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# Resolution and Purification of Three Periplasmic Phosphatases of *Salmonella typhimurium*

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A survey of *Salmonella typhimurium* enzymes possessing phosphatase or phosphodiesterase activity was made using several different growth conditions. These studies revealed the presence of three major enzymes, all of which were subsequently purified: a cyclic 2',3'-nucleotide phosphodiesterase (EC 3.1.4.d), an acid hexose phosphatase (EC 3.1.3.2), and a nonspecific acid phosphatase (EC 3.1.3.2). A fourth enzyme hydrolyzed bis-(*p*-nitrophenyl)phosphate but none of the other substrates tested. No evidence was found for the existence of an alkaline phosphatase (EC 3.1.3.1) or a specific 5'-nucleotidase (EC 3.1.3.5) in *S. typhimurium* LT2. All three phosphatases could be measured efficiently in intact cells, which suggested a periplasmic location; however, they were not readily released by osmotic shock procedures. The three major phosphatases were purified by column chromatography. The nonspecific acid phosphatase, which was purified to apparent homogeneity, yielded a single polypeptide band on both sodium dodecyl sulfate and acidic urea gel electrophoretic systems.

Bacterial periplasmic phosphatases are believed to be located between the inner cell membrane and the outer wall or membrane (evidence is reviewed by Heppel [19]). The location of these enzymes permits them to hydrolyze phosphate esters to inorganic phosphate and the corresponding alcohol outside the cell's main permeability barrier, the cytoplasmic membrane. It appears that bacteria are relatively impermeable to phosphate esters that are not actively transported (21, 24). Thus, the periplasmic phosphatases could act as scavenging enzymes, hydrolyzing nontransportable phosphate esters into components that could then be transported and utilized by the cell. We were interested in the regulation of these enzyme activities in *Salmonella typhimurium*, but before the regulation of the enzymes could be studied it was necessary to examine the properties of the phosphatase enzymes in *Salmonella*.

Studies of the phosphatases of *Escherichia coli* had established that several phosphatase enzymes were present (32, 39). Among the first of these enzymes to be studied was alkaline phosphatase (EC 3.1.3.1), originally purified and characterized by Garen and Levinthal (15). The enzyme hydrolyzes a wide variety of phosphate esters as well as pyrophosphate bonds (14, 20). Anraku purified a cyclic 2',3'-nucleotide phosphodiesterase that also hydrolyzed 3'-nucleotides (7, 8). A 5'-nucleotidase activity was discovered by Neu and Heppel (30) and purified by Neu (27). In addition to hydrolyzing 5'-nucleotides, this enzyme also acts on nucleotide diphosphosugar compounds (16). Dvorak et al. described and purified an acid hexose phosphatase (12). They also reported partial purification of a nonspecific acid phosphatase with activity towards a wide variety of phosphate esters.

We have examined the phosphatases of *S. typhimurium* with regard to the types of phosphatase or phosphodiesterase activity present in this organism, as well as the cellular location of the enzymes. After resolving the enzyme activities that were present, the activities were purified to establish that each type of enzyme activity was due to a single protein species. The latter was especially important in the case of the nonspecific acid phosphatase activity, which had such a catholic substrate specificity that it was possible that more than one enzymatic species could have been responsible for the observed activity. This enzyme activity has not been previously purified to homogeneity from an enteric bacterial source.

## MATERIALS AND METHODS

**Bacterial strains.** *S. typhimurium* LT2 was used as the wild-type strain. TA1014 contains the *dhuA1* mutation and produces elevated levels of the J histidine-binding protein (6). TA2367 is a mutant derived from LT2 which produces elevated levels of nonspe-

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cific acid phosphatase (L.D. Kier, R. Weppelman, N. Schonbeck, and B.N. Ames, manuscript in preparation).

**Growth media and culture conditions.** For studies of enzyme levels of cells grown on poor carbon and nitrogen sources, the nitrogen and carbon-free salts medium ($N^-C^-$) used by Gutnick et al. was used (17). This medium was supplemented with 0.4% (wt/vol) carbon source and 10 mM nitrogen source. Nitrogen-, carbon-, and phosphate-free medium ($N^-C^-P^-$) contains 0.2 M triethanolamine-HCl, pH 7.0, and the following amounts of salts per liter: KCl, 5 g; $Na_2SO_4$, 0.43 g; $MgCl_2 \cdot 6H_2O$, 0.2 g; and $CaCl_2$, 0.11 g. Carbon sources were added to this medium to make a concentration of 0.4% (wt/vol), nitrogen sources to make a concentration of 10 mM, and phosphate sources to make a concentration of 1 mM. Minimal glucose medium is minimal medium E of Vogel and Bonner (38) supplemented with 0.4% glucose (wt/vol).

Maximal yields of the phosphatase and phosphodiesterase enzymes for purification were obtained by growing TA2367 under glucose-limited conditions. TA2367, grown to midexponential phase on minimal glucose medium, was inoculated into two 10-liter batches of $N^-C^-$ salts medium containing 10 mM $NH_4Cl$. A sterile glucose solution was added in exponentially increasing amounts to the cultures, using a programmed gradient pump (model 190, Instrument Specialties Co., Lincoln, Neb.), which was modified to drive a syringe pump rather than the original diaphragm pump. The rate of glucose delivery to the culture doubled every 12 h. The bacteria, therefore, were growing exponentially with a doubling time of 12 h. All cultures were grown at 37°C with vigorous aeration.

**Materials.** Phosphate esters and diesters used as substrates were purchased from Sigma Chemical Co. (St. Louis, Mo.) as the sodium salts. Sources of materials used in polyacrylamide gel electrophoresis are described in reference 5. Urea (ultrapure) was purchased from Schwarz/Mann (Orangeburg, N.Y.). All other chemicals used were reagent grade or the highest purity obtainable from commercial sources. Materials for column chromatography were obtained from the following sources: Whatman P11 cellulose phosphate, Reeve-Angel (Clifton, N.J.); diethylaminoethyl (DEAE)-cellulose, standard grade, Schleicher and Schuell (Keene, N.H.); hydroxylapatite (Bio-Gel HTP), Bio-Rad (Richmond, Calif.); and Sephadex column materials, Pharmacia (Piscataway, N.J.).

