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In Vitro Excitation of Purified Membrane Fragments by Cholinergic Agonists

III. Comparison of the Dose-Response Curves to Decamethonium with the Corresponding Binding Curves of Decamethonium to the Cholinergic Receptor

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Summary. The reversible binding of ^{14}C-decamethonium (Deca) to excitable microsacs prepared from the electric tissue of *Electrophorus electricus* is followed by an ultracentrifugal assay. α-Bungarotoxin, a snake venom toxin, blocks irreversibly the binding of ^{14}C-Deca. The displacement is partial. The fraction of ^{14}C-Deca displaced by α-bungarotoxin corresponds to molecules of Deca bound to the cholinergic receptor site, whereas the fraction of ^{14}C-Deca bound in the presence of α-bungarotoxin corresponds to molecules bound to the catalytic site of acetylcholinesterase (AcChE). The total number of cholinergic receptor sites is found to be close but not identical to the total number of catalytic sites of AcChE.

On the same preparation of microsacs, the binding of ^{14}C-Deca and the permeability response corresponding to a given concentration of Deca are measured as a function of increased concentration of Deca. The dose-response curve and the binding curve superimpose almost exactly; in other words, the "apparent" affinity of Deca coincides with its "real" affinity. Displacement of ^{14}C-Deca by *d*-tubocurarine gives an "apparent" affinity for *d*-tubocurarine which coincides as well with its "real" affinity.

The transport properties of the ionophore controlled by one Deca binding site are estimated.

It has always been a challenge for pharmacologists to correlate the observed response of a tissue or of a cell to a chemical signal with the actual binding of the signal to its specific receptor site. In general, a sequence of numerous events occurs between the initial interaction of the effector with its pharmacological receptor and the observed effect. It is a particular convenience in using the excitable microsacs that, on the same preparation, the binding of a cholinergic agonist *and* the permeability response, which is believed to be the primary response of the excitable membrane to the cholinergic agonist, can be followed in parallel.

The measure of the reversible binding of a cholinergic agonist to the cholinergic receptor does not appear at present to be a task as formidable as it was thought for many years. In particular, the use of equilibrium dialysis techniques (Changeux, Leuzinger & Huchet, 1968; O'Brien & Gilmour, 1969; O'Brien, Gilmour & Eldefrawi, 1970; Changeux, Kasai, Huchet & Meunier, 1970*a*) rendered possible the detection of significant and reproducible binding of cholinergic agonist to either subcellular fragments or soluble extracts of electric organ. In order to demonstrate that this binding occurs specifically and exclusively at the level of the cholinergic receptor, Changeux, Kasai and Lee (1970*b*) took advantage of the exceptional properties of a snake venom toxin, α-bungarotoxin, which was shown to act like an irreversible curare at the neuromuscular junction (Lee & Chang, 1966), on the isolated electroplax or on excitable microsacs (Changeux, Kasai & Lee, 1970*b*). By chance, the purified toxin, a polypeptide of molecular weight 8,000, has no detectable effect on the catalytic activity and affinity of acetylcholinesterase (AcChE). On the other hand, it blocks the binding of a cholinergic agonist, decamethonium (Deca), to a protein present in crude extracts of electric organ which binds as well (and with a high affinity), two typical cholinergic antagonists, *d*-tubocurarine and flaxedil. This protein is considered to be the cholinergic receptor. Changeux *et al.* (1970*b*) concluded that α-bungarotoxin (α-Bgt), as well as other α-toxins from different snakes (Boquet & Changeux, *unpublished results*), is a specific and exclusive reagent for the physiological receptor of acetylcholine.

In this paper, using an ultracentrifugal assay (*see* O'Brien & Gilmour, 1969), we show that Deca binds reversibly to the excitable microsacs and that a large amount of bound Deca is displaced by α-Bgt. The amount displaced thus corresponds to molecules of Deca attached to the cholinergic receptor site. A binding curve of Deca to the receptor sites present in the microsacs membrane is then established and compared to the response curve of the same microsacs to Deca. The two curves superimpose almost exactly. We are thus in a position to measure the permeability change which corresponds to a given occupancy of cholinergic receptor sites by Deca and, as a consequence, to propose an estimate of the transport properties of the ionophore(s) associated with a single Deca binding site. The results are finally discussed in terms of the allosteric transition of the cholinergic protomer.

Materials and Methods

Excitable microsacs were prepared, following exactly the method described in paper I, by ultracentrifugation of 25 ml of a low-speed supernatant on top of a discontinous gradient made of 5 ml of 1.4 M sucrose and 5 ml of 0.4 M sucrose. The only

modification introduced was that, in order to increase the yield, the crude Virtis homogenate was submitted to ultrasonic vibrations in a Mullard apparatus for 1 min at 0 °C before ultracentrifugation.

Binding of ^{14}C-Deca (methyl ^{14}C-decamethonium bromide, 20.9 mC/mmole, from the Radiochemical Centre, Amersham, England) to excitable microsacs in the presence of physiological Ringer's solution was measured by ultracentrifugation in the following manner. The suspension of excitable microsacs in approximately 0.7 M sucrose and containing, for example, 6.8 mg of membrane protein per ml, was first diluted at room temperature in an equal volume of a saline solution containing the constituent ions of the Ringer's solution but at twice the concentration.

The salt composition of the diluted microsac suspension was thus that of the physiological solution. It contained 1.6×10^{-1} M NaCl, 5×10^{-3} M KCl, 2×10^{-3} M $CaCl_2$, 2×10^{-3} M $MgCl_2$, 2.5×10^{-3} M sodium phosphate, buffered at pH 7.0 *and* 0.35 M sucrose. In a typical experiment, the final protein concentration was 3.4 mg/ml and that of AcChE, 4.65 moles of acetylthiocholine hydrolyzed per hr per g protein at 27 °C, i.e.: 8.1×10^{-8} moles of AcChE per g protein, assuming a molecular weight of 260,000 and a turnover number of 750 moles of acetylthiocholine per hr per g protein. The microsac suspension was supplemented with ^{14}C-Deca at the indicated concentration. Then 0.5 ml of the radioactive suspension was centrifuged in a Rotor 40 of a Beckman model L ultracentrifuge at 40,000 RPM for $1^1/_2$ hr, at 20 °C, in polycarbonate tubes, type "Oak Ridge", with a screw cap of IEL. After centrifugation the supernatant was carefully separated from the pellet. The inside walls of the centrifuge tube were then washed with Ringer's solution and dried by air. The volume of the pellet was estimated by weighing the tube and was found to be, under our experimental conditions, close to 20 μliters, assuming for the pellet a density of one. The pellet was finally solubilized by incubation in 0.4 ml of 5% sodium dodecyl sulfate overnight at 40 °C. Counting of the radioactive suspension *before* centrifugation gave an estimate of the total Deca concentration. The *free* concentration of Deca was obtained by counting the radioactivity of the supernatant *after* centrifugation. The amount of Deca bound was given by the difference of the above values; it was also measured directly by counting the radioactivity of the solubilized pellet, knowing the volume of the pellet and therefore the free concentration of Deca within the pellet. Under these conditions, almost 3% of total AcChE and 5% of total protein were found in the supernatant solution after centrifugation, which means that the separation of the membrane fragments from the medium in which they were suspended was excellent.

