

# Electrophoretic Studies of Cholinesterases in Brain and Muscle of the Developing Chicken

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**ABSTRACT** Acrylamide gel electrophoresis of aqueous homogenates from whole brain of perfused chick embryos of 18 days' incubation results in three anodally migrating zones having the properties of cholinesterases (ChE's 1, 2 and 3). These sites hydrolyze acetylthiocholine, alpha-naphthyl acetate and alpha-naphthyl propionate. Their activity is inhibited by  $10^{-5}$  M eserine sulfate, by  $10^{-4}$  M di-isopropylfluorophosphate, and by a  $10^{-4}$  M solution of Burroughs-Wellcome compound 284c51j dibromide. Homogenization with 0.1% Triton X-100 selectively increases activity of ChE's 1 and 3, especially the former.

The three ChE zones occur in all sub-regions of brain which were examined: optic tectum, medulla, cerebellum, telencephalon, and optic nerve, although activities in the last area are very low. In homogenates of adult brain, ChE 1 (the most rapidly migrating form) is considerably less active, relative to ChE's 2 and 3, than in embryos of 18 days' incubation.

Aqueous homogenates of heads of embryos at stages 17-18 (3rd day of incubation) show a weak ChE 2. Inclusion of Triton X-100 in the homogenate results in appearance of all three ChE zones in this tissue.

Electrophoresis of blood plasma from embryos of 18 days' incubation produces two zones of activity comparable in mobility and substrate affinity to ChE's 2 and 3 of brain. The relative intensities of the reactions in electrophoretic preparations of the two tissues plus the results from perfused brains indicate that residual blood is not the source for the brain enzymes.

Electrophoresis of homogenates of skeletal muscle from embryos of 18 days' incubation produces three zones of ChE activity similar to those of brain. In adult muscle, the ChE's 2 and 3 are present, although less intense, and ChE 1 is not detectable. Electrophoretic preparations of limbs from embryos of stages 20-22 show the fastest migrating ChE (ChE 1) as the predominant form. All three ChE's are detectable in limbs at stages 26-27.

The results are discussed in relation to literature in the fields of manometric and microscopic studies of cholinesterases in the developing chicken.

Recent reports of multiple, electrophoretically separable forms of cholinesterase in neural tissues of adult animals (Bernsohn et al., '62; Barron et al., '63; Bernsohn and Barron, '64; Maynard, '64) led to the present investigation of these enzymes during ontogeny. It seemed pertinent to examine electrophoretically several tissues in which cholinergic mechanisms are known to function, to establish the characteristics of the various separable cholinesterases, and then to compare the development of these enzyme forms in the different sites. Brain, skeletal muscle, and cardiac muscle of chick embryos incubated 18 days were used; the observed patterns of electrophoretically separable cholinesterases were compared with those in the adult and in embryos ranging from the third to the fourteenth day of incubation.

This work has been presented in abstract form (Maynard, '65).

## MATERIALS AND METHODS

Chicken embryos incubated for 3 to 19 days and chickens 4 to 6 months of age were used. Tissues examined included the following: the whole brain or parts of the brain from adult chickens and from embryos of seven days' incubation and older; the head minus the eyes from embryos incubated five days (3-4 heads per experiment); the whole head from embryos incubated three and four days (6-10 heads per experiment); cardiac muscle, liver, blood plasma, skeletal muscle (*semispinalis capitis*) from embryos of 17 to 19

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days' incubation and from adults; limb buds and limbs from embryos of 3 to 5 days' incubation (Hamburger-Hamilton stages 20 through 27 (Hamburger and Hamilton, '51)—limbs from 6–12 embryos used per experiment). Extra-embryonic membranes were removed and tissues were rinsed briefly in 0.88% NaCl. Blood was drawn from the exposed heart into a heparanized syringe; the cells were removed by centrifugation at 3,000 rpm for ten minutes.

Freshly dissected tissues were homogenized in ice cold distilled water, at concentrations ranging from 1:4 to 1:20 (mg wet tissue per  $\mu$ l water). The homogenates were centrifuged for 15 minutes at 8–15°C, at about 12,000 g, in an International microcapillary centrifuge or a Lourdes Model AB centrifuge. The resulting supernatant was used as sample (see below). At times, indicated below, 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, to insure adequate polymerization of the sample gel. This procedure was not necessary for brain or muscle but was helpful in the cases of liver and blood plasma.

Acrylamide gel electrophoresis was performed according to a modification (Maynard, '64) of the method of Ornstein and Davis ('62). 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 10% (occasionally 7.5%). 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% methylenebisacrylamide. Three hundred microliters of spacer gel were used, and 150  $\mu$ l of sample gel solution. The latter consisted of one part sample (supernatant after centrifugation of homogenate) with two parts spacer gel stock solution (containing 5% acrylamide). Electrophoresis was conducted in the cold (8–15°C), and a current of 2.5 ma per gel tube was maintained until the tracking dye (brom phenol blue) had moved 27 mm

into the lower gel (about one and one-quarter hours).

Following electrophoresis, individual acrylamide gels were placed in test tubes containing 3.5 ml of one of the following reaction mixtures: (1) Modified Koelle thiocholine method (Maynard, '64): (a) Pre-incubation for one-half hour at 0°C in a freshly prepared solution containing  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , glycine,  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ , and maleate — NaOH buffer (pH 6.1) in the proportions used in Koelle's ('51) reaction mixture. (b) Incubation for 1 to 20 hours at room temperature (25°C) in freshly prepared reaction mixture containing the above ingredients plus acetyl- or butyrylthiocholine iodide, all in the proportions used in Koelle's mixture. Following incubation, gels were washed in cold water and were photographed under constant conditions against a black background. This provided optimum visualization of the white reaction product (copper thiocholine sulfate, Malmgren and Sylvén, '55). The procedure used 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. Pre-incubation was a necessary step; its omission resulted in uniformly weaker reactions with the thiocholine substrates. Pre-incubation in the solution described resulted in more crisply defined reaction sites than pre-incubation in distilled water of equivalent temperature. The sharpness of the reaction sites could be improved and the speed of the reaction increased somewhat by renewing the pre-incubation solution twice during the half hour. (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. (b) Incubation for one-half or one hour, at 37°C, in a fresh reaction mixture containing 0.02% alpha-naphthyl acetate or alpha-naphthyl propionate,<sup>2</sup> 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.

<sup>2</sup> Alpha-naphthyl propionate was generously supplied by Dr. Robert L. Hunter.

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-isopropyl-fluorophosphate (DFP, Merck) ( $10^{-6}$  M to  $10^{-3}$  M), BW 284c51j dibromide (1-5 bis (4-allyl dimethylammoniumphenyl) pentan-3-one dibromide, Burroughs-Wellcome Co., Tuckahoe, New York) ( $10^{-6}$  M and  $10^{-5}$  M). When the reversible inhibitors (eserine and the BW compound) were 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 surface is the boundary between lower gel and spacer gel. The photographs are at magnifications of two times, except in figure 2, which is 1:1.

Unless otherwise indicated, the acrylamide gels figured were made with the lower gel at an initial pH of 8.2 and an acrylamide concentration of 10%. Tissues were diluted 1:8 with water at homogenization, unless stated otherwise. Electrophoretic separations were made in duplicate on any given day and were repeated on subsequent days with fresh material. In a case where the small volume of the tissue homogenate precluded duplication on the same day (with the youngest limbs, for example) the experiment was repeated an appropriate number of times. Comparisons among several tissues were always made when those tissues were run in the same experiment.

## RESULTS

### *I. Brain of chicken embryos from 17 through 19 days' incubation*

*A. Whole brain: acetylcholinesterases.* Following electrophoresis in acrylamide gel, aqueous homogenates of whole brain from embryos of 17, 18, or 19 days' incubation show three anodally migrating

zones of activity against acetylthiocholine (Acthch) (sites 1, 2 and 3, in fig. 1a). Placing the homogenate for two minutes in a water bath at 60°C results in no hydrolysis of Acthch after 18 hours' incubation of acrylamide gels in the reaction mixture. The three sites of activity persist when acrylamide concentration, initial pH and ionic strength of the lower gel are varied (fig. 2a, b, c, d).

