Studies in the Enzyme Make-up of *Vibrio cholerae*: Part V— Nucleotidase Activity of Vibrios

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Vibrio cholerae **and related water vibrios readily dephosphorylate nucleotides. General properties of the enzyme extracted from the cells are reported.**

THE deamination of nucleotides by resting cells of vibrios has been shown to be accompanied by the release of inorganic phosphate[1]. The consistent failure to detect any phosphatase activity in the organism led us to a study of the factors influencing the dephosphorylation of nucleotides both by intact vibrios and by enzyme extracts obtained from the cells.

Experimental procedure

The organisms were grown on nutrient agar (*p*H 8·0) and the methods employed for the preparation and standardization of cell suspensions have been described elsewhere[2].

Preparation of cell-free enzyme extract — The organism (49514 Og.) grown in one litre Roux bottles was harvested after 24 hr. and washed twice with saline on the centrifuge. The sedimented cells were treated with ten volumes of ice-cold acetone and immediately filtered under suction. The filter pad with the cells was evenly spread on a glass plate and dried over calcium chloride and sulphuric acid kept separately in a vacuum desiccator. If absolute acetone was used for dehydration of the cells, a crisp whitish powder could be obtained by drying for *c*. 2 hr.

The powder was ground with 100 mesh acid-washed sea sand for 5 min. in a cooled glass mortar, portions of ice-cold saline containing a fifth of the volume of 0·1M phosphate buffer (*p*H 7) being added at intervals. The mixture was centrifuged at 3,000 r.p.m. for 15 min. and the supernatant was set aside for further treatment. The residue was again ground up with buffered saline and the extract obtained by centrifugation was combined with the first, cooled down to about 5°C. and added with thorough mixing to 3·5 volumes of absolute alcohol kept at —10°C. The mixture was left in the deep freeze for 30 min., the alcoholic supernatant siphoned off and the sediment centrifuged to remove the remaining solvent.

The precipitate thus obtained was dispersed in saline containing a fifth in volume of a 1·5 per cent potassium chloride solution. The turbid dispersion was centrifuged at 2,500 r.p.m. for 15 min. The stringy residue that settled down was found to be devoid of dephosphorylase activity, but gave a strongly positive Molisch's test. This was discarded. Ammonium sulphate was added to full saturation to the supernatant and the resulting precipitate, collected by centrifugation, was dispersed in saline and dialysed against cold distilled water for 8 hr. A tenfold purification on dry weight basis was achieved by this procedure, although the overall recovery of activity accounted for only 32 per cent of the original value.

Assay of enzyme activity — Dephosphorylation was studied in a total volume of 1·5 cc. by incubating aliquots of cell suspension or cell-free enzyme, veronal buffer of appropriate *p*H value and specified substrates. At a fixed time, the reaction was arrested by the addition of 1·5 cc. of 10 per cent trichloroacetic acid. The tubes were left overnight in the cold, contents filtered through Whatman No. 42 paper and inorganic phosphate estimated in the filtrate by the colorimetric method of Fiske and Subbarow[3]. While using phenolphthalein phosphate as substrate, the activity was measured by estimating the intensity of the colour of phenolphthalein by addition of glycine buffer[4]. With diphenyl

phosphate, however, the phenol released was estimated by the Folin-Cicolateau method as adopted by Daniel *et al.*[5]. The substrates (Nutritional Biochemicals Corp., Cleveland, Ohio) were dispersed in distilled water and adjusted to *p*H 7·0. Excepting guanylic acid all the substances gave clear solutions by this method. Phenolphthalein phosphate used for the phosphatase test was prepared according to the procedure of Fishman *et al.*[6]. Other chemicals used were of reagent grade.

Results

Phosphatase activity — In order to test for phosphatase activity both the intact cells as well as culture filtrates were allowed to act upon a variety of substrates over a *p*H range of 4-10. None of the vibrio culture tested was found to hydrolyse the following substrates: sodium-β-glycerophosphate, sodium phenolphthalein phosphate, sodium diphenyl phosphate, sodium pyrophosphate, sodium hexametaphosphate, urea phosphate, glucose-1-phosphate, fructose-6-phosphate, fructose-1-6-diphosphate, ribo and desoxyribonucleic acids.

