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

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ABSTRACT: The cyclic phosphodiesterases (3′-nucleotidases) of the various members of the *Enterobacteriaceae* have been purified and characterized. All of the enzymes hydrolyze 3′-nucleotides, 2′,3′-cyclic nucleotides, bis(*p*-nitrophenyl)phosphate, and *p*-nitrophenyl phosphate. The ratios of specific activity of the hydrolysis of 3′-nucleotides and cyclic nucleotides remain constant through various purification procedures. There is parallel loss of cyclic phosphodiesterase and 3′-nucleotidase activity upon heating. No activation at 45 or 55° was seen.

The pH optimum for both cyclic phosphodiesterase and 3′-nucleotidase activity is 7.8. The pH optima of the cyclic phosphodiesterases (3′-nucleotidases) of the *Enterobacteriaceae* tested were similar. Ion stimulation was greatest with Co^{2+}. Polyacrylamide electrophoresis and molecular sieve chromatography with Sephadex indicated that the enzymes from different members of the *Enterobacteriaceae* are similar in physical as well as hydrolytic properties.

Anraku (1964a) described a cyclic phosphodiesterase having 3′-nucleotidase activity from *Escherichia coli* B. Neu and Heppel (1964a,b) discovered that the cyclic phosphodiesterase of *E. coli* was a surface enzyme. Subsequent studies of surface enzymes using the osmotic shock technique (Neu and Chou, 1967) showed that only 50% of this cyclic phosphodiesterase (3′-nucleotidase) activity could be released from members of *Klebsiellae* such as *Enterobacter aerogenes* and *Serratia marcescens* or various strains of *Salmonella*. Although *Proteus* and *Providencia* possessed high levels of the enzyme, the cyclic phosphodiesterase was not released by osmotic shock. Subsequent studies of the effect of various growth conditions on the properties and activity of cyclic phosphodiesterases (Neu, 1968a,b) failed to show any differences among the members of the *Enterobacteriaceae*. This study was undertaken to determine if there were differences in properties or size of the cyclic phosphodiesterases (3′-nucleotidases) which accounted for the different response to osmotic shock.

This paper presents methods of purification of the enzymes from a number of the *Enterobacteriaceae* and shows that the enzymes are similar in respect to pH optima, metal ion requirements, heat stability, electrophoretic mobility, and molecular weight as determined by molecular sieve chromatography.

Experimental Procedures

Materials. Bis(*p*-nitrophenyl)phosphate, *p*-nitrophenyl phosphate, and nucleotides were purchased from commercial sources. DEAE-cellulose was obtained from Reeve Angel, Clifton, N. J., Sephadex from Pharmacia and hydroxylapatite from 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 (1962).

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

Enzyme Assays. Cyclic phosphodiesterase activity was determined from the hydrolysis of uridine 2′,3′-cyclic phosphate. The assay consisted of 2 mM 2′,3′-cyclic UMP,[1] 5 mM $CoCl_2$, 1 mM $MgCl_2$, 50 mM Tris-maleate (pH 7.8), and enzyme at 37° for 20 min in a volume of 0.1 ml. The reaction was stopped with 0.2 ml of 0.05 N HCl and the phosphate was determined by the method of Ames and Dubin (1960). A unit is 1 μmole of phosphate released/hr at 37°. Bis(*p*-nitrophenyl)phosphate assay consisted of incubating 5 mM $CoCl_2$, 100 mM Trismaleate (pH 6.8), 0.1 mg of calcium bis(*p*-nitrophenyl)-phosphate, and enzyme at 37° for 20 min in a volume of 0.1 ml. The reaction was stopped with 1.0 ml of 0.1 N NaOH, and the absorbance at 410 mμ was measured. A unit is defined as an optical density change of 2.0/20 min. The 3′-nucleotidase activity was determined from phosphate release from 3′-AMP. The 3′-AMP assay consisted of 2 mM 3′-AMP, 10 mM $CoCl_2$, and either 50 μM sodium acetate (pH 6.0) or 100 mM Tris-maleate (pH 7.8) in a final volume of 0.1 ml. The reaction was stopped with 0.2 ml of 0.05 N HCl and the phosphate released was determined by the method of Ames and Dubin (1960). A unit is 1 μmole of phosphate released/hr at 37°. Hydrolysis of *p*-nitrophenyl phosphate was determined with 5 mM *p*-nitrophenyl phosphate, 5 mM $CoCl_2$, and 100 mM sodium acetate (pH 6.0) in a

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

total volume of 0.1 ml at 37°. The reaction was stopped with NaOH and activity was determined as for the bis(*p*-nitrophenyl)phosphate.

