Esterases in Crustacean Nervous System

I. ELECTROPHORETIC STUDIES IN LOBSTERS

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Biochemical and pharmacological studies have established that crustacean nervous system contains fairly large amounts of cholinesterase and acetylcholine or acetylcholine-like compounds (Nachmansohn and Rothenburg, '45; Schallek, '45; Augustinsson, '48; Walop, '50, '51; Walop and Boot, '50). The ability of lobster central ganglia to hydrolyze acetylcholine, and the effectiveness of extracts of these tissues in standard bio-assays for acetylcholine, are of magnitudes comparable to those in mammalian sympathetic ganglia (Prosser, '46; Welsh, '61). However, in crustacean central nervous system there is no conclusive evidence to support the existence of cholinergic transmission, and no information to establish any other role of acetylcholine (Prosser, '40; Schallek and Wiersma, '48, '49; Turner et al., '50; Welsh, '61; Wiersma, '61). In the peripheral nervous system, neuromuscular transmission is not cholinergic (Grundfest et al., '59), and the acetylcholine of peripheral nerve is associated with sensory fibers rather than with motor elements (Florey and Biederman, '60).

Several years ago, a study of the localization of cholinesterase in cryostat sections of ganglia and nerves in lobsters (*Homarus americanus, Panulirus argus, Panulirus interruptus*) was initiated, using the Koelle ('51) thiocholine method and several more general esterase methods. These results have been partially reported (Maynard and Maynard, '60a, '60b; Maynard, '60) and will be presented in detail elsewhere (Maynard and Maynard, '64). Eserine-sensitive hydrolysis of acetyl- and butyrylthiocholine occurred in certain neural and non-neural, or glial, elements, both in regions known to contain many synapses, and in non-synaptic sites, such as the glial sheaths enveloping neuron somata or surrounding axons in peripheral nerve. Cytochemical studies using thiocholine substrates and various cholinesterase inhibitors did not firmly establish, as in mammalian nervous system (Holmstedt, '57), the presence of more than one type of cholinesterase in this tissue. Resolution of this problem was attempted by electrophoretic studies using starch and acrylamide matrices (Maynard, '61, '64a). The present paper describes two electrophoretically separable cholinesterases and some other esterases in central and peripheral nervous systems of three species of lobsters. A second paper (Maynard and Maynard, '64) describes the histochemically localizable cholinesterases in central and peripheral ganglia and nerves of these species and attempts to correlate that data with electrophoretic preparations from various sub-regions of the nervous system.

MATERIALS AND METHODS

Two species of Bermuda lobsters were used in these studies, the spiny lobster, *Panulirus argus*, and the guinea chick lobster, *Panulirus guttatus*. Studies of these forms were conducted at the Bermuda Biological Station. In addition, some Maine lobsters (*Homarus americanus*) were examined, in Ann Arbor.

Homogenates of freshly dissected tissues were prepared in ice cold distilled water. Homogenate concentrations for acrylamide electrophoresis ranged from 1:4 to 1:30 (mg wet tissue per μl water). For starch electrophoresis 1:1 homogenates

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(w/v) were used. The homogenates were centrifuged for 15 minutes at about 12,000 g, in an International microcapillary centrifuge, at 8–15°C, and the resulting supernatant used as sample (see below). In the case of *Homarus*, adequate polymerization of the sample gel for acrylamide gel electrophoresis was obtained only when the homogenates were dialyzed for two hours at 6°C, against a Tris-glycine buffer (5 mM tris(hydroxymethyl)aminomethane and 38 mM glycine) at pH 8.3.

Acrylamide gel electrophoresis. The method of Ornstein and Davis ('62) was used, with certain modifications. The gels were cast in soft glass tubes 64 mm long and 5 mm inside diameter. The lower gel was 35 mm high and contained acrylamide at a final concentration of 7.5, 10, or 12.5%. The final concentration of Tris in the lower gel was set at one of three levels: 0.35 M (pH of lower gel before polymerization was 8.9), or 0.125 M (lower gel initial pH 8.2), or 0.063 M (lower gel initial pH 7.1). The spacer and sample gels contained 3.3% acrylamide and 0.83% methylene-bisacrylamide. Three hundred µl of spacer gel solution were used, and 150 µl of sample gel solution. The latter consisted of one part sample with two parts spacer gel stock solution (containing 5% acrylamide). Electrophoresis was conducted in the cold (8–15°C), using a set of buffer chambers and electrodes designed by Dr. John Allen [3] of the Zoology Department of this University. A current of 2.5 ma per gel tube was maintained until the tracking dye (brom phenol blue) had moved 25 mm into the lower gel (about one hour).

Following electrophoresis, individual acrylamide gels were placed in test tubes containing 3.5 ml of one of the following reaction mixtures:

(1) Modified Koelle ('51) thiocholine method: (a) Pre-incubation for one half hour at 0°C in a freshly prepared solution containing $CuSO_4 \cdot 5H_2O$, glycine, $MgCl_2 \cdot 6H_2O$, and maleate-NaOH buffer (pH 6.1) in the proportions used in Koelle's reaction mixture. (b) Incubation for 1 to 20 hours at room temperature (28°C) in freshly prepared substrate solution containing the above ingredients plus acetyl- or butyrylthiocholine iodide, all in the proportions used in Koelle's reaction mixture. Following incubation, gels were washed in cold water and were photographed under constant conditions against a black background, for best visualization of the white reaction product (copper thiocholine sulfate, Malmgren and Sylvén, '55). This procedure differed from the histochemical method of Koelle in that sodium sulfate was not included in pre-incubation or in substrate solutions, and conversion of the white copper thiocholine sulfate precipitate to copper sulfide was omitted. Initial attempts to convert to copper sulfide resulted in weakening or loss of bands and this step was therefore discarded. Pre-saturation of substrate solution with copper thiocholine sulfate was also omitted (Holmstedt, '57). Pre-incubation was necessary when using acrylamide gels; its omission resulted in uniformly weaker reactions with the thiocholine substrates. In the case of starch gels, pre-incubation was utilized only in inhibitor studies, but was then applied to the controls as well as to the experimental material.

(2) Azo dye method: (a) Pre-incubation for one half hour at 0°C in 8 mM tris(hydroxymethyl)aminomethane — HCl buffer, pH 7.4. This step was always included when inhibitor studies were performed, but was omitted at other times, both for acrylamide and for starch gels. (b) Incubation for one half or one hour, at 28 or 18°C, in a fresh reaction mixture containing 0.02% α-naphthyl acetate, α-naphthyl propionate, or α-naphthyl butyrate and 0.05% Fast Blue RR salt (Dajac) in an 8 mM Tris-HCl buffer, pH 7.4. After incubation, gels were rinsed in ice water and photographed under transillumination.

(3) Bromoindoxyl acetate (modified from Holt, '58); (a) Pre-incubation for one half hour at 0°C in 20 mM Tris-HCl buffer, pH 7.4, containing 0.55 mM potassium ferrocyanide and 0.55 mM potassium ferricyanide. (b) Incubation for 2 to 4 hours at room temperature (28°C) in a reaction mixture prepared as in 3a, but containing in addition 1.15 mM 5-bromoindoxyl acetate. Following incubation

[3] The advice of Dr. Allen on the electrophoretic procedure is gratefully acknowledged.

gels were washed and photographed as in 2. Use of the halogenated substrate instead of indoxyl acetate gave a faster and more intense reaction. Also, inclusion of the catalysts in the pre-incubation solution decreased the required incubation time.