For *counting of radioactivity*, 100 μliters of radioactive solution was added to 10 ml of Bray solution (naphthalene 60 g, PPO 4 g, POPOP 0.2 g, methanol 100 ml, ethyleneglycol 20 ml, dioxane up to 1 liter). The flasks were subsequently counted in a Packard liquid scintillation counter (gain 25%, window width 0.050 to 1.000). The self quenching by the proteins was found to be less than 1%.

Efflux of $^{22}Na^+$ from excitable microsacs was measured as previously described (*see* paper I).

Proteins were measured by the Folin reagent using bovine serum albumin as the standard.

Results and Discussion

The Two Classes of Deca Binding Sites Present in Excitable Microsacs

In Fig. 1 is represented a binding curve of ^{14}C-Deca to excitable microsacs. In this experiment the binding of Deca was followed by an ultracentrifugal assay (described in Methods) which, in many respects, resembles

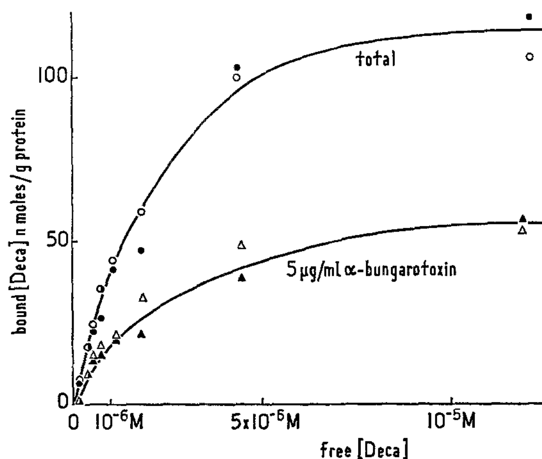

Fig. 1. Reversible binding of ^{14}C-Deca to excitable microsacs as measured by the ultracentrifugal assay. Conditions are given in Methods. *Upper curve*, the total amount of Deca bound is plotted as a function of free Deca concentration. *Lower curve*, 30 min before addition of ^{14}C-Deca, the membrane suspension was supplemented with 5 µg/ml (6.25×10^{-7} M) α-bungarotoxin and then centrifuged. ○ or △, bound Deca was estimated from measurements of the radioactivity of the pellet. ▲ or •, bound Deca was estimated from measurements of the radioactivity of the supernatant. Preparation no. 24

the one used by O'Brien and Gilmour (1969). With membrane fragments, this technique appeared to be more reliable and convenient than millipore filtration or equilibrium dialysis, this latter method being very useful with soluble preparations of receptor. In a typical experiment, the membrane fragments were first equilibrated with Deca in a medium which had the same ionic composition as the physiological Ringer's solution. They were then centrifuged at high speed, and the amount of Deca bound to the membrane fragments at a given concentration of free Deca was readily computed from the radioactivity of the supernatant and from that of the pellet. Under our assay conditions, the displacements were large enough to be measured with some accuracy. For example, with a suspension of microsacs containing 3.4 mg of membrane portein per ml, 1,800 counts/10 min/100 µliters of suspension were displaced over 13,200 counts/10 min/100 µliters of the corresponding solution of free Deca (5×10^{-7} M).

Let us first consider the total quantity of Deca bound to the membrane fragments as a function of increasing concentrations of free Deca (Fig. 1, *upper curve*). The curve drawn from these data tends to a plateau at high Deca concentration, corresponding to the saturation by Deca of a limited number of binding sites. However, the double reciprocal plot as well as the Scatchard plot of the same data significantly deviate from the straight line

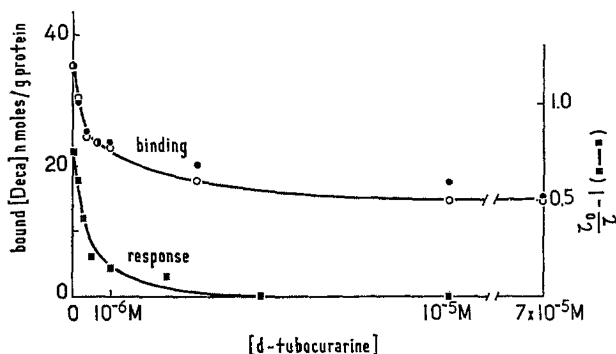

Fig. 2. Displacement of ^{14}C-Deca bound to excitable microsacs by *d*-tubo. The conditions are given in Methods. Symbols are the same as for Fig. 1. The concentration of ^{14}C-Deca added to the suspension was 8×10^{-7} M. Also in this figure we have plotted the permeability response of the excitable microsacs as measured by following $^{22}Na^{+}$ efflux according to the standard method with the same membrane fragments and in the presence of the same concentration of Deca. Preparation no. 24

expected for a simple Langmuir isotherm. The analysis of this systematic deviation suggested to us that *several classes* of Deca binding sites were present in the excitable microsacs.

In order to distinguish between these different classes of sites, we performed the same binding experiment after exposure of the membrane fragments to α-Bgt.