In order to see whether phosphatase could be produced by adaptation[7] of the organism the following experiment was carried out. A 0·55 per cent solution of phenolphthalein phosphate sterilized by Seitz filtration was aseptically added to a sterile medium consisting of Lab Lemco 1 per cent, peptone 1 per cent, and sodium chloride (*p*H 8·0) 0·5 per cent. The tubes were inoculated with *V. cholerae* (52 Og.) and a water vibrio (Water Tank). At intervals of 24 hr. 5 cc. glycine buffer (*p*H 10·0) were added to the tubes to detect the production of phenolphthalein by the action of phosphatase. The test was negative up to 96 hr. indicating absence of phosphatase activity in the organism.

Nucleotidase activity of different strains — Preliminary screening with a number of representative strains belonging to different antigenic groups of *Vibrio cholerae* and non-pathogenic water vibrios showed that they could readily attack the phosphoric acid ester linkage of purine and pyrimidine nucleotides. The results presented in Table 1 show that the activity was demonstrable in all the strains tested. Of the substrates tested ATP showed minimum cleavage. There was, however, a considerable variation in the activity of different strains and no correlation could, therefore, be found between dephosphorylase and antigenic structure of the organism.

Optimal conditions for dephosphorylation by cell suspensions — The action of a cholera vibrio (52 Og.) and a water vibrio was tested at various *p*H levels on six substrates. The data (Table 2) revealed that inorganic phosphorus was released from all the substrates and there was no sharp optimum *p*H. In general, the dephosphorylation was more

TABLE 1 — NUCLEOTIDASE ACTIVITY OF REPRESENTATIVE STRAINS OF VIBRIOS

[*Activity expressed as μg. P released from* 500 *μg. substrate by* 0·5 *cc. cells (opacity equivalent* 40% *transmission in Lumetron) in* 2 *hr.*]

CULTURE	ATP	AMP^3	AMP^5	CMP	GMP	UMP
Ogawa 52	37·5	46·8	33·7	40·0	42·5	42·5
Ogawa 123	17·5	36·3	40·0	40·0	33·7	36·3
Ogawa 49514	17·5	33·8	38·8	38·8	28·8	48·5
Inaba 52	10·0	36·3	36·3	43·2	25·0	38·8
Inaba 123	19·6	34·5	42·4	39·6	32·8	37·4
R 49514	17·6	41·3	36·3	41·3	37·5	48·8
El Tor 31	22·5	47·5	37·5	48·8	27·5	42·5
Nag 1	30·0	32·5	41·2	36·2	46·2	43·0
Nag 2	35·0	35·0	42·4	46·4	43·4	43·8
Water Tank	34·2	41·4	39·9	42·8	44·3	47·2

ATP, adenosine triphosphoric acid; AMP^3, adenylic acid yeast; AMP^5, adenylic acid muscle; CMP, cytidylic acid; GMP, guanylic acid; UMP, uridylic acid.

TABLE 2 — EFFECT OF *p*H ON NUCLEOTIDASE ACTIVITY OF VIBRIOS

[*μg. P liberated from* 250 *μg. substrate by* 0·5 *cells (opacity equivalent* 40% *transmission in Lumetron) in* 2 *hr.*]

*p*H	ATP		AMP^3		AMP^5		CMP		GMP		UMP	
	Og. 52	W. tank	Og. 52	W. tank	Og. 52	W. tank	Og. 52	W. tank	Og. 52	W. tank	Og. 52	W. tank
4·9	7·5	15·0	3·8	10·5	—	14·0	12·0	14·0	10·5	8·5	13·3	8·9
5·9	8·0	16·0	15·0	10·4	15·0	14·4	21·0	14·5	18·0	8·9	23·0	12·3
6·6	9·0	15·0	15·0	11·3	16·2	15·1	27·0	15·0	21·0	10·1	21·0	15·0
7·0	10·5	16·2	18·0	15·2	16·2	16·7	30·0	15·0	21·0	10·4	23·5	15·3
7·4	12·0	16·4	18·0	16·4	16·5	16·2	30·1	14·8	18·5	10·4	19·5	16·0
8·0	15·0	16·2	21·0	16·8	15·2	17·4	30·2	14·9	20·2	10·2	22·4	15·3
8·5	18·0	17·3	18·0	17·0	15·3	17·4	30·4	15·0	21·0	10·8	21·4	14·3
9·2	17·9	17·3	18·0	17·4	15·0	17·4	30·0	15·0	21·0	11·0	23·0	14·3

TABLE 3 — EFFECT OF CONCENTRATION OF CELL SUSPENSION ON NUCLEOTIDASE ACTIVITY

(*μg. P released from 250 μg. substrate in 2 hr.*)