Hydrolysis of Acthch is completely inhibited by  $10^{-5}$  M eserine sulfate (fig. 1b), by  $10^{-4}$  M di-isopropylfluorophosphate (DFP) (fig. 1c, d, e), and by a  $10^{-4}$  M solution of Burroughs-Wellcome anti-acetylcholinesterase compound 284c51j dibromide (fig. 1f, g, h, i). With the last inhibitor, a slight reaction at site 3 is still visible at an inhibitor concentration of  $10^{-4}$  M. This persistence of site 3 is even more apparent with alpha-naphthyl acetate as substrate (fig. 3j, k). In brain at this age the usual gradation of activities of the three sites with thiocholine substrates as well as with the naphtholic ester is such that site 3 normally appears relatively more active than site 2 and the latter more active than site 1 (as judged by time of initial appearance of bands and by their final densities — see dilution series in fig. 4d-f). Thus, it is impossible, without precise kinetic studies using the various substrates and this reversible inhibitor, to say whether site 3 may be less sensitive to the BW compound than are the other two sites, or whether site 3 may have a greater affinity for the naphtholic ester than sites 2 and 1.

Butyrylthiocholine (Buthch) is hydrolyzed, but to a much lesser extent, at the same sites and with the same relative intensities, as Acthch (fig. 1j, compare with Acthch reaction in fig. 1m) and this reaction is completely inhibited by  $10^{-5}$  M eserine sulfate (fig. 1k).

Incubation of gels with alpha-naphthyl acetate or alpha-naphthyl propionate results in a number of zones of activity, three of which correspond, in electrophoretic mobility and in sensitivity to  $10^{-5}$  M eserine, to the three sites hydrolyzing Acthch (sites 1, 2 and 3, fig. 3a, b, c, e). Inhibition of activity at these sites, by DFP and by the Burroughs-Wellcome compound, is comparable to that seen with acetylthiocholine (fig. 3h, i, j, k).

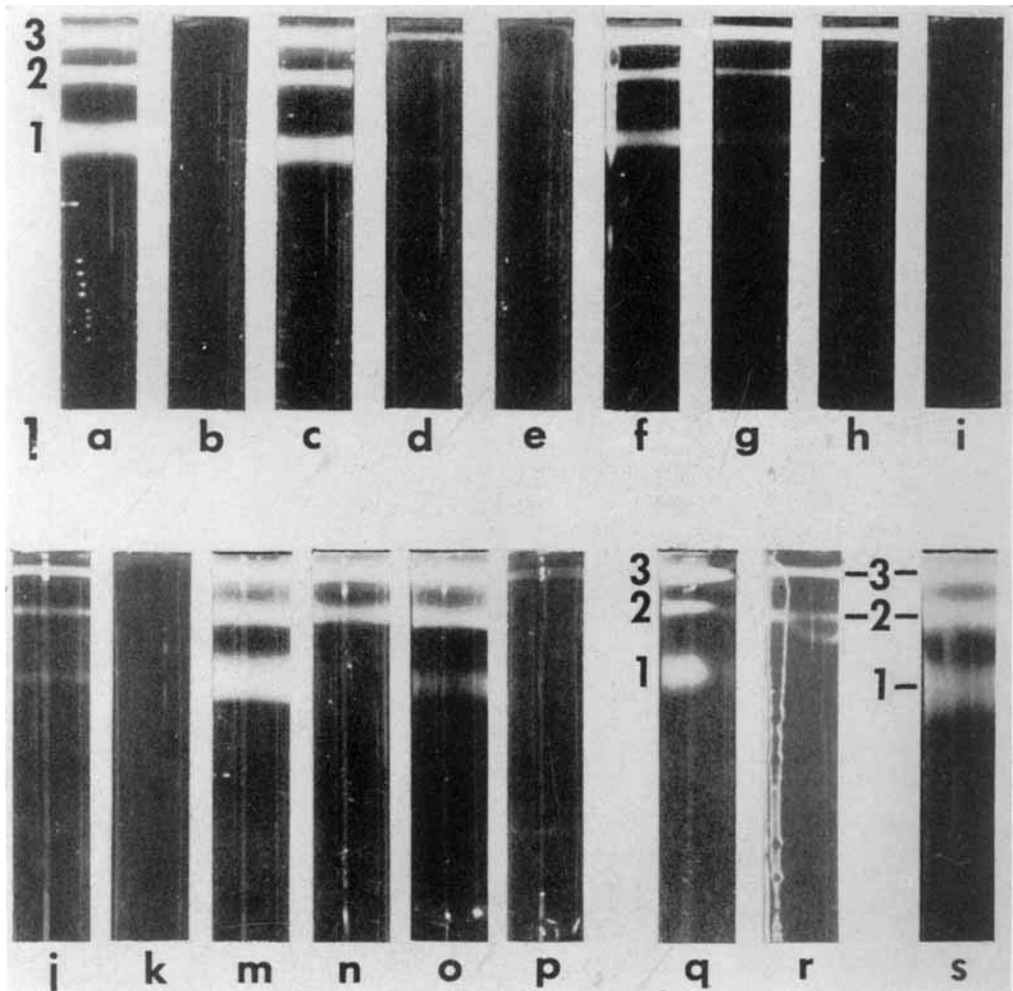


Fig. 1 *a*, brain, 19-day embryo, Acthch one hour. ("Acthch" refers to the acetylthiocholine reaction mixture and the subsequent time is the length of incubation of the gel in this mixture). *b*, the same as *a*, but with  $10^{-5}$  M eserine sulfate. *c*, *d*, *e*, brain, 19-day embryo, Acthch one hour, with increasing concentrations of di-isopropylfluorophosphate (DFP); *c*,  $10^{-6}$  M DFP, *d*,  $10^{-5}$  M DFP, *e*,  $10^{-4}$  M DFP; compare with control preparation from same experiment, in *a*, above. *f*, *g*, *h*, *i*, brain, 17-day embryo, Acthch one hour; *f*, control preparation; *g*, with  $10^{-6}$  M BW 284c51j dibromide; *h*, with  $10^{-5}$  M BW 284c51j dibromide; *i*, with  $10^{-4}$  M BW 284c51j dibromide. *j*, *k*, *m*, brain, 18-day embryo, from a single experiment, for comparison of reactions in the two thiocholine substrates; *j*, Butchh, 20 hours; *k*, Butchh plus  $10^{-5}$  M eserine sulfate, 20 hours; *m*, Acthch one hour. ("Butchh" refers to butyrylthiocholine reaction mixture.) *n*, *o*, *p*, brain, adult chicken, from the same experiment as *j*, *k*, and *m*, for comparison of adult and embryo; *n*, Acthch one hour; *o*, Acthch two hours; *p*, Butchh 20 hours. *q*, electrophoretic preparation from a slice of optic tectum, nine-day chick embryo, Acthch five hours (see text for details). *r* and *s*, optic nerve, 18-day embryo, Acthch one hour; *r*, 1:8 aqueous homogenate; *s*, 1:8 homogenate in 0.1% Triton X-100.

