2nd International Conference on Pyridoxal Catalysis, Moscow, Sept (in press).
Nishimura, J. S., and Greenberg, D. M. (1961), *J. Biol. Chem. 236*, 2684.
Novogrodsky, A., and Meister, A. (1964), *J. Biol. Chem. 239*, 879.
Novogrodsky, A., Nishimura, J. S., and Meister, A. (1963), *J. Biol. Chem. 238*, PC1903.
Osbond, J. M. (1964), *Vitamins Hormones 22*, 367.
Passmann, J. M., Radin, N. S., and Cooper, J. A. D. (1956), *Anal. Chem. 28*, 484.
Rosen, H. (1957), *Arch. Biochem. Biophys. 67*, 10.
Smyrniotis, P. Z., Miles, H. T., and Stadtman, E. R. (1958), *J. Am. Chem. Soc. 80*, 2541.
Soda, K., Novogrodsky, A., and Meister, A. (1964), *Biochemistry 3*, 1450.
Srinivasan, N. G., Corrigan, J. J., and Meister, A. (1965), *J. Biol. Chem. 240*, 796.
Torchinsky, Yu. M., and Koreneva, L. G. (1964), *Biokhimya 29*, 780.
Wada, H., and Snell, E. E. (1961), *J. Biol. Chem. 236*, 2089.
Wang, C. H., and Jones, D. E. (1959), *Biochem. Biophys. Res. Commun. 1*, 203.
Wilson, E. M. (1963), *Biochim. Biophys. Acta 67*, 345.
Wilson, E. M., and Kornberg, H. L. (1963), *Biochem. J. 88*, 578.
Wilson, E. M., and Meister, A. (1966), *Biochemistry 5*, 1166.

Purification and Properties of Two Acid Phosphatase Fractions Isolated from Osmotic Shock Fluid of *Escherichia coli**

Harold F. Dvorak, Robert W. Brockman,† and Leon A. Heppel

ABSTRACT: An osmotic shock procedure causes the specific release from *Escherichia coli* of a group of hydrolytic enzymes considered to be localized near the surface of the cell. Three distinct acid phosphatase fractions have been purified and examined. One of these, accounting for about 70% of the activity against glucose 6-phosphate of cell extracts, was purified 124-fold from the shock fluid and was homogeneous on disc gel electrophoresis. It had a striking specificity for hexose phosphates, although ribose 5-phosphate was also split at a substantial rate. The second fraction hydrolyzed a number of naturally occurring phosphate monoesters but was most active against *p*-nitrophenyl phosphate. The third fraction also hydrolyzed *p*-nitrophenyl phosphate, but evidence is presented that this represents a new activity for an enzyme previously described in the literature, namely cyclic phosphodiesterase. Growth conditions and media are described by which the concentration of hexose phosphatase may be varied over a 100-fold range.

The acid phosphatase activity of *Escherichia coli* has been incompletely characterized. Rogers and Reithel (1960) described two major phosphatases active at an acid pH but only one of these was extensively purified; this was a nucleoside 2′- or 3′-phosphatase which was virtually inactive against sugar phosphates. Von Hofsten and Porath (1962) purified an acid phosphatase from extracts of *E. coli* and found it to be very active against hexose phosphate monoesters although a complete summary of substrate specificity has not been published. Neu and Heppel (1964, 1965) found that acid phosphatase was among the group of enzymes released into the medium when *E. coli* was converted into spheroplasts by treatment with lysozyme and EDTA, or when cells were subjected to osmotic shock. Osmotic shock provides a convenient first step in the fractionation of released enzymes, capable of giving up to a 30-fold purification compared with sonic extracts.

In the present report we describe two acid phosphatase fractions obtained from shock fluid. One of these was partially purified by Neu and Heppel (1964) and we have added additional steps in order to obtain a preparation that is homogeneous on disc gel electrophoresis. This enzyme is now shown to be highly specific for certain sugar phosphate esters and is referred to as the acid hexose phosphatase. Conditions for its suppression will be discussed. The second fraction differs in its specificity from any acid phosphatase previously described in *E. coli;* it has thus far resisted substantial purification and is designated as the nonspecific acid phosphatase. Evidence is also presented

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that PNPP,[1] a favorite substrate in phosphomonoesterase assays, is hydrolyzed in *E. coli* mainly by the enzyme cyclic phosphodiesterase.

Materials and Methods

Growth of Cells. *E. coli*, strain K37 (a derivative of K10, obtained from Dr. N. D. Zinder), was grown on a rotary shaker at 37°, in order to obtain cells for work on osmotic shock and purification of the enzymes. Growth medium A consisted of 0.04 M K_2HPO_4, 0.022 M KH_2PO_4, 0.08 M NaCl, 0.02 M NH_4Cl, 0.003 M Na_2SO_4, 1×10^{-3} M $MgCl_2$, 2×10^{-4} M $CaCl_2$, 2×10^{-6} M $ZnCl_2$, and 0.5% Difco Bacto-Peptone, adjusted to pH 7.1. It was supplemented with 0.6% glycerol.[2] We have confirmed the observation of Von Hofsten and Porath (1961) that glucose represses the formation of acid phosphatase. Cells were harvested in late exponential phase (about 1×10^9 cells/ml). The formation of alkaline phosphatase is completely suppressed by inorganic phosphate present in the growth medium.

