The 5′-Nucleotidases (Uridine Diphosphate Sugar Hydrolases) of the *Enterobacteriaceae**

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ABSTRACT: The 5′-nucleotidases (nucleotide diphosphate sugar hydrolases) of various *Enterobacteriaceae* have been purified and characterized. Previous studies had shown that 5′-nucleotidases of *Escherichia coli*, *Shigella*, and *Citrobacter* were released from the cells by the technique of osmotic shock which releases periplasmic or surface enzymes. Fifty per cent of the 5′-nucleotidase of *Klebsiella–Enterobacter* groups was released, and the 5′-nucleotidase of *Proteus* species could not be released by osmotic shock. These studies show that the enzymes from all *Enterobacteriaceae* exhibit similar properties in regard to pH optimum, ion stimulation, substrate specificity, and physical properties. The 5′-nucleotidases hydrolyze all 5′ ribo- and deoxyribonucleotides in which there is an unsubstituted hydroxyl on the 3′ carbon. Nucleoside di- and triphosphates are hydrolyzed to the nucleoside and free phosphate without the formation of pyrophosphate. Uridine diphosphoglucose is hydrolyzed to uridine, glucose 1-phosphate, and phosphate. The greatest stimulation of hydrolysis is caused by Co^{2+} and Mn^{2+}. Zn^{2+} and chelating agents are inhibitory. Phosphate does not inhibit. The pH optimum for hydrolysis of 5′-nucleotides is 5.8–6.1. The pH optimum for hydrolysis of uridine diphosphate glucose is 7–8. All *Enterobacteriaceae* contain a protein inhibitor of the enzyme. The 5′-nucleotidase inhibitor of one organism partially inhibits hydrolytic activity of the 5′-nucleotidase of another species.

Although 5′-nucleotidases (5′-ribonucleotide phosphohydrolases) have been found in a variety of mammalian tissues (Heppel and Hilmoe, 1951; Segal and Brenner, 1960; Center and Behal, 1966; Itoh *et al.*, 1967), they have been minimally studied in bacteria. Recently we described the purification of the 5′-nucleotidase of *Escherichia coli* (Neu, 1967a). This enzyme is located in the periplasmic space (Neu, 1967b) and also acts as a uridine diphosphate sugar hydrolase (Glaser *et al.*, 1967; Neu, 1967a). Neu and Chou (1967) showed that all *Enterobacteriaceae* contain 5′ nucleotidases but that members of the *Klebsiella–Enterobacter* group release only 50% of the enzyme activity when subjected to an osmotic shock which normally releases periplasmic enzymes. *Proteus* species contain a 5′-nucleotidase, but it cannot be released by osmotic shock. Neu (1968) demonstrated that the 5′-nucleotidase activity of various *Enterobacteriaceae* was similar in regard to lack of control by phosphate level or catabolite repression. It seemed of value to purify the 5′-nucleotidases of a number of the *Enterobacteriaceae* to compare the enzymes in more detail.

This paper outlines the purification of the 5′-nucleotidases of the *Enterobacteriaceae*. In all cases they show similar properties in regard to pH optimum, ion stimulation, substrate specificity, and molecular size.

Experimental Procedures

Materials. Bis(*p*-nitrophenyl)phosphate, *p*-nitrophenyl phosphate, and nucleotides were purchased from commercial sources. *E. coli* tRNA was purchased from General Biochemicals. DEAE-cellulose was obtained from Reeve Angel and Sephadex from Pharmacia. Hydroxylapatite was a product of Clarksen Chemical Co.

Organisms. Isolates from the diagnostic laboratory of the Presbyterian Hospital were used. Identification was based on the methods of Edwards and Ewing.

Culture Conditions. Organisms were grown to early stationary phase on the previously described phosphate medium (Neu and Chou, 1967) or Penassay broth (Difco).

Enzyme Assays. The standard assay for 5′-nucleotidase contained 5 mM 5′-AMP,[1] 5 mM $CoCl_2$, 20 mM $CaCl_2$, 100 mM sodium acetate (pH 5.8), and 10 μg of bovine serum albumin/ml. After 20 min at 37° the reaction was stopped with 0.05 N HCl and the phosphate was assayed by the method of Chen *et al.* (1956). Activity is expressed as micromoles of phosphate hydrolyzed per hour at 37°. The assay was modified as noted in the text for comparison of enzymes from various sources. The assays for hydrolysis of ATP, uridine diphosphoglucose, and bis(*p*-nitrophenyl)phosphate are those previously described by Neu (1967a). The methods for 3′-nucleotidase (Neu, 1968), acid hexose phosphatase (Dvorak *et al.*, 1967), alkaline phosphatase and RNase I (Neu and Heppel, 1964a), RNase II (Singer and Tolbert, 1965), and DNA–endonuclease I (Weissbach and Korn, 1963) were those published. Protein

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[1] Abbreviations are listed in *Biochemistry* 5, 1445 (1966).