The three zones hydrolyzing thiocholine substrates are considered cholinesterases, because of their affinity for choline esters and their inhibition by  $10^{-5}$  M eserine sulfate (Augustinsson, '48, '57). They are

also inhibited by two anticholinesterase agents, DFP and the Burroughs-Wellcome compound, at concentrations which are ineffective against the eserine-resistant complex of esterases (complex A) described

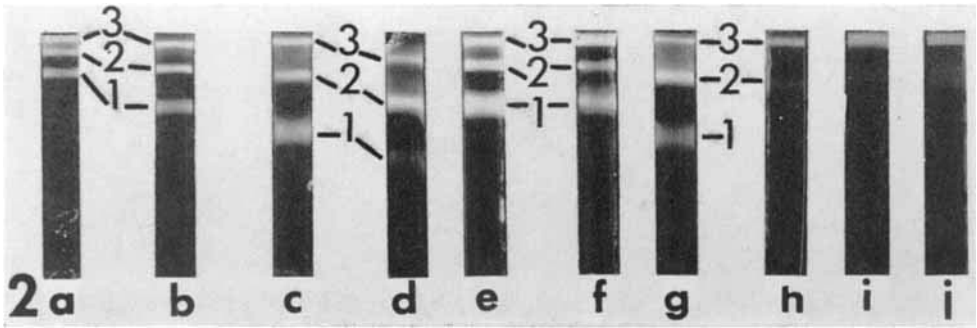


Fig. 2 *a, b, c, d*, brain, 18-day embryo, variations in pH and ionic strength of lower gel; *a*, lower gel 10% acrylamide, initial pH 8.9, incubation in Acthch two hours; *b*, 10% acrylamide, initial pH 8.2, Acthch one hour; *c*, 10% acrylamide, initial pH 7.1, Acthch one hour; *d*, 7.5% acrylamide, initial pH 8.2, Acthch two hours. *e*, brain from 18-day embryo in which the vascular system had been perfused with saline, 1:3 homogenate diluted with an equal volume of water, Acthch one hour; *f*, same as *e*, except the brain homogenate was diluted with an equal volume of blood plasma. *g*, brain, 18-day embryo, 1:8 aqueous homogenate, Acthch one hour, lower gel 10% acrylamide, initial pH 7.1 for comparison with *h*, plasma (1:1 aqueous dilution) of same animal, Acthch two hours, same lower gel as *g*. *i* and *j*, liver, 19-day embryo, for comparison of the two thiocholine substrates; *i*, Acthch 18 hours; *j*, Buthch 18 hours; one slowly migrating zone, comparable in mobility to ChE 3 of brain, but the band in liver appears equally active against Acthch and Buthch.

below. Further, the preference of these cholinesterases for acetylthiocholine over butyrylthiocholine suggests that the enzymes are more similar to acetylcholinesterases than to "non-specific" or butyrylcholinesterases. The electrophoretically separable cholinesterases will hereafter be referred to as ChE 3 (the most slowly migrating form), ChE 2 (the intermediate zone), and ChE 1 (the most rapidly migrating form).

**B. Whole brain: effects on ChE's of variations in tissue processing prior to electrophoresis.** (1) Avoidance of homogenization. The three ChE's are demonstrable in acrylamide gels when homogenization is avoided, through use of a thin, fresh tissue slice as sample instead of the homogenate. The tissue slice, in this case from optic tectum, is of an area approximately equal to a cross section of the acrylamide gel; it is placed on a small piece of filter paper above the polymerized spacer gel, and 150  $\mu$ l of additional spacer gel are pipetted over it. The positions of the three zones of ChE activity, and their relative intensities, are maintained in this preparation (fig. 1q), suggesting that the three forms are not artifacts of the homogenization procedure.

(2) Effect of dialysis. Dialysis of a brain homogenate for two hours at 6°C

against a Tris-glycine buffer (5 mM Tris, 38 mM glycine, pH 8.3) results in selective diminution of the activity of ChE 1 after electrophoresis in acrylamide gel (fig. 4a, b, c). The other zones of ChE activity are not diminished, and may in fact be increased, when compared with the results from a non-dialyzed control homogenate kept at the same temperature for two hours. It is unlikely that this effect of dialysis on ChE 1 represents a differential sensitivity of this zone to a simple loss of magnesium ions since the presence of such ions in the standard Acthch reaction mixture would presumably obscure such an effect. Also, omission of magnesium chloride from the pre-incubation and reaction mixtures has no selective effect but rather results in slower and weaker reactions at all three ChE sites in preparations from non-dialyzed homogenates. It remains possible that zone 1 fails to rebind or utilize magnesium ions after dialysis or that some other ion is involved.

(3) Triton X-100. Homogenization of brain with 0.1% Triton X-100 instead of water results in a preferential increase in activity of ChE 1 and possibly also of ChE 3; activity of ChE 2 is not increased (fig. 4d through i). Use of a higher concentration of Triton X-100 in the homogenate (1%) results in very minimal increase of