Assays for alkaline phosphatase, acid phenylphosphatase, acid hexose phosphatase, RNase I, RNase II, DNase, and 5′-nucleotidase (uridine diphosphate sugar hydrolase) are previously published (Neu and Heppel, 1964a,b; Neu, 1967). Protein concentration was determined by the method of Lowry *et al.* (1951).

Purification of the Enzymes

Preparation of Cyclic Phosphodiesterase (3′-Nucleotidase) When the Enzyme Is Released by Osmotic Shock. Shigella sonnei is used as the example. *S. sonnei* were grown overnight to early stationary phase in high phosphate medium (Neu and Chou, 1967). They were washed and subjected to osmotic shock as described (Neu, 1968b). The osmotic shock fluid was concentrated to one-tenth the original volume by means of an Amicon membrane dialysis apparatus using filter no. 1. The concentrated osmotic shock fluid was applied to 2.5 × 40 cm DEAE-cellulose column (Whatman 32) prepared with 0.01 M Tris-HCl (pH 7.5). The cyclic phosphodiesterase was eluted with a linear gradient (1000 ml) of 0–0.2 M NaCl in 0.01 M Tris (pH 7.5). Flow rate was 50 ml/hr and fractions of 6 ml were collected. The enzyme was eluted as a sharp peak one-third of the way in the gradient. Active fractions (90 ml) were pooled and dialyzed against three changes of 2 l. of 0.01 M potassium phosphate (pH 7.2). The dialyzed material was applied to a 2 × 10 cm hydroxylapatite column at a rate of 0.5 ml/min. The enzyme was eluted with a 0.01–0.2 M potassium phosphate gradient of 400 ml. Fractions of 3 ml were collected and pooled after activity was determined by the bis(*p*-nitrophenyl)phosphate assay. Active fractions (28 ml) were dialyzed against four changes of 2 l. of 0.01 M Tris-HCl (pH 7.5) over 12 hr. The dialyzed material was applied to a 1.4 × 10 cm DEAE-cellulose column (Whatman No. 32) which had been preequilibrated with 0.01 M Tris-HCl (pH 7.5). A gradient of NaCl 0–0.2 M in 0.01 M Tris-HCl was used. Fractions (2 ml) were collected at a rate of 0.5 ml/min. This yielded material that in the peak tube had a specific activity of 5000 and the pooled average specific activity (10 ml) was 2500 with 3′-AMP as substrate.

The osmotic shock fluids of *S. marcescens. E. aerogenes, Salmonella heidelberg*, and *E. coli* were processed in a similar manner. Over-all yield was about 40% of the starting material.

Preparation of Cyclic Phosphodiesterase (3′-Nucleotidase) When the Enzyme Is Not Released by Osmotic Shock. Proteus vulgaris was grown to early stationary phase in the high phosphate medium. The cells were washed, resuspended in 0.01 M Tris-HCl (pH 7.5)–0.01 M $MgCl_2$ at a ratio of 3 g (wet weight) to 10 ml, and sonically disrupted. The disrupted cells were centrifuged for 30 min at 19,000 rpm in an SS34 head of a Sorvall RC2B centrifuge. The pellet was discarded and the supernatant fluid was brought to 40% saturation with solid $(NH_4)_2SO_4$ (Mann purified) and the pH was adjusted to 7.2 with 3 N NH_4OH. After 30 min at 3° the material was recentrifuged for 15 min as before. The pellet was discarded and the supernatant was brought to 70% $(NH_4)_2SO_4$ saturation. After 1 hr at 3° the material was centrifuged and the pellet was resuspended in 110 ml of 0.01 M Tris-HCl (pH 7.5). This was dialyzed against three changes of 4 l. overnight. The dialyzed material was applied to a Whatman No. 32 DEAE-cellulose column (2.5 × 35) cm which had been preequilibrated with Tris-HCl. A linear 0–0.2 M NaCl gradient (1000 ml) in 0.01 M Tris-HCl was used and 6-ml fractions were collected. The peak tubes (106 ml) were pooled and dialyzed against three changes of 2 l. of 0.01 M potassium phosphate (pH 7.2). This fraction was applied to a 2 × 15 cm hydroxylapatite column at a rate of 0.5 ml/min. A 300-ml gradient of 0–0.2 M potassium phosphate (pH 7.2) was used and 3-ml fractions were collected. The pooled fractions (60 ml) were dialyzed against a total of 10 l. of 0.01 M Tris-HCl (pH 7.5) overnight and then applied to a 1 × 15 cm DEAE-cellulose (Whatman No. 32) column. A gradient of 0–0.2 M NaCl (200 ml) in 0.01 M Tris-HCl was employed and 2-ml fractions were collected. The peak tube had a specific activity of 6000 and the pool (12 ml) 3200.