For studies on suppression of hexose phosphatase synthesis, strain E15 (obtained from Dr. H. H. Winkler) was used; this carries a deletion for alkaline phosphatase. The basal growth medium B contained 0.12 M Tris, 0.08 M NaCl, 0.02 M KCl, 0.02 M NH_4Cl, 3×10^{-3} M Na_2SO_4, 1×10^{-3} M $MgCl_2$, 2×10^{-4} M $CaCl_2$, and 2×10^{-6} M $ZnCl_2$. It was supplemented with a variety of carbon sources. Suppression was also studied using medium C which differed from B only in the replacement of 0.12 M Tris by 0.04 M K_2HPO_4 and 0.022 M KH_2PO_4. Cells were harvested at different stages of growth, washed with cold 0.01 M Tris-HCl (pH 7.3)–0.03M NaCl, and resuspended in 0.33 M Tris (pH 7.3) at a concentration of about 8×10^9 cells/ml. Sonic extracts were made by treatment for 90 sec with a Branson Sonifier, Model LS75. Extracts were also prepared by successively treating cell suspensions for 10 min at 37° with 10 μl/ml of $CHCl_3$, 20 μg/ml of lysozyme, and 2 μmoles/ml of EDTA.

Procedure for Osmotic Shock. The cell suspensions were quickly chilled, centrifuged in the cold, and washed three times with about 40 volumes of cold 0.01 M Tris-HCl (pH 7.3)[3]–0.03 M NaCl. The pellets were suspended in 0.033 M Tris-HCl (pH 7.3) containing 20% sucrose and 2×10^{-3} M EDTA at a concentration of 1 g wet wt/40 ml at room temperature. The mixture was stirred for 10 min and centrifuged. The supernatant fluid was discarded and the cells were subjected to a sudden osmotic transition by being rapidly dispersed in cold water (40 ml/g). The mixture was stirred for 10 min and centrifuged in the cold. The supernatant "shock fluid" containing the released enzymes was concentrated to dryness by lyophilization. Acid phosphatase, cyclic phosphodiesterase, and 5′-nucleotidase were recovered in yields exceeding 95% after this treatment. The dry powder was dissolved in a volume of distilled water equivalent to one-fortieth of the original volume and dialyzed for several hours against 5×10^{-3} M Tris-HCl (pH 7.4)–1×10^{-3} M $MgCl_2$–5×10^{-4} M $CoCl_2$.

Chromatography. DEAE- (Selectacel, type 20, 1 mequiv/g) and CM-cellulose were obtained from Brown Co., hydroxylapatite (Bio-Gel HT) from Calbiochem, and Sephadex G-100 from Pharmacia. Pooled enzyme fractions were frequently concentrated and desalted by negative pressure dialysis in collodion bags (Carl Schleicher & Schuell Co.). Disc electrophoresis was performed in 7.0% polyacrylamide gel at 4° in Tris-glycine buffer (pH 9.5) for 2 hr, using the general procedure of Reisfeld *et al.* (1962).

Enzyme Assays. Acid phosphatase activity was assayed in several ways. Since all fractions described here were active with *p*-nitrophenyl phosphate, this substrate could be used in a screening procedure to locate active fractions. The assay mixture (0.1 ml) contained 0.1 M sodium acetate buffer (pH 5.8), 5×10^{-3} M *p*-nitrophenyl phosphate, 1×10^{-2} M $MgCl_2$, 0.1 mg/ml of albumin, and enzyme. Incubation was for 20 min at 37°, after which the reaction was stopped with 1.0 ml of 0.1 N NaOH. One unit of activity was defined as that amount which results in an increase in optical density at 410 mμ of 1.0.

The acid hexose phosphatase was measured in the presence of inorganic phosphate as follows. The reaction mixture (1.0 ml) contained 0.9 ml of 1 M sodium acetate buffer (pH 4.6), 0.16 mg of *O*-carboxyphenyl phosphate, and enzyme. After incubation at 37° for 30 min, the reaction was stopped by addition of 0.15 ml of 1 M Tris-HCl (pH 8.8), and the absorbancy was measured at 298 mμ. For most of the samples, the following more sensitive assay could be used. The reaction mixture (0.1 ml) contained 0.5 μmole of glucose 6-phosphate, 10 μg of albumin, and 0.1 M sodium acetate buffer (pH 5.8). Incubation was for 30 min at 37° and P_i was measured as described by Ames and Dubin (1960) or, in the case of more labile esters, by the method of Fiske and Subbarow (1925). Enzyme units were expressed as micromoles hydrolyzed per hour. Assays for cyclic phosphodiesterase were as described by Neu and Heppel (1965). When this enzyme was tested with uridine 2′,3′-cyclic phosphate activities were expressed as micromoles hydrolyzed per hour; when BisPNPP was the substrate, one unit was taken as a change in absorbance at 410 mμ of 2.0/20 min.

When the nonspecific acid phosphatase was tested with different phosphate esters the reaction mixture (0.1 ml) contained 0.5 μmole of the ester, 10 μg of albumin, 0.01 M $MgCl_2$, 1×10^{-3} M $CoCl_2$, 0.1 M sodium acetate buffer (pH 5.7), and enzyme. Incubation was

[1] Abbreviations used: PNPP, *p*-nitrophenyl phosphate; Bis-PNPP, bis(*p*-nitrophenyl) phosphate; UcP uridine 2′,3′-cyclic phosphate; 3′-AMP, adenosine 3′-phosphate; ADP and ATP, adenosine di- and triphosphates.