Fig. 1 *(lower curve)* shows the result of this experiment: α-Bgt blocked the binding of Deca but blocked it only partially, even at high levels of α-Bgt. We then tested the effect of two typical reversible cholinergic antagonists, *d*-tubocurarine (*d*-tubo) and flaxedil. As illustrated in Figs. 2, 3 and 4, *d*-tubo or flaxedil, although not structurally related to α-Bgt, blocked Deca binding exactly to the *same* maximal extent. With the particular membrane preparation used, in the presence of 5×10^{-7} M free Deca, only 65% of the total quantity of Deca bound was displaced by saturating levels of any one of these effectors. In addition, after exposure to an excess of α-Bgt, no further displacement of bound Deca beyond 65% was observed in the presence of up to 5×10^{-5} M *d*-tubo or flaxedil.

The only common property of α-Bgt, *d*-tubo and flaxedil is, as already discussed, that they bind tightly to the cholinergic receptor site. It is thus very likely, and this will be confirmed later, that the quantity of Deca which is bound to the membrane fragments and displaced by α-Bgt, *d*-tubo or flaxedil does correspond to molecules of Deca which are actually associated with the cholinergic receptor sites.

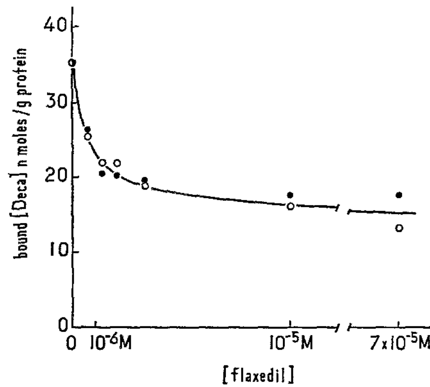

Fig. 3. Displacement by flaxedil of ^{14}C-Deca bound to excitable microsacs as measured by the ultracentrifugal assay. Same conditions and symbols as for Fig. 2. Preparation no. 24

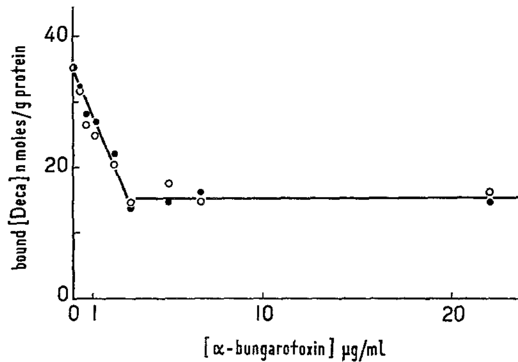

Fig. 4. Irreversible blocking by α-bungarotoxin of ^{14}C-Deca binding to excitable microsacs. Same conditions and symbols as for Fig. 2 except that the membrane suspension was incubated 30 min before centrifugation with the indicated concentration of α-Bgt. 1 μg/ml α-Bgt $= 1.25 \times 10^{-7}$ M. Preparation no. 24

We then tried an identification of the membrane macromolecule to which Deca binds in the presence of α-Bgt or *d*-tubo. The membrane fragments contain high levels of AcChE (up to 1 % of the proteins). It is well established both by measurements of enzymatic activity and by equilibrium dialysis that the catalytic center of AcChE presents, under our assay conditions (high salts), a high affinity for Deca but a very low one for *d*-tubo or flaxedil (*see* Table 1). In addition, as already mentioned, α-Bgt has strictly no effect on the catalytic activity and affinity of AcChE for its substrates. We immediately thought that this "residual" amount of bound Deca was associated with the active site of AcChE. We shall present here two additional observations which confirm this interpretation.

Table 1. *Real or apparent dissociation constants of a few cholinergic effectors from the cholinergic receptor site and from the catalytic site of AcChE*

Effector	Binding of ^{14}C-Deca to microsacs at 22 °C in the presence or absence of effector	Efflux of $^{22}Na^{+}$ from microsacs at 22 °C	Receptor protein in solution at 4 °C	AcChE with acetyl-thiocholine as the substrate at 22 °C
Deca-methonium	Receptor 1.3×10^{-6} M	1.2×10^{-6} M	0.8×10^{-6} M	
	AcChE 2.5×10^{-6} M			2.5×10^{-6} M
Carbamyl-choline	Receptor 2.2×10^{-5} M	3.3×10^{-5} M	1.83×10^{-5} M	1.7×10^{-5} M
d-Tubo-curarine	Receptor 2.0×10^{-7} M	1.5×10^{-7} M	2.6×10^{-6} M	5.0×10^{-5} M
Flaxedil	Receptor 4.0×10^{-7} M	3.3×10^{-7} M	2.2×10^{-6} M	1.3×10^{-4} M

Data from Figs. 1, 2, 3 and 5. The fraction of bound Deca which is displaced by α-Bgt is, as discussed in the text, assumed to belong to the cholinergic receptor site, the rest to the catalytic site of AcChE. The response of the microsacs to cholinergic effectors was measured by following $^{22}Na^{+}$ efflux as described in paper I. The data concerning the cholinergic receptor protein in solution and the catalytic center of AcChE are those of Changeux, Kasai, Huchet & Meunier, 1970. Preparation no. 24, submitted to ultrasounds. The dissociation constant for effects other than Deca were always estimated *indirectly* by displacement of ^{14}C-Deca bound to the membrane fragments.

(1) There exists a clear-cut correlation, illustrated in Fig. 6 (right), between the content of the microsacs in AcChE measured by an enzymatic test and the quantity of radioactive Deca which remained bound to the membrane fragments in the presence of α-Bgt (the "residual" Deca or "bound (Deca) α-B" in the figure).

(2) Carbamylcholine (Carb), which is known to bind strongly to the *catalytic center* of AcChE, *completely* displaced Deca bound to the membrane fragments in the presence of α-Bgt (Fig. 5) and at concentrations very close to those which displace the substrate from the catalytic site of the enzyme (see the exact values of the dissociation constants in Table 1).

