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Enzyme Histochemistry of *Brugia pahangi* 2. Localization of Acetylcholinesterase Activity in Developing Larvae in Mosquito (*Aedes aegypti*)

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Introduction

It is generally accepted that acetylcholinesterase (AChE) is localized in the nervous system of parasitic helminths. AChE has been detected in the sensory organs of helminths (Rohde, 1960; McLaren, 1972; Bruckner and Voge, 1974). It has been also reported that many species of intestinal nematodes secrete AChE (Bremner *et al.*, 1973; Ogilvie *et al.*, 1973; Rothwell *et al.*, 1973). Isozymes of AChE have been found in *Nippostrongylus brasiliensis* (Edwards *et al.*, 1971) and in *Fasciola hepatica* (Probert and Durrani, 1977).

In the preliminary study on histochemical demonstration of AChE in *Brugia pahangi* microfilaria, we have found at least two types of staining which probably indicate the existence of the isozymes in *B. pahangi* (unpublished data). One is the granular reaction products in the microfilaria demonstrated with the substrate concentration of $1.7\text{-}3.5\times10^{-3}$M at pH 6.8-7.0. The other is the staining of amphids, nerve ring, phasmids and/or nerve cord with the substrate concentration of $3.5\text{-}5.2\times10^{-2}$M at pH 5.5-6.8. While the latter seems to be closely related to the nervous system, the exact localization and the physiological role of the former remain obscure.

In this paper a report is made on the localization of AChE in the developing stages of *B. pahangi*, from the microfilaria in canine peripheral blood to the infective larvae in mosquitoes.

Materials and Methods

The microfilariae were obtained from an infected dog with *Brugia pahangi* microfilaremia. Venous blood, 1 ml was taken from the dog with a heparinized plastic syringe and was mixed with cold 1% saponin solution to attain hemolysis. After centrifugtion, the sediment was resuspended in cold physiological saline and recentrifuged. The washing procedure was repeated three times. One drop of microfilarial suspension was put on a cover slip and air dried.

The mosquitoes used were Liverpool strain of *Aedes aegypti* maintained in our laboratory. A group of mosquitoes were fed on the infected dog for 15 min. The mosquitoes were dissected in a drop of saline on cover slips at appropriate intervals and air dried.

For the demonstration of AChE, the specimens were processed following the method described by Bueding *et al.* (1967), using acetylthiocholine iodide as the substrate. The specimens were fixed in 4% ice-cold formaldehyde for 1 min. The specimens were preincubated in Gomori's stock solution for 2 hours and incubated in the substrate mixture for 30 min at 25°C. The substrate concentration of 0.5 mg/ml (1.7×10^{-3}M) at pH 6.8