**Buffers used in purification.** Tris(hydroxymethyl)aminomethane (Tris)-salts buffer (buffer A) contains 10 mM Tris-hydrochloride, pH 7.4, 1 mM $MgCl_2$, 1 mM $MnCl_2$, and 0.1 mM $CoCl_2$. Additions to this buffer, such as bovine serum albumin (1 mg/ml) or dithiothreitol (0.1 mM), had no effect on the stability or activity of any of the three enzymes studies. Buffer B contains 10 mM Tris-hydrochloride, pH 7.4, and no additional salts. Buffer C is 10 mM sodium phosphate, pH 7.4.

**Cell disruption.** Cells were disrupted by discontinuous sonication with a Branson model W165D sonicator operated at 75 W. Cell suspensions were placed in a Rosette cell (Branson Instruments), which was immersed in a salt-ice-water bath (−4°C). Sonication periods of 20 s were alternated with 40-s cooling periods until the optical absorbance at 650 nm was 10% of the absorbance of the intact cells. Toluene treatment of cells was performed as described (4).

**Preparation of extracts and Sephadex chromatography.** To determine phosphatases present under different growth conditions, cell extracts were prepared from *S. typhimurium* grown to midexponential phase in 500 ml of media. Cells were harvested by centrifugation and resuspended in buffer A to a protein concentration of about 10 mg/ml. Cell extract was prepared by sonication, centrifugation at 6,000 × *g*, and addition of solid KCl to make a 0.5 M solution. Five milliliters of this solution was applied to a Sephadex G-200 column (2 by 100 cm) equilibrated and eluted with buffer A. Samples from all of the 4-ml fractions collected were assayed for activity towards adenosine 5′-monophosphate (5′-AMP) and β-glycerophosphate (pH 5.5), glucose 6-phosphate (pH 6.0), cyclic 2′,3′-uridine monophosphate (pH 7.5), bis-(*p*-nitrophenyl)phosphate (bis-PNPP), and PNPP (pH 5.5 and 7.5).

**Enzyme assays.** Enzyme assays, except as indicated, measured the release of inorganic phosphate from phosphate ester substrates. All assays were performed in a volume of 0.5 ml. The standard nonspecific acid phosphatase assay mix contained 0.1 M sodium acetate, pH 5.5, and 5 mM 5′-AMP as substrate. The acid hexose phosphatase assay mix contained 0.1 M sodium acetate, pH 6.0, and 5 mM glucose 6-phosphate as substrate. The cyclic phosphodiesterase assay mix contained 0.1 M Tris-hydrochloride, pH 7.5, 5 mM cyclic 2′,3′-AMP, enzyme, and excess (usually 1 U) *E. coli* alkaline phosphatase (Sigma). All assays were initiated by addition of substrate and conducted at 37°C. At various times after substrate addition, 0.1-ml samples were removed and inorganic phosphate in these samples was determined (3). Absorbance of the reduced phosphomolybdate complex was measured at 820 nm in a spectrophotometer using cuvettes of 1-cm path length. The molar absorptivity of the phosphomolybdate complex at 820 nm under the conditions of the assay for inorganic phosphate is 25,400 $M^{-1}$ $cm^{-1}$. One unit of enzyme activity for each enzyme is defined as that amount of enzyme that releases 1 nmol of inorganic phosphate per min from the substrates used in the standard assays under the assay conditions.

Whole cells were prepared for assay by centrifuging the cells twice at 1,000 × *g* and resuspending them each time in 0.9% saline to remove inorganic phosphate present in the growth medium. The cell suspension was added in 0.1-ml portions to 0.4 ml of the standard assay buffer for each enzyme. The washing procedure did not release detectable amounts of the enzymes into the supernatant solution. Enzyme activities of either purified enzymes or whole cells were independent of the dilution of assay buffer and of the level of addition of NaCl.

Enzyme activities were detected in the presence of inorganic phosphate by measuring the hydrolysis of PNPP for nonspecific acid phosphatase and acid

hexose phosphatase activity or bis-PNPP for cyclic phosphodiesterase activity. Substrate concentrations of 5 mM in the assay mix were used. The procedures were the same as for the standard assays, except the 0.1-ml samples of assay mix were removed after various times and added to 0.5 ml of 0.2 N NaOH. The *p*-nitrophenylate ion concentration was determined by measuring optical absorbance at 410 nm.

**Concentration of protein samples.** Solutions to be concentrated were placed in dialysis tubing (Union Carbide) and packed in polyethylene glycol flakes (Aquacide III, Calbiochem). A 10-fold reduction in volume was obtained in 2 or 3 h, with a routine recovery of 95 to 100% of enzyme activity. It was found that some polyethylene glycol entered the dialysis bag during the concentration procedure. This could be removed either by a subsequent purification step or by dialysis.

**Polyacrylamide gel electrophoresis.** Slab gel electrophoreses were performed using the same apparatus, gels, and techniques as described by G.F. Ames (5) for sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. The components used to prepare basic gels were those described by Davis (11). Basic urea gels used the same components as the basic gels, with urea being added to make an 8 M solution in the resolving gel and a 5 M solution in the stacking gel. Solutions were warmed briefly at 45°C to dissolve the urea. Acidic urea gels were prepared using the same components described by Takayama et al. (35). Polymerization of the acidic urea gels was conducted at 37°C rather than at room temperature. Basic native gel electrophoresis was conducted by applying a current of 10 mA for 45 min (until the tracking dye entered the resolving gel), followed by a current of 25 mA for 3 h.

Samples were prepared for SDS-polyacrylamide gel electrophoresis by boiling in the sample buffer of Laemmli (22) for 2 min. Samples were prepared for electrophoresis in the basic gels by mixing equal volumes of sample and double-strength Laemmli sample buffer made without SDS or 2-mercaptoethanol. Samples for basic urea electrophoresis were prepared by mixing equal volumes of sample and double-strength Laemmli sample buffer made without SDS or 2-mercaptoethanol and containing 5 M urea. Solid urea was added to make a concentration of 2 M to samples that were subjected to electrophoresis on the acidic urea system.

After the positions of the major protein and activity bands were determined on basic gels, 1-mm sections in the same mobility region were cut from unstained gel sections, and 20 μl of Laemmli sample buffer was added to the sections. After crushing the gel pieces in the sample buffer and boiling the mixture for 2 min, the samples were applied to SDS-polyacrylamide gels, which were run as usual.

