THE JOURNAL OF HISTOCHEMISTRY AND CYTOCHEMISTRY
Vol. 21, No. 9, pp. 769-778, 1973

Printed in U.S.A.

SIZES OF END PLATE COMPARTMENTS, DENSITIES OF ACETYLCHOLINE RECEPTOR AND OTHER QUANTITATIVE ASPECTS OF NEUROMUSCULAR TRANSMISSION[1]

MIRIAM M. SALPETER AND MOHYEE E. ELDEFRAWI

Section of Neurobiology and Behavior (M.M.S., M.E.E.), and School of Applied and Engineering Physics (M.M.S.), Cornell University, Ithaca, New York 14850

Received for publication February 8, 1973

The area of the postsynaptic membrane and the volume of the synaptic cleft were calculated for the end plates of the diaphragm and sternomastoid of mouse and rat. From these dimensions we were able to extrapolate, from data given by others on acetylcholine (ACh) released during neuromuscular transmission and on ACh receptor per whole end plate, to densities in the postneural compartments. The concentration of ACh in the cleft per nerve impulse was found to be $\sim 10^{-5}$ M and the density of ACh receptor was between 5 and $10 \times 10^3/\mu^2$ of postsynaptic membrane. (This is approximately a factor of 2 to 3 higher than that for acetylcholinesterase at this site.) From these values we conclude that the concentration of ACh receptor in the plane of the postsynaptic membrane is considerably higher than that of ACh in the cleft during neuromuscular transmission. In addition the ACh itself is present at considerably higher concentrations than necessary to give optimal response. We calculated that the acytelcholinesterase plus ACh receptor together would occupy about 25% of the surface area of the postsynaptic membrane.

Evidence from physiologic, biochemical and histologic studies has led to the formulation of the widely accepted hypothesis of neuromuscular transmission at cholinergic end plates. According to this hypothesis, the sequence of events in the end plate starts by the arrival of a nerve impulse at the neuromuscular junction, causing the quantal release of acetylcholine (ACh) from the presynaptic nerve ending into the synaptic cleft. Acetylcholine is believed to bind to a proteinaceous receptor molecule in the postsynaptic membrane. As a result, the permeability of the postsynaptic membrane to ions is increased and it becomes depolarized causing an end plate potential (EPP). The action of ACh is terminated by the hydrolytic activity of acetylcholinesterase (AChE), another component of the postneural region of the end plate.

In order to understand the interaction between the various molecules involved, several studies have been aimed at determining the numbers of these molecules present during neuromuscular transmission. Although the critical events occur in the postneural region of the end plate (defined as the cleft and postsynaptic membrane) (Fig. 1), most of the quantitative data regarding these molecules (*e.g.*, amount of ACh released per nerve impulse or numbers of ACh receptor molecules) are available per whole end plate. Indirect estimates of the sizes of the postneural compartments have usually been necessary to arrive at molecular densities at the "business" end of the end plate.

In the present study we calculate directly the volume of the cleft and surface area of the postsynaptic membrane for two cholinergic end plates, diaphragm and sternomastoid, in the mouse and rat, from the simple arithmetic relation:

$$\frac{\text{Total sites per whole compartment}}{\text{Sites per } \mu^3 \text{ (or } \mu^2\text{) of that compartment}} = \text{Size of compartment in } \mu^3 \text{ (or } \mu^2\text{)} \quad (1)$$

Several studies using light and electron microscope autoradiography have provided the information giving both the numerator and denominator of this equation. We were then able to determine the local concentrations of ACh in the cleft during neuromuscular trans-

[1] This work was supported in part by U.S. Public Health Service Grant NS09315 from the Institute of Neurological Diseases and Stroke.

mission and of the ACh receptor at the postsynaptic membrane from data published by others on the numbers of these molecules present per whole end plate. When compared with the density of AChE sites measured previously, the quantitative relationships between these three molecules were reassessed.

SIZES OF END PLATE COMPARTMENTS

Values are available both for the numerator and denominator of equation 1 from studies on the numbers and densities of diisopropyl fluorophosphate (DFP)-reactive sites. Using light microscope autoradiography, Rogers *et al.* (48, 49) and Barnard, Rymaszewska and Wieckowski (6) gave the number of DFP sites present per whole end plate. Salpeter (51, 52) showed that about 90% of these are present in the postneural region (*i.e.*, cleft and/or postjunctional membrane). Thus 90% of the whole end plate value gives the numerator of equation 1. Using electron microscope (EM) autoradiography Salpeter, Plattner and Rogers (53) have obtained the number of total sites present per cubic micron of cleft and per square micron of postsynaptic membrane (Reference 53, Table II). These values were used for the denominator of equation 1.[2]

It must be emphasized that the use of DFP-reactive sites is fortuitous, and the results are independent of that fact. Similar results would be obtained using any other parameter, if information were available, on total numbers per whole compartment and on the number per unit volume (or area) of that compartment. We must also emphasize that the ratios of DFP sites per cubic micron of cleft or square micron of postsynaptic membrane do not imply localization of the sites within these compartments. They are mere correlations. Their use in the formula is valid, however, under the conditions used for collecting the data; *i.e.*, the two compartments are anatomically linked, the autoradiograms were random sections through many end plates and all of the autoradiograms contained the full image of both compartments plus enough area around them to include all of the developed grains which could have arisen as a result of radioactivity in either compartment.

