Cholinesterase in the Development of *Ciona intestinalis* (Ascidia)

Research carried out in this laboratory on the developing Ascidian egg has determined the localization and time of appearance of various enzymes. This work has demonstrated that certain enzymes (cytochrome-oxidase[1]; succino-dehydrogenase[2]) are present from the very beginning and are localized very early, while others (phosphatase[3]; dopa-oxidase[4]) appear rather late in the development.

The *Ciona* egg has a very rapid development; at 20° C an actively swimming larva is obtained in less than 24 h. This larva has, in respect to the adult, a rather complex structure; above all possessing a nervous system with brain and spinal cord and powerful tail muscles. After the larva becomes attached, it enters into metamorphosis, the tail atrophies and the muscular cells are reabsorbed. The presence of well-developed nervous and muscular systems during a brief period of larval development, and their rapid disappearance, give rise to various questions: (a) whether or not cholinesterase is present, (b) the time of its possible appearance, (c) its location. At present, comparative studies on this enzyme in the development are lacking; such studies should lead to the solution of many problems as yet unanswered.

In our work, we have used eggs of *Ciona intestinalis*, *Phallusia mamillata* and *Clavelina lepadiformis*; however, the data here presented refer exclusively to the Ciona eggs. With the other two species, different results were obtained.

The histochemical technique of KOELLE, as modified by GOMORI[5], was used to indicate the presence of cholinesterase. The eggs at various stages of development were fixed for a few seconds in 80% ethanol; they were incubated *in toto* at 37° C for 1 h, with acetylthiocholine as a substrate. The following results were obtained:

(1) In the undivided egg, and in the first stages of development, there are no traces of cholinesterase.

(2) It appears for the first time only at the formation of the neurula.

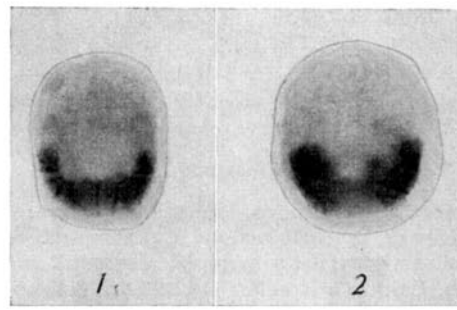
(3) At this stage the cholinesterase is localized in 2 masses of presumptive muscular cells which are situated laterally and posteriorly in the embryo. No other area contains cholinesterase; particularly, it is completely lacking in the neural plate.

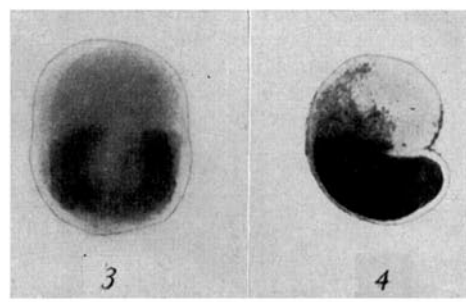
(4) The cholinesterase remains localized exclusively in the muscle cells during all the development until the end of the larval stage. In the Figures 1–4, embryos at successive stages of development are shown: the reaction appears in the muscle cells which are not yet differentiated. In the actively moving larva (having pigmented sensory organs), the reaction occurs in the muscle cells which are already elongated and differentiated (Fig. 5). On the other hand, there is no trace of cholinesterase in the brain and spinal cord.

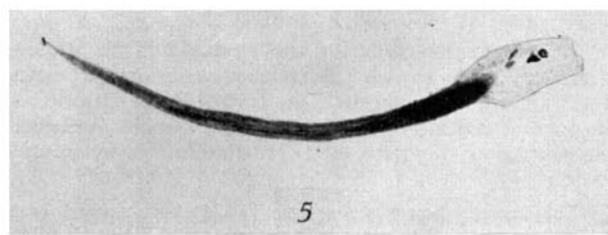
(5) In the larva in metamorphosis, in which the tail has just become reabsorbed, the cholinesterase appears localized in the posterior region (Fig. 6). This is explained by the fact that, during the reabsorption of the tail, the muscle cells migrate to this portion of the trunk.

(6) In the metamorphosized larva having 2 siphons, there is no more cholinesterase.

(7) The treatment of the developing egg with eserine (0·005% solution) or with neostigmine (0·01% solution) blocks the activity of the enzyme. The treatment with di-isopropylfluorophosphate (0·002%) does not cause any inhibition.







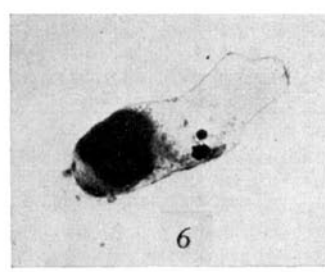


Fig. 1–6. – Reaction of cholinesterase in *Ciona intestinalis*; incubation in acetylthiocholine for 1 h at 37° C. – 1. – Neurula. 2. – Advanced neurula. 3. – Young tail bud. 4. – Tail bud, side view. 4. – Swimming larva. 6. – Larva in metamorphosis.

