The 5'-Nucleotidase of Escherichia coli

I. PURIFICATION AND PROPERTIES*

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SUMMARY

An enzyme, 5'-nucleotidase, was purified from Escherichia coli. With the use of the osmotic shock technique, a 5000-fold purification was achieved with DEAE-cellulose chromatography, hydroxylapatite chromatography, and repeat DEAE-cellulose chromatography. This enzyme hydrolyzes 5'-AMP, ATP, uridine diphosphate glucose and bis(p-nitrophenyl)phosphate. The ratios of specific activities remain constant for the various substrates throughout the purification, and the heat inactivation curves parallel each other. The purified 5'-nucleotidase of E. coli hydrolyzed all 5'-ribo- and deoxyribonucleotides with optimal activity against 5'-AMP. Substitution by a phosphoryl group as in pXp makes a compound resistant, as does substitution by another nucleotide as in pXpY. The enzyme is inactive against all sugar phosphates but will hydrolyze UDP-glucose.

The 5'-AMP activity has a pH optimum of 6.0, the ATP activity 6.8, and UDP-glucose activity 7.4. The 5'-AMP activity is stimulated 100-fold by 5 × 10⁻³ M Co⁺⁺ and 200-fold by the further addition of Ca⁺++. The enzyme is inhibited by 0.1 M Zn⁺⁺ and chelating agents. Only Mn⁺⁺ is effective in replacing the Co⁺⁺ stimulation of 5'-AMP or ATP hydrolysis, but Co⁺⁺ is not needed for the UDP-glucose hydrolysis. The enzyme has been shown to be pure, as judged by molecular sieve chromatography, polyacrylamide disk gel electrophoresis, and ultracentrifugation. An s_{av} value of 4.2 has been obtained. A molecular weight of 52,000 was found. The amino acid composition showed no unusual distribution.

During the course of studies on the release of enzymes from the surface of Escherichia coli (1, 2), we discovered a Co⁺⁺-stimulated 5'-nucleotidase. Previous studies of bacterial nucleotidases had shown an Fe⁺⁺-stimulated 5'-nucleotidase in Clostridium sticklandii (3) and in an unidentified soil bacterium (4), a heat-activated one in Proteus vulgaris (5), and an uncharacterized 5'-nucleotide-degrading ability of Bacillus subtilis (6). Kohn and Reis (7) found nucleotidases in a variety of Proteus species but had not characterized them. They found only minimal 5'-AMP hydrolysis by E. coli. It is now apparent from these studies that a 5'-nucleotidase is present in E. coli as a constitutive enzyme in high amounts. It is also evident that a number of species of bacteria possess a 5'-nucleotidase activity similar in properties to E. coli.

This 5'-nucleotidase of E. coli has been purified some 5000-fold with the osmotic shock method of Neu and Heppel (2). Although the enzyme has been purified to relative homogeneity, as judged by disk gel electrophoresis, molecular sieve column chromatography, and ultracentrifugation, it cleaves uridine diphosphate glucose, ATP, and bis(p-nitrophenyl)phosphate, as well as 5'-AMP. The ratios of specific activity have remained constant throughout the purification, and heat inactivation curves parallel each other.

We have also partially purified a specific protein inhibitor from E. coli which has also been found by Melo and Glaser (8) and by Dvorak, Anraku, and Heppel (9).

In these papers we will describe the purification of the 5'-nucleotidase and its properties and physical characteristics, as well as additional evidence concerning its surface location. The partial purification and characteristics of the 5'-nucleotidase inhibitor will also be described.

EXPERIMENTAL PROCEDURE

Materials

Nucleotides were purchased from commercial sources as was calcium bis(p-nitrophenyl)phosphate, p-nitrophenylphosphate, and sugar phosphates. Tritium-labeled DNA from E. coli K-12 (λ) was a gift of Dr. Arthur Weissbach. E. coli-soluble RNA was purchased from General Biochemicals. DEAE-cellulose used in column chromatography was obtained from both Bio-Rad and Reeve Angel. Hydroxylapatite (Hyapatite C) was obtained from the Clarkson Chemical Company, Inc. Sephadex was purchased from Pharmacia Fine Chemicals. Alkaline phos-
Enzymes were used for polyacrylamide gel electrophoresis were obtained from Canal Industrial Corporation, Bethesda, Maryland.

**Methods**

**Cells and Medium—**

*E. coli*, K-372, a mutant of a strain originally derived from K-10 by Zinder and Cooper (10) was used for large-scale purification. This strain is particularly low in cyclic phosphodiesterase, glucose 6-phosphatase, and DNA-endonuclease activities.

The medium used was the high phosphate medium of Neu and Hoppel (2) with 0.2% glucose and 0.6% glycerol as the carbon source. Each sample was measured. Exclusion volume was determined with dextran blue. Lysozyme, albumin, ribonuclease, and hemoglobin were used as standards.