Starch gel electrophoresis (Markert and Hunter, '59). For some experiments the starch gel was prepared in a 30 mM boric acid — NaOH buffer, pH 7.5, using hydrolyzed potato starch (Connaught Laboratories) in the concentration given on the Connaught label. Alternatively, the gel was prepared in a tris(hydroxymethyl) aminomethane — citric acid buffer (30 or 15 mM Tris) at pH 7.5 or at pH 8.5. The lucite starch molds were 22.4 cm long, 21 mm wide and 6 mm deep (inside dimensions). Gels were stored 12 hours at 10°C prior to use. Five or 10 µl aliquots of the supernatant were pipetted onto pieces of Whatman no. 1 filter paper measuring 5 mm × 6.5 mm or 5 mm × 13 mm, respectively (the former for two samples being run in the same gel, the latter for a single sample). The filter papers were inserted into a cut in the gel 5 cm from the cathodal end. Gels were enclosed in Saran wrap centrally, suspended horizontally, and the ends connected by filter paper strips to double trays of buffer (0.3 M boric acid — NaOH, pH 7.5, for borate-buffered gels, 0.15 M Tris-citric acid, pH 7.5 or 8.5, for Tris-citric acid buffered gels). Electrophoresis was performed for four or five hours at room temperature (28–30°C), using 8 v/cm with borate buffer and 3.5 or 4 v/cm with Tris-citric acid buffer. Following electrophoresis the gels were sliced horizontally into halves (3 mm thick). The pieces of gel were placed in the solutions described in (1) and (2) above, except that incubation times were from seven hours to overnight (room temperature) for the thiocholine method, and from 1 to 12 hours for the azo dye method. The latter was routinely used at 10°C; this gave crisper bands than incubating at room temperature. In the thiocholine method, following incubation, the gels were rinsed one half hour in water and were then placed in 4% ammonium sulfide for five minutes, rinsed, and photographed. The white copper thiocholine sulfate deposit was often visible in the starch gels, but its conversion to copper sulfide permitted easier recognition of bands of activity.

Inhibitors. At various times the following inhibitors, at the concentrations indicated, were included both in the pre-incubation solution and in the reaction mixture; eserine sulfate (10^{-6} M to 10^{-3} M), di-isopropylfluorophosphate (DFP, Merck) (10^{-6} M to 10^{-3} M), BW 284c51j dibromide (Burroughs Wellcome Co., Tuckahoe, New York) (10^{-6} M and 10^{-5} M), parahydroxymercuribenzoate (10^{-4} M). When the reversible inhibitor, eserine, was used with the azo dye method, it was necessary, following incubation, to place the gels in water containing the appropriate concentration of the inhibitor, to avoid hydrolysis of any remaining substrate by the reactivated enzyme. In inhibitor studies, control gels were always pre-incubated and incubated for times equivalent to the inhibited gels; solutions were of identical composition except for the inhibitor.

All figures are photographs of gels, with the origin (cathodal end) at the top. This point represents the site of sample insertion in the case of starch gels and the boundary between lower gel and spacer in the case of acrylamide preparations. Photographs of the latter are at a magnification of 1.9 times, and those of starch gels at 0.95 times.

Unless otherwise indicated, the acrylamide gels figured were made with the lower gel at an initial pH of 8.9 and an acrylamide concentration of 10%. Tissues for acrylamide matrices were diluted 1:20 with water at homogenization, unless stated otherwise.

RESULTS

I. Cholinesterases

Substrate preferences and inhibitor sensitivities. After electrophoresis in acrylamide gel, homogenates of brain, thoracic or abdominal chain ganglia, or the entire nerve cord show two anodally migrating zones of activity against acetylthiocholine (Acthch) (bands 1 and 2 in fig. 1a, b, c). These bands persist, with altered electrophoretic mobilities, as the initial pH, ionic strength, and acrylamide concentration of the lower gel are varied (fig. 1c, d, e, f).

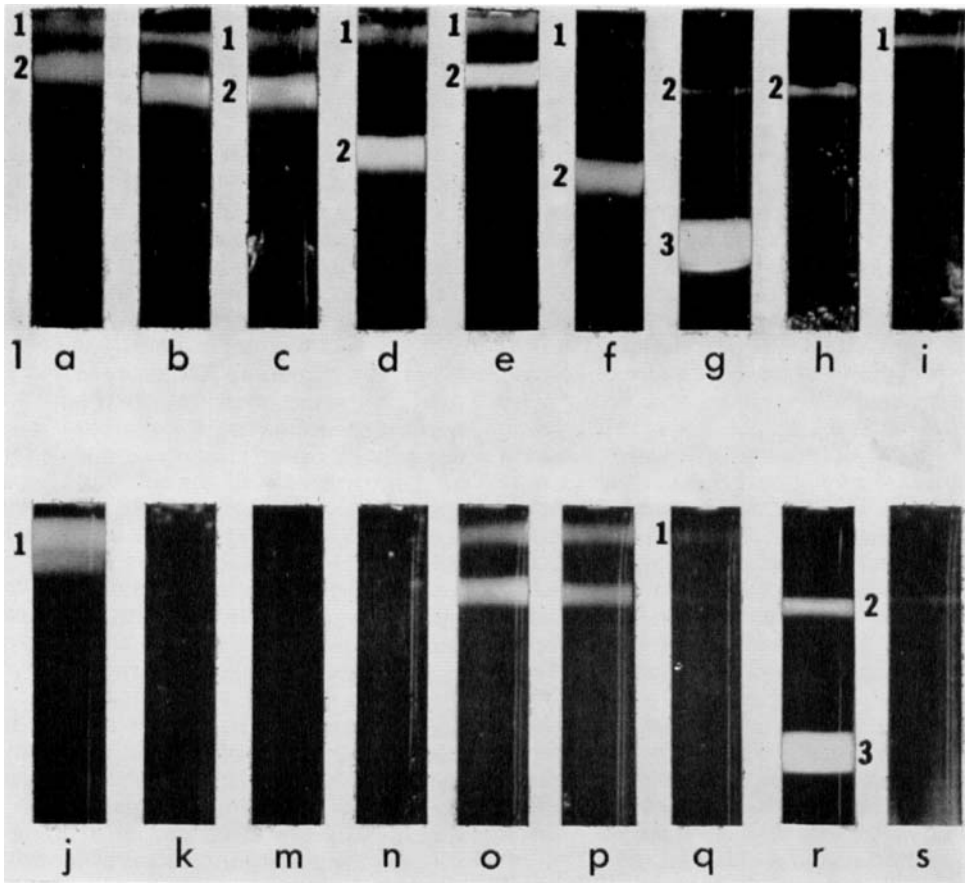

Fig. 1 Acrylamide matrices. *a*, *Homarus* thoracic and abdominal nerve cord, 1:5 homogenate, Acthch 2 hrs ("Acthch" represents acetylthiocholine; "Buthch" represents butyrylthiocholine. Times noted are for incubation in reaction mixture indicated). *b*, *P. guttatus* brain, Acthch 2 hrs. *c*, *P. argus* brain and thoracic nerve mass, Acthch 2 hrs. *d*, *e*, and *f*, same as *c*, except lower gel composition is 10% acrylamide, 0.125 M Tris, initial pH 8.2 in *d*, 12.5%, 0.35 M Tris, pH 8.9 in *e*, and 7.5%, 0.125 M Tris, pH 8.2 in *f*. Compare with standard gel used in this work (10% acrylamide, 0.35 M Tris, pH 8.9) in *c*. *g*, *P. argus* brain, and *h*, *P. guttatus* brain, both in Buthch, 2 hrs. *i*, *P. argus* antennular nerve, Buthch, 16 hrs, and *j*, the same tissue in Acthch one and one-quarter hours. *k*, *P. argus* brain and thoracic nerve mass, Acthch and 10^{-5} M eserine, 7 hrs (compare with control in *c*). *m*, *P. argus* brain, Buthch and 10^{-5} M eserine, 7 hrs (compare with control in *g*). *n*, *P. argus* brain and thoracic nerve mass, Acthch and 10^{-5} M BW284C51j dibromide, seven and one-half hours. *o*, *P. argus* circumesophageal connective, Acthch one and one-quarter hours. *p*, same, but with 10^{-6} M DFP. *q*, same, but with 10^{-5} M DFP. *r*, *P. argus* circumesophageal connective, Buthch, 15 hrs. *s*, same but with 10^{-6} M DFP.

Both bands will also hydrolyze butyrylthiocholine (Buthch), although in this case the reaction is much weaker and slower than with Acthch (fig. 1g,h,i,j). In addition, the central ganglia and interganglionic connectives of *Panulirus argus* contain a third, much more rapidly migrating enzyme, which hydrolyzes Buthch readily and does not attack Acthch (band 3, in fig. 1g; compare with h and i).

