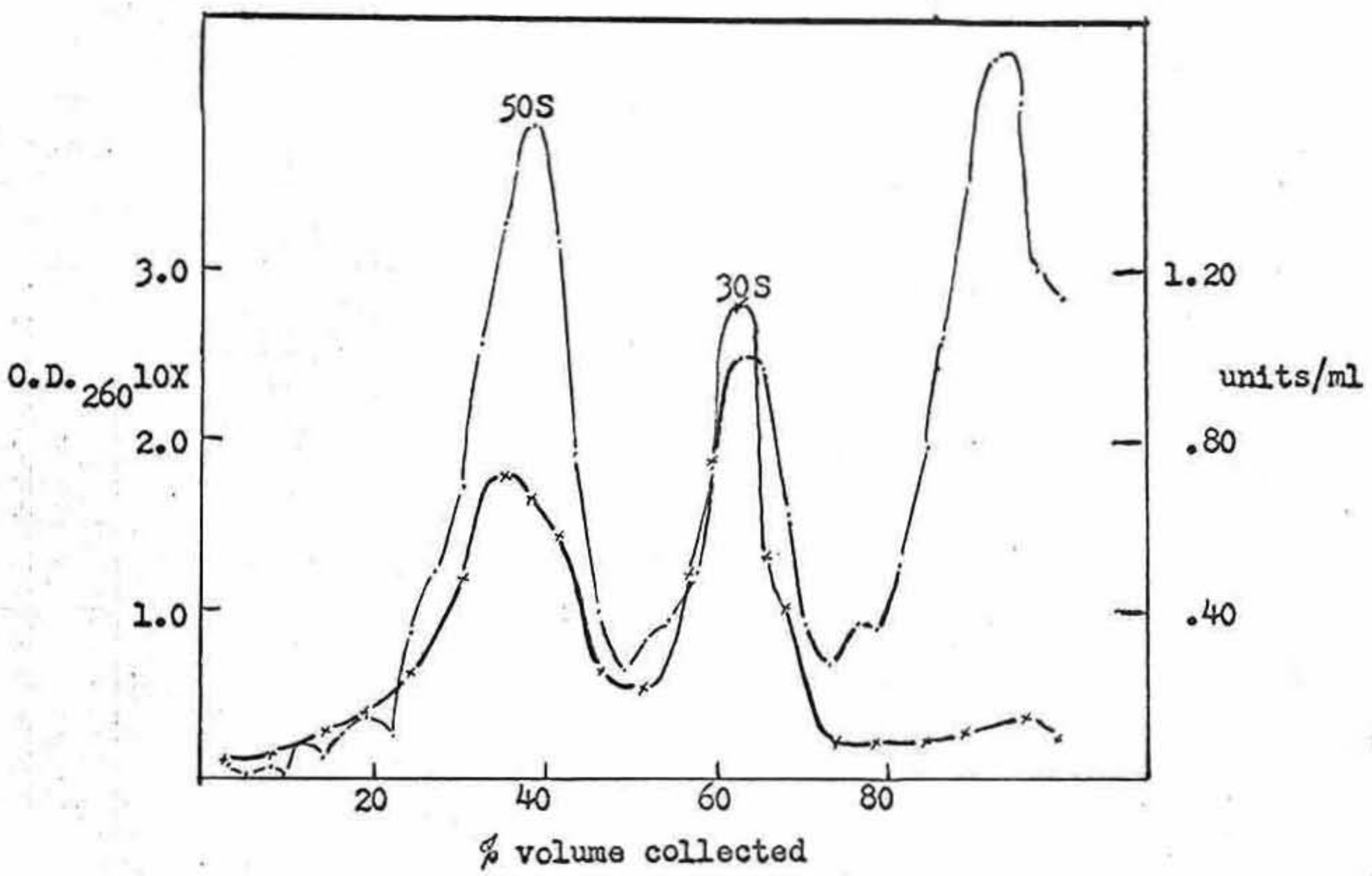

Figure 20. Sucrose gradient analysis of a crude extract prepared in 10^{-4}M Mg^{++} TSM buffer. Conditions as described under Methods.
x—x enzyme ·—· O.D.$_{260}$ 10X