**Enzyme Assays—**

The standard assay for 5'-nucleotidase contained 5 mM 5'-AMP, 5 mM CoCl₂, 20 mM CaCl₂, 100 mM sodium acetate buffer, pH 6.0, and 10 mg of bovine serum albumin in a total volume of 0.1 ml. Enzyme dilutions were made in a solution of 0.1 mg of albumin per ml. After 20 min at 37°C the reaction was stopped with 0.05 N HCl, and the assay was performed by the method of Chen, Toribara, and Warner (11). Activity is expressed as micromoles of phosphate released per hour at 37°C. The assay for hydrolysis of ATP contained 100 mM Tris-maleate, pH 6.8, 5 mM CoCl₂, 10 mM CaCl₂ and 2 mM ATP, in a total volume of 0.1 ml. The reaction was incubated for 15 min at 37°C and stopped with 0.01 N H₂SO₄. Phosphate was assayed by a modification of the Fiske and SubbaRow procedure (12). Activity is expressed as micromoles of phosphate hydrolyzed per hour. For assay of the UDP-glucose hydrodrolase activity the reaction mixture contained 1.4 mM UDP-glucose, 100 mM Tris-Cl, pH 7.4, 10 mM MgCl₂, and excess purified alkaline phosphatase, in a total volume of 0.1 ml. Activity is expressed as micromoles of phosphate released. Hydrolysis of bis(p-nitrophenyl)phosphate was measured by incubating 5 mM CoCl₂, 100 mM Tris-maleate, pH 6.7, 0.1 mg of calcium bis(p-nitrophenyl)phosphate, and enzyme at 37°C for 20 min, in a total volume of 0.1 ml. The reaction was stopped with 1.0 ml of 0.1 N NaOH, and the absorbance at 410 nm was measured. A unit is defined as an optical density change of 2.0 per 20 min. Acid phosphatase activity was measured by the hydrolysis of p-nitrophenyl phosphate. The reaction mixture (0.1 ml) contained 25 mM sodium p-nitrophenyl phosphate, 200 mM sodium acetate buffer, pH 4.1. After a 20-min incubation at 37°C, the reaction was stopped by addition of 1.0 ml of 0.1 N NaOH, and the absorbance at 410 nm was read. A unit is defined as an optical density change of 410 nm of 2.0 per 20 min.

The methods for cyclic phosphodiesterase (1), acid hexose phosphatase (2), alkaline phosphatase (13), RNase I (14), RNase II (15), and DNA-endonuclease I (16) are those previously published. Protein concentration was determined by the method of Lowry et al. (17).

**Polyacrylamide Electrophoresis—**

All polyacrylamide gel electrophoresis experiments were run at both 5°C and 25°C with 7.5% standard gel with glycine buffer at pH 8.6, as described in the manual supplied by the Canal Industrial Corporation. All samples were applied in 0.005 ml Tris-Cl buffer, pH 7.5, and run with a constant current of 5 ma per tube.

**Sucrose Gradient Centrifugation—**

Sucrose gradient centrifugation was carried out by the method of Martin and Ames (18). Gradients (4.8 ml) from 20 to 5% sucrose in 0.01 M Tris-Cl (pH 7.4)-10⁻⁴ M MgCl₂ were used. Purified enzyme, 0.1 mg in the same buffer, was layered on top of the gradient. Centrifugation was at 39,000 rpm in a Spinco model L ultracentrifuge with an SW34 rotor for 16 hours at 4°C. Five-drop fractions were collected from the bottom of the tube and assayed for enzyme content. Molecular weight calculations were based upon comparison of sedimentation of lysozyme, hemoglobin, and albumin.

**Gel Filtration—**

Sephadex gel filtration was done with a modification of the method of Andrews (19). Sephadex G-100 in a 0.4 M NaCl-0.06 Tris, pH 7.4, at 3°C was used with a column 1.6 × 110 cm. Proteins were dissolved in 0.5 ml of the equilibration buffer and with sucrose layered on the top of the column. Samples were collected with a Gilson fraction collector with a 2-ml sample. Each sample was measured. Exclusion volume was determined with dextran blue. Lysozyme, albumin, ribonuclease, and hemoglobin were used as standards.

**RESULTS**

**Purification of Enzyme**

All operations were carried out at 0–5°C unless otherwise indicated. The pH of all buffer systems is that obtained at the ionic strength and temperature specified. The procedure is summarized in Table I. All subsequent studies employed the most purified material unless specified.

