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Enzymatic Liberation of Inorganic Phosphate from Adenosinetriphosphate by Tissues of *Molgula manhattensis.*

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One of the important sources of chemical energy for various forms of work in cells and tissues is the enzymatic liberation of inorganic phosphate from adenosinetriphosphate (ATP).[1] The enzymatic systems which effect the liberation of the first two phosphate groups from ATP have been designated as apyrase.[2] A better understanding of the functional role of this important enzyme system may be possible through a survey of its comparative biochemistry. The following observations on the apyrase activity in the tissues of the ascidian, *Molgula manhattensis* (DeKay), are here reported as part of a contribution to such a survey.

Material and methods. The ascidians were collected during the fall at Milford (Conn.) harbor where they are common fouling organisms on wharf piles from mean low water to a depth of about 2 meters. The animals were transported to New Haven where the experiments were performed. Their physiological condition was checked before use by noting the contraction of the inhalent and exhalent siphons and the flow of blood through the heart.

After removal of the tough external wall of the tunic the internal organs were separated into the following fractions for analysis: (1) gonads (ovary and testis), (2) excretory organ, (3) pharynx, (4) stomach and intestine. In later experiments the digestive tract was divided into 3 parts: (1) the anterior folded portion or stomach, (2) the first half of the more tubular and less folded portion of the intestine (called henceforth the "midgut"), (3) the posterior half of the intestine, designated as "hindgut." In several experiments the entire tubular section was used removing the "stomach" but without separation into "mid" and "hindgut." In these instances the tissue used was simply designated as "gut."

After dissection and pooling the organs from at least 3-5 animals, 250 mg of the tissue (in each case) was homogenized[3] for 1 to 2 minutes with 5 ml of chilled distilled water. The homogenate was poured into 20 ml of chilled distilled water and portions of 0.1-0.3 ml of this suspension were used for determination of enzymatic activity according to the method of Dubois and Potter.[4] For the dry weight determinations the entire remaining quantity of suspension was precipitated by 10% trichloroacetic acid; the precipitate was washed in the centrifuge with 95% alcohol and dried overnight at about 100°C. Our substrates were obtained from commercial sources. ATP was supplied by Armour and Co. as the dibarium salt which contained 8.9% total N, 11.9% total P and 63% hydrolyzable P. The slight excess of total P (Theor. 10.9%) could be accounted for almost entirely by inorganic phosphate. Small volumes of solutions of the sodium salt were prepared by adding an equivalent amount of solid Na_2SO_4 to a solution of the barium salt in HCl. After removal of the barium sulfate the clear solution was neutralized with a small amount of 2N NaOH. Hexosediphosphate

[1] Potter, V. R., *Adv. Enzymology*, 1944, **4**, 201; Engelhardt, V. A., *Adv. Enzymology*, 1946, **6**, 147; Szent-Györgyi, A., *Chemistry of Muscular Contraction*, 1947, New York, 1-150; Sandow, A., *et al.*, *Ann. New York Acad. Sci.*, 1947, **47**, 665.

[2] Meyerhof, O., *J. Biol. Chem.*, 1945, **157**, 105.

[3] Potter, V. R., in Umbreit, W. W., Burris, R. H., and Stauffer, J. F., *Manometric Techniques and Related Methods for the Study of Tissue Metabolism*, 1945, Minneapolis, 92-98.

[4] Dubois, K. P., and Potter, V. R., *J. Biol. Chem.*, 1943, **150**, 185.

TABLE I.

Liberation of Inorganic Phosphate from Adenosinetriphosphate and Other Phosphate Esters by Homogenates of the Tissues of *Molgula manhattensis.*

The results are given as micrograms of P liberated per minute per mg dry weight during an incubation period of 15 min. at 38°C at a pH of 7.5 (if not indicated otherwise). Each figure gives the value obtained with a single sample of pooled tissue.

Tissue	Substrate	μg P
Pharynx	ATP	0.8
Excretory Organ	"	0.4
Gonads	"	0.7
Total Intestine	"	11.3
"Stomach"	"	3.8
Midgut	"	18.5
Hindgut	"	16.0
Gut, dry wt per sample:		
0.052 mg	ATP	15.5
0.112 "	"	20.1
0.156 "	"	18.6
Gut with Ca	"	14.5
	"	13.8
Gut without Ca	"	0.5
	"	0.2
Gut	Hexosediphosphate	0.8
	Beta glycerophosphate	0.9
	Ribonucleate	0.1
Gut pH 7.0	ATP	14.7
7.5	"	18.5
8.5	"	24.2
9.0	"	29.3
9.5	"	28.7
10.0	"	29.0

was obtained from Schwartz Laboratories, New York, as the Ca salt which was converted into the sodium salt by addition of the equivalent amount of sodium oxalate. The sodium salt of ribonucleic acid was obtained from the same source. It was prepared from yeast and contained 15.15% N and 9.04% P. Sodium glycerophosphate was prepared from a 25% solution of β-glycerophosphoric acid (Eimer and Amend). The solutions of the substrates were adjusted to a pH of 7-7.5 before use.

