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Acetylcholine Receptor Oligomers from Electroplax of *Torpedo* Species†

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ABSTRACT: Sedimentation in sucrose gradients of α-bungarotoxin-labeled crude and pure acetylcholine receptor preparations from *Torpedo californica* showed two major oligomers. The molecular weights, corrected for the bound Triton X-100 by comparing sedimentation in H_2O and in D_2O, were 330 000 for the heavy (H) oligomer and 190 000 for the light (L) oligomer. Lesser peaks were found in preparations of *T. marmorata* and purified preparations of *T. californica* with molecular weights of 500 000 (HH) and 80 000 (LL). These molecular weights are based upon the assumption of globularity, and may require adjustment if the assumption is wrong. The H and L peaks have similar drug sensitivities, but at pH 10 the L peak was stable whereas the H peak dissociated to components sedimenting as LL. Treatments with *p*-chloromercuribenzoate, which blocks acetylcholine binding partially without affecting α-bungarotoxin binding, had no effect upon the pattern of sedimentation. This and other evidence suggested that the heterogeneity of oligomers was unrelated to the heterogeneity of site affinities for acetylcholine and nicotinic drugs.

The great specificity of α-bungarotoxin for acetylcholine receptor (AChR)[1] was demonstrated by the physiological studies of Chang and Lee (1963). Subsequently, radioactive α-bungarotoxin has been shown to react irreversibly with both particulate and soluble receptors from *Torpedo marmorata* (Miledi et al., 1971); preparations solubilized by 1% Triton X-100 showed, on sucrose gradients, one major peak and a smaller, more rapidly sedimenting peak of one-fifth the height of the major peak. Subsequently, Raftery et al. (1972) fractionated similar material from *T. californica* on Sepharose 6B, and then showed that different fractions contained differing amounts of a rapidly and slowly sedimenting peak. The major binding component sedimented at 9.0 S and appeared to predominate at high toxin concentrations; at lower toxin concentrations, a minor component of 13.7 S was present. They pointed to the possibility that the two classes of binding site might correspond to one form reversibly associated with, and another irreversibly bound to, bungarotoxin; and also pointed to a possible parallel with the high and low affinity sites for acetylcholine binding which have been reported (Eldefrawi et al., 1971; O'Brien and Gibson, 1974).

In the present study, we have used various conditions to demonstrate a series of differently sedimenting forms of receptor–α-bungarotoxin complex, and have interpreted them in terms of a series of oligomers. We show that the oligomeric heterogeneity bears no relation to the two kinds of acetylcholine binding site.

Materials and Methods

Sucrose gradients were prepared over the range of 5–20% sucrose in a Ringer solution containing the indicated percentage of Triton X-100, and the following millimolar concentrations of salts: NaCl, 115; KCl, 4.6; $CaCl_2$, 0.65; $MgSO_4$, 1.15; and Na_2HPO_4, 15.7. The gradients were 12 ml in volume, and were made with a Beckman gradient former. The receptor preparation was incubated for 15 min at 0 °C with the indicated radioactive toxin at a ratio ("toxin–receptor ratio") of 1 except as indicated; the ratio is that of the calculated number of moles of toxin to the moles of acetylcholine binding sites in the receptor preparation, measured by equilibrium dialysis against acetylcholine (O'Brien and Gibson, 1974).

The receptor preparations were as follows. "Stored" *Torpedo marmorata* electroplax was collected in Naples, Italy, in 1968 and the heavy membrane fraction was prepared there, lyophilized, and stored at −25 °C (O'Brien et al., 1970). A concentration of 10 mg or 40 mg/ml, as indicated (corresponding to 0.73 or 2.92 g of original electroplax per ml), was prepared in the above Ringer with 1% Triton X-100, and the supernatant after centrifuging at 100 000*g* for 90 min was prepared as previously described (O'Brien and Gibson, 1974).

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[1] The following abbreviations are used: ACh, acetylcholine; AChR, acetylcholine receptor; *M*, molecular weight; pD, −log [D_3O^+]; ClHgBzO, *p*-chloromercuribenzoate.

Fresh *T. marmorata* and *T. ocellata* obtained in Alexandria, Egypt, in Dec 1974 and stored as frozen electroplax for 2 months were also used. The heavy membrane fractions of fresh *T. marmorata* and *T. ocellata* were prepared according to the method of Miledi et al. (1971) and then solubilized in 1% Triton X-100 and Ringer at a concentration of 1.5 g of original tissue per ml of Ringer. The heavy membrane fraction from *Torpedo californica* electroplax was prepared as described for fresh *T. marmorata* from freshly killed fish. The fraction was lyophilized and stored at −25 °C. The lyophilized material was then solubilized and stored at −25 °C. The lyophilized material was then solubilized in Triton–Ringer at a rate of 10 mg of lyophilized material per ml (corresponding to 1.5 g of original tissue per ml) and centrifuged as described above, the supernatant being used.

Purified *T. californica* receptor was prepared precisely as described for *T. marmorata* by Eldefrawi and Eldefrawi (1973) but using preparations of *T. californica* described above. Its Triton content was very small (0.0007%, equal to 0.113 mg of Triton bound per mg of receptor protein (Edelstein et al., 1975)). A volume of 0.3 ml of a preparation containing 0.052 mg of protein per ml in 5 mM phosphate buffer was incubated with 6 μl of toxin (35 nmol per ml) and layered on the gradient as described below. The specific binding activity was 10 nmol of acetylcholine or α-bungarotoxin per mg of protein.