The same scheme was used for purification of *Proteus mirabilis* and *Providencia* enzymes. In order to purify the cyclic phosphodiesterase activity which could not be released from *S. marcescens, S. heidelberg*, and *E. aerogenes* by osmotic shock the cells were sonically disrupted. The crude extract was treated in the same scheme used for *P. vulgaris*. The material was brought to 40% $(NH_4)_2SO_4$ saturation and the precipitate that formed was discarded. The precipitate obtained between 40 and 70% $(NH_4)_2SO_4$ saturation was retained and dialyzed against 0.01 M Tris-HCl (pH 7.5). This material was then subjected to DEAE-cellulose chromatography followed by hydroxylapatite column chromatography and repeat DEAE-cellulose chromatography. Enzymes obtained by this method had specific activities of 3000–4000 with 2′,3′ > UMP as substrate.

Results

Properties of the Enzymes

Contamination with Other Enzymes. DNase activity was absent as measured by hydrolysis of both native and denatured *E. coli* K12λ DNA. There was no RNase I or II activity using both *E. coli* tRNA and poly A with the assays of Neu and Heppel (1964b) and Singer and Tolbert (1965). After the second DEAE-cellulose step, traces of 5′-nucleotidase (Neu, 1968b), acid hexose phosphatase (Dvorak *et al.*, 1967) were removed.

Stability. There was a loss of cyclic phosphodiesterase activity in the osmotic shock fluid stored at 3°. Frozen samples were stable for 1 year. The enzyme after the hydroxylapatite step showed rapid decay even if frozen at −40°. The purified cyclic phosphodiesterase is stable for 6 months at 40° but loses 10–20% of activity each time it is thawed.

Proportionality to Time and Enzyme Concentration. Under the assay conditions used, the release of inorganic phosphate from 3′-AMP and 2′,3′-cyclic UMP was proportional to enzyme concentration over a 100-fold range. The assays were linear for 30 min. The re-

lease of *p*-nitrophenol from bis(*p*-nitrophenyl)phosphate and *p*-nitrophenyl phosphate was also proportional with respect to enzyme concentration and time. Figure 1 shows that the release of P_i from 3′-UMP and 2′,3′-cyclic UMP and a combination of the two is linear. There is no lag of P_i release from 2′,3′-cyclic UMP. The increase in velocity of P_i release with the increasing amount of 3′-UMP in the reaction suggests that the cleavage of the cyclic phosphodiesterase bond is rapid and not inhibited by 3′-UMP. Thus, in assaying for cyclic phosphodiesterase activity, the 3′-nucleotidase activity was not rate limiting. Valid determinations of phosphodiesterase activity could be made measuring inorganic phosphate release from cyclic nucleotides in the absence of phosphatase.

TABLE I: Purification of Cyclic Phosphodiesterases (3′-Nucleotidases).[a]

Fractions	Units	Protein	Sp Act.
S. sonnei			
Sonic extract	5.8	2.9	2
I Osmotic fluid	6.1	0.11	55
II DEAE-cellulose	62	0.12	516
III Hydroxylapatite	96	0.1	960
IV DEAE-cellulose	124	0.05	2480
S. heidelberg			
Sonic extract	12.35	1.59	7.9
I Osmotic fluid	13.5	0.07	193
II DEAE-cellulose	160	0.1	1600
III Hydroxylapatite	339	0.1	3390
IV DEAE-cellulose	213	0.04	5322
E. coli Ca48			
Sonic extract	9.05	2.2	4.1
I Osmotic fluid	8.1	0.15	54
II DEAE-cellulose	192	0.2	960
III Hydroxylapatite	233	0.15	1555
IV DEAE-cellulose	268	0.06	4466
P. vulgaris			
I Sonic extract	242	27.1	8.9
II $(NH_4)_2SO_4$ (40–70%)	686	20	34.3
III DEAE-cellulose	132	0.43	308
IV Hydroxylapatite	71	0.4	177
V DEAE-cellulose	625	0.19	3270

[a] Details of the procedure are in the text.