The accuracy of the calculated dimensions is estimated to be well within a factor of 2. This depends on several factors. First is the accuracy of the quantitative light and EM autoradiographic procedures, which we estimate to be within about 30%. Second is the accuracy in obtaining a representative sampling. The values for the densities were obtained from numerous random sections through ~100 end plates, of both sternomastoid and diaphragm, and can thus be considered to be representative of these end plates. Subsamples treated separately gave similar densities. Third is the possible volume changes which could be introduced during processing of the tissue for electron microscopy. This would affect the calculated densities used in the denominator of equation 1.

Various studies have attempted to give information on the amount of shrinkage or swelling introduced by the fixation and embedding procedures for electron microscopy, but unfortunately none has used identical preparatory conditions. By combining the published values for glutaraldehyde and OsO_4 fixation, alcohol dehydration and Epon embedding, we arrive at a figure for final shrinkage of tissue prepared for electron microscopy varying from 0 to 15% (4, 29, 31, 39). We made an independent estimate of shrinkage from the EM autoradiographs which were used in the study of the submicroscopic densities of DFP by Salpeter *et al.* (53), which was the material from which the denominator in equation 1 was derived. We compared the major myelin period in the micrographs with that reported in the literature from x-ray diffraction studies (9) and found them to match within 10%.

Table I gives the calculated sizes for the

[2] Values for total sites are available for both mouse (48, 49) and rat end plates (8), yet that for postneural densities is available only for the mouse (52, 53). However, it was found that the postneural densities are the same in the two mouse end plates studied (*i.e.*, the diaphragm and sternomastoid) while the total sites per end plate varied with the surface area of the end plate. Since the functional characteristics and fine structural features of these rat end plates are similar to those for the mouse, we assumed that the postneural densities found in the mouse are applicable to the rat end plates. The validity of this assumption was strengthened when we found that the total sites per end plate of the rat as well as the mouse muscles varied as the surface area of the end plates. (Total sites are 3×10^7 and 8.8×10^7 for mouse diaphragm and sternomastoid end plates, respectively (48, 49), and are 6.6×10^7 and 8.8×10^7 for these end plates in the rat (6); for surface dimensions of the end plates, see Table I.) Since the general shapes of the end plates are rather irregular, the two surface dimensions only approximate their true size.

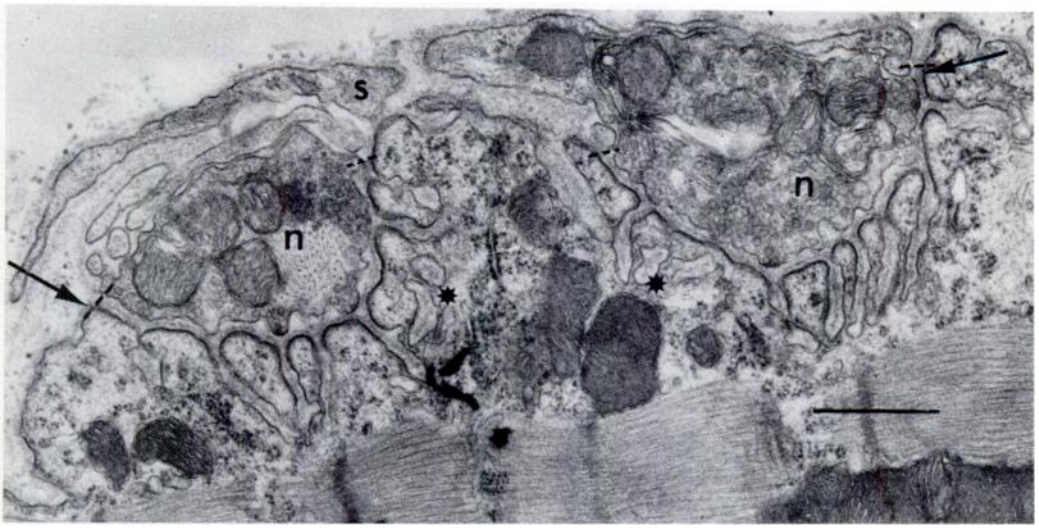

FIG. 1. Section through a part of an end plate of mouse diaphragm from the autoradiographic studies on DFP binding by Salpeter *et al.* (53) illustrating and identifying the end plate compartments. The overlying Schwann cytoplasm (teloglial cap) is marked s and the axonal twigs n. The postsynaptic membrane is the full length of sarcolemma containing the junctional folds as delimited by the two arrows. Profiles of junctional folds isolated from the rest of the postsynaptic membrane due to the plane of sectioning (asterisks) are included. (If junctional folds appear in the sarcolemma between two adjacent axonal twigs (as in this picture) this membrane segment is also included with the postsynaptic membrane.) We found, in agreement with Fambrough and Hartzell (23), that the ratio of postsynaptic to presynaptic membrane is ~5:1. The presynaptic membrane includes only that portion of the axolemma apposed to the postsynaptic membrane. The cleft includes the entire space bounded by the presynaptic and postsynaptic membranes and the dashed lines (marked on either side of each axonal twig).

One important morphologic feature emphasized by such an electron micrograph is the scalloped nature of the postneural surface of the end plate due to its numerous axonal twigs. Any estimate of postsynaptic membrane surface area based on the assumption that the end plate "approximates a disc," as was done by Fambrough and Hartzell, would tend to give a value which is too low. This may account in part for the discrepancy between the 3000-μ^2 estimate by Fambrough and Hartzell and the 7000-μ^2 value obtained in the present study for the rat postsynaptic membrane area. ×16,500.

