BACTERIAL NUCLEOTIDASES

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ABSTRACT

KOHN, J. (Queen Mary's Hospital, London, England) AND J. L. REIS. Bacterial nucleotidases. J. Bacteriol. **86**:713–716. 1963.—The 3- and 5-nucleotidase activity in various bacterial species was investigated. Both enzymes were found in bacterial extracts in varying proportions. The nucleotidases were found to be very active in *Proteus vulgaris*, in which organism they were studied in detail. The relative activities, the pH optima, and the effect of metal ions were investigated. It was concluded that bacterial 3- and 5-nucleotidases are distinct and separate enzymes.

Bacterial phosphatases have been studied by several authors, but comparatively little attention has been paid to the problem of specific phosphatases and to nucleotidases in particular. Wang (1954) found that a soil bacterium (strain U 1) showed a considerable activity of 5-nucleotidase; Swartz, Kaplan, and Frech (1956) studied the heat activation of 5-nucleotidase in *Proteus vulgaris*. As 5-nucleotidase is widely distributed in animal tissues (Reis, 1940, 1951) and a "b" nucleotidase (probably 3-nucleotidase) in plants (Shuster and Kaplan, 1953), it seemed worthwhile to investigate these enzymes in bacteria.

MATERIALS AND METHODS

Cultures. Bacterial strains were isolated from human sources, and some cultures were obtained from the National Collection of Type Cultures. The organisms were grown on nutrient agar, harvested, washed in normal saline, and then suspended in distilled water in concentrations ranging from 5 to 10 mg (dry weight) per ml.

Preparation of bacterial extracts for enzyme studies. It was found that after addition of chloroform to the bacterial suspension approximately 90% of the enzyme activity could be found in the supernatant fluid after centrifugation. Extracts were, therefore, prepared from bacterial suspensions treated with 1 drop of chloroform per ml, left for 2 days at 4 C, and then centrifuged. The clear supernatant was used for enzyme studies. Extracts kept at 4 C with chloroform as preservative showed no loss of activity even after a period of 1 year. The dry weight of the residue obtained after evaporation of the supernatant amounted to about 20% of the original dry weight of the bacterial mass.

Estimation of enzyme activities. The enzyme activity was estimated by a method described in a previous paper (Reis, 1951). For determination at pH 7.5, a 0.05 M Veronal buffer was used. At other pH values, phthalate or carbonate-bicarbonate buffers were used.

Substrates. Substrates used were (i) adenosine 5′-phosphoric acid ("muscle" adenylic acid), (ii) yeast adenylic acid (mixture of adenosine 3′-phosphoric acid and adenosine 2′-phosphoric acid), (iii) pure adenosine 2′-phosphoric acid, (iv) adenosine 3′-phosphoric acid, and (v) sodium phenyl phosphate (all obtained from L. Light & Co., Ltd., Colnbrook, Bucks., England). The concentration of the substrate solutions was 0.01 M, and the pH was adjusted to 7.5.

Techniques. A 0.2-ml portion of the bacterial enzyme extract, prepared as above, was added to a tube containing 1.6 ml of the buffer solution and 0.2 ml of the substrate. The enzyme extract was further diluted if necessary, to avoid hydrolysis of more than 50% for pure nucleotides and 25% for yeast adenylic acid. The tubes were incubated at 38 C, usually for 0.5 hr. To stop enzyme activity, 4 ml of 5% trichloroacetic acid were added. The free phosphate was then estimated by the Fiske and SubbaRow (1929) method, modified to suit our requirements. After centrifugation, to each sample were added 0.2 ml of 10% ammonium molybdate in 30% (v/v) sulfuric acid, 0.1 ml of 0.2% amino-napthol-sulfonic acid in a solution of 12% sodium metabisulfite, and 2.4% sodium sulfite ($Na_2SO_3 \cdot 7H_2O$). The color was developed for 5 min at 38 C, and was estimated in a photoelectric absorptiometer.

The enzyme activity was expressed in μg of phosphorus hydrolyzed by an equivalent of 1 mg of dried bacterial mass.

It must be borne in mind, of course, that any nonspecific phosphatase present also acts on the adenosine phosphoric acids used in our experiments; the nonspecific phosphatase activity was therefore estimated concurrently by using phenyl-phosphate as substrate. The difference between the adenosine phosphoric acid hydrolysis and the phenyl phosphate hydrolysis gives the approximate value of the specific nucleotidase activity.