**Visualization of protein and enzyme activities of gels.** Gels of all systems used were stained for protein by the method described by Fairbanks et al. (13). Enzyme activity was detected on gels by a post-incubation capture method described by Allen and Hyncik (2). Gel pieces were soaked for 10 min (30 min for gels containing urea) in the standard assay buffers for the nonspecific acid phosphatase and acid hexose phosphatase, or in 0.1 M Tris-acetate, pH 7.5, for the cyclic phosphodiesterase. Gels were then rinsed with distilled water, and a solution of the same buffer containing 5 mM substrate was added. After incubation at 25°C for 10 to 60 min, depending on the amount of enzyme applied, phosphate released from the substrates was detected as described by Allen and Hyncik (2).

**Protein determination.** Protein levels were determined by the method of Lowry et al. (25). Bovine serum albumin (fraction V, Sigma) was used as a protein standard.

## RESULTS

**Number and types of phosphatase enzymes in *S. typhimurium*.** For carbon limitation *S. typhimurium* LT2 was grown on $N^-C^-$ medium containing succinate as a carbon source (doubling time, 4 h) and $NH_4Cl$ as nitrogen source. For nitrogen limitation LT2 was grown on $N^-C^-$ medium containing glucose as carbon source and L-proline as nitrogen source (doubling time, 11 h). Phosphate limitation was obtained by growth on $N^-C^-P^-$ medium containing glucose, $NH_4Cl$, and β-glycerophosphate as phosphate source (doubling time, 2.9 h). A doubling time of 1.2 h was observed when *Salmonella* were grown on $N^-C^-P^-$ medium supplemented with glucose, $NH_4Cl$, and sodium phosphate. These growth conditions were chosen to permit expression of any phosphatase species that were regulated by catabolite repression, nitrogen repression or phosphate repression. Figure 1 shows a typical Sephadex G-200 chromatogram of phosphatase activity assayed with several substrates. Three major types of activity were observed. An activity that hydrolyzed cyclic 2′,3′-nucleotides and bis-PNPP (peak 2) was partially resolved from an activity that hydrolyzed glucose 6-phosphate (peak 3). These activities presumably correspond to the cyclic 2′,3′-nucleotide phosphodiesterase originally discovered in *E. coli* by Anraku (7) and the acid hexose phosphatase purified from *E. coli* by Dvorak et al. (12). The same nomenclature has been used for the *Salmonella* enzymes. A third peak of activity (peak 4) chromatographed adjacent to inorganic phosphate. This activity hydrolyzed β-glycerophosphate, glucose 6-phosphate, PNPP, and 5′-AMP (not shown) and resembled the nonspecific acid phosphatase activity of *E. coli* described by Dvorak et al. (12). A fourth peak of activity (peak 1) hydrolyzed bis-PNPP only and not the other substrates shown or 5′-AMP. This activity was extremely labile under the conditions used. After 18 h at 4°C, no activity towards bis-PNPP could be detected. The activity in these fractions was not studied further. The same

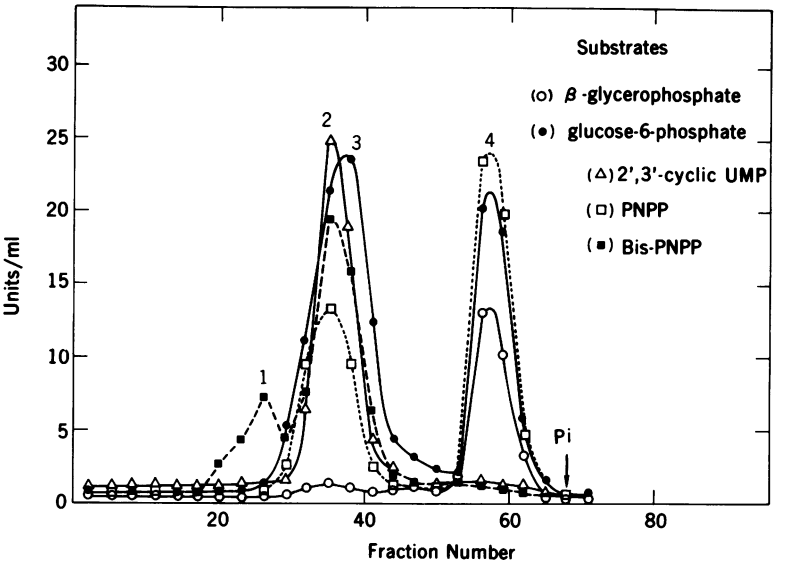


FIG. 1. *Profile of Salmonella phosphatase activity in Sephadex G-200 column fractions. Extract, prepared from S. typhimurium LT2 grown on glucose, $NH_4Cl$, and β-glycerophosphate, was chromatographed on Sephadex G-200 as described in the text. Activity towards 5'-AMP (not shown) was coincident with the activity that hydrolyzed β-glycerophosphate. The arrow denotes the position of the peak of inorganic phosphate eluted from the column.*

four types of activity were also observed when extracts were prepared from cells grown on succinate as carbon source or L-proline as nitrogen source and chromatographed on Sephadex G-200.

Two phosphatase activities observed in *E. coli* were not observed in *S. typhimurium*. Under these conditions of limited phosphate availability, no alkaline phosphatase species was observed. Schlesinger and Olsen have also reported the absence of alkaline phosphatase activity in *S. typhimurium* (33). Also, in agreement with Neu (28), we found no evidence for the presence of a specific 5'-nucleotidase in *S. typhimurium* LT2. The only activity towards 5'-AMP in the fractions from Sephadex G-200 chromatography was associated with the nonspecific acid phosphatase. Activity towards 5'-AMP measured in whole cells was not stimulated by those metal ions, $Co^{2+}$ and $Ca^{2+}$, reported to stimulate *E. coli* 5'-nucleotidase (30). No decrease in 5'-AMP hydrolyzing activity was observed when activity in sonic extracts prepared from whole cells was compared with activity in whole cells using the assay conditions of Neu for 5'-nucleotidase (27). This apparently precludes the possibility that 5'-nucleotidase activity was present but masked in extracts by the presence of a cytoplasmic inhibitor, as has been observed for *E. coli* 5'-nucleotidase (26, 29).

