Some Components of the Cholinergic System in the Prawn *Palaemonetes varians* (Leach)

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1. An enzyme similar to mammalian acetylcholinesterase is found in high activity in the nervous tissue of *Palaemonetes varians*, i.e. eyes plus stalks, brain, suboesophageal ganglion and ventral cord. Acetylcholinesterase is also found associated with the abdominal muscles. Multiple enzyme forms are found in extracts of nervous tissues and muscles by electrophoresis and isoelectric focusing. 2. Cholinesterase is present in high activity in the stomatogastric system of *P. varians*. Three electrophoretically separable forms are found, having isoelectric points at pH 4.2, 4.5 and 5.4. 3. Approx. 50% of the total acetylcholinesterase activity, approx. 80% of the choline acetyltransferase activity and 100% of the acetylcholine equivalents are found associated with the nervous tissue. Among the tissues examined, eyes plus stalks were the richest source of both choline acetyltransferase and acetylcholine equivalents. Suboesophageal ganglion and brain also contained large amounts of these components. 4. The distribution of these components could support the function of acetylcholine as a central and/or sensory transmitter in *P. varians*.

Numerous studies have been carried out on the highly purified acetylcholinesterase (acetylcholine acetylhydrolase, EC 3.1.1.7), from the electric organ of the eel (see review by Nachmansohn, 1970) as well as on the less pure preparations from a number of mammalian tissues (see review by Augustinsson, 1971); however, the crustacean enzyme has been little studied. Acetylcholinesterase has been demonstrated in high activities in the nervous tissues of both the lobster *Homarus americanus* (Nachmansohn & Rothenberg, 1945) and the crab *Carcinus maenas* (Walop & Boot, 1950). The presence of choline acetyltransferase (acetyl-CoA–choline *O*-acetyltransferase, EC 2.3.1.6) in a number of different species of crab has also been described (Easton, 1950). Pharmacological studies have indicated that large amounts of acetylcholine-like substances are present in the nervous tissues of a number of different Crustacea (Welsh, 1961). At relatively low concentrations, inhibitors of ‘cholinesterase’ [for the purpose of the present paper the term ‘cholinesterase’ is used to cover both cholinesterase (EC 3.1.1.8) and acetylcholinesterase (EC 3.1.1.7)] paralyse and kill the shrimp *Gammarus pulex* (Callaway *et al.*, 1952), but there is no conclusive evidence to support the existence of cholinergic transmission in the crustacean nervous system (Wiersma, 1961).

Florey & Biederman (1960) found that sensory bundles from crab chelipeds contained acetylcholine-like substances, and Florey (1967) suggested that acetylcholine might be the sensory transmitter in

crustacean nervous tissue. Barker *et al.* (1972), using *Homarus americanus* sensory neurons, have obtained evidence that supports this suggestion.

The work outlined in the present paper concerns some of the properties of acetylcholinesterase and cholinesterase (acetylcholine acetylhydrolase, EC 3.1.1.8) in the decapod crustacean *Palaemonetes varians*. The regional distributions of these enzymes as well as choline acetyltransferase and acetylcholine equivalents are also described and discussed.

Experimental

Animals

The brackish-water prawn *P. varians* (Leach) was used in all experiments. Animals were either caught in Zeeland or obtained from the Nederlands Instituut voor Onderzoek der Zee, Texel. Prawns were maintained in aquaria provided with circulating seawater at 12–14°C.

Preparation of homogenates and extracts

Animals were cooled in seawater at 0–4°C for 10–15 min and were then killed by cutting off the cephalothorax from the abdomen. All dissections were carried out at 0–4°C. As much of the exoskeleton as possible was removed and discarded. Organs and tissues were separated, by eye, and the pooled samples (usually from four to eight animals) were homogenized in a

Teflon-glass homogenizer (800–1000 rev./min), in 2–5 ml of glass-distilled water.

Eyes plus stalks were prepared by cutting the base of the stalk from the cephalothorax of the animal. The brain or supraoesophageal ganglion was then readily scooped out from its cavity. At this stage the suboesophageal ganglion plus connectives was also readily removable when approached from the dorsal side after removal of the organs lying above it. Ventral cords were removed by severing the roots connecting ganglia to peripheral tissue.