Complementary evidence is also offered by the recent experiments performed by Meunier, Huchet, Boquet and Changeux (1971) on soluble extracts of electric organ. The same two classes of Deca binding sites are present. By selective heat treatment these authors have obtained a soluble preparation which has lost more than 99% of its AcChE activity but still binds substantial amounts of Deca. Interestingly, Deca bound to this heat-treated preparation is *completely* displaced by α-Bgt or *d*-tubo. In addition, the kinetics of inactivation of AcChE strictly parallel the kinetics of disappearance of the sites which bind Deca in the presence of α-Bgt or *d*-tubo. In this case, and there are excellent reasons to extend this conclusion to

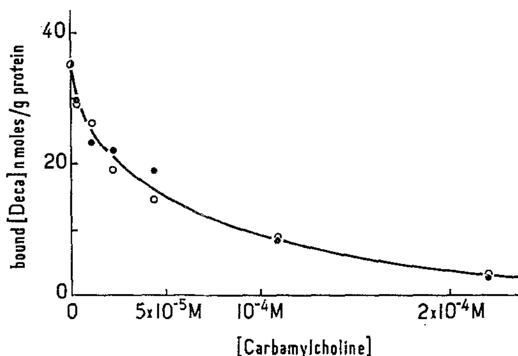

Fig. 5. Complete displacement by Carb of ^{14}C-Deca bound to excitable microsacs. Same conditions and symbols as for Fig. 2. Preparation no. 24

the membrane fragments from which the soluble extracts were made, Deca binds, in the presence of high concentration of salts[1], to only two classes of sites: the cholinergic receptor site and the catalytic center of AcChE. On the basis of their differential affinities for α-Bgt or *d*-tubo, these two classes of sites can easily be distinguished from each other.

Comparison of the Binding Properties, and of the Numbers, of Catalytic Centers of AcChE and of Cholinergic Receptor Sites

Catalytic Centers of AcChE. We have presented a number of arguments which strongly support the conclusion that ^{14}C-Deca bound to the microsacs in the presence of α-Bgt is, in fact, bound by the catalytic site of AcChE. The Scatchard plot or the Lineweaver-Burk plot of the data obtained at increasing concentrations of free Deca are now fitted by straight lines; the population of Deca binding sites studied under these conditions is thus homogeneous with respect to affinity for Deca. As expected, the dissociation constant of Deca measured by this method (2.5×10^{-6} M) was found to be very close to the K_i (2.6×10^{-6} M) of Deca measured by following the hydrolysis of acetylthiocholine by membrane-bound AcChE (Table 1). By extrapolation of the binding data at infinite concentration of substrate (in the two different plots considered), we obtained a preliminary estimate of the number of Deca binding sites carried by AcChE present in the membrane fragments. Values obtained with three different preparations are given in

1 In a previous work (Changeux, Leuzinger & Huchet, 1969) binding of acetylcholine or Deca to extracts of electric organ was performed in the presence of a buffer of low ionic strength. Recent experiments (Changeux *et al.*, 1970*a*) have shown that, under these conditions, a considerable amount of nonspecific binding occurs. At high ionic strengths, this nonspecific binding of Deca is completely eliminated. The same is true for the allosteric sites of AcChE (*see* Changeux, Podleski & Meunier, 1966) which seem to be operative *in vitro* only in conditions of low ionic strength.

Table 2. *Numbers and properties of cholinergic receptor sites and of AcChE catalytic centers present in three preparations of excitable microsacs*

Property	Preparation		
	A	B	C
AcChE, specific activity (mole ATCh/hr/g protein)	4.65	2.9	5.2
AcChE, number of molecules from specific activity (nmole/g protein)	24	15	27
AcChE, number of ^{14}C-Deca binding sites in the presence of α-Bgt (nmole/g protein)	71	44	52
Cholinergic receptor sites number of ^{14}C-Deca binding sites blocked by α-Bgt (nmole/g protein)	59	13	39
α-Bgt binding sites (nmole/g protein)	108	—	—
Kd for ^{14}C-Deca displaced by α-Bgt (cholinergic receptor) $\times 10^{-6}$ M	1.3	1.0	0.8
Kd for ^{14}C-Deca in the presence of α-Bgt (AcChE) $\times 10^{-6}$ M	2.5	2.4	2.4

The three preparations A, B and C were made following the technique described in paper I. Measurements of ^{14}C-Deca binding were performed as described in "Methods". Assay of AcChE was carried out at 27 °C by following the change of optical density at 412 nm of a reaction mixture containing, in addition to the enzyme: 5×10^{-4} M acetylthiocholine (from Sigma), 5×10^{-5} M dithio-bis-dinitrobenzoic acid, and 5×10^{-2} M sodium phosphate at pH 7.0. The number of α-Bgt binding sites was estimated from the titration curve of ^{14}C-Deca binding sites by α-Bgt (Fig. 4). A, preparation no. 24; B, preparation, no. 22; C, preparation no. 23; all were submitted to ultrasounds.

Table 2. We then compared these values to those estimated by measuring the specific activity of AcChE in the membrane fragments. The specific activities given in Table 2 are expressed in moles of *acetylthiocholine* hydrolyzed per hour per gram protein (measured by the method of Folin) at 27 °C. In order to convert these specific activities into numbers of AcChE molecules, several essential assumptions were made. According to Leuzinger and associates (Leuzinger, Goldberg & Cauvin, 1969; Leuzinger, Baker & Cauvin, 1968; Leuzinger & Baker, 1967), the molecular weight of the so-called pure AcChE is 260,000 daltons, and its turnover number 750 moles of *acetylcholine* hydrolyzed per gram protein per hour at a temperature which is not mentioned by the authors. We assume, here, that the molecular weight of the enzyme is correct [although the presence of isozymes might have to be considered seriously (*see* Massoulié & Riéger, 1969)], and that the turnover of the membrane-bound enzyme is still 750, when acetylthiocholine (instead of acetylcholine) is used as the substrate, at 27 °C. Interestingly, we found values which are consistent with those given by the binding of radioactive Deca in the presence of α-Bgt. The accuracy was good enough to allow a comparison of the two sets of results on a quantita-

tive basis. From the results given in Table 2 for three preparations studied, we found 2.9, 2.9 and 1.9 Deca binding sites per molecule of AcChE. The regression line drawn from similar binding and enzymatic data obtained with different preparations gave an average value of 2.7. We thus find that there are about three sites for Deca per molecule of AcChE, a finding which is similar to that reported by O'Brien, Gilmour and Eldefrawi (1970) with a subcellular preparation of *Torpedo* electric organ.