Dependence of Rate of Hydrolysis upon pH. The pH optimum of the cyclic phosphodiesterases using the 2′,3′-cyclic UMP as substrate and Tris-maleate as the buffer system was 7.8. A similar situation existed for hydrolysis of 3′-AMP. No significant differences in pH optima of the cyclic phosphodiesterases of *E. coli* Ca38, *S. sonnei*, *S. marcescens*, *S. heidelberg*, and *P. vulgaris* were noted. All were most active in a range of 7.2–8.0.

TABLE II: Effect of Temperature on Inactivation of Enzymes.[a]

Temp (°C)	Rel Act. (%) *S. sonnei* 3′-AMP	*S. sonnei* U > P	*P. vulgaris* 3′-AMP	*P. vulgaris* U > P
3	100	100	100	100
37	98	96	111	98
45	103	78	116	89
55	53	32	33	31
80	27	38	21	21

[a] The enzyme preparation was heated in the presence of 0.1 M Tris-maleate buffer (pH 7.8) for 5 min at the designated temperature. The samples were rapidly chilled to 3° and aliquots were assayed immediately by the usual methods.

At pH 5.7 *Proteus* and *Salmonella* cyclic phosphodiesterases showed 40% of the cyclic phosphodiesterase activity at pH 7.8. *E. coli*, *Shigella*, *Serratia*, and *Enterobacter* cyclic phosphodiesterases showed 60% of optimal cyclic phosphodiesterase activity at pH 5.7. Hydrolytic activity with 3′-AMP as substrate at pH 8.4 for all organisms was 50% of that at pH 7.8.

Heat Stability. In all cases studied the cyclic phosphodiesterase and 3′-nucleotidase activity showed parallel decay. Table II illustrates this for the enzyme re-

TABLE III: Effect of Various Metal Ions upon Hydrolysis of 3′-AMP.[a]

Compound	Organism and Source of Enzyme: *S. Sonnei* Osmotic Shock	*S. heidelberg* Osmotic Shock	*S. marcescens* Sonic Extract	*P. vulgaris* Sonic Extract
None	59	38	35	70
Co^{2+}	100	100	100	100
Ca^{2+}	55	40	37	95
Mg^{2+}	57	52	33	90
Mn^{2+}	102	88	122	100
Zn^{2+}	20	7	28	36
Co^{2+}, Ca^{2+}	99	97	100	96
Co^{2+}, Mg^{2+}	94	98		103

[a] The assay contained 2 mM 3′-AMP (total volume 0.1 ml), 100 mM Tris-maleate (pH 7.8), enzyme, and the noted cations at 10 mM. In the case of two cations both were at the same concentration. Relative activity is expressed in terms of the assay done in the presence of $CoCl_2$.

TABLE IV: Effect of Various Compounds on Hydrolysis of 3′-AMP.[a]

Compound	Concn (M)	Rel Act. (%) *S. sonnei*	Rel Act. (%) *P. vulgaris*
None		100	100
EDTA	10^{-4}	81	89
	10^{-2}	28	40
Urea	10^{-2}	100	81
	4.0	20	18
KF	10^{-3}	100	100
NaN_3	10^{-3}	98	93
Mercaptoethanol	10^{-3}	107	103
Iodoacetate	10^{-4}	57	70
p-Hydroxymercuribenzoate	10^{-5}	72	100
$CuSO_4$	10^{-3}	13	10

[a] The standard assay was used. The enzyme was incubated for 30 min at 3° before the substrate (3′-AMP) was added.