TABLE I

Muscle Fiber and End Plate Dimensions of Mouse and Rat End Plates

Species and Muscle	Average[a] Cleft Volume	Average[a] Postsynaptic Membrane Area	Muscle Fiber Diameter	Surface Dimensions of Stained End Plate
	μ^3	μ^2	μ	μ
Mouse diaphragm	200	3000	18–25[b, c]	23 × 26[b]
Rat diaphragm	450	7000	25–50[b, c, d]	30 × 40[b, e]
Mouse sternomastoid	800	9000	40–80[b, f]	40 × 65[b]
Rat sternomastoid	800	9000	50–70[b]	40 × 60[b]

[a] Calculated from equation 1 in text.

[b] Our own measurements of muscle fibers stained for AChE by the method of Karnovsky and Roots (34). They are based on more than 25 determinations each. The surface dimensions of the stained end plates represent averages of the length and width of these end plates after approximating the irregularly shaped surface to an oval.

[c] From Gauthier and Padykula (27).

[d] From Krnjevic and Miledi (37) and Padykula and Gauthier (45).

[e] Our measurements are consistent with stained end plates in Cole (Reference 15, Figs. 13–16) and Fambrough and Hartzell (Reference 23, Fig. 1), although in the text Cole gives somewhat lower values.

[f] From Hegarty and Hooper (30).

postneural end plate compartments. It also gives the diameters of the muscle fibers and the surface dimensions of the end plates, seen by light microscopy after staining for AChE. We see that the differences in the sizes of the postneural compartments of the various end plates are in line with the differences in their surface dimensions. There also appears to be a positive correlation between the surface area of these end plates and the diameter of the muscle fiber they innervate. As we pointed out in footnote 2, the surface area of the end plates is also correlated with total DFP sites (6, 49) while the postneural densities remain constant (53).

DENSITY OF ACH RECEPTOR

As a consequence of having calculated the dimensions of the end plate compartments, we were next able to determine local concentrations of ACh receptor and ACh. There is currently a surge of activity which started only 5 years ago to isolate ACh receptor molecules, mainly from electric tissue of fish (28, 41, 44, 47). The identification of these macromolecules *in vitro* relies mainly on either the reversible binding of cholinergic ligands or the binding of neurotoxins from snake venoms. Because of its relatively irreversible binding to nicotinic receptors (13), a member of the latter class, α-bungarotoxin, has been widely used to label ACh receptors *in situ* and to calculate the number of their binding sites. In rat and mouse diaphragms these data have been obtained primarily as binding sites either per whole diaphragm or per whole end plate (7, 8, 23, 42). On the basis of our calculated values for the surface area of postsynaptic membrane, we have extended the published data to densities per square micron of postsynaptic membrane. These values are given in Table II. In the case of two studies, that of Fambrough and Hartzell (23) and Barnard, Wieckowski and Chiu (7), in which the authors themselves extrapolated their data to receptor densities per square micron of membrane, their values were somewhat higher than those that we calculated from their data, for the following reasons. Fambrough and Hartzell (23) had found that there were $\sim 4 \times 10^7$ α-bungarotoxin binding sites/rat diaphragm end plate. On the basis of a simplified model for the shape of the end plate (*cf.* Fig. 1) they then estimated that the area of the postsynaptic membrane in the rat diaphragm was

TABLE II

Density of α-Bungarotoxin Binding Sites per Square Micron of Postsynaptic Membrane[a]

Species and Muscle	Average No. of Sites/ Whole End Plate ($\times 10^7$)	Postsynaptic Membrane Area	Sites/μ^2 Membrane ($\times 10^3$)
		μ^2	
Rat diaphragm	4.0[b]	7000	5.7
	4.7[c]		6.7
	0.9[d]		1.3
Mouse diaphragm ...	1.6[c]	3000	5.3
	3.0[e]		10.0

[a] Calculated from sites per diaphragm or whole end plate published by others divided by size of postsynaptic membrane in Table I.

[b] From Fambrough and Hartzell (23) using ^{125}I-α-bungarotoxin.

[c] From Miledi and Potter (42) using ^{131}I-α-bungarotoxin.

[d] Berg *et al.* (8), using ^{125}I-α-bungarotoxin, reported 0.2–0.4 pmole sites/end plate region of whole rat diaphragm. We used the estimate of 10,000 end plates/hemidiaphragm (37) to arrive at this value.

[e] Barnard *et al.* (7) reported this value per mouse diaphragm end plate since they found that there was an equivalent number of binding sites per end plate after ^{3}H-acetyl-α-bungarotoxin as after ^{3}H-DFP. The value given per end plate then represents a previously published value for the DFP sites obtained from light autoradiography (see Table 1 in Barnard *et al.* (7)).

3000 μ^2 and thus calculated a value of 1.3×10^4 receptor molecules/μ^2 of membrane. Using our value of $\sim$7000 μ^2 for the postsynaptic membrane in this end plate and Fambrough and Hartzell's whole end plate data, we get 5.7×10^3 receptor molecules/μ^2 of postsynaptic membrane (reported in Table II).