In all three species of lobsters, 10^{-5} M eserine sulfate completely inhibits the two enzymes active against Acthch; hydrolysis of Buthch in *P. argus* is also prevented (fig. 1k and m; compare with controls in fig. 1c and g). Partial inhibition occurs with 10^{-6} M eserine. A specific inhibitor of vertebrate acetylcholinesterase, the Burroughs Wellcome compound 284c51j dibromide, at 10^{-5} M, completely abolishes

activity against Acthch, in *P. argus* (fig. 1n). There is partial inhibition at an inhibitor concentration of 10^{-6} M. The effect of this compound on the enzyme preferring Buthch, or in the other species of lobsters, was not examined.

In *P. argus*, 10^{-6} M di-isopropylfluorophosphate (DFP) partially inhibits hydrolysis of Acthch, especially in band 2 (fig. 1o and p). At a concentration of 10^{-5} M DFP (fig. 1q), band 2 is completely inhibited whereas band 1, although less reactive than band 2 in the control preparation, is still present. This suggests that the enzyme in band 2 is more sensitive to DFP than that in band 1. Band 3, preferentially hydrolyzing Buthch, is completely inhibited at 10^{-6} M DFP, while band 2, despite its only minimal hydrolysis of Buthch, is still present in the same gel (fig. 1r and s).

The three bands hydrolyzing thiocholine substrates also react with bromoindoxyl acetate (fig. 2a). In the panulirids, there is an additional band present between bands 1 and 2 (x, in fig. 2c), which is more easily distinguished when the acrylamide concentration in the lower gel is reduced to 7.5%. Band x and a portion of the zone corresponding to band 3 are re-

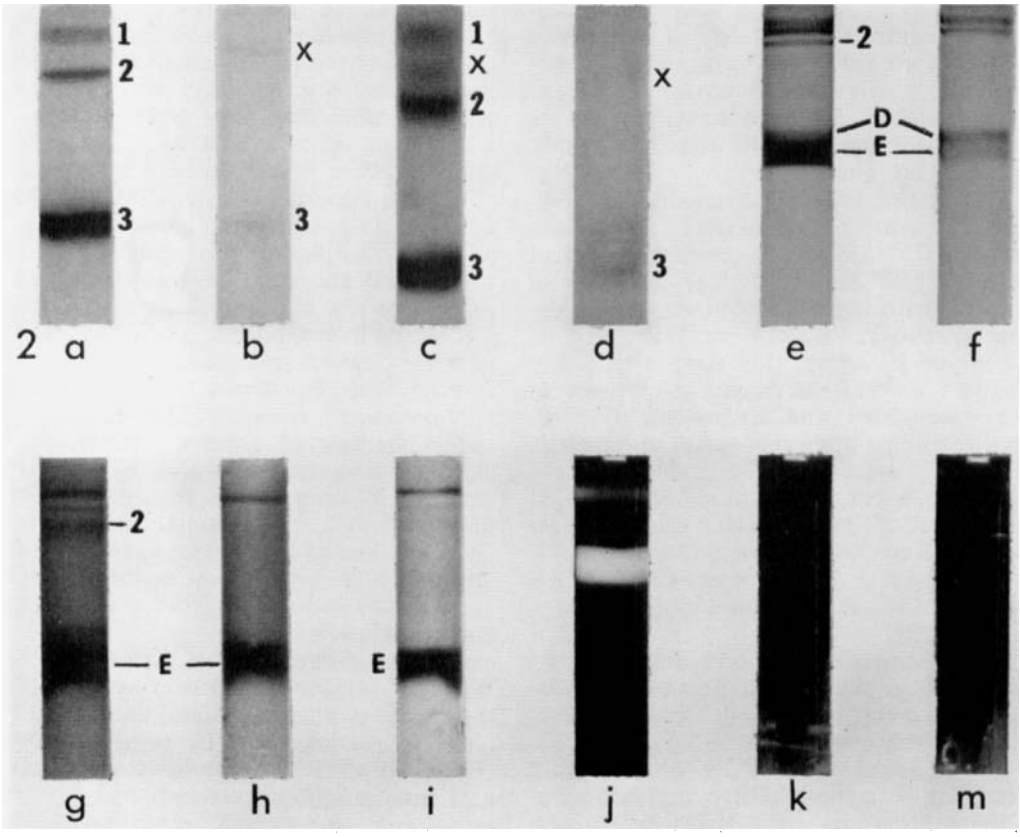

Fig. 2 Acrylamide matrices. *a*, *P. argus* brain and thoracic nerve mass, bromoindoxyl acetate 8 hrs. *b*, same but with 10^{-4} M eserine. *c*, *P. argus* brain and thoracic nerve mass, bromoindoxyl acetate 7 hrs. *d*, same but with 10^{-5} M eserine. (Both *c* and *d* have lower gels with 7.5% acrylamide, 0.35 M Tris, initial pH 8.9.) *e* and *f*, *P. argus* brain and thoracic nerve mass, lower gel 12.5% acrylamide, 0.35 M Tris, pH 8.9, α-naphthyl acetate one-half hour, *e*, the control and *f* with 10^{-4} M eserine, showing inhibition of band 2 and part of band E. The bands cathodal to band 2 are not clearly differentiable in this concentrated lower gel. *g*, *h*, and *i*, *P. argus* brain and thoracic nerve mass incubated 50 min in α-naphthyl acetate, propionate and butyrate respectively. *j*, *P. argus* circumesophageal connective, 1:30 homogenate, Acthch 2 hrs, for comparison with *k*, cardiac muscle and *m*, blood, both at 1:10 dilutions and incubated in Acthch 15 hrs.

sistant to 10^{-4} M eserine whereas bands 1 and 2 are completely inhibited, as with the thiocholine substrates, by 10^{-5} M eserine (fig. 2b and d). The eserine-sensitive and eserine-resistant components of the fastest band will be considered in detail below.

Alpha-naphthyl acetate is hydrolyzed to some extent by band 2; this is best demonstrated with an increased acrylamid concentration (12.5%) in the lower gel, although a diffuse reaction is also present in the 10% gel (fig. 2e and g). The identity of this band with band 2 hydrolyzing Acthch is on the basis of comparable distances from the origin in matched gels, as well as selective inhibition by eserine (fig. 2f). Band 1 does not hydrolyze α-naphthyl acetate, even in tissues where this band is extremely active against Acthch. Alpha-naphthyl propionate and butyrate are not hydrolyzed by bands 1 and 2 (fig. 2h and i).

Thiocholine band 3 corresponds in position to a zone which actively hydrolyzes α-naphthyl acetate, propionate and butyrate ("E" in fig. 2g,h,i), as well as reacting with bromoindoxyl acetate as described above. In the central nervous system of *P. argus*, this zone apparently contains several enzymes: one which is eserine-sensitive and hydrolyzes Buthch, another (or possibly the same) component which is eserine-sensitive and hydrolyzes the other esters, and a third well-defined component which is eserine-resistant and does not react with Buthch (fig. 2e and f; fig. 7b and c). Further discussion of this zone is included below under "Other Esterases."

The three sites hydrolyzing thiocholine substrates in acrylamide gels can also be seen following starch gel electrophoresis (0.03 M Tris-citric acid buffer, pH 8.5). In the panulirids, there are two anodally migrating, eserine-sensitive bands hydrolyzing Acthch (fig. 3b,c,d); these are presumably identical with bands 1 and 2 in the acrylamide preparations. No bands occur cathodal to the origin, with Acthch as substrate.

In starch gels, bands 1 and 2 are visible when homogenates (1:1) are made with water, but the reaction is much stronger, especially in the case of band 1, when the homogenate is prepared in 0.1% aqueous Triton X-100 (fig. 3b and c). With Buthch, homogenates of *P. argus* central nervous system show an eserine-sensitive band comparable to band 3 seen in acrylamide gels. This band is readily seen even in aqueous homogenates (fig. 3e and f). In starch, as in acrylamide, band 3 is at a site of heavy reaction with α-naphthyl acetate (fig. 3g and h); bands 1 and 2 do not coincide with any of the prominent zones hydrolyzing naphtholic esters (fig. 3a and b).

From these results, it is apparent that the nervous system of three species of lobsters contains two electrophoretically separable zones of activity (bands 1 and 2) having substrate affinities and inhibitor sensitivities resembling those of acetylcholinesterase. Subsequently sites 1 and 2 will be called the "slow acetylcholinesterase" (slow AChE) and the "fast acetylcholinesterase" (fast AChE) respectively.