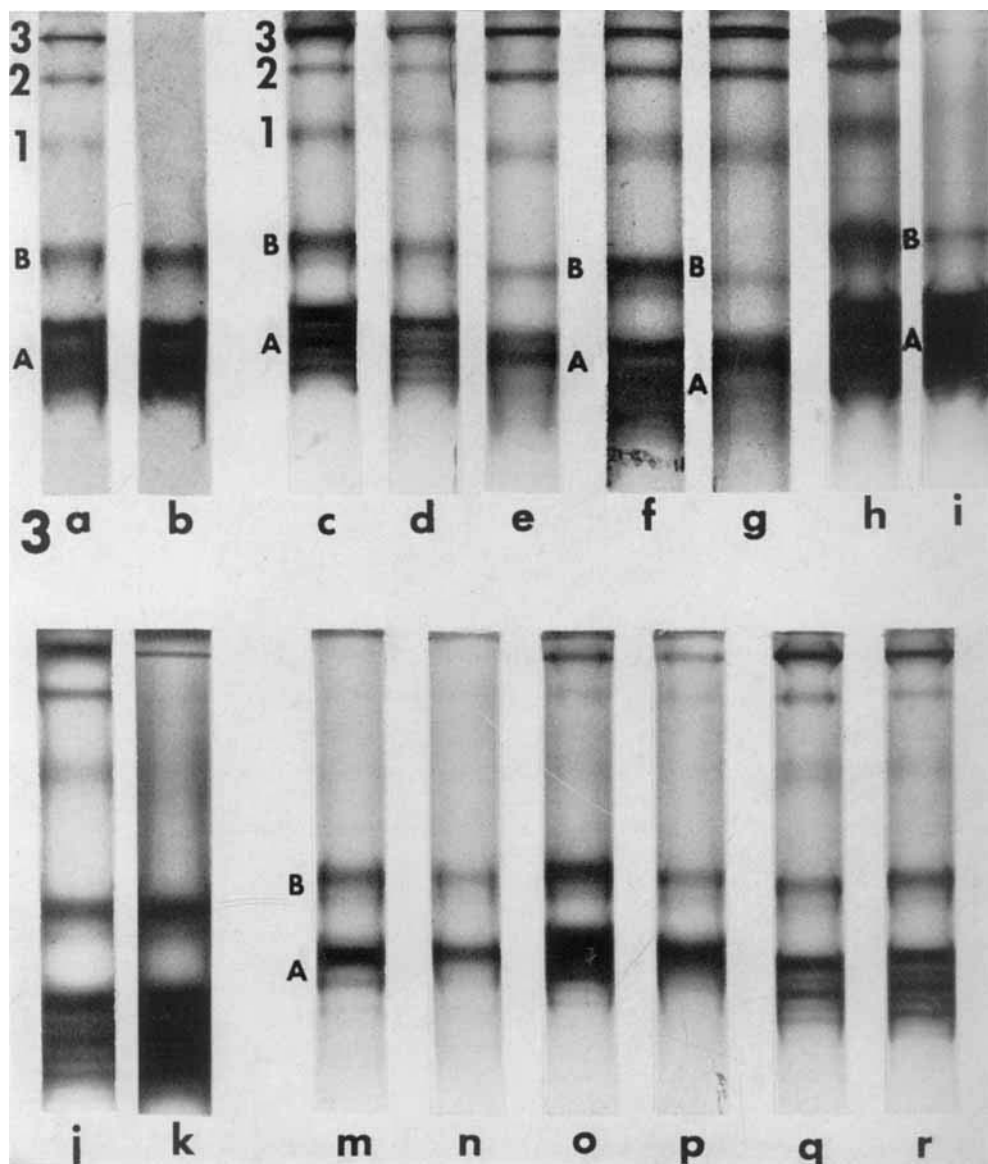


Fig. 3 *a* and *b*, brain, 14-day embryo, alpha-naphthyl acetate, one-half hour; *b* with  $10^{-5}$  M eserine sulfate. *c* and *d*, brain, 17-day embryo, alpha-naphthyl acetate one-half hour; *c*, a 1:9 homogenate, and *d* a 1:19 homogenate. *e*, brain, 17-day embryo, alpha-naphthyl propionate, one-half hour, for comparison with *c*. *f* and *g*, optic tectum, 18-day embryo; *f*, alpha-naphthyl acetate, and *g*, alpha-naphthyl propionate, both one-half hour. *h* and *i*, brain, 18-day embryo, alpha-naphthyl acetate one hour; *h*, control, and *i* with  $10^{-4}$  M DFP. *j*, and *k*, brain 17-day embryo, alpha-naphthyl acetate one hour; *j*, the control and *k* with  $10^{-4}$  M BW 284c51j dibromide. *m*, whole heads, 1:4 homogenate, from chick embryos on third day of incubation, alpha-naphthyl acetate, one-half hour. *n*, same as *m*, but a 1:9 homogenate. *o* and *p*, whole heads (minus the eyes) of embryos incubated seven days, alpha-naphthyl acetate one-half hour; *o* a 1:4 homogenate and *p* a 1:9 homogenate. *q*, brain, ten-day embryo, 1:9 homogenate, alpha-naphthyl acetate one-half hour. *r*, brain, 14-day embryo, 1:9 homogenate, alpha-naphthyl acetate one-half hour.

activity over that observed with a 0.1% solution. Increasing the detergent to 10% results in decreased activity of all three ChE's after electrophoresis. The inclusion of 0.1% Triton X-100 in the reaction mixture used to incubate gels from aqueous homogenates is without effect or else appears to be slightly inhibitory to all three sites. The data from these studies using Triton X-100 indicate that in the brain of 18-day embryos the three ChE's vary in the degree of binding within the tissues.

(4) n-butanol. Homogenization of brain with 0.5% or 1.0% n-butanol results in a slight increase of activity at all three ChE sites.

(5) Effect of vascular perfusion. Perfusion of chick embryos through the left ventricle of the heart with 0.88% NaCl prior to electrophoresis of brain homogenates does not diminish activity at any of the three cholinesterase zones, either with Acthch or with the naphtholic esters.

C. *Whole brain: variation in incubation of gels.* Pre-incubation for one-half hour in 10% neutral buffered formalin diminishes uniformly the reaction of the three ChE's and also decreases the activity of the eserine-resistant esterases (see below).

D. *Whole brain: eserine-resistant esterases.* Using alpha-naphthyl acetate, the eserine-resistant sites of activity which migrate further towards the anode than the eserine-sensitive enzymes appear under conditions of optimal resolution (fig. 3c and d) as a single moderately rapidly migrating zone (Zone B, in fig. 3a through d, f) and a faster moving complex containing five narrow zones (complex A in fig. 3c, d, f, i).

Zone B hydrolyzes alpha-naphthyl acetate in preference to alpha-naphthyl propionate (fig. 3c and e, f and g). Its activity is extensively inhibited by  $10^{-4}$  M DFP (fig. 3h and i), and is unaffected by a  $10^{-4}$  M solution of the Burroughs-Wellcome compound (fig. 3j and k). This enzyme may thus be comparable to one of the eserine-resistant, DFP-sensitive esterases described in manometric studies of chicken brain by Poulsen and Aldridge ('64).

The two most slowly migrating zones within complex A react with alpha-naphthyl propionate to a much greater extent

than do the other zones (fig. 3, compare c and e, f and g). The activity of complex A is not affected by any of the three inhibitors used (fig. 3a and b, h and i, j and k).

E. *Cholinesterases in sub-regions of brain.* The following regions were examined using acetylthiocholine substrate in embryos of 17 to 19 days' incubation: optic tectum, medulla, cerebellum, telen-cephalon (cerebrum and corpus striatum), optic nerve. With the exception of optic nerve, all three ChE zones appear readily after electrophoresis of 1:8 aqueous homogenates of these tissues (fig. 5u through x). There are suggestions of consistent differences in relative intensity of zones from one brain region to another, but these have not yet been reliably confirmed. In 1:8 aqueous homogenates of optic nerve, ChE 3 and ChE 2 are seen after one hour's incubation of the gel in Acthch; when 0.1% Triton X-100 is used in the homogenate, all three ChE's are visible (fig. 1r and s).

## II. Brain of adult chickens: *Cholinesterases*

Homogenates of whole, adult chicken brain possess three ChE's comparable to those described above. However, the ChE 1 zone appears less active, relative to ChE's 3 and 2, in homogenates of whole brain from hens than in the 18-day embryo (fig. 1j, m, n, o, p). This decreased activity of ChE 1 in adult brain is indicated by experiments in which homogenates were progressively diluted before electrophoresis. In a typical experiment using the normal 1:8 homogenate, preparations from brain of 18-day embryos required incubation in the Acthch reaction mixture for 10, 20 and 30 minutes before the first appearance of reaction product at zones 3, 2 and 1 respectively. For comparable homogenates of adult brain, in the same experiment, the times required were 20, 30 and 120 minutes respectively. When a sample of embryonic brain was diluted (1:18) such that the incubation times required for appearance of zones 3 and 2 were equal to those for a 1:8 homogenate of adult brain, zone 1 was visible much earlier than in the adult, that is, after 60 rather than 120 minutes in reaction mixture.

### III. Brain in embryos younger than 17 days' incubation

A. *Cholinesterases*. Electrophoretic preparations from aqueous (1:4) homogenates of whole heads (amnion removed) from embryos of Hamburger - Hamilton stages 17-18 (29-36 somites, during the third day of incubation) show a faint ChE 2 after 24 hours in the reaction mixture (fig. 4j). When the homogenate (1:4) is made with 0.1% Triton X-100, ChE's 3 and 2 are faintly visible after 18 hours in the reaction mixture. Using 1.0% Triton X-100 in a 1:4 homogenate, all three ChE's are present after six hours in the substrate and ChE 2 is the most reactive (fig. 4k).

Aqueous homogenates (1:4) of heads from animals one day older, that is, taken on the fourth day of incubation (Hamburger-Hamilton stages 20-22), show increased reactions of the ChE's (fig. 4m), with ChE 2 predominant. Figure 4n demonstrates the inhibition of these enzymes by  $10^{-5}$  M eserine sulfate. The band marked "X" is a weakly reactive eserine-resistant zone which was not further characterized in this study. It occurs also in liver and skeletal muscle. A similar zone has been observed in human brain (Barron et al., '63; Bernsohn and Barron, '64) and was identified there as a copper-binding protein.

At five and one-half days' incubation, in 1:4 aqueous homogenates of heads, the three ChE zones are readily seen after only one and one-half hours in substrate (fig. 4o); the predominance of zone 2, observed at earlier stages, is still apparent although less obvious.

Figures 5, i, j, k, and m, taken from a single experiment, show the appearance of the ChE zones in homogenates of whole brain from chicks at intervals from six and one-half days' incubation through the period just prior to hatching. At six and one-half days, ChE 3 is the most active zone and ChE 2 the least active (fig. 5i and j). The apparent decrease in relative activity of ChE 2, from five and one-half to six and one-half days' incubation (fig. 4o and fig. 5i) is observed regardless of whether whole heads or brains are used as specimens at the older age. Repeated observa-

tions of preparations from whole brains of animals incubated six and one-half to seven days show clearly that ChE 2 is the least active zone at this stage, as judged by visual examination of bands and by the times required in substrate before first appearance of reaction product at the three sites.

In brains of chicks incubated 10 to 11 days, ChE 2 appears increased in its relative activity (fig. 5k), and at 14 days' incubation the relative activities of the three ChE zones appear similar to those in the 18-day embryo (zone 3 more active than zone 2, and the latter more active than zone 1). During the time period from 7 to 19 days' incubation all the ChE zones are greatly increasing their activity in the acrylamide gels (fig. 5i through m).