TABLE I: Purification Schema for the Various 5′-Nucleotidases.[a]

Organism		Fraction	Units/ml	Protein (mg/ml)	Sp Act. (units/mg of protein)
S. sonnei		Sonic extract	20.3	3.94	5.2
	I	Osmotic shock fluid	60	0.1108	556
	II	First DEAE-cellulose	341	0.117	2,920
	III	Hydroxylapatite	995	0.109	9,140
	IV	Second DEAE-cellulose	803	0.048	16,000
E. aerogenes		Sonic extract	2.6	4.62	0.56
	I	Osmotic shock fluid	24	0.123	182
	II	First DEAE-cellulose	204	0.04	5,100
	III	Hydroxylapatite	218	0.04	5,650
	IV	Second DEAE-celllulose	414	0.038	10,900
S. heidelberg		Sonic extract	27	1.59	17
	I	Osmotic shock fluid	38	0.10	380
	II	DEAE-cellulose	55	0.10	550
	III	Sephadex G-100	56	0.006	9,330
P. vulgaris	I	Sonic extract	264	27.1	9.7
	II	First DEAE-cellulose	78	0.82	95
	III	Second DEAE-cellulose	105	0.54	133
	IV	Sephadex G-100	324	0.2	1,620

[a] The various steps are described in Methods.

concentration was determined by the method of Lowry *et al.* (1951).

Purification of the Enzymes. All operations were carried out at 0–5°. The pH of the buffer systems is that obtained at the ionic strength and temperature specified.

Preparation of 5′-Nucleotidase Released by Osmotic Shock. Shigella sonnei were grown overnight to early stationary phase in the high phosphate medium. The cells were harvested and washed with 0.01 M Tris-HCl (pH 7.3)–0.03 M NaCl. Washed cells were suspended in 0.03 M Tris-HCl (pH 7.3) at a ratio of 1 g (wet weight) to 40 ml. An equal volume of 1 M sucrose in 0.03 M Tris-HCl was added. The suspension was made 1 mM with respect to EDTA and mixed at 21° for 10 min. The cells were removed by centrifugation at 0°. The pellet of cells was resuspended in cold water for 10 min. The cells were centrifuged and the supernatant osmotic shock fluid was used as the starting material.

The osmotic shock fluid (2000 ml) was applied to a DEAE-cellulose chromatographic column (Whatman No. 23) which was 2.5 × 40 cm and had been prepared with 0.01 M Tris-HCl (pH 7.5). The 5′-nucleotidase was eluted with a linear gradient (1000 ml) of 0–0.2 M NaCl. Flow rate was 50 ml/hr and fractions of 6 ml were collected. The enzyme was eluted as a sharp peak in the middle of the gradient. Active fractions were pooled and dialyzed for 6 hr at 3° against two 4-l. changes of 0.01 M potassium phosphate buffer (pH 7.2). The dialyzed material (42 ml) was applied to a (2 × 20 cm) column of hydroxylapatite which had been preequilibrated with 0.01 M potassium phosphate buffer (pH 7.2). The rate of application was 0.5 ml/min. The enzyme was eluted with a linear 0.01–0.2 M potassium phosphate gradient of 300 ml. Fractions of 3 ml were collected at a rate of 0.5 ml/min. Activity was determined by use of the bis-(*p*-nitrophenyl)phosphate assay. Active fractions (18 ml) were pooled and dialyzed for 8 hr against four changes of 2-l. each of 0.01 Tris-HCl (pH 7.5). This material was then applied at a rate of 1 ml/min to a 1 × 15 cm microgranular DEAE-cellulose column (Whatman DEAE-32). A gradient of 0–0.2 M NaCl–0.01 M Tris-HCl (pH 7.5) was used. Total volume was 200 ml and 2-ml fractions were collected. Over-all recovery of enzyme by this procedure ranged from 25 to 40%. Peak tubes after the second DEAE-cellulose chromatography in the case of *E. coli* had specific activities of about 100,000. An identical procedure was followed for *Klebsiella*, *Citrobacter*, and *Serratia* to obtain the enzyme released by osmotic shock. Specific activities ranged from 10,000 to 20,000 (Table I).

The *Salmonella heidelberg* 5′-nucleotidase did not hydrolyze bis(*p*-nitrophenyl)phosphate. Thus, after the first DEAE-cellulose chromatography, the material was concentrated by negative pressure using collodion bags (Carl Schleicher & Schuell Co.). The concentrated material was then applied to a 2.5 × 90 cm Sephadex G-100 column and eluted with 0.4 M NaCl–0.05 M Tris-HCl (pH 7.5) (Table I). A specific activity of 10,000 was obtained and the enzyme gave a single band on polyacrylamide gel electrophoresis.