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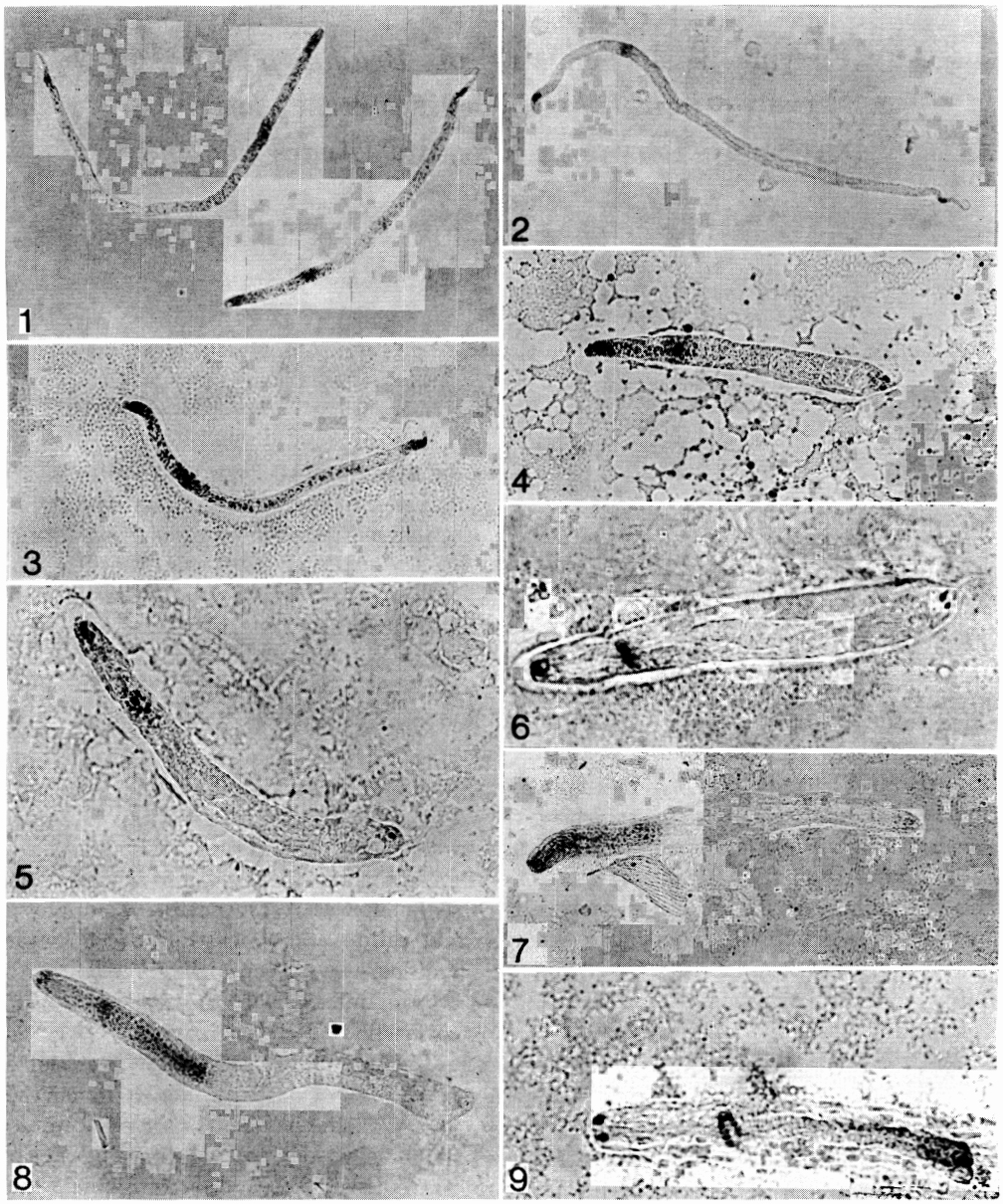

Fig. 1 With 2 mg/ml substrate, fine granules are distributed in the whole body of microfilariae besides congregated granules at the amphids, nerve ring and phasmids. (×250)

Fig. 2 The microfilarial amphids, nerve ring and phasmids are well stained with 10 mg/ml substrate at pH 5.5. Note a fine thread extending from the nerve ring. (×300)

Fig. 3 The 1st stage larva on the 1st day after the infection to the mosquito, incubated with 0.5 mg/ml substrate. Coarse granules are found at the tip of head, nerve ring and phasmids. Fine granules are distributed sparsely behind the nerve ring. (×250)

Fig. 4 The 3rd day's 1st stage larva developed in the mosquito, incubated with 0.5 mg/ml substrate. Granular reactions are decreasing. Note a pair of well-stained phasmids. (×250)

Fig. 5 The 4th day's 1st stage larva in the mosquito reveals much weaker granular reactions than those in the ones on the earlier days. (×250)

was used for the demonstration of granular reactions in microfilariae and larvae. Specimens were also incubated in the solution containing substrate of 10 or 15 mg/ml (3.5×10^{-2} or 5.2×10^{-2}M) at pH 6.8. Some microfilariae were incubated with 2 mg/ml substrate at pH 6.8 and 10–15 mg/ml substrate at pH 5.5. To check the specificity of reaction, controls were incubated in the stock solution without substrate and with inhibitors. 10^{-5}M eserine or 10^{-6}M di-isopropylfluorophosphate (DFP).

Results

Numerous fine granules were observed in the microfilariae from canine peripheral blood which were incubated with the substrate concentration of 0.5 mg/ml. The amphids and nerve ring were usually not stained. Congregated granules were found at the phasmids. When the substrate concentration was increased to 2 mg/ml, weak reaction was found at the amphids and nerve ring, besides the positive phasmids and the fine granules distributed in the whole body (Fig. 1).

In the preparation incubated with the substrate concentration of 10–15 mg/ml, the amphids, nerve ring and phasmids were clearly stained without such granules as found in the microfilariae which were incubated with lower substrate concentration. When the pH was lowered to 5.5 at the substrate concentration of 10–15 mg/ml, a fine thread, which seemed to be the longitudinal nerve cord, was found to extend posteriorly from the never ring, besides three well stained nervous structures mentioned above (Fig. 2).

With 0.5 mg/ml substrate, coarse granules were found at the tip of head, nerve ring and phasmids in the first stage larvae on the first day in the mosquito. Fine granules were distributed thickly between the tip of head and the nerve ring, while sparsely behind the nerve ring (Fig. 3). The reaction of amphids and nerve ring was obscure due to the coarse granules. In some larvae the phasmids were found to be stained clearly. When the substrate concentration was increased to 10–15 mg/ml, only the amphids, nerve ring and phasmids were well stained as in the microfilaria.

