*ANTIBODIES TO ACETYLCHOLINESTERASE**

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Abstract.—A rabbit antiserum against commercially available *Electrophorus electricus* acetylcholinesterase has been prepared. Five precipitation bands were distinguished by immunoelectrophoresis, but only three of these contained demonstrable enzyme activity. In an *in vitro* system, activity of the commercial enzyme or of highly purified acetylcholinesterase was inhibited by 70–82 per cent after incubation with antiserum. Antibody specificity was demonstrated by the absence of serological cross reactions or enzyme inhibition with bovine erythrocyte acetylcholinesterase or horse serum cholinesterase.

The importance of membrane-associated acetylcholinesterase in electrically responsive nerve and muscle cells is well documented,[1, 2] and several investigators are interested in the conformational changes postulated for membrane macromolecules.[3–7] Purified preparations of acetylcholinesterase from the electric organ of *Electrophorus electricus* are commercially available,[8] and this enzyme has recently been crystallized[9] and separated into subunits.[10] In electron-microscope studies, enzyme activity has been associated with membranes,[11] but previous techniques have not permitted localization of the enzyme protein without hydrolysis of a substrate. In view of these developments and limitations, preparation of specific antibody to acetylcholinesterase seemed desirable.

Materials and Methods.—Antigen: Acetylcholinesterase (acetylcholine acetyl-hydrolase, 3.1.1.7) from the electric organ of *Electrophorus electricus* was purchased from Worthington Biochemical Corp., Freehold, New Jersey. Enzyme preparations purified by ammonium sulfate fractionation (ECH, 100 units/mg; 1 unit hydrolyzes 1 μmole of acetylcholine/min at 25°C) or by chromatography and gel filtration (ECHp, 1100 units/mg) were dissolved in 0.02 *M* $Na_2H_2PO_4$, pH 7.0, and used in parallel experiments.

Immunization: Pairs of male albino rabbits were injected in each hind footpad on day 0, with 250 μg ECHp (rabbits 1 and 2) or ECH (rabbits 3 and 4) emulsified in complete Freund's adjuvant. Thirty-three days later, rabbit 1 received a booster of 100 μg ECHp subcutaneously, and rabbit 2 received the same dose intravenously. Rabbits 3 and 4 received 50 μg ECH subcutaneously on day 32 and 100 μg ECH intravenously on day 33. Rabbits 1 and 2 received 200 μg ECH intravenously 6 months after immunization, and rabbits 3 and 4 were given the same dose of ECH 5 months after immunization. Blood was removed from the marginal ear vein prior to injection, 10 and 12 days after the first booster, and 1 week after the last injection of ECH. Antiserum samples were stored at −20°C and tested separately.

Immunoelectrophoresis and immunodiffusion: Immunoelectrophoresis was performed by the method of Van Orden and Treffers[12] in 0.9% agar (Purified Difco, Detroit, Mich.), prepared in ethylenediamineacetic acid buffer (5.56 ml ethylenediamine plus 4.58 ml acetic acid in 2 liters H_2O, adjusted to pH 8.6 with 1.0 *N* HCl). Immunodiffusion (Ouchterlony) was done in 0.9% agar as above, or in 2.0% agar prepared in 0.15 *M* phosphate-buffered saline, pH 7.6. Goat anti-rabbit globulin was purchased from Microbiological Associates, Bethesda, Md.

Additional cholinesterases: Other cholinesterases tested for serological cross reactivity and enzyme inhibition included bovine erythrocyte acetylcholinesterase (Sigma, lot

#76B-0040-1, 1.74 μM units/mg; 1 μM unit will hydrolyze 1 μmole of acetylcholine/min at 37°C) and horse serum cholinesterase (Sigma, lot #127B-1680, 5.5 μM units/mg). A highly purified solution of *Electrophorus electricus* acetylcholinesterase (prepared by W. Leuzinger) was also tested for inhibition by anti-ECH serum.

Protein stain: Slides overlaid with agar gel were dried at 42°C and stained with acid fuchsin (1 gm in 250 ml MeOH + 50 ml acetic acid + 200 ml H_2O).

Enzyme stain: After precipitation bands had formed for 1 week, slides were washed in 0.85% NaCl for 4 days and in distilled water for another 4 days. Strips of agar were removed from the slides and incubated for 90 min in the following substrate-indicator reagent: 5 mg of 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl-tetrazolium chloride dissolved in 1 ml ethanol plus 19 ml 0.05 *M* barbital buffer, pH 8.2, with 70 μmoles acetylthiolcholine or butyrylthiolcholine added as substrate immediately before use.