Cholinergic Receptor Sites. The quantity of Deca which is bound by the microsacs and displaced by α-Bgt or *d*-tubo corresponds, as already discussed, to Deca associated with the cholinergic receptor sites. A plot of the binding data as a function of increasing concentration of Deca again approximates roughly a Langmuir isotherm. A dissociation constant could be readily computed from this plot. The values obtained are given in Tables 1 and 2. Interestingly, these values are significantly smaller than those given for the K_i of Deca for the catalytic site of AcChE. In contrast, they are very close to those given in paper I for the "apparent" affinity of Deca as measured by following the response either of the excitable microsacs or of the whole electroplax. They are consistent as well with the values found with the receptor protein isolated in solution. This confirms our earlier conclusion that we are indeed measuring binding of Deca to the cholinergic receptor site.

Additional support for this conclusion is offered by the study of the effect of cholinergic antagonists. After subtraction, from the total amount of Deca bound, of the residual amount which is still bound in the presence of α-Bgt (or of an excess of *d*-tubo or flaxedil), we got curves of displacement by cholinergic antagonists which again did not deviate, within the limit of the accuracy of our experiments, from the simple laws of Henri and Michaelis for competitive antagonism. The K_i measured falls very close to the "apparent" K_i measured on the microsacs or the isolated electroplax. There is still agreement, although a significant difference exists, with the values obtained by equilibrium dialysis with the preparation of soluble receptor protein[2].

Confident in the conclusion that we are indeed measuring binding of Deca to the cholinergic receptor protein integrated in the microsac membrane, we propose values for the total number of cholinergic receptor sites by extrapolating the binding data at infinite ligand concentration (*see* Table 2).

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2 It is likely that in the experiments with the soluble receptor protein the affinity of the antagonists was underestimated: the K_i's were calculated as a function of the total concentration of *d*-tubo or flaxedil added and not as a function of free concentration of antagonists. In addition, the receptor might take, in solution, a different "conformation" than the one favored within the membrane.

We then compare these numbers with those given by another method based on the use of α-Bgt as a specific and irreversible reagent of the cholinergic receptor sites. The titration curve represented in Fig. 4 resembles the one proposed by Changeux *et al.* (1970*b*). However, these authors followed the permeability response as a function of increasing α-Bgt concentration. Here, we measured directly the displacement of ^{14}C-Deca bound to the microsacs as a function of added α-Bgt. Assuming with Changeux *et al.* (1970*b*) (1) that α-Bgt is an irreversible and highly specific reagent of the cholinergic receptor site and (2) that under our experimental conditions, the reaction between α-Bgt and the cholinergic receptor site is complete, we then get the number of α-Bgt molecules required for full titration of the cholinergic receptor sites. Interestingly, this number (Table 2) was consistent with the number of cholinergic receptor sites estimated by ^{14}C-Deca binding. The results are good enough to allow a close comparison of the numbers obtained. In the experiment represented in Fig. 4, we found that approximately two molecules of α-Bgt are necessary to block one Deca receptor site.

Comparison of the number of cholinergic receptor sites and of AcChE catalytic centers in the microsac membrane. Having in hand the number of catalytic sites of AcChE and the number of cholinergic receptor sites in a variety of membrane preparations, we compared both series of numbers to see if some correlation existed between them. First of all, whatever the preparation tested, the two numbers were always on the same order of magnitude. In Fig. 6 we have plotted, for each preparation tested, the number of Deca binding sites blocked by α-Bgt (receptor sites) against the number of binding sites which were *not* blocked by α-Bgt (catalytic centers of AcChE). Line (a) in Fig. 6 (left) corresponds to a ratio of Deca receptor sites/catalytic sites of 0.75, line (b) of 0.41. The numbers of receptor sites and of AcChE catalytic sites present in the membrane fragments are thus very close. Some correlation exists between them but is not absolute: it is not as good as the one we found when we compared the number of AcChE catalytic sites obtained from enzymatic measurements and from binding of ^{14}C-Deca in the presence of α-Bgt (Fig. 6, right).

Comparison of the Dose-Response Curve to Deca with Its Binding Curve

With the microsacs it is possible to measure on the same preparation the binding of a cholinergic agonist and the permeability response to this agonist. In Fig. 7, we have plotted the two sets of data obtained with Deca as the agonist. In order to make the comparison easier, the binding and response curves were normalized to the same maximum value obtained, in both cases, by extrapolation of a Scatchard plot of the data at infinite Deca

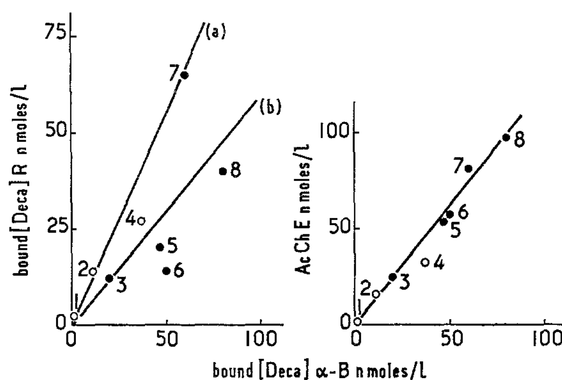

Fig. 6. Comparison of the concentrations of cholinergic receptor sites and of AcChE molecules or catalytic centers in eight microsac preparations of different specific activities. *Right*: The concentration of AcChE molecules in the considered microsac suspension is plotted as a function of the concentration of ^{14}C-Deca bound to the membrane fragments in the presence of 5 µg/ml α-Bgt [bound (Deca) α-B] and 10^{-6} M Deca. The concentration of AcChE molecules was estimated from standard enzymatic measurements using acetylthiocholine (AcTCh) as the substrate (*see* paper I) at 27 °C in the presence of sodium phosphate, 5×10^{-3} M, pH 7.0. We assumed a mol. wt. of 260,000 Daltons and a turnover number of the pure protein of 750 moles AcTCh per g protein per hour. ^{14}C-Deca binding was followed by the standard ultracentrifugal assay in the presence of a 10^{-6} M free ^{14}C-Deca. From these measurements, the total number of Deca binding sites associated with AcChE was calculated on the basis of a dissociation constant of 2.4×10^{-6} M. The slope of the straight line obtained is 1.25; this corresponds to 2.7 binding sites for Deca (at saturation of Deca) per mole of AcChE. *Left*: The difference between the total quantity of ^{14}C-Deca bound to the membrane fragments and the quantity bound in the presence of 5 µg/ml α-Bgt (i.e., ^{14}C-Deca bound to the cholinergic receptor sites) is plotted as a function of ^{14}C-Deca bound to membrane fragments in the presence of 5 µg/ml α-Bgt, (i.e. ^{14}C-Deca bound to the catalytic centers of AcChE). The binding is followed by the ultracentrifugal assay in the presence of 10^{-6} M ^{14}C-Deca. The specific activities of the various fractions used were respectively: 1, 0.4; 2, 1.5; 3, 1.6; 4, 1.7; 5, 4.4; 6, 6.9; 7, 4.7; 8, 4.4 moles ATCh per g protein per hour at 27 °C. The slope of (a) is 1.1, that of (b) 0.6. Assuming that the dissociation constant of Deca for the cholinergic receptor is 1.3×10^{-6} M, that for the catalytic center of AcChE 2.4×10^{-6} M, then the ratio of the numbers of receptor to catalytic sites becomes 0.75 for (a) and 0.41 for (b)