Conc. of cell suspension opacity equivalent % transmission	ATP		AMP[3]		AMP[5]		CMP		GMP		UMP	
	Og.	WT	Og.	WT	Og.	WT	Og.	WT	Og.	WT	Og.	WT
80	2·5	2·5	16·3	11·2	13·3	13·2	15·0	10·0	3·8	7·2	10·5	10·2
60	8·7	6·8	18·4	19·3	18·4	17·8	23·0	20·0	6·5	8·1	15·8	14·8
40	14·6	10·0	28·4	21·7	23·4	21·3	28·5	22·4	14·2	12·6	30·0	26·2
20	16·4	15·0	38·5	23·8	26·5	24·2	28·7	27·6	16·1	16·8	38·0	31·4
10	17·5	16·8	37·5	24·8	26·4	24·6	29·0	28·9	17·4	17·1	37·5	34·0

TABLE 4 — EFFECT OF SUBSTRATE CONCENTRATION ON NUCLEOTIDASE ACTIVITY

(*μg. P released from different amounts of substrate by 0·5 cc. of 40% transmission cells in 2 hr.*)

Substrate μg.	ATP		AMP[3]		AMP[5]		CMP		GMP		UMP	
	Og.	WT	Og.	WT	Og.	WT	Og.	WT	Og.	WT	Og.	WT
125	8·7	6·4	17·5	12·5	18·8	17·4	17·4	7·5	11·2	6·3	15·0	11·3
250	16·2	11·2	35·0	18·5	45·0	35·0	33·8	8·8	21·8	10·0	28·8	28·8
500	38·7	20·0	60·0	31·2	81·3	68·8	68·8	20·0	35·0	27·5	63·8	43·4
1250	51·3	26·2	135·0	76·8	187·5	177·5	168·8	63·8	82·5	65·0	153·8	147·5
2500	63·8	35·0	216·0	155·0	211·3	202·5	235·0	127·5	156·8	131·3	257·5	282·5

pronounced when *p*H shifted towards the alkaline side. In Tables 3 and 4 are given the results obtained in experiments on the effect of concentrations of cell suspension and substrates respectively on the amount of phosphate liberated from six nucleotides by the same two cultures. The results showed that the amount of phosphorus released steadily increased with the concentrations of cells and substrates. To find out the rate of hydrolysis of the six nucleotides by the organism, cell suspensions, veronal buffer and substrates were incubated at 37°C. and activity assayed at 30 min. intervals up to 5 hr. It was observed that equilibrium state was reached within the first hour of reaction, complete dephosphorylation occurring in all nucleotides excepting ATP.

Effect of activators and inhibitors on the activity of intact cells — A cell suspension with an opacity equivalent of 20 per cent transmission in the Lumetron was incubated at 37°C. for 30 min. with an equal volume of a 0·01M solution of the following substances: potassium fluoride, 8-hydroxy quinoline, iodoacetate, cysteine, sodium azide, magnesium sulphate, calcium chloride and sodium cyanide. On testing these mixtures against the six substrates neither stimulation nor depression of enzyme activity could be observed as compared to the control suspension incubated with distilled water for the same period.

Properties of the cell-free extract — The buffered saline extract from the ground cells could catalyse the dephosphorylation of all the six nucleotides. During the final stages of purification, however, it was found that the enzyme retained activity only towards yeast adenylic acid (adenosine-3-phosphoric