B. *Other esterases*. In a series of electrophoretic preparations from heads or

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Fig. 4 *a, b, c*, brain, 18-day embryo, Acthch one hour; *a*, homogenate dialyzed two hours at 6°C (see text); *b*, homogenate stored for two hours at 6°C without dialysis; *c*, tissue freshly removed, no storage. Note diminution of zone 1 in *a*. *d, e, f*, brain, 18-day embryo, Acthch one and one-half hours, serial dilutions of aqueous homogenates; *d*, the usual 1:8 homogenate; *e*, 1:18 homogenate; *f*, 1:27 homogenate. *g, h, and i*, same experiment as *d, e, and f*, brain, 18-day embryo, Acthch one and one-half hours, serial dilutions of homogenates in 0.1% Triton X-100; *g*, 1:8 homogenate; *h*, 1:18 homogenate; *i*, 1:27 homogenate. Note zones 1 and 3 are much stronger in *i* than in *f, j*, heads, embryos on the third day of incubation (stages 17-18), 1:4 aqueous homogenate, Acthch 24 hours; *k*, same tissue as in *j*, but a 1:4 homogenate in 1.0% Triton X-100, Acthch six hours. *m*, heads, embryos on the fourth day of incubation (stages 20-22), 1:4 aqueous homogenate, Acthch three hours. Note density of zone 2 in *k* and *m*. *n*, same as *m*, but with  $10^{-5}$  M eserine sulfate. *o*, heads, embryos incubated five and one-half days (stages 26-27), 1:4 aqueous homogenate, Acthch one and one-half hours. Zone 2 is predominant. *p*, limbs, embryos on the fourth day of incubation (stages 20-22), 1:4 aqueous homogenate, Acthch seven hours. Zone 1 is predominant. Compare with preparations from the heads of the same animals, same experiment, in *m* above, and in *q*. "X" is an eserine-resistant zone similar to that shown in heads above (*m* and *n*). *q*, heads from same embryos as in *p*, same treatment, Acthch seven hours, for comparison with *p* and *m*. *r, s, and t*, limbs from embryos incubated five and one-half days (stages 26-27), 1:4 aqueous homogenates; *r*, Acthch one and one-half hours, for comparison with heads from same animals in *o* above; *s*, Acthch two hours; *t*, same as *s*, but with  $10^{-5}$  M eserine sulfate.



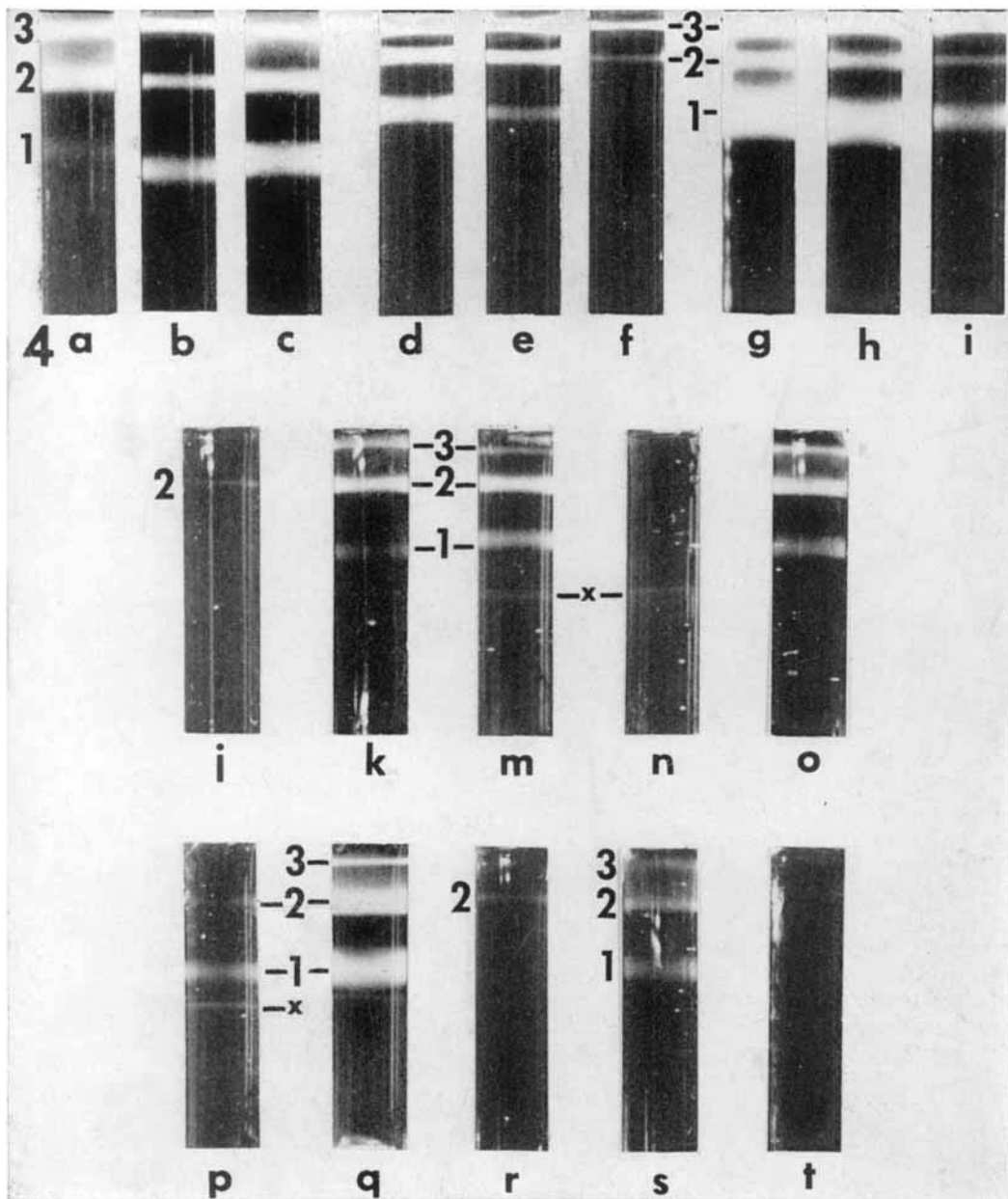


Figure 4

brains of embryos from 3 through 17 days' incubation, there is a progressive increase, with age, in the reactivity of all the eserine-resistant zones, without any qualitative changes being apparent (fig. 3m through r, c). In the youngest embryos (fig. 3m,

n), the number of components visualized in zone A is fewer than that seen in older animals, but the missing elements are the weakest ones and the method may not be sensitive enough to detect them. In agreement with these observations, Bernsohn