In studies with D_2O gradients, the receptor preparation and the gradient were made up in 99.5% D_2O, containing the above molarities of salts and Triton X-100, but using minimally hydrated salts, i.e. $CaCl_2 \cdot 2H_2O$, and anhydrous $MgSO_4$ and Na_2HPO_4. The pH was adjusted to 7.4 with 40 μl of concentrated HCl. The value for pD is different from the value determined by a glass–calomel electrode and is obtained by adding 0.4 to the pH thus giving a value of pD 7.8 (Appel and Yang, 1965). This solution contained a maximum of 2.4% of H_2O, assuming the hydroxyl protons of sucrose were all exchangeable.

Two kinds of toxin preparations were used. That referred to as [^{125}I]bungarotoxin was prepared by iodinating purified α-bungarotoxin by the method of Eldefrawi and Fertuck (1973); it had a specific activity of 0.7 Ci/nmol. It bound to *Torpedo marmorata* electroplax membranes, and the binding was blocked 76% by 10^{-4} M *d*-tubocurarine and 71% by 10^{-4} M acetylcholine (O'Brien et al., 1974). Any physiologically inactive material would not have been bound and would remain as free toxin in sucrose gradients. The [^{3}H]bungarotoxin was prepared (Thompson, unpublished results) by treating pyridoxal with α-bungarotoxin and reducing the Schiff base with BT_3, in a manner precisely analogous to that used for labeling *Naja* toxin by Cooper and Reich (1972). The unpurified material (specific activities, in two preparations, 0.85 and 1.0 Ci/mmol) contained a reversibly binding polypyridoxylated fraction and three equiactive irreversibly binding monopyridoxylated components. It was purified by cellulose phosphate chromatography, using elution by a sodium phosphate gradient from 0.02 to 0.3 M. The fourth peak eluted consisted of one of the monopyridoxylated components (specific activity 1.2 Ci/mmol), which bound to receptor in the amount of one per acetylcholine binding site, and the binding was blocked by nicotinic drugs. All of the work with pH gradients, and about half of the controls, was performed with the monopyridoxylated toxin; the results were the same with both toxins except that the impure material gave a larger free toxin peak. After incubation with toxin, 0.2 ml of toxin–receptor preparation was layered onto the gradient, which was then centrifuged in an SW-40 head in a Beckman L265B centrifuge at 4 °C for 17 h at 39 000 rpm (190 000*g*). The tubes were removed and 20-drop fractions for Triton-containing or 10-drop fractions for Triton-free preparations were collected in scintillation counting vials, using a Beckman tube-piercing apparatus. Then 10 ml of a toluene scintillation counting solution, containing Biosolv BBS-3 at the rate of 39.5 ml/l. was added. Samples were counted on a Tri-Carb 3375 scintillation counter for 2 min.

In the usual run (using 0.2 ml of 10 mg/ml of crude Triton extract, with a toxin–receptor ratio of 1) the toxin concentration was 1.7 μM during the incubation period. In work with unpurified α-[^{3}H]bungarotoxin, there was always some fraction that did not bind to receptor. With monopyridoxylated receptor, free toxin was greatly reduced.

The effect of pH on the AChR–toxin complex was examined by incubating the receptor and α-bungarotoxin at pH 7.4 followed by centrifugation on sucrose gradients prepared at the desired pH values. Phosphate-buffered Ringer was used for pH 2.0–7.4 and Tris-buffered Ringer (0.01 M Tris, other salts as in phosphate Ringer) was used for pH 7.4–11. The pH of the gradient was not affected by the incubation buffer or the p*I* of the proteins.

Marker proteins were used as follows, with the assumed molecular weights shown parenthetically. Aldolase (Pharmacia) (160 000) and human serum albumin (Sigma) (68 000) were assayed as protein by the method of Lowry et al. (1951). Catalase (Sigma) (232 000) was assayed enzymatically (Bergmeyer, 1965).

The sedimentation coefficients for the acetylcholine receptor were calculated from eq 1, which is derived from eq 2 and 4 of Martin and Ames (1961), where $s^{(1)}_{20,w}$ is the sedimentation coefficient of the AChR and r_1 the fractional distance travelled by the protein from the meniscus; ρ is the density of H_2O at 4 °C and $\rho_{20,w}$ is the density of H_2O at 20 °C. The partial specific volume of the AChR in 1% Triton, $\bar{v}_1$, was determined by the differential centrifugation procedure described below. The corresponding values for aldolase are $s^{(2)}_{20,w}$, r_2, ρ, and $\bar{v}_2$; $s_{20,w}$ for aldolase is 7.9 (Stellwagen and Schachman, 1962). The validity of eq 1 was confirmed by theoretical calculations of the sedimentation of proteins through a 5–20% sucrose gradient using the solution for eq 3 of Martin and Ames (1961). The molecular weight of the receptor–toxin complex was estimated from eq 2 derived from the Svedberg equation and assuming globular proteins, where M_1 and M_2 are the molecular weights of the receptor and aldolase, respectively, and ρ is the density of water at 4 °C.