When acetylcholine equivalents were to be measured, tissues were homogenized (by hand) in cold 10% (w/v) trichloroacetic acid immediately after dissection. The homogenates were left at 22°C for 1 h, after which they were centrifuged and the supernatants extracted at least six times with 2 vol. of water-saturated ether. The aqueous phase (approx. pH 6) was stored at 4°C until the assay could be performed, usually within 2 h.

When *P. varians* is kept at 4°C for any length of time, blood seems to concentrate in the area around the heart, which becomes blue. Blood was obtained from the cephalothorax of a number of cooled animals by allowing it to pump out into the assay medium immediately after the abdomen had been severed from the cephalothorax.

Assay methods

Acetylcholinesterase and cholinesterase were assayed at pH 8.0 and 25°C in a medium containing 150 mM-NaCl and 20 mM-MgCl₂ by using a Radiometer (Copenhagen, Denmark) Titrigraph with a 0.25 ml Autoburette AB 12 attachment. The pH was maintained with 15 mM-NaOH. Initial rates were measured and corrected for blank rates due to CO₂ uptake and spontaneous acid production by the sample. Spontaneous hydrolysis of substrate was always subtracted at substrate concentrations where it made any significant contribution. At the lower substrate concentrations used, rates were constant for at least 30 s. Substrate, freshly prepared each day, was added in μ l quantities from Hamilton syringes to start the reaction.

Choline acetyltransferase was assayed by the method of McCaman & Hunt (1965) as modified by Goldberg *et al.* (1969). Water homogenates (nervous tissue, 3 mg/ml; remainder, 15 mg/ml) were used and incubation times were 20 and 40 min for each sample. Product formation was linear with time for at least 40 min under these conditions.

Acetylcholine equivalents were assayed biologically by using the isolated guinea-pig ileum. The ileum was immersed in a 5 ml organ bath containing Mg²⁺-free Tyrode solution (147 mM-NaCl, 2.7 mM-KCl, 1.8 mM-CaCl₂, 0.4 mM-NaH₂PO₄, 1.2 mM-NaHCO₃ and 5.6 mM-glucose), gassed with CO₂+O₂ (5:95) at

34°C. The antihistamine mepyramine was added to the bathing fluid to a concentration of 0.6 μ M. The contractions produced by the tissue extracts were compared with those produced by standard solutions of acetylcholine iodide (Gaddum, 1936).

Protein was measured by the method of Lowry *et al.* (1951), with dry bovine serum albumin as standard.

Electrophoresis and isoelectric focusing

Discontinuous disc electrophoresis was carried out at 4°C in 6% (w/v) polyacrylamide gels, pH 8.9, in the system described by Maurer (1968). The gels were stained for enzyme activity by using 2 mM-acetylthiocholine or 4 mM-butyrylthiocholine as substrate. The staining method used was that described by Maynard (1964b) with the modification that the white precipitate of copper thiocholine was finally stained deep green with a saturated solution of dithio-oxamide (Juul, 1968).

Isoelectric focusing was carried out in polyacrylamide gels as described by Dale & Latner (1968). Gels were stained for enzyme activity in exactly the same way as described for electrophoresis.

Histochemical studies

Acetylcholinesterase activity was detected by the method of Karnovsky & Roots (1964). Incubations were carried out in the presence of both 10 and 100 μ M-tetraisopropyl pyrophosphortetramide to inhibit any cholinesterase that might be present.

Materials

Substrates used were: acetylcholine iodide and butyrylcholine iodide (BDH Chemicals Ltd., Poole, Dorset, U.K.); acetyl- β -methylcholine iodide, Mann Research Laboratories Inc. (New York, N.Y., U.S.A.); [¹⁴C]acetyl-CoA, Philips-Duphar (Amsterdam, The Netherlands); acetyl-CoA, lithium salt (93% pure), Mann Research Laboratories Inc.