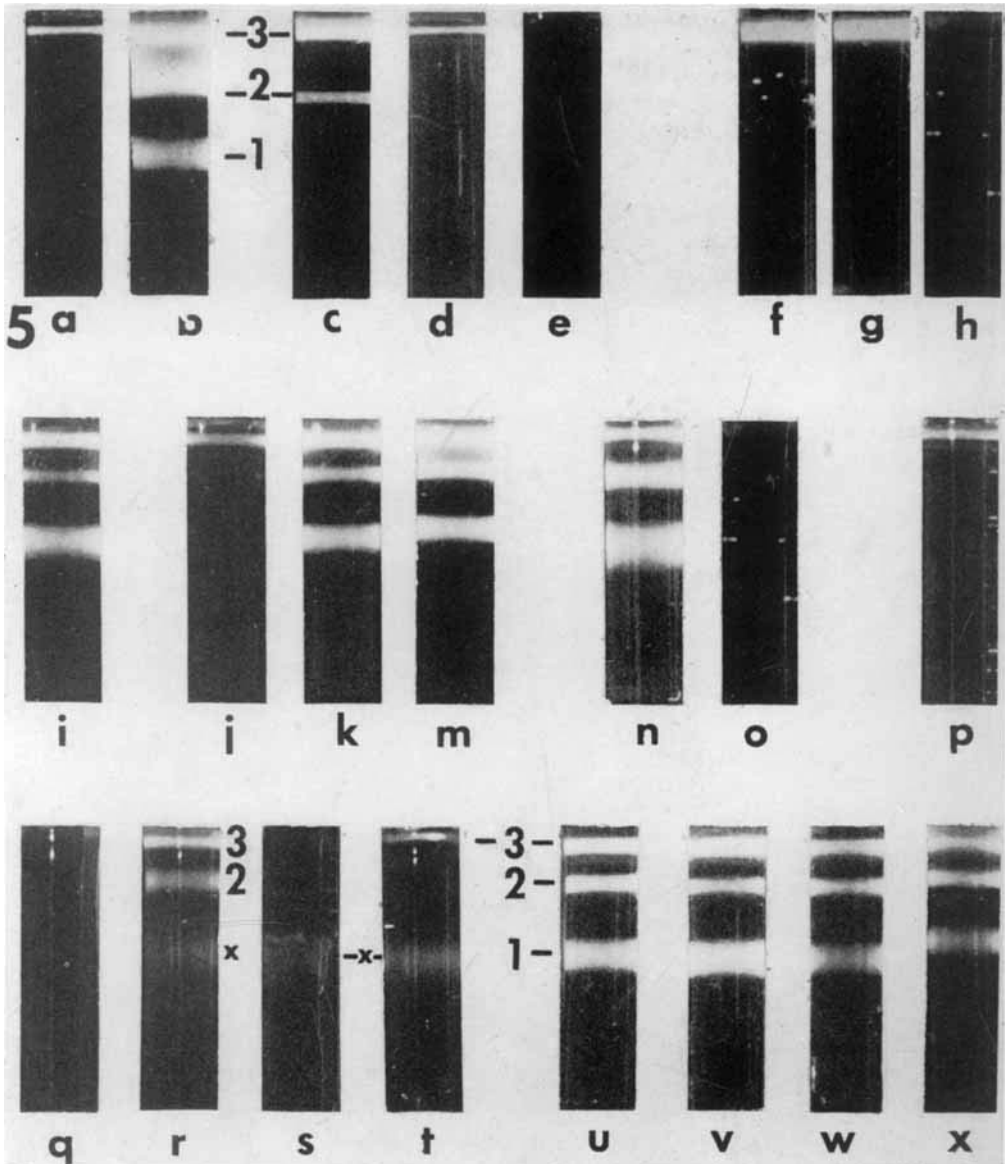


Fig. 5 *a* through *e*, comparison of brain (1:4 aqueous homogenate) and blood plasma (1:4 aqueous dilution) from 18-day embryos; *a*, plasma, Acthch one hour; *b*, brain, Acthch one hour; *c*, plasma, Acthch 18 hours; *d*, plasma, Buthch 18 hours, compare with *c*; *e*, plasma, Acthch 18 hours, with  $10^{-5}$  M eserine sulfate. *f*, *g*, and *h*, blood plasma from adult chicken, 1:4 aqueous dilution; *f*, Acthch two hours; *g*, Buthch two hours. Compare with brain in figure 1, *o* and *p* for differences in substrate affinities; *h*, Acthch two hours, with  $10^{-5}$  M eserine sulfate. *i* through *m*, brain from embryos of varying incubation times, Acthch two hours; *i*, six and one-half days' incubation, 1:4 aqueous homogenate; *j*, same age, 1:9 homogenate; *k*, 11 days' incubation, 1:9 homogenate; *m*, 18 days' incubation, 1:9 homogenate. *n* and *o*, skeletal muscle, 19-day embryo, 1:4 aqueous homogenate; *n*, Acthch one hour (compare with equivalent preparation of brain in *b* above); *o*, Acthch one hour with  $10^{-5}$  M eserine sulfate. *p*, cardiac muscle, 17-day embryo, 1:4 aqueous homogenate, Acthch 16 hours, compare with skeletal muscle in *n*. *q* through *t*, skeletal muscle from adult chicken, 1:4 aqueous homogenate; *q*, Acthch one hour; *r*, Acthch 20 hours; *s*, Acthch 20 hours, with  $10^{-5}$  M eserine sulfate; *t*, Buthch 20 hours. *u* through *x*, sub-regions of brain from 19-day embryos, Acthch one hour; *u*, optic tectum; *v*, telencephalon (cerebral hemisphere and corpus striatum); *w*, cerebellum; *x*, medulla.

and Barron ('64), in starch gel electrophoretic studies of esterases in chick embryo brains, noted that the zones hydrolyzing  $\alpha$ -naphthyl acetate did not change qualitatively from 9 to 20 days' incubation or to the hatched chick.

#### IV. Blood plasma and liver

These were studied primarily for comparison with brain and muscle. In embryos incubated 17, 18, or 19 days, electrophoretic preparations of blood plasma show two zones of activity against Acthch, comparable in mobility to ChE's 3 and 2 of brain, but relatively much less reactive (fig. 5a, b, c). The plasma enzymes are similar to those of brain in their weak hydrolysis of Butchch (compared with Acthch, fig. 5d and c), in their activity against the naphtholic esters, and in their sensitivity to eserine (fig. 5e) and to DFP. In mixed samples consisting of equal parts of plasma and homogenate from perfused brain there is no evidence of bands in addition to the three ChE's seen in brain. The enzymes of plasma and of brain appear to migrate together (fig. 2e, f). Likewise, when the composition of the lower gel is varied, such that electrophoretic mobilities are altered, the two enzymes of plasma continue to migrate for distances comparable to the ChE's 3 and 2 of brain (fig. 2g, h).

Studies of plasma from adult chickens show an eserine-sensitive enzyme apparently comparable in electrophoretic mobility to the more slowly migrating of the two forms described in embryonic plasma (fig. 5f and h). The adult form, however, hydrolyzes Butchch just as readily as Acthch, in contrast to the hydrolysis in embryonic plasma and in brain at both ages (fig. 5f and g). No data are available concerning the presence of a second, more rapidly migrating cholinesterase in adult blood.

An eserine-sensitive enzyme similar to that of adult plasma is found in liver homogenates of embryos incubated 17-19 days (fig. 2i and j). These animals were perfused with saline prior to tissue removal. When the speeds of reactions in gels from liver homogenates were carefully compared with those from equivalent dilutions (1:8) of brain, the liver ChE zone

was first visible in Acthch and Butchch substrates after 55 and 45 minutes' incubation, respectively, whereas in the same experiment, the ChE 3 of brain was first seen at 15 minutes' incubation in Acthch, but not until after five hours in Butchch. The cholinesterases of adult plasma and embryonic liver thus resemble butyrylcholinesterase rather than acetylcholinesterase.

#### V. Muscle

A. *Skeletal muscle.* Electrophoretic preparations from aqueous homogenates (1:4) of skeletal muscle (*semispinalis capitis*) of embryos incubated 17, 18, or 19 days show three zones of enzymatic activity comparable in mobility, substrate affinity, and eserine-sensitivity to the three ChE's of brain (fig. 5n and o). These enzymes are undiminished in activity when muscle from saline-perfused animals is used.

In adult chickens 1:4 aqueous homogenates of skeletal muscle show no activity against Acthch after one hour in substrate (fig. 5q). With an identical reaction time, comparable preparations of embryonic muscle (19-day embryo, fig. 5n) show three very reactive zones. After 20 hours in substrate adult muscle has the two more slowly migrating ChE's (fig. 5r), but the fastest enzyme is not detectable. A weak zone of eserine-resistant activity (see above) against Acthch and Butchch is also present ("X" in fig. 5r, s and t).

B. *Cardiac muscle.* In contrast to skeletal muscle, 1:4 aqueous homogenates of cardiac muscle from embryos incubated 17-19 days show only a very weak zone of eserine-sensitivity against Acthch, at a site comparable to that of the most slowly migrating ChE in skeletal muscle and in brain (fig. 5p). This site does not hydrolyze Butchch, or does so only minimally. When cardiac muscle from saline-perfused chicks is used, there is no hydrolysis of Acthch after 21 hours in substrate, suggesting that the single band described above may be caused by residual blood. However, the use of 0.1% Triton X-100 instead of water in homogenizing a sample of the same perfused cardiac muscle results in appearance of the slow cholinesterase zone. Further work is needed to clarify the nature of this cardiac enzyme.