Mixing experiments in acrylamide gels (fig. 5m,n,o), as well as electrophoresis of adjacent samples in the same starch column, indicate that the fast and slow AChE's of the two *Panulirus* species have identical electrophoretic mobilities. Comparable experiments have not been performed with *Homarus*.

Non-neural tissues. Acrylamide gel electrophoresis of cardiac muscle (1:10 aqueous homogenate) and blood (1:10 aqueous dilution) from *P. argus* resolves no sites which hydrolyze Acthch after incubation for 15 hours (fig. 2j,k,m). Several bands hydrolyzing α-naphthyl acetate are present and those of blood will be discussed below.

General distribution of AChE's in central and peripheral nervous systems. In the three species of lobsters, the fast AChE band is predominant in central ganglia (brain, thoracic and abdominal ganglia) and interganglionic connectives (circumesophageal connective, optic peduncle), whereas the slow AChE is more active in preparations of those peripheral nerves which have been examined (antennal, antennular, and walking leg nerves in the panulirids; claw nerve and leg nerves in *Homarus*) (fig. 4a,f,j,k,m,n,o; fig. 1j). The fast AChE of peripheral nerve frequently does not appear unless long incubation is

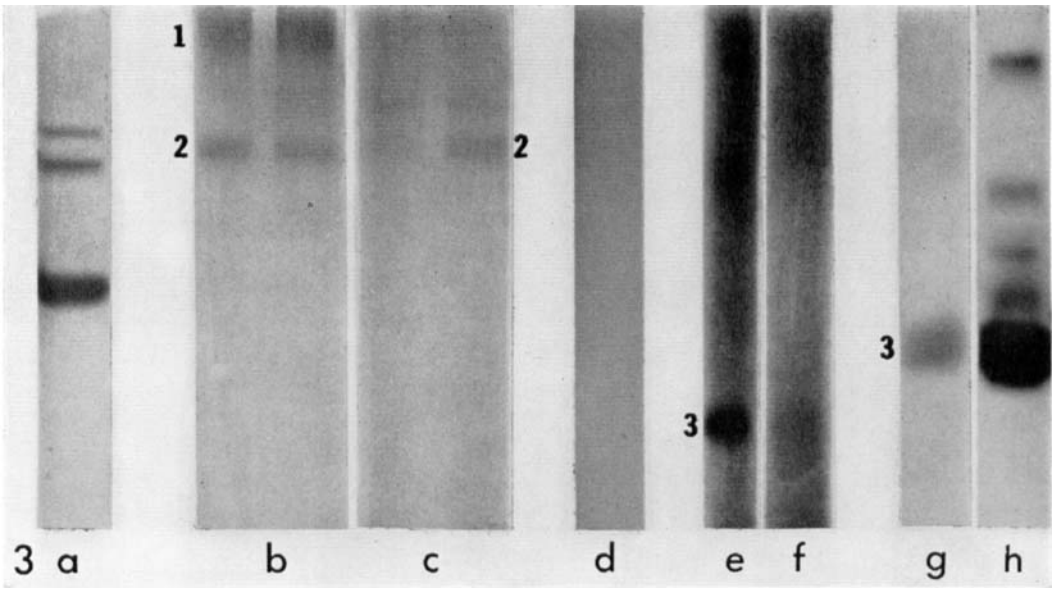

Fig. 3 Starch gel matrices. P. *argus*. *a*, circumesophageal connective, homogenized 1:1 in 0.1% Triton X-100, incubated in α-naphthyl acetate five and one-half hours at 7°C. *b*, at left, connective treated with Triton as above, and at right, antennal nerve similarly treated; Acthch, 7 hrs. *c*, same but using 1:1 aqueous homogenates for comparison with detergent-treated specimens in *b*; antennal nerve at left, circumesophageal connective at right side of gel. *d*, circumesophageal connective, 1:1 aqueous homogenate, Acthch and 10^{-5} M eserine, 7 hrs. Gels in *a* through *d* made with 0.03 M Tris-citric acid buffer, pH 8.5, and run 3 hrs at 3.5 v/cm. *e* through *h*, nerve cord; 1:1 aqueous homogenates; *e* and *f*, halves of same gel, made with 0.03 M Tris-citric acid buffer, pH 7.45, run at 3.5 v/cm for 4 hrs; *e*, Buthch, 16 hrs. *f*, same with 10^{-5} M eserine. *g* and *h*, halves of same gel, made with 0.03 M Tris-citric acid buffer, pH 7.45, and run three and one-half hours at 3.5 v/cm; *g*, Buthch, 8 hrs. *h*, α-naphthyl acetate, 4 hrs.

used (compare fig. 4f, with fig. 1j). The relative differences between electrophoretic preparations from central nervous system (CNS) and peripheral nerve (PN) are seen with bromoindoxyl acetate as well as with Acthch (fig. 4p and q) and can be expressed semi-quantitatively when serial dilutions of homogenates are examined (fig. 4a through i). Thus, the slow AChE of antennal nerve is no longer visible at a final sample dilution of 1:240 (fig. 4i), whereas in brain it disappears at 1:120 dilution (fig. 4c). The fast AChE of antennal nerve is not visible in the 1:90 dilution (fig. 4g), while that of brain persists through the 1:480 dilution (fig. 4e).

In acrylamide gels, the two AChE's of peripheral nerve have substrate and inhibitor characteristics comparable to those described above for the AChE's of central nervous system. Electrophoretic mobilities of the respective AChE's appear identical when CNS and PN samples are run adjacent to one another in the same starch gel (fig. 3b). In addition, electrophoretic preparations of mixed homogenates of equal parts of CNS and PN have the appearance of arithmetic sums of non-mixed homogenates. In the mixture, the two bands are roughly equal in activity against Acthch or bromoindoxyl acetate, and there is no apparent dislocation of the bands, or appearance of new zones of activity (fig. 5a,b,c).

Electrophoretic preparations have also been made of mixtures of (a) native homogenates from central nervous system with heat-inactivated (30 minutes in boiling water) homogenates of peripheral nerve, or (b) heat-inactivated homogenates of CNS with native homogenates of PN (fig. 5d through k). The heat-treated preparations have no activity against Acthch (fig. 5e and i). Mixing inactivated CNS with native PN results in a slow AChE of moderate reactivity (fig. 5g),