**Growth of Cells—**

*E. coli* K-372 was grown overnight in 2-liter flasks (1000 ml) in the high phosphate medium (2) with 0.2% glucose-0.3% glycerol as the carbon source. This was done to lower the level of acid phosphatase and acid hexose phosphatase, which are repressed by glucose. Cells were harvested by use of the "Szent-Gyorgyi and Blum" eight-tube continuous flow system for the RC2-B Sorvall centrifuge. Generally the yield was 4 g per liter of culture. The phosphate culture of the medium prevented formation of inducible alkaline phosphatase of these cells, and the use of stationary phase cells and strain K-372 resulted in very low levels of the DNA endonuclease I (20) and no appreciable release of RNase I (2).
at 3° in a volume of 10 ml of buffer per g of cell wet weight. This was done in the GSA head of the Sorvall centrifuge. The washed cells were then resuspended in 0.03 M Tris-Cl buffer, pH 7.3, at room temperature at a concentration of 1 g of cells to 20 ml of buffer. To this was added, with continued gentle swirling, an equal volume of 40% sucrose-0.03 M Tris, pH 7.3. The final concentration of cells was 1 g to 40 ml of 20% sucrose-0.03 M Tris, pH 7.3. Use of more concentrated cell preparations (1 g to 20 ml of 20% sucrose) resulted in a significant decrease in the release of the enzyme. Disodium-EDTA, 0.2 M, pH 7.4, was added with continued swirling so that the final EDTA concentration was 10-4 M. The cell suspension was divided into 1000-ml parts for each 2-liter flask. After 10 min on a rotary shaker the cells were then resuspended in 0.03 M Tris, pH 7.5, over 8 hours. Fractions of 6 ml were collected. The enzyme was dialyzed for 6 hours at 3° against 4-liter volumes of 0.01 M potassium phosphate buffer, pH 7.1, in three changes.

**Hydroxylapatite Column Chromatography**—The dialyzed material (54 ml) was applied to a (2.5 x 20 cm) column of hydroxylapatite which had been pre-equilibrated with 0.01 M potassium phosphate buffer, pH 7.1. The rate of application was 0.5 ml per min. The enzyme was eluted with a 0.01 to 0.2 M potassium phosphate gradient of 400 ml total volume. Fractions of 3 ml were collected with a flow rate of 15 ml per hour. Activity was determined by use of the bis(p-nitrophenyl)phosphate assay. Active fractions were pooled and dialyzed against four changes of 2 liters of 0.005 Tris, pH 7.5, over 8 hours.

**Second DEAE-cellulose Chromatography**—Fraction IV (24 ml) was applied at the rate of 0.5 ml per min to a column (2.5 x 30 cm). The material was washed into the column with 30 ml of the starting buffer, and a gradient of NaCl, 0 to 0.2 M, was used. The total volume was 300 ml, and 2.5-ml fractions were collected at a rate of 1 ml per min. This yielded material in which the peak tube had a specific activity of 106,000, and the pooled average (12 ml) was 66,600.

Attempts at further purification of the enzyme and crystallization were made. However, use of dialysis against a 70% ammonium sulfate solution, pre-evaporation techniques, and use of Biogel resulted in marked loss of activity. Use of a DEAE-Sephadex column for the second DEAE-cellulose chromatograph did not yield material of either higher specific activity or homogeneity.

**Properties of the Enzyme**

**Stability**—The osmotic shock fluid (Fraction I) and the heat-treated material (Fraction II) were stable for several months at -20°. However, there was always some loss of activity if these fractions were thawed and frozen several times. The DEAE-cellulose fractions were stable frozen at -40° for up to 6 months, but showed rapid loss with repeated freezing and thawing. At 0-5° the first DEAE-cellulose fraction began to lose about 5% of the activity each week after the 1st week. The second DEAE-cellulose fraction was stable at 0-5° for about 3 weeks. Thereafter a slow decay occurred. It could be stabilized by making the solution 0.1 mg per ml with bovine albumin. At room temperature the purified enzyme lost 80% of the activity in 1 week. The enzyme was most stable at pH 7.0 to 7.6. Fractions stored at -20° at pH 6.0 in acetate showed fairly rapid deterioration.

Dilution of the enzyme results in rapid loss of activity if the diluting solution fails to contain 0.1 mg per ml of albumin. When the purified enzyme was diluted 10-fold into sodium acetate buffer, 0.05 M, pH 6.0, it lost 60% of the activity in 3 hours at 3°. Dilution in sodium succinate, pH 6.0, also resulted in loss of 50% of the activity in 3 hours. Dilution into 0.01 M Tris, pH 7.4, showed a loss of 25% in 3 hours, but 400-fold dilution into 0.1 mg per ml of albumin showed only a 3% loss in 6 hours and 5% in 24 hours at 3°. Addition of β-mercaptoethanol to the fractions did not prevent decay. Use of 20% glycerol as a stabilizing solution was almost as satisfactory as albumin for long term 0-5° storage, but did not halt eventual decay or change the rate of decay.