Inhibitors used were: mepyramine (*N*-4-methoxybenzyl-*N'*-dimethyl-*N*-2-pyridylethylenediamine), Specia (Paris, France); atropine sulphate, Brocades (Maarssen, The Netherlands); compound BW 284 [1,5-bis-(*N*-allyl-*NN*-dimethyl-4-ammoniumphenyl)-pentan-3-one dibromide], Burroughs Wellcome (Beckenham, Kent, U.K.); ethopropazine [10-(2-diethylaminopropyl)phenothiazine] hydrochloride, Specia; tetraisopropyl pyrophosphortetramide, Koch-Light (Colnbrook, Bucks., U.K.); eserine (Merck, Darmstadt, Germany).

Dithio-oxamide was from BDH Chemicals Ltd., acrylamide from Fluka (Buchs, Switzerland), ampholine from LKB (Stockholm, Sweden) and crystalline bovine serum albumin from BDH Chemicals Ltd.

All remaining chemicals were of the purest grade available and were purchased from Merck.

Results

Distribution and characterization of 'cholinesterase' activity

The distribution of 'cholinesterase' activities in *P. varians* in the presence of 0.75 mM-acetylcholine or 10 mM-acetyl- β -methylcholine as substrates was similar, approximately 50% of the total activity being associated with the nervous tissue (eyes plus stalks, brain, suboesophageal ganglion and ventral cord) (Table 1). No activity was detectable in the pooled blood obtained from six *P. varians*. Little activity was found associated with the stomatogastric tissue; however, a substantial activity was found in the abdominal-muscle preparations.

The effect of substrate concentration on the activity of a nervous-tissue homogenate was measured (Fig. 1). Acetylcholine was rapidly hydrolysed at low substrate concentrations, but at higher concentrations substrate inhibition was observed (substrate optimum, approx. 0.75 mM). Acetyl- β -methylcholine was also readily hydrolysed; however, no substrate inhibition was found up to a concentration of 50 mM. Butyrylcholine was hydrolysed only very slowly. In an experiment with an abdominal-muscle homogenate, the results (Fig. 2) were similar to those obtained with the mixed nerve preparation, except that acetyl- β -methylcholine was hydrolysed more rapidly than acetylcholine at the same concentration.

Histology of an abdominal-muscle preparation indicated that visceral nerve was present; however, the activity of visceral-nerve homogenates was too low to account for the enzyme activity associated with the muscle preparation. Histochemistry of the abdominal muscles demonstrated a high acetylcholin-

esterase activity in nervous tissue lying between the muscle fibres.

The distribution of 'cholinesterase' activity in the tissues of *P. varians* in the presence of 10 mM-butrylcholine was quite different from that found in the presence of 0.75 mM-acetylcholine or 10 mM-acetyl- β -methylcholine (see Table 1). By far the greatest amount of activity was associated with the stomatogastric system. The effect of substrate concentration on the activity of a stomatogastric-tissue homogenate

Fig. 1. Effect of substrate concentration on the activity of a homogenate of nervous tissue from *P. varians*

Nervous tissue used: eyes plus stalks, brain, suboesophageal ganglion and ventral cord. Assay was carried out at pH 8.0 and 25°C, in the presence of different concentrations of acetylcholine iodide (○), acetyl- β -methylcholine iodide (●) and butyrylcholine iodide (△) as substrates. Protein concentration, 0.15 mg/ml.

Table 1. Distribution of 'cholinesterase' activity in the various tissues of *P. varians*

Activity was assayed titrimetrically at pH 8.0 and 25°C, in the presence of three different choline esters. In each case 100% is equivalent to the sum of the total activities of the individual homogenates. Results are mean values $\pm$ S.D. for the numbers of observations in parentheses.

