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The 5′-Nucleotidases and Cyclic Phosphodiesterases (3′-Nucleotidases) of the *Enterobacteriaceae*

HAROLD C. NEU

Department of Medicine, College of Physicians and Surgeons, Columbia University, New York, New York 10032

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All members of the *Enterobacteriaceae* possess distinct 5′-nucleotidases and cyclic phosphodiesterases (3′-nucleotidases) that can be differentiated from the acid and alkaline phosphatases and the acid sugar hydrolases. The nucleotidases and cyclic phosphodiesterases of the various *Enterobacteriaceae* are remarkably similar in properties. All of the 5′-nucleotidases hydrolyze 5′-nucleotides, adenosine triphosphate, and uridine diphosphoglucose. Their *p*H optimum is from 5.7 to 6.1. The cyclic phosphodiesterases hydrolyze 3′-nucleotides, cyclic phosphonucleotides, bis-(*p*-nitrophenyl)phosphate, and *p*-nitrophenylphosphate. Their *p*H optimum is from 7.2 to 7.8. For both enzymes, cobalt showed optimal metal stimulation. An intracellular protein inhibitor for the 5′-nucleotidase is present in all of the *Enterobacteriaceae*. No inhibitor of cyclic phosphodiesterase activity exists, although hydrolysis of both cyclic phosphonucleotides and 3′-nucleotides is inhibited by ribonucleic acid. Neither of the enzymes is subject to control by phosphate level or by catabolite repression. Of the other bacteria studied, only *Haemophilus* and *Bacillus subtilis* contained significant 3′- or 5′-nucleotidase activity.

Although 5′-nucleotidases are widely distributed in animal tissues (3, 10, 23) and the 3′-nucleotidases of plants have been purified (22), bacterial nucleotidases have not been investigated to any degree. In the course of studying the existence of surface enzymes in *Escherichia coli* (19, 20), a cobalt-stimulated 5′-nucleotidase was discovered (20, 16), which has been shown to be identical to the diphosphate sugar hydrolase of *E. coli* (9, 16). Anraku (1) has shown that *E. coli* B contains a 3′-nucleotidase which is identical to the ribonucleic acid (RNA)-inhibited cyclic phosphodiesterase of *E. coli*. *E. coli* contains an intracellular protein which inhibits the 5′-nucleotidase (6, 9, 17). No such activity has been seen for the cyclic phosphodiesterase of *E. coli*. All members of the *Enterobacteriaceae*, except *Proteus* strains, have been shown to release these enzymes when subjected to the osmotic shock method of Neu and Heppel (17). It seemed worthwhile to study these enzymes in *Enterobacteriaceae* to determine their actual distribution and growth characteristics.

MATERIALS AND METHODS

Organisms. E. coli strains were gifts from numerous sources. All the other organisms were isolates from the microbiology laboratory of the Presbyterian Hospital. All organisms were classified by the chemical and serological methods outlined by Edwards and Ewing (8).

Techniques of cultivation. Organisms were maintained on Penassay agar slants. The basic phosphate medium contained 0.04 M K_2HPO_4, 0.02 M KH_2PO_4, 0.08 M NaCl, 0.02 M NH_4Cl, 3 mM Na_2SO_4, 1 mM $MgCl_2$, and 0.1 mM $CaCl_2$. The low phosphate medium contained 0.12 M tris(hydroxymethyl)aminomethane (Tris), 0.08 M NaCl, 0.02 M KCl, 0.02 M NH_4Cl, 3 mM Na_2SO_4, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, and 2 μM $ZnCl_2$. Both media were supplemented with peptone (Difco) in concentrations varying from 0.05 to 0.5%. The *p*H in both cases was adjusted to 7.3. The carbon content of the media was 0.5% glucose, 0.5% glycerol, or 0.3% sodium succinate. Penassay Broth (Difco) was also used. Organisms were incubated at 35 C on a rapid rotary shaker, and growth was followed by changes in optical density at 600 mμ in a Beckman DU spectrophotometer.