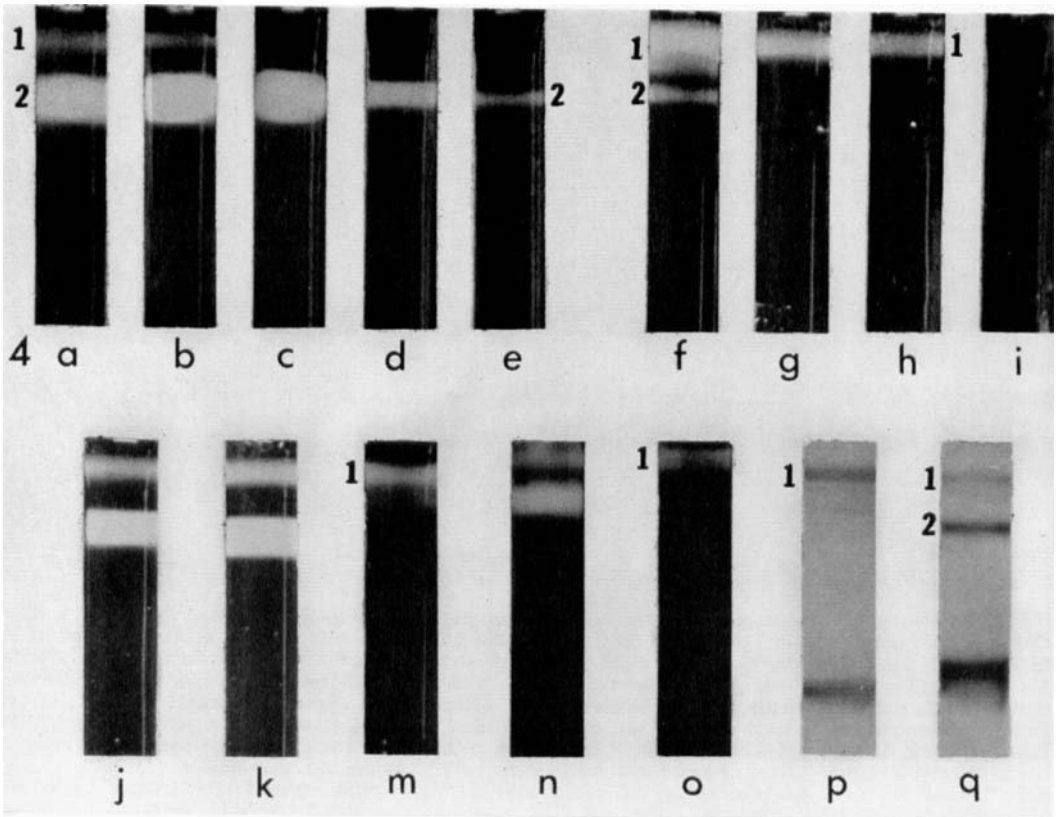

Fig. 4 Acrylamide matrices. *a* through *e*, P. *argus* brain, Acthch, one and one-half hours. Serial dilutions of sample. Final dilution in sample gel (mg/μl) as follows: *a*, 1:60; *b*, 1:90; *c*, 1:120; *d*, 1:240; *e*, 1:480. *f* through *i*, P. *argus* antennal nerve, Acthch, one and one-half hours. Serial dilutions of sample for comparison with those of brain. Final dilution in sample gel is as follows: *f*, 1:60; *g*, 1:90; *h*, 1:120; *i*, 1:240. *j*, P. *argus* circumesophageal connective, Acthch, one and one-half hours. *k*, P. *argus* optic peduncle, Acthch, three and one-half hours. *m*, P. *argus* leg nerve, Acthch, one and one-half hours. *n*, *Homarus* nerve cord, 1:5 homogenate, Acthch, 2 hrs. *o*, *Homarus* claw nerve, 1:5 homogenate, Acthch, 2 hrs, for comparison with *n*. *p*, P. *argus* antennal nerve, bromoindoxyl acetate 2 hrs. *q*, P. *argus* brain, bromoindoxyl acetate 2 hrs.

comparable to that seen in the control situation, where inactivated PN is mixed with native PN (fig. 5f). Likewise, a mixture of native CNS with inactivated PN produces a moderately active fast AChE band (fig. 5k), like that of the control mixture of native and inactivated CNS (fig. 5j).

Effects of variations in homogenization. It was mentioned above that band 3 migrates with relative ease from aqueous homogenates into starch gel, whereas the fast and slow AChE's, especially the latter, apparently migrate more readily when homogenates are made with 0.1% Triton X-100. The preferential effect of Triton X-100 on the slow AChE occurs both in central nervous system and in peripheral nerve (fig. 6a,b,c,d), and is present in all three species of lobsters. This large increase in activity of the slow AChE is produced only when the detergent is included in the homogenate, and does not occur when 0.1% Triton X-100 is added to the substrate solution after electrophoresis of aqueous homogenates. In the latter case, the detergent appears to have a slight inhibitory effect on both AChE bands.

The relative ease with which band 3 is released from the tissue is demonstrated also by its intense reactivity in the supernatant after gentle maceration of the specimen (diluted 1:4 with water) using a test

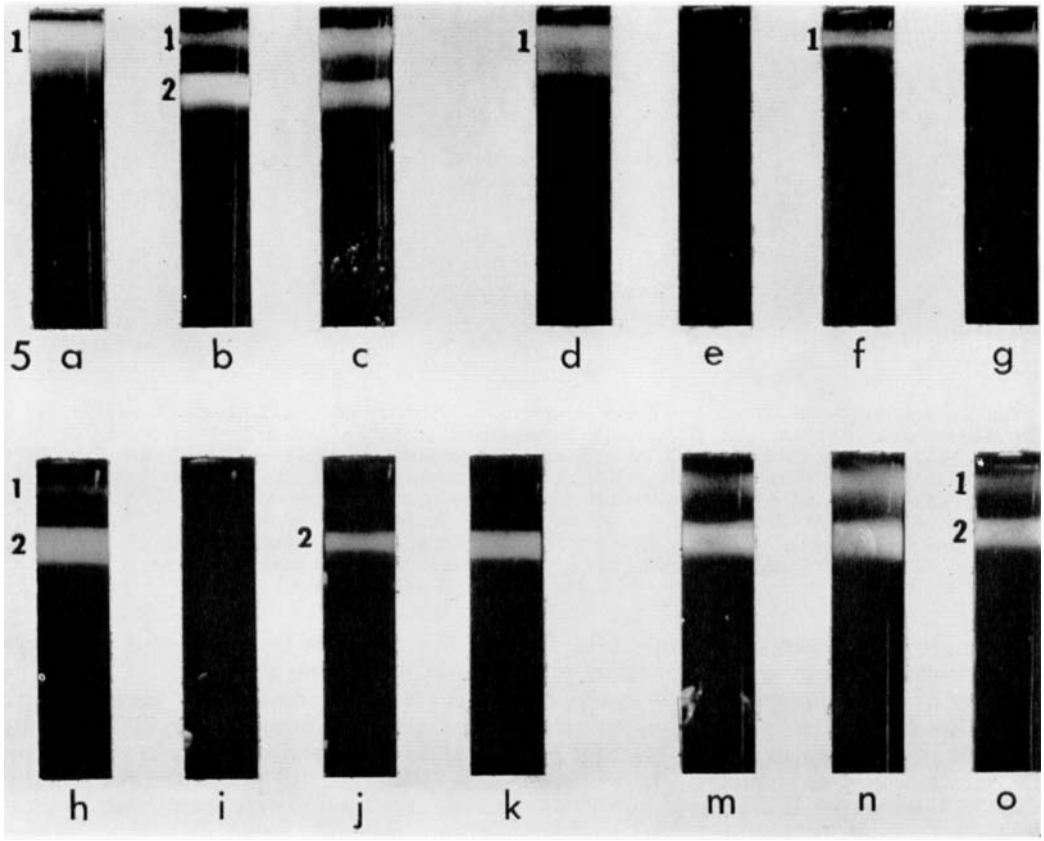

Fig. 5 Acrylamide matrices. *a* through *c*, mixing experiment, in *P. argus*. *a*, antennal nerve, Acthch 2 hrs. *b*, circumesophageal connective, Acthch 2 hrs. *c*, equal parts antennal nerve and circumesophageal connective, Acthch 2 hrs. *d* through *k*, experiments mixing native and heat-inactivated central nervous system (circumesophageal connective, "CNS") and native and heat-inactivated peripheral nerve (a mixture of antennal, antennular, and tegumentary nerves, "PN"), all in Acthch, three and one-half hours (*P. argus*). *d*, PN, native. *e*, PN, heat-inactivated. *f*. equal parts native and heat-activated PN. *g*, native PN and heat-inactivated CNS. *h*, CNS, native. *i*, CNS, heat-inactivated. *j*, equal parts native and heat-inactivated CNS. *k*, native CNS and heat-inactivated PN. *m* through *o*, experiment mixing brain and thoracic nerve mass of *P. argus* with those of *P. guttatus*, all in Acthch, 2 hrs. *m*, *P. argus* alone. *n*, *P. guttatus* alone. *o*, equal parts of *P. argus* and *P. guttatus* tissues.

tube and a very losely fitting smooth glass rod (fig. 6e). The fast AChE is barely visible after this treatment and the slow AChE is not seen, in homogenates of CNS (fig. 6f). However, when the sediment from such a preparation is re-homogenized in a standard type of ground glass homogenizer, the resulting supernatant contains fast and slow AChE's and band 3 is reduced in activity (fig. 6g and h).

In *P. argus*, repeated freezing and thawing of the homogenate (up to six times), or storage at 9°C for 48 hours, or exposure to room temperature (28°C) for three and one-half hours, are without effect on the electrophoretic mobilities and relative reactivities (Acthch) of the two AChE's in central nervous system. In this case the room temperature is within the normal range of water temperatures (18–30°C) to which these animals are accustomed. In *Homarus*, homogenization in 0.5 N KCl has no effect on the pattern or relative activities of the AChE's from central or peripheral nervous system.