On the basis of the results, it can be stated that the enzyme considered here corresponds to the "specific" cholinesterase.

Conclusions. – In the Ciona egg cholinesterase appears at a rather late stage of development (neurula stage) and is localized exclusively and permanently in the muscular territory. It appears before the differentiation and functioning of the muscles. It is yet to be investigated whe-

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[5] G. GOMORI, *Microscopic Histochemistry* (Chicago Univ. Press, 1952), p. 210.

ther the quantity or activity increases with the muscular differentiation. The cholinesterase disappears entirely in the larva which has undergone metamorphosis and in which the muscular system has been reabsorbed. The central nervous system does not show the presence of cholinesterase in any stage of development. The treatment with specific inhibitors demonstrates that the enzyme described corresponds to specific cholinesterase or acetylcholinesterase.

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Riassunto

Con metodo istochimico, sono state effettuate ricerche sulla presenza, la localizzazione, e il tempo di comparsa della colinesterasi nello sviluppo di *Ciona intestinalis* (Ascidie). È stato visto che: 1) l'attività colinesterasica, negativa negli stati precoci di sviluppo, si palese durante e dopo la neurulazione; 2) il territorio in cui si localizza la reazione è esclusivamente quello muscolare; 3) esiste una relazione tra la presenza dell'enzima e la capacità funzionale dell'embrione; 4) nella reazione è interessato un solo tipo di enzima e precisamente la colinesterasi specifica o acetilcolinesterasi.

Trace Element Stimulation of Keratin (Hair) Degradation by Oral Keratinolytic Microflora[1]

The proteolysis-chelation theory explains the etiology of dental caries as two interrelated reactions occurring simultaneously on enamel: (a) microbial destruction of the organic matrix which is composed largely of keratin, and (b) loss of apatite through dissolution by organic chelators some of which originate as matrix breakdown products[2]. This theory was derived in part from consideration of numerous systems throughout nature where ordinarily insoluble inorganic materials are mobilized and transported as soluble sequestration complexes[3]. It was also suggested by the Ca activation and stabilization of enzymes and other proteins, the role of Ca in proteolysis, and the ability of proteins and their derivatives to form water-soluble chelate complexes with alkaline earths[4]. The fact that $CaCO_3$ and $Ca_3(PO_4)_2$ stimulate metabolism of oral keratin-digesting microflora[2] motivated the present studies to determine whether Ca, PO_4, and other trace elements influence keratinolysis, and whether this proteolysis dissolves enamel apatite.

Experimental.—The method of cell production, conditions for Warburg respirometry, preparation of enamel and hair, and composition of the trace element supplement have already been reported[2]. Finely cut human hair was a convenient source of keratin since it is impractical to obtain purified yet undenatured enamel keratin in adequate amounts.

Results.—Both autorespiration and hair oxidation were stimulated by $CaCl_2$ (Table). The complete trace element supplement depressed autorespiration but accelerated O_2 uptake with keratin. Even without Ca and PO_4, the supplement increased O_2 consumption with hair. Thus other elements may participate in the catabolism of this material. The maximum O_2 uptake observed with the Ca-free supplement cannot mean that Ca inhibited since $CaCl_2$ enhanced the oxidation, but probably reflects trace metal unbalance or ion antagonism[5]. The enamel supplied sufficient trace elements to stimulate hair catabolism, but not enough organic matter to in-

[1] This investigation was supported in part by a research grant D-182(C) from the National Institute of Dental Research of the National Institutes of Health, United States Public Health Service.

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Trace element stimulation of keratin (hair) degradation by oral keratinolytic microorganisms

Distilled H_2O cell suspension supplemented with	400 min Warburg experiment			
	Autorespiration (no hair present)		100·0 mg hair added to each vessel	
	μl O_2 uptake	μl O_2 stimulation over control due to trace elements	μl O_2 uptake	μl O_2 stimulation over control due to trace elements
Ca as $CaCl_2$ (5·0 mg%)	78	30	832	95
Trace element supplement	37	– 11	1025	288
Trace element supplement minus Ca and phosphate	55	7	850	113
Trace element supplement minus Ca	58	10	1042	305
Human enamel as source of trace elements (50·0 mg added to each vessel)	47	– 1	950	213
No minerals added (controls)	48		737	

pH of cell suspension initially adjusted to 7·0; KOH in each center well; 30°C. Trace element supplement includes Na, K, Ca, Mg, Mn, Zn, Mo, Fe, Co, Cu, P, S, and Cl in the same concentrations employed in the basal culture medium for growing keratinolytic microorganisms[2]. The data have not been corrected by subtracting autorespiration.