Purification of 5′-Nucleotidase Not Released by Osmotic Shock. Purification of the *Proteus vulgaris* 5′-nucleotidase was performed using sonically disrupted cells. Washed cells (58 g) were resuspended in 290 ml of 0.01

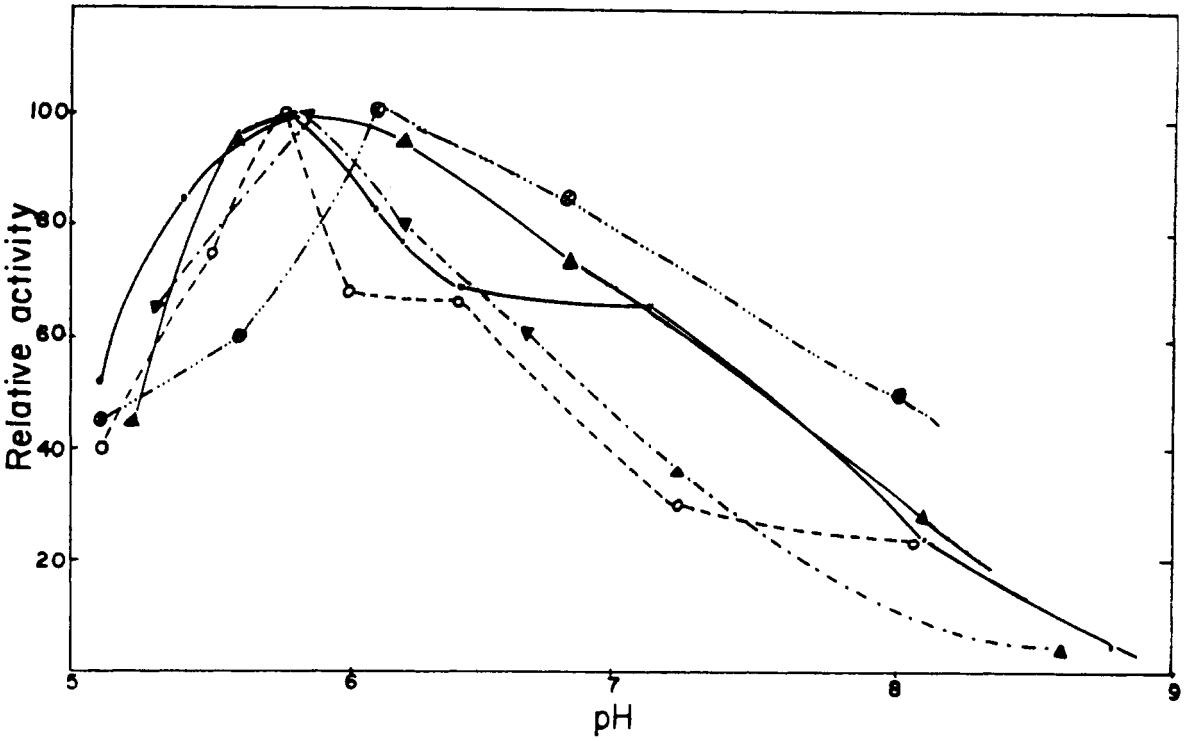

FIGURE 1: Effect of pH on the activity of various *Enterobacteriaceae* 5′-nucleotidase activity. Activity was measured in the usual 5′-AMP assay with Tris-maleate as buffer. The pH was determined at 37°. *S. sonnei* (purified from osmotic shock fluid), ●; *S. heidelberg* (osmotic shock), ○; *E. aerogenes* (osmotic shock), ▲; *E. aerogenes* (sonic extract), ▼; *P. vulgaris* (sonic extract).

M Tris-HCl (pH 7.5). The cells were sonically disrupted. The cell debris and intact cells were removed by 20-min centrifugation at 19,000 rpm in a Sorvall RC2B centrifuge. The supernatant fluid was brought to 40% ammonium sulfate saturation with solid $(NH_4)_2SO_4$. The pH was adjusted to 7.5 with 3 N NH_4OH. After standing for 30 min at 0°, the material was centrifuged for 20 min at 19,000 rpm. The pellet was discarded and the supernatant solution was brought to 70% $(NH_4)_2SO_4$ saturation and left at 0° for 60 min. The pellet recovered after 30-min centrifugation was resuspended in 110 ml of 0.01 M Tris-HCl (pH 7.5) and dialyzed against three changes of 4 l. of the same buffer. The enzyme was applied to a DEAE-cellulose column (2.5 × 35 cm). A linear gradient (total volume 1000 ml) of 0–0.25 M NaCl in 0.01 M Tris-HCl (pH 7.5) was used and 6-ml fractions were collected. In contrast to the chromatography of the osmotic shock fluid, the enzyme was eluted at a higher salt concentration and the separation of 5′-nucleotidase and acid hexose phosphatase was less distinct. The fractions were pooled (112 ml) and dialyzed against 4 l. of 0.01 M Tris-HCl (pH 7.5). The dialyzed material was applied to a 2 × 15 cm DEAE-cellulose column (Whatman No. 32) and eluted with a linear 0–0.25 M NaCl gradient. A distinct peak was obtained (31 ml) which was concentrated by negative pressure using a collodion bag. The concentrated enzyme fraction was applied to a Sephadex G-100 column (2.5 × 90 cm) and 2-ml fractions were collected eluting with 0.4 M NaCl–0.05 Tris-HCl (pH 7.5). Table I shows the purification.