In *Homarus* and in *P. argus*, the fast and slow AChE's of central nervous system and peripheral nerve are demonstrable in

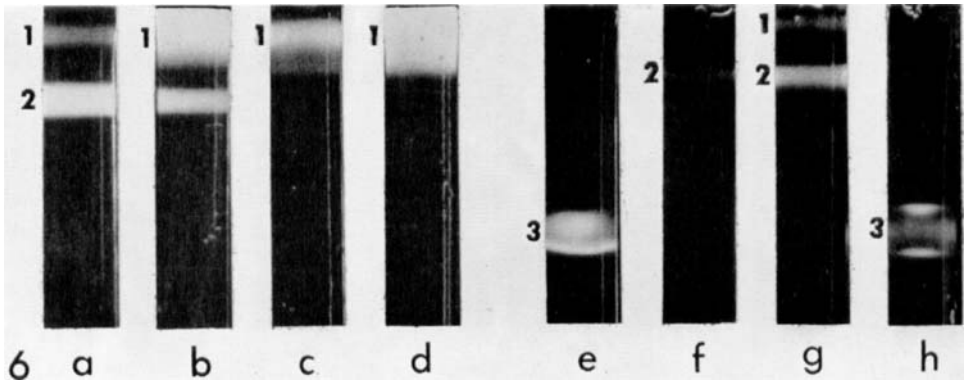

Fig. 6 Acrylamide matrices. *a* through *d*, effect of Triton X-100, in *P. argus*. Acthch, 1 hr. *a*, circumesophageal connective, 1:20 aqueous homogenate. *b*, same, but 1:20 homogenate in 0.1% Triton X-100. *c*, antennal nerve, 1:20 aqueous homogenate. *d*, same, but 1:20 homogenate in 0.1% Triton X-100. *e* through *h*, effect of variation in homogenization, in *P. argus* brain and thoracic nerve mass. *e* and *f*, 1:4 aqueous dilutions, tissue gently macerated, supernatant used as sample. *e*, Buthch, 80 min. *f*, Acthch, 80 min. *g* and *h*, sediment from *e* and *f*, diluted 1:4 in water, homogenized in ground glass homogenizer, centrifuged, and supernatant used as sample. *g*, Acthch, 80 min. *h*, Buthch, 80 min (Lower gel concentration in *e* through *h* was 12.5% acrylamide, initial pH 8.9.)

acrylamide gels when homogenization is avoided, through use of a thin, fresh tissue slice as sample instead of the homogenate. The tissue slice, which is of an area approximately equal to a cross section of the acrylamide gel, is placed on a small piece of filter paper above the polymerized spacer gel, and 150 µl of additional spacer gel are pipetted over it. The relative differences in activities of the fast and slow AChE's in CNS *vs.* PN are maintained in these preparations (fig. 7i,j,k).

II. Other esterases in the panulirids

Substrate preferences and inhibitor sensitivities in neural tissues. In acrylamide and starch gel preparations from the central nervous system of *P. argus*, there are five major zones of activity (bands A through E) against α-naphthyl acetate. The appearance of these bands in the two types of matrix and their positions relative to the two AChE's are shown in figure 7a,b,d, and figure 8b and f. (The fast AChE, where visible, is indicated by "2".)

Bands C and D do not readily hydrolyze α-naphthyl propionate or butyrate; the other bands do react with these substrates (fig. 8,b,c,e).

Bands C and D are resistant to 10^{-4} M eserine sulfate, while bands A and B are only partially inhibited; band E has one portion which is inhibited and this corresponds in general position to the eserine-sensitive zone (band 3) described above as hydrolyzing Buthch (fig. 2e and f; fig. 7b and c). Bands C and D are also resistant to DFP at 10^{-4} M, whereas the other bands are completely inhibited (fig. 8f and g).

Regional variations within the nervous system (central ganglia, interganglionic connectives and peripheral nerves). Circumesophageal connective contains a relatively stronger band B, when compared with brain, and lacks band D (fig. 7d and e). Peripheral nerves exhibit a strong band C, in comparison with brain and connective (fig. 7d through h). The same figures demonstrate that band E is consistently narrower in peripheral nerves than in central nervous system; this is presumably correlated with the absence in PN of the component hydrolyzing Buthch (see above). Leg nerve has a weaker band E than either antennular or antennal nerves.

Inter-specific comparison. Central ganglia of *P. guttatus* possess a zone comparable to band C of *P. argus* in regard to electrophoretic mobility, preference for α-naphthyl acetate, and resistance to eserine and DFP (fig. 8a–d, h–k). This band is inhibited by 10^{-4} M parahydroxymercuribenzoate (fig. 8j and m).

A group of slowly migrating esterolytic bands occurs in *P. guttatus* (fig. 8a and d), but it is not clear at present how similar these are to bands A and B in *P. argus*. *P. guttatus* also has a rapidly migrating complex of bands hydrolyzing all three naphtholic esters (fig. 8a and d). The electrophoretic mobility of this complex is close but not identical to that of bands D and E of *P. argus;* also, a portion of the complex is eserine-sensitive (fig. 8h,i,q,r), despite the lack of hydrolysis of Buthch in this zone. Further separation and characterization of the elements in this complex have not been completed.

Blood. The fast moving complex of bands in the nervous system of the panulirids is not directly comparable to any zones of esterase activity in blood (fig. 8n through s). There is a band in blood (N,

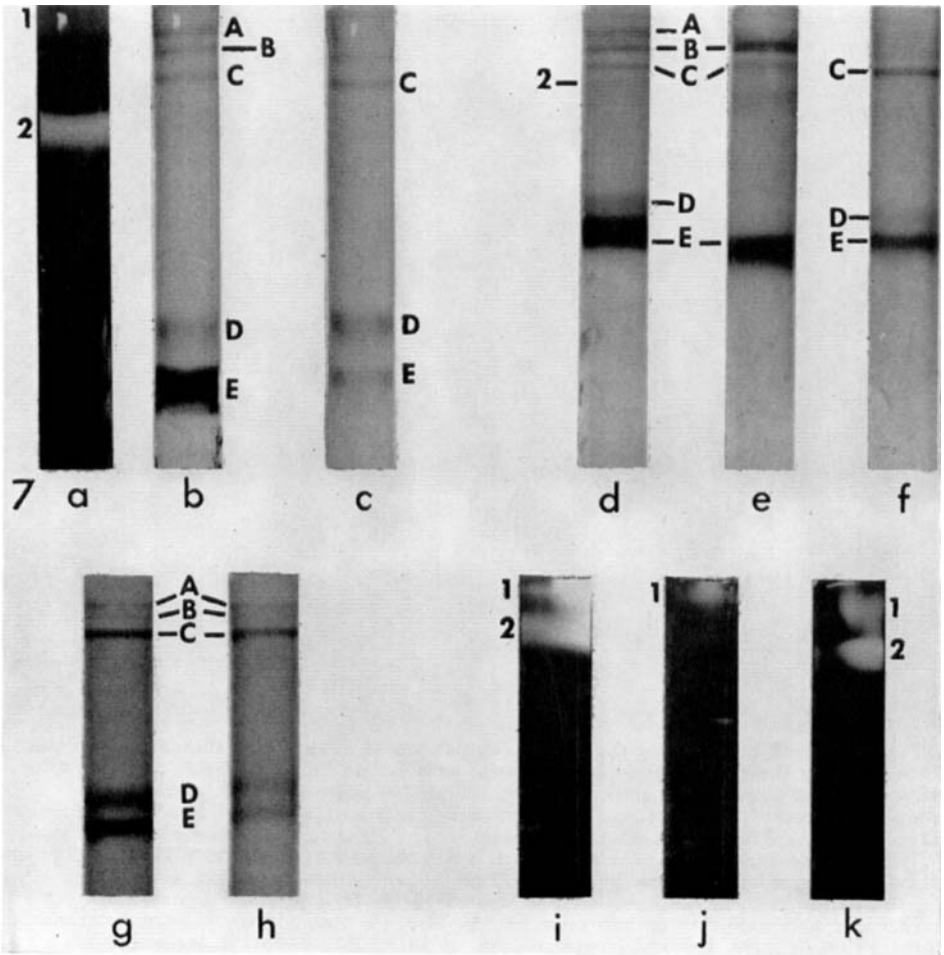

Fig. 7 Acrylamide matrices. *a*, *b*, and *c*, *P. argus* brain and thoracic nerve mass, lower gel initial pH 8.2, with 10% acrylamide. *a*, Acthch, 2 hrs. Compare positions of bands 1 and 2 with those of the esterases (A through E) in *b*, which is a matched gel run in the same experiment as *a*, and incubated one-half hour in α-naphthyl acetate. *c*, same as *b*, but with 10^{-5} M eserine. *d* through *h*, comparison of central nervous system with peripheral nerves, α-naphthyl acetate, 45 min (*P. argus*). *d*, brain. *e*, circumesophageal connective. *f*, antennal nerve. *g*, antennular nerve. *h*, leg nerve. *i*, *j*, and *k*, avoidance of homogenization by the use of thin tissue slices as sample. Acthch, 1 hr. *i*, *Homarus*, brain slice. *j*, *Homarus*, antennular nerve slice (cross section). *k*, *P. argus*, slice from core of accessory lobe (glomerular neuropil) of brain.