**Contamination with Other Enzymes**—The purified enzyme (Fraction V) was tested for a number of contaminating enzymes.
DNase activity was measured with both native and denatured E. coli K-12 λ-DNA. Enough enzyme to hydrolyze 72 μmoles of 5′-AMP per hour was used. There was no detectable hydrolysis of either DNA after 3 hours. Therefore, DNase activity is essentially absent. E. coli RNase I was shown to be absent by incubating an amount of enzyme sufficient to hydrolyze 140 μmoles of 5′-AMP with E. coli tRNA and poly A for 1 hour under the optimal conditions for RNase I with the assay of Neu and Heppel (14). RNase II was shown to be absent by use of the assay of Singer and Tolbert (15). The absence of the E. coli cyclic phosphodiesterase (23), pH 4, p-nitrophenyl phosphatase activity and the acid hexose phosphatase activity was shown by incubation of enough purified enzyme to cleave 200 μmoles of 5′-AMP with each of these with the use of both optimal conditions for each of the other activities. Adenosine deaminase activity was shown to be absent by incubation of sufficient enzyme to cleave 100 μmoles of 5′-AMP with adenine at pH 6.0, 7.0, and 7.5 in succinate, Tris, and phosphate for 2 hours at 37°. No change in the extinction at 265 μμ or 240 μμ was noted (24).

Comparison of Ratios of Specific Activities against Various Substrates—Table II shows the ratio of specific activities for the cleavage of 5′-AMP against ATP, bis(p-nitrophenyl)phosphate, and UDP-glucose. The ratio of specific activity remains essentially constant throughout the purification. The variation seen with the bis(p-nitrophenyl)phosphate is due to the fact that the osmotic shock fluid contains a small amount of cyclic phosphodiesterase which also cleaves this substrate. In another purification, in which the procedure was altered so that the first DEAE-cellulose chromatography was followed by chromatography on DEAE-Sephadex and then on hydroxylapatite, which resulted in a homogeneous protein with a specific activity for 5′-AMP of 42,300, the ratio of AMP:UDP-glucose was 28.8 and of AMP:ATP, 1.39. In preparations which have decayed because of repeated freezing and thawing, the ratios of specific activities have remained constant.

Effect of Enzyme Concentration and Incubation Time—The rate of the reaction for the hydrolyses of both 5′-AMP and ATP was proportional to enzyme concentration and was constant for at least 30 min at 37°. In the absence of albumin there was a marked fall in the rate of hydrolysis of 5′-AMP, which is consistent with the stabilization of the enzyme by albumin. At 21° the linearity of the assay was less well maintained, with some lag seen in the initial 5 min. The release of p-nitrophenol from bis(p-nitrophenyl)phosphate at 37° was similar to the hydrolysis of 5′-AMP.

Effect of pH on Rate of Reaction—The enzyme had a pH optimum for 5′-AMP between 5.7 and 6.1 with sodium acetate, sodium succinate, or Tris-maleate buffer systems at a concentration of 0.1 M. The pH optimum for ATP was slightly higher at 6.7 to 7.1 with Tris-maleate. The enzyme showed less than 20% of optimal activity below pH 4.5. Unlike the bull semen

![Graph](http://www.jbc.org/)

**Table II**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>AMP:ATP</th>
<th>AMP:UDP-glucose</th>
<th>AMP: bis(p-nitrophenyl)phosphate</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Osmotic shock fluid</td>
<td>1.86</td>
<td>27.3</td>
<td>49.3</td>
</tr>
<tr>
<td>II. Heated osmotic shock fluid</td>
<td>1.41</td>
<td>32.7</td>
<td>48.6</td>
</tr>
<tr>
<td>III. First DEAE-cellulose</td>
<td>1.65</td>
<td>31.4</td>
<td>46.5</td>
</tr>
<tr>
<td>IV. Hydroxylapatite</td>
<td>1.45</td>
<td>34.6</td>
<td>58.0</td>
</tr>
<tr>
<td>V. Second DEAE-cellulose</td>
<td>1.30</td>
<td>34.7</td>
<td>64.0</td>
</tr>
</tbody>
</table>
5'-nucleotidase (25, 26), no other pH optimum at an alkaline pH was found. The pH optimum for hydrolysis of bis(p-nitrophenyl)phosphate was broad (from pH 6.0 to 7.4). The optimal pH for UDP-glucose pyrophosphatase activity was, as reported by Molo and Glaser (8), between 7 and 8.