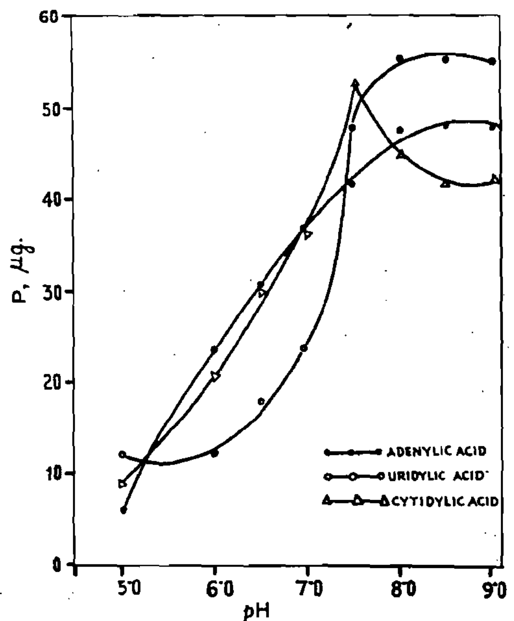

FIG. 1 — EFFECT OF *p*H ON THE CELL-FREE EXTRACT OF VIBRIO NUCLEOTIDASE

acid), cytidylic acid and uridylic acid. The conditions favouring optimal reaction with these substrates were, therefore, studied using the dialysed enzyme preparation. The *p*H-activity curves are presented in Fig. 1. Cytidylic acid has a sharp optimum *p*H at 7·5. Maximum activity with adenylic and uridylic acids was observed at *p*H 8·0 and above. The time-activity curves represented in Fig. 2 indicate that equilibrium is reached within 60 min. with all the three substrates. Maximum activity was observed at a concentration of 0·25 mg. of substrate (Fig. 3) beyond which it levelled off giving half-maximum substrate concentrations of $2{\cdot}6 \times 10^{-4}$, $2{\cdot}8 \times 10^{-4}$ and $2{\cdot}8 \times 10^{-4}$M respectively for the three substrates. The enzyme concentration-activity graphs were linear (Fig. 4) for the concentrations tested, indicating the typical catalytic nature of the reaction mediated by the cell-free extract.

Effect of activators and inhibitors on cell-free enzyme — The action of the different reagents tried on the cell suspension was also examined on the cell-free extract under identical conditions. It was found that the enzyme preparation was equally resistant to the action of the compounds tested excepting iodoacetate which, in a concentration of 0·01M, produced a 25-30 per cent inhibition.