**Location of the *Salmonella* phosphatases.** In *E. coli* the three phosphatase activities analogous to those observed in *Salmonella* are believed to be periplasmic, i.e., located between the cytoplasmic membrane and the cell wall. Evidence for this location is the hydrolysis of nontransported substrates by intact cells and the release of the enzymes by osmotic shock or spheroplast procedures (reviewed in reference 19). We have analyzed the ability of intact cells to hydrolyze phosphate esters, which are believed not to penetrate the cytoplasmic membrane readily. Table 1 compares rates of hydrolysis of several substrates in intact cells, sonic extracts, and toluene-treated cells. Only the hydrolysis of cyclic 2',3'-nucleotides and bis-PNPP, measured by the assay coupled with alkaline phosphatase, is substantially increased by sonication. This is probably due to the inaccessibility of the periplasmic space to the alkaline phosphatase added in the assay. Similar effects on the rate of hydrolysis of cyclic 2',3'-nucleotides in a coupled assay system for measuring cyclic phosphodiesterase were observed by Brockman and Heppel (9).

Although the phosphatase enzymes of *Salmonella* behave like the corresponding *E. coli* enzymes by exhibiting activity in intact cells, they are not efficiently released by osmotic shock procedures. Table 2 demonstrates the inability of an osmotic shock procedure to quanti-

TABLE 1. *Expression of phosphatase activity in whole cells and disrupted cells*[a]

| Cell treatment | Substrate[b] | Phosphatase(s) that hydrolyzes substrate[c] | Whole-cell activity (%) |
|---|---|---|---|
| Sonication | 5'-AMP | NAP | 116 |
| | β-Glycerophosphate | NAP | 105 |
| | Glucose 6-phosphate | NAP + AHP | 91 |
| | 3'-AMP | NAP + CPDE | 110 |
| | Cyclic 2',3'-AMP | CPDE | 277 |
| | Bis-PNPP | CPDE | 186 |
| Toluene treatment | 5'-AMP | NAP | 91 |
| | β-Glycerophosphate | NAP | 94 |
| | Glucose 6-phosphate | NAP + AHP | 95 |
| | 3'-AMP | NAP + CPDE | 87 |

[a] LT2, grown to midexponential stage in minimal glucose medium, was washed twice by centrifugation and resuspended in 0.9% saline prior to cell disruption (see text).
[b] Assays were conducted at pH 5.5 (5'-AMP, β-glycerophosphate, and 3'-AMP), pH 6.0 (glucose 6-phosphate), and at pH 7.5 in the presence of 1 U of alkaline phosphatase (bis-PNPP and cyclic 2',3'-AMP).
[c] NAP, Nonspecific acid phosphatase; AHP, acid hexose phosphatase; CPDE, cyclic phosphodiesterase.

tatively release the *Salmonella* phosphatases. The effectiveness of the osmotic shock procedure was monitored by the release of the J histidine-binding protein (17, 23), which was released in normal amounts in this experiment. The amount of enzyme recovered from osmotic shock fluid and sonically treated, shocked cells is lower than that obtained from sonically treated whole cells in the case of all three phosphatases. This could be due to damage of the enzymes during the osmotic shock procedure or an increased lability of enzymes during sonication of the shocked cells as compared to whole cells.

**Preliminary steps in purification of the phosphatases.** A mutant producing high levels of nonspecific acid phosphatase, TA2367, was grown under carbon-limited conditions as described in Materials and Methods. Growth under these conditions gave increases in specific activity of the phosphatases of fivefold for the nonspecific acid phosphatase, fourfold for the acid hexose phosphatase, and threefold for the cyclic phosphodiesterase when compared with specific activities of LT2 grown on the same medium with excess glucose. When a cell concentration of $10^9$ cells/ml was reached, cells were harvested by centrifugation in a Sharples centrifuge and resuspended in buffer A to a protein concentration of 60 mg/ml. All operations, except as specified, were conducted at 0 to 4°C. Cells were washed by centrifugation at 6,000 × *g* in a Sorvall RC2-B centrifuge and resuspended in buffer A to the same protein concentration. Cells were then disrupted by discontinuous sonication as described in Materials and Methods and centrifuged at 6,000 × *g* for 20 min to remove unbroken cells and debris. The supernatant solution (low-speed supernatant) was brought to 0.5 M KCl by addition of solid KCl, incubated for 20 min at 37°C, and centrifuged at 120,000 × *g* for 20 min to further fractionate the extract.

TABLE 2. *Effects of osmotic shock on the release of the Salmonella phosphatases*[a]

| Activity measured | Total units of activity[b] | | |
|---|---|---|---|
| | Sonic extract of whole cells | Sonic extract of shocked cells | Shock fluid |
| Nonspecific acid phosphatase | 4,500 | 2,200 | 400 |
| Acid hexose phosphatase | 17,200 | 10,300 | 200 |
| Cyclic phosphodiesterase | 6,200 | 4,300 | 1,300 |
| J histidine-binding protein[c] | | 60 | 950 |

[a] TA1014 was grown to late log phase in minimal glucose medium. A portion of the cells was subjected to sonication and another portion was subjected to osmotic shock.
[b] Total units equivalent to 138 mg (dry weight) of cells.
[c] Dialysis units, described in reference 23.

Before treatment with KCl the phosphatases appear to be associated with high-molecular-weight material. When supernatant solutions from low-speed centrifugation of sonic extracts were centrifuged at high speeds to remove ribosomes and membrane fragments, part of the activity of all three phosphatases was observed in the pelleted material. The extent of the pelleted activity was 5 to 10%, 20 to 25%, and 30 to 40% of the total activity for the cyclic phosphodiesterase, acid hexose phosphatase, and nonspecific acid phosphatase, respectively. On Sephadex G-200 columns, nonspecific acid phos-

phatase remaining in the supernatant co-chromatographed with blue dextran 2000 (Pharmacia), indicating that the enzyme was associated with high-molecular-weight particles or was of a molecular weight in excess of 450,000. On phosphocellulose columns eluted with NaCl gradients, two peaks of nonspecific acid phosphatase activity were observed, one that did not bind to the column and one that eluted at 0.1 M NaCl. Both peaks had the same relative rates of hydrolysis towards 5'-AMP, glucose 6-phosphate, and β-glycerophosphate. The cyclic phosphodiesterase and acid hexose phosphatase from high-speed supernatant solutions (not treated with KCl) chromatographed as single peaks on Sephadex G-200. Addition of KCl and incubation for 20 min at 37°C prevented pelleting of all three enzymes during high-speed centrifugations and eliminated the aberrant chromatographic properties of the nonspecific phosphatase. Treatment of low-speed supernatant solutions with KCl cause no significant loss of enzyme activity of any of the three phosphatases, and there was no significant alteration of the enzymatic properties of the phosphatases when enzyme properties in whole cells were compared with purified enzymes.