On the second day, coarse and fine granules were distributed in the larvae incubated with 0.5 mg/ml substrate as observed on the first day. During from the third day to the fifth day, the coarse and fine granular reactions decreased gradually. In some larvae the phasmids were stained as a pair of dots (Figs. 4, 5). The amphids and nerve ring were still obscure. In the preparation incubated with 15 mg/ml substrate, the late first stage larvae revealed a pair of well stained amphids and two nerve cells besides a pair of phasmids (Fig. 6).

The granular AChE activity demonstrated with 0.5 mg/ml substrate became again strong in the second stage larvae on the sixth day. Coarse granules were densely distributed around the amphids and nerve ring. The granules were less densely found between the two structures and at the glandular region of esophagus (Fig. 7). On the seventh day, the amphids, nerve ring and phasmids were weakly stained. Granules of equal size were located on the glandular esophagus (Fig. 8). When the substrate concentration was increased to 10–15 mg/ml, a pair of amphids and phasmids were clearly stained besides the ring-formed nerve ring (Fig. 9). Although the glandular region of esophagus was well stained, the granules of equal size were not detected in the preparations incubated with high con-

Fig. 6 With 15 mg/ml substrate, a pair of amphids, two nerve cells and a pair of phasmids are well demonstrated in the 4th day's 1st stage larva developed in the mosquito. (×250)

Fig. 7 The 2nd stage larva on the 6th day after the infection to the mosquito, incubated with 0.5 mg/ml substrate reveals coarse granules at the amphids and nerve ring. (×100)

Fig. 8 The 7th day's 2nd stage larva developed in the mosquito, incubated with 0.5 mg/ml substrate. Note the equal-sized granules on the glandular esophagus. (×100)

Fig. 9 The larva on the same day that is shown in Fig. 8, incubated with 10 mg/ml substrate. A pair of amphids and a ring-formed nerve ring are clearly stained. (×200)