Barnard *et al.* (7) had arrived at a value of 1.2×10^4 receptor molecules/μ^2 of postsynaptic membrane of mouse diaphragm in a more indirect manner. In fact, what they found was that in light autoradiographic sections of mouse diaphragm end plate the number of α-bungarotoxin binding sites equalled that of total DFP binding sites. Then, since Salpeter (52) had reported $\sim 1.2 \times 10^4$ DFP binding sites/μ^2 of sternomastoid postsynaptic membrane, Barnard *et al.* (7) used this value for the α-bungarotoxin sites/μ^2 of postsynaptic membrane in the diaphragm end plate. However, Salpeter has recently revised this value downward by

almost 30% because of a recently discovered "dose dependence" in EM autoradiography when using Microdol X development (54). A corrected value is given in Salpeter *et al.* (53) as $\sim 9 \times 10^3$ DFP sites/μ^2. The density of ACh receptors given by Barnard *et al.* (7) must thus also be reduced accordingly. Since one would expect all of the α-bungarotoxin binding sites to reside on the postsynaptic membrane, the final density should be somewhat higher than that for the DFP sites. These considerations are consistent with the value of 10,000/μ^2 reported in Table II, which we calculated from Barnard's whole end plate data and our postsynaptic membrane size. (The dose dependence does not affect the values for DFP bound to whole end plates, since these were obtained by light autoradiography under different photographic conditions.) Of interest is a recent paper by Styckowski, Vogel and Nirenberg (58) giving a similar value (*i.e.*, 9,000 ACh receptors/μ^2) for ACh receptor sites (as measured by α-bungarotoxin binding) in clusters on the surface of myotubes of cultured chick embryo skeletal muscle cells.

From Table II we see that with the exception of Berg *et al.* (8), when we apply our values for the surface area of the postsynaptic membrane to values of α-bungarotoxin bound per end plate published by others, we conclude that the ACh receptor (*i.e.*, α-bungarotoxin binding sites) in mouse and rat end plates is present at a density of 5–10 $\times$ $10^3/\mu^2$ of postsynaptic membrane (average $\sim 7.2 \times 10^3$). The accuracy of these values depends, of course, both on the accuracy of our surface area calculation and on that of the original published whole end plate values. It is noteworthy that, once related to square micron of membrane, the difference between the mouse and rat end plate is no longer as great as it appeared from the whole end plate data (42). The densities calculated here are within a factor of 2 of those estimated by several other authors for ACh receptor packing in excitable membranes (*e.g.*, References 7, 23, 41 and 58) but differ considerably from the 100,000/μ^2 estimated for frog end plates by Miledi and Potter (42) and the 33,000/μ^2 for *Electrophorus* electroplax by Bourgeois *et al.* (10). For comparison, there are approximately 9×10^3 DFP binding sites/μ^2 (53). Since only $\sim 2.5 \times 10^3$ of these can be reactivated by pyridine-2-aldoxime methiodide (63), it is assumed that only about one-fourth to one-third of these DFP sites are AChE and the rest are butyrylcholinesterase and nonspecific esterases (43, 52, 53).

One may want to estimate the total area occupied by all of these molecules if they were embedded in a single plane. The molecular weight of the monomeric form of the ACh receptor has been suggested to be about 40,000 daltons (33), that for an AChE molecule having two catalytic sites as 260,000 daltons (40). (This AChE molecule may possibly have three catalytic sites (50).) Assuming a molecule to be a sphere, one can estimate its diameter (D) in centimeters from the following equation:

$$\rho = \frac{MW/N_0}{(\pi/6)\, D^3} \qquad (2)$$

where ρ = the density in grams per milliliters, MW = the molecular weight and N_0 = Avogadro's number (6.02×10^{23}). (This formula gives values for molecules consistent with those obtained by x-ray diffraction. For instance for cytochrome *c*, molecular weight 12,400, we get a value for D = 32 Å, whereas x-ray diffraction gives dimension of 25 x 25 x 37 A (18).)

Using equation 2, and assuming a density of 1.3, we get for the receptor molecule (molecular weight 40,000) (33) a diameter of 46 Å, and for an AChE molecule of molecular weight 260,000 (40), we get a diameter of 87 Å. If each catalytic site were an independent sphere, then for two catalytic sites/molecule (40), each would be 69 Å in diameter, and for three catalytic sites (50), each would be 60 Å. Thus the 7.2×10^3 receptor sites/μ^2 of postsynaptic membrane (average value calculated above), if arranged in a simple cubic array, would occupy $\sim$15% of the surface area. The 2.5×10^3 AChE sites/μ^2 of membrane (assuming two catalytic sites/molecular weight 260,000 (40) and thus 69 Å diameter/site) would occupy $\sim$12% of the surface area. With such tight packing, the ACh receptor plus AChE occupy only about one-fourth to one-third of the membrane surface. Even if the maximal number of ACh receptor sites prevailed (*i.e.*, $10^4/\mu^2$), there is theoretically still enough space in the same plane of the membrane to accommodate the $6.5 \times 10^3/\mu^2$ esterase sites which are not AChE (53), assuming a similar molecular weight per esterase site. Such tight packing is unlikely however. Histochemical (17) as well as EM autoradiographic studies (53) have recently suggested a broader distribution for the non-AChE

sites. This discussion does not assume insertion of the molecule into the membrane, merely the occupation of space on the surface plane of the membrane.

Our estimate of the percent surface area occupied by the receptor (*i.e.*, ~15%, or at most ~20%) is lower than that made for frog sartorius and mouse diaphragm muscles (7, 42). On the other hand in *Electrophorus* electroplax, the estimates varied from 5% (33) to almost 100% of the postsynaptic membrane (10) depending largely on each group's calculations of the percentage of the area of the innervated membrane that is postsynaptic.