After centrifugation of the KCl-treated solution, the resultant supernatant solution (high-speed supernatant) was desalted by passage through a Sephadex G-50 column (3 by 35 cm) equilibrated with buffer A. This could be accomplished without any apparent reassociation of the phosphatases or cyclic phosphodiesterase with high-molecular-weight material. Resolution of the three activities was accomplished by DEAE-cellulose chromatography (Fig. 2). Fractions (Fig. 2) were pooled for further purification.

**Purification of cyclic phosphodiesterase.** Pooled fractions from DEAE-cellulose chromatography were concentrated by polyethylene glycol treatment to a volume of 6 ml and applied to a Sephadex G-100 column (2 by 40 cm) equilibrated with buffer A. A single peak of activity eluted at 50% of the elution volume of orthophosphate. Fractions containing more than 600 U/ml were pooled (90% of total units) and dialyzed against 10 mM sodium phosphate buffer, pH 7.4. The dialyzed sample was applied to a hydroxylapatite column (1.6 by 1.5 cm) equilibrated with buffer C. After washing the column with 30 ml of buffer C, a linear gradient of 0.01 to 0.01 M sodium phosphate buffer was applied to the column. The profiles of enzyme activity and protein eluted from the hydroxylapatite column are shown in Fig. 3A. Fractions, as indicated, were pooled for study of enzyme

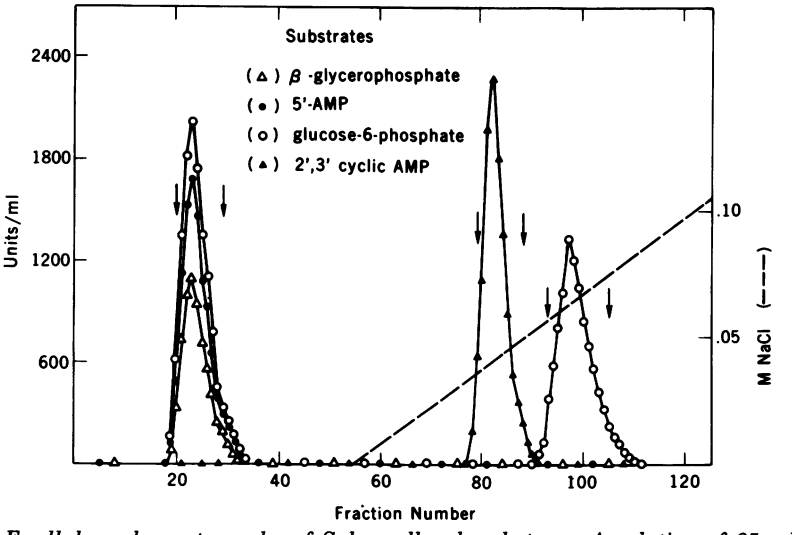


FIG. 2. *DEAE-cellulose chromatography of Salmonella phosphatases. A solution of 65 ml (26 mg of protein per ml) of cell extract prepared as described in the text was applied to a DEAE-cellulose column (4.2 by 25 cm) equilibrated with buffer A. The column was eluted with 360 ml of buffer A followed by a linear gradient of NaCl in buffer A. Each of the 10-ml fractions (collected from the beginning of sample application) was assayed with the substrates indicated at pH 5.5 (β-glycerophosphate and 5'-AMP), pH 6.0 (glucose 6-phosphate), or pH 7.5 (cyclic 2',3'-AMP). For presentation, the actual units of acid hexose phosphatase and cyclic phosphodiesterase per milliliter have been divided by 3 and 5, respectively. The arrows indicate the inclusive fractions pooled for further purification.*

properties. Purification data are summarized in Table 3. Cyclic phosphodiesterase, could be stored at −20°C in a 50% glycerol solution for several months without significant loss of activity. These storage conditions are also appropriate for the nonspecific acid phosphatase and the acid hexose phosphatase.

**Purification of acid hexose phosphatase.** Fractions from DEAE-cellulose chromatography were pooled and dialyzed against buffer B to remove NaCl. The dialyzed material was applied to a phosphocellulose column (2 by 15 cm) equilibrated with buffer B. The column was washed with 150 ml of buffer B, followed by application of a linear 0 to 0.2 M NaCl gradient. Acid hexose phosphatase activity eluted as a single peak of activity at 0.08 M NaCl. Fractions containing activity were pooled, concentrated with polyethylene glycol treatment, and chromatographed on Sephadex G-100 column (2 by 60 cm) equilibrated with buffer A. The enzyme eluted as a single peak of activity at 45% of the elution volume of orthophosphate. Fractions containing more than 400 U/ml were pooled (98% of total units) and dialyzed exhaustively against buffer C. The dialyzed sample

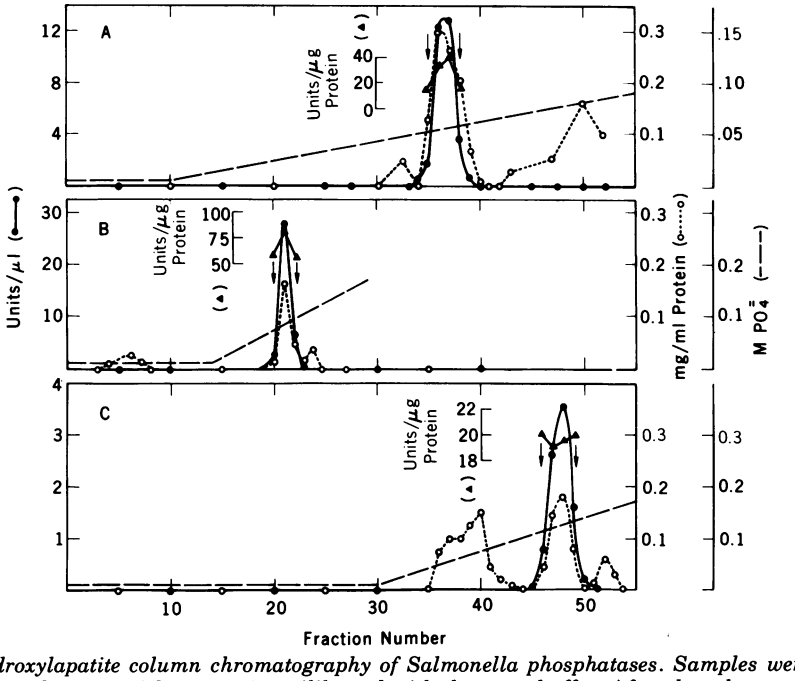


FIG. 3. *Hydroxylapatite column chromatography of Salmonella phosphatases. Samples were applied to hydroxylapatite columns (1.6 by 1.5 cm) equilibrated with the same buffer. After the columns were washed with 30 to 70 ml of buffer C, linear gradients of sodium phosphate buffer, pH 7.4, were applied. Each of the 3-ml fractions of the columns was assayed for activity against PNPP, and samples of active fractions were dialyzed against buffer A and assayed by the standard assay procedures. (A) Purification of cyclic phosphodiesterase; (B) purification of acid hexose phosphatase; (C) purification of nonspecific acid phosphatase.*