Tissue	Activity (% of total)		
	0.75 mM-Acetylcholine	10 mM-Acetyl- β -methylcholine	10 mM-Butyrylcholine
Eyes + stalks	14 $\pm$ 3.3 (7)	12.5 $\pm$ 1.9 (6)	1.3 $\pm$ 0.7 (6)
Brain	11.3 $\pm$ 4 (7)	10.5 $\pm$ 2.6 (6)	1 $\pm$ 1.3 (6)
Suboesophageal ganglion	17.9 $\pm$ 3.2 (7)	17 $\pm$ 2.1 (6)	1.2 $\pm$ 0.4 (6)
Ventral cord	9 $\pm$ 2.6 (7)	7.8 $\pm$ 1.9 (6)	1.3 $\pm$ 0.5 (6)
Stomatogastric system	5.7 $\pm$ 3 (7)	6 $\pm$ 3.5 (6)	81.3 $\pm$ 2.9 (6)
Abdominal muscle	20 $\pm$ 6.1 (7)	23.5 $\pm$ 8 (6)	7.5 $\pm$ 4.8 (6)
Remainder of animal	22 $\pm$ 3.4 (7)	22.2 $\pm$ 5.8 (6)	6.5 $\pm$ 4.1 (6)

Fig. 2. Effect of substrate concentration on the activity of a homogenate of abdominal muscle from *P. varians*

Activity was measured at pH 8.0 and 25°C in the presence of different concentrations of acetylcholine iodide (○), acetyl-β-methylcholine iodide (●) and butyrylcholine iodide (△). Protein concentration, 1.8 mg/ml.

Fig. 3. Effect of substrate concentration on the activity of a homogenate of stomatogastric tissue from *P. varians*

Activity was assayed at pH 8.0 and 25°C in the presence of acetylcholine iodide (○), acetyl-β-methylcholine iodide (●) and butyrylcholine iodide (△). Protein concentration, 0.3 mg/ml.

is shown in Fig. 3. Of the three substrates used butyrylcholine gave the highest activity. Both acetyl- and acetyl-β-methylcholine were hydrolysed to a small extent, but no evidence of substrate inhibition was found.

The activities of these various tissue homogenates were also tested in the presence of a number of inhi-

bitors (Table 2). Eserine (100 μM), an inhibitor of all 'cholinesterases' (Augustinsson, 1971), completely inhibited the activity of all of the homogenates. The stomatogastric preparation was 41% inhibited by 0.5 μM of compound BW 284, a selective inhibitor of acetylcholinesterase (Austin & Berry, 1953), and all the other tissue homogenates showed between 63 and 100% inhibition. Selective inhibitors of cholinesterase, namely 33 μM-ethopropazine hydrochloride (Klingman *et al.*, 1968) and 500 μM-tetraiso-propyl pyrophosphortetramide (Austin & Berry, 1953), gave 88–100% inhibition of the stomatogastric preparation activity, whereas the other tissue homogenates showed 16–48% inhibition.

Most of the stomatogastric activity was soluble, in direct contrast with the nervous-tissue activity (Table 3). The amount of nervous-tissue or abdominal-muscle activity in the supernatant after centrifuging was increased to 47 and 65% respectively by homogenizing the tissues in 0.1% Triton X-100.

Polyacrylamide-gel-disc electrophoresis was carried out with extracts prepared in 0.1% Triton X-100, and the gels were stained for enzyme activity by using acetyl- and butyryl-thiocholine as substrates. Enzyme activity which is inhibited by 100 μM-eserine (cf. Table 2) and which stains preferentially with acetylthiocholine is due to acetylcholinesterase, whereas that which stains preferentially with butyrylthiocholine is due to cholinesterase.

The results indicate that the stomatogastric system contains three electrophoretically separable forms of enzyme which stain strongly with butyrylthiocholine and not with acetylthiocholine (bands B, C and D; Fig. 4c). Nervous-tissue extracts (Fig. 4a) contained two forms of enzyme which enter the gel and which stained preferentially with acetylthiocholine (bands 1 and 4). Quite a large amount of activity remained at the origin. Abdominal-muscle extract (Fig. 4b) contains three forms of enzyme which stain with acetylthiocholine only (bands 2, 3 and 4), one form which stains with butyrylthiocholine only (band C) and one form which stains with either acetyl- or butyrylthiocholine (band A). Control gels were run for every fraction and carried through the staining procedure in the absence of substrate. With both nervous-tissue and abdominal-muscle extracts bands of colour were obtained, which were probably due to haemocyanin or some other copper-containing protein, and were easily distinguishable from the bands due to enzyme activity. Sometimes nervous-tissue extracts contained traces of two of the bands staining with butyrylthiocholine which are found in the stomatogastric tissue (bands B and C, Fig. 4c). These may perhaps be due to contamination by stomatogastric tissue during dissection.