$$\frac{s^{(1)}_{20,w}}{s^{(2)}_{20,w}} = \frac{r_1(1 - \bar{v}_2\rho)(1 - \bar{v}_1\rho_{20,w})}{r_2(1 - \bar{v}_1\rho)(1 - \bar{v}_2\rho_{20,w})} \quad (1)$$

$$\frac{M_1}{M_2} = \left[\frac{s_1(1 - \bar{v}_2\rho)(\bar{v}_2)^{1/3}}{s_2(1 - \bar{v}_1\rho)(\bar{v}_1)^{1/3}}\right]^{3/2} \quad (2)$$

When Triton X-100 was used, corrections had to be applied for the weight contribution and buoyancy effect of the detergent, which binds to AChR (Meunier et al., 1972a). The molecular weight of the protein–Triton complex (M_c) is related to the molecular weight of the uncomplexed protein (M_p) by

$$M_c = M_p(1 + x) \quad (3)$$

where x is the grams of detergent bound per gram of AChR, and is calculated from the equation:

$$x = \frac{\bar{v}_c - \bar{v}_p}{\bar{v}_T - \bar{v}_c} \quad (4)$$

where $\bar{v}_c$, $\bar{v}_p$, and $\bar{v}_T$ are the partial specific volumes of the

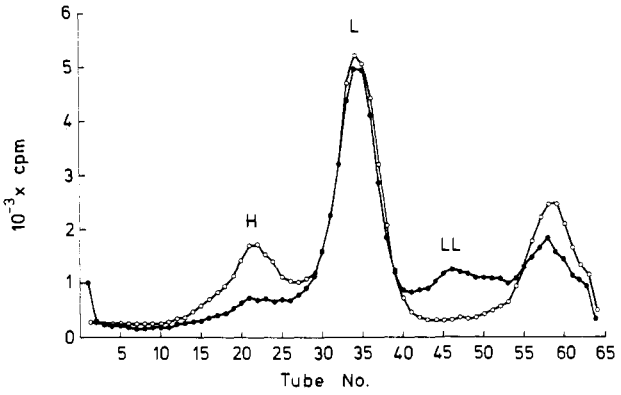

FIGURE 1: Sedimentation of [^{3}H]pyridoxyl toxin (monopyridoxylated) labeled extracts of fresh *T. marmorata* (●) and *T. ocellata* (○). Samples were 0.2 ml of 1.5 g of electroplax per ml of phosphate Ringer. Note the LL peak in *T. marmorata* and lack of LL in *T. ocellata*.

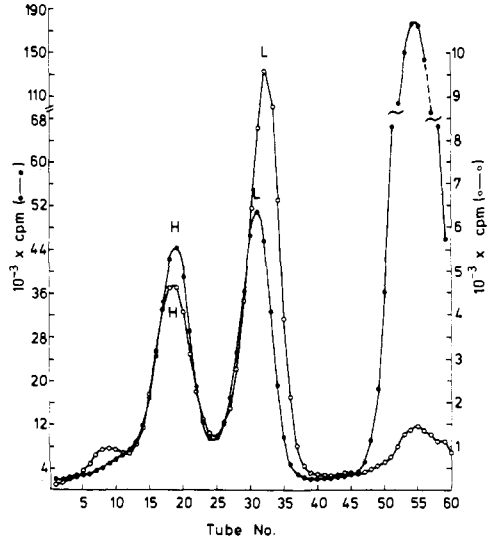

FIGURE 2: Sedimentation of 1% Triton unpurified extracts of *T. californica* electroplax membranes (●) labeled with [^{3}H]pyridoxyl toxin (mixed mono- and polypyridoxylated). Sample was 0.2 ml of 40 mg of lyophilized membrane per ml of phosphate Ringer. Note absence of LL and HH peaks. Sedimentation of highly purified receptor from *T. californica* (○) with added 1% Triton in sample and gradient, labeled with ^{3}H-labeled monopyridoxylated toxin. The sample was 0.2 ml of 0.05 mg of protein per ml of 20 mM phosphate buffer.

complex, the uncomplexed protein, and Triton, respectively. For Triton we used 0.945 for $\bar{v}_T$ at 4 °C, based upon the average of the range (0.937–0.952) provided by the manufacturer, Rohm and Haas. The essential problem was to measure $\bar{v}_c$, in order to estimate x; this we did by comparing sedimentation rates of the AChR peaks and aldolase ($\bar{v}_2 = 0.742$) in water and in D_2O, using a variant of eq 8 of Bon et al. (1973):

$$\bar{v}_c = \frac{k(1 - \bar{v}_2\rho) - a(k - \bar{v}_2\rho')}{\rho'(1 - \bar{v}_2\rho) - \rho a(k - \bar{v}_2\rho')} \quad (5)$$

where k is the ratio of the molecular weights of proteins in H_2O and D_2O, which is relatively constant for all proteins at 1.0155 (Martin et al., 1959; Hvidt and Nielsen, 1966); $\bar{v}_2$ is the partial specific volume of the standard protein (0.742 for aldolase); a is defined by:

$$a = (r_1'/r_1)(r_2/r_2') \quad (6)$$

r_1 and r_1' are the fractional distances travelled by the AChR peak in H_2O and D_2O, respectively; r_2 and r_2' are the corresponding values for aldolase; and ρ and ρ' are the densities of H_2O and D_2O, respectively, at 4 °C.