Heat Stability—The heat stability of the 5'-nucleotidase is highly dependent upon the protein concentration of the enzyme and the pH of the suspending buffer. In the osmotic shock fluid the enzyme is stable for up to 20 min at 55°. This is true both in the presence and absence of Co++ and Mg++. A dilute solution of the purified enzyme was stable in albumin at 55° for 10 min, but in acetate buffer, pH 6.0, or Tris-HCl at pH 8.0, 90% of the activity was lost. The enzyme is effectively destroyed by 5 min at 90°. When 83 μg were used in the incubation, 82% of the activity remained at pH 7.2 in 0.05 M Tris-HCl at 60° for 10 min, and 50% at pH 5.8 and 8.0. All activity was lost at pH 5.1 in sodium succinate. Cobalt, calcium, and magnesium all failed to stabilize the purified enzyme when it was treated in the absence of albumin. A parallel decay of activity for hydrolysis of 5'-AMP, ATP, and UDP-glucose was found when the enzyme was heated in albumin, Tris-HCl, and acetae at 50°.

In contrast to the case with the cyclic phosphodiesterase of E. coli (23), we did not see any consistent activation by exposure to heat at 45°, 50°, and 60°. Although some activation occurs in the early fractions, this could be due to traces of inhibitor which may be present. The 5'-nucleotidase does not show recovery from heat denaturation when incubated at 0° or 25°, as does the E. coli alkaline phosphatase (27).

Effect of Divalent Cations on Enzyme Activity—Cobalt is the most effective cation in stimulating the 5'-nucleotidase (Table III) in its effect on both 5'-AMP and ATP. Only Mn++ is almost as effective. The addition of Ca++ to the system results in a doubling of the stimulation when 5'-AMP is the substrate. This doubling was not seen with ATP. Since the calcium salt of bis(p-nitrophenyl)phosphate was used, no attempt was made to evaluate the role of calcium. A number of cations are inhibitory as is shown by the effect of Ni++, Fe++, and Zn++. This occurs in the presence of Co++ also. Zn++ at 0.1 M results in total inhibition. The cation effect was shown not to be pH-dependent since Mg++, Ni++, and Fe++ failed to replace the Co++ effect at pH 6.0, 7.4, and 8.0 in 0.1 M Tris-maleate buffer. The hydrolysis of UDP-glucose, on the other hand, as previously shown by Molo and Glaser (8), is fully Mg++-dependent and Co++ is not needed.

The dependence of the reaction upon Co++ for the cleavage of both 5'-AMP and ATP is shown in Fig. 2. The optimal concentration of Co++ for 5'-AMP was 5 mM. This was true in the presence and absence of both albumin and calcium. The optimal Co++ concentration for ATP was also 5 mM.

In the case of both 5'-AMP and ATP, variation of the amount of substrate over a 10-fold range did not alter the optimal Co++ concentration. This trend would tend to speak against a stoichiometric combination of the cobalt with the phosphate of the nucleotides, but this has not been completely eliminated.

Effect of Various Compounds on Enzyme Activity—Metal chelating agents such as EDTA, citrate, or ascorbic acid caused marked inhibition of the enzyme activity against both 5'-AMP and ATP. Urea (0.6 M) results in total inhibition of the enzyme, but the effect is completely reversible by removal of the urea through dialysis or lowering the concentration by dilution. At 0.4 M urea, 90% of the activity was present. Mercaptoethanol had no significant effect on enzymatic activity of the purified enzyme. Of several other enzyme inhibitors, 1 mM, NaCN caused 20% inhibition; 1 mM, KF, 40% inhibition; 1 mM, NaN3, 15% inhibition; 1 mM, CuSO4, 90% inhibition; iodoacetate, 90% inhibition. The effects of the compounds were seen both at pH 6.0 in sodium succinate and pH 7.2 in Tris-maleate. Dinitrophenol and oligomycin had no significant effect on hydrolysis of either ATP or 5'-AMP. The effect of albumin is felt to be stabilization of the enzyme, which is rapidly denatured by glass surfaces. If albumin is added during the reaction, no specific stimulation is seen.

Effect of Ionic Environment—Although the alkaline (27, 28) and acid phosphatases (29) of E. coli are affected by the ionic environment of the assay system, the 5'-nucleotidase shows no stimulation with a variety of salts. NaCl, NH4Cl, (NH4)2SO4, and KCl in a range from 0 to 100 mM, failed to increase activity; in fact, KCl appears to show a minimal inhibitory effect. Buffer concentration with Tris-maleate and sodium acetate could be varied from 0.02 M to 0.1 M without significant effect.

Substrate Specificity—A number of nucleotides and sugar phosphates have been examined (Table IV). In all cases the enzyme is free of nonspecific acid and alkaline phosphatase. No pyrophosphatase activity (30) was seen by the failure to cleave PPi or polyphosphates. Cyclic phosphodiesterase activity (23) has been eliminated, for 3'-AMP and 2',3'-cyclic phosphonucleotides are not hydrolyzed. Substitution of position 3' of a
5'-nucleotide, as pAp or pTp, results in inhibition of hydrolysis. Oligonucleotides with free 5'-phosphates are not cleaved. Nucleoside diphosphates are cleaved in a manner identical with that of ATP. Methylation of 5'-CMP did not inhibit its use as a substrate. UDP-glucose is hydrolyzed to give ultimately uridine and glucose, but no other nucleotide sugars were investigated.

