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Size of Acetylcholine Receptors in the Membrane. An Improved Version of the Radiation Inactivation Method†

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ABSTRACT: The radiation inactivation method was used to study the size of acetylcholine receptors in the intact membrane-bound state. This technique was reinvestigated, and modifications were made which remove substantial difficulties affecting previous applications of it to such proteins. The molecular size was deduced here by reference to a set of protein standards: an inactivation ratio was defined relative to a given internal enzyme molecular weight standard, and a linear calibration plot for the inactivation ratios of the protein standards was constructed and applied. The acetylcholine receptor in *Torpedo* electric organ, cat denervated muscle, and chick embryonic muscle was found by this method to exist in the membrane as a homogeneous population of the same size in each case. This receptor, when identified thus as the α-neurotoxin-binding target structure, has an apparent molecular weight of 300 000 or a molecular volume of about 350 nm^3. In comparison, the molecular weight of the cat muscle receptor when solubilized, as analyzed by gel electrophoresis after extensive cross-linking, was found to be 270 000 ± 20 000. These two values are thought to be equivalent by virtue of the situation and structure of the receptor protein in the cell membrane. If a disulfide-bridge dimeric receptor exists in the membrane (as other evidence has indicated for *Torpedo*), each monomer acts independently there in binding α-neurotoxin, since the monomers can be inactivated independently by irradiation in the *Torpedo* membrane. In the muscle membrane no evidence for the existence of receptor dimers, of any kind, has been found.

The molecular size of nicotinic AcCh[1] receptors has hitherto been studied only in detergent solutions. The receptor, as extracted thus from *Torpedo* electric organ, exists as stable monomeric and dimeric molecules, with sedimentation coefficients of about 9 S and 13 S and Stokes' radii of 7 and 9 nm, respectively; when extracted from eel electric organ, it is found only as the smaller of these [for references, see Karlin (1980)]. The *Torpedo* receptor dimer in detergent solution contains those 9S monomers joined by a specific disulfide bridge (Chang & Bock, 1977; Hucho et al., 1978; Hamilton et al., 1977); reduction (e.g., with dithiothreitol) converts the dimers present to monomers. The dimers have been clearly seen in electron microscopy of the 13S fraction (Wise et al., 1981). Reduction of *Torpedo* membranes (Witzemann & Raftery, 1978), and chemical reoxidation thereof (Hamilton et al., 1979), produces (in subsequent analysis in detergent solution) those same monomers and dimers, respectively. The extent or significance of the dimer occurrence in the native membrane is uncertain.

For the related AcCh receptors of mammalian skeletal muscles, only the 9S/7-nm form is found as the native form in detergent solutions (Chiu et al., 1973; Brockes & Hall, 1975; Merlie et al., 1978; Shorr et al., 1981). The treatments with dithiothreitol or *N*-ethylmaleimide which change the size distribution of the *Torpedo* AcCh receptors (Chang & Bock, 1977) do not change this single native size of muscle AcCh receptors (Barnard et al., 1978; Lo et al., 1981).

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[1] Abbreviations: AcCh, acetylcholine; ADH, alcohol dehydrogenase; α-BuTX, α-bungarotoxin; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

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The molecular weight of the 9S *Torpedo* species in solution has been determined—considering only analyses which would not be affected (Tanford & Reynolds, 1976) by the binding of detergent to the molecule—by sedimentation equilibrium with adjustment of solvent density by D_2O as 250 000 (Reynolds & Karlin, 1978), by membrane osmometry as 270 000 (Martinez-Carrion et al., 1975), or by cross-linking of the oligomer and $NaDodSO_4$–polyacrylamide gel electrophoresis (for *Torpedo* or eel purified AcCh receptors) as 230 000–260 000 (Hucho & Changeux, 1973; Hucho et al., 1978). Likewise, neutron scattering in detergent/18% D_2O solutions yielded a molecular weight of the monomer of 240 000 ± 40 000 and a radius of gyration of 4.6 ± 0.1 nm, showing that the molecule is asymmetric and likely to be of complex shape (Wise et al., 1979, 1981). Finally, electrophoretic estimation of the subunit composition [although subject to possible errors in the molecular weights of the subunits, arising from their glycosylation (Leach et al., 1980)] yields additively a molecular weight of 255 000 (Raftery et al., 1980).

We have sought to confirm these molecular weights for AcCh receptors from *Torpedo* and other sources in the intact membrane, where the size of the functional unit is of direct relevance to the mechanism of activation by the transmitter. The only method known so far to be capable of providing this information in situ is that of high-energy radiation inactivation. This method has hitherto been used for some enzymes (Kempner & Schlegel, 1979) and for a glucagon receptor (Houslay et al., 1977; Martin et al., 1979; Schlegel et al., 1979) and an insulin receptor (Harmon et al., 1980) in situ. High-energy radiation (preferably an electron beam) is applied; according to well-established radiation target theory (Pollard et al., 1955; Augenstein, 1963), the biological activity of the protein is then destroyed by one high energy "hit" occurring within its volume, V (the molecular volume of the target carrying the activity), according to Poisson probability, so that

$$A = A_0 e^{-VD} \tag{1}$$

where A_0 is the initial activity and A that after a radiation dose D (in inactivating events per centimeter), and by an obvious extension the molecular weight (M_r) of that unit is obtained:

$$M_r = \frac{N_0 V}{\bar{v}} \tag{2}$$

where N_0 is Avogadro's number and $\bar{v}$ is the partial specific volume of the protein molecule.