TABLE V: Substrate Specificity of Cyclic Phosphodiesterase (3′-Nucleotidase).[a]

Substrate	Rel Act. (%) *S. sonnei*	Rel Act. (%) *S. marcescens*	Rel Act. (%) *P. vulgaris*
3′-AMP	100	100	100
3′(2′)-UMP	90	100	100
3′(2′)-CMP	88	*b*	90
3′(2′)-GMP	95	*b*	103
2′,3′ > AMP	50	*b*	55
2′,3′ > UMP	68	65	65
2′,3′ > CMP	52	*b*	60
2′-AMP	0.001	0	0.001
pUp	0.02	*b*	0.02
ATP	0	0	0
5′-AMP	0	0	0
3′,5′ > AMP	0	0	0
PNPP[c]	28	36	22
Bis-PNPP	32	41	38
NADP	0	0	0
Ribose-5-P	0	0	0
Glucose-6-P	0	0	0
UDP-glucose	0	0	0
dAMP	0	0	0

[a] A value of 100 is assigned to activity against 3′-AMP. Assay conditions are as indicated in Methods. [b] No assay was performed. [c] PNPP.

leased by osmotic shock from *S. sonnei* and the enzyme from *P. vulgaris*. We failed to see significant activation of the enzymes when heated at 50° as had been reported by Anraku (1964a) with *E. coli* B. Activation was not seen with either the crude extracts or with the purified enzymes of any of our *E. coli* strains. No reactivation of enzymatic activity occurred on prolonged standing at 3 or 23°.

Effect of Divalent Cations on Enzymatic Activity. The influence of various cations on the hydrolysis of 3′-AMP is seen in Table III. Cation influence on the hydrolysis of 2′,3′-cyclic UMP, bis(*p*-nitrophenyl)phosphate, and *p*-nitrophenyl phosphate was similar. In contrast to the 5′-nucleotidases of the *Enterobacteriaceae* no significant increase in stimulation of hydrolysis occurred with multiple cations. The optimal cobalt concentration for all 3′-nucleotidase activity was 5 mM. For all organisms except *Proteus*, Mg^{2+} and Ca^{2+} caused less stimulation of hydrolysis than Co^{2+} or Mn^{2+}. Zinc and nickel inhibited all hydrolytic activities of the enzymes. At pH 5.7 there was no significant difference among Co^{2+}, Mn^{2+}, Ca^{2+}, and Mg^{2+} in causing stimulation of hydrolysis of 3′-UMP or 2′,3′ > UMP.

Effect of Phosphate and Various Compounds upon Enzymatic Activity. In contrast to the acid and alkaline phosphatases of the *Enterobacteriaceae*, the cyclic phosphodiesterases of *P. vulgaris*, *S. sonnei*, and *S. marcescens* were not inhibited by the presence of phosphate. In the presence of 10^{-3} M potassium phosphate using the bis(*p*-nitrophenyl)phosphate assay, 100% activity was present. At 0.03 M, 58% activity remained. Similarly, no inhibition of hydrolysis of 3′-AMP was seen.

Urea (4.0 M) resulted in a significant inhibition of 3′-nucleotidase activity of enzymes obtained from organisms that release the nucleotidases as well as those that do not (Table IV). Hydrolysis of *p*-nitrophenyl phosphate was also inhibited. EDTA seemed to be more effective in inhibiting the hydrolysis of 3′-AMP by the *E. coli* and *Shigella* 3′-nucleotidases than it was in inhibiting the *Proteus* 3′-nucleotidase activity.

Substrate Specificity. The relative activity of these enzymes toward a number of nucleotides is shown in Table V. All enzymes hydrolyze 3′-nucleotides, 2′,3′-cyclic nucleotides, bis(*p*-nitrophenyl)phosphate, and *p*-nitrophenyl phosphate. There is no base specificity in the case of either 3′-nucleotides or cyclic nucleotides. Substitution by a phosphoryl group as in pTp makes a compound relatively resistant as are compounds like ApAp. Oligonucleotides are not dephosphorylated by the cyclic phosphodiesterase but the cyclic phosphodiester bond is opened. Incubation of the enzyme with a cyclic oligonucleotide such as ApApA > p in the presence of alkaline phosphatase results in the slow release of phosphate but no nucleosides. This occurs with only a fraction of the activity against cyclic nucleotides. Nucleotide sugars are not hydrolyzed nor are 3′,5′-cyclic nucleotides. Ribose and hexose phosphates are resistant at pH 5.7 and 7.8.