5′-Nucleotidase which was not released by osmotic shock from *Enterobacter aerogenes*, *Serratia marcescens*, and *S. heidelberg* was purified by sonically disrupting the cells after osmotic shock and using the purification system described for the *Proteus* enzyme.

Results

Properties of the Enzymes

Stability. The osmotic shock fractions and the purified enzymes of all organisms were stable frozen at −40° for up to 1 year. Freezing and thawing resulted in marked loss of activity. Hydroxylapatite fractions in phosphate buffer were unstable even when frozen. The enzyme showed least decay at pH 7.5 in Tris-HCl supplemented with glycerol or albumin.

Marked dilution of the enzymes caused loss of activity probably with absorption to glass surfaces. Use of 0.1 mg/ml of albumin or 20% glycerol stabilized the enzymes. Addition of β-mercaptoethanol to fractions did not reduce the decay. There were no differences in stability between enzymes released by osmotic shock and those not released by osmotic shock.

Proportionality to Time and Enzyme Concentration. Under the assay conditions described, the release of inorganic phosphate from 5′-AMP, ATP, and uridine diphosphoglucose was proportional to time for at least 30 min. The release of product was proportional to enzyme concentration over a wide range provided that dilutions of the enzyme were made in 0.1 mg/ml of bovine serum albumin. Addition of albumin in the course of the reaction did not alter the rate of the reaction and was consistent only with stabilization of the enzyme.

Dependence of Rate of Hydrolysis upon pH. Figure 1

TABLE II: Effect of Various Metal Ions and Other Reagents on Hydrolysis of 5′-AMP.[a]

Compound	Concn (M)	Organisms and Source of Enzyme						
		S. sonnei Osmotic Shock	*S. heidelberg* Osmotic Shock	*P. vulgaris* Sonic Extract	*E. aerogenes* Osmotic Shock	*E. aerogenes* Sonic Extract	*S. marcescens* Osmotic Shock	*S. marcescens* Sonic Extract
None		3	9	32	11	8	15	18
Co^{2+}	0.001	100	100	100	100	100	100	100
Ca^{2+}	0.001	20	20	38	15	11	20	28
Mg^{2+}	0.001	25	10	28	20	8	11	15
Mn^{2+}	0.001	100	95	77	100	72	100	90
Co^{2+}, Ca^{2+}[b]	0.001	380	190	105	140	108		
Co^{2+}, Mg^{2+}	0.001	135			103	101		
Zn^{2+}	0.001	18		30	20	24		37
EDTA	0.001	5	3	10	10	15		
Urea	0.4	4	11	60	20	15	10	15
	0.04	95	99	98				
Mercapto-ethanol	0.0001	95	115	100	95	100		

[a] A value of 100 is assigned to the hydrolysis in the presence of $CoCl_2$. [b] Where two ions are used, both are at a concentration of 0.01 M.

shows the effect of pH on the rate of hydrolysis of 5′-AMP for the 5′-nucleotidases of *Shigella sonnei*, *P. vulgaris*, and *E. aerogenes*. No significant differences were noted and the pH optimum of all organisms studied (*Klebsiella pneumoniae*, *S. heidelberg*, *Serratia marcescens*, *Citrobacter freundi*, and *Proteus mirabilis*) was 5.7–6.2. The pH optimum for hydrolysis of ATP was slightly higher at 6.5–7.2. Bis(*p*-nitrophenyl)phosphate was hydrolyzed over a broad pH range of 6–7.5. The optimal pH for UDP-glucose pyrophosphatase activity was 7–8 for all enzymes.

Influence of Metal Ions on Enzyme Activity. The influence of various metal ions on the 5′-AMP hydrolysis of the various enzymes is shown in Table II. The only striking difference is afforded by both Co^{2+} and Ca^{2+}. The enzyme that is released by osmotic shock shows greater stimulation of hydrolytic activity than the enzyme left in the cells (*Enterobacter*) or never released (*Proteus*). The optimal cobalt concentration for all 5′-nucleotidases was 5 mM for hydrolysis of both 5′-AMP and ATP. The cation effect in our hands was somewhat pH dependent since a different influence was seen at pH 5.7, 7.4, and 8.0 in sodium acetate, Tris-maleate, and Tris-HCl buffers. At pH 5.7 Co^{2+} showed the greatest stimulation of hydrolytic activity, but at pH 8.0 Mn^{2+} was best for all enzymes. At pH 8.0 Mn^{2+} caused optimal hydrolysis with an order of stimulation of hydrolytic activity of Mn > Mg > Ca > Co. The actual activity at pH 8.0 is only 25–50% of that seen at pH 5.7. The ion effect at pH 8.0 is subject to question since Co^{2+} will complex with Tris-HCl at this pH. The 5′-nucleotidases of all the organisms studied showed inhibition of activity in the presence of Zn^{2+}.