It is very difficult to determine D in those terms, however, and in the studies cited above of other proteins the analysis has employed for convenience an empirical equation derived by Kepner & Macey (1968) from the observed inactivation rates of a range of molecules:

$$M_r = \frac{6.4 \times 10^{11}}{D_{37}} \tag{3}$$

where (at any given temperature) D_{37} is the (macroscopic) radiation dose in rads needed to reduce the activity measured to 37% of its initial value. Further, an empirical correction factor (Kempner & Schlegel, 1979; Schlegel et al., 1979) of 250–300% is applied in studies where cooling during the irradiation is obtained by liquid N_2 vapor, to correct it to 30 °C. This modified approach, however, has yielded variable results with enzymes (Kempner & Schlegel, 1979) and has not been validated for receptors of any type. We find that there are considerable practical problems in the methods previously employed and that the equations used are not soundly based. We have employed, therefore, an alternative approach which avoids the assumption that eq 3 applies to membrane-bound receptors or in any particular experimental conditions of irradiation. A general method of radiation inactivation is described for determining the functional molecular weight of a protein in the membrane and is applied to the AcCh receptors in both *Torpedo* electric organ and denervated and embryonic muscle membranes.

The molecular weight determined is the target size, i.e., the weight of the active structure which is compact enough for significant energy transfer to occur within all parts of it. A multisubunit structure would therefore be expected to behave as a single molecule for this purpose, even if there is more than one active site in it, so that the true oligomeric molecular weight should in principle be measured.

Materials and Methods

Preparation of Membrane Samples. All operations were at 0–4 °C. Fresh *Torpedo marmorata* electric organ was homogenized and fractionated, with separation of the receptor-rich membranes on a discontinuous sucrose gradient, by using the methods of Sobel et al. (1977), with modifications as follows. The homogenization buffer contained (as protease inhibitors) bacitracin (50 μg/mL), EDTA (2 mM), leupeptin (4 μg/mL), phenylmethanesulfonyl fluoride (10 mM), and soybean trypsin inhibitor (10 μg/mL). These additives were also present, at half those concentrations overall, together with 3 mM NaN_3, in the gradient centrifugation and in 5 mM sodium phosphate buffer (pH 7.0) used for the final washes and suspension. Sonication was omitted. One sample of the washed purified membranes was, where specified, treated at pH 11 by a modification of the method of Neubig et al. (1979); the membranes, suspended in 10% (w/w) sucrose solution in water (with the above-mentioned protease inhibitors present), were brought to pH 11 at 2 °C by the addition of 0.2 M NaOH, held thus at 2 °C for 30 min, and centrifuged at 250000*g* for 45 min. The pellet was resuspended in the same medium, and the pH 11 extraction was repeated. Another sample was, where specified, reduced by incubation in 5 mM dithiothreitol/5 mM sodium phosphate (pH 7) (plus the inhibitors) for 1 h at 2 °C. All membrane samples were washed twice in water and resuspended in 5 mM sodium phosphate (pH 7.0), again with the protease inhibitors and 3 mM sodium azide present.

Cats were subjected to unilateral sciatic nerve scission (close to the muscle) and the denervated leg muscles dissected after 14–18 days, as described elsewhere (Shorr et al., 1981). The fresh muscle was minced and homogenized (Polytron PC-U2, 5 × 50 s bursts) in 10 volumes of 50 mM sodium phosphate (pH 7) containing the above-mentioned protease inhibitors plus benzethonium chloride (0.1 mM) and NaN_3 (3 mM). The homogenate was centrifuged at 150000*g* for 40 min, and the pellet was washed with the same medium and finally with 5 mM sodium phosphate (pH 7.0) containing the inhibitors (less benzethonium) and resuspended in the latter medium. The final volume of the suspension was such that there were about 10 pmol of receptor (in terms of α-BuTX binding sites) per mL.

Preparation of Samples for Irradiation. Horse liver ADH or β-galactosidase was added to the membrane suspension at 4 °C, at 0.2 unit of each enzyme per pmol of receptor present, and the mixture was rehomogenized with a glass Potter homogenizer (0.2-mm clearance) to give a uniform suspension. Aliquots (50 or 100 μL) were transferred to hard-glass tubes and were frozen immediately at −80 °C. The samples were lyophilized for 15 h and then sealed under high vacuum (<2 × 10^{-2} mmHg), taking care to keep the dried membranes unheated. The samples were stored at −80 °C before being

Table I: Molecular Size of Membrane-Bound AcCh Receptors Determined by Radiation Inactivation

source	pretreatment	inactivation ratio[a] I_H	I_g	n	target mol wt	mol vol[b] (nm³)
Torpedo marmorata	none	2.535	0.747	4	303 000 ± 5 900[d]	362 ± 14[d]
	reduced[c]		0.708	1	300 000	
	pH 11[c]	2.537		1	292 000	
cat denervated muscle	none	2.563	0.666	3	290 000 ± 12 600[d]	346 ± 27[d]
chick embryo muscle	none	2.570		1	301 000	359