TABLE 3. *Purification of cyclic phosphodiesterase*

| Purification step | Vol (ml) | Protein (mg/ml) | Units of enzyme/mg of protein | Total units ($\times 10^3$) | Fold purification | Yield (%) |
|---|---|---|---|---|---|---|
| Whole cells | 85 | 60 | 88 | 450 | | |
| Sonic extract | 85 | 61 | 158 | 819 | 1 | 100 |
| Low-speed supernatant | 75 | 56 | 178 | 748 | 1.1 | 91 |
| High-speed supernatant | 60 | 36 | 305 | 659 | 1.9 | 80 |
| Sephadex G-50 column | 65 | 26 | 392 | 662 | 2.5 | 81 |
| DEAE-cellulose chromatography | 100 | 0.57 | 10,100 | 575 | 64 | 70 |
| Sephadex G-100 chromatography | 36 | 0.64 | 15,700 | 362 | 99 | 44 |
| Hydroxylapatite chromatography | 12 | 0.23 | 72,000 | 199 | 460 | 24 |

was then applied to a hydroxylapatite column (1.6 by 1.5 cm) and chromatographed as shown in Fig. 3B. Data for the purification of the acid hexose phosphatase are shown in Table 4.

**Nonspecific acid phosphatase purification.** The pooled nonspecific acid phosphatase fractions from the DEAE-cellulose column were applied directly to a phosphocellulose column (2 by 15 cm) equilibrated with buffer B. Chromatography was conducted by applying a linear NaCl gradient of 0 to 0.2 M NaCl. Nonspecific acid phosphatase activity eluted as a single peak at 0.1 M NaCl. Fractions containing activity were concentrated by polyethylene glycol treatment and chromatographed on a Sephadex G-100 column (2 by 60 cm) equilibrated with buffer B, containing 0.5 M KCl. The presence of KCl was necessary to eliminate binding of the nonspecific acid phosphatase to Sephadex. Fractions containing activity that eluted as a single peak at 54% of the elution volume of orthophosphate were pooled and dialyzed against buffer C. Hydroxylapatite chromatography was then conducted as shown in Fig. 3C. Data for purification of the nonspecific acid phosphatase are presented in Table 5.

**Gel electrophoresis of enzyme preparation.** SDS-polyacrylamide gel electrophoretograms of the purified, pooled enzyme fractions from hydroxylapatite columns are shown in Fig. 4. The nonspecific acid phosphatase preparation exhibited a single protein band. The cyclic phosphodiesterase preparation showed two bands and acid hexose phosphatase preparations had several bands. The same pattern of two protein bands for the cyclic phosphodiesterase was obtained when samples were boiled in Laemmli sample buffer for up to 60 min before electrophoresis. When each of these bands was cut from unstained gels, boiled for 2 min in Laemmli sample buffer, and again subjected to electrophoresis on SDS-polyacrylamide gels, each band migrated as it had on the original gel. Apparently, the two bands were due to different polypeptides rather than to aggregation of a single species of polypeptide. On acidic polyacrylamide gels containing urea, the nonspecific acid phosphatase appeared as a single protein band, but the cyclic phosphodiesterase and acid hexose phosphatase preparations appeared as multiple protein bands (not shown).

**Enzyme activity and protein detection on polyacrylamide gels.** To identify which of the polypeptides present in cyclic phosphodiesterase and hexose phosphatase preparations were responsible for activity, these preparations were subjected to basic gel electrophoresis. Gels were cut into three sections, which were either stained for protein, stained for activity, or left unstained. Figure 5 demonstrates that the acid

TABLE 4. *Purification of acid hexose phosphatase*

| Purification step | Vol (ml) | Protein (mg/ml) | Units of enzyme/mg of protein | Total units ($\times 10^3$) | Fold purification | Yield (%) |
|---|---|---|---|---|---|---|
| Whole cells | 85 | 60 | 146 | 745 | | |
| Sonic extract | 85 | 61 | 158 | 819 | 1 | 100 |
| Low-speed supernatant | 75 | 56 | 194 | 815 | 1.2 | 100 |
| High-speed supernatant | 60 | 36 | 377 | 814 | 2.4 | 99 |
| Sephadex G-50 column | 65 | 26 | 400 | 676 | 2.5 | 82 |
| DEAE-cellulose chromatography | 130 | 0.73 | 4,270 | 405 | 27 | 49 |
| Phosphocellulose chromatography | 90 | 0.105 | 19,400 | 183 | 123 | 22 |
| Sephadex G-100 chromatography | 39 | 0.169 | 23,700 | 156 | 150 | 19 |
| Hydroxylapatite chromatography | 9 | 0.320 | 37,500 | 108 | 237 | 10 |

TABLE 5. *Purification of nonspecific acid phosphatase*

| Purification step | Vol (ml) | Protein (mg/ml) | Units of enzyme/mg of protein | Total units ($\times 10^3$) | Fold purification | Yield (%) |
|---|---|---|---|---|---|---|
| Whole cells | 85 | 60 | 55 | 280 | | |
| Sonic extract | 85 | 61 | 66 | 342 | 1 | 100 |
| Low-speed supernatant | 75 | 56 | 71 | 298 | 1.1 | 87 |
| High-speed supernatant | 60 | 36 | 121 | 261 | 1.8 | 76 |
| Sephadex G-50 column | 65 | 26 | 125 | 211 | 1.9 | 62 |
| DEAE-cellulose chromatography | 100 | 0.90 | 1,120 | 101 | 17 | 30 |
| Phosphocellulose chromatography | 75 | 0.137 | 4,290 | 44.1 | 65 | 13 |
| Sephadex G-100 chromatography | 35 | 0.184 | 5,270 | 33.9 | 80 | 10 |
| Hydroxylapatite chromatography | 12 | 0.087 | 21,700 | 22.7 | 330 | 7 |