Preparation of cell extracts. Intact cells were harvested by centrifugation and were washed twice with 40 volumes of cold 0.03 M NaCl, 0.01 M Tris-chloride, *p*H 7.3, per gram (wet weight) of cells. Washed cells were suspended in 0.01 M $MgCl_2$, 0.01 M Tris-chloride, *p*H 7.5, at 3 C, and were sonically disrupted with a model 75 Branson Sonifier using 15-sec bursts over a 2-min period. Samples were kept at 0 to 3 C. Intact cells and cell debris were removed by centrifugation for 10 min at 16,000 rev/min in a Sorvall RC2B centrifuge. The supernatant fluid was stored frozen at −20 C when it was not assayed immediately. Cells were subjected to osmotic shock by the procedure described for the various members of the *Enterobacteriaceae* (18).

Enzyme assays. The standard assay for 5′-nucleoti-

dase contained (in 0.1 ml) 5 mM 5′-adenosine monophosphate (AMP), 5 mM $CoCl_2$, 20 mM $CaCl_2$, 100 mM sodium acetate buffer, *p*H 5.7, and 10 μg of bovine serum albumin. 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 phosphate was assayed by the method of Chen, Toribara, and Warner (4). Activity is expressed as micromoles of phosphate hydrolyzed per hour at 37 C. The standard assay for 3′-nucleotidase contained (in 0.1 ml) 2 mM 3′-AMP, 10 mM $CoCl_2$, and 100 mM Tris-maleate buffer, *p*H 7.7. After 20 min at 37 C, the assay was stopped with HCl, and the phosphate was determined as for the 5′-nucleotidase. The assays for hydrolysis of bis-(*p*-nitrophenyl)phosphate and *p*-nitrophenylphosphate (16), and those for cyclic phosphodiesterase (19), adenosine triphosphate (ATP) hydrolysis (16), and uridine diphosphoglucosidase (16) have been previously published. Protein concentration was determined by the method of Lowry et al. (13), with bovine serum albumin as the standard.

Materials. Nucleotides were purchased from commercial sources as were calcium bis-(*p*-nitrophenyl) phosphate, *p*-nitrophenylphosphate, and sugar phosphates. The diethylaminoethyl (DEAE)-cellulose used in column chromatography was obtained from Reeve Angel.

RESULTS

In agreement with studies on the alkaline phosphatase (25) and the acid phosphatases of *E. coli* (11, 21), it was found that the nonspecific alkaline phosphatases of other *Enterobacteriaceae* were repressible by phosphate. Since acid phosphatases are subject to catabolite repression (7, 11), a medium of high phosphate and glucose content could be used to study the bacterial nucleotidases. Although it was possible to reduce the effect of the various nonspecific phosphohydrolases, it was necessary to document the existence of specific nucleotidases. In the course of studies on osmotic shock (17, 18, 20), all of the 5′-nucleotidases and cyclic phosphodiesterases of the *Enterobacteriaceae* have been purified. Enzymes have been prepared which were judged to be pure by molecular sieve chromatography and acrylamide disc gel electrophoresis (H. Neu, *in preparation*).

Properties of the enzymes. It was possible to use sonically prepared extracts of the various organisms to screen for the presence of the enzymes. All extracts were assayed after preparation and after preheating at 45 C for 15 min. Freezing and thawing of the sonic extracts did not cause appreciable decay of either the 5′-nucleotidases or the cyclic phosphodiesterases. The 5′-nucleotidase inhibitor was markedly damaged by freezing and thawing. Inhibition of the 5′-nucleotide hydrolysis could also be abolished by diluting the sonic extracts in water or in a dilute albumin solution. Hydrolysis of 5′-AMP and 3′-AMP was linear over a wide protein range and with respect to time (for 30 min).

The *p*H optimum for the 5′-nucleotidase of all the organisms studied was from 5.7 to 6.1. The cyclic phosphodiesterase *p*H optimum was from 7.2 to 7.8. Purification of the enzymes to single homogeneous proteins did not alter the *p*H optima. Similarly, the intact cells showed a *p*H optimum for hydrolysis of 3′-AMP and 5′-AMP, as did bacterial extracts.