[a] Measured relative to horse liver ADH (I_H) or β-galactosidase (I_G), as defined. The mean, where applicable, for a number (n) of independent experiments, each in duplicate analyses, is shown. [b] Target molecular volume, V, calculated from eq 2, taking $\bar{v}$, the partial specific volume, to be 0.74 cm³ g⁻¹, as calculated from the known (Raftery et al., 1980) amino acid composition of the pure *Torpedo* receptor. [c] For details, see Materials and Methods. After each pretreatment, the total receptor activity was quantitatively unchanged. [d] Mean ± SE, for all experiments on that species.

irradiated. For the calibration experiments, the mixture of enzyme standards (as listed under Results) was dissolved similarly in the same medium and lyophilized and sealed as described above for the receptor.

Irradiation Procedure. Irradiation was performed with the 15 MeV Phillips-MEL SL75-20 linear accelerator at the Department of Radiotherapeutics, University of Cambridge. A uniform beam 10 cm in diameter was produced by a diffuser screen. The specimens were held in this beam in a holder against the lead target block and were cooled with a continuous stream of compressed air which had passed through solid CO_2 in a Dewar flask. Calibration of the radiation dosage at the target block was routinely performed by using the Perspex optical density method (Berry & Marshall, 1969). The dosage there was maintained at 2 Mrad min⁻¹, and samples were exposed in the sample chamber for increasing lengths of time to give various integrated radiation dosages incident on the sample tubes. Irradiation was performed with the highest dose first, to allow temperature equilibrium to be reached; this was about 30 °C in a control irradiated tube, and the temperature was controlled to be in the range 25–35 °C in all cases used.

The sealed tubes were stored at −80 °C for 1–2 days after irradiation. The samples were then suspended in 50 mM sodium phosphate (pH 8.0) containing 2% Triton X-100. After extraction on a rotary shaker for 1 h at 5 °C, appropriate aliquots were taken and assayed for receptor or enzyme activities or protein content. Control extractions showed that the activity which can be solubilized reached a maximum after about 30 min under these conditions.

Enzymes. The enzyme markers used were all the purest grades from Sigma. All assays were adapted to automated sampling and recording of activity on the LKB 8600 reaction rate analyzer. Creatine kinase and pyruvate kinase assays therein were as described elsewhere (Liu et al., 1980). ADH assay was an automated version of that of Vallee & Hoch (1955) and β-galactosidase that of Craven et al. (1965).

Polyacrylamide Gradient Gel Electrophoresis. Gels were prepared by using 4% and 25% acrylamide solutions in equal volumes in a linear gradient mixer, with other procedures as in the method of Laemmli (1970), for native proteins. A 4% acrylamide stacking gel was used in all cases. The Coomassie blue stained gels were scanned at 500 nm. For calibration, the native protein standards were bovine serum albumin, lactate dehydrogenase, catalase, ferritin, and thyroglobulin (Pharmacia molecular weight standards); when the logs of their M_r values were plotted against the positions of the centers of their peaks, all the points lay on a straight line.

Electrophoresis of denatured samples in the presence of $NaDodSO_4$ was performed as described by Shorr et al. (1981), except that the gels were polyacrylamide gradient gels prepared as described above.

Other Methods. The receptor assay was by the method of Dolly & Barnard (1977), using as the ligand pure mono-[³H]propionyl-α-BuTX (Dolly et al., 1981). The latter was also used for labeling the receptors for analysis by gradient centrifugation (Lo et al., 1981). The complete purification and ^{125}I labeling of homogeneous cat muscle AcCh receptor and procedures for the extraction of receptors, together with their analyses on sucrose density gradient centrifugation, were as described elsewhere (Shorr et al., 1981; Lo et al., 1981), as were all other methods and materials not specified.

Results

Requirements for Radiation Inactivation Analysis of AcCh Receptors. The requirements for the reliable application of this technique to a protein such as the membrane-bound AcCh receptor were first investigated. It was necessary to establish that the following conditions hold: (i) The AcCh receptors, both in electric organ and in muscle membranes, are completely resistant to freeze-drying and rethawing, so that anhydrous, active specimens may be irradiated. (ii) Irradiation inactivation occurs at a temperature within the specimen well below (despite the heat dissipation which can arise at the highest doses used) that where any thermal inactivation of the receptor commences. (iii) The large empirical correction factor for temperature (see above) is eliminated, since it is not known whether it would apply to the particular receptor and physical specimen involved in every case. (iv) The determination should not depend upon knowledge of the absolute dose of radiation, since this is difficult to determine within the specimen at the time of irradiation and is affected by radiation quenching therein to an unknown degree.