Results

Molecular Weights of AChR Oligomers. The principal peaks of toxin–receptor complex observed have been heavy (H) and light (L) components, with much smaller amounts of very heavy (HH) and very light (LL) components. The term toxin–receptor ratio will be used to describe the number of moles of α-bungarotoxin added per mole of acetylcholine binding sites measured (in independent studies) in the preparation. (It is not implied that all toxin preparations were fully labelled or fully active; some of the iodinated preparations may not have been. The term is introduced in order to describe the effects of varying the proportions to toxin and receptor.) In runs with a toxin–receptor ratio of 1, performed in 1% Triton at pH 7.4, the results were as follows. In seven runs with crude extracts of heavy membranes of "stored" *T. marmorata* electroplax, a large L peak was found with $s = 8.6$ S ± 0.8 (standard deviation), with a shoulder (H) of $s = 12.5$ S ± 1.3, and a small (LL) peak of $s = 5.1$ S ± 0.7. Virtually identical results were obtained from preparations of *T. marmorata* and *T. ocellata* stored as frozen whole electroplax for 2 months before use (Figure 1). Note, however, the lack of the LL peak in the *T. ocellata*. In 12 runs with crude extracts of heavy membranes from freshly lyophilized *Torpedo californica* electroplax, two large peaks were found, with s values for L of 9.1 S ± 0.8 and for H of 13.1 S ± 1.1, the L peak being larger than the H peak (in its area under the curve) by a factor of 1.3 ± 0.36 (Figure 2). In addition there was a small HH peak which was only clearly seen in three runs, with $s = 17.6$ S ± 0.1. In ten runs with highly purified *Torpedo californica* receptor in 1% Triton, a virtually identical picture was seen: the H peak had $s = 13.2$ S ± 0.4, the L peak had $s = 8.9$ S ± 0.3, and the L peaks were larger than the H by a factor of 1.6 ± 0.68; the small HH peak was seen in six runs and had $s = 16.6$ S ± 6.5 (Figure 2). A plot of r against $s_{20,w}(1 - \bar{v}_1\rho)/(1 - \bar{v}_1\rho_{20,w})$ gives a straight line for the standard proteins and four receptor oligomers (correlation coefficient 0.9965).

All the variances described are for the total set of control runs which extended in the case of the major series, *T. californica* crude, over a period of 1 year. For 24 crude *T. californica* runs (including 12 runs in the pH study) $s_H/s_L = 1.44$ ± 0.04, indicating that the higher errors for the determination of the sedimentation coefficients (and the resultant molecular weights below) were due to systematic errors and not to changes in the relative peak positions.

In experiments in which 1% Triton extracts of fresh *T. californica* receptor–toxin complex were centrifuged on sucrose gradients prepared in D_2O with 1% Triton, the peaks were shifted from $s = 13.0$ ± 0.2 to 7.1 ± 0.2 for H and from $s = 8.9$ ± 0.2 to 4.9 ± 0.2 for L. Applying the values obtained in H_2O gradients (s_1) and D_2O gradients (s_2) to eq 5 we calculated $\bar{v}_c$, the partial specific volume of the receptor–toxin complex in Triton, as 0.77 for H and L. We assumed the other receptor peaks would exhibit the same $\bar{v}_c$. From eq 2 the molecular weights of the receptor–toxin peaks in Triton (with respect to aldolase) were LL = 97 000 (*T. marmorata*); L = 230 000; H = 400 000; and HH = 620 000. Using $\bar{v}_c = 0.77$, $\bar{v}_T = 0.945$, and $\bar{v}_p = 0.73$ (see Materials and Methods) in eq 4 we determined $x = 0.23$ where x is the grams of detergent bound per gram of receptor. Since $M_p = M_c(1 + x)$, where M_c is the molecular weight of the receptor–toxin–detergent complex, the Triton bound to the receptor accounted for 19%

Table I: Comparison of Molecular Weights (in Thousands) of Acetylcholine Receptor Oligomers or Subunits.

This Paper[f]			Edelstein et al. (1975),	Hucho and Changeux (1973),
T. californica Unpurified	Purified	*T. marmorata*	*T. californica* or *T. marmorata*	*E. electricus*
		80		95[b]
				145[b]
190	180	170		190[b]
330	330	300	330[a]	230,[c] 275[d] Meunier et al. (1972a,b)
				360[e]
500	460		660[a]	
			1300[a]	

[a] Sedimentation equilibrium (analytical ultracentrifugation) on *T. californica* in 2% sucrose. [b] Partial suberimidate cross-linking, then sodium dodecyl sulfate electrophoresis. [c] Extensive subimidate cross-linking, then sodium dodecyl sulfate electrophoresis, uncorrected. [d] As *c*, but corrected for "consistent underestimation" associated with sodium dodecyl sulfate electrophoresis. [e] Sucrose gradient centrifugation in Triton, corrected for Triton contribution by the D_2O method. [f] Smaller subunits are excluded (see text).