Effect of Various Compounds on Enzyme Activity. Metal chelating agents such as EDTA, citrate, or ascorbic acid caused marked inhibition of enzyme activity against 5′-AMP, ATP, and UDP-glucose in all cases. Table II shows a comparison of activity inhibition with 5′-AMP as substrate. No specific stimulation of hydrolytic activity was obtained by alteration of the ionic environment with NaCl, KCl, and $(NH_4)_2SO_4$ over a wide range.

Substrate Specificity. The enzymes are free of nonspecific acid and alkaline phosphatases as is shown by inability to cleave β-glycerol phosphate, galactose 6-phosphate, ribose 5-phosphate, or *p*-nitrophenyl phosphate at pH 4 and 8. No pyrophosphatase activity is present since PP_i and polyphosphates are not hydrolyzed. Cyclic phosphodiesterase (3′-nucleotidase) activity is eliminated except for a trace in the *Proteus* enzyme. RNA endonuclease and RNA exonuclease activity of the *E. coli* RNase I and II types are absent. All the 5′-nucleotidases do not hydrolyze 3′-substituted 5′-nucleotides such as pAp or pTp and oligonucleotides such as ApApAp (Table III). Nucleoside diphosphates are cleaved in a manner analogous to the hydrolysis of ATP. In the case of both ATP and ADP, no PP_i is formed. Methylation of 5′-nucleotides does not inhibit the removal of the 5′-phosphate. UDP-glucose is hydrolyzed to yield uridine and glucose. ADP-glucose was not hydrolyzed by purified enzymes. *S. heidelberg* 5′-nucleotidase showed no appreciable activity against bis(*p*-nitrophenyl)phosphate. The *Shigella*, *Enterobacter*, and *Proteus* 5′-nucleotidases hydrolyzed bis(*p*-nitrophenyl)phosphate.

Effect of Substrate Analogs upon 5′-Nucleotidase Activity. Previous studies on the 5′-nucleotidase of K37[2] showed no effect of various substrate analogs (Neu,

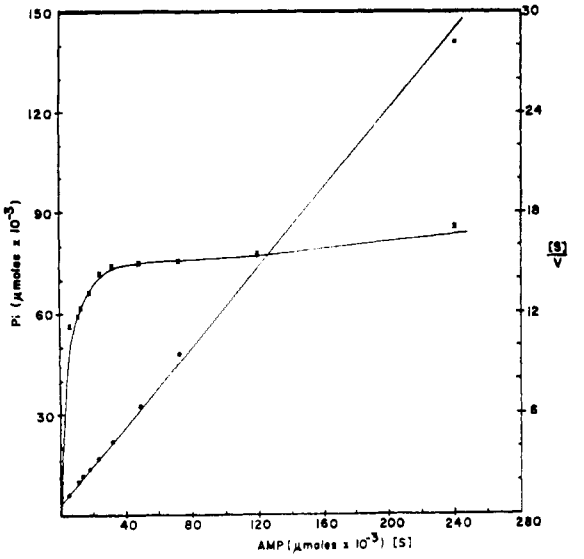

FIGURE 2: Lineweaver–Burk plot for determination of K_m of 5′-nucleotidase of *S. sonnei* with 5′-AMP as substrate.

1967a). Adenosine, 3′-AMP, 2′-AMP, 3′,5′ > AMP, and 5′-adenosine monosulfate all failed to show an inhibitory effect on the hydrolysis of 5′-AMP or ATP by the 5′-nucleotidases of *P. vulgaris*, both *E. aerogenes* enzymes, and the nucleotidases of *S. heidelberg*, *S. marcescens*, and *S. sonnei*. Poly A, RNA, and DNA showed only minimal inhibition, less than 15%, which may be merely polyanionic.

Effect of Phosphate. The 5′-nucleotidase of *E. coli* (Neu, 1967a) was previously shown not to be inhibited by phosphate. Both of the enzymes obtained from *Enterobacter* strains, those from *Proteus* and *Providencia*, and the enzymes from *Shigella* and *Salmonella* were not inhibited by up to 0.1 M potassium phosphate. In higher concentrations inhibition was due to precipitation of cobalt–phosphate complexes since this could be overcome by dilution. Phosphate had no inhibitory effect on the hydrolysis of ATP or UDP-glucose.