Requirement i arises because the presence of liquid water is, of course, incompatible with this method due to the large indirect effect of its radiolytic products. Either freeze-dried preparations or frozen samples have, therefore, been used in the past in such studies on enzymes, but with variable results (Kempner & Schlegel, 1979). We have employed only lyophilized membrane-containing preparations, for two reasons. First, in some other cases frozen preparations have been found to exhibit a considerably reduced radiation sensitivity over the same preparations lyophilized, for some soluble enzymes (Kepner & Macey, 1968), or for membrane-bound adenylate cyclase (Schlegel et al., 1979). Second, if frozen aqueous suspensions were to be used, errors would arise due to local melting in the beam unless a very low temperature cooling system is constructed, but this means that requirement iii cannot be met. It was, in fact, possible to use lyophilized membranes since it was found that both the muscle AcCh receptor and the *Torpedo* receptor lost no toxin-binding activity upon rapid freezing and lyophilization (Table I). Hence, each type was studied in that state.

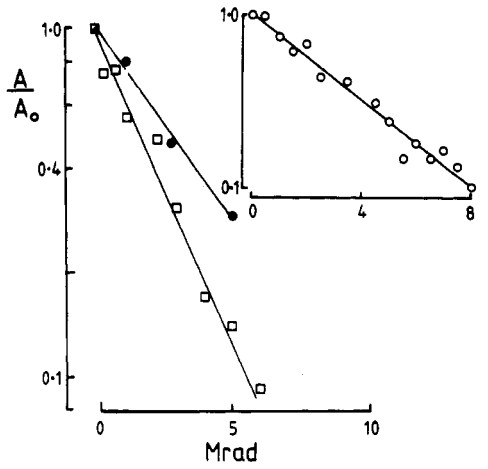

FIGURE 1: Radiation dose dependences. A/A_0 is the activity (toxin binding or enzymic) relative to that (A_0) prior to irradiation, plotted on a logarithmic scale. All data are expressed for an equal amount of protein; means of triplicates are shown, with lines fitted by least squares. Illustrated here are dose-dependence plots for a β-galactosidase standard (□) and the *Torpedo* AcCh receptor (●) in the same specimen; the slope of the latter plot relative to the standard plot is the inactivation ratio (relative to β-galactosidase, I_G). (Insert) Another irradiation, of yeast ADH, with A/A_0 and Mrad plotted on a different scale. Yeast ADH when used as a standard was kept in 0.3 mM EDTA since it is otherwise unstable (Leskovac & Parkov-Pericin, 1975).

Requirement ii arises because of the heat dissipated within the specimen by the high radiation doses involved. The limits involved were found by incubating samples of the freeze-dried receptors at different temperatures (5–100 °C) for 15 min, twice the maximum period using in obtaining the radiation doses required. There was no loss of toxin-binding activity in these conditions up to 50 °C, but a sharp decrease occurred above that temperature (data not shown). Accordingly, the specimen temperature was kept in the range 25–35 °C (to give a margin of safety) as described under Materials and Methods. This procedure also then covered factor iii above.

Requirement iv was met by the use of proteins of known molecular weights as internal standards, i.e., irradiated in the same system. First, several suitable pure enzymes were chosen as such standards. Each was lyophilized, redissolved, and assayed (to verify the retention of activity; this was 100% for all except liver ADH, which was about 93%). The molecular weight of each was checked after lyophilization by gradient gel electrophoresis. In a gel calibrated by five other (non-lyophilized) standard molecular weight markers (see Materials and Methods), the positions of all of the enzyme internal standards corresponded precisely to their reported molecular weights, except that the mobility of rabbit muscle creatine kinase corresponded to twice the size of the molecule of nonlyophilized creatine kinase, i.e., to 160 000 daltons (data not shown). This preparation was also denatured and analyzed in $NaDodSO_4$–polyacrylamide gel electrophoresis (data not shown), when it showed the presence of a single subunit of M_r 41 000, agreeing with reported values (e.g., Degani & Degani, 1979). This formation of a stable, active tetramer from the native dimer upon lyophilization has been confirmed on other samples by using the sedimentation coefficients on sucrose gradients but does not occur with chicken muscle creatine kinase (J. M. Lyles and E. A. Barnard, unpublished data). Hence, for each standard enzyme to be used in such a method, it is necessary to determine both the activity and the molecular weight after lyophilization.

Behavior of Standard Enzymes upon Irradiation. When each of the five standard enzymes was irradiated in vacuo in the lyophilized state, the enzymic activity declined with total dose following a single exponential, as illustrated in Figure 1, in every case. Each enzyme was then irradiated mixed with either horse liver ADH or β-galactosidase as a standard, and the dose dependence of its inactivation relative to that of the standard (the *inactivation ratio*), given by the ratio of the slopes of the semilog plots of the type of Figure 2, I_H or I_G, respectively, was determined. The relative dose dependence for one enzyme did not differ between such experiments on eight different occasions by more than 5%, whereas the absolute slopes for one enzyme on different occasions differed by much more than this, due to variations in the beam strength, inaccuracy in its calibration, or variations between the samples or in quenching by the glass containers.