RESULTS

Specificity. In our experiments, in addition to hydrolysis of muscle adenylic acid (adenosine 5′-phosphoric acid) due to a 5-nucleotidase, we also found hydrolysis of yeast adenylic acid. The latter is a mixture of adenosine 2′- and adenosine 3′-phosphoric acids. It was easily demonstrated (Fig. 1) that only about half of yeast adenylic acid is hydrolyzed by the bacterial nucleotidases. This was also found by Shuster and Kaplan (1953), working on Rye grass extracts. When yeast guanylic acid (i.e., a mixture of guanosine 2′- and 3′-phosphoric acids with a predominance of the latter) was used as substrate, its maximal hydrolysis was higher, but still was not more than 62%. To decide which of the two adenylic acid compounds was hydrolyzed in our case, we used pure adenosine 2′-phosphoric and adenosine 3′-phosphoric acids. It was found that only the adenosine 3′-phosphoric acid was hydrolyzed. The very low degree of hydrolysis of adenosine

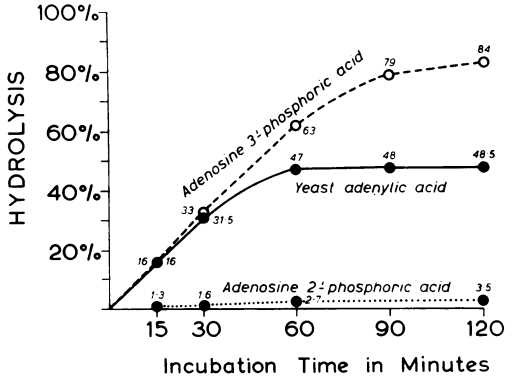

FIG. 1. *Hydrolysis of yeast adenylic acid and its two component nucleotides by Proteus vulgaris. Enzyme extract: 0.2 ml corresponding to 0.12 mg of dry bacteria. Temperature, 38 C; pH 7.5.*

TABLE 1. *Hydrolytic activity of nucleotidases and nonspecific phosphatase in various bacterial species**

Organism	Phenyl-phos-phate	Adenosine 3′-phos-phate†	A denosine 5′-phos-phate
Proteus vulgaris	4	200	270
P. vulgaris	10	212	483
P. vulgaris	10	374	150
P. vulgaris	17	380	46
P. vulgaris	1	42	46
P. vulgaris	2	64	158
P. rettgeri	2	72	51
P. mirabilis	2	137	120
P. mirabilis	4	75	83
P. morganii	41	33	63
P. inconstans 9207	3	36	108
P. inconstans 8893	1	13	34
P. inconstans 9262	2	13	28
P. inconstans 9249	20	25	94
P. inconstans 9559	5	254	123
Haemophilus influenzae	70	50	590
H. influenzae	10	22	208
H. influenzae	18	62	106
Staphylococcus aureus	50	11	20
S. aureus	75	19	24
S. epidermidis	1	0	0
Escherichia coli	1	3	6
Pseudomonas aeruginosa	0	0	0
Clostridium sporogenes	0	0	0
C. welchii	1	8	65
Bacillus cereus	1	1	1
Serratia marcescens	3	3	2
Diplococcus pneumoniae	0	0	0
Salmonella typhimurium	3	4	1
Shigella dysenteriae	2	3	3

* The hydrolytic activity is represented as μg of phosphorus hydrolyzed by 1 mg of dry bacterial mass in 1 hr at pH 7.5 at 38 C.

† Yeast adenylic acid was used.

2′-phosphoric acid detected could be due to a nonspecific phosphatase, or possibly to some adenosine 3′-phosphoric impurities present in the adenosine 2′-phosphoric acid preparation.

Distribution. Table 1 shows the enzyme activities of various bacterial species. It can be seen that there is a great variation among different species, and even within one species there are strain variations. Nevertheless, some species characteristics could be observed. We found that the nucleotidase activity of *Haemophilus influenzae* and of *P. vulgaris* differs greatly from most of the other bacterial species examined.

H. influenzae has a very high 5-nucleotidase and a very low 3-nucleotidase activity, whereas in *P. vulgaris* both the 5-nucleotidase and 3-nucleotidase activity are usually very high. The relative activities are variable; sometimes the 5- and sometimes the 3-nucleotidase predominates. Other members of the *Proteus* group appear to behave similarly to *P. vulgaris*, although the overall activities are less pronounced. They all show very little nonspecific phosphatase activity with the exception of *P. morganii*, in which nonspecific phosphatase activity is always considerable.