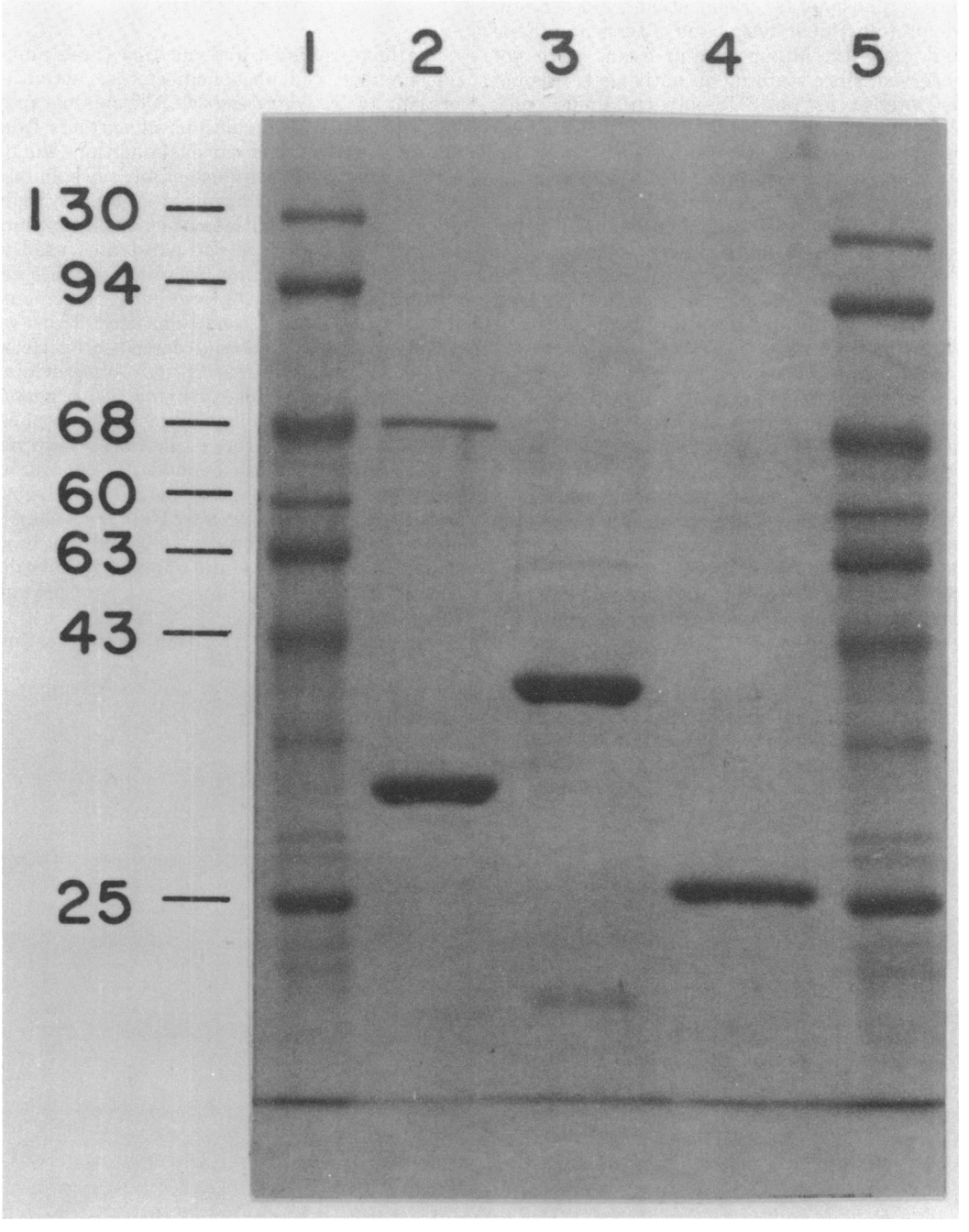


FIG. 4. *SDS-poylacrylamide gel electrophoresis of purified phosphatase enzymes and molecular weight standards. SDS-polyacrylamide gels (9% acrylamide) were run as described in reference 4. Sample 1, Molecular weight standards (5) with $10^3$ molecular weights as indicated; sample 2, purified cyclic phosphodiesterase (2.3 μg of protein applied); sample 3, purified acid hexose phosphatase (2.5 μg of protein); sample 4, purified nonspecific acid phosphatase (2.0 μg of protein); sample 5, molecular weight standards.*

hexose phosphatase preparation yielded a single protein and activity band of the same shape and mobility. Minor protein bands were not observed, since staining on native gels was not as intense as on SDS-polyacrylamide gels. When the section corresponding to the acid hexose phosphatase band was cut from the unstained gel and examined by SDS-polyacrylamide gel electrophoresis, only one band, corresponding to the major band in Fig. 4, was observed. At the staining intensity obtained for the major band on the SDS-polyacrylamide gel shown in Fig. 5, minor proteins, if present, probably would have been detected (cf. Fig. 4 with Fig. 5).

The cyclic phosphodiesterase preparation also yielded a single protein and activity band on basic native gels. When this band was subjected to SDS-gel electrophoresis, two bands resulted (Fig. 5), corresponding to the two bands shown on the SDS electrophoretogram of the purified preparation (Fig. 4). Either the cyclic phosphodiesterase consists of two nonidentical polypeptides or the enzyme preparation contains a persistent contaminant that cannot be resolved by basic polyacrylamide gel electrophoresis.

## DISCUSSION

We have resolved and purified three major phosphatase and phosphodiesterase activities present in *S. typhimurium*. Chromatography on Sephadex G-200 columns of extracts from cultures grown under various conditions should have revealed the presence of any phosphatase species except those enzymes that bind to Sephadex under the conditions used, those enzymes that do not hydrolyze the substrates used to characterize the column fractions, or those enzymes that have cofactor or other requirements not met by the assay conditions used. Thus, we did not detect the activity described by Uerkvitz et al., which primarily hydrolyzes uridine 5'-monophosphate, deoxythymidine 5'-monophosphate, and deoxyuridine 5'-monophosphate (37), because these substrates were not used to characterize the Sephadex G-200 column fractions. If a phosphatase activity analogous to that described in *E. coli* by Hafkenscheid (18) exists in *S. typhimurium*, it would not have been detected, because the enzyme activity described by Hafkenscheid is very low at pH values greater than 4.