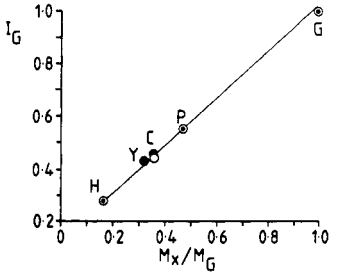

FIGURE 2: Relationship between the inactivation ratio and molecular weight (M_x). I_G is the inactivation ratio relative to β-galactosidase (○), as defined in Figure 1. Values of I_H for the same enzymes were also determined independently; these are shown (●) after conversion to the I_G scale by multiplying by the I_G value of horse ADH (concentric circles show that the two values coincided). Five molecular weight standards are used [their abbreviations and molecular weights (M_r) are given in parentheses]: horse liver ADH (H; 84 000), yeast ADH (Y; 160 000), rabbit muscle creatine kinase (C; 170 000), rabbit muscle pyruvate kinase (P; 224 000), and *E. coli* β-galactosidase (G; 464 000). The ordinate shows M_x relative to the molecular weight of β-galactosidase. The (least-squares) slope of the line is 0.90.

The value of I_G for each of the standards (x) was related to its molecular weight, M_x, normalized relative to the molecular weight (M_G) of the reference, β-galactosidase; i.e., a plot was made of I_G against M_x/M_G. This gave a good linear fit (Figure 2). This is based upon eq 1 and 2, which give, for an enzyme x

$$2.3 \log A/A_0 = -\frac{\bar{v}}{N_0} M_x D$$

so that the slope of the plot of log A/A_0 vs. D (slope_x) is proportional to M_x. Hence, the inactivation ratio for x equals

$$I_G(x) = \frac{\text{slope}_x}{\text{slope}_G} = \frac{M_x}{M_G} \qquad (4)$$

so that the molecular weight of any other enzyme can be determined from this plot. The absolute value of D is eliminated (since only relative values of the macroscopic radiation dose are required for the slope of the plots of Figure 1).

This method of ratio plotting allows direct comparison with other data obtained when another enzyme is used as the reference [in this case horse liver ADH (H) instead of β-galactosidase] by using the obvious relationship (from eq 4)

$$I_G(x) = I_H(x) I_G(H) \qquad (5)$$

and the inactivation dose dependences of the two standards, G and H.

The slope of the calibration plot (Figure 2) obtained is 0.90. The values for I_G fall on precisely the same line as those found for I_H.

Radiation Inactivation of AcCh Receptors. For the toxin-binding activity of AcCh receptors, similar linear semilog plots were obtained on irradiation, as illustrated for the

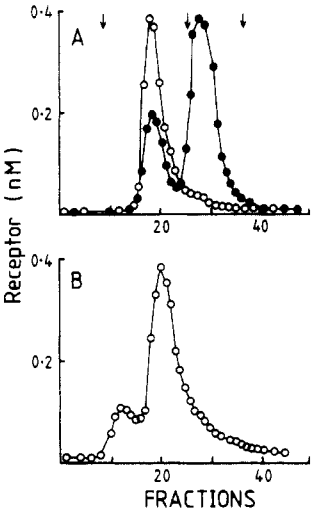

FIGURE 3: Sedimentation analysis of oligomeric forms of AcCh receptors solubilized from lyophilized membranes from *Torpedo marmorata* and cat denervated muscle. Parallel samples of lyophilized membranes used for irradiation experiments were extracted in 2% Triton X-100 buffer containing protease inhibitors. Receptor–[^{3}H]-α-BuTX complexes were formed by incubation of the extracts with a molar excess of [^{3}H]-α-BuTX, followed by gel filtration to remove unbound toxin. Aliquots were sedimented on 5–20% sucrose gradients containing 0.5% Triton as previously described (Lo et al., 1981); radioactive contents of fractions from the gradient were measured. Arrows show the positions of standard molecular weight markers (from left to right): alcohol dehydrogenase, catalase, and β-galactosidase. (A) Extracts of lyophilized *Torpedo* membranes before (●) and after (○) treatment with 5 mM dithiothreitol for 2 h at 5 °C. (B) Triton extract of lyophilized membranes from cat denervated leg muscles.

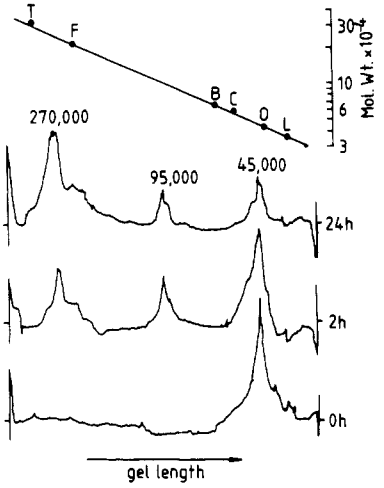

FIGURE 4: NaDodSO$_4$–polyacrylamide gradient gel electrophoresis of cross-linked AcCh receptor from cat denervated muscle. Receptor was purified by adsorption to α-toxin–Sepharose gel followed by extensive washing; it was radioiodinated while bound on the gel, washed, and then eluted with carbamoylcholine and anti-toxin antibodies as previously described (Shorr et al., 1981). ^{125}I-Labeled receptor was reacted with 10 mM dimethyl suberimidate in 50 mM sodium phosphate (pH 8)/0.2% Triton at 23 °C for the periods shown. After the reaction, each sample was treated with 1% NaDodSO$_4$ prior to lyophilization; samples were denatured therein at 100 °C for 2 min in 0.1 M Tris-HCl, pH 8. Electrophoresis was performed in a 4–25% polyacrylamide gradient gel (containing 0.1% NaDodSO$_4$) for 24 h at constant 150 V, and the gel was processed for autoradiography. Scans of the autoradiograms at 500 nm are shown. At the top is a semilog plot of the size and relative mobility of molecular weight standards: T, thyroglobulin; F, ferritin; B, bovine serum albumin; C, catalase; O, ovalbumin; L, lactate dehydrogenase.