Effect of Substrate Concentration—The calculated $K_m$ values are $3.3 \times 10^{-5}$ for 5'-AMP and $1.2 \times 10^{-4}$ M for ATP. At higher concentrations of ATP, definite substrate inhibition is seen. This is not due simply to formation of complexes with the cobalt, for increasing the Co$^{2+}$ concentration did not alter the inhibition. Over a wide range the initial substrate concentration has no effect on the over-all rate as regards the hydrolysis of 5'-AMP. The kinetic plots for the 5'-AMP and ATP activity, when the assays are run in the presence of cobalt, do not fit a Lineweaver-Burk plot (31). This may be a reflection of the lack of sensitivity of the phosphatase assay systems at very low concentrations of substrate or be due to an allosteric effect of the enzyme, substrate, and cobalt. Attempts are being made to couple an adenosine deaminase assay with 5'-nucleotidase hydrolysis in a manner sensitive enough to answer this question.

Effect of Substrate Analogues and Nucleic Acids on 5'-Nucleotidase Activity—Adenosine, 3'-AMP, and 2'-AMP do not show an inhibitory effect on the hydrolysis of either 5'-AMP or ATP. However, poly A, transfer RNA, and DNA inhibit to some extent. Although the effect may be merely polyanionic, the poly A inhibition may have other mechanisms.

Effect of Phosphate—In contrast to the acid and alkaline phosphatases of E. coli (27-29), the 5'-nucleotidase was uninhibited by the presence of phosphate. At 0.1 M potassium phosphate there was no significant inhibition of activity. Inhibition at higher concentrations is undoubtedly due to precipitation of a phosphate-cobalt complex. Similar results were seen with 5'-UMP and 5'-CMP. Phosphate had no inhibitory effect on the hydrolysis of ATP or UDP-glucose. The bis(p-nitrophenyl)-phosphate assay was run in the presence of phosphate. Assay for 5'-AMP and ATP cleavage could be achieved with a coupled assay with adenosine deaminase (24).

Products of ATP Cleavage—Although no pyrophosphatase activity was seen with the purified enzyme, as evidenced by failure to cleave PP$_i$, it was possible that the cleavage of ATP resulted in AMP and PP$_i$. This was unlikely since nucleoside diphosphates of the ADP type were hydrolyzed in the presence of Co$^{2+}$. To exclude the formation of PP$_i$, the hydrolysis of ATP was stopped before completion. The nucleotide material was adsorbed to acid-washed Norit and separated by centrifugation. The supernatant was then assayed for both total free phosphate and acid-labile phosphate. No acid-labile phosphate was found.

In another experiment, cleavage of ATP was halted before completion, and the reaction mixture was applied to a DEAE-cellulose column. The products were eluted with a linear gradient of 0.01 to 0.15 M NH$_4$HCO$_3$ pH 8.6, at room temperature (32), and the peaks of adenosine, 5'-AMP, ADP, and ATP were separated. The amount of ATP remaining was 26% of that in the incubation. The percentage composition of the hydrolysis was ADP, 11%; 5'-AMP, 26%; and adenosine, 37%. These data suggest that the reaction from ATP→ADP→AMP is more rapid than the AMP to adenosine reaction; but the exact kinetics with labeled substrates is yet to be solved. It is postulated that the 5'-nucleotidase is able to hydrolyze ATP and ADP only in the presence of cobalt because of a cobalt-phosphate chelate formed. However, with the use of column chromatography and paper chromatography, thus far, we have been unable to isolate such intermediates.

Characterization of Purified Enzyme

Polyacrylamide Gel—Electrophoresis of the material from the osmotic shock fluid and second DEAE cellulose fraction is shown in Fig. 3. This protein band corresponded to the position...
TABLE IV
Activity of E. coli 5'-nucleotidase against different substrates

The assays for 5'-nucleotides contained (in a total volume of 0.1 ml) 5 mM substrate, 5 mM CoCl₂, 20 mM CaCl₂, 100 mM sodium acetate buffer, pH 6.0, and 10 μg of bovine serum albumin. The triphosphate assays contained (0.1 ml) 1 mM substrate, 5 mM CoCl₂, and 100 mM Tris-maleate buffer, pH 6.8. In addition to the substrates listed in the table, the following nucleotides and sugar phosphates were tested and showed no hydrolysis: 2',3' > UMP, 3',5' > AMP, uridine 2'(3')5'-diphosphate, thymidine 3',5' diphosphate, cytosine 3',5' diphosphate, pApApAp, ApApAp, β-glycerol phosphate, galactose 6-phosphate, fructose 1-phosphate, glucose 1-phosphate, glucose 6-phosphate, ribose 5-phosphate, ethanolamine phosphate, p-nitrophenyl phosphate at pH 4 and pH 8, and o-carboxyphenyl phosphate. All substrates were assayed at both pH 6.0 and 7.0.