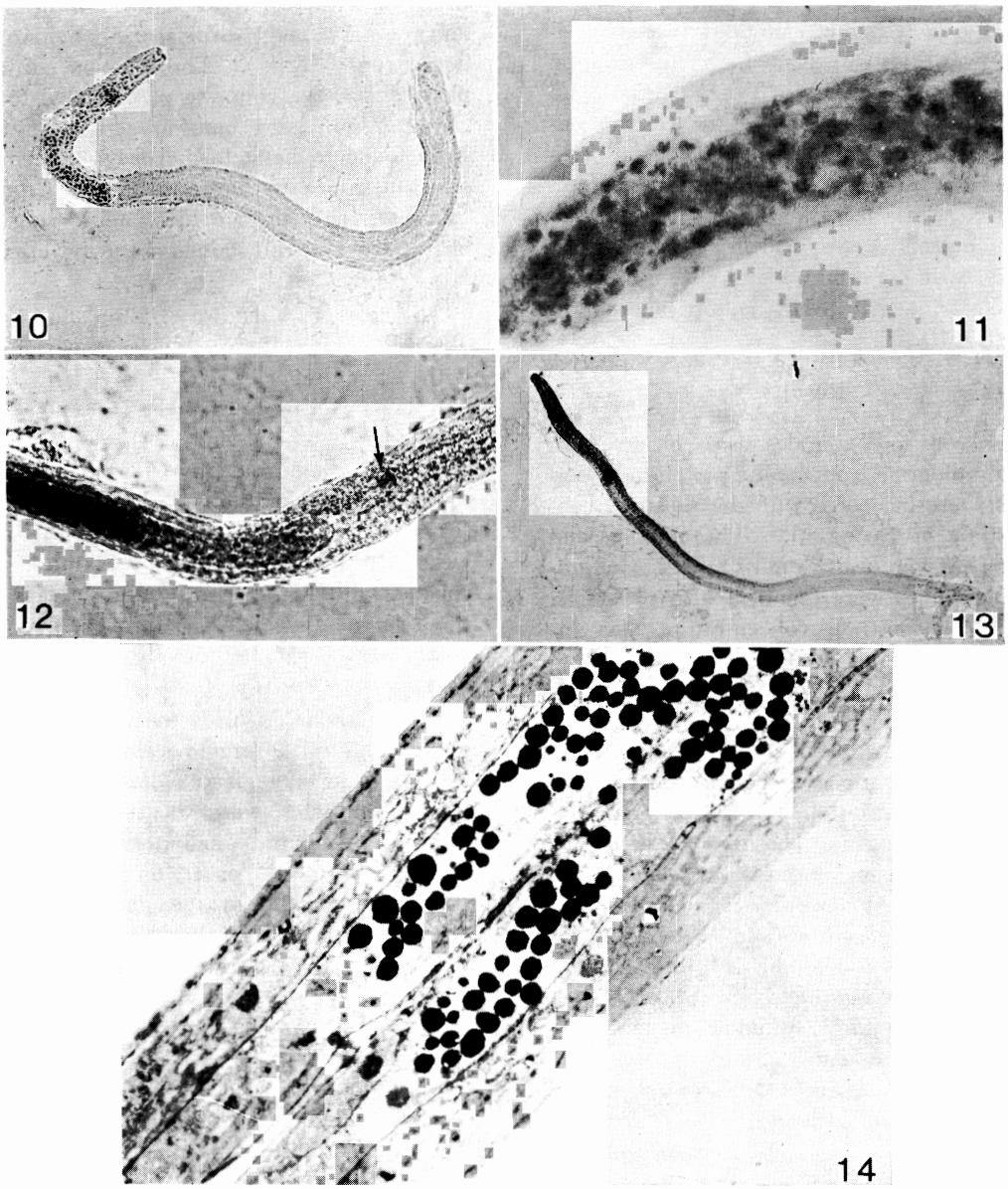

Fig. 10 The 8th day's 2nd stage larva developed in the mosquito, incubated with 0.5 mg/ml substrate. Note strong granular reaction at the glandular esophagus. (×100)

Fig. 11 The 9th day's 2nd stage larva in the mosquito, incubated with 0.5 mg/ml substrate reveals granules, 1.2-1.8 μm in diameter, at the glandular esophagus. (×1,000)

Fig. 12 Granules are found at the male genital primordium (an arrow) and the glandular esophagus in the 9th day's 2nd stage larva developed in the mosquito, incubated with 0.5 mg/ml substrate. (×250)

Fig. 13 The 10th day's 3rd stage larva after the infection to the mosquito, incubated with 0.5 mg/ml substrate. The internal structure is not well stained. (×50)

Fig. 14 The electron micrograph reveals electron-dense secreting granules, 1.9 μm or less in diameter, at the glandular esophagus of the infective larva. (×2,000)

centration of substrate after the seventh day.

The granular reaction detected with 0.5 mg/ml substrate at the glandular region of esophagus became clearer on the eighth and ninth days (Fig. 10). In high magnification, granules, 1.2–1.8 μm in diameter, were observed at the glandular region (Fig. 11). In some larvae, a well-stained structure which seemed to be the male genital primordium was found behind the esophago-intestinal junction (Fig. 12).

After the tenth day, fine granules were found throughout the body of the third stage larvae which stained brownish in color. The internal structure was not well stained probably due to the development of cuticle (Fig. 13). An electron micrograph revealed secreting granules, 1.9 μm or less in diameter, at the glandular esophagus of infective larvae (Fig. 14).

Control specimens without substrate were all negative. Eserine, 10^{-5}M inhibited the reaction completely. The reaction was not reduced by 10^{-6}M DFP. The results of controls also supported the fact that the positive staining and granules were produced by the activities of specific AChE.

Discussion

In the microfilaria of *Wuchereria bancrofti*, AChE activity was reported to be positive at the amphids, phasmids, excretory vesicle and anal vesicle, but negative at the nerve ring after such long incubation time as 3-6 hours in substrate (Omar and Kuhlow, 1977). However, positive reaction was observed by other workers at the nerve ring besides the amphids, phasmids and anal vesicle in the microfilaria (Chandrasekaran *et al.*, 1981). Positive reaction was detected at the amphids, nerve ring, phasmids and cephalic papillae in adult *Dipetalonema viteae* (McLaren, 1972). In the present study, the staining of such nervous organs as the amphids, nerve ring and phasmids were observed in the microfilariae and larvae of *B. pahangi*, when the specimens were incubated with the substrate concontration of 10–15 mg/ml ($3.5–5.2 \times 10^{-2}$M). The excretory and anal vesicles were not stained with the substrae concentration.

In the second stage larvae incubated with the substrate concentration of 0.5 mg/ml (1.7×10^{-3}M) from the sixth to ninth day, the granular reaction at the glandular region of esophagus became gradually stronger in course of time. The granules were 1.2–1.8 μm in diameter. In the third stage larvae, the internal structure was not stained well probably due to the development of cuticle. Secreting granules were reported to be present at the glandular esophagus of *B. pahangi* infective larvae (Collin, 1971). In our specimen, secreting granules of 1.9 μm or less in diameter were found by electron microscope. It seems likely that the electron-dense granules at glandular esophagus contain AChE.