concentration. Although there was still an appreciable scattering in our measurements, it can be said that, within the present accuracy of our methods, there was no major difference between the response curve and the binding curve. In particular, the midpoints were approximately the same. The "apparent" affinity of Deca measured with the response curve thus coincides with the "real" affinity of Deca for the cholinergic receptor site. Although, as mentioned above, it is clear that cooperative effects are present when the permeability response is followed as a function of Deca concentration, the same cannot be stated without ambiguity for the binding of Deca.

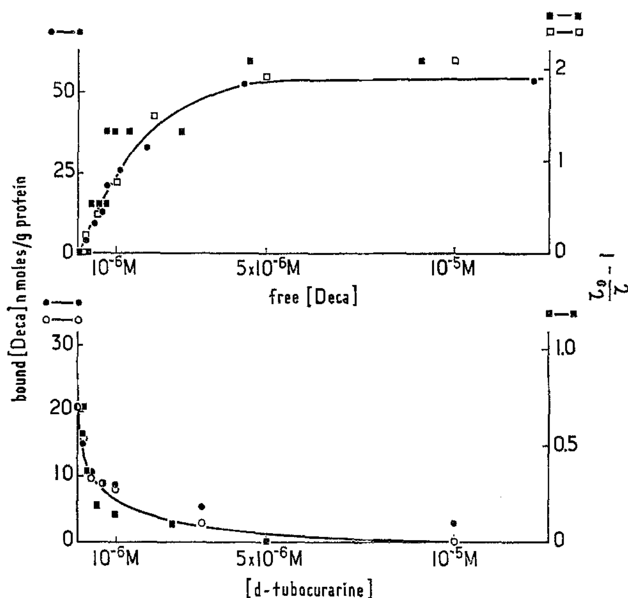

Fig. 7. Comparison of *the dose-response curves* (squares) to Deca and *d*-tubo with the corresponding *binding curves* (dots) of ^{14}C-Deca to the cholinergic receptor sites. The response was followed by the increase of permeability of the excitable microsacs to $^{22}Na^{+}$. ^{14}C-Deca binding was measured by the ultracentrifugal assay. We plotted here, as ^{14}C-Deca bound to the cholinergic receptor, the difference between the total amount bound to the membrane fragments and the amount which is *not* displaced by 5 μg/ml α-Bgt (there is no more displacement in the presence of 20 μg/ml α-Bgt). ▪——▪ the response curve was measured with the same membrane fragments as those used to make the binding curve (preparation no. 24); ▫——▫ we have replotted here the data given in paper I, preparation no. 1. Antagonism by *d*-tubo was followed in the presence of a constant concentration of Deca (8×10^{-7} M)

Presence of cooperative interactions for Deca binding are highly probable, but the imprecision of measurement, particularly in the region of very low site occupancy, was such to prevent any definitive conclusion in this respect.

In Fig. 7, we have compared the effect of an antagonist, *d*-tubo, on the binding of Deca and on the permeability response to Deca. It is clear from Fig. 2 that the response to Deca completely blocked by *d*-tubo; on the other hand, *d*-tubo displaces bound Deca only partially. As extensively discussed before, the residual binding at high *d*-tubo concentration was interpreted as due to the association of Deca with the catalytic site of AcChE. Subtraction of this value from the whole binding curve gives a curve of antagonism which superimposes on the response curve. The antagonism between Deca and *d*-tubo on the permeability response is thus directly associated with the displacement of Deca by *d*-tubo from the cholinergic receptor site. Here again, within the limits of accuracy of our measurements, a superimposition of the response curve and of the binding curve is obtained.

"Apparent" and "real" affinities thus coincide almost exactly (Table 1). This result further strengthens our earlier conclusion that, under the conditions of our centrifugal assay, we are indeed following the binding of ^{14}C-Deca to the cholinergic receptor sites present in the microsac membrane.

The Transport Properties of One Cholinergic Ionophore

Knowing the absolute value of the increase of $^{22}Na^{+}$ efflux from a population of excitable microsacs and the corresponding occupancy of cholinergic receptor sites, it becomes easy to figure out what are, in these conditions, the transport properties of the ionophore (or of the group of ionophores) which is under the control of a single cholinergic receptor site.

In Fig. 8 we have plotted the selective increase of $^{22}Na^{+}$ efflux as a function of the number of receptor sites occupied by Deca. The concentration of free Deca in this experiment was 10^{-6} M, which is precisely the value of both the apparent and real K_D of Deca for the cholinergic receptor site.

Under these conditions, half of the total number of sites should be occupied and half of the maximal response should be reached. The data reported in Fig. 8 were obtained with seven different preparations, and it is clear that from one preparation to another an important scattering exists. Nevertheless, we drew two lines: line (a) corresponds to the *maximal* increase of $^{22}Na^{+}$ volume flow recorded, and line (b) to an *average* value. The slopes

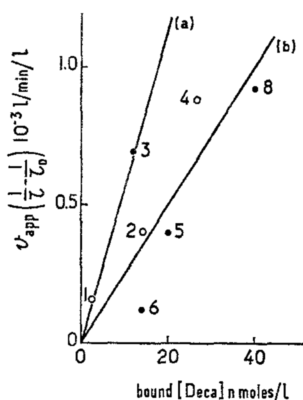

Fig. 8. The selective increase of ^{22}Na efflux caused by Deca studied as a function of the *number* of cholinergic receptor sites occupied by Deca. In this experiment, six different preparations (*see* Fig. 6) with different specific activities of AcChE were used. ∘, the suspensions were not sonicated (preparation no. 20); •, the suspensions were sonicated for 1 min in a Mullard apparatus (preparation no. 24). V_{app} is the apparent volume as defined in paper II; τ and τ_0 are defined in paper I. For all points, the concentration of free Deca was 10^{-6} M. The slope of (a) is 9.3×10^5 ml/sec · mole Deca, that of (b) is 4.2×10^5 ml/sec · mole Deca bound

of (a) and (b) are, respectively, 9.3 and 4.2×10^5 ml/sec·mole of Deca bound to the cholinergic receptor site.