Torpedo membranes in Figure 1. In fact, the inactivation of the AcCh receptor from *Torpedo* electric organ, cat denervated muscle, or chick embryo muscle exhibited only a single exponent over the loss of at least 90% of the initial receptor activity. When either of two enzymes (ADH or β-galactosidase) was used as the internal standard lyophilized with the receptor membranes, the respective inactivation ratios (as defined above) for each receptor were obtained (Table I). The molecular weights of the AcCh receptors were determined from these relative inactivation dose dependences by using the calibration line of Figure 2. As shown in Table I, these apparent molecular weights are all close to 300 000. The molecular volume of the receptor, V, which is the parameter directly determined here, is close to 350 nm^3 in each case. Since these values for the three different receptors were obtained by using in each case standards irradiated within the same specimen, they are not affected by variations in the actual internal radiation intensity which may occur between specimens or experiments. Samples of *Torpedo* membranes were also pretreated with 5 mM dithiothreitol to reduce any 13S dimeric receptor present to the monomer (see introduction). Other samples were preextracted at pH 11, a treatment which preserves the receptors but removes extrinsic proteins (Neubig et al., 1979). Neither of these two pretreatments affected the target size of the receptor in the membrane (Table I).

Molecular Size of AcCh Receptors in Solution. The receptors in Triton extracts of lyophilized membranes were analyzed by sucrose density gradient centrifugation in 0.5% Triton medium by using their complexes with [^{3}H]BuTX (Lo et al., 1981). The *Torpedo* receptor was shown thus to exist—as is generally found (see introduction)—as two forms with sedimentation coefficients of about 13 S (predominant) and 9 S, precisely as when fresh tissue is used (Figure 3A, closed circles). Treatment of the membranes and the extract with 5 mM dithiothreitol converted virtually all of the receptor to the 9S form (Figure 3A, open circles), as found by Hamilton et al. (1979) after such treatment of fresh *Torpedo californica* tissue.

Similar extraction and analysis of the receptor in the lyophilized membranes from cat muscle showed the same result as obtained with fresh muscle, i.e., a major 9S receptor–toxin complex and a minor 4.6S complex (Figure 3B). Traces of heavier forms are usually present; no significant difference in distribution occurred on lyophilization of the muscle membranes and resolubilization. A very similar profile is obtained with the 14-day-old chick embryo muscle, as shown elsewhere (Lo et al., 1981). The addition of 5 mM *N*-ethylmaleimide (Chang & Bock, 1977) in the homogenization and solubilization stages did not change appreciably the proportions of 9S and 13S receptor complexes obtained from fresh electric organ. When the receptor–toxin complex is formed in muscle membranes, as we have shown elsewhere (Lo et al., 1981), the treatment with *N*-ethylmaleimide leaves almost all of the complex in the 9S form.

The molecular size of the cat muscle AcCh receptor was also determined by chemical means. The receptor in the fully purified, ^{125}I-labeled state (Shorr et al., 1981) was reacted with dimethyl suberimidate for varying periods of time, to achieve full cross-linking of the subunits. Electrophoretic analysis of the subunits separable in denaturing conditions (Figure 4) showed that, initially [as found previously (Shorr et al., 1981) in nongradient gel NaDodSO$_4$ electrophoresis], a major subunit of about M_r 45 000 (in the gradient gel system) is present, plus a shoulder in the M_r range 50 000–56 000. After cross-linking for 2 h, a component of M_r 95 000 ± 10 000 and others of M_r 210 000–270 000 are also found. After maximal cross-linking, the major peak had M_r 270 000 ± 20 000, with the other species mentioned still present to a lesser degree,

including a minor component of M_r about 220 000 (Figure 4, upper trace). No band corresponding to a higher molecular weight than 270 000 was seen in such gradient gels, even after 24-h reaction with 10 mM cross-linking reagent and running of the gels under conditions where a heavier band would have been discerned. The cross-linking was performed on extremely dilute receptor solutions, <1 μg/mL, so intermolecular cross-linking was assumed to be absent, and the maximum value of M_r 270 000 seen must represent the molecular weight of the monomeric protein.

Discussion

The principle of functional molecular size determination using ionizing radiation has been known for over 30 years. This method can be an important tool in membrane biochemistry, since it is the only one known capable of measuring the molecular size in situ of receptors and membrane-bound enzymes. However, its application there has hitherto relied upon the assumption of an empirical relationship which had been deduced for globular proteins (itself unreliable even for those, as discussed below) and upon large and arbitrary correction factors (see introduction). The method has given in the past very variable results for some enzymes, as the published data compiled by Kempner & Schlegel (1979) show. A recent example for a membrane protein is that of the adenylate cyclase (associated with the glucagon receptor) of the rat hepatocyte, where the size of the unit giving ground-state activity in the plasma membrane has been determined by such target analysis (using eq 3) to be 1 300 000 daltons by Schegel et al. (1979) but 328 000 daltons by Martin et al. (1980).