The additional activity that hydrolyzes bis-

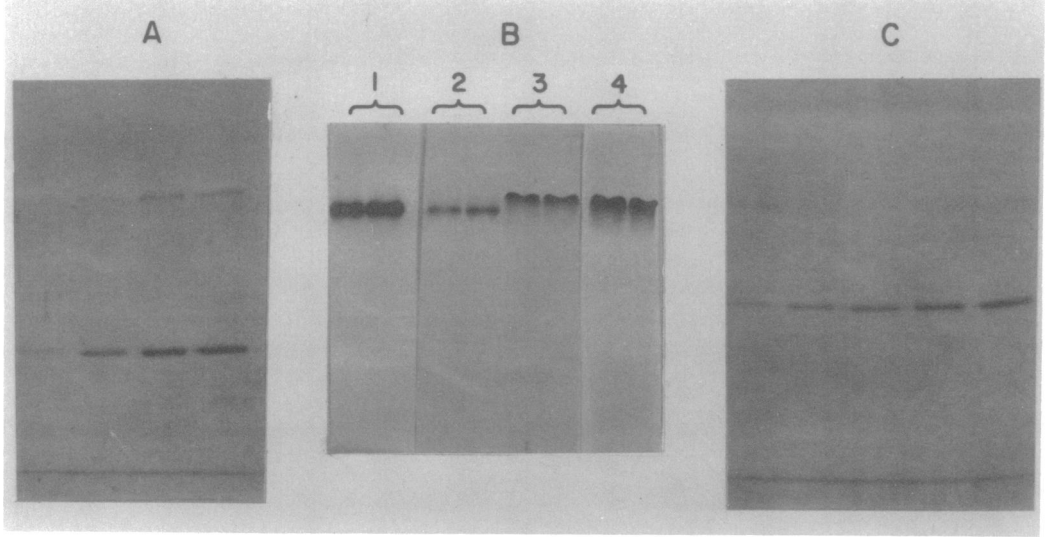


FIG. 5. *Basic native gel electrophoresis of acid hexose phosphatase and cyclic phosphodiesterase. Basic gel electrophoresis was conducted with 2.5-μg samples of purified acid hexose phosphatase and 2.3-μg samples of purified cyclic phosphodiesterase. The gel was cut into pieces and stained for protein or enzyme activity as in the text. Substrates used in the staining were 3'-AMP for cyclic phosphodiesterase and glucose 6-phosphate for acid hexose phosphatase. (B) Results of the activity and protein stain. Sample 1, cyclic phosphodiesterase samples stained for activity; sample 2, cyclic phosphodiesterase samples stained for protein; sample 3, acid hexose phosphatase samples stained for protein; sample 4, acid hexose phosphatase samples stained for activity. SDS-polyacrylamide electrophoresis of gel sections corresponding to the major protein band are presented in (A) for the cyclic phosphodiesterase and in (C) for the acid hexose phosphatase.*

PNPP and none of the other substrates tested presumably hydrolyzes some other phosphodiester substrate(s). It is possible that this activity hydrolyzes nucleotide diphosphosugar compounds. Such types of activity have been observed in extracts of *S. typhimurium* by Melo and Glaser (26). The 5'-nucleotidase of *E. coli* that hydrolyzes nucleotide diphosphosugar compounds also hydrolyzes bis-PNPP (12). Because of the lability of this enzyme under the conditions used, this activity was not characterized further.

The primary evidence for the periplasmic location of the *Salmonella* phosphatases is the ability of intact cells to efficiently hydrolyze phosphate ester and diester substrates. Similar observations on the hydrolysis of substrates by *E. coli* phosphatases were made by Brockman and Heppel (9) and Torriani (36). The *Salmonella* phosphatases, however, are not efficiently released by osmotic shock procedures. Similar observations on *Salmonella* phosphatases were made by Nossal and Heppel (31).

An unusual property of the *Salmonella* phosphatases, especially the nonspecific acid phosphatase, is their adhesion to particles of high molecular weight. Some periplasmic enzymes are bound to ribosomes when sonic extracts are prepared. Ribonuclease I (EC 2.7.7.h) has been shown to be periplasmic by virtue of its release from cells by osmotic shock procedures (1, 34); yet, when whole cell extracts are prepared, most of the enzyme is bound to ribosomes, specifically 30*S* ribosomal subunits in *E. coli* (34) and *Salmonella* (10). Spahr and Hollingsworth noted that a PNPP and glucose 6-phosphate hydrolyzing activity was present in 30*S* ribosomal subunits of *E. coli* preparations (34). If the *Salmonella* enzymes, especially the nonspecific acid phosphatase, are associated with ribosomes in cell extracts, then this association is presumably of limited extent in vivo because most of the phosphatase activity is measurable in intact cells and is presumably periplasmic and inaccessible to cytoplasmic ribosomes. A second possibility is that the phosphatases have some affinity for cell wall or membrane fragments produced during sonication. If this affinity were greater for *Salmonella* phosphatases than for *E. coli* phosphatases, it could explain the inefficient release of the *Salmonella* phosphatases by osmotic shock procedures.

The observed enzyme activities have been purified to establish the number of protein species responsible for each enzymatic activity. In the purification procedure, phosphocellulose chromatography may have functioned as an affinity chromatography step for the nonspecific acid phosphatase and acid hexose phosphatase since both enzymes can hydrolyze glucose 6-phosphate. Cyclic phosphodiesterase, which does not hydrolyze sugar phosphates, did not bind to the phosphocellulose column under the conditions used.

The use of polyacrylamide gel electrophoresis has proven to be a useful analytical tool. Although acid hexose phosphatase was not completely purified, it was possible to demonstrate that the major protein band observed on SDS-polyacrylamide gel electrophoresis was the protein responsible for the enzyme activity. The purified cyclic phosphodiesterase preparation, examined by basic electrophoresis, possessed a single protein and activity band. However, this single band gave rise to two protein bands when it was subsequently subjected to electrophoresis on SDS-polyacrylamide gels. Either the cyclic phosphodiesterase consists of two nonidentical polypeptides or the preparation contained a persistent contaminant. Nonspecific acid phosphatase purified as a single protein species. The specific activity of the fractions, which comprises the protein peak in hydroxylapatite chromatography, the final purification step, was constant, and the purified preparation yielded only one protein band in SDS or acidic urea-polyacrylamide gel electrophoresis. The nonspecific acid phosphatase activity from *S. typhimurium* appears to be due to a single protein consisting of a single type of polypeptide.

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