Effect of Substrate Concentration. The calculated K_m values for various enzymes are seen in Table IV. Over a wide range the initial substrate concentration (Figure 2) has no effect on the over-all rate of hydrolysis of 5′-AMP. At higher concentrations of ATP, definite substrate inhibition is seen (Figure 3). This is not due to formation of cobalt complexes and consequent reduction of Co^{2+} concentration because adding Co^{2+} does not alter the inhibition.

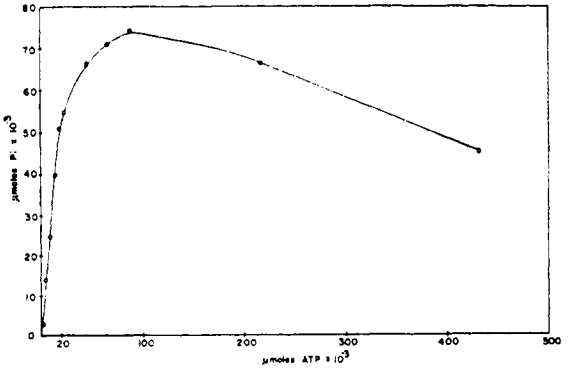

FIGURE 3: Substrate concentration plot of the 5′-nucleotidase of *S. sonnei* with ATP as substrate.

TABLE III: Relative Activities of 5′-Nucleotidases against Various Nucleotides.[a]

	S. sonnei	*E. aerogenes*	*P. vulgaris*
Substrate			
5′-AMP	100	100	100
5′-UMP	50	85	39
5′-GMP	81	42	42
5′-CMP	48	38	39
dTMP	37		35
IMP	80		65
dAMP	40	25	17
dCMP	42		42
dGMP	29		21
5′-Methyl-CMP	42	35	28
2′-AMP	0	0.002	0.5
3′-AMP	0	0.03	0.2
3′,5′-Cyclic AMP	0	0	1
2′,3′-Cyclic UMP	0	0	1
ATP	100	100	100
ADP	95	92	74
NADH	0	0	5
PP_i	0	0	0

[a] A value of 100 is assigned to the activity of 5′-nucleotidase against 5′-AMP and ATP in the different assay conditions outlined. Substrate concentration was 5×10^{-3} M.

Heat Stability. Exposure of the 5′-nucleotidases to heat under similar conditions revealed no differences between those enzymes that were released by osmotic shock and those that were not released. Dilute solutions of purified enzymes were stable at 55° for 10 min when incubated in bovine serum albumin but by 20 min at 55° showed only 50% activity. Incubation of 5′-nucleotidases of *Shigella*, *Proteus*, *Enterobacter*, and *Salmonella* in pH 5.1 sodium acetate for 10 min at 55° resulted in loss of 90% of activity (hydrolysis of 5′-AMP, ATP, and UDP-glucose). Incubation in Tris-HCl at pH 7.5 resulted in loss of 75% of activity by 30 min. The presence of cobalt, calcium, or magnesium during heating of the 5′-nucleotidases did not stabilize the enzymes. Incubation of heated fractions at 0 and 25° did not result in recovery of hydrolytic activity.

Molecular Sieve Chromatography. All purified enzymes were subjected to chromatography on Sephadex G-100 in 0.4 M NaCl–0.05 M Tris-HCl (pH 7.5). Enzymes released by osmotic shock from *E. coli*, *S. sonnei*, *S. heidelberg*, *S. marcescens*, and *E. aerogenes* showed elution volumes identical with those of enzymes from *E. aerogenes* and *P. vulgaris*. Combination of the enzymes (Figure 4) showed a single peak of activity for protein and