Conclusions

On the same preparation of microsacs, we measure, in parallel, the binding of radioactive Deca and the response to Deca. For the first time we compare the "apparent" and the "real" affinity of an excitable membrane for its chemical regulatory signal. From the superimposition of the binding and response curves, it is concluded that, in the case of the excitable microsacs, the apparent affinity for Deca is very close to its real affinity.

The accuracy of our binding measurements is not yet good enough to let us detect a systematic deviation of the binding curve from a Langmuir isotherm. If cooperative effects of the same order as those observed with the dose-response curve were present (*see* paper I), they would remain undetected. We would not discriminate small differences either in shape or in position between the response curve and the binding curve.

To simplify the rest of the discussion, we shall neglect possible deviation from a straight absorption isotherm to a single class of non-interacting sites. We shall nevertheless keep in mind that these deviations might exist, and, as discussed in paper I, possibly reflect a cooperative association of cholinergic protomers in oligomeric clusters.

Extrapolation to infinite concentration of the binding curves gives estimates of the numbers of cholinergic receptor sites present in the microsac membrane: from 10 to 60 nmoles of Deca receptor sites are present per gram of membrane protein. This number seems to be in the same range as the one given by O'Brien and Gilmour (1969) or by Karlin, Prives, Deal and Winnik (1970). However, the use by these authors of different cholinergic ligands and the expression of their results in units which cannot be simply converted to ours render the comparison difficult. On the other hand, the agreement is excellent with the value of 60 nmoles/g protein found by Changeux *et al.* (1970*a*) with soluble extracts of electric organ.

Interestingly, the number of cholinergic receptor sites obtained by measuring the binding of ^{14}C-Deca is very close to the one found by the titration of the cholinergic receptor sites with α-Bgt. The number of α-Bgt molecules blocking 100% of Deca binding to the cholinergic receptor is found, however, to be slightly higher – twice as much in the experiment reported in Table 2. Is this just a coincidence or should we relate this result to the fact that Deca possesses *two* quaternary ammoniums? For example, should we consider seriously the eventuality that the receptor site for Deca on the receptor protein possesses two anionic centers and that each anionic

center might react with one molecule of α-Bgt? The comparative measurements of the number of binding sites for monoquaternary and bisquaternary agonists should give an answer to this question.

The number of catalytic centers of AcChE present in the membrane fragments is estimated by two distinct methods: (1) the measurement of the specific activity of the enzyme in the microsacs, and (2) the binding of ^{14}C-Deca to the membrane fragments when the cholinergic receptor site is blocked by α-Bgt[3].

The numbers obtained coincide quite well. Making several assumptions about the physicochemical properties of the pure enzyme (some of which are still uncertain), we find about three sites for Deca per molecule of molecular weight 260,000. According to our present knowledge of the pure enzyme, AcChE is believed to be an $\alpha_2\beta_2$ tetramer, and the only possible numbers of Deca binding sites per molecule of AcChE are either 2 or 4 but not 3.[4] Several causes of errors, frequent in this type of study, might account for the ambiguity of our results; in addition to the inaccuracy of our measurements, the presence of nonspecific binding sites for Deca or the inadequacy of some of our assumptions on the pure protein might have to be invoked.

Some correlation exists between the number of cholinergic receptor sites and the number of catalytic centers of AcChE present in our preparations of microsacs. The two numbers are always in the same range, although it appears that the number of cholinergic receptor sites measured by ^{14}C-Deca binding is always lower than the number of AcChE catalytic centers. It is clear, however, that the correlation between the two series of data is not as perfect as one would expect if the two classes of sites were carried by the same macromolecule. This is not due to the imprecision of our measurements, which give excellent correlations when sites of the same class (the catalytic center of AcChE) are measured by two different methods. This absence of strict stoichiometry suggests, in fact, that the catalytic center of AcChE and the cholinergic receptor sites are carried by different proteins. Such a proposal, which has been made for years by pharmacologists (*see* Nachmansohn, 1959; Karlin, 1969; O'Brien *et al.*, 1970), is in agreement with the recent experiments of Meunier *et al.* (1971) performed with soluble extracts of electric organ. These authors have shown, on a soluble extract

3 The comparison between binding data and measurements of specific activity is legitimate since it has never been possible to detect any change in turnover number in the course of either AcChE solubilization from the microsacs or transformation from a high molecular weight species to a low one (Massoulié & Rieger, 1969).

4 Since we perform our experiments in the presence of high salts, we cannot measure under these conditions binding of Deca to the allosteric sites present on the enzyme molecule.

of electric organ, that the two classes of sites, and thus the proteins which carry the sites, can be separated by various physicochemical procedures.[5]

Knowing the concentration of AcChE receptor sites or of cholinergic receptor sites, one might calculate an average *distance* between sites in the electroplax membrane. Obviously, the proposed value is a function of the state of purity of the membrane fragments. Our preparations contain from 0.1 to 0.7% of their protein as AcChE or receptor protein, but preparations with higher specific activities might be obtained. Under our conditions, assuming the membrane made exclusively of proteins with a density of 1 and a thickness of 100 Å, we get an area per cholinergic receptor site (or per catalytic site of AcChE) of 4.3 to 0.62×10^6 Å^2. Since our membrane fragments are not pure, it is plausible that in the living cell this area is much smaller.