The metal requirements (Table 1) of all of the organisms studied were remarkably similar. Cobalt gave the highest stimulation for the 5′-nucleotidase at *p*H 5.7 in sodium acetate buffer. It was not possible to show that Mg^{++}, Co^{++}, and Mn^{++} had similar activities at *p*H 8.0, as was suggested by Glaser et al. (9). In fact, at *p*H 8.0, Tris-chloride and Co^{++} tended to form a complex.

The metal stimulation of the cyclic phosphodiesterase varied with the *p*H of the reaction. At *p*H 5.7 in sodium acetate, $CoCl_2$ gave the greatest stimulation; at *p*H 8.0 in Tris-chloride, Co^{++} and Mg^{++} were equivalent. This may be due to the

TABLE 1. *Stimulation by metals*[a]

Organism	5′-Nucleotidase					Cyclic phosphodiesterase				
	None	Co^{++}	Mn^{++}	Mg^{++}	Ca^{++}	None	Co^{++}	Mn^{++}	Mg^{++}	Ca^{++}
Proteus vulgaris	32	100	77	28	33	100	142	143	128	145
Shigella sonnei	3	100	100	10	20	100	159	162	97	95
Enterobacter aerogenes	11	100	100	20	15	100	155	170	105	110
Serratia marcescens	42	100	110	35	33	—	—	—	—	—
Salmonella heidelberg	—	—	—	—	—	100	342	175	110	100

[a] Bacterial extracts were prepared as outlined. They were dialyzed against 1 mM ethylenediaminetetraacetic acid and then 0.01 M Tris-chloride, *p*H 7.5. Assays were performed at *p*H 5.7 for the 5′-nucleotidase and at *p*H 7.7 for the 3′-nucleotidase. Concentration of cations was 10 mM for each cation. The 5′-nucleotidase activity in the presence of cobalt is taken as 100, and the 3′-nucleotidase activity without added metals is 100; other values are relative to these. Dashes indicate that no assay was performed.

complexing of cobalt. The 5′-nucleotidases and the cyclic phosphodiesterases of all organisms were inhibited by Zn^{++}. The *E. coli* nucleotidases showed greater stimulation than did the nucleotidases of other *Enterobacteriaceae*. *Proteus* nucleotidases showed the least stimulation.

Substrate specificity. The 5′-nucleotidase of *E. coli* has been shown to cleave 5′-AMP, ATP, and uridine diphosphoglucose (9, 16). The 5′-nucleotidases of *Shigella sonnei*, *Salmonella heidelberg*, *Serratia marcescens*, *Klebsiella pneumoniae*, *Enterobacter aerogenes*, *P. mirabilis*, *P. vulgaris*, and *Citrobacter freundii* have identical properties. A number of strains of *Salmonella typhimurium* contain sugar diphosphate hydrolases but lack 5′-nucleotidase activity. The cyclic phosphodiesterase of *E. coli* B (1) was shown to hydrolyze cyclic phosphates, 3′-nucleotides, and bis-(*p*-nitrophenyl)phosphate. All of the above organisms have a 3′-nucleotidase with these properties, as well as the ability to hydrolyze *p*-nitrophenylphosphate at *p*H 6.0.

Distribution of the nucleotidases. Table 2 tabulates the enzymatic activities of both 5′- and 3′-nucleotidases of a number of *Enterobacteriaceae*. Variation between organisms of the same group is moderately great when compared to the situation with *E. coli* (Table 3). In most organisms, there was a greater level of 5′-nucleotidase activity than cyclic phosphodiesterase-3′-nucleotidase activity. However, some *Proteus* strains showed greater 3′-nucleotidase activity.