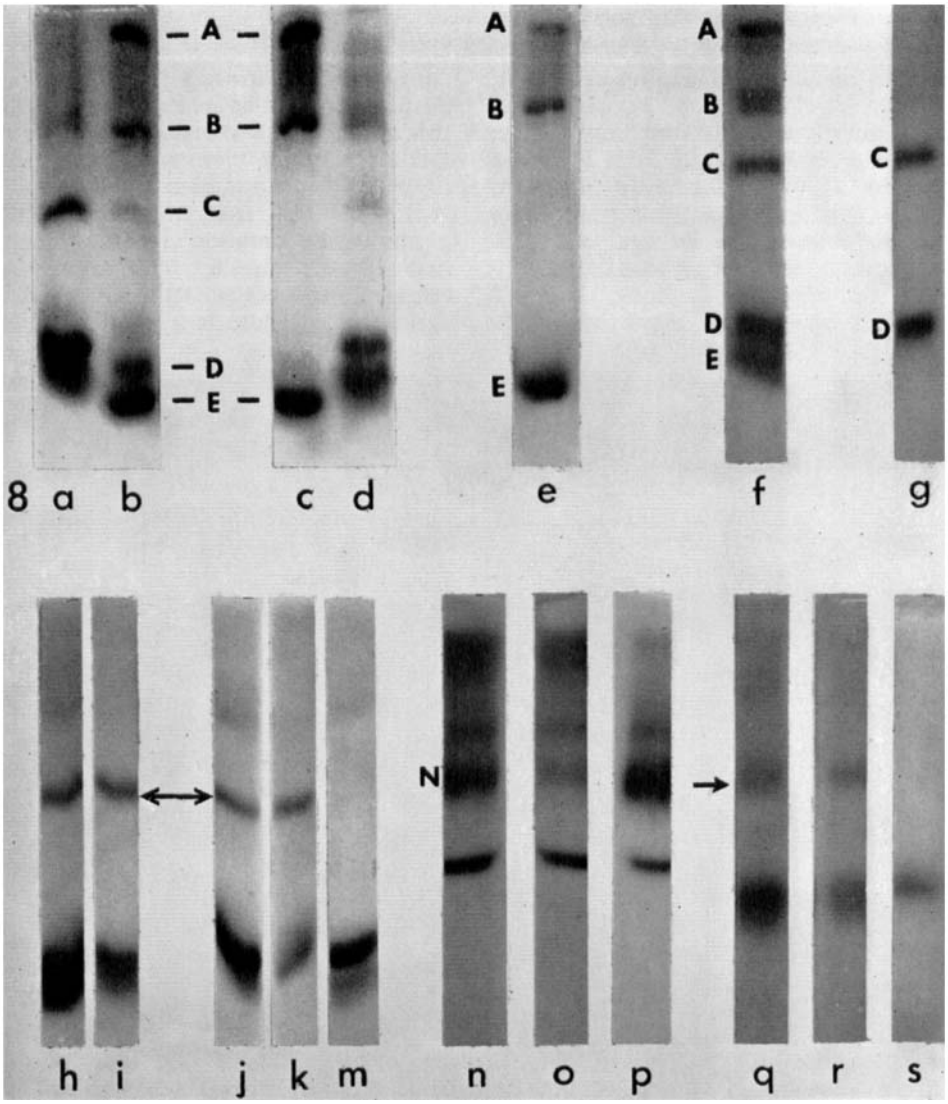

Fig. 8 Starch matrices. *a* through *d*, comparison of esterases in thoracic nerve mass of *P. argus* with those in *P. guttatus*, gel made with borate buffer, pH 7.5, and run 4 hrs at 8 v/cm. *a* and *b*, parallel insertions of two samples in same gel. *a*, *P. guttatus*; *b*, *P. argus*; α-naphthyl acetate, 11 hrs. *c* and *d* represent the other half of the gel in *a* and *b*, incubated 11 hrs in α-naphthyl propionate. *c*, *P. argus*; *d*, *P. guttatus*. *e*, thoracic nerve mass of *P. argus*, same experiment as *a* through *d*, but incubated 11 hours in α-naphthyl butyrate. *f* and *g*, *P. argus* brain, borate buffered gel, pH 7.5, run 4 hrs at 8 v/cm, gel split lengthwise as well as horizontally, and portion *f* incubated 12 hrs in α-naphthyl acetate, and *g* for the same time in α-naphthyl acetate plus 10^{-4} M DFP. *h*, through *m*, *P. guttatus*, brain and thoracic nerve mass, gel and electrophoresis as in *a*. *h*, α-naphthyl acetate, 7 hrs. *i*, the same, but with 10^{-4} M eserine. *j*, α-naphthyl acetate, 10 hrs. *k*, the same, but with 10^{-4} M DFP. *m*, the same, but with 10^{-4} M parahydroxymercuribenzoate. Arrow indicates band comparable to band C of *P. argus* CNS; it is resistant to eserine and DFP, and inhibited by parahydroxymercuribenozoate. *n* through *s*, comparison of esterases in blood and circumesophageal connective of *P. guttatus*; gel made with Tris-citric acid buffer, pH 7.5, and run 4 hrs at 3.8 v/cm. *n*, *o*, and *p*, blood, incubated five and one-half hours in α-naphthyl acetate (*n*), α-naphthyl acetate plus 10^{-4} M eserine (*o*), and α-naphthyl butyrate (*p*) respectively. *n*, *o*, and *p* are from one gel column. "N" indicates a band which hydrolyzes α-naphthyl butyrate strongly and is partly inhibited by 10^{-4} M eserine. *q*, *r*, and *s*, circumesophageal connective, all from the same gel, incubated five and one-half hours in α-naphthyl acetate (*q*), α-naphthyl acetate plus 10^{-4} M eserine (*r*), and α-naphthyl butyrate (*s*), respectively. Arrow indicates band comparable in position to band N of blood, but differing in substrate preference and resistance to eserine.

in fig. 8n) with about the same mobility as the slower DFP-resistant band of CNS (arrow, fig. 8q), but the former hydrolyzes α-naphthyl butyrate at least as well as acetate and is partially inhibited by 10^{-4} M eserine, whereas the latter definitely prefers alpha-naphthyl acetate and is resistant to eserine (fig. 8n through s).

DISCUSSION

The material presented indicates that the nervous systems in three macruran decapod Crustacea (*Panulirus argus, P. guttatus,* and *Homarus americanus*) possess two electrophoretically distinct enzymes which hydrolyze acetylthiocholine. Because of the preference for this substrate over butyrylthiocholine and the preference for the choline esters over the naphtholic esters, as well as the inhibition by 10^{-5} M eserine, these zones have been designated as acetylcholinesterases (AChE's) (Augustinsson, '48, '57). The data further demonstrate a difference between central nervous system (ganglia and interganglionic connectives) and peripheral nerve, the fast AChE being the more active form in CNS and the slow AChE predominating in peripheral nerve.

Experiments using intact tissue slices or frozen thawed (or aged) homogenates as samples suggest that the presence of two electrophoretically separable AChE's is not the result of a particular treatment during homogenization. Within the framework of the variations employed here, neither of these enzyme forms is converted into the other. No concerted attempt was made to determine whether the observed AChE's may be polymers of some smaller unit. In the case of electric organ in *Electrophorus*, a very large polymer of AChE has been described (Lawler, '61, '63), but electrophoretic data on the purified polymer *vs.* the smaller molecule were not presented. No intermediate polymers were observed in sedimentation studies, and the polymer apparently broke down readily when electric organ was frozen prior to homogenization (Lawler, '63).