All of the above calculations on the number of ACh receptor sites assume that they are equivalent to the number of bound α-bungarotoxin. Recent findings suggest that this neurotoxin binds to and affects the catalytic reaction of membrane-bound AChE of the mouse diaphragm (56). This probably means that the receptor and esterase molecules are closely associated to the point of affecting each other's reactions. The possibility that AChE has α-bungarotoxin binding sites which are different from those of the ACh receptor, although originally entertained, is unlikely on the basis of the separation of AChE from the α-bungarotoxin binding molecule.[3]

RELATIONSHIP BETWEEN CONCENTRATIONS OF ACHE, ACH RECEPTOR AND ACH RELEASED DURING NEUROMUSCULAR TRANSMISSION

The sizes of the end plate compartments also allow us to speculate on the relationship between ACh released per nerve end plate which produces an EPP and the active sites of AChE and ACh receptor. Potter (46) reported that there are about 3×10^6 molecules (5×10^{-18} moles) of ACh released/nerve impulse/rat diaphragm end plate—a value slightly lower than that of 10^{-17} moles given previously by Krnjevic and Mitchell (38). If we use the simplifying assumption that the released ACh follows a square pulse with no leakage of ACh or hydrolysis and gets uniformly distributed throughout the cleft, we can use Potter's value for released ACh (*i.e.*, 3×10^6 molecules) and the volume of the cleft in the rat diaphragm end plate given in Table I (*i.e.*, 450 μ^3) to arrive at a concentration of 1.1×10^{-5} *M* for ACh in the cleft. If we consider the 7.2×10^3 ACh receptor/μ^2 and the 2.5×10^3 AChE/μ^2 as being in the plane of the membrane (*i.e.*, being within a zone less than 100 Å thick), rather than distributed throughout the cleft, then the local concentration of ACh receptor and AChE would be about 1.2×10^{-3} and 5.7×10^{-4} *M*, respectively. This is 100- and 50-fold higher in concentration, respectively, than that of the local concentration of ACh. With loss of ACh by lateral diffusion, this excess of ACh receptor and AChE over ACh could be expected to be even higher.

A similar ratio for ACh to ACh receptor may prevail during a miniature end plate potential (MEPP), which is a localized depolarization (~0.5 mV) (24), giving a membrane conductance of $1\text{–}2 \times 10^{-7}$ mho (59). This occurs in the end plate as a result of the spontaneous release of a quantum of ACh (10^4 molecules) (35). A quantum is thus ~0.3% of the total ACh needed to produce an EPP in the rat diaphragm. Eccles (19) has suggested that a quantum of ACh diffuses to only 10–20 μ^2 of the postsynaptic surface, which in the rat diaphragm represents 0.2% of the total area calculated here. If Eccles' estimate is correct, the molar concentration of ACh at the receptor sites would be similar during an MEPP or an EPP. If true, this may explain why desensitization is produced not only by high dosages of ACh or agonists (36, 60) or by repetitive nerve stimulation (61) but also may result from maintaining for 10–20 sec the iontophoretic application of a small dosage of ACh producing a constant localized depolarization of only 0.5–1 mV (36). This would mean that lateral diffusion of the applied ACh is limited.

Our conclusion that there is considerable excess of AChE over ACh is contrary to the arguments of Barnard and Rogers (5) but more in line with the later views expressed by Barnard *et al.* (7). Regarding the ratio of ACh to ACh receptor we agree with the models of Ariens (1), Stephenson (57) and Furchgott (25), which assume the presence of spare receptor reserve and suggest that a maximal response may be produced with only a small fraction of the receptors occupied by the activator. By contrast Negrette *et al.* (43) claim that, at least for an MEPP, the ACh supersaturates the local receptor level. Barnard *et al.* (7) recognized a

[3] Eldefrawi ME: Unpublished data.

6-fold excess of ACh receptor to ACh but estimated that functionally this excess was less than 2-fold, while Miledi and Potter (42) estimated a 1000-fold excess of ACh receptors. Our data lead us to an intermediate position. At present we have no good estimate to what extent these excess sites are required for optimal function and to what extent they merely represent a nonfunctional reserve.

Having calculated a concentration of ACh prevailing in the cleft during neuromuscular transmission, we can consider the kinetics of the interaction between ACh and ACh receptor. The affinity (or association constant, K_A) of ACh for its receptor is the reciprocal of the dissociation constant, K_D (*i.e.*, the concentration of ACh that gives 50% response). Using the electrophysiologically obtained figures relating the concentration of applied ACh to the observed response, apparent K_D values for ACh can be calculated. Such calculated K_D values are 5×10^{-8} M in smooth muscles (12, 55) and 10^{-7} M in skeletal muscles (32). By comparison, the K_D of ACh from particulate fractions of *Torpedo* electroplax, containing ACh receptors determined directly by the *in vitro* binding of ACh to the major portion of receptor sites, is 6.8×10^{-8} M (21).

These K_D values are about two orders of magnitude smaller than our calculated concentration for ACh ($\sim 10^{-5}$ M) prevailing in the end plate following nerve stimulation. Our calculation is in line with the observed dose response curve for ACh on the frog rectus abdominis (62), where K_D is approximately one order of magnitude less than the ACh concentration required to give maximal response. The affinity data thus indicate that the concentration of ACh released in the cleft is in excess of that needed for maximal response in the postsynaptic membrane. We should also note that these K_D values are at least 100-fold lower than the estimated K_m values of AChE for ACh (3), which would indicate that the percentage of receptor sites occupied by ACh would be higher than those of AChE.