Manometric data of Sippel ('55) indicate, on the basis of DFP inhibition studies, that heart from chick embryos incubated 16 days contains both specific and non-specific cholinesterases.

Table 1 summarizes the data from embryos of 17 to 19 days' incubation and from adults.

#### VI. Limbs from young embryos

Electrophoresis of aqueous homogenates (1:4) of limbs (amnion removed) from embryos of Hamburger-Hamilton stages 20-22 (three to three and one-half days' incubation) results in two zones of eserine-sensitive activity against AChCh (zones 1 and 2, fig. 4p). These zones appear comparable in mobility to ChE's 1 and 2 of brain and muscle in the 18-day embryo. In contrast to preparations of heads from stages 20-22, ChE 1 is the dominant form in limbs. The overall intensity of the re-

actions is much weaker in limb homogenates than in the heads of the same embryos (compare fig. 4p with fig. 4m and q). At about five and one-half days' incubation (stages 26-27), three ChE's are detectable in limb preparations. ChE 2 appears slightly more active than the others (fig. 4r, s, t; compare with heads from same animals in fig. 4o).

#### DISCUSSION

The experiments reported above describe electrophoretically separable zones of cholinesterase activity in brain and skeletal muscle of chick embryos and adults. Multiple forms of cholinesterase have previously been demonstrated in the nervous systems of other animal species. In human brain, three eserine-sensitive zones of activity against acetylthiocholine were seen following starch gel electrophoresis (Baron et al., '63; Bernsohn et al., '62). The

TABLE 1  
*Electrophoretically separable cholinesterases in several tissues of embryonic and adult chickens*

Specimen	Zones reacting more intensely with acetylthiocholine than with butyrylthiocholine			Zone reacting equally readily with acetyl- and butyrylthiocholine (electrophoretic migration similar to ChE 3)
	ChE 3	ChE 2	ChE 1	
<b>Whole brain</b>				
Embryo, 1:8 aq. hom.	×××	×××	×××	
Adult, 1:8 aq. hom.	×××	×××	××	
<b>Skeletal muscle</b>				
Embryo, 1:4 aq. hom.	×××	×××	×××	
Adult, 1:4 aq. hom.	××	×	—	
<b>Cardiac muscle</b>				
Perfused embryo, 1:4 aq. hom.	—	—	—	
Perfused embryo, 1:4 hom. in 0.1% Triton X-100	×	—	—	
<b>Liver</b>				
Perfused embryo, 1:8 aq. hom.	—	—	—	×××
<b>Blood plasma</b>				
Embryonic, dil. 1:4	×××	×	—	
Adult, dil. 1:4	(—)	(—)	(—)	×××

#### Explanation of table:

×××, Activity detectable in acrylamide gel during the first hour in reaction mixture. The time of first appearance of reaction product is a good index to the visually observed density of a zone, the shorter time intervals corresponding to the more intense bands. The relative time relationships, between various zones and tissues, remain quite constant in carefully performed experiments.

××, Activity detectable after 1-5 hours in reaction mixture.

×, Activity detectable after 5-20 hours in reaction mixture.

—, No activity detectable after 20-24 hours in reaction mixture.

(—), No activity detectable after 2 hours in reaction mixture.

"Embryo" refers to chicks incubated 17, 18, or 19 days.

"ChE 3", the most slowly migrating cholinesterase of brain; "ChE 1", the most rapidly migrating form; and "ChE 2", the form intermediate in position.

"Hom." (1:8 or 1:4) indicates the dilution of the homogenate (mg wet tissue/ $\mu$ l).

nervous system in certain lobsters contains two electrophoretically separable acetylcholinesterases, one of which is the predominant form in the central nervous system, while the other is most active in peripheral nerve (Maynard, '64).

Common to the studies in human and crustacean material, referred to above, and also to the present work in the chick, is the fact that the multiple forms of cholinesterase are all demonstrable in non-synaptic regions of the nervous system (optic nerve in the case of chick), making it impossible for one of the forms to be associated exclusively with synapses. Likewise, it is apparent, from the presence of all three chick cholinesterases in preparations from embryonic skeletal muscle and from optic nerve, that neuron somata cannot represent the sole locus of any one of the enzyme forms.

#### *Types of cholinesterase*

**A. Plasma.** The relationships between cholinesterases demonstrated in brain and those in embryonic and adult plasma are of interest. Electrophoresis of brains from perfused embryos indicates that residual blood in the brain is not the source of the cholinesterases in non-perfused brain homogenates. It remains possible that the cholinesterases demonstrated in embryonic plasma, which resemble those of brain in electrophoretic mobility and substrate affinity, may be derived from the nervous system. Another potential source for the plasma enzymes is the yolk sac, in which cholinesterase has been demonstrated manometrically (Ammon and Schütte, '35). The present studies, although taking care to remove all the extra-embryonic material from the specimens, to avoid possible contamination, did not examine yolk sac or amnion electrophoretically. Attempts to make electrophoretic preparations from yolk have so far been unsuccessful.

The cholinesterase present in electrophoretic preparations of adult plasma, like that of embryonic liver, resembles ChE 3 of brain in mobility, but differs from the brain enzyme in its affinity for butyrylthiocholine. In contrast to brain, the reactions of the adult plasma enzyme appear equally intense in the acetyl- and the

butyrylthiocholine substrates. This plasma enzyme resembles the single, "intermediate" type cholinesterase found in manometric studies of adult fowl plasma by Blaber and Cuthbert ('62).

**B. Brain and skeletal muscle.** The three cholinesterases demonstrated electrophoretically in chicken brain and skeletal muscle resemble acetylcholinesterases in their preference for acetyl- over butyrylthiocholine. This is in agreement with the manometric studies of Blaber and Cuthbert ('62), who showed that adult chicken brain and skeletal muscle, in contrast to plasma, contained a typical acetylcholinesterase with only minimal hydrolysis of butyrylcholine.

In addition to the acetylcholinesterase Blaber and Cuthbert suggested, on the basis of inhibitor studies, that chicken brain and muscle contain a relatively small proportion of a second type of cholinesterase, possibly similar to the "intermediate" type cholinesterase (a propionylcholinesterase) which they found in adult plasma. The present acrylamide gel studies of chick brain have not revealed any zone having typical propionyl- or butyrylcholinesterase activity although such enzymes have been demonstrated electrophoretically in other neural tissues (Barron et al., '63; Bernsohn and Barron, '64; Maynard, '64). Failure to observe this type of cholinesterase in the present studies may have resulted from any of several factors: (1) the enzyme did not migrate into the acrylamide, or (2) its activity in whole brain is too weak to detect with the methods used here, or (3) its electrophoretic mobility, like that of the adult plasma cholinesterase, is so similar to that of ChE 3 in brain that its existence is masked by the latter, more active zone. Possibly further manipulation of the conditions of electrophoresis or use of a propionylthiocholine substrate might reveal a "non-specific" type of enzyme in brain.

#### *Comparison of electrophoretic and quantitative studies*

**A. Brain.** It is of interest to compare the developmental changes in quantitative measures of brain cholinesterase activity with the electrophoretic preparations of similar material. Nachmansohn ('38), studying whole chicken brains manome-

trically, found that cholinesterase levels per mg tissue were extremely low in the six-day embryo (1.38 mg acetylcholine hydrolyzed/100 mg tissue/60 min.), rose rapidly to about 15 times this value at hatching (20.8 mg acetylcholine hydrolyzed/100 mg tissue/60 min.), and continued to rise slowly during the next week, until at eight days post-hatching the adult level was reached (25–26 mg acetylcholine hydrolyzed/100 mg tissue/60 min.). This progressive increase in cholinesterase activity of whole brain during the embryo's development is mirrored in the present study by the gradual increase in reactivity of all three electrophoretically separable enzymes, up through the 19-day embryo (fig. 5i, j, k, m).