Anraku (2) has shown that the cyclic phosphodiesterase-3′-nucleotidase activity of a number of strains of *E. coli* varies only slightly. The same is true of the 5′-nucleotidases of *E. coli* (Table 3). Surface characteristics of organisms do not affect the level of enzyme, as the level is similar in F^+, Hfr, and F^- strains. The absence of 3′-nucleotidase (K 37_2), ribonuclease I (MRE 600), and alkaline phosphatase (U 24) and the constitutive presence of alkaline phosphatase (C_4F_1) do not change the level of 5′-nucleotidase or its inhibitor.

Presence of intracellular inhibitors. Several investigators have shown that there is an intracellular protein inhibitor of the 5′-nucleotidase (diphosphate sugar hydrolase) of *E. coli* (6, 9, 17). An intracellular inhibitor of the 5′-nucleotidase activity of *P. vulgaris* had been shown long before (24). If the hydrolysis of 5′-AMP is carried out at 21 C, it is possible to demonstrate the presence of inhibitors in all bacteria studied (Table 4). The intracellular inhibitor prevents hydrolysis of 5′-AMP, ATP, and uridine diphosphoglucose. The partially purified *E. coli* inhibitor can inhibit other 5′-nucleotidases (17). It has been difficult to clarify the inhibitory activities of *Enterobacter* enzyme, as only 50% of the free enzyme is released by osmotic shock (18) and the *Proteus* strains contain the inhibitor and nucleotidase bound tightly together. Thus far, the labile nature of the protein inhibitor has made its purification and study difficult.

TABLE 2. *Nucleotidases in various strains of Enterobacteriaceae*[a]

Organism	5′-Nucleotidase	Cyclic phosphodiesterase
	units/mg	*units/mg*
Enterobacter aerogenes	29.8	5.92
E. aerogenes	11.6	2.27
E. aerogenes	33.4	7.75
Klebsiella pneumoniae	2.54	1.13
Serratia marcescens, A	9.7	6.0
S. marcescens, B	4.0	1.73
Proteus mirabilis, A	13.2	31.0
P. mirabilis, B	18.8	16.8
P. mirabilis, C	1.8	0.53
P. vulgaris	24.6	86.5
P. vulgaris	63.3	28.9
Providencia	28.9	7.27
Citrobacter freundii	16.25	5.94
C. freundii	4.26	2.34
Shigella sonnei	9.34	4.93
S. flexneri	9.7	3.27
S. shiga	2.95	2.28
Salmonella typhimurium, A	1.07	7.57
S. typhimurium, B	0.86	0.32
S. typhimurium, C	2.42	4.67
S. typhimurium LT 2	0	1.63
S. typhimurium ADe 97	0	2.8
S. typhimurium Leu 126	0	2.07
S. anatrum	28	8.93
S. derby	15	4.74
S. heidelberg	35	9.45
S. montevideo	50	20.7
S. oranienberg	7.0	9.55
S. enteriditis	4.33	8.95
Haemophilus influenzae	3.81	16.7
Caulobacter AC 48	6.0	14.0

[a] Organisms were grown in the phosphate-glucose medium. Activity is expressed as micromoles of phosphate hydrolyzed per hour per mg of protein. 5′-AMP at *p*H 5.7 was used as the substrate for 5′-nucleotidase. 3′-AMP at *p*H 7.7 was used as the substrate for 3′-nucleotidase.

No inhibitor of the cyclic phosphodiesterase or 3′-nucleotidase activity was found in any of the organisms. The cyclic phosphodiesterase was not activated on heating at 50 C.

Effect of growth conditions. The effect of phosphate upon the level of alkaline phosphatase in *E. coli* is well known (25). The acid hexose phosphatases of *E. coli* are subject to catabolite repression (7, 11). Since all of these are surface enzymes, it was of interest to see if the nucleotidases showed similar effects. Anraku (1) found that in

TABLE 3. *Specific activities of various Escherichia coli 5'-nucleotidases*[a]

Bacterial strain	Specific activity (units/mg of protein)	
	Osmotic shock fluid	Extract
K-12 (F^-)	301	16
AB 282 (F^+)	356	20
Hfr (Hayes)	352	12
K 37_2	380	20
MRE 600	308	18
U 24	385	16
B	310	17
Ca 48 (Col E)	325	15
C_4F_1	325	18
K 245 (Col K)	295	12

[a] Hydrolytic activity is expressed as the micromoles of phosphate released from 5'-AMP per hour at 37 C per mg of protein.