It is obvious that AChE should be capable of hydrolyzing almost all of the ACh released into the cleft within 1–2 msec of the opening of ion channels, otherwise a sustained depolarization would ensue from occupation of additional ACh receptors. Negrette *et al.* (43) have shown that the activation of the end plate in response to iontophoretic application of small pulses of ACh spreads when AChE is inhibited by prior treatment with eserine sulfate.

The turnover number for purified AChE from *Electrophorus* electroplax was given as 7.6–8.8 $\times 10^5$ molecules/catalytic site/min at 25°C (50) or an average of 13.7 molecules/catalytic site/msec at the optimal ACh concentration of $2.7 \times 10^{-3} M$. However, based on the size of the cleft calculated above (Table I) and the value of Potter for the number of ACh molecules released per impulse, the concentration of ACh in the cleft during transmission is only 1.1×10^{-5} M. At this low concentration, the rate of hydrolysis should be lower than the maximal rate, as shown by the following Michaelis equation:

$$v = \frac{V}{K_s} \cdot S \qquad (3)$$

where v = the hydrolysis rate, V = the maximal hydrolysis rate (*i.e.*, at optimal ACh concentration), K_s = the dissociation constant and S = the existing ACh concentration. Assuming that $K_s = K_m$ (where K_m is the substrate concentration giving one-half the maximal rate), we arrive at a hydrolysis rate (when S of ACh = 1.1×10^{-5} M) equal to 0.3 ACh molecules hydrolyzed/catalytic site/msec (*i.e.*, only ~2% of the maximal rate).

We attempted to test this relationship experimentally using the Ellman *et al.* (22) method for determining hydrolysis of acetylthiocholine by AChE from *Electrophorus* electroplax (from Worthington Biochemicals). At a substrate concentration of 10^{-5} M, the K_m value was 5×10^{-4} M and the observed hydrolysis rate was 2% of the maximal rate. Therefore, if the AChE in the end plate were in solution, then the 1.7×10^7 AChE-active sites present in the postneural region of the rat diaphragm end plate (*i.e.*, 2.5×10^3 sites/μ^2 (53) $\times$ 7000 μ^2 of postsynaptic membrane (Table I)) would be capable of hydrolyzing $\sim 5 \times 10^6$ ACh molecules in 1 msec. Therefore, the AChE of the end plate would hydrolyze all of the released ACh in less than 1 msec. If the nonspecific cholinesterases of the end plate are also contributing, the removal of ACh would be even faster. However, it should be noted that AChE of the end plate is membrane-bound, which may have different kinetic

properties than if in solution (20). Also in studies on the activity of AChE, the concentration of substrate used is several orders of magnitude higher than the enzyme concentration, while the opposite is true in the end plate during transmission.

The fastest vertebrate muscles on record, the extraocular muscles, can contract at 350–400 times/sec before attaining mechanical fusion and have contraction times of about 6–7.5 msec, with similar relaxation times (11, 16). In other muscles these contraction and relaxation times are considerably larger (14, 16, 26). The rat diaphragm muscle can contract at stimulation rates of 20 times/sec (37) and sustains tetany at a stimulation rate up to 200 stimuli/sec (2). At the turnover rate of soluble AChE the concentration of AChE prevailing in the diaphragm cleft is sufficient to hydrolyze the released ACh in less time than is required even by the very fast eye muscles. Even with the expected decrease in catalytic rate of the bound enzyme, removal of ACh is probably not a limiting factor in determining speed of muscle response in either the diaphragm or sternomastoid. The excess of AChE may be required for optimal function in sustaining high frequency tetanic response (2).

CONCLUDING REMARKS

Using the simple relation (*i.e.*, total sites divided by density of sites gives size) we have calculated the area of postsynaptic membrane and the volume of the cleft in several mouse and rat end plates. These values enabled us to convert data on ACh receptor (α-bungarotoxin binding sites) in rat and mouse diaphragm to densities per square micron of postsynaptic membrane and to compare these with previously published densities of AChE at this membrane. The receptor density varied less between different size end plates than was suggested by published whole end plate data.

We conclude that the prevailing concentration of AChE is sufficient to clear the cleft of ACh released during neuromuscular transmission within 1 msec, that both AChE sites and ACh receptor sites are present in considerable excess of released ACh and that the ACh is itself released in excess of that needed for optimal response and finally that there is sufficient room in the plane of the postsynaptic membrane to accommodate ACh receptor, AChE and other esterase sites.

We must emphasize that the above calculations were based on several assumptions. First is the assumption that the ACh receptors, which are used during neuromuscular transmission, are uniformly distributed over the postsynaptic membrane. Such an assumption ignores the not unlikely possibility of a gradient of receptors within the junctional folds. Second is the assumption that the ACh released during an EPP becomes uniformly distributed within the cleft. Furthermore, since the value of ACh molecules released per neuromuscular EPP is unfortunately available for only the rat diaphragm, all our detailed calculations relating ACh with its receptor and AChE were restricted to this end plate. The general applicability of this discussion must await more knowledge on ACh released at different end plates and on the true distribution of ACh receptor in the postsynaptic junctional folds.

ACKNOWLEDGMENTS

We wish to thank Ms. Teresa Feng, Ms. Julie Matthews and Dr. A. T. Eldefrawi for their help.