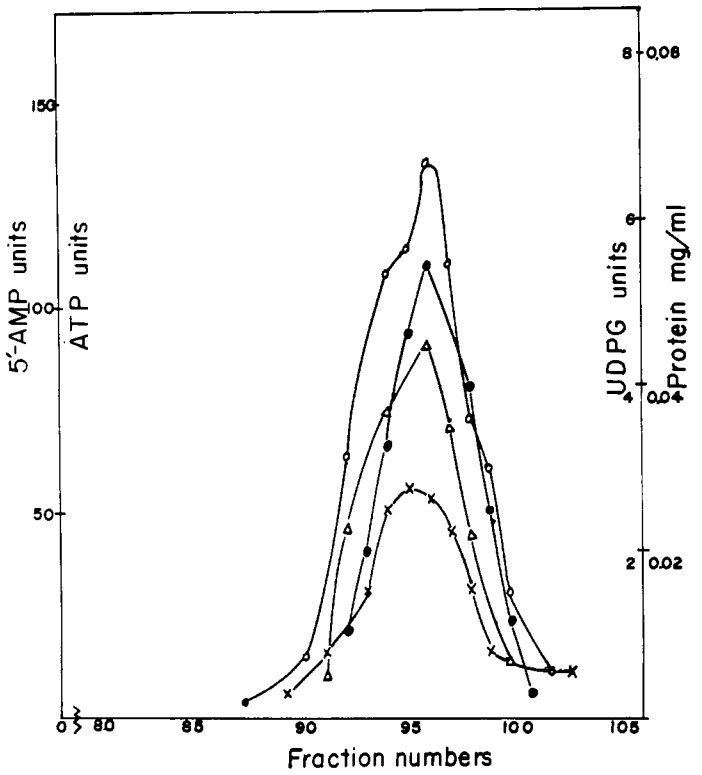

FIGURE 4: 5′-nucleotidases purified from *E. coli*, *S. sonnei*, *S. heidelberg*, *S. marcescens*, *E. aerogenes*, and *P. vulgaris* were combined in equal activity and applied to a Sephadex G-100 column (2.5 × 95 cm). They were eluted with 0.4 M NaCl–0.05 M Tris-HCl, and 2-ml fractions were collected. Assays were as indicated in Methods. 5′-AMP (○), UDPG (●), ATP (△), and protein (X).

TABLE IV: Michaelis Constants for Various 5′-Nucleotidases.[a]

Organism	K_m		
	5′-AMP	ATP	UDPG
S. sonnei	1.2×10^{-5}	1.3×10^{-4}	1.1×10^{-4}
E. aerogenes	2.4×10^{-5}	*b*	*b*
S. heidelberg	1.2×10^{-5}	*b*	7.2×10^{-3}
P. vulgaris	8.4×10^{-4}	1.14×10^{-4}	1.35×10^{-3}

[a] Assays were as outlined in Methods. [b] No determination was made.

hydrolysis of 5′-AMP and ATP. Molecular weights calculated from such runs using pancreatic RNase, human lysozyme, bovine albumin, and *E. coli* alkaline phosphatase as standards gave values that ranged from 44,000 to 53,000. These values are compatible with the errors inherent in the method.

Polyacrylamide Gel Electrophoresis. Electrophoresis of the purified enzymes of *E. coli*, *S. sonnei*, *E. aerogenes*, and *P. vulgaris* gave protein bands of similar location that corresponded to the enzymatic activity for hydrolysis of 5′-AMP and ATP. The *Proteus* enzyme contains several other faint bands in addition to the main band. These showed no hydrolytic activity against 5′-AMP nor did they increase the activity of the main band when combined with it.

Intracellular Protein Inhibition. Previous studies showed that a variety of strains of *E. coli* contained an internal protein inhibitor of the 5′-nucleotidase (Neu, 1967a,b; Glaser *et al.*, 1967). The existence of the inhibitor protein is shown by heating sonically prepared cell extracts and demonstrating an increase in activity. All *Enterobacteriaceae* (Table V) show a one- to threefold increase in activity on heating. This, however, totally inactivates the inhibitor. The presence of inhibitor is also demonstrated by the altered location on polyacrylamide electrophoresis or Sephadex G-100 chromatography of crude extracts as compared with osmotically released enzyme or heated extracts.

In contrast to the case of the 5′-nucleotidase inhibitor of *E. coli* we have been unsuccessful in purifying the pro-

TABLE V: Crypticity of 5′-Nucleotidase Activity.[a]

Organism	Ratio of Act. (heated cell extracts to unheated cell extracts)
S. sonnei	3.0
S. heidelberg	1.9
E. aerogenes	1.1
P. vulgaris	2.4
P. mirabilis	1.8
S. marcescens	1.5

[a] Washed cells of the various bacteria were sonically disrupted and assayed at 21° using 5′-AMP as substrate. A matched sample of sonically disrupted cells was heated at 45° for 20 min. The ratio of activity of heated to unheated cell extracts is calculated.

TABLE VI: Effect of 5′-Nucleotidase Inhibitors.[a]

Inhibitor	Source of Nucleotidase (units/ml)			
	E. coli K12	*E. coli* Ca38	*S. sonnei*	*P. vulgaris*
None	69	68	89	12.3
E. coli Ca38	6.1	10	8	9.0
E. coli K12	0	1.0	0	6.1
S. sonnei	1	6.9	1.7	8.0

[a] 5′-Nucleotidase inhibitors were incubated with the 5′-nucleotidases and 5 μg of albumin at 21° for 10 min. Then a mixture containing 5 mM 5′-AMP, 5 mM $CoCl_2$, 20 mM $CaCl_2$, and 100 mM sodium acetate was added. After 10 min at 21° the assay was stopped and the phosphate release was determined.

tein inhibitor in those organisms in which the 5′-nucleotidase is not totally released by osmotic shock. The partially purified 5′-nucleotidase inhibitors of two strains of *E. coli* and one *Shigella* showed excellent cross reaction of inhibition with each other (Table VI). But the inhibition of the *Proteus* 5′-nucleotidase was much less for all inhibitors. The inhibitor proteins inhibit all hydrolytic action of the 5′-nucleotidases (5′-AMP, ATP, and UDP-glucose).