Effect of Substrate Analogs. Although Anraku (1964b) had not seen any inhibitory effect of uridine on the *E. coli* B cyclic phosphodiesterase, as Table VI shows, we

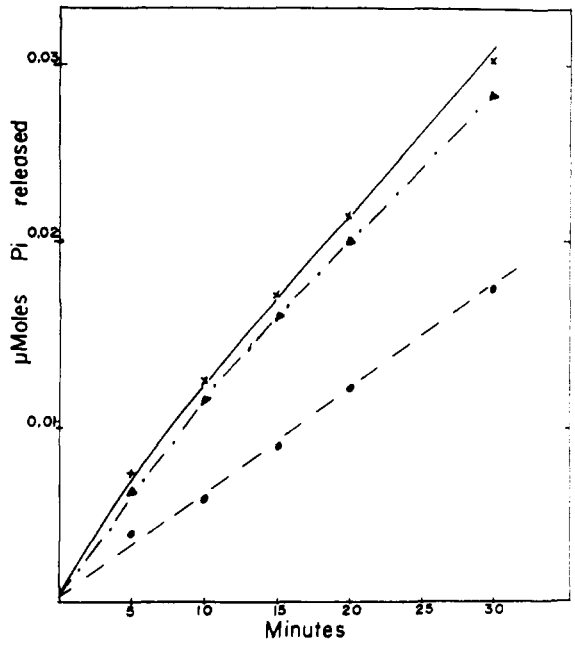

FIGURE 1: Release of P_i from the substrate as a function of time of incubation. The reaction mixture contained 0.1 µM 2',3' > UMP or 0.1 µM 3'-UMP, or 0.05 µM 2',3' > UMP, and 0.05 µM 3'-UMP, 100 mM Tris-maleate (pH 7.8), 5 mM $CoCl_2$, and enzyme. The reaction was stopped with 0.2 ml of 0.1 N HCl, and the phosphate released was determined by the method of Ames and Dubin (1960). (●) P_i released from 0.1 µM of 2',3'-cyclic UMP. (×) P_i released from 0.1 µM of 3'-UMP. (▼) P_i released from a mixture of 0.05 µM 2',3'-cyclic UMP and 0.05 µM 3'-UMP.

noted inhibitory effect on both *Shigella* and *Proteus* enzymes. Our assays were run in the absence of Co^{2+} and at pH 7.8 rather than 5.8. It is clear that both cyclic phosphodiesterase and 3'-nucleotidase activities are inhibited by tRNA and ribopolynucleotides. The effect of nucleotides upon hydrolysis of bis(*p*-nitrophenyl)phosphate and *p*-nitrophenyl phosphate was not determined.

Effect of Substrate Concentration. The calculated K_m values for the various enzymes are seen in Table VII. Over a wide range the initial substrate concentration had no effect on the over-all hydrolysis of 3'-nucleotides or cyclic phosphonucleotides. The only difference that could be seen between enzymes from bacteria that released their cyclic phosphodiesterases (3'-nucleotidases) by osmotic shock and those that did not were in the V_{max} values. The *Shigella* showed values of 1680 µmoles/hr per mg of protein as compared with 1055 µmoles/hr per mg of protein for *Proteus*. A V_{max} of 1100 µmoles for 2',3'-cyclic UMP was also found for *Proteus*. These figures probably do not reflect any significant differences.

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 *Citrobacter freundii*, *S. sonnei*, and *S. heidelberg* showed identical elution volumes as compared with enzymes prepared from sonic extracts of *E. aerogenes* (enzyme left in the cells after shock) and *P. vulgaris* (Figure 2). A combination of enzymes from the five bacteria gave a single peak of protein and hydrolytic activity against 3'-AMP and 2',3'-cyclic UMP. Molecular weights calculated from such runs using RNase, human lysozyme, bovine serum albumin, and *E. coli* alkaline phosphatase as standards gave values of 57,400–64,200.

