CHAPTER - VI

ACETYLCHOLINESTERASE IN EMBRYONIC STAGES OF CYPRINUS CARPIO

INTRODUCTION

The importance of acetylcholine and acetylcholinesterase in neural transmission is too well known. Action of acetylcholine in nervous tissue has to be brief. Acetylcholinesterase hydrolyzes and inactivates acetylcholine quickly. Their occurrence in nonnervous tissues like serum and RBC (Broede & Wilson, 1971) has led to assignment of other roles, such as permeability control, sodium transport and glucose uptake (Schwabe, 1959; Schwabe et al, 1961 and Lee et al, 1963). Sawyer (1943) contended that neuromuscular development

and increased cholinesterase activity are closely correlated.

Gustafson and Toneby (1970) have attributed them a morphogenetic role. While studying the role of cellular motility in morphogenesis of sea urchin embryos, they observed that increased motility caused decrease in adhesion of cells, leading to their detachment and movement. Vegetal region at gastrulation was thought as the site of metabolism of aromatic amines. Serotonin and acetylcholine produced by the vegetal cells were said to elicit their pulsatory movements; serotonin to play a role in the release of primary mesenchyme during primary invagination (G_1 phase) and perhaps also at the end of G_1 phase, whereas acetylcholine-acetylcholinesterase system possibly had to play a role in basic function of ectoderm cells, may be in relation to their membrane activities and in contraction of attached pseudopods during second phase of gastrulation. Synthesis of serotonin, acetylcholine and other receptor molecules were considered to be under genic control.

Uesugi and Yamazoe (1964) found an abrupt increase in acetylcholinesterase activity at the eyed stage in *Salmo gairdnerii*; other than this, there is no work so far on the occurrence and importance of acetylcholinesterase in a fish embryo.

The role of acetylcholine-acetylcholinesterase system in developmental physiology has acquired tremendous potential in the background of Gustafson and Toneby's (1970) work on sea urchin embryos. The present Chapter includes study on activities of acetylcholinesterase in unfertilized eggs and in different embryonic stages of *Cyprinus carpio*.

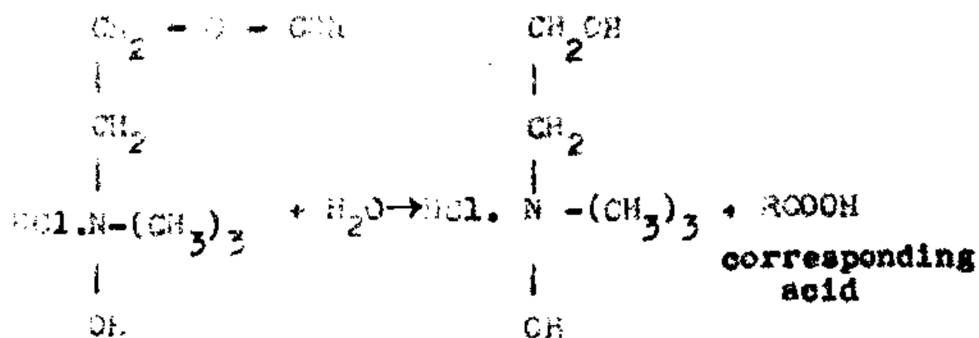
METHOD

ACETYLCHOLINESTERASE (E.C. 3.1.1.7)

Fresh samples of different embryonic stages were homogenized in icecold 0.25 M sucrose solution to approximately 10% w/v; centrifuged at 3000 rpm at $4^{o}C$ for 15 minutes; supernatant was used for enzyme assay by the colorimetric method of Metcalf (1951), as modified by Murali Krishnadas (1967).

PRINCIPLE:

Acetylcholinesterases catalyze the following reaction:



Acetylcholine chloride Choline

Removal of acetylcholine per unit time is measured by comparison of its initial concentration in a reference tube with the final concentration in experimental tube. Acetylcholine reacts with hydroxylamine to form corresponding acyl hydroxamic acid, which forms a strongly coloured ferric hydroxamate with ferric salts.

Reagents:

1. M/15 Phosphate buffer(pH 7.4): 11.72 g of disodium hydrogen phosphate (Na_2HPO_4) and 2.72 g of monopotassium dihydrogen phosphate (KH_2PO_4) dissolved in 1 litre of double distilled water.

2. 0.004 M Acetylcholine solution: Stock solution (0.04 M) was prepared by dissolving 0.7466 g of acetylcholine chloride in 100 ml of HCl of pH 4.5. Stock solution was diluted with 9 volumes of phosphate buffer (1) before use.

3. 2 M Hydroxylamine hydrochloride: 13.9 g of hydroxylamine hydrochloride dissolved in 100 ml of distilled water.

4. 3.5 N Sodium hydroxide: 140 g of sodium hydroxide (NaOH) dissolved in 100 ml of distilled water.

5. Alkaline hydroxylamine solution: Equal volumes of hydroxylamine hydrochloride solution (3) and 3.5 N NaOH solution (4) mixed together before use.

6. <u>Hydrochloric acid</u> (1:1): **Concentrated** HCl (sp. gravity 1.18) **diluted with equal volume of** distilled **water.**

7. <u>0.37 M Ferric chloride solution</u>: 10 g of ferric chloride dissolved in 100 ml of 0.1 N HCl.

8. <u>0.25 M Sucrose solution</u>: 8.70 g of sucrose dissolved in 100 ml of distilled water.