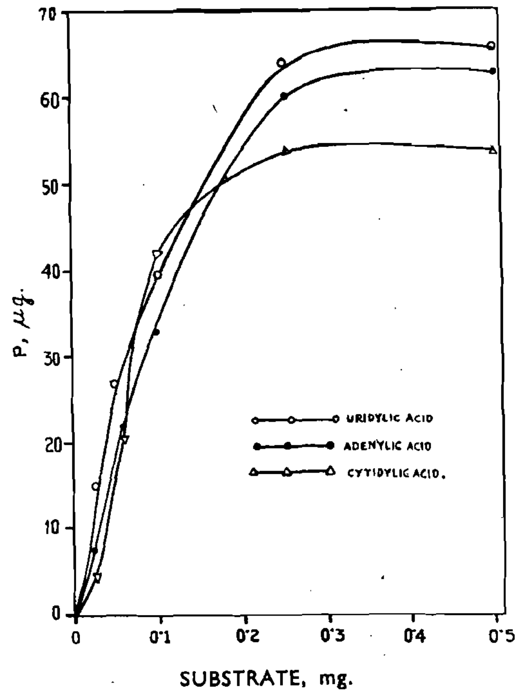

FIG. 3 — EFFECT OF SUBSTRATE CONCENTRATION ON THE ACTIVITY OF CELL-FREE NUCLEOTIDASE OF *Vibrio cholerae*

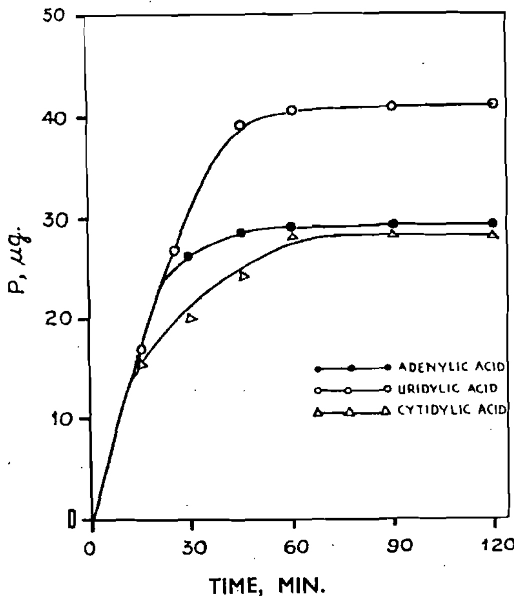

FIG. 2 — TIME-ACTIVITY CURVES FOR CELL-FREE NUCLEOTIDASE OF *Vibrio cholerae*

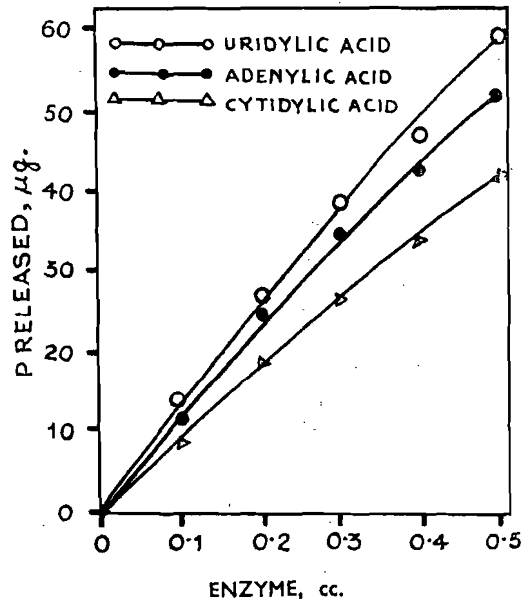

FIG. 4 — CONCENTRATION OF CELL-FREE EXTRACT *vs.* ENZYME ACTIVITY

Discussion

5-Nucleotidase is reported to be widely distributed in mammalian tissues[8-9]. Its presence has also been demonstrated in certain microbial cultures invariably in association with the conventional alkaline phosphatases[10-11]. Our results, which are in striking contrast, show that the vibrio cultures very readily dephosphorylated ATP, A-3-P, I-5-P, C-5-P and U-5-P, but are completely unreactive on the phosphoric esters of glycerol, phenol, phenolphthalein and hexoses and inorganic meta and pyrophosphates. The only observation of this kind, as far as we are aware, is on the venom of a number of species of snakes which have a similar preferential action on the phosphoric acid ester linkages of nucleotides and not on phosphate monoesters[12].

The dephosphorylation of nucleotides as mediated by the resting cells of vibrios and the cell-free enzyme preparation made from them proceeds to completion under optimal conditions. The rate of hydrolysis is not affected by a number of common enzyme activators and inhibitors. The only exception to this was iodoacetate which partially inhibited the cell-free enzyme. This would indicate that the vibrio-5-nucleotidase is apparently different from the mammalian enzyme which has been shown to be considerably activated by magnesium ions and inhibited by sodium fluoride[13]. Another difference noticed between the two enzymes is with respect to the action of the cell-free enzyme of *Vibrio cholerae* on the 5 and 3 phosphate esters of adenosine. On purification of animal tissue fluids like bull semen or snake venom, action on A-3-P is lost whereas the cell-free enzyme of *Vibrio cholerae* was found to have only limited action on A-5-P but much greater affinity towards A-3-P.

The function of nucleotides and the role played by 5-nucleotidases in the metabolism of bacterial cultures are not clearly understood. By using microspectrophotography in the ultraviolet and the Feulgen staining technique, Malmgren and Heden[14,15] have shown that in several Gram-negative and positive organisms the nucleotides are intimately associated with the growth of the cell. If one were to view against this background the observations of Lampen[16] on the role of phosphate in the transfer reactions involved in the intricate mechanism of purine and pyrimidine interconversions, the principal finding reported in this paper on the high specificity of nucleotidase can be considered to be of significance in the biosynthesis of nucleic acids by vibrios. This, together with the resistance of the enzyme to common inhibitors and activators, might point out a fruitful site of attack by suitably designed metabolic antagonists.

Summary

1. Resting cells of vibrios and culture filtrates have no phosphatase activity against glycerophosphate, phenolphthalein phosphate, diphenyl phosphate, sodium meta and pyrophosphates, glucose-1-phosphate, fructose-6-phosphate and fructose-1-6-diphosphate. A cholera vibrio and a water vibrio were found to produce no phosphatase activity when grown in a medium in which a chromogenic substrate was incorporated.

2. The cells have high reactivity towards the phosphate attached to the 5 carbon of ribose in nucleotides. The optimal conditions of dephosphorylation by the cells have been studied.

3. The enzyme can be isolated from acetone-dried cells by extraction with buffered saline and successive precipitation by ethanol and ammonium sulphate. A tenfold purification is achieved by this method. The cell-free enzyme, however, was active only towards adenosine-3-phosphate, cytidine-5-phosphate and uridine-5-phosphate.

4. Magnesium sulphate, calcium chloride, potassium fluoride, sodium azide, sodium cyanide, dinitrophenol, cysteine and 8-hydroxyquinoline had no action on the intact cells and the cell-free enzyme. Iodoacetate exerted 30 per cent inhibition on the cell-free enzyme.

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