Photography: After being stained for protein or enzyme activity, the agar was dried onto a clean microscope slide, which was later used as a negative for contact prints. All slides were exposed together, so the contrast of the white band in the photograph reflects the relative intensity of the stained (red) bands.

Enzyme assay: Enzyme activity at pH 7.5 and 25°C was measured on the "pH-stat" (autotitrator). Aliquots of 2–10 μl enzyme solution were added to 10 ml of 0.5 mM acetylcholine bromide in 0.1 *M* NaCl under an argon atmosphere. The pH was kept constant by the stepwise automatic addition of 0.01 *M* NaOH, and the rates were recorded in arbitrary units as the slope of total NaOH added versus time. In this system, 2 μl of 50 μg/ml ECH yielded a V_{max} of 2.00 arbitrary units with a K_m of 10^{-4} *M*.

Solutions of enzyme were added to equal volumes of undiluted rabbit anti-ECH serum or control serum. After incubation for 15 min at 25°C, an aliquot (2–10 μl) of this solution was added to the substrate. Enzyme activity was also measured by the Ellman technique[13] with similar results, but this method was less specific and required a correction for cholinesterase activity in the rabbit serum.

Results.—All serum samples from immunized rabbits inhibited enzyme activity and formed lines of identity by Ouchterlony diffusion against *both* ECH and ECHp. By serological techniques, the two enzyme preparations (ECH and ECHp) were indistinguishable; that is, there were no detectable differences between anti-ECH and anti-ECHp sera. Serum from unimmunized rabbits or rabbit anti-bovine gamma globulin did not inhibit enzyme activity or precipitate enzyme in agar gel. For the immunoelectrophoretic analysis and enzyme inhibition described in Table 1, 45-day anti-ECH serum from rabbit 4 was used.

Precipitating antibody: As shown in Figure 1, anti-ECH sera were specific for eel acetylcholinesterase and did not cross-react with bovine erythrocyte acetylcholinesterase or horse serum cholinesterase. As a further check for weak cross reactions, agar gels were washed and goat anti-rabbit globulin was added to the original bovine erythrocyte AcCh-esterase and horse serum cholinesterase wells. Washing removes all nonprecipitated rabbit γ-globulin, so the goat anti-rabbit globulin could have formed precipitation lines only with the rabbit anti-ECH serum that cross-reacted with bovine erythrocyte AcCh-esterase or horse serum cholinesterase. No remaining rabbit globulin and therefore no cross reactions were detected (Fig. 1*c* and *d*).

When the eel acetylcholinesterase preparation was separated into components by electrophoresis, developed with anti-ECH serum, and stained for protein, five precipitation bands could be distinguished (Fig. 2*a* and *g*). If these lines of precipitate were stained for enzyme activity (Fig. 2 *b*, *d*, and *g*), only three could be seen, and the intensity of staining did not correspond to the protein-

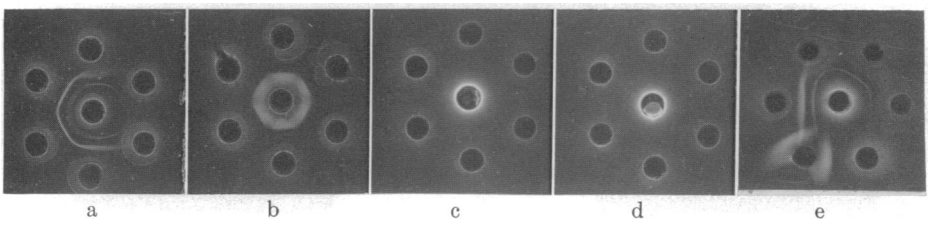

FIG. 1.—Immunochemical specificity of rabbit antibody against eel acetylcholinesterase (ECH).

(*a–d*) *Outside wells:* Various anti-ECH sera from rabbits 1–4 (same relative position in *a–d*). *Center wells:* (*a*) ECH, 10 mg/ml; (*b*) goat anti-rabbit globulin; (*c*) bovine erythrocyte-acetylcholinesterase (agar plate was incubated at 4°C for 1 week, and washed, then goat anti-rabbit globulin was added to the center well; see text for rationale and details); (*d*) horse serum cholinesterase followed by goat anti-rabbit globulin, as described in (*c*).

(*e*) *Center well:* ECH. *Outside wells:* *bottom left,* goat anti-rabbit globulin; *bottom right,* rabbit 4 preimmunization serum; *left center,* anti-ECH serum from rabbit 4; *other outside wells,* antiserum from rabbit 3 or rabbits 3 and 4 pooled anti-ECH serum after the last injection of the enzyme.