We have found that a major cause of inaccuracy is uncertainty in the absolute value of the radiation dose (required for eq 3 above) within the experimental sample. This uncertainty arises both from the mode of operation of equipment of this type and from the unknown degrees of quenching within different specimens and assemblies (e.g., cooling systems for sample holders). We avoided the requirement of knowing the absolute values of the radiation dosages by the use of internal molecular weight standards, to determine the inactivation ratio as defined here, and a linear (*not* logarithmic) calibration plot (Figure 2) using the standards in a relative mode only. We likewise avoided conditions (e.g., frozen solutions) where temperature factors must be assumed, for the reasons noted earlier.

The physical theory for inactivation by fast electrons (Pollard et al., 1955) gives rise to eq 4, which predicts a slope of unity for the plot of Figure 2. In practice, the slope is 0.90. It is not known if this is due to experimental inaccuracy or to some systematic effect in the electron inactivation of proteins: the latter is possible, since the values for all of the enzymes tested fell significantly below a theoretical line of slope 1.0, rather than being scattered above and below it. An important requirement here is a set of accurate molecular weight values for the standards: enzymes were chosen where these are well established, in several cases by their known amino acid sequence. The samples used were checked for conformity to these. Corrections for their slightly different $\bar{v}$ values would not alter the fit to the slope of 0.90. Repeated determinations for given standards on different occasions give the same relative values (I_G), although the absolute slopes (as in Figure 1) varied. The same I_G value was obtained for a standard enzyme irradiated (with its reference) mixed with a membrane sample or alone.

Reasons, given above, why eq 3 should not be employed are reinforced by examination of the data on which Kepner & Macey (1968) empirically based it. These were collected from the earlier literature and covered samples ranging in molecular weight from a few hundreds upward, giving a double logarithmic plot of inactivating dose [D_{37}; see eq 3] against molecular weight, which spanned 5 orders of magnitude. The radiation doses in the samples were assumed implicitly to have been known absolutely in a variety of published studies (using not only different methods and sample conditions but also in some cases even different types of radiation, e.g., α particles). The molecular weights used for the proteins are, in about half the cases, far from the values now accepted. They included such molecular weights as those of "*Escherichia coli* ribosome" (2.6×10^6) and various poorly characterized nucleic acid specimens, assessed by methods now discarded. The same enzyme (e.g., trypsin or ribonuclease) gave values covering a 3–4-fold range in different determinations, and all were used. The small molecules (e.g., penicillin) which were included would certainly not show the same probability of "inactivation" by local ionizations as would the proteins used and should have, in the solid state used, a much higher susceptibility (Pollard et al., 1955). The linear relationship, expressed as eq 3, appears to arise from the great range compressed into the log–log plot, from ignoring the wide scatter in that plot, and from a fortuitous relationship between incompatible data.

By use of Figure 2 or eq 5, the values (Table I) of M_r for the three types of AcCh receptor studied here in the membrane-bound state lay in the range 290 000–303 000. This can be compared with the values for the *Torpedo* AcCh receptor monomer in solution, as determined by a number of reliable methods, of about M_r 260 000 (see introduction) and that for the purified cat muscle receptor determined here by cross-linking as M_r 270 000 (Figure 4).

The results show, first, that the entire molecular assembly, having five subunits in the *Torpedo* case (Reynolds & Karlin, 1978; Raftery et al., 1980), constitutes the toxin-binding structure. There are two toxin-binding sites and two sites for AcCh per 260 000 daltons in the receptors from *Torpedo* (Blanchard et al., 1979; Neubig & Cohen, 1979; Wolosin et al., 1980) and mammalian muscle (Wolosin et al., 1980; Sine & Taylor, 1980); it is seen that these must be on a single molecule in situ, since all of the toxin-binding capacity is destroyed in a single event.

Second, by the present method the AcCh receptor (as defined by its toxin binding) is seen to be of the same size (within experimental error) in the membrane in the three diverse sources sampled. This size, moreover, is that corresponding to the 9S solution form, i.e., "monomer", and not the "dimer" (13 S) seen in extracts of *Torpedo* electric organ (Figure 3A). The chemical molecular weight of the *Torpedo* dimer in solution is >500 000 (Reynolds & Karlin, 1978), and this is far beyond the range of possible experimental error in the data obtained here, whereas the latter fit the monomeric molecular weight in the membranes. No evidence for the 13S dimer in solution has been found for the muscle receptors from any species. Furthermore, reduction of disulfides in the membranes did not decrease the target size of the receptors (Table I). Therefore, either the receptor is monomeric in situ in *Torpedo* membranes as well as in muscle, and the disulfide bridging within the soluble *Torpedo* dimer (see introduction) occurs on homogenization and solubilization of the membranes, or a high-energy electron impact on half of such a dimer in this case cannot spread to, and inactivate, the second half. The evidence for an intersubunit bridge, from gel electrophoresis of denatured *Torpedo* membranes (Hucho et al., 1978; Witzemann & Raftery, 1978; Hamilton et al., 1979), argues