[2] As indicated in a later section, other carbon sources would have given higher levels of acid hexose phosphatase than obtained with the combination of glycerol and Bacto-Peptone.

[3] In all cases, the pH of Tris buffers was measured at 23° in a Beckman Model G pH meter, even though the solutions were sometimes used at 3°.

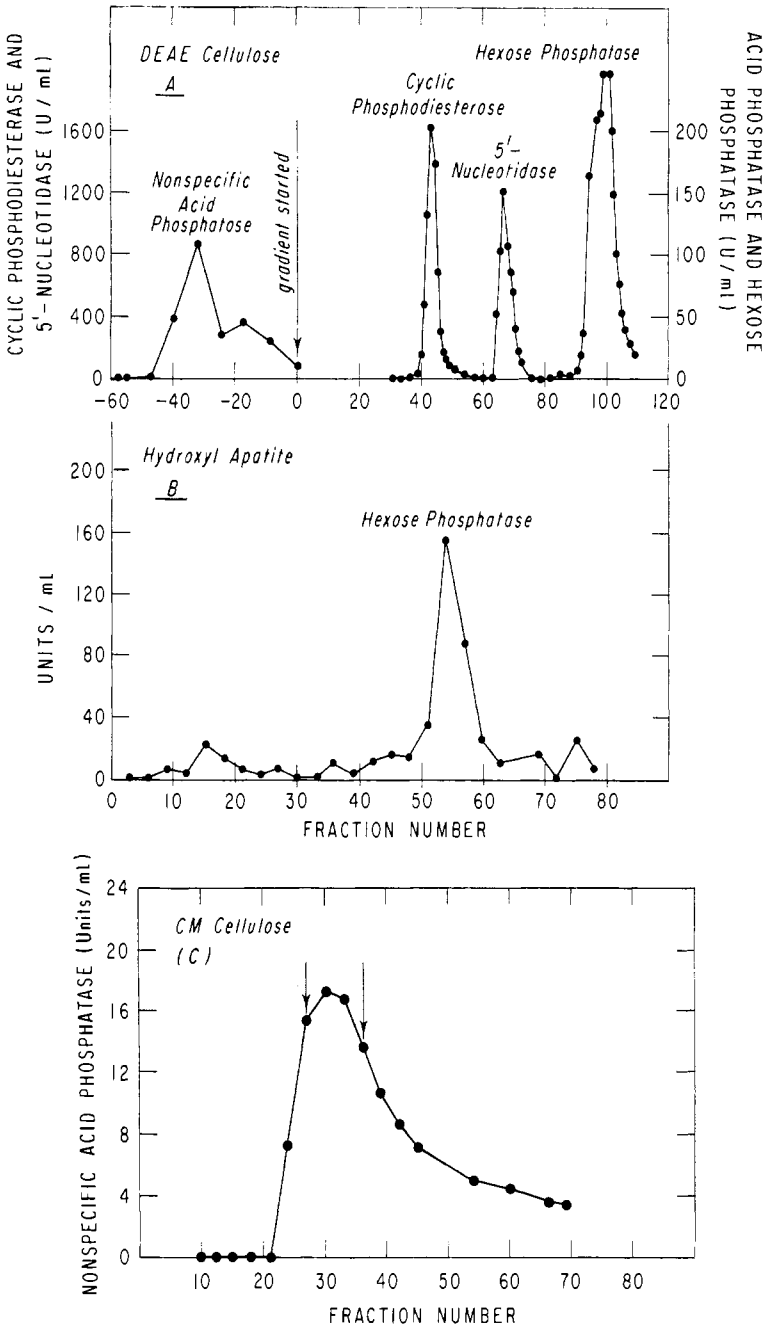

FIGURE 1: (A) Chromatography of shock fluid on DEAE-cellulose, (B) hexose phosphatase activity on hydroxylapatite, and (C) CM-cellulose chromatography of the nonspecific acid phosphatase. (A) The column (2.5 × 26 cm) was equilibrated with a buffer mixture consisting of 5×10^{-3} M Tris-HCl (pH 7.4)–1×10^{-3} M $MgCl_2$–5×10^{-4} M $CoCl_2$. Lyophilized shock fluid (33.5 ml, 0.2 g of protein) was applied and the column was washed with 315 ml of the buffer mixture. A linear gradient was begun as indicated by the arrow. The mixing vessel contained 300 ml of the buffer mixture. The reservoir contained 300 ml of the buffer mixture also containing 0.2 M NaCl. The fraction size was 4.8 ml. (B) Material concentrated from the hexose phosphatase peak fractions eluted from DEAE-cellulose (3 mg of protein) was rechromatographed on a column of hydroxylapatite (2 × 12 cm), equilibrated with 5×10^{-3} M Na-phosphate buffer (pH 7.3). A linear gradient was established with 150 ml of starting buffer in the mixing vessel and an equal volume of 0.15 M Na-phosphate buffer (pH 7.3) in the reservoir. The fraction size was 3.9 ml. Enzyme activity is expressed as the increase in absorbancy at 298 mμ obtained with 0.01 ml of enzyme fraction, using the *O*-carboxyphenyl phosphate assay. (C) Pooled, concentrated enzyme that did not adhere to DEAE-cellulose (6.4 mg) was applied to a column (2 × 13 cm) equilibrated with 5×10^{-3} M Na-acetate (pH 6.0)–1×10^{-3} M $MgCl_2$. Elution was with a linear gradient of 0–0.15 M NaCl. Each reservoir contained 150 ml. The fraction size was 3.9 ml.