All slides were stained for protein, washed, and photographed in the same manner. Rings around outside wells in *a* and *b* result from peeling agar.

stained bands. In particular, the strongest line after protein staining was the weakest of the three enzyme-stained lines. These lines were only slightly darker when acetylthiolcholine (Fig. 2*b*) as opposed to butyrylthiolcholine (Fig. 2*d*) was the substrate.

After electrophoresis of anti-ECH serum, no precipitation lines could be detected against bovine erythrocyte AcCh-esterase or horse serum cholinesterase (Fig. 2*c*), and the two lines against ECH corresponded to the rabbit γ-globulin component developed with goat anti-rabbit globulin (Fig. 2*e*). Both rabbit anti-ECH precipitation lines contained an enzyme which hydrolyzed acetylthiolcholine (Fig. 2*f*).

Enzyme inhibition: All serum samples from immunized rabbits inhibited ECH activity *in vitro* by 70–82 per cent. This was true even for samples obtained just prior to the second injection of antigen. Rabbits 3 and 4 received a subcutaneous desensitizing dose of 50 μg ECH before the intravenous boosting dose of 100 μg ECH on day 33, because rabbit 2 had exhibited symptoms of anaphylaxis after the intravenous dose of 100 μg ECHp.

Some results for a serum sample removed from rabbit 4 on day 45 are given in Table 1. Inhibition of ECH activity was approximately 77 per cent when enzyme solution (ECH, 100 μg/ml) was incubated with an equal volume of undiluted anti-ECH serum for 15 minutes at 25°C, and there was no further reduction of enzyme activity after incubation for two hours. Neither normal rabbit serum nor rabbit anti-bovine gamma globulin inhibited activity after incubation with enzyme, and no inhibition was seen when anti-ECH serum and enzyme (without preincubation together) were added directly to the substrate solution.

Just as there were no cross reactions between anti-ECH serum and other esterases tested, preincubation with anti-ECH serum had no effect upon the