Isoelectric focusing of the same extracts was also done in polyacrylamide gels (Table 4). The stomatogastric extract contained three forms of enzyme stain-

Table 2. *Inhibition of 'cholinesterase' activity in P. varians tissue homogenates at pH 8.0 and 25°C by a number of different inhibitors*

Assays were done as described in Fig. 1. Samples were preincubated in the assay medium at 25°C, with and without inhibitor for 30 min before addition of substrate except in the case of 'remainder of animal' where preincubation destroyed the enzyme activity. The activities (uninhibited) of the fractions were: nervous tissue 0.032, stomatogastric system 0.09, abdominal muscle 0.0024 and remainder of animal 0.0013 $\mu\text{mol/min}$ per mg wet wt.

Tissue	Substrate	Inhibition (%)			
		100 μM -Eserine	0.15 μM -Compound BW 284	33 μM -Ethopropazine hydrochloride	500 μM -Tetraisopropyl pyrophosphortetramide
Stomatogastric system	10 mM-Butyrylcholine	100	41	88	100
Mixed nervous tissue	0.75 mM-Acetylcholine	100	100	48	16
Abdominal muscle	0.75 mM-Acetylcholine	100	100	42	25
Remainder of animal*	0.75 mM-Acetylcholine	100	63	24	20

* No preincubation with inhibitor.

Table 3. *Solubility of P. varians 'cholinesterase' in water*

Homogenates (stomatogastric system, 50 mg/ml; nervous tissue, 15 mg/ml; abdominal muscle, 150 mg/ml) in water were centrifuged at 50000g for 30 min at 4°C (r_{av} 9.5 cm). The activity of each fraction was measured at pH 8.0 and 25°C with 10 mM-butyrylcholine (stomatogastric system) or 0.75 mM-acetylcholine (mixed nervous tissue and abdominal muscle) as substrates.

Tissue	Activity (% of total)		
	Homogenate	Sediment	Supernatant
Stomatogastric system	100	30	74
Mixed nervous tissue	100	88	12
Abdominal muscle	100	49	51

ing preferentially with butyrylthiocholine and with isoelectric points at pH 5.4, 4.5 and 4.2 (major form). The nervous-tissue and abdominal-muscle extracts contained a number of forms of enzyme staining preferentially with acetylthiocholine and with isoelectric points close to pH 7.0. The bands staining preferentially with acetylthiocholine in the abdominal-muscle extract seemed to have different isoelectric points from those in the mixed-nerve extract. The abdominal-muscle extracts also contain a number of enzyme forms staining preferentially with butyrylthiocholine, and these also seem to have different pI values from those present in the stomatogastric extracts.

Specific activities of 'cholinesterase'

A wide range of values was found (Table 5). There seemed to be some correlation between the season and the activity, the highest values being found in

mid-winter (see Fig. 5). Welsch & Dettbarn (1970) have also reported finding seasonal variations in the components of the cholinergic system in lobster walking-leg nerve. However, no obvious difference in the percentage distribution in the various tissues has been noted (see Table 1), even though the measurements were carried out at various times of the year.

Distribution of choline acetyltransferase

Nervous tissues accounted for approx. 80% of the choline acetyltransferase activity in the whole animal (Table 6). Eyes plus stalks contained approx. 40% of the latter.