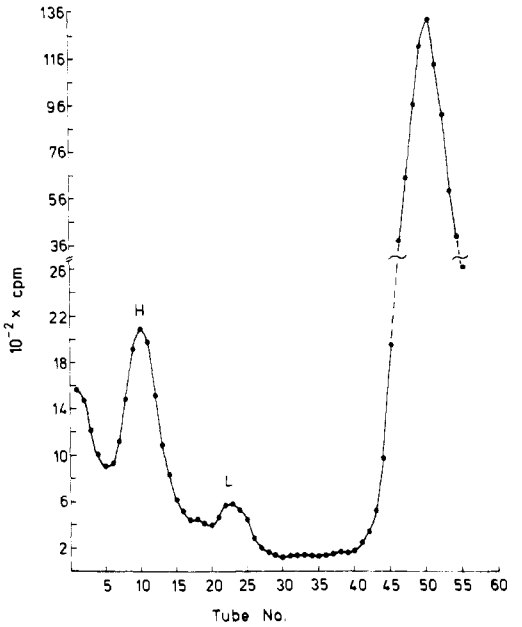

FIGURE 3: Sedimentation of highly purified receptor precisely as in Figure 2, but with no added Triton in sample and 0.01% Triton in the gradient; the toxin used was [^{3}H]pyridoxylate (mixed mono- and polypyridoxylated).

of the effective molecular weight, and the receptor–toxin molecular weights in the absence of Triton can be calculated (Table I).

The above calculations all assume the receptor to be globular. But receptor from *Electrophorus electricus* has been reported (Meunier et al., 1972a) to have an apparent frictional ratio of 1.4 in Triton X-100, corresponding to an apparent axial ratio of about 8 (Schachman, 1959; p 239). This axial ratio seems extraordinarily high in light of the electron micrographs of receptor (Eldefrawi et al., 1976) and it is more likely that the high frictional ratio is due to extensive hydration of the receptor–detergent complex. Preliminary experiments with Sepharose 6B chromatography in this laboratory indicate that H and L, like the AChR from eel, have frictional ratios of about 1.5, and that their corrected molecular weights would be 410 000 and 260 000, as calculated by the method of Siegel and Monty (1966).

Factors Affecting the Relative Contributions and Sedimentation Velocities of the Oligomers. Several studies were made of the effects which varying the toxin–receptor ratio had upon the factor by which the L peak was larger than the H. But with the above *T. mamorata* preparations, and also with crude or highly purified preparations of *T. californica,* the factor was unaffected by varying the toxin–receptor ratio from 0.1 to 10.

We considered the possibility that labeling the receptor with toxin in situ might produce a substantially different sedimentation profile. However, when *T. californica* electroplax which had been stored under liquid nitrogen was incubated with toxin at a toxin–receptor ratio of 10 for 4 h at 0 °C, and the soluble fraction was prepared as usual with Triton–Ringer, the L peak was the larger of the two; the area ratio of L/H was 2.9, averaged from two studies.

We next explored the effects of varying the detergent concentration. These studies could only be readily done with the highly purified *T. californica* preparations in which the detergent concentration was reduced to 0.01% of the original medium, or 0.11 mg of detergent per mg of protein. As the Triton concentration in the gradient was increased, the usual H and L curves were obtained, but the migration of the peaks decreased somewhat; the H peak had average *r* values (averages of duplicate runs) of 0.828, 0.792, 0.774, and 0.692 in 0.01, 0.03, 0.10, and 1.0% Triton, respectively; the L peaks had average *r* values of 0.595, 0.574, 0.520, and 0.462, respectively ($s_{20,w}$ values could not be calculated as $\bar{v}_c$ was not determined for the different Triton concentrations). The H peak was little affected in size by these changes, but the L peak became proportionately greater as the detergent concentration was increased with the L/H ratio being 0.31, 0.89, 1.3, and 1.6 for the respective Triton X-100 concentrations. Furthermore, a very heavy peak (either HH or even heavier) which was conspicuous in very low concentrations of detergent (Figure 3) was reduced in peak area as the detergent concentration was increased; the reduction approximated the increase in the peak area of L.

When 0.01% sodium dodecyl sulfate was used in place of 1% Triton for a crude *T. californica* preparation, the usual H and L peaks were seen, with little change in *s* values (H was 13.3 S and L was 9.2 S) or in peak-area ratio, but a clear LL peak of s = 5.3 S was seen, as previously observed only with *T. marmorata.*

Another question considered was, to what extent were the sedimentation properties those of the receptor–toxin complex, and thus possibly different from those of the uncomplexed receptor? We therefore ran highly purified receptor from *T. californica* in a gradient containing 1% Triton, and subsequently assayed the fractions for ability to bind α-bungarotoxin, using the Millipore membrane procedure of Franklin and Potter (1972). The results were essentially the same as those in Figure 2 with two clear peaks at s = 13.4 and 8.7 S.

Differences in Properties of the Major Oligomers. A variety of treatments was explored to find whether the L and H peaks were derived from components which had different properties, for instance with respect to drug sensitivity, heat stability, or pH.