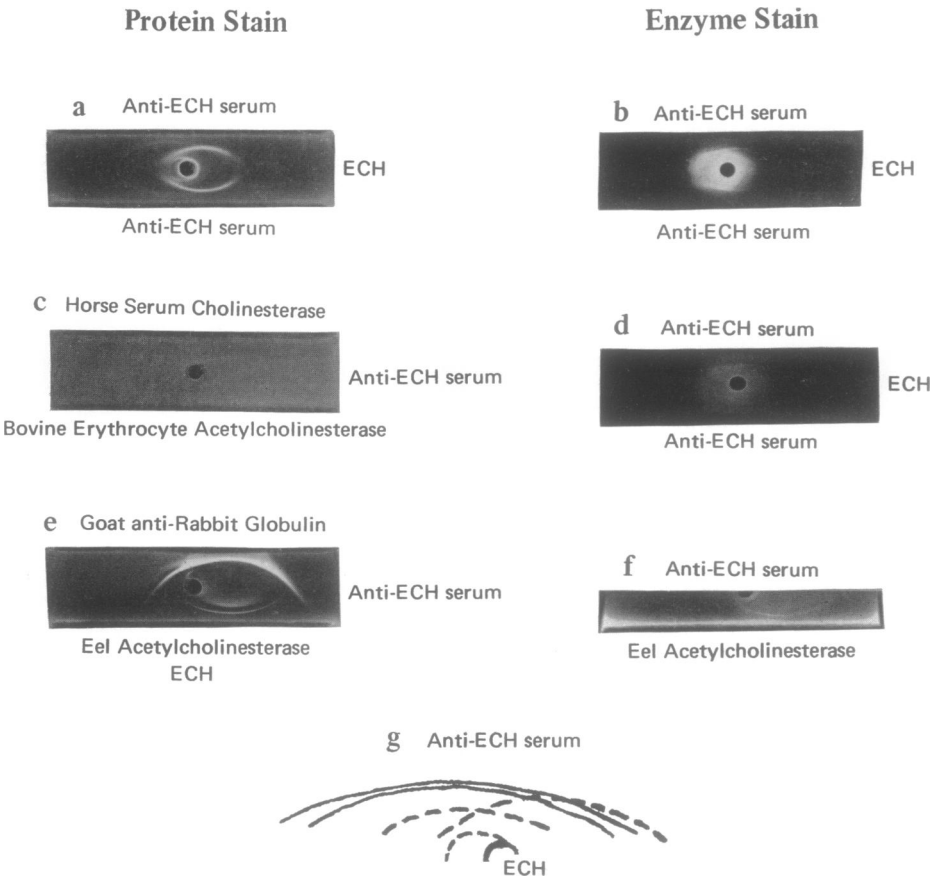

FIG. 2.—Immunoelectrophoretic analysis of eel acetylcholinesterase (ECH) and specific anti-ECH serum. The diagram (*g*) depicts the five ECH-anti-ECH serum precipitation bands which could be distinguished after staining for protein. The dashed lines represent the three of these bands which contained enzyme activity with acetylthiolcholine (*b* and *f*) or butyryl-thiolcholine (*d*) as substrate. In *a*, *b*, *d*, and *g* the electric field was applied to ECH, while in *c*, *e*, and *f* the electric field was applied to anti-ECH serum. The anode is on the right.

activity of either the bovine erythrocyte AcCh-esterase or the horse serum cholinesterase (Table 1).

Discussion.—The population of antibodies formed after immunization with an enzyme antigen may be quite heterogeneous and dynamic.[14] Anti-enzyme antibodies may combine with enzyme with or without inhibiting or enhancing activity, and this competitive population may change with time after immunization. In these experiments, the first anti-ECH serum samples were obtained relatively late (32 days) after immunization, and the inhibition was essentially the same (82%) with antiserum obtained after four injections of the enzyme. It should be emphasized that the inhibitory capacities of these antisera have not been titrated. It may still be possible to achieve 100 per cent enzyme inhibition by antiserum, but with the system described, all undiluted antisera inhibited ECH activity by 70–82 per cent.

TABLE 1. *Inhibition of eel acetylcholinesterase activity by rabbit anti-ECH serum.*

	Rate	Inhibition
ECH (eel acetylcholinesterase, Worthington)	1.77 ± 0.10 (Mean ± SD,* N = 10)	—
ECH + anti-ECH serum (without preincubation)	1.70	†
ECH + anti-ECH serum	0.40 ± 0.06	77.4%
ECH + normal rabbit serum	1.80	0
ECH + rabbit anti-bovine gamma globulin	1.95	0
Highly purified eel acetylcholinesterase (prepared by W. Leuzinger)	2.46	—
Highly purified eel AcCh-esterase + anti-ECH serum	0.60	75.6%
Horse serum cholinesterase	0.76	—
Horse serum cholinesterase + anti-ECH serum	0.76	0
Bovine erythrocyte acetylcholinesterase	0.97	—
Bovine erythrocyte AcCh-esterase + anti-ECH serum	0.91	†

Rabbit-anti-ECH serum or other rabbit sera were incubated with an equal volume of enzyme solution for 15 min at 25°C. To measure normal enzyme activity, samples of enzyme solution were incubated with equal volumes of diluent. After incubation, aliquots (2–10 μl) of the solutions were added to 10 ml of 0.5 mM acetylcholine in 10 ml 0.1 *M* NaCl. Rates, in arbitrary units, represent the slope of the total NaOH added versus time as recorded directly with an autotitrator.

* Standard deviation. † Not significant.

Only three of the five anti-ECH precipitation lines had enzyme activity (Fig. 2*g*). The other lines may represent contaminants, denatured enzyme, or both. The three enzyme-antibody lines representing enzyme activity could reflect different forms of the enzyme or active enzyme bound to an antigenic contaminant.

When highly purified crystallized acetylcholinesterase and isolated α- and β-chains become available, antibodies to these preparations will be valuable tools for investigating the molecular physiology of acetylcholinesterase and excitable membranes. With fluorescent-antibody, ferritin-labeling, or enzyme-labeling[15] techniques plus anti-ECH serum, it should be possible to localize the enzyme without requiring the hydrolysis of substrate.

The "acetylcholinesterase" isolated from the eel electric organ may be a combination of specific enzyme and acetylcholine receptor protein, or the α- and β-chains of the enzyme may act in a coordinated fashion to recognize and to hydrolyze acetylcholine. Changeux recently postulated that the tetramer, acetylcholinesterase, is an allosteric enzyme with a catalytic site on each of two α-chains and an allosteric site on each of two β-chains.[16] Regardless of how the mechanism for reception and hydrolysis of acetylcholine is finally resolved, antibody specific for α-chains alone, β-chains alone, or "acetylcholinesterase" could be valuable in distinguishing between acetylcholinesterase and acetylcholine receptor protein on a cellular and molecular level.

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