TABLE VI: Effect of Substrate Analogs on Cyclic Phosphodiesterase and 3'-Nucleotidase Activity.[a]

Substrate	Analog and Concn	Rel Act. (%) *Proteus*	Rel Act. (%) *Shigella*
3'-AMP	None	100	100
	Adenosine (0.08 mM)	86	102
	Adenosine (0.4 mM)	80	97
	5'-AMP (4 mM)	99	100
	2'-AMP (4 mM)	82	82
	tRNA (1 µg)	55	84
	Poly A (1 µg)	37	66
3'-UMP	None	100	
	Uridine (0.4 mM)	83	
	Uridine (4 mM)	58	
	5'-UMP (5 mM)	92	
	Poly U (1 µg)	100	
	Poly A (1 µg)	83	
	tRNA (1 µg)	82	
	DNA (1 µg)	93	
2',3'-Cylic UMP	None	100	
	Uridine (0.4 mM)	71	
	Uridine (4 mM)	43	
	5'-UMP (5 mM)	93	
	Poly U (1 µg)	100	
	Poly A (1 µg)	69	
	tRNA (1 µg)	64	
	DNA (1 µg)	84	

[a] The reaction contained 2 mM substrate, 100 mM Tris-maleate (pH 7.8), 5 mM $CoCl_2$, and enzyme. Test compounds were added to achieve concentration noted.

Polyacrylamide Gel Electrophoresis. Acrylamide gel electrophoresis was performed with 7% polyacrylamide at 5 or 23° in Tris-glycine buffer (pH 8.9). Electrophoresis of the purified cyclic phosphodiesterases (3'-nucleotidases) or *E. coli*, *S. sonnei*, *S. heidelberg*, *S. marcescens*, and *P. vulgaris* gave single protein bands that migrated identical distances and corresponded to the enzymatic activity for hydrolysis of bis(*p*-nitrophenyl)-phosphate, 3'-AMP, and 2',3'-cyclic UMP. We found that trace bands were present after the hydroxylapatite chromatography and these were removed by the second DEAE-cellulose chromatography.

Activity of Intact Cells. Interpretation of hydrolytic activities of whole cells incubated with nucleotide substrates are open to question. But it is generally agreed that these nucleotides do not enter cells. To determine whether intact *Proteus* cells that did not release the 3'-

TABLE VII: Michaelis Constant for Cyclic Phosphodiesterase and 3′-Nucleotidase Activities.[a]

Organism	K_m of Substrate		
	3′-AMP	3′-UMP	2′,3′-Cyclic UMP
S. sonnei	4.5×10^{-5}	2.2×10^{-5}	2×10^{-5}
P. vulgaris	5×10^{-5}	7×10^{-4}	6.75×10^{-4}
S. heidelberg	*b*	2.4×10^{-4}	2×10^{-4}

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

nucleotidases on osmotic shock could hydrolyze nucleotides, we incubated intact cells with 3′-AMP in the presence of $MgCl_2$ and $CoCl_2$. All organisms can hydrolyze *in vivo* 3′-AMP, *i.e.*, *Shigella*, *Serratia*, and *Proteus*. In fact, intact organisms can hydrolyze nucleotides at rates comparable with sonically disrupted cells.

Discussion

This report describes the characteristics of the cyclic phosphodiesterases of the *Enterobacteriaceae*. Previous studies from our laboratory (Neu and Chou, 1967) had shown that this enzyme was released by the process of osmotic shock from *E. coli*, *Citrobacter*, and *S. sonnei*, but that only half of the enzyme was released from members of the *Enterobacter*, *Serratia*, and *Salmonella* groups. The enzyme was not released from *Proteus* species or *Providencia*. In the present study no differences in the enzymes from the different bacteria were discernible by the methods employed. All of the enzymes hydrolyze 3′-nucleotides, 2′,3′-cyclic nucleotides, and two chromogens, *p*-nitrophenyl phosphate at pH 6.0, and bis(*p*-nitrophenyl)phosphate. The constant ratios of specific activities for 3′-UMP and 2′,3′-cyclic UMP 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. The pH optima, ion stimulation, and substrate specificity of the nucleotidases showed only minor differences among the bacteria studied. The results obtained by polyacrylamide gel electrophoresis and molecular sieve chromatography with Sephadex seem to indicate that the proteins from different members of the *Enterobacteriaceae* are similar if not identical. Since intact *Proteus* cells can hydrolyze 3′-AMP, it is probable that the cyclic phosphodiesterase (3′-nucleotidase) is a surface enzyme in all *Enterobacteriaceae*. Studies of the histochemical electronmicroscopic localization of the enzyme are in process. The fact that osmotic shock fails to release all the enzyme from *Enterobacter* strains and releases none of the *Proteus* enzyme is more likely due to differences in cell wall protoplasmic membrane relationships.