TABLE 4. *Effect of preheating of sonic extracts on 5'-nucleotidase and cyclic phosphodiesterase activity*[a]

Organism	5'-Nucleotidase (units/mg)		Cyclic phosphodiesterase (units/mg)	
	Unheated	Heated	Unheated	Heated
Escherichia coli Hfr Hayes	2.6	24.6	2.4	3.96
Proteus vulgaris	33	96	59	68
Shigella sonnei	7.5	17.8	4.9	5.5
Enterobacter aerogenes	1.8	3.2	1.2	1.7
Salmonella heidelberg	3.8	18.6	11.4	12.5

[a] Extracts of the organisms were prepared as outlined. Unheated samples were assayed at 21 C against 5'-AMP and 3'-AMP. Heated samples were heated for 10 min at 45 C before being assayed.

E. coli B glucose did not repress the cyclic phosphodiesterase, and Neu (17) found that glucose or phosphate did not affect the 5'-nucleotidase of K 37_2 and U 24 (phosphatase-negative mutant). Tables 5 and 6 show that the levels of 5'-nucleotidase and cyclic phosphodiesterase are minimally affected by the sugar content of the medium in any of the *Enterobacteriaceae*. The effect of phosphate could be produced only on partially purified enzymes, for alkaline phosphatase is synthesized at low phosphate levels. No differences were seen in enzymes purified through DEAE chromatography when the organisms had been grown on the high phosphate or low phosphate (Tris-chloride) medium.

In all of the organisms studied, the specific activities of the nucleotidases increased with time and became optimal in the late exponential phase (Table 7). Significant decay did not occur in the late stationary phase. Indeed, both 5'- and 3'-nucleotidase activity were at a very low level in the early exponential phase of growth.

Other bacteria. Kohn and Reis (12) studied the nucleotidase activity in a number of bacteria. The presence of such enzymes in *Haemophilus influenzae*, which has a high level of cyclic phosphodiesterase-3'-nucleotidase activity, has

TABLE 5. *Effect of growth conditions on 5'-nucleotidases*[a]

Medium	5'-Nucleotidase activity (units/mg)				
	Escherichia coli Hfr Hayes	*Enterobacter aerogenes*	*Salmonella heidelberg*	*Shigella sonnei*	*Proteus vulgaris*
Penassay Broth	25	2.9	23.3	6.4	40
Phosphate-glycerol	16	3.3	19.7	10.0	39
Phosphate-glucose	15	4.5	16.3	6.8	33
Phosphate-succinate	21	—	—	12.2	40

[a] Each organism was grown to early stationary phase in the designated medium. Extracts were prepared, and the 5'-nucleotidase activity against 5'-AMP was determined. Activity refers to phosphate released from 5'-AMP per hour per mg of protein.

TABLE 6. *Effect of growth conditions on cyclic phosphodiesterases*[a]

Medium	Cyclic phosphodiesterase activity (units/mg)				
	Escherichia coli Hfr Hayes	*Enterobacter aerogenes*	*Salmonella heidelberg*	*Shigella sonnei*	*Proteus vulgaris*
Penassay Broth	2.7	4.9	1.8	4.3	59
Phosphate-glycerol	6.1	5.6	1.9	8.8	91
Phosphate-glucose	3.2	3.1	1.3	5.4	33
Phosphate-succinate	—	—	—	11.5	49

[a] Each organism was grown to early stationary phase in the designated medium. Extracts were prepared, and the 3'-AMP hydrolysis was determined.