Results. The figures for apyrase activity in the organs of *Molgula* are compiled in Table I. The pharynx, the gonads and the excretory organ are low and of the same order of magnitude. The activities in the digestive tract are considerably higher. The highest values were obtained in the intestinal portion; the values for the stomach are significantly lower. The results in Table I show that the amount of inorganic phosphate liberated per mg dry weight is approximately constant for, and thus roughly proportional to, varying tissue concentrations. The activity of the enzyme system is dependent upon the presence of Ca ions and is practically inactive in their absence.

The specificity of the enzyme was tested with β-glycerophosphate, hexosediphosphate and ribonucleate as substrate. When used in a final concentration of 0.02 M and correcting for small blank values obtained with control samples containing substrate without tissue and tissue without substrate, the liberation of inorganic P from these substrates was found to be insignificant compared with ATP. An attempt to characterize the enzyme by the dependence of its activity upon the pH of the solution showed an increase in activity from pH 7 up to a pH of 9, but no significant change was observed on further increase up to a pH of 10. In a preliminary experiment the intestine was homogenized at pH 7.5 in 0.5 M KCl and extracted by centrifuging at 3,000 r.p.m. for 30 minutes. The supernatant was poured into 4 vol. of cold distilled water and a small precipitate was centrifuged off. Both the residue obtained after the first centrifugation as well as the second precipitate were resuspended in small volumes of distilled water and the suspensions tested for enzymatic activity. Only about 20% of the activity of the unfractionated tissue was recovered in the residue while the precipitate was devoid of any activity. This indicates either that the enzyme system is very unstable and inactivated by the extraction, or that the enzyme is more soluble than other animal apyrase preparations such as the myosin fraction of muscle which is one of the richest sources of this enzyme system. The activity of the intestinal homogenate was found to be completely lost after 10 minutes in a boiling water bath.

Discussion. In comparing the apyrase activity in the gut of *Molgula* with that of other tissues, it should be pointed out that the activity in the gut exceeds apparently about twofold the highest activities found so far in animal tissues as well as that of purified myosin.[4,7] This observation and other data

[5] Schneider, W. C., *J. Biol. Chem.*, 1946, **165**, 585.

of other authors indicate that a high activity of apyrase can be found in tissues which are free of muscular elements and also in cell structures which do not contain myosin (Mitochondria, nucleus).[5,6] However, it should be pointed out that purified myosin splits off only the first one of the phosphate groups of ATP (Adenosinetriphosphatase). Apyrases of other tissues liberate two phosphate groups in the form of inorganic P; but even if half of the inorganic phosphate liberated by *Molgula* intestine were due to the removal of the second phosphate group from ATP the rate at which the first phosphate is split off by homogenates of the gut would still be as high as that of pure myosin. Considering that the dry weight of the intestinal preparations includes the entire cell material and since only a small fraction of it can be responsible for the apyrase activity, its activity in purified state must exceed considerably the activity of purified myosin. The higher activities of apyrases of plant tissues should be mentioned here[8] since a second similarity between plant and ascidian apyrase could possibly be expected in their solubility properties, considering our failure to find intestinal apyrase in the less soluble protein fractions.

The high activity of apyrase in the intestinal cells of *Molgula* suggests an important role of the enzyme in the physiology of this tissue. The mucosa of the intestine of higher animals is known to contain high activities of phosphatases and in this case enzymatic activity was correlated with the phosphorylations of food stuffs in the course of absorption and utilization. However, these phosphatases seem to hydrolyse mono- and di-esters at a much faster rate than ATP[9] while the reverse holds for the enzyme in *Molgula* intestine. Unfortunately, apart from the work of Yonge on *Ciona*[10] very little is known about the physiology and biochemistry of digestion of ascidians and therefore no pertinent suggestion can be made at present as to the possible role of apyrase in the physiology of the intestine of *Molgula*. A possible clue for future investigations of this question can be expected from the difference in the activities of apyrase in the stomach and the mid- and hindgut of this organism.

Summary. The enzymatic liberation of inorganic phosphate from adenosine-triphosphate was tested with homogenates of the pharynx, the gonads, the excretory organ, the stomach and the gut of the tunicate *Molgula manhattensis* (DeKay). The activity in the gut was found to be more than 4 times as high as in the stomach and 20-40 times as high as in the other tissues. The activity in the gut of *Molgula* was compared with the activities observed by other authors in vertebrate tissues and it was found to be twice that observed in mammalian muscle.

6 Steinbach, H. B., and Moog, F., *J. Cell. and Comp. Physiol.*, 1945, **26**, 175.

7 Bailey, K., *Biochem. J.*, 1942, **36**, 121.

8 Kalckar, H. M., *J. Biol. Chem.*, 1944, **153**, 355.

9 Schmidt, G., and Thannhauser, S. J., *J. Biol. Chem.*, 1943, **149**, 369.

10 Yonge, C. M., *J. Exp. Biol.*, 1924-25, **2**, 373.