All the other bacterial species investigated had either very low nucleotidase activity or none at all, except *Clostridium welchii*, which revealed moderate 5-nucleotidase activity. *Staphylococcus aureus* is an example of a microorganism without any demonstrable nucleotidase activity, but with abundant nonspecific phosphatase with an optimum at pH 5.6. It is interesting to compare the activity of nucleotidases in bacteria with that in other known sources of these enzymes. The highest 5-nucleotidase activity in animal tissue has been found in bovine testis, in which the specific activity is about 1000 μg of phosphorus per mg of dry tissue, whereas the highest activity found in *P. vulgaris* did not exceed 500 μg of phosphorus per mg of dried bacterial mass.

Influence of pH. Figure 2 shows the effect of pH on the nucleotidase activity. The 5-nucleotidase of *P. vulgaris* has its optimum at about pH 6,

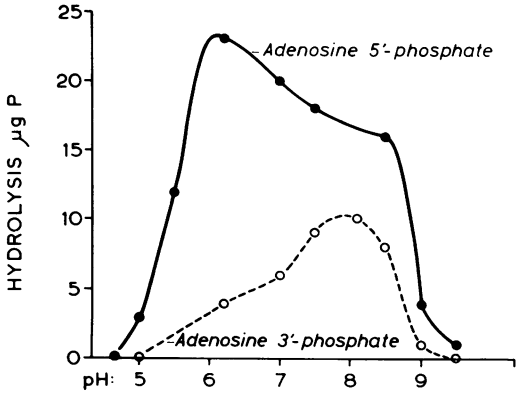

FIG. 2. *Effect of pH on nucleotide hydrolysis by Proteus vulgaris. Enzyme extract: 0.2 ml corresponding to 0.25 mg of dry bacteria; incubation time, 30 min; temperature, 38 C. (Hydrolysis of phenyl phosphate was less than 1 μg of phosphorus.)*

TABLE 2. *Activation and inhibition of Proteus vulgaris nucleotidases by metal ions**

Substrate	Hydrolysis in control†	Hydrolysis after addition of ions (0.001 M)‡			
		Mg^{++}	Mn^{++}	Zn^{++}	Ni^{++}
Adenosine 3'-phosphate (yeast adenylic acid)	18	102	94	26	9
Adenosine 5'-phosphate	26	92	138	61	26
Phenyl phosphate	1	—	—	—	—

* Enzyme extract: 0.2 ml corresponding to 0.19 mg of dry bacterial mass. Incubation time: 30 min at 38 C (pH 7.5).

† Expressed as μg of phosphorus.

‡ Expressed as percentage of control values.

TABLE 3. *Hydrolysis of mixed substrates by Proteus vulgaris nucleotidases**

Substrate	Final substrate concn (M)		
	0.001	0.002	0.004
Adenosine 3'-phosphate (yeast adenylic acid)	6.9	7.4	8.1
Adenosine 5'-phosphate	14.6	14.6	14.6
Mixture of both substrates (1:1)	19.6	20.3	—

* Results are expressed as μg of phosphorus hydrolyzed. Enzyme extract: 0.2 ml corresponding to 0.31 mg of dry bacteria. Incubation time: 30 min at 38 C (pH 7.5).

and the activity diminishes slowly with increasing pH, whereas the optimum for the 3-nucleotidase is in the region of pH 8.

Effect of metal ions on enzyme activities. The effects of metal ions are shown in Table 2. Manganese enhances the 5-nucleotidase activity but does not appear to affect the 3-nucleotidase. Zinc and, to a greater extent, nickel inhibit both enzymes. Magnesium has no influence on the activities of the two nucleotidases.

Mixed substrates experiments. The enzyme activity on a mixture of two substrates, namely, the "muscle" adenylic acid and the yeast adenylic acid, was estimated. The result was very near the sum of the activities of each substrate separately (Table 3).

DISCUSSION

Our investigations pointed to the presence of two separate nucleotidases, each of them specific. This was borne out by the different pH optima, by the variable activities against the 5′- and 3′-nucleotides, by the selective activation by manganese ions, and by the mixed substrates experiments. All these data appear to corroborate the assumption that the 3- and 5-nucleotidases are indeed two distinct and separate specific enzymes.

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