<table>
<thead>
<tr>
<th>Substrate Relative activity</th>
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<tbody>
<tr>
<td>5'-AMP .......................... 100</td>
</tr>
<tr>
<td>5'-GMP .......................... 86</td>
</tr>
<tr>
<td>5'-CMP .......................... 56</td>
</tr>
<tr>
<td>5'-UMP .......................... 48</td>
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</tr>
<tr>
<td>dCMP ........................... 56</td>
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<tr>
<td>dGMP ........................... 60</td>
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<tr>
<td>5'-methyl-CMP ................... 48</td>
</tr>
<tr>
<td>IMP ............................. 79</td>
</tr>
<tr>
<td>2'-AMP ........................ 0</td>
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</tr>
<tr>
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</tr>
<tr>
<td>PPP' ............................ 0</td>
</tr>
<tr>
<td>Polymetaphosphateb ........... 0</td>
</tr>
</tbody>
</table>

a Assays against ATP, etc. are related to ATP as a 100 rather than to 5'-AMP.
b Average chain length, 8.

of the enzymatic activity. The ratios of relative activity for cleavage of 5'-AMP, UDP-glucose, and ATP were unchanged. Some preparations of the enzyme showed an extremely faint band just cathodal to the 5'-nucleotidase, but this was variable. Polycrylamide gel electrophoresis at pH 4.5 also produced only a single band.

Sucrose Gradient Centrifugation—Sedimentation of the 5'-nucleotidase through a sucrose density gradient showed a uniform peak containing 5'-AMP, ATP, and UDP-glucose activity. Molecular weight calculations with the method of Martin and Ames (18) gave values of 53,300 and 51,200.

Molecular Sieve Chromatography—With the use of Sephadex G-100, according to a method of Andrews (19) modified so as to avoid any ionic interaction by use of 0.4 M NaCl, a molecular weight of 52,000 was obtained. Fig. 4 shows that the enzyme activity for 5'-AMP, ATP, and the protein exactly coincide.

Sedimentation Behavior—With the use of ultraviolet optics the sedimentation coefficient of the 5'-nucleotidase was obtained. Fig. 5 shows a tracing of the photograph of the ultraviolet absorption at 44, 60, 76, 92, and 108 min after reaching speed. Readings were taken from 0 to 116 min at 4-min intervals, and an s₂₀,₅ of 4.2 was calculated. Considering the size of the molecule the preparation shows excellent homogeneity.

FIG. 3. Diagrammatic representation of polycrylamide electrophoresis patterns of E. coli K-12 5'-nucleotidase. Each gel represents 100 μg of material in 0.005 M Tris, pH 7.5, run in the standard method of Canal Industrial Corporation for 120 min at 5° using a Buchler temperature-regulated electrophoresis apparatus. ---, trace material seen in some preparations run after the enzyme has been frozen and thawed; it has been inconstant.

FIG. 4. Sephadex G-100 chromatography of the purified 5'-nucleotidase of E. coli. X, 5'-AMP units; O, UDPG units; ▲, absorbance at 280 μₐ. (For details, see the text.)
**Table V**

**Amino acid composition of 5'-nucleotidase of E. coli**

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Amount of amino acid</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine</td>
<td>0.211</td>
<td>6.1</td>
</tr>
<tr>
<td>Histidine</td>
<td>0.0386</td>
<td>1.6</td>
</tr>
<tr>
<td>Arginine</td>
<td>0.087</td>
<td>20.7</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>0.322</td>
<td>9.7</td>
</tr>
<tr>
<td>Threonine</td>
<td>0.153</td>
<td>4.6</td>
</tr>
<tr>
<td>Serine</td>
<td>0.120</td>
<td>3.6</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>0.306</td>
<td>9.2</td>
</tr>
<tr>
<td>Proline</td>
<td>0.128</td>
<td>3.8</td>
</tr>
<tr>
<td>Glycine</td>
<td>0.229</td>
<td>6.9</td>
</tr>
<tr>
<td>Alanine</td>
<td>0.377</td>
<td>8.3</td>
</tr>
<tr>
<td>Half-cystine</td>
<td>0.235</td>
<td>7.1</td>
</tr>
<tr>
<td>Valine</td>
<td>0.204</td>
<td>6.1</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.448</td>
<td>13.5</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>0.113</td>
<td>3.4</td>
</tr>
<tr>
<td>Leucine</td>
<td>0.219</td>
<td>6.6</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.0676</td>
<td>2.0</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>0.0863</td>
<td>3.0</td>
</tr>
<tr>
<td>Ammonia</td>
<td>0.699</td>
<td>21</td>
</tr>
</tbody>
</table>

**Amino Acid Composition**—Table V shows the amino acid composition of the purified 5'-nucleotidase. The values are presented as micromoles and molar per cent of the sample. The amount of tryptophan was not calculated. No unusual aspects are noted that would offer suggestions either as to the role of Co++ in the enzyme or its surface location.