Heating the crude preparation of *T. californica* in 1% Triton X-100, at 40 °C for 40 min (a procedure which almost eliminates the high-affinity binding of acetylcholine (O'Brien and Gibson, 1974)), had virtually no effect on the L peak, but typically halved the area of the H peak and led to a consider-

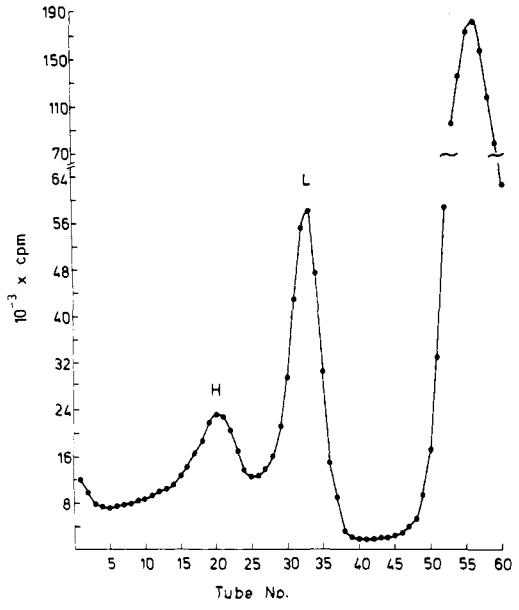

FIGURE 4: Effect of heating (40 °C, 40 min) on sedimentation profiles of *T. californica* electroplax membranes labeled with pyridoxyl toxin. The toxin and sample were identified with those described in Figure 2, and were run on the same day.

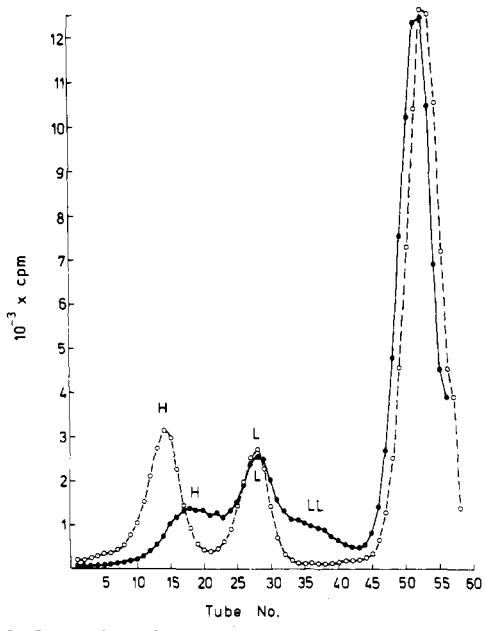

FIGURE 5: Comparison of sedimentation at pH 7.0 (○) and 10.0 (●). The samples were 0.2 ml of 10 mg of lyophilized membrane per ml in Tris buffer (pH 7.4). Gradients were prepared at pH 7.0 and 10.0 and were unaffected by sample buffer. Total area under peaks is the same for both pH values. Note shift of H peak and appearance of LL peak.

able increase in fractions representing higher molecular weights (Figure 4). However, treatment with 0.1 mM *p*-chloromercuribenzoate (which also eliminates high-affinity binding of acetylcholine) was without effect on peaks H and L.

By contrast, when purified AChR was heated to 40 °C for 40 min in the absence of detergent (a procedure which had no effect on the relative amounts of high- and low-affinity ACh binding but resulted in a modest reduction in the total binding) and then run in gradients prepared in 1% Triton X-100, the heat treatment reduced the L peak by an average of 32% compared to controls. The H peak was not affected and the HH peaks increased by the amount lost in the L peak.

Cholinergic drugs at 0.1 mM were preincubated with crude *T. californica* in 1% Triton at room temperature prior to addition of toxin at a toxin-receptor ratio of 1. Gradients were prepared containing 1 mM drug. Carbamylcholine, *d*-tubocurarine, and nicotine all severely reduced the labeling of H and L, and essentially to the same extent. In duplicate runs, the average reductions by the three drugs were, for H, 77, 81, and 76%, and, for L, 68, 70, and 67%. By contrast, the muscarinic agent pilocarpine reduced H by only 25% and L by 21%.

A series of studies was next performed with *T. californica* on the influence of pH upon sedimentation, using sucrose gradients from 5 to 16%. At extreme pH values (2.0 or 11.0) only a free toxin peak was found, indicating that the toxin-receptor complex was fully dissociated. Over a middle range of values (pH 5.5–9.5) the results were substantially as in the standard buffer as described above. But at pH 10 (Figure 5) there was a reduction in the area of the H peak by 11.6 ± 4.0 pmol and in the sedimentation coefficient to s = 12.3 S ± 1.4 (compared to 14.0 S ± 1.3 for controls) and a concurrent appearance of a new peak tentatively identified with the LL peak, s = 6.4 S ± 0.7 and a peak area of 12.2 ± 4.6 pmol, the amount lost by H. These observations suggest that the H peak was dissociating to LL units. By contrast, the L peak was unaffected, suggesting that L did not dissociate. The quantitative evidence for these statements is at the 0.05 level of significance.

At pH 10.5 the H and L peaks disappeared altogether, and a broad band was seen at s = 5.8 S ± 1.2, which may represent an unresolved mixture.