Multiple forms of acetylcholinesterase have been reported in mammalian brain. Three electrophoretically separable zones of eserine-sensitive activity against acetylthiocholine were identified in starch gel matrices (Bernsohn et al., '62; Barron et al., '63). Studies in my own laboratory on the chick embryo, using acrylamide gel electrophoresis, have demonstrated three acetylcholinesterases in embryonic brain and skeletal muscle, as well as in adult chicken brain (Maynard, '64b). As in the Crustacea, the acetylcholinesterases of vertebrates are among the more slowly migrating esterases present in the tissue extracts.

Certain inferences may be made concerning the degree of binding within the tissue of some of the enzymes studied here. For example, band 3 in *P. argus* central nervous system, a rapidly migrating enzyme which hydrolyzes butyrylthiocholine, must be much less tightly bound to tissue components than either of the two AChE's. This conclusion is appropriate since the enzyme migrates adequately into starch gel without the use of Triton X-100 in the homogenate, and since band 3 occurs at full strength in acrylamide preparations of the supernatant from gently macerated tissues, where the AChE's are still largely in the sediment of centrifuged samples. The preferential effect of Triton X-100 on release of the slow AChE implies that this enzyme is more firmly bound than the fast AChE, both in CNS and in peripheral nerve. A similar, but more complete, distinction between an enzyme form released by physical means and one requiring a detergent has been described in the case of electrophoretically separable acid phosphatases (Allen and Gockerman, '64).

The evidence from electrophoresis of mixed homogenates of peripheral nerve and CNS, and from electrophoresis of adjacent samples in starch gel, as well as from studies of substrate and inhibitor characteristics, indicates that the fast and slow AChE's of CNS and PN are identical enzymes. The mixing experiments and the studies combining native with heat-inactivated homogenates suggest that the observed difference in predominance of the fast or the slow AChE, in CNS *vs.* PN, is a real distinction. These experiments offer no evidence for the presence, in brain, of a hypothetical enzyme which might be "producing" the fast AChE by breaking off some terminal portions of the slow AChE. Likewise, the studies do

not support the possibility of some non-enzymatic factor occurring in peripheral nerve and combining with the fast AChE to "produce" the slow form.

One of the most important questions arising from the present study concerns the cellular localization of the two AChE's. It is pertinent to inquire whether both may occur within a single cell, or whether they represent the synthetic activities of two types of cells. The latter possibility is perhaps more likely, since microscopic examination of tissue sections reveals eserine-sensitive hydrolysis of acetylthiocholine in neural as well as in glial elements of these lobsters' brains and nerves (Maynard and Maynard, '64). The problem is complicated, however, by the fact that, for a wide variety of reasons, differential inhibitor sensitivities or substrate preferences observed for enzymes in electrophoretic preparations cannot always be applied directly to microscopic sections, where conditions of substrate and inhibitor penetration, and binding phenomena, to name but two factors, may be widely divergent from those in gels. The question of correlation between histochemical localization and electrophoretic data will be considered in detail in a subsequent paper (Maynard and Maynard, '64). One conclusion from the present work may be noted: the possibility that the fast AChE is associated primarily with synaptic regions can be discarded since this form is the predominant one in interganglionic connectives where synapses are lacking.

Manometric studies of crustacean neural tissues have disclosed cholinesterase activity comparable to that of "specific" or acetylcholinesterase in vertebrate nervous systems. Nachmansohn and Rothenburg ('45) found that cholinesterase activity in the abdominal ganglia of *Homarus* resembled that of cat caudate nucleus and rat brain in several significant (Augustinsson, '48) respects. No other substrate was hydrolyzed at a higher rate than acetylcholine, butyrylcholine and methyl butyrate were hydrolyzed only minimally, and benzoylcholine was not hydrolyzed. The enzyme was inhibited by high concentrations of acetylcholine. Walop and Boot ('50) reported similar results in the ganglia of a crab (*Carcinus maenas*), and reported 50% inhibition of the ganglionic cholinesterase with 10^{-6} M physostigmine. The present data from electrophoretic studies are compatible with the results of these manometric measurements, in that the slow and fast AChE's appear similar, in substrate preference and inhibitor sensitivity, to the cholinesterase described in the quantitative experiments. Precise kinetic studies of the electrophoretically separable enzymes would be pertinent.

No activity comparable to the vertebrate "pseudo"- or "butyryl"-cholinesterase has been described in crustacean neural tissues. The one species (*P. argus*) from the present work which does have an enzyme hydrolyzing butyrylthiocholine in preference to acetylthiocholine, has not been examined in manometric studies of cholinesterase activity.

The cholinesterase activity of crustacean blood has been reported to be extremely low (2-5 mg acetylcholine hydrolyzed/gm tissue/hour, as opposed to 200 mg/gm tissue/hour for nerve cord, in *Homarus gammarus*, Marnay and Nachmansohn, '37) or absent (*Carcinus maenas*, Walop and Boot, '50). This is in general agreement with the negative findings in the present study, when acetylthiocholine was used as substrate for electrophoretic preparations of blood from the panulirids. The crustacean heart, on the other hand, does have small amounts of cholinesterase, according to several authors (*Libinia* and *Pagurus*, Smith and Glick, '39; *Carcinus*, Walop and Boot, '50), and very minimal amounts according to Florey ('63, *Pacifastacus leciusculus*). Where this activity has been compared with that of central ganglia, the latter have from 5 to 28 times the cholinesterase activity of the heart (Walop and Boot, '50). It is impossible to say whether the failure to observe acetylthiocholine hydrolysis after electrophoresis of heart muscle is due to inadequate sensitivity of the method or to a species difference.

Esterase band C of *P. argus* and its counterpart in *P. guttatus* have properties similar to those of "aromatic" or A-type esterases (Aldridge, '53; Pearse, '60), that is, preference for acetate over butyrate as substrate, resistance to DFP and eserine, and inhibition by parahydroxymercuriben-

zoate. The other esterases in *P. argus* have not been studied sufficiently to characterize them other than to say that bands A, B, and E are DFP-sensitive and band D is resistant.

Both the rapidly migrating esterase complex, band E, of *P. argus* central nervous system, and the similar zone in *P. guttatus* CNS possess an eserine-sensitive component. This component hydrolyzes Buthch in addition to the naphtholic esters, in *P. argus*, but does not hydrolyze the thiocholine compound in *P. guttatus*. This failure is not due to any methodological factor since the fast AChE (band 2) in this tissue does show minimal hydrolysis of Buthch in those gels (fig. 1h) where no band 3 is present. Absence of hydrolysis of Buthch by a fast component in *P. guttatus* CNS is observed in starch as well as in acrylamide. Thus, there is a distinct species difference between the two panulirids, with regard to the fast esterase complex and Buthch as substrate. When comparing the entire series of electrophoretically separable esterases in these two species, it is apparent that some enzymes, like the acetylcholinesterases and the A-type esterase, may be very similar in the two animals whereas others, like the fast-migrating esterase complex, have more divergent properties.

SUMMARY

The esterases in the nervous systems of three species of lobsters (*Panulirus argus, Panulirus guttatus, Homarus americanus*) have been studied after electrophoretic separation in acrylamide or starch gel matrices, using naphtholic esters with an azo dye, or the Koelle thiocholine method. There are two anodally migrating zones of enzymatic activity which have substrate affinities (acetylthiocholine) and inhibitor sensitivities like those of acetylcholinesterases. The more rapidly migrating of these is the predominant form in central ganglia and interganglionic connectives, while the slower enzyme is the principal one active in peripheral nerves. Various experiments are described which suggest that the two forms of acetylcholinesterase are not artifacts of homogenization and that the observed difference in relative activities, between central nervous system and peripheral nerve, is a real one.

A rapidly migrating complex hydrolyzing naphtholic esters is described, in the central nervous system of the panulirids; this complex has an eserine-sensitive component and in *P. argus* it also hydrolyzes butyrylthiocholine, although not acetylthiocholine. An esterase of the A type is also present in the CNS of the panulirids.[4]

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