Distribution of acetylcholine equivalents

The guinea-pig ileum was used as a test organ; however, because of its lack of specificity (see review by Whittaker, 1963), the contractions may be caused

Fig. 4. Electrophoretic separation of cholinesterase and acetylcholinesterase from *P. varians*

P. varians tissue extracts were electrophoresed in polyacrylamide gels (6%, w/v) at pH 8.9 for 3.5 h at 4°C, 7 mA/gel and 100 mV. Gels were stained for enzyme activity with acetylthiocholine (2 mM) and butyrylthiocholine (5 mM) as substrates. Staining times varied with the extracts used; however, gels containing the same extracts were stained for the same length of time with acetylthiocholine (gels ii) or butyrylthiocholine (gels iii). Gels (i) are control blanks without substrate. Extracts of nervous tissues (a) and abdominal muscle (b) were prepared in 0.1% Triton X-100. Stomatogastric-tissue extracts (c) were prepared in distilled water. ---- represents the point of attachment of the spacer and separation gels. For details of bands A–D and 1–4 see the text.

by some compound other than acetylcholine, and have thus been defined as due to acetylcholine equivalents. The contractions were not affected by mepyramine (0.6 μ M), and were completely abolished by atropine (0.3 μ M). When the extracts were kept at pH 10 for 20 min at 70°C and then reneutralized, contractions were abolished. This is to be expected when the response is due to a choline ester.

Wide variations in the contents of acetylcholine equivalents were obtained, even though these experiments were carried out during two winter months, i.e. November and December (Table 7). The eyes plus stalks and the suboesophageal ganglia each contained

Fig. 5. Seasonal variations in the acetylcholinesterase activity of nervous tissue from *P. varians*

Activity was assayed at pH 8.0, 25°C with 0.75 mM-acetylcholine as substrate. Each point represents the mean of duplicate measurements of the activity of a homogenate of the pooled nervous tissues from three animals. Tissues used: eyes plus stalks, brain, suboesophageal ganglion and ventral cord. *P. varians* were caught in large quantities, approx. once a year (usually in August) and were maintained for at least 3 weeks under laboratory conditions (as described in the Experimental section) before any measurements were carried out. No discrimination was made according to sex, size or moulting time.

Table 4. Isoelectric focusing of *P. varians* tissue extracts in polyacrylamide gels

For experimental details see the text. Focusing time, 18 h. mf, Major form.

Tissue	Substrate	pI
Mixed nervous tissue	Acetylthiocholine	7.7, 7.2 (mf), 5.7
	Butyrylthiocholine	—
Abdominal muscle	Acetylthiocholine	>9.5, 8.8, 6.9 (mf), 6.8 (mf)
	Butyrylthiocholine	6.5, 6.2, 5.7
Stomatogastric system	Acetylthiocholine	—
	Butyrylthiocholine	5.4, 4.5, 4.2 (mf)

Table 5. *Range of specific activity of 'cholinesterase' at pH 8.0 and 25°C*

Homogenates of a number of different *P. varians* tissues were assayed as described in the Experimental section. Experiments were carried out in February, March, April, December (2×) 1971 and January February 1972. The ranges of activities are quoted.

Tissue	Acid produced (μmol/min per mg of protein)		
	0.75 mM-Acetylcholine (n, 7)	10 mM-Acetyl-β-methylcholine (n, 4)	10 mM-Butyrylcholine (n, 5)
Eyes plus stalks	0.13–1.04	0.22–0.89	0.07–0.21
Brain	0.31–0.97	0.44–0.67	0.02–0.15
Suboesophageal ganglion	0.73–7.26	0.71–7.46	0.02–0.47
Ventral cord	0.55–6.05	0.56–8.1	0.04–0.5
Stomatogastric system	0.01–0.1	0.01–0.09	0.1–1.16
Abdominal muscle	0.02–0.05	0.03–0.17	0.01–0.02
Remainder of animal	0.04–0.13	0.03–0.15	0.01–0.07

Table 6. *Choline acetyltransferase activity in homogenates of various P. varians tissues at pH 7.4 and 25°C*

Activity was measured, as described in the Experimental section, in November 1970 (first values) and June 1971.