TABLE 7. *Activities of nucleotidases at different stages of growth*[a]

Organism	5'-Nucleotidase (units/mg)		Cyclic phosphodiesterase (units/mg)	
	Exponential	Stationary	Exponential	Stationary
Proteus vulgaris	33.3	24.6	17.6	9.8
Shigella sonnei	9.3	18.8	3.42	3.7
Enterobacter aerogenes	2.98	3.36	1.71	1.85
Citrobacter freundii	16.0	24.3	1.63	3.4
Serratia marcescens	9.7	7.2	2.96	2.58
Salmonella heidelberg	16.3	21.1	1.97	2.34

[a] Organisms were grown in Penassay Broth and were harvested in either exponential or early stationary phase. Extracts were prepared and assayed against 5'-AMP and 3'-AMP at 37 C.

been confirmed. Nucleotidase activity was not found in *Pseudomonas aeruginosa*, *P. fluorescens*, *Herellea vaginocola*, *Alcaligenes faecalis*, *Pasteurella pestis*, or in *Mima polymorpha*. Of the gram-positive organisms studied, *Bacillus subtilis* and *B. cereus* possessed 5'-nucleotidase activity. However, specific nucleotidase activity was not found in *Staphylococcus aureus*, *Micrococcus lysodeikticus*, or in *Streptococcus faecalis*.

DISCUSSION

Nucleotidases have generally been overlooked in bacteria because of the presence of acid and alkaline phosphatases. Although mammalian enzymes have their *p*H optima at alkaline levels, bacterial nucleotidases are active at neutral or slightly acid *p*H. In all members of the *Enterobacteriaceae*, cyclic phosphodiesterases and 5'-nucleotidases are constitutive enzymes which are not sensitive to the regulatory mechanisms affecting phosphatase production. The physiological roles of the two enzymes in the *Enterobacteriaceae* remain unclear.

The previous demonstration that both enzymes can be released by osmotic shock from all members of the *Enterobacteriaceae* except *Proteus* and *Providencia* (18) has led to the suggestion that they are surface enzymes (18, 19). However, *Proteus* strains can hydrolyze nucleotide substrates that should not be able to enter the cells. Detailed studies (*in preparation*) have not revealed any differences among enzymes from all strains purified to homogeneity. The kinetic and physical properties are identical.

It has been suggested that these enzymes cleave exogenously supplied nucleotides and nucleotide diphosphate sugars (17). Alternately, it has been suggested that the 5'-nucleotidase acts to control nucleotide diphosphate sugar levels (9). The K_m (9, 16) studies might support the existence of such a mechanism for *E. coli*, but this has not been clarified in other bacteria. The cyclic phosphodiesterase also acts primarily against cyclic nucleotides and secondarily against 3'-nucleotides (1, 2). It is interesting that strains of *E. coli* with very low levels of this enzyme can be obtained, in contrast to the situation with the 5'-nucleotidase. The 5'-nucleotidases of *Enterobacteriaceae* have intracellular protein inhibitors to prevent attack on cellular contents. The cyclic phosphodiesterases do not possess protein inhibitors, but are inhibited by RNA in *E. coli* (1) and in the other *Enterobacteriaceae* (*unpublished data*). It has not been possible to correlate the presence or absence of ribonuclease and deoxyribonuclease with the levels of nucleotidases in *E. coli*. It is of note, however, that *Pseudomonas*, *A. faecalis*, and *Pasteurella*, all of which lack ribonuclease I, do not have these nucleotidases.

In previous studies, it has been postulated (19) that the surface enzyme phenomenon is seen in gram-negative organisms and not in gram-positive organisms because of differences in the cell wall structure. However, we have been unable to find nucleotidases excreted into the growth medium of *B. subtilis*, *S. aureus*, or *M. lysodeikticus* species. There is a 5'-nucleotidase in *B. subtilis* (5, 15), but it is not released by any osmotic transition or by KCl elution techniques (*unpublished data*), which do elute the acid phosphatases of *S. aureus* (14). If these nucleotidases are concerned with the turnover of nucleotides in the cell wall as an alternative to feedback inhibition, this may explain the differences between the gram-negative and gram-positive organisms.

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