<u>PROCEDURE</u>:

1.0 ml of enzyme extract added to 1.0 ml of buffered substrate, incubated for exactly 30 minutes at 37°C; reaction stopped by the addition of 2.0 ml of alkaline hydroxylamine hydrochloride; contents mixed thoroughly; 1.0 ml of HCl:H_2O (1:1) added with further shaking and the whole centrifuged. 2.5 ml of supernatant was taken in a test tube and 0.5 ml of ferric chloride solution was added. Contents were again mixed thoroughly. Zero time controls were maintained by adding 2.0 ml of alkaline hydroxylamine hydrochloride to the buffered substrate prior to the addition of enzyme extract. Colour density of ferric hydroxamate formed was read at 540 nm against blank, which contained 1.0 ml of buffer and 1 ml of distilled water in place of buffer substrate mixture and enzyme extract.

Enzyme activity was determined by the amount of unreacted acetylcholine chloride left after 30 minutes of incubation. The intensity of colour obtained at the end of incubation period gave the amount of acetylcholine left unhydrolysed. On the basis of difference between initial substrate concentration and substrate concentration left unhydrolysed, enzyme activity was estimated and expressed as μg acetylcholine hydrolyzed/mg protein/hour. Protein content of the extract was measured by the method of Lowry et al (1951) as described earlier on page 34 .

RESULTS

Acetylcholinesterase activity (Table 23; Fig. 44) was found to increase following fertilization, during late morula, when cell size reduced considerably, and at gastrula onward in increasing order until eye stage, when it attained peak activity. Enzyme activity thereafter decreased.

DISCUSSION

Schwabe (1959), Schwabe et al (1961) and Lee et al (1963) implicated acetylcholinesterase activity in permeability control and sodium transport, Ca^{++} is said to promote release of acetylcholine (Prosser & Brown, 1965). Chetty and Agarwal (under communication, also in Chapter VII , p. 149) observed rise in Ca^{++} and fall in Na^{+}

TABLE 7.42

Acetylcholinesterase activity (µg acetylcholine hydrolyzed/mg protein/hr) in unfertilized eggs and in different embryonic stages of *Cyprinus carpio*

S.No.	Stages									
	Unfertilized eggs	Blastodisc	Early morula	Late morula	Blastula	Gastrula	Closing of blastopore	Comma	Eyed	Prior to hatching
1	57.22	56.70	41.43	53.58	43.76	50.90	52.66	54.54	62.00	39.20
2	45.72	56.70	41.48	42.85	43.76	50.90	57.44	54.54	58.00	49.44
3	45.72	56.70	41.48	53.58	-	40.10	47.88	54.54	62.00	49.44
4	51.22	56.70	41.48	58.92	43.76	50.90	47.88	54.54	58.00	54.54
5	45.72	56.70	41.48	58.92	39.06	-	52.66	49.44	62.00	54.54
6	45.72	56.70	35.70	53.58	39.06	50.90	52.66	54.54	62.00	-
7	45.72	56.70	41.48	58.92	43.76	45.54	52.66	54.54	62.00	-
8	45.72	51.22	41.48	53.58	43.76	50.90	47.88	54.54	62.00	49.44
Mean ± SD	47.09 ±2.55	56.01 ±1.94	40.88 ±1.59	54.24 ±5.30	42.42 ±2.29	48.60 ±4.22	51.46 ±2.38	53.90 ±1.80	63.50 ±2.78	49.43 ±5.60

levels, following fertilization in *C. carpio*, which is thus intelligible in view of changed permeability relations of the egg membrane at this stage.

Willmer (1960) visualized acetylcholine-acetylcholinesterase system to have evolved in embryonic cells for some basic membrane functions. Following fertilization, changes in permeability relations of egg are well known (Brachet, 1960). Eggs of *C. carpio* swell considerably soon after fertilization due to incorporation of water and ions. Correlated acetyl-cholinesterase activity soon after fertilization, as observed by the present author, is thoroughly understandable, more so, in view of the rise in levels of Ca^{++} (Chapter VII ; p. 149) soon after fertilization in *C. carpio*. Increased acetylcholinesterase activity during late morula in *C. carpio* is possibly on account of rapidly dividing cells with greatly increased surface tension and tendency to adhere to hyaline membrane at this stage, preparatory to blastula formation.

Gustafson and Toneby (1970) visualized acetylcholine-acetylcholinesterase involvement in the second phase of gastrulation of sea urchin embryo and cited in support the marked or rather strong inhibition of gastrulation following exposure of embryos to lipid soluble cholinolytics, such as

PAD (Pyridinaldoxine dodecyl iodide) and several other tertiary cholinolytics, or to cholinesterase inhibitors. Increase in cholinesterase activity during gastrulation and closing of blastopore stages in C. carpio lends further support to the theory of acetylcholine-cholinesterase involvement in movement of cells during gastrulation.

Increased acetylcholinesterase activity at the comma stage is obviously the result of formation of tail musculature and nerves and commencement of neuromuscular activity. The observation is fully in accord with Sawyer (1943), who correlated increased acetylcholinesterase activity with increase in neuromuscular development.

At the eye stage, however, with the brain almost fully differentiated, eyes formed, with melanoblasts differentiated into pigment containing cells and tail twitching movements having commenced, the steep rise in enzyme activity, as observed in C. carpio, is a natural consequence. This contention gets further support by the observation of Uesugi & Yamazoe (1964) of abrupt increase in acetylcholinesterase activity at the eyed stage in Salmo gairdnerii.

This study brings home that acetylcholine-cholinesterase system, besides their importance in neurotransmission, is evolved for specific purposes at the different stages of embryonic development in C. carpio.