Activity of Intact Cells. Although caution must be exercised in regard to interpretation of activity measurements with whole cells, use of $MgCl_2$ in the buffer seems to prevent loss of permeability (Neu *et al.*, 1967). We incubated intact cells of all the *Enterobacteriaceae* with 5′-AMP and ATP, neither of which enters intact cells. All organisms, including *Proteus vulgaris* and *P. mirabilis*, were able to hydrolyze the substrates. No enzyme was released into the medium. These studies show that *in vivo* the 5′-nucleotidase and inhibitor are separated even in cells which fail to release the enzyme on being subjected to osmotic shock. Indeed intact cells hydrolyze 5′-AMP at a rate greater than that of sonically disrupted cells.

Discussion

We have described the properties and purification of the 5′-nucleotidases (uridine diphosphate sugar hydrolases) of the various members of the *Enterobacteriaceae*. It is possible to purify enzymes that are homogeneous on acrylamide gel electrophoresis and yet hydrolyze 5′-AMP, ATP, and UDP-glucose. The various enzymatic activities are not separable by heat denaturation, ion stimulation, or molecular sieve chromatography.

The 5′-nucleotide hydrolases of all *Enterobacteriaceae* require that the carbon 1′ of the ribose possess a nitrogenous base and that the hydroxyl group of the carbon atom 3′ be free. Bacterial nucleotidases, in contrast with mammalian and reptile nucleotidases, all seem to hydrolyze nucleoside di- and triphosphates. The greatest stimulation of hydrolysis of all substrates results from the presence of Co^{2+} or Mn^{2+}. Mg^{2+} is generally of lesser importance. There is no phosphate inhibition of the 5′-nucleotidases, and substrate analogs and nucleic acids do not exert an inhibitory effect.

Previous studies of the effect of various growth conditions on the production of the 5′-nucleotidases (Neu, 1968) showed that the enzymes in all the *Enterobacteriaceae* were not subject to phosphate repression as is the alkaline phosphatase or to catabolite (glucose) repression as is the acid hexose phosphatase.

We had previously demonstrated that the 5′-nucleotidases of *E. coli*, *Citrobacter freundi*, and *S. sonnei* were completely released by osmotic shock (Neu and Chou, 1967) but only 50% of the 5′-nucleotidases of *Enterobacter*, *Serratia*, and some strains of *Salmonella*. The 5′-nucleotidases of *Proteus* and *Providencia* strains were not released by osmotic shock. These studies fail to show any significant differences in the 5′-nucleotidases isolated from all of these organisms. All are of the same molecular size and there is nothing to suggest that the unreleased enzymes are dimers or polymers. Intact *Proteus* cells can hydrolyze 5′-AMP and ATP. These studies suggest that the differences in enzyme release encountered in osmotic shock are due to cell wall cytoplasmic membrane differences rather than to differences in the enzymes.

All *Enterobacteriaceae* possess intracellular protein inhibitors which inhibit all hydrolytic activities of these hydrolases. However, purification of the inhibitors from those organisms that fail to release the enzyme on osmotic shock has not been possible due to the lability of the inhibitor protein. In all cases the inhibitor proteins are intracellular by all criteria. This intracellular protein inhibition which is found for the 5′-nucleotidase of *P. vulgaris* is similar to that described by Swartz *et al.* (1958) for DPNase activity.

Histochemical electron microscopic data show the 5′-nucleotidase of a number of strains of *E. coli* to be at the surface of the cell (I. I. Nisonson and H. C. Neu,

in preparation). These enzymes may act on both sides of the cell membrane with intracellular hydrolysis inhibited so as to avoid competing for substrate with biosynthetic enzymes. This would be especially important in the case of the hydrolysis of ATP. Since the reaction is totally dependent upon the presence of Co^{2+} or Mn^{2+}, this may occur *in vivo* only at a site where these ions might be concentrated, such as at the cell membrane. The nucleoside phosphate sugar hydrolase system with the acid hexose phosphatase constitutes a most effective means of allowing phosphorylated compounds which ordinarily cannot enter the cell to be utilized rapidly.

Nonetheless, it is still not possible to assign to these enzymes or their inhibitors a specific function in the overall cellular metabolism. The inability to find mutants lacking these enzymes in a wide variety of strains in the *Enterobacteriaceae* suggests that they may play a significant role in nucleotide pool turnover at the cell surface. On the other hand, the fact must be considered that *Salmonella typhimurium* LT_2 lacks this particular enzyme but contains a UDP-glucose hydrolase lacking 5′-nucleotidase activity. Through the study of mutants of *Salmonella* and antibodies to purified 5′-nucleotidase we may be able to get more unequivocal information as to their physiologic role.

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