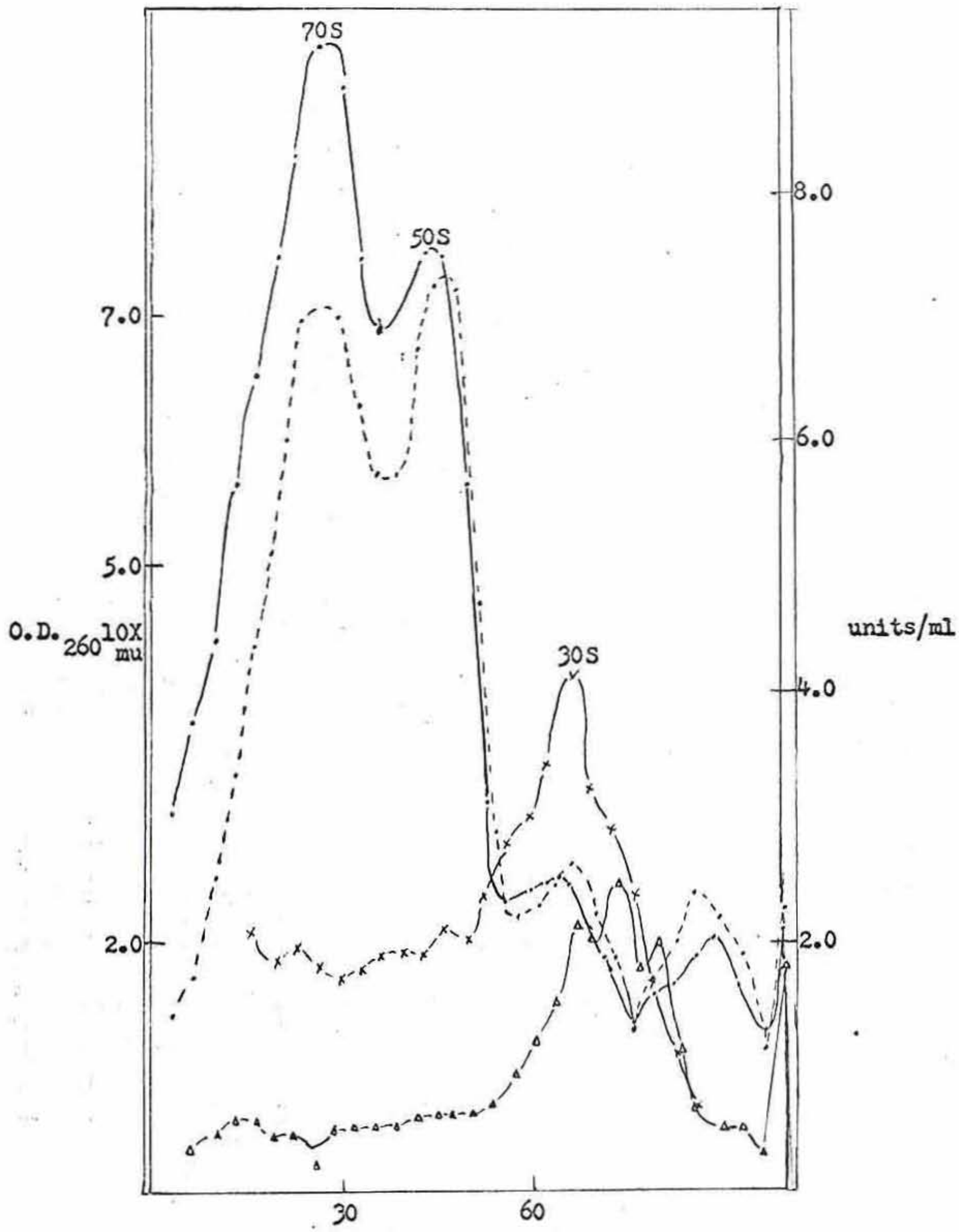

Figure 21. Sucrose gradient analysis of Sephadex fractions. The fractions were prepared as described in Methods. ·—· $O.D._{260}^{10X}$ mu Sephadex A; ×–× enzymatic activity, Sephadex A; ---- $O.D._{260}$mu 10X, Sephadex B; ▲–▲ enzymatic activity, Sephadex B.

SUMMARY

The properties of alkaline phosphatase from two strains of B. subtilis, K and 6, were compared with those reported for the E. coli enzyme. Differences in the conditions necessary for optimum activity (Tris buffer concentration and pH), in the effects of inhibitors such as Mg^{++} and Na^{+}, and in the Michaelis constants were observed between the three enzymes.

The localization of alkaline phosphatase was studied in B. subtilis strain 6. During logarithmic growth phase, most of the enzyme was excreted into the medium while during stationary phase most of the activity was retained inside the cells. Eighty per cent of the enzyme within the cell was particulate with 44% ribosomal bound and 36% membrane bound. Most of the ribosomal bound enzyme was associated with the 30s ribosome. Preliminary evidence suggested that the enzyme was nonspecifically bound to the ribosomes. Activation of the particle bound enzyme was observed when it was incubated with a soluble fraction or when it was dialyzed from 10^{-2} M Mg^{++} to 10^{-4} M Mg^{++} and centrifuged in a sucrose gradient. Possible mechanisms for this activation were discussed.