There are several differences in our results and those of Anraku (1964a,b) on *E. coli* B. In our hands the pH optimum with 3′-nucleotides and 2′,3′-cyclic nucleotides was 7.5–8.0 rather than 6.4. We confirmed the stimulation of hydrolysis by Co^{2+} but noted that stimulation of enzymatic activity by Co^{2+} was greatest in those bacteria which totally release the enzyme on osmotic shock, *i.e.*, *E. coli* and *Shigella*. We were unable to confirm the activation of any of the enzymes by heating at 45 or 55°. We also noted inhibition of activity by ribonucleosides but not by the 5′-nucleotides. These differences might be explained by differences in our assay procedures from those of Anraku (1964a,b). Although the majority of the data in this paper deals with the cyclic phosphodiesterase (3′-nucleotidase) of *P. vulgaris*, we purified the enzyme from two strains of *P. mirabilis* and could detect no differences.

The fact that the enzyme hydrolyzes the cyclic phosphodiester bond of oligonucleotides (*i.e.*, ApApApAp) but not the phosphate bond and also poorly hydrolyzes compounds such as pTp suggests that the cyclic phosphodiesterase and 3′-nucleotidase are at separate sites.

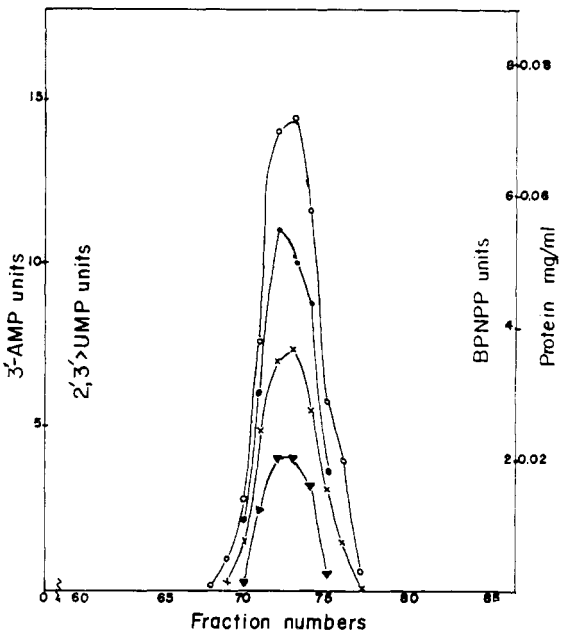

FIGURE 2: Sephadex G-100 chromatography of a mixture of cyclic phosphodiesterases of *Citrobacter freundi*, *S. sonnei*, *E. aerogenes*, *S. heidelberg*, and *P. vulgaris*. (○) 3′-AMP units, (×) 2′,3′-cyclic UMP, (●) bis(*p*-nitrophenyl)phosphate units, and (▼) protein. A 2.5 × 95 cm Sephadex G-100 column in 0.4 M NaCl–0.05 M Tris-HCl (pH 7.5) was layered with 2.0 ml of enzymes. Fractions of 2 ml were collected at a rate of 2 ml/hr.

The greater inhibition of cyclic phosphodiesterase activity by RNA also suggests this.

Neu (1968a,b) showed that the cyclic phosphodiesterases (3′-nucleotidases) of the *Enterobacteriaceae* are constitutive enzymes not sensitive to the regulatory mechanisms affecting phosphatase production such as phosphate repression and catabolite inhibition. The precise role of these enzymes in cellular metabolism remains to be elucidated. In view of the slow cyclic phosphodiesterase activity of RNase I which is also a so-called periplasmic or surface enzyme it could be postulated that they act to cleave the nucleotides produced by RNase so that the nucleosides can enter the cell. However, *Pseudomonas*, *Alcaligenes fecalis*, and *Pasteurella*, all of which lack RNase I, do not have this enzyme (Neu, 1968a). However, *E. coli* MRE600, an RNase I deficient strain, has normal amounts of the cyclic phosphodiesterase. Hopefully some insight into the biological role of these proteins will come from mutants of *E. coli* showing marked decrease of the enzymes.

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