Discussion

The primary finding in our study is that all *T. californica* preparations contain two major oligomers in roughly equal amounts of molecular weight 330 000 (H) and 190 000 (L). These oligomers are also present in preparations of *T. ocellata* and *T. marmorata* although in differing proportions. Two additional species of molecular weight 80 000 (LL) and 500 000 (HH) contribute significantly when preparations are treated by heating, with high pH, or varying concentrations of Triton detergent. The following comparisons with the literature are summarized in Table I. Findings from gel filtration have been omitted, because all receptor preparations contain bound detergent, and the consequent change in partial specific volume frequently causes apparent molecular weight to be far different from the true.

For fresh *T. marmorata*, Miledi et al. (1971) reported that in 1% Triton, the toxin-receptor complex moved essentially as one peak, with a heavier shoulder. Values of s were not given, but the sedimentation profile looks much like our *T. marmorata* in Figure 1. Similarly, with α-bungarotoxin labeled *T. californica* in 1% Triton, Raftery et al. (1972) found a major peak of 9.0 S (probably our L peak) and a minor peak of 13.7 S (probably our H peak). However, their minor peak was only seen "if the membrane extract was labelled submaximally". Our studies indicated no difference in the H/L ratio over a 100-fold range of toxin concentrations.

The work of Meunier et al. (1972a) on the properties of AChR of *Electrophorus* in detergents provides striking comparisons with our results on Torpedo. By differential centrifugation in H_2O and D_2O with sodium cholate as the detergent, they found a single peak only, and determined $\bar{v}_p$ as 0.73, the same value calculated above for purified *Torpedo* AChR from its amino acid composition. Our value of $\bar{v}_c$ of 0.77 for the AChR-Triton complex compares well with their value of 0.78. Although they observed only one peak in 1% Triton at 12.5 S,

the peak position relative to their marker proteins suggests that that peak corresponds to our L peak at $s = 9.1$ S. In an earlier study, Meunier et al. (1972b) reported one peak for *Electrophorus* AChR with $s = 9.5$ S which corresponds well with our L peak. The difference between their two studies appears to be the method of calculating the $s_{20,w}$.

The most relevant results are those of Edelstein et al. (1975) in sedimentation equilibrium studies on purified *T. californica* receptors. They observed, with no added detergent, approximately equal amounts of species with molecular weights of 330 000 and 660 000. At Triton concentrations above 0.1% their 330 000 species was the principal component. It seems plausible that these correspond to our L and H peaks. If so, the estimates of the molecular weights of H and L from our sucrose gradients (190 000 and 330 000) differ substantially from those of Edelstein et al. and somewhat less substantially from our own findings with Sepharose (260 000 and 410 000). These sharp discrepancies are probably related to the fact that we are dealing with a molecule which is highly heterogeneous in that it has a sheath of light detergent which is presumably extensively hydrated. This factor is probably related to the extraordinarily high frictional ratio of about 1.5. Consequently, the different inherent assumptions in the three methods may be the cause of the discrepancies in the three different conclusions. They also calculated, from data on acetylcholine binding per weight of protein, a molecular weight of the minimal acetylcholine-binding protomer as 90 000, fairly close to our value of 80 000 for the complex peak LL.

For *Electrophorus,* Bieseker (1973) used cross-linking studies to find a monomer of 44 000 daltons, giving a single toxin binding oligomer of 260 000 daltons, a value intermediate between our L and H values. Hucho and Changeux (1973) reported from cross-linking studies for *Electrophorus* two fast subunits (45 000 and 54 000) and three oligomers of 190 000, 145 000, and 95 000 daltons. Of these, only the 95 000 and certainly the 190 000 oligomers come fairly close to our values of 80 000 for the complex in LL and 190 000 for L.

We have previously reported what we may call affinity heterogeneity in *Torpedo* receptors: particulate or 1% Triton extracts from fresh *T. californica* display only a high-affinity binding form (form A), but particulates or 1% Triton extracts from "stored" *T. mamorata,* or highly purified receptor from fresh *T. californica,* invariably contain 30–80% of a low-affinity binding form (form B) as well as the A form. Both forms were about equally reactive to α-bungarotoxin (O'Brien and Gibson, 1974, 1975). In view of the fact that aged *T. marmorata* has 80% of B form, and almost all its α-bungarotoxin complex migrates as the L peak, we considered the possibility that the L peak represents the B form, and the H peak represents the A form. The following evidence rules out this possibility.

(a) Fresh *T. californica* contains only the A form, but gives about equal H and L peaks. (b) Heating fresh Triton extracts of *T. californica* for 40 min at 40 °C converts it from 100% A form to 95% B form. But gradient fractionation of these heated extracts shows little change in peak L, and only partial disappearance of peak H to produce extra material in peak HH. (c) Treatment of fresh Triton extracts of *T. californica* with 1 mM Cl-HgBzO converts it from 100% A form to 95% B form. But gradient fractionation of these treated extracts shows the treatment to have absolutely no effect on the sedimentation profile. (d) Exposure of fresh Triton extracts of *T. californica* to carbamylcholine, *d*-tubocurarine, and nicotine prior to α-bungarotoxin treatment reduced the peak heights of H and L about equally. But the affinity of these drugs for A and B forms differs by factors of 50, 1.6, and 24, respectively (O'Brien and Gibson, 1974). These observations are supported by recent work of Sugiyama and Changeux (1975).