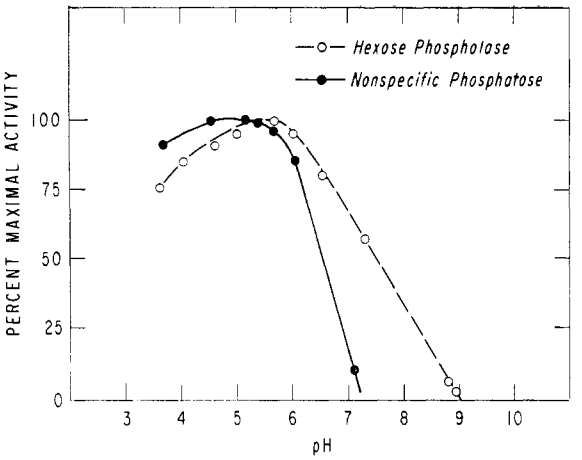

FIGURE 2: Effect of pH on the activity of the purified hexose phosphatase against glucose 6-phosphate and on the rate of hydrolysis of PNPP by the nonspecific acid phosphatase fraction. Sodium acetate buffers were used below pH 7.0; Tris-HCl buffers above pH 7.0.

for 30 min at 37°. Protein was determined by a micro-modification of the method of Lowry *et al.* (1951).

Other Materials. The phosphate esters were obtained from Sigma Chemical Co. except for α-lactose 1-phosphate which was supplied by Dr. J. Kanfer. Bacto-Peptone and Casamino Acids were Difco products.

Results

Purification of Acid Phosphatases. The acid phosphatase activity measured with PNPP was separated into three distinct fractions by chromatography of concentrated shock fluid on DEAE-cellulose (Figure 1). Depending on the size of column and amount of protein applied, the first fraction was eluted either with the washing buffer (within four column volumes), as shown in Figure 1A, or in the early tubes shortly after a linear salt gradient was begun. We refer to this fraction as the nonspecific acid phosphatase. The second peak of PNPP activity was coincident with cyclic phosphodiesterase and we believe that hydrolysis of PNPP represents a newly reported activity due to this enzyme. The third fraction active against PNPP was eluted as a discrete peak at a concentration of approximately 0.15 M NaCl, following a peak for 5′-nucleotidase. It will be referred to as the acid hexose phosphatase. These three fractions will now be discussed in turn.

Nonspecific Acid Phosphatase. The initial acid phosphatase fraction obtained on DEAE-cellulose chromatography resisted further purification. Several protein species are found on disc gel electrophoresis and the designation "nonspecific" does not imply one enzyme of wide specificity, since several separate phosphatases may be present. Rechromatography of this material on CM-cellulose resulted in a single peak of activity with extensive tailing that began to elute at a concentration of 0.05–0.07 M NaCl (Figure 1C). Fractionation on CM-cellulose increased the specific activity very little.

TABLE I: Relative Activities of Hexose Phosphatase and the Nonspecific Acid Phosphatase against Different Phosphate Esters.[a]

Compound	Hexose Phosphatase	Nonspecific Acid Phosphatase
Glucose-6-P	100	8.2
Fructose-6-P	86	16
Fructose-1,6-diP	93	—
α-D-Glucose-1-P	102	1.6
α-D-Galactose-1-P	95	2
Mannose-6-P	46	—
Ribose-5-P	36	—
Sedoheptulose-7-P	1.8	—
α-L-Arabinose-1-P	0	7.2
α-Lactose-1-P	0	0
DL-α-Glycero-P	0.3	—
DL-β-Glycero-P	—	5.6
3-Phosphoglyceric acid	0.7	0.1
ATP	0	3
ADP	0	2
5′-AMP	0.9	3
3′-AMP	0.2	21
2′-AMP	0.9	—
Phenylphosphate	0	—
PNPP	7	100
Phosphoserine	0.05	0
Phosphocholine	0.1	0.3
NADP	0	—
Histidinol-P	0.05	0
Inorganic pyrophosphate	0	0
Phosphoethanolamine	—	0

[a] A value of 100 is assigned to the activity of hexose phosphatase against glucose 6-phosphate and to the activity of the nonspecific acid phosphatase against PNPP. Assay conditions are presented in Methods. Blank spaces indicate that no assay was carried out. Concentration of substrate was 5×10^{-3} M.