When one compares the electrophoretic studies of adult whole brain with those of the very late embryos, however, the results do not reflect the quantitative measurements quoted above. The general impression from electrophoretic studies in adults is of a decreased cholinesterase activity, especially in zone 1, as compared to the activity in embryos just prior to hatching. The manometric data show some increase in activity. The differences are small enough, and the manometric measurements vary sufficiently so that technical factors in methods of sampling could be responsible for the discrepancy. Another possibility, under investigation at present, is that enzymes of adult brain may be more tightly bound in the tissues, less readily available for electrophoresis than are those of the embryo. Such a progressive increase in the proportion of bound (sedimentable) enzyme has been reported for esterases in developing brain of rat and human (Bernsohn et al., '63; Bernsohn and Barron, '64). Triton X-100 which preferentially releases ChE 1 in chicken embryo brains was not used on adult material in the present study.

**B. Skeletal muscle.** Quantitative measures of cholinesterase activity in skeletal muscle may also be compared with the results from electrophoretic preparations. Manometric studies of embryonic chicken skeletal muscle (Domini, '38; Nachmansohn, '39) indicate moderately low values at the earliest stages examined (2.8 mg acetylcholine hydrolyzed/100 mg hindlimb

muscle/60 min., at 9–10 days' incubation), followed by a rapid rise in enzyme activity until just before hatching (8.2 mg acetylcholine hydrolyzed/100 mg tissue/60 min. at 16 days' incubation). Following this, the cholinesterase levels decrease fairly rapidly during the first two weeks after hatching and the adult value in skeletal muscle is extremely low, less than that in the nine-day embryo (0.5 mg acetylcholine hydrolyzed/100 mg tissue/60 min., in adult hindlimb muscle, Nachmansohn, '39). Nachmansohn attributed this decrease of cholinesterase activity between hatching and adulthood to the preferential growth of the non-junctional parts of the muscle cells relative to the cholinesterase-rich areas of the myoneural junctions. It is noteworthy that the intensities of reaction in the electrophoretically separable cholinesterase zones of adult muscle are much weaker than those of the 18-day embryo (fig. 5n, q, r), thus mirroring the results of the quantitative analyses.

#### *Comparison of electrophoretic and microscopic studies*

**A. Brain.** Several microscopic histochemical studies afford ample evidence for the presence of cholinesterase at very early stages of the chick embryo's development. Zacks ('54), using 6-brom-2n-acetate as substrate in whole mounts of young embryos, found a physostigmine-sensitive enzyme in Hensen's node and the primitive streak of chicks incubated fifteen hours. At 21 hours the surfaces of the developing neural folds contained this enzyme and at the 8-somite stage activity was seen in the prosencephalon and mesencephalon. Bonichon ('58) and Filogamo ('60), using acetylthiocholine, found cholinesterase activity in presumptive neuroblasts within or just leaving the germinal layer in sections of the chick's mesencephalic vesicle, at stages 20 through 24. These last studies are at stages of development roughly comparable to the earliest whole-head preparations examined electrophoretically in the present studies and thus provide independent evidence for the presence of cholinesterase in these tissues.

**B. Muscle.** Microscopic studies localizing cholinesterase in developing skeletal muscle are also pertinent to the present

electrophoretic data. Studies of paravertebral muscles in the chick embryo (Bonichon, '57; Mumenthaler and Engel, '61) demonstrate cholinesterase throughout the sarcoplasm of the earliest myoblasts, at times in development (stage 24, fourth day of incubation) prior to the appearance of silver-stainable nerve fibers between the muscle cells. According to these reports as well as tissue culture studies (Engel, '61), only after neural elements have established contact with individual muscle fibers does one see focal zones of more intense cholinesterase activity in the sarcoplasm, namely at the poles of each myoblast and at the central region of the cell, representing future loci of myotendinous and myoneural junctions respectively. These foci of enzymatic activity increase in reactivity and become more discrete up to the time of hatching, while the general sarcoplasmic cholinesterase gradually decreases.

With respect to chick embryo limbs, however, the published studies of cholinesterase localization do not consider animals younger than eight days' incubation (Mumenthaler and Engel, '61). Our preliminary attempts to localize the enzyme microscopically in limbs comparable to the earliest stages used for electrophoresis (stages 20-22, fourth day of incubation) demonstrate the enzyme throughout the cytoplasm of cells judged by their shape and position to be myoblasts; this represents almost the entire complement of detectable activity in these limbs.

Presumably limbs at this stage are not yet innervated. According to Tello's ('17) silver studies nerve fibers are first seen between myoblasts of chick embryo leg during the sixth day of incubation and the axons make contact with the central zones of individual muscle cells on the seventh day. The electrophoretic preparations of the youngest limbs (fourth day) which in contrast to brain show ChE 1 as the dominant form, may thus represent primarily the cholinesterase pattern of the sarcoplasm of myoblasts. However, direct confirmation of the absence of nerve fibers in our material by silver methods or by electron microscopy has not been completed; nerve trunks are not apparent within these limb buds, in Masson-stained sections. It

is also of interest to note that ChE 1, the predominant electrophoretically separable form in these youngest limbs, was not detectable in adult skeletal muscle. Studies in progress are attempting to relate the developmental changes in the electrophoretically separable ChE's of muscle to the changes in microscopic localization and quantitative measures of the enzyme and to the process of innervation.

*General remarks.* A certain degree of confidence in the relative extent to which the enzymes are free to migrate into the electrophoretic matrix is afforded by the comparisons of electrophoretic results with quantitative biochemical data, as discussed above for embryonic brain at various ages and for skeletal muscle in adult vs. embryo. However, there are a number of findings in the present study which are difficult to interpret without attempting cholinesterase determinations and kinetic studies in whole homogenates, in whole acrylamide gels, and in material eluted from the several ChE zones or collected after preparative electrophoresis. Among these problems are included: (1) the possibility of changes in degree of enzyme-binding during the course of development; (2) the possibility of tissue components peculiar to heads or limbs, for example, in the early embryos, which could preferentially bind certain of the ChE forms and create an apparent difference in pattern; (3) the difficulty of interpreting negative results (such as the apparent absence of ChE 1 from adult muscle), when one is not sure of the sensitivity of the method or of the completeness of migration into the gel; (4) the need for more precise measures of relative activities, substrate affinities, and inhibitor sensitivities of the multiple forms of chicken cholinesterases, comparable to the kinetic studies on human ChE's (Bernsohn and Barron, '64). Certain aspects of the present work are thus suggestive rather than conclusive.

At present the significance of the multiple forms of cholinesterase can only be speculated upon. Possibly the electrophoretically separable forms represent enzyme existing at various sites within the cell, perhaps in different states of polymerization or with the molecules secondarily modified or bound in several ways. In

such speculation it is of interest to recall that neuronal acetylcholinesterase has been demonstrated at multiple intracellular loci. In neuron somata and nerve fibers of sympathetic ganglia, for example, *in vivo* inhibitor studies with irreversible, lipid-soluble and reversible, lipid-insoluble anticholinesterases, followed by histochemical localization, show an "internal," "reserve" form of cholinesterase, which may be associated with endoplasmic reticulum, and an "external," "functional" form, associated with the cell membrane (Koelle and Steiner, '56; Koelle, '57; Fukuda and Koelle, '59). Electron microscopy likewise has demonstrated cholinesterase at various sites within the neuron: in association with membranes of endoplasmic reticulum, in the cell membrane, and in the membranes of small cytoplasmic vesicles (Torack and Barrnett, '62). At the adult neuromuscular junction portions of the synaptic vesicles and the presynaptic membrane as well as larger areas of the post-synaptic membrane and the synaptic clefts, contain cholinesterase (Barrnett, '62). It is conceivable that this multiplicity of intracellular loci is reflected in the several molecular forms of acetylcholinesterase.

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