against the first alternative. For the second alternative, if two protein molecules are linked only by a single disulfide bridge, and each is biologically active independently, then it may be that each presents a separate target for an inactivating electron: this question has not been resolved (or even raised) in previous irradiation studies on proteins. We presume that this method recognizes an oligomer as a single target molecule when the subunit (or monomer) surfaces are in good contact, with a number of bonding interactions occurring between them (as generally is the case in such proteins); this will permit the ionization energy on impact to be dissipated through the entire structure. Indeed, the enzymes used as standards here were all inactivated according to the sizes of their oligomers (and not of their subunits, even though these are active separately). An instructive example is that of alcohol dehydrogenase, where the physicochemical evidence in solution shows the molecule from horse liver to be a dimer and from yeast to be a tetramer, of similar active subunits. The radiation method shows (Figure 2) their target sizes to be in this ratio of 1:2 and having the predicted oligomeric values relative to β-galactosidase. That the target size is the oligomer has held true, also, for several other oligomeric globular enzymes since tested (unpublished results). However, in a supramolecular assembly where the surfaces of units are not in good contact, as would be the case if two neighboring cylindrical receptors touched only through one disulfide bond, an exception may occur. We conclude that if, as generally described, the dimer exists in the *Torpedo* membrane, the two receptors in it have very little contact other than through the disulfide bond.

We have seen, then, that if dimers exist, each monomer therein functions independently in the binding of toxin. This independence is known to extend also to the ion channel function of electric organ receptors, since reduction of the disulfide bridge in the membrane state does not remove the agonist-induced cation translocation function (Hamilton et al., 1979) and reconstitution in an artificial lipid system of either the 9S or the 13S form has yielded equivalent channels for each (Anholt et al., 1980; Wu & Raftery, 1981).

Third, the value obtained for the molecular size cannot be affected by the fairly close packing of the receptors in the synaptic membrane, since it is the same also for the embryonic and denervated muscle receptors. In the *Torpedo* membranes, the receptors are packed at about 10000 molecules/μm^2 (Cartaud et al., 1978; Klymkowsky & Stroud, 1979; Heuser & Salpeter, 1980; Lo et al., 1980). For synaptic AcCh receptors at muscle end plates, taking two toxin-binding sites per muscle receptor (Wolosin et al., 1980), a very similar surface density holds at the fold crests (Porter & Barnard, 1975; Fertuck & Salpeter, 1976). However, for the 14-day-old chick embryonic muscle and denervated mammalian muscle, the receptors are very largely extrajunctional and at a very much lower surface density (Porter & Barnard, 1976; Burden, 1977; Fambrough, 1979). The independence of each receptor is clearly not affected by these very different receptor densities.

Fourth, our results indicate a rather higher (by ~15%) functional molecular weight for the receptor in the membrane than the value known from accurate chemical and hydrodynamic data. This difference appears to be significant. We think it unlikely that it arises from experimental error, since it was found in the same direction in every determination made here and was about the same for all three sources, although the specimens varied greatly in composition and bulk between these sources. Further, if the specific volume, $\bar{v}$, were to be changed from the value of 0.745 (taken from the amino acid composition of the pure receptor protein) to a low value of 0.73, this would produce only a 2% change in M_r, instead of the 15% in question. It seems probable that some systematic error occurs to increase by this modest but consistent amount the apparent molecular weight of the AcCh receptor in the membrane. The following are factors which should be considered as plausible causes of this:

(1) There may be membrane lipid molecules which are tightly bound to the receptor protein. A shell of such lipid for the AcCh receptor in the *Torpedo* membrane was deduced by Allen et al. (1977) from freeze–fracture evidence, and a fraction of associated immobile lipid was seen by Rousselet et al. (1979) in spin-labeling studies. Whether such lipid binding could be so intimate as to increase the target size of the protein is not known; it would be desirable to compare with Figure 3 the behavior upon irradiation of enzymes of accurately known size which are integral membrane proteins, at present very few in number.

(2) The discrepancy may reflect a truly enlarged effective molecular domain of the AcCh receptor as a transmembrane protein, compared to that predicted from its molecular weight. Since the induced ionizations are random in the specimen volume, target theory (Pollard et al., 1955) predicts the overall volume, and not (directly) the molecular weight, irrespective of molecular shape. The theoretical molecular volume for a molecule of 260000 weight is 311 nm^3, for its calculated $\bar{v}$ value of 0.745 (or 304 nm^3 for $\bar{v} = 0.73$). The molecular volume of the *Torpedo* AcCh receptor in solution has been determined by Reynolds & Karlin (1978) by rigorous sedimentation analysis to be 305 nm^3. The value of 362 ± 14 nm^3 which we find (corresponding to the higher molecular weight found) in the membrane might have been increased due to a different molecular structure there compared to that in solution, involving transmembrane subunits and a gated ion channel within the structure, giving an overall lower density because of different internal packing.

In conclusion, we suggest that the methods employed here, and the possibilities considered in interpretation, are likely to be applicable to other receptors in their membranes.

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