REFERENCES

1. Ariens EJ: Affinity and intrinsic activity in the theory of competitive inhibition. Part I. Problems and theory. Arch Int Pharmacodyn 99:32, 1954
2. Ariens AT, Meeter E, Wolthuis OL, van Benthem RMJ: Reversible necrosis at the end-plate region in striated muscles of the rat poisoned with cholinesterase inhibitors. Experientia 25:57, 1969
3. Augustinsson KB: Classification and comparative enzymology of the cholinesterases and methods for their determination, Handbuch der Experimentelle Pharmakologie. Edited by GB Koelle. Vol 15. Springer-Verlag, Berlin, 1963, p 89
4. Bahr GF, Bloom G, Friberg U: Volume changes of tissues in physiological fluids during fixation in osmium tetroxide or formaldehyde and during subsequent treatment. Exp Cell Res 12:342, 1957
5. Barnard EA, Rogers AW: Determination of the number, distribution and some in situ properties of cholinesterase molecules in the motor endplates, using labeled inhibitor methods. Ann NY Acad Sci 144:584, 1967
6. Barnard EA, Rymaszewska T, Wieckowski J: Cholinesterases at individual neuromuscular junctions, Cholinergic Ligand Interactions. Edited by DJ Triggle, JF Moran, EA Barnard. Academic Press, New York, 1971, p 175
7. Barnard EA, Wieckowski J, Chiu TH: Cholinergic

receptor molecules and cholinesterase molecules at mouse skeletal muscle junctions. Nature (Lond) 234:207, 1971
8. Berg DK, Kelly RB, Sargent PB, Williamson P, Hall ZW: Binding of α-bungarotoxin to acetylcholine receptors in mammalian muscle. Proc Natl Acad Sci USA 69:147, 1972
9. Blaurock AE, Worthington CR: Low-angle x-ray diffraction patterns from a variety of myelinated nerves. Biochim Biophys Acta 173:419, 1969
10. Bourgeois JP, Ryter A, Menez A, Fromageot P, Boguet P, Changeux JP: Localization of the cholinergic receptor protein in Electrophorus electroplax by high resolution autoradiography. FEBS Letters 25:127, 1972
11. Brown GL, Harvey AM: Neuro-muscular transmission in the extrinsic muscles of the eye. J Physiol 99:379, 1941
12. Burgen ASV: The role of ionic interaction at the muscarinic receptor. Br J Pharmacol 25:4, 1965
13. Chang CC, Lee CY: Isolation of neurotoxins from the venom of Bungarus multicinctus and their modes of neuromuscular blocking action. Arch Int Pharmacodyn 144:241, 1963
14. Close R: Properties of motor units in fast or slow skeletal muscles of the rat. J Physiol 193:45, 1967
15. Cole WV: Structural variations of nerve endings in the striated muscles of the rat. J Comp Neurol 108:445, 1957
16. Cooper S, Eccles JC: The isometric responses of mammalian muscles. J Physiol 69:25, 1930
17. Csillik B, Knyihar E: On the effect of motor nerve degeneration on the fine-structural localization of esterases in the mammalian end-plate. J Cell Sci 3:529, 1968
18. Dickerson RE, Kopka ML, Weinzierl JE, Varnum JC, Eisenberg D, Margoliash E: Location of the heme in the horse heart ferricytochrome c by x-ray diffraction. J Biol Chem 242:3015, 1967
19. Eccles JC: The ionic mechanisms of excitatory and inhibitory synaptic action. Ann NY Acad Sci 137:473, 1966
20. Ehrenpreis S, Hehir RM, Mittag TW: Assay and properties of essential (junctional) cholinesterases of the rat diaphragm, Cholinergic Ligand Interactions. Edited by DJ Triggle, JF Moran, EA Barnard. Academic Press, New York, 1971, p 67
21. Eldefrawi ME, Eldefrawi AT, Gilmour LP, O'Brien RD: Multiple affinities for binding of cholinergic ligands to a particulate fraction of Torpedo electroplax. Mol Pharmacol 7:429, 1971
22. Ellman GL, Courtney KD, Andreas V Jr, Featherstone RM: A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol 7:88, 1961
23. Fambrough DM, Hartzell HC: Acetylcholine receptors: number and distributions at neuromuscular junctions in rat diaphragm. Science 176:189, 1972
24. Fatt P, Katz B: Spontaneous subthreshold activity at motor nerve endings. J Physiol 117:109, 1952
25. Furchgott RF: The use of β-haloalkylamines in the differentiation of receptors and in the determination of dissociation constants of receptor-agonist complexes, Advances in Drug Research. Edited by NJ Harper, AB Simmonds. Vol 3. Academic Press, New York, 1966, p 21
26. Gainer H, Klancher JE: Neuromuscular junctions in a fast-contracting fish muscle. Comp Biochem Physiol 15:159, 1965
27. Gauthier FG, Padykula HA: Cytological studies of fiber types in skeletal muscle. A comparative study of the mammalian diaphragm. J Cell Biol 28:333, 1966
28. Hall ZW: Release of neurotransmitters and their interaction with receptors. Ann Rev Biochem 41:925, 1972
29. Hampton JC: Effects of fixation on the morphology of Paneth cell granules. Stain Technol 40:283, 1965
30. Hegarty PM, Hooper AC: Sarconerve lengths and fiber diameter distribution in four different mouse skeletal muscles. J Anat 110:249, 1971
31. Hopwood D: Some aspects of fixation with glutaraldehyde. J Anat 101:83, 1967
32. Jenkinson DH: The antagonism between tubocurarine and substances which depolarize the motor end-plate. J Physiol 152:309, 1960
33. Karlin A, Prives J, Deal W, Winnik M: Affinity labeling of the acetylcholine receptor in the electroplax. J Mol Biol 61:175, 1971
34. Karnovsky MJ, Roots L: A "direct-coloring" thiocholine method for cholinesterases. J Histochem Cytochem 12:219, 1964
35. Katz B: The Release of Neural Transmitter Substances. Charles C Thomas Publisher, Springfield, Ill., 1969, p 10
36. Katz B, Thesleff S: A study of the "desensitization" produced by acetylcholine at the motor endplate. J Physiol 138:63, 1957
37. Krnjevic K, Miledi R: Motor units in the rat diaphragm. J Physiol 140:427, 1958
38. Krnjevic K, Mitchell JF: The release of acetylcholine in the isolated rat diaphragm. J Physiol 155:246, 1961
39. Kushida H: A study of cellular swelling and shrinkage during fixation, dehydration and embedding in various standard media, Proceedings of the 5th International Congress on Electron Microscopy, Philadelphia, 1962, P-10
40. Leuzinger W: The number of catalytic sites in acetylcholinesterase. Biochem J 123:139, 1971
41. Miledi R, Molinoff P, Potter LT: Isolation of the cholinergic receptor protein of Torpedo electric tissue. Nature (Lond) 229:554, 1971
42. Miledi R, Potter LT: Acetylcholine receptors in muscle fibres. Nature (Lond) 233:599, 1971
43. Negrette J, Del Castillo J, Escobar I, Yankelevich G: Spreading activation of end-plate receptors by single transmitter quanta. Nature [New Biol] 235:158, 1972
44. O'Brien RD, Eldefrawi ME, Eldefrawi AT: Isolation of acetylcholine receptors. Ann Rev Pharmacol 12:19, 1972
45. Padykula HA, Gauthier GF: The ultrastructure of