The nonspecific acid phosphatase fraction accounted for 25–35% of the PNPP-splitting activity of the osmotic shock fluid and it hydrolyzed this substrate five times more rapidly than any other phosphate ester that was tested (Table I). A substantial rate of hydrolysis was also observed with 3′-AMP, fructose 6-phosphate, glucose 6-phosphate, α-L-arabinose 1-phosphate, and β-glycerophosphate. The pH optimum was near 5.0 (Figure 2), and no striking effects of metal ions were observed. Thus 1×10^{-2} M Mg^{2+} and 1×10^{-3} M Co^{2+} together stimulated activity by 10%, while

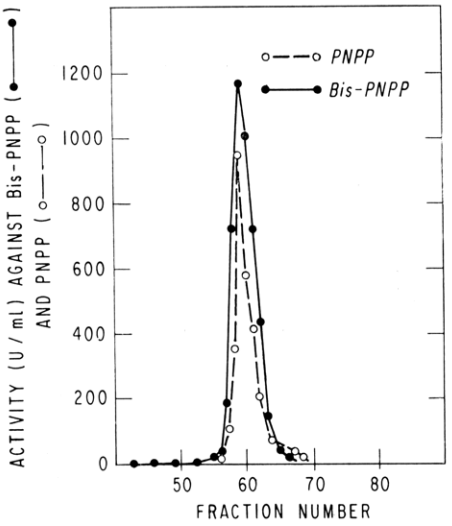

FIGURE 3: Chromatography of cyclic phosphodiesterase on hydroxylapatite. Peak tubes from a DEAE-cellulose column (2.3 mg of protein) were pooled, concentrated, and applied to a column (2 × 14 cm) equilibrated with 1×10^{-3} M $MgCl_2$–1×10^{-3} M β-mercaptoethanol. A linear gradient of Na-phosphate buffer (pH 7.5) from 1×10^{-3} to 0.15 M was applied. The fraction size was 3.9 ml.

inhibitions of 15% by 1×10^{-3} M Zn^{2+} and 37% by 1×10^{-2} M Ca^{2+} were noted. A concentration of 1×10^{-2} M EDTA reduced enzyme activity by 18%.

Cyclic Phosphodiesterase. The second peak of PNPP activity showed a pH optimum of 6.0 and accounted for the bulk of the PNPPase activity of *E. coli* extracts under conditions of alkaline phosphatase suppression.

TABLE II: Summary of Purification of Cyclic Phosphodiesterase from Osmotic Shock Fluid.[a]

Step	% Over-All Yield	Specific Activity (units/mg of protein) BisPNPP	PNPP
Sonic extract		10.1	8.8
Shock fluid	100	226	152
DEAE-cellulose	62	3,400	1,500
Hydroxylapatite	53	19,200[b]	10,100

[a] The procedure for obtaining shock fluid is described in Methods and the purification of cyclic phosphodiesterase from shock fluid is outlined in the legends of Figures 1 and 3. Assays on a sonic extract are shown for comparison. [b] This fraction had a specific activity of 25,000 units/mg when tested with uridine 2′,3′-cyclic phosphate.

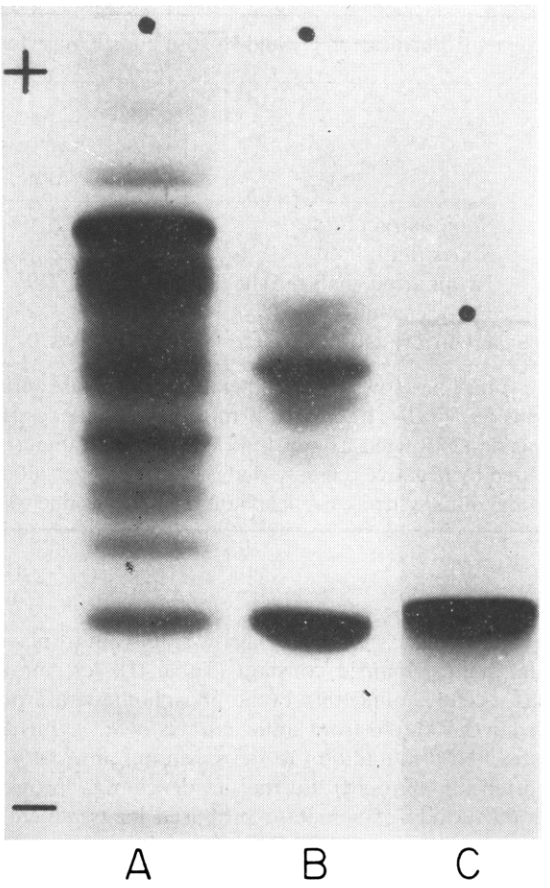

FIGURE 4: Disc gel electrophoresis. (A) Lyophilized cold water wash (46 μg of protein) showing at least 23 discrete bands. (B, C) Hexose phosphatase after DEAE-cellulose (34 μg of protein) (B) and hydroxylapatite (28 μg of protein) (C) chromatography. Despite heavy application of protein, only a single band, with some "tailing," is visible. The black dots indicate the origin. In order to accommodate larger volumes of relatively dilute solution, purified enzyme (C) was applied in a denser sample gel than in A or B; as a result, migration in the elution field was somewhat retarded. Smaller volumes of purified enzyme, applied in usual sample gels, gave a single band of protein and enzyme activity with a mobility as in A or B.

Its behavior on DEAE-cellulose was similar to that of the acid phosphatase I described by Rogers and Reithel (1960). We conclude that this fraction is cyclic phosphodiesterase, which hydrolyzes PNPP in addition to the substrates described by Anraku (1964a,b), namely, ribonucleoside 2′,3′-cyclic phosphates, 3′-ribonucleotides, and bis(*p*-nitrophenyl) phosphate. The evidence for this is as follows. (a) Coincident peaks of activity were obtained by chromatography on DEAE-cellulose, DEAE-Sephadex, and hydroxylapatite (Figure 3). (b) The material was purified almost 200-fold from osmotic shock fluid, and during the course of this purification the ratio of activity

TABLE III: Purification of Acid Hexose Phosphatase from Osmotic Shock Fluid.