Tissue	Activity (% of whole animal)	Specific activity (μmol of acetylcholine produced/min per g of protein)
Eyes plus stalks	38, 40	3.3, 2.5
Brain	23, 17	0.69, 0.92
Suboesophageal ganglion	15, 14	2.64, 1.72
Ventral cord	10, 12	1.81, 1.0
Remainder of animal	14, 18	0.08, 0.03

Table 7. *Acetylcholine equivalents extracted from P. varians tissues*

Experiments were carried out in November (2) and December (3), i.e. total number of experiments was 5. Acetylcholine was assayed as described in the Experimental section and values are means ± s.d.

Tissue	Acetylcholine equivalents (% of whole animal)	Range of concentrations (μmol/g of protein)
Eyes plus stalks	39.2 ± 3.8	0.58–1.13
Brain	13.8 ± 2.2	1.16–2.29
Suboesophageal ganglion	40 ± 6.6	1.69–4.17
Ventral cord	7 ± 1.9	0.15–0.43
Remainder of animal	not detectable	—

about 40% of the whole animal content; the highest content was found in the suboesophageal ganglion. No activity of the test system was detected in the remainder of the animal.

Distribution of components of the cholinergic system

Each of the nervous tissues studied contain each of the three components measured (Table 8). Neither acetylcholine equivalents nor choline acetyltrans-

ferase are as widely distributed as acetylcholinesterase. Perhaps the most obvious point is the very high content of both acetylcholine equivalents and choline acetyltransferase found in eyes plus stalks.

Discussion

Enzymes hydrolysing choline esters are present in the various tissues of *P. varians*. The hydrolysis of 0.75 mM-acetylcholine or 10 mM-butyrylcholine was

Table 8. Summary of the percentage distribution of three components of the cholinergic system within the tissues of *P. varians*

For details see Tables 1, 6 and 7.

Tissue	Percentage distribution		
	Choline acetyltransferase	Acetylcholine equivalents	Acetylcholinesterase
Eyes plus stalks	39	39	14
Brain	20	14	11
Suboesophageal ganglion	14	40	18
Ventral cord	11	7	9
Remainder of animal	16	not detectable	48

completely inhibited by 100 μ M- eserine (Table 2). Since practically all 'cholinesterases' are inhibited by 100 μ M- eserine (see review by Augustinsson, 1971), it may be concluded that in general only 'cholinesterases' contribute to the hydrolysis of 0.75 mM- acetylcholine or 10 mM- butyrylcholine by homogenates of *P. varians* tissues.

In mammalian tissues both cholinesterase and acetylcholinesterase hydrolyse acetylcholine; however, the latter enzyme is inhibited by excess of acetylcholine and readily hydrolyses acetyl- β -methylcholine (Alles & Hawes, 1940). Butyrylcholine is rapidly hydrolysed by cholinesterase, but it is split at a very low rate by acetylcholinesterase (Nachmansohn & Rothenberg, 1945). Determinations in the presence of a number of different substrates indicate that stomatogastric tissue contains cholinesterase (Fig. 1), whereas both nervous tissue and abdominal muscle contain acetylcholinesterase (Figs. 2 and 3 respectively). Studies in the presence of compound BW 284, a selective inhibitor of acetylcholinesterase, and tetraisopropyl pyrophosphortetramide (Austin & Berry, 1953) or ethopropazine hydrochloride (Klingman *et al.*, 1968), selective inhibitors of cholinesterase, support this conclusion (see Table 2).

Acetylcholinesterase in the abdominal muscles may be different from that in nervous tissue, since the ratio of the activity in the presence of acetylcholine at the optimum substrate concentration to the activity in the presence of 10 mM- acetyl- β -methylcholine is different (compare Figs. 2 and 3). The nervous-tissue enzyme is also much less soluble when homogenized in water (Table 3).

The presence of four forms of acetylcholinesterase on electrophoresis, two of the forms being associated with the nervous-tissue extract and three with the abdominal-muscle extract (Fig. 4), may explain the difference observed between the acetylcholinesterase in these tissues. However, it should be emphasized that not all of the enzyme activity is solubilized by 0.1 % Triton X-100, and that not all of the enzyme in

the Triton X-100 solution enters the polyacrylamide gel.