We now consider the probable oligomeric constitution of the receptor–toxin complexes. The pH-induced disaggregation of the H peak to the LL peak strongly suggests that the H peak is an oligomer of LL. Although the results of the studies on heat treatment, Triton concentrations, and pH suggest that the H and L peaks are not interconvertible, such a conclusion may not be warranted, and H may be a dimer of L. If so, the differing responses of H and L to heat, pH, and detergent may indicate that dimerization of the L peak significantly affects the properties of the dimer.

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Hydrophobic Chromatography and Fractionation of Enzymes from Extremely Halophilic Bacteria Using Decreasing Concentration Gradients of Ammonium Sulfate†

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ABSTRACT: Ammonium sulfate fractionation of proteins from extremely halophilic bacteria on Sepharose 4B, carboxymethylcellulose, diethylaminoethylcellulose, and hexamethylenediamine-Agarose is described. Halophilic proteins are adsorbed on these gels at 2.5 M ammonium sulfate and eluted by decreasing concentration gradients of this salt. The method has enabled the separation of malate dehydrogenase from glutamate dehydrogenase and aspartate aminotransferase on Sepharose 4B and the additional 15-fold purification of glutamate dehydrogenase on DEAE-cellulose. The technique is simple and convenient, operates at low cost, and possesses great power of resolution. The mechanism of adsorption is discussed and compared to previous instances of "hydrophobic chromatography". It is concluded that the retention of halophilic proteins on the polysaccharide gels at 2.5 M ammonium sulfate is due to hydrophobic interactions.

Enzymes from extremely halophilic bacteria constitute a fascinating example of biochemical adaptation. Thus, these enzymes which, both in vivo and in vitro, perform their catalytic functions at 4–5 M NaCl and KCl, lose their activity rapidly when exposed to low salt concentrations (Lanyi, 1974). They constitute therefore a unique system for inactivation–reactivation studies, which can shed light on the type of interactions involved in the maintenance of the integrity of native structures. In order to perform these studies it is indispensable to obtain pure enzyme preparations. However, the lability of these enzymes at low salt concentrations imposes many restrictions on the choice of purification techniques.

Recently, we have discovered that Agarose gels adsorb halophilic proteins from 2.5 M ammonium sulfate solutions. There were several previous attempts to use gradients of ammonium sulfate on solid supports for the fractionation of proteins—on Celite (King, 1972), on DEAE-cellulose[1] (Mayhew and Howell, 1971), on alkylamino-Agaroses (Rimerman and Hatfield, 1973; Doellgast and Fishman, 1974); and of transfer RNA species—on Sepharose (Holmes et al., 1975).

This communication deals with the chromatographic behavior of halophilic enzymes on several gels under the conditions of decreasing concentration gradients of ammonium sulfate. The results show the general utility of this technique, employing Sepharose and DEAE-cellulose as the solid supports, for the separation and purification of halophilic enzymes. The applications of this procedure to the separation of halophilic MDH from GDH and AAT on Sepharose 4B and to the purification of GDH on DEAE-cellulose are described.

Experimental Section

Chemicals. Oxaloacetic, α-ketoglutaric, and aspartic acids and NADH and NADPH were purchased from Sigma. All the salts employed were of analytical grade and their solutions were filtered through 0.45-μm Millipore filters before use. Sepharose 4B was obtained from Pharmacia, DEAE-cellulose (DE-52), and CM-cellulose (CM-52) were from Whatman. Celite 545 was a product of Fisher Scientific Co. and was used without further treatment. HMD-Agarose was prepared from Bio-Gel A-0.5m (a Bio-Rad Laboratories product) according to Jost and Yaron (1974) and contained 10.5 μmol of diamine/ml of gel.

Bacterial Extract. The bacteria of the species *Halobacterium* (Ginzburg et al., 1970) were a gift from Drs. M. and B. Z. Ginzburg. The bacteria were grown in 6-l. Erlenmyer flasks containing 1.5 l. with the following content (per l.): 208 g of NaCl, 46.6 g of $MgSO_4$ (anhydrous), 0.5 g of $CaCl_2$, 0.125 g of $MnCl_2$, and 10 g of Difco yeast extract. After 4 days of shaking the bacteria were harvested by centrifugation in a Sorvall GSA rotor. The yield was about 9 g of wet bacteria/l. of medium. The packed bacteria (50 g) were then resuspended in 4.3 M NaCl–0.01 M Na-phosphate, pH 7.3, and sonicated for 4 min at 0 °C in a Branson sonicator equipped with a microtip. The sonicated suspension was centrifuged for 1 h in a Spinco ultracentrifuge at 30 000 rpm at 25 °C. The supernatant was dialyzed against 1.6 M $(NH_4)_2SO_4$–0.05 M Na-phosphate, pH 6.6, and then centrifuged as above. Solid am-

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[1] Abbreviations used are: AAT, aspartate aminotransferase; CM-cellulose, carboxymethylcellulose; DEAE-cellulose, diethylaminoethylcellulose; GDH, glutamate dehydrogenase; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; HMD-Agarose, hexamethylenediamine-Agarose; MDH, malate dehydrogenase; NADH, reduced nicotinamide adenine dinucleotide; NADPH, reduced nicotinamide adenine dinucleotide phosphate; Tris, tris(hydroxymethyl)aminomethane; OD, optical density.