Step	Vol. (ml)	Activity (units/ml)	Activity (total units)	% Over-All Yield	Protein (mg/ml)	Sp Act. (units/mg)
Sonic extract, 1:40[a]	222	20.1	4450		2.02	9.9
Shock fluid, 1:40	222	18.1	4000	90	0.26	70
Lyophilized, dialyzed shock fluid	7.9	494	3900	88	6.6	75
DEAE-cellulose[b]	4.2	642	2700	61	0.46	1400
Hydroxylapatite[b]	3.0	485	1455	33	0.056	8650[c]

[a] This line shows the total hexose phosphatase activity and specific activity of a sonic extract made from a 1:40 suspension of cells (1 g wet wt/40 ml) and in a sense represents the starting conditions. In the actual purification, the enzyme was first extracted from a 1:40 suspension of intact cells, using osmotic shock with cold water. [b] Peak tubes concentrated by negative pressure dialysis. [c] The over-all purification is greater than here indicated because as much as 10% of the initial glucose 6-phosphatase activity is due to the nonspecific acid phosphatase fraction described in this paper.

against PNPP to that against bis(*p*-nitrophenyl) phosphate remained constant (Table II). (c) Anraku[4] has recently found that cyclic phosphodiesterase purified over 900-fold from sonic extracts of *E. coli* hydrolyzes PNPP in addition to the substrates previously reported by him. (d) Further evidence was provided from a study of a mutant produced by treatment of *E. coli* K37 with nitrosoguanidine.[5] Sonic extracts of the mutant showed only 5% as much activity against UcP as was present in extracts of K37 when both cultures were harvested in stationary phase. Shock fluids obtained with cold water showed the same difference in levels of enzyme. Concentrated shock fluid was chromatographed on DEAE-cellulose and a single, small peak of UcP activity was obtained, which was coincident with peaks of both PNPP and bis(*p*-nitrophenyl) phosphate activity. Comparison of the peak tubes obtained with the mutant and the parent organism showed a comparable reduction for all three activities.

Anraku has suggested that the enzyme has two active sites, one for the hydrolysis of the 2′,3′-cyclic phosphate linkage and the other for the splitting of phosphomonoesters. In this connection it is of interest that the hydrolysis of PNPP was stimulated up to 60-fold by 1×10^{-3} M Co^{2+} whereas no more than a three- to fourfold stimulation was obtained with the other substrates.

Acid Hexose Phosphatase. The third peak of PNPP activity obtained on DEAE-cellulose chromatography was found to account for the bulk of the hexose phosphatase activity of *E. coli*. It was concentrated by negative pressure dialysis and emerged as a single discrete peak when rechromatographed on hydroxylapatite (Figure 1B). Enzyme recovered from hydroxylapatite was purified 870-fold as compared with sonic extracts of intact cells and 124-fold as compared with osmotic shock fluid (Table III). The over-all yield was 33%. The purified fraction was slightly retarded on G-100 Sephadex. It gave a single band of activity and protein on disc gel electrophoresis (Figure 4), with 60% recovery of activity from the gel.

This enzyme showed a striking specificity for hexose phosphate esters (Table I), although ribose 5-phosphate was also split at a substantial rate. Ribulose 1,5-diphosphate was more slowly hydrolyzed while the rate for sedoheptulose 7-phosphate was only 2% of that noted for glucose 6-phosphate. The hydrolysis of the sedoheptulose ester was linear with time until more than one-half of the substrate had been utilized. Many other phosphate esters were tested with the highly purified fraction and found to be split at less than 1% of the rate observed with glucose 6-phosphate. Thus, the substantial activities previously reported (Neu and Heppel, 1965) against β-glycerophosphate and 5′-AMP have been removed by further purification. We have confirmed the earlier observation (Neu and Heppel, 1965), that aldohexose phosphates must be in the α configuration to serve as substrates.

The pH optimum was between 5.5 and 6.0 (Figure 2) and K_m was 3.3×10^{-4} for glucose 6-phosphate (Figure 5). No effect of metal ions could be demonstrated, and the enzyme was fully active in the presence of 1×10^{-2} M EDTA. The purified enzyme was stable for more than 1 year when stored at −20°.

Effect of Composition of Growth Medium on the Level of Hexose Phosphatase. Hexose phosphatase is subject to catabolite repression (Von Hofsten, 1961), a fact which we have confirmed. Thus, the lowest levels of activity were observed with glucose as a carbon source and the highest levels resulted when the basal medium was supplemented with Casamino Acids. Several strains of *E. coli* were examined but most of the work was carried out with E15, which carries a deletion for the P_i-repressible alkaline phosphatase. This allows measure-

[4] Private communication.

[5] This mutant was isolated in the laboratory of Dr. N. D. Zinder, and his help is gratefully acknowledged. Dr. Zinder also established that the mutant is a derivative of K37.