Isoelectric focusing of these Triton X-100 extracts also demonstrates the presence of seven different forms of acetylcholinesterase (Table 4). In general, the isoelectric points of these forms are close to or greater than pH 7. Maynard (1964a,b) studying the nervous tissues of the lobsters *Panulirus argus* and *Panulirus guttatus* found two forms of acetylcholinesterase moving towards the anode. The more rapidly migrating enzyme was predominant in central ganglia, whereas the slower form was predominant in peripheral nerves. Comparable results were obtained with extracts of tissues from *P. varians* (see Fig. 4).

Electrophoretically separable forms of acetylcholinesterase have also been reported in extracts of nervous tissue from a number of insects; three forms in the fly *Musca autumnalis* (Knowles & Arurkar, 1969), three from the nerve cords of the cricket *Acheta domesticus* (Edwards & Gomez, 1966), four in the head of the housefly *Musca domestica* and two in the head of the American cockroach *Periplaneta americana* (Eldefrawi *et al.*, 1970).

Cholinesterase in human serum has been extensively studied and the electrophoretically separable forms which are present can be designated as isoenzymes since they are genetically controlled (see review by LaMotta & Woronik, 1971). Thus it is possible that the three electrophoretically separable forms of cholinesterase found in *P. varians* stomatogastric tissue are isoenzymes. Human serum cholinesterase isoenzymes have isoelectric points at approx. pH 4 (LaMotta & Woronik, 1971), and this is in the same range as the values found for the three forms of prawn cholinesterase, i.e. pH 4.2, 4.5 and 5.4 (see Table 4).

Acetylcholinesterase is present in *P. varians* in high concentrations in each of the nervous tissues examined. However, it is not confined to the nervous tissues alone. This agrees with Maynard (1971), who found that 'cholinesterase' activity is present in the

neural and glial elements of both the central and peripheral nervous systems of two species of lobster. Preliminary studies on acetylcholinesterase in the abdominal muscles of *P. varians* agree with these observations.

No correlation was found between the distribution of acetylcholinesterase and choline acetyltransferase in *P. varians*. Barker *et al.* (1972) have postulated that each enzyme responsible for the biosynthesis of a transmitter may accumulate only in those cells destined to employ its product as transmitter, whereas the transmitter-degrading enzyme may be made and retained by many types of neurons (see Amano *et al.*, 1972; Giller & Schwartz, 1971a,b). If this is true, then in *P. varians* cholinergic transmission should occur in each of the nervous tissues examined (Table 6). The low choline acetyltransferase activities found in the remainder of the animal would support the lack of cholinergic activity in motor fibres in Crustacea (Grundfest *et al.*, 1959). However, the presence of components of the cholinergic system within nervous tissue is not sufficient to establish cholinergic transmission.

The distribution of acetylcholine equivalents bears more resemblance to that of choline acetyltransferase than to acetylcholinesterase (Table 8). The highest contents of acetylcholine equivalents occur in suboesophageal ganglion and brain (Table 7). No acetylcholine equivalents were detectable in the remainder of the animal. This disagrees with results obtained by other authors, who have found large amounts of acetylcholine (by biological assay) in lobster walking-leg nerves (Dettbarn, 1963; Davis & Nachmansohn, 1964; Dettbarn & Rosenberg, 1967; Welsch & Dettbarn, 1970).

Lasotta *et al.* (1972) have reported that the choline ester present in high concentrations in Lepidoptera is acetyl- β -methylcholine, although acetylcholine was present in low amounts. If the same thing was true for Crustacea, then the assay method used in the present work would not distinguish between acetylcholine and acetyl- β -methylcholine.

In general the results obtained agree with the idea of Florey (1967) that acetylcholine may be the sensory transmitter in crustaceans, and with the findings of Barker *et al.* (1972) that the brain and suboesophageal ganglia which contain the nerve terminals of many sensory elements are among the richest sources of choline acetyltransferase. The richest source of this enzyme found in the present work was eyes plus stalks, which would contain, among other types, primary sensory fibres (Wiersma & Yamaguchi, 1966). However, the results found in the present work could also support the action of acetylcholine as a central transmitter.

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