**Discussion**

There are few reports dealing with bacterial nucleotidases (3, 5-7), in contrast to the numerous studies of mammalian enzymes of this type (25, 33-36). In most reports the enzyme has been active in the absence of added metal ions, and the degree of metal stimulation varies from less than double to about 10-fold. The mammalian enzymes further have differed in that their pH optima have generally been alkaline. For the most part, Mg++ or rarely Mn++, has been the most effective metal activator. When Co++ or Ca++ have been used, they have often been of lesser importance. It has been noted that Zn++ is a good inhibitor of such enzymes of both mammalian and bacterial origin, in contrast to the stimulatory effect of Zn++ on alkaline phosphatases (27).

The 5'-nucleotidase of E. coli has two definite requirements for hydrolysis: (a) the carbon 1' of the ribose must possess a nitrogenous base; and (b) the hydroxy group on carbon atom 3' must be free. Removal of the nitrogenous base of ribose makes the compound resistant. Substitution of deoxyribo-AMP for ribo-AMP results in a 60% decrease in hydrolysis, whereas the change from ribose to deoxyribose of the other bases was much less marked. Substitution by a phosphoryl group as in pXp makes the compound resistant, as does substitution by another nucleotide as in pXpY. This is analogous to the situation seen with the 5'-nucleotidase from the venom of Bothrops atrox (37). The hydrolysis of ATP and ADP shown by the E. coli 5'-nucleotidase is similar to that of Clostridium sticklandi (3), but in contrast to the mammalian (25) and reptilian (37) 5'-nucleotidases.

The 5'-nucleotidase, in contrast to E. coli alkaline phosphatase (27, 28), is not inhibited by phosphate, and in this regard resembles the inorganic pyrophosphatase of E. coli (30). This makes it a useful reagent in the quantitative analysis of mononucleotides occurring in a nucleic acid digest.

The fact that this enzyme has activity against several substrates is unusual, since the hydrolysis of 5'-AMP and the hydrolysis of UDP-glucose are quite unrelated. The constant ratios of specific activities for AMP, ATP, UDP-glucose, and bis(p-nitrophenyl)phosphate speak for one enzyme activity. The parallel losses of activity on heating at several temperatures and in different buffer systems also strongly suggest that the various enzyme activities are associated with the same protein. Other examples of enzymes with several activities are seen in E. coli (23, 38, 39). The metal stimulation by Co++ and other ions is highly similar for 5'-AMP and ATP hydrolysis. Certainly the results obtained by polyacrylamide gel electrophoresis, ultracentrifugation, sucrose density centrifugation, and molecular sieve chromatography speak for one enzyme protein.

The fact the bis(p-nitrophenyl)phosphate, a synthetic chromogenic compound, is a substrate for this enzyme is rather unusual, since this substrate is usually used for phosphodiesterase activity. This should point out the nonspecific nature of the hydrolysis of bis(p-nitrophenyl)phosphate and the hazards of using it as a specific diesterase substrate.

The purification of this enzyme with the osmotic shock technique illustrates the high purity that can be achieved. In the case of this enzyme, the use of a sonic extract as the starting material would be complicated by the presence of an intracellular inhibitor for the enzyme (40).

The molecular weight, gel electrophoresis characteristics, and amino acid composition do not offer any suitable explanation for the surface location (1, 2, 40) of this enzyme or for its several enzymatic properties. The molecular weight of 52,000 is considerably above that of galactoside transacetylase and only slightly less than that of the 5'-nucleotidase inhibitor (40), neither of which is released by osmotic shock.

It is possible that the role of the enzyme in vitro is the hydrolysis of both UDP-glucose and mononucleotides such as 5'-AMP that would exist in the periplasmic space, and that the hydrolysis of nucleoside di- and triphosphates is seen only with the system in vitro. It remains unknown, however, whether concentrations of cobalt or manganese could exist at a cytoplasmic location which would allow these activities to occur.

**Acknowledgments**—The author wishes to express his thanks to Mr. James Chou for excellent technical assistance, to Dr. R. Canfield for the amino acid analysis, to Dr. H. Rosenkranz for the analytical ultracentrifugation, and to Dr. Leon A. Heppel for critical analysis of the manuscript.

**References**


The 5'-Nucleotidase of *Escherichia coli*: I. PURIFICATION AND PROPERTIES
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