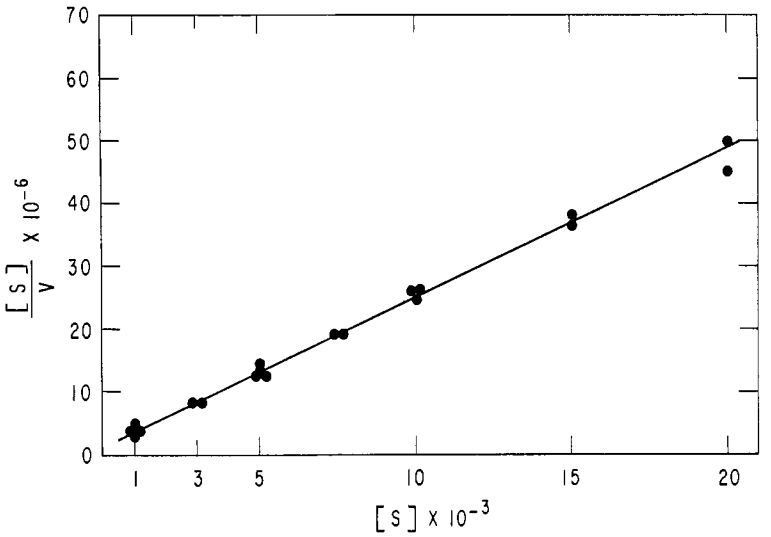

FIGURE 5: Lineweaver–Burk plot for determination of K_m of hexose phosphatase, with glucose 6-phosphate as the substrate. Substrate concentrations are in molar units.

TABLE IV: Effect of Growth Conditions on the Hexose Phosphatase Activity of Extracts of *E. coli* E15.[a]

Medium	Supplement	Hexose Phosphatase Activity (units/mg)			
		1st Growth Cycle	2nd Growth Cycle	3rd Growth Cycle	4th Growth Cycle
B	1% Casamino Acid	13[b]	20	21	20
B[c]	0.085 M glucose + 0.25% Casamino Acid	0.2			
C	0.085 M glucose + 0.5% Bacto-Peptone	0.2[d]	0.25	0.25	
C	0.17 M glucose + 0.5% Bacto-Peptone	0.25	0.19		

[a] Strain E15 has a deletion for alkaline phosphatase, so that no interference is encountered in assays for hexose phosphatase, regardless of the P_i content of the growth medium. Cells were grown to stationary phase and subcultured to give several cycles of growth. Chloroform–lysozyme–EDTA extracts were made of washed cells (see Methods). The composition of the basal medium B is given in Methods. Activity was assayed with glucose 6-phosphate as the substrate, and is given as units per milligram of protein. Cells were harvested in stationary phase. [b] An extract from a sample harvested in mid-exponential phase showed 7.5 units/mg of protein. [c] Growth on these media was limited by the low concentration of P_i. However, when they were supplemented with 1×10^{-2} M P_i the results were similar. [d] Similar activities were recorded in early and mid-exponential phase.

ment of the cellular content of acid hexose phosphatase regardless of the concentration of P_i in the medium. Increasing the concentration of glucose in the medium from 0.03 to as much as 0.17 M did not cause further suppression, and the level of enzyme did not change significantly when cells were subjected to three cycles of growth to stationary phase in a glucose-containing medium (Table IV). However, when a supplement of 1% Casamino Acids was used, the greatest specific activity of hexose phosphatase was not obtained until two cycles of growth had been carried out. In the presence of glucose the specific activity of acid hexose phosphatase was reduced to 1% of that found with Casamino Acids (Table IV).

The effect of other supplements added to medium B was also studied. Suppression was observed with 0.01 M glycerol and 0.1% yeast extract and was overcome by increasing the concentration of yeast extract of 0.4%. Bacto-Peptone (1–2%) resulted in an enzyme level in stationary phase about one-half that observed with Casamino Acids. Succinate (0.01 M) gave results similar to those obtained with Bacto-Peptone. In general, activity of the enzyme was greatest early in stationary phase when more favorable carbon sources were used,

TABLE V: Effect of Growth Conditions on Hexose Phosphatase and β-Galactosidase in Extracts of *E. coli* E15.[a]

Additions	Activities at Several Stages of Growth (units/mg)			
	Early Exponential	Mid Exponential	Late Exponential	Stationary
	A. Hexose Phosphatase			
0.9% Casamino Acids	5.2	8.4	7.3	10.0
0.01 M glucose + 0.01 M P_i	0.8	1.0	0.8	2.6
0.01 M glucose 6-phosphate	1.0	0.7	3.3	5.7
	B. β-Galactosidase			
0.9% Casamino Acids	43	69	75	78
0.01 M glucose + 0.01 M P_i	6.4	10.5	8.5	12.9
0.01 M glucose 6-phosphate	4.0	4.5	13.0	22

[a] Cells of strain E15 (deletion for alkaline phosphatase) were grown on basal medium B (see Methods) with 0.1% Casamino Acids and other additions shown below. In our hands, growth was poor unless at least 0.1% Casamino Acids was present. Samples of washed cells were extracted with $CHCl_3$–lysozyme–EDTA. Activities are expressed as units per milligram of protein. Similar rates of growth were obtained with the various supplements.

although there was little change for some hours thereafter. When sonic extracts derived from suppressed and nonsuppressed cells were mixed and assayed, no evidence for an inhibitor or activator was found.

Most of the experiments on metabolite suppression were carried out both under conditions where P_i limited the extent of growth and also in the presence of 0.01 M P_i. The results, at least in the exponential phase of growth, were similar. Assays for β-galactosidase were frequently done (see Table V) and the well-known suppression of this enzyme was roughly parallel to that of hexose phosphatase.