Positive AChE reaction was reported in the genital organs of adult ascarid nematodes (Lui *et al.*, 1964). In the present study, the male genital primordium was found to be positive. The reason why the positive reaction was not seen on the female genital primordium remains obscure.

AChE has been detected in the nervous system of parasitic helminths (Rohde, 1960; McLaren, 1972; Bruckner and Voge, 1974). AChE was also demonstrated in the mid-gut, esophageal glands and/or subventral excretory glands of many species of intestinal nematodes (Bremner *et al.*, 1973; Ogilvie *et al.*, 1973; Rothwell *et al.*, 1973; McLaren *et al.*, 1974). Isozymes of AChE were found in *Nippostrongylus brasiliensis* (Edwards *et al.*, 1971; Ogilvie *et al.*, 1973) and *Fasciola hepatica* (Probert and Durrani, 1977). In the present study, with the substrate at a concentration of 0.5 mg/ml (1.7×10^{-3}M), the amphids and nerve ring were weakly stained after the seventh day in the mosquito, while the phasmids of some of the larvae showed positive reaction from as early as the first day. As stated above, when the substrate concentration was increased to 10–15 mg/ml ($3.5–5.2 \times 10^{-2}$M), the nervous organs of both microfilariae and developing larvae were clearly stained without granular reactions which were found in the specimens incubated with

the substrate concentration of 0.5 mg/ml. The positive AChE reaction, which was well demonstrated with higher concentration of substrate in the amphids, nerve ring and phasmids of *B. pahangi*, seems to be rather related to the nervous function than to the secretion. On the other hand, the granular reaction at the glandular region of esophagus seems to be related to the secretion as stated above. The granular reactions were well demonstrated with lower concentration of substrate. There is a large body of evidence to support the idea that enzyme could be inhibited by the excess of substrate (Bergmeyer, 1974; Bergmeyer and Bernt, 1974). Brody and Engel (1964) reported the inhibition of lactate dehydrogenase isozymes in mammalian muscles by high concentration of substrate using a histochemical technique. No reports have been available as to the inhibition of AChE isozymes in nematodes by the excess of substrate. It seems likely that the AChE in *B. pahangi* larvae producing granular reactions at the glandular region of esophagus was inhibited by $3.5\text{-}5.2\times10^{-2}$M substrate, while the AChE in the nervous organs was well demonstrated by the same substrate concentration. It is generally accepted that differences are observed among isozymes not only in pH optimum, or in sensitivity to inhibitors or to denaturing agents, but also in affinity constants for the substrate (Wieme and Moss, 1983). If the findings mentioned above imply the existence of difference in affinity constant for acetylthiocholine iodide, there seems to be, at least, two types of AChE isozymes in *B. pahangi* larvae.

The exact localization of granules in the microfilaria, first stage larvae and third stage larvae are not known. Another unanswered question concerns the decrease of granular reaction in late first stage larvae. Further studies into these problems are necessary to be done.

Summary

Histochemical localization of acetylcholinesterase was reported on *Brugia pahangi* microfilariae and their larvae developing in *Aedes aegypti*. With the low concentration (1.7×10^{-3}M) of substrate (*i.e.* acetylthiocholine iodide), granular reaction was detected. With substrate concentration of $3.5\text{-}5.2\times10^{-2}$M, the amphids, nerve ring and phasmids were well stained without granular products. The granular reaction at the glandular region of esophagus became stronger in the course of development of the second stage larvae. Findings suggest that there are two types of isozymes in the larvae: one is related to nervous activities and the other to secretion.

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Brugia pahangi の酵素組織化学
2. 蚊 (*Aedes aegypti*) 内発育幼虫におけるアセチルコリンエステラーゼ活性の局在

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Brugia pahangi のミクロフィラリアと *Aedes aegypti* (Liverpool strain) 内で発育中の幼虫のアセチルコリンエステラーゼの組織化学的分布を報告した．基質のヨウ化アセチルチオコリンの濃度を低下 (1.7×10^{-3} M)すると，顆粒状の反応を認める．基質濃度を3.5-5.2 $\times10^{-2}$M と高くするとアンフィド，神経輪，ファスミッドが好染し，顆粒状の反応は起らない．第2期幼虫の発育と共に glandular esophagus の顆粒状の反応は次第に増強する．神経機能と関連するアイソザイムと，分泌と関連するアイソザイムの存在が示唆される．