the neuromuscular junctions of mammalian red, white and intermediate skeletal muscle fibers. J Cell Biol 46:27, 1970
46. Potter LT: Synthesis, storage and release of C^{14} acetylcholine in isolated rat diaphragm muscles. J Physiol (Lond) 206:145, 1970
47. Potter LT, Molinoff PB: Isolation of cholinergic receptor proteins, Perspectives in Neuropharmacology. Edited by SH Snyder. Oxford University Press, London, 1972, p 9
48. Rogers AW, Darzynkiewicz Z, Barnard EA, Salpeter MM: Number and location of acetylcholinesterase molecules at motor endplates of the mouse. Nature (Lond) 210:1003, 1966
49. Rogers AW, Darzynkiewicz Z, Salpeter MM, Ostrowski K, Barnard EA: Quantitative studies on enzymes in structures in striated muscles by labeled inhibitor methods. I. The number of acetylcholinesterase molecules and of other DFP-reactive sites at motor endplates measured by radioautography. J Cell Biol 41:665, 1969
50. Rosenberry TL, Chang HW, Chen YT: Purification of acetylcholinesterase by affinity chromatography and determination of active site stoichiometry. J Biol Chem 247:5, 1972
51. Salpeter MM: Electron microscope radioautography as a quantitative tool in enzyme cytochemistry. I. The distribution of acetylcholinesterase at motor endplates of a vertebrate twitch muscle. J Cell Biol 32:379, 1967
52. Salpeter MM: Electron microscope radioautography as a quantitative tool in enzyme cytochemistry. II. The distribution of DFP-reactive sites at motor endplates of a vertebrate twitch muscle. J Cell Biol 42:122, 1969
53. Salpeter MM, Plattner H, Rogers AW: Quantitative assay of esterases in end plates of mouse diaphragm by electron microscope autoradiography. J Histochem Cytochem 20:1059, 1972
54. Salpeter MM, Szabo M: Sensitivity in electron microscope autoradiography. I. Effect of radiation dose. J Histochem Cytochem 20:425. 1972
55. Sastry BVR, Cheng HC: Dissociation constants of D- and L-lactoylcholines and related compounds at cholinergic receptors. J Pharmacol Exp Ther 180:326, 1972
56. Stalc A, Zupancic AO: Effect of α-bungarotoxin on acetylcholinesterase bound to mouse diaphragm end plates. Nature [New Biol] 239:91, 1972
57. Stephenson RP: A modification of receptor theory. Br J Pharmocol Chemother 11:379, 1956
58. Styckowski AJ, Vogel Z, Nirenberg MW: Development of acetylcholine receptor clusters on cultured muscle cells. Proc Natl Acad Sci USA 70:270, 1973
59. Takeuchi A, Takeuchi N: On the permeability of end-plate membrane during the action of transmitter. J Physiol 154:52, 1960
60. Thesleff S: The mode of neuromuscular block caused by acetylcholine, nicotine, decamethonium and succinylcholine. Acta Physiol Scand 34:218, 1955
61. Thesleff S: Motor end-plate "desensitization" by repetitive nerve stimuli. J Physiol 148:659, 1959
62. Van Maanen EF: The antagonsim between acetylcholine and the curare alkaloids D-tubocurarine, c-curarine-I, c-toxiferine-II and β-erythroidine in the rectus abdominis of the frog. J Pharmacol Exp Ther 99:255. 1950
63. Wilson IB, Froede HC: The design of reactivators for irreversibly blocked acetylcholinesterase, Drug Design. Vol 2. Edited by EJ Ariens. Academic Press, New York, 1971, p 213