Glucose 6-phosphate is taken up by intact cells (Fraenkel *et al.*, 1964; Winkler, 1966), and this occurs without preliminary hydrolysis at the cell surface. It was considered worthwhile to examine whether glucose 6-phosphate would act as an inducer for hexose phosphatase at some stage of growth. However, when compared with equivalent concentrations of free glucose and P_i, no significant differences in the levels of acid hexose phosphatase were noted at any stage of growth; the ester acted like free glucose in suppressing enzyme activity (Table V). These results agree with preliminary data for glucose 1-phosphate reported by Von Hofsten (1961). Several experiments were performed using α-glycerophosphate, another phosphate ester for which a transport system has been described (Hayashi *et al.*, 1964). Its behavior was quite comparable to that of glycerol in that formation of hexose phosphatase was suppressed.

Discussion

This report describes the properties of two acid phosphatase fractions that are released from *E. coli* by osmotic shock and presents a relatively simple scheme for the purification of one of them. The purification resulted in material that was homogeneous on disc gel electrophoresis and was highly specific for certain hexose and pentose phosphate esters. The enzyme accounts for approximately 90% of the glucose 6-phosphate splitting activity found in extracts of cells lacking the P_i-repressible alkaline phosphomonoesterase. We use the trivial name acid hexose phosphatase since the most rapid rate of hydrolysis was observed with hexose phosphates. The second acid phosphatase fraction described in this paper was less well defined in its specificity.

Acid phosphatase fractions of *E. coli* have previously been obtained from extracts of whole cells. The acid hexose phosphatase described here probably corresponds to one or all of the phosphatase II group described by Rogers and Reithel (1960), but certain differences in chromatographic behavior and pH optima are apparent. These differences may be due to the strains of *E. coli* that were used or the nature of the starting material. Their phosphatase I fraction, which hydrolyzes 3′-AMP and PNPP, corresponds most closely in its properties to cyclic phosphodiesterase, an enzyme that was discovered after the work of Rogers and Reithel. We have shown in this paper that PNPP is among the compounds hydrolyzed by cyclic phosphodiesterase.

The fraction obtained by Von Hofsten and Porath (1962) was poorly retained on DEAE-cellulose and in this respect resembles our nonspecific acid phosphatase. However, in pH optimum and substrate specificity their preparation does not match either of our fractions.

Histochemical data[6] suggest that the hexose phos-

[6] Unpublished observations of Drs. B. Wetzel and S. Spicer. See also Spicer *et al.* (1966).

phatase is localized near the cell surface of *E. coli*. This conclusion is supported by the fact that glucose 6-phosphate is hydrolyzed nearly as rapidly by intact cells as by an equivalent sonic extract, even in the case of mutants unable to transport this ester into the cell.[7] The function of this enzyme is obscure, but its location suggests that it may be used for the degradation of hexose phosphates present in the medium. It has also been postulated that transport of certain sugars may involve formation of a phosphorylated intermediate which is hydrolyzed by hexose phosphatase in the final step of the transport process (Kundig *et al.*, 1966). Finally, it has been proposed by Melo and Glaser (1966) that hexose phosphatase and related enzymes may serve to regulate the intracellular levels of hexose phosphate and of coenzymes containing this moiety. This is an interesting idea but, as pointed out by these authors, difficulties arise if the coenzymes and the phosphatase are indeed in separate compartments.

Some insight into the function of hexose phosphatase might be gained by examination of cells in which the level of enzyme is varied over extremely wide limits. Mutants deficient in hexose phosphatase have not yet been reported. However, as shown in this paper, by proper choice of growth conditions and age of cultures the concentration of enzyme can be varied over a 100-fold range.

[7] Unpublished observations of Dr. R. W. Brockman.

References

Ames, B. N., and Dubin, D. T. (1960), *J. Biol. Chem. 235*, 769.

Anraku, Y. (1964a), *J. Biol. Chem. 239*, 3412.

Anraku, Y. (1964b), *J. Biol. Chem. 239*, 3420.

Fiske, C. H., and Subbarow, Y. (1925), *J. Biol. Chem. 66*, 375.

Fraenkel, D. G., Falcoz-Kelly, F., and Horecker, B. L. (1964), *Proc. Natl. Acad. Sci. U. S. 52*, 1207.

Hayashi, S., Koch, J. P., and Lin, E. C. C. (1964), *J. Biol. Chem. 239*, 3098.

Kundig, W., Kundig, F. D., Anderson, B., and Roseman, S. (1966), *J. Biol. Chem. 241*, PC3243.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.

Melo, A., and Glaser, L. (1966), *Biochem. Biophys. Res. Commun. 22*, 524.

Neu, H. C., and Heppel, L. A. (1964), *Biochem. Biophys. Res. Commun. 17*, 215.

Neu, H. C., and Heppel, L. A. (1965), *J. Biol. Chem. 240*, 3685.

Reisfeld, R. A., Lewis, U. J., and Williams, D. E. (1962), *Nature 195*, 281.

Rogers, D., and Reithel, F. J. (1960), *Arch. Biochem. Biophys. 89*, 97.

Spicer, S. S., Wetzel, B. K., and Heppel, L. A. (1966), *Federation Proc. 25*, 539.

Von Hofsten, B. (1961), *Biochim. Biophys. Acta 48*, 171.

Von Hofsten, B., and Porath, J. (1962), *Biochim. Biophys. Acta 64*, 1.

Winkler, H. H. (1966), *Biochim. Biophys. Acta 117*, 231.