From the measurement of the permeability response to Deca, which corresponds to the binding of a given number of Deca molecules, we get an estimate of the transport properties of the ionophore(s) controlled by a single cholinergic receptor site. It should be emphasized, however, that we do not consider this estimate to be a definitive one. Indeed, it cannot be ascertained that all the microsacs which contain cholinergic receptor sites and thus bind ^{14}C-Deca are (1) closed and (2) functional; it is conceivable that some cholinergic protomers present in our preparations can bind ^{14}C-Deca but have lost their ability to mediate the interaction between the cholinergic receptor site and the ionophore. Finally, we have never been able to detect any electrical effect with our excitable microsacs although it is well established that, *in vivo*, the actual transport of ions (not the permeability) is under the control of the high electric fields established across the subsynaptic membrane. It is expected that improvement of our methods, in particular of our fractionation procedure, shall be followed by a revision of the proposed numbers. Nevertheless, we found that the volume flow increase per mole of Deca bound is from 10 to 4×10^5 ml · sec.

In order to have an intuitive notion of the capacity of transport that these values represent, we have compared these data with those obtained by Bangham and associates with liposomes charged with the polypeptide antibiotic valinomycin. Johnson and Bangham (1969) found that the increase of permeability to K^+ caused by the incorporation of 1 μmole of valinomycin into 1 mole of lipid is $p = 10^{-11}$ cm/sec. Under their conditions, the calculated flow of K^+ through the liposome bilayer would be about 4.3×10^4 ml/sec · mole of valinomycin. This number is quite close to the one found with the microsacs for one mole of Deca receptor site, al-

5 *Note Added in Proof.* This result has been recently confirmed by R. Miledi, P. Molinoff and L. Potter (Nature, **229**:554, 1971).

though in our case we followed the transport of $^{22}Na^{+}$ instead of the transport of $^{42}K^{+}$. That the efficiency of transport of a cholinergic ionophore in its active state is of the same order of magnitude as the one of an antibiotic carrier is further supported by our results obtained with gramicidin A (Gra; *see* paper I of this series). We found that in the presence of Gra the permeability of the microsacs to $^{22}Na^{+}$ increases. An increase of permeability which is of the same amplitude as the one obtained in the presence of saturating levels of Deca is reached when 0.5 µg Gra is added per mg microsacs protein. If all the molecules of Gra added were efficient as Na^{+} carriers, then the capacity of transport of a cholinergic ionophore would be equivalent to about 10 Gra molecules. It is very probable that an appreciable fraction of Gra molecules either is bound by the membrane fragments without being efficient as ionic carrier or is not bound at all. This value of 10 should thus be too high. In any case, it is clear that the transport properties of the ionophore associated with a single cholinergic receptor site are still in the same range as those found with typical antibiotic carriers.

Under these conditions, taking 5×10^{5} ml/sec · mole of Deca for the volume flow of Na^{+} and 0.17 M for the inside concentration of Na^{+}, the turnover number of the cholinergic ionophore is found to be about 5×10^{3} Na^{+} ions transported per min per Deca binding site. This value is close to that found for the permease of β-galactoside transport (Kennedy, 1969) or for the active transport of Na^{+} or K^{+} erythrocytes (Post, 1970) (in both cases, on the order of 10^{4} molecules/min).

In order to extend the comparison of our results with those obtained *in vivo* by electrophysiological methods, we tried to compute the equivalent conductance of a single cholinergic ionophore in its active state in an excitable microsac. Making the same assumptions as those stated earlier in similar calculations (see the legend of Table 5 in paper II), in particular neglecting the effect of electrical potential, we find a conductance which is of the order of 10^{-15} mho per cholinergic receptor site or ionophore.[6] There

6 *Note Added in Proof:* To avoid confusion it is worth emphasizing that the value reported for the conductance of a single cholinergic ionophore is an *average* one, measured under conditions of *steady-state* ion flow. Hladky and Haydon (Nature, **225**:451, 1970; and Proceedings of the Symposium on Molecular Mechanisms of Antibiotic Action on Protein Biosynthesis and Membranes, Granada, June 1971) have determined the conductance of single channels created by gramicidin A in artificial bilayers. In 5×10^{-1} M NaCl this unit conductance is 1.7×10^{-11} mho. However, their elegant studies reveal that only a small fraction (possibly one thousandth) is opened at any given time. Their data might thus be consistent with ours. This might also be true for the most recent findings of Katz and Miledi (Nature, **232**:124, 1971) on "acetylcholine noise", although these authors do not give any estimate of the fraction of acetylcholine "channels" opened at a given time in the excitable membrane.

are no data in the literature yet available for the conductance of a single cholinergic ionophore, but values have been published for the so-called Na^+ channels in the squid axon (*see* Hille, 1970) and for the "channels" caused by various excitability-inducing materials such as the protein of Müller and Rudin (1968) or antibiotics (alamethicin or nystatin) in black film membranes (reference in Ehrenstein, Lecar & Nossal, 1970). In both cases, the authors agree on a value of about 10^{-10} mho per ionic channel. This value is 10^5 times larger than the one found for the ionophore associated with a single cholinergic receptor site. A large difference thus exists between our results and those obtained with electrically excitable membranes.

Either there exists a major misinterpretation of the experimental results, in particular because we always neglected electrical field effects, or radically different mechanisms for ion transport are involved in electrical excitation and in chemical excitation. There is no question that our values are *minimal* estimates; they might be too low by one order of magnitude, possibly by two, hardly by five. On the other hand, the enumeration of Na^+ channels in nerves has always been made indirectly (Moore, Narahashi & Shaw, 1967), and what is believed to be a *single channel* in an electrically excitable membrane might, in fact, be a large domain of membrane surface consisting of *hundreds or thousands of units* with transport properties similar to those of the cholinergic ionophore. The discrete, quantized fluctuations of the membrane potential, which were in the past attributed to the transition of a single channel, would then correspond to the all-or-none transition of the ensemble of channels or ganized in a lattice structure or any type of large cooperative assembly. It is, however, premature to decide what is the correct mechanism involved.

Finally we would like to discuss to what extent the present results are consistent with a theory for chemical excitation which was presented a few years ago by one of us and already extensively discussed (Changeux, 1966; Changeux, Thiery, Tung & Kittel, 1967; Karlin, 1967; Changeux & Podleski, 1968; and Changeux, 1969). In short, chemical excitation is viewed as an *indirect*, and thus allosteric, control of the properties of a selective *ionophore* (Changeux *et al.*, 1969) by a chemical signal, a cholinergic agonist for instance. Binding of the chemical signal to the specific *receptor protein* triggers the change of permeability through a structural transition (*see* Nachmansohn, 1969 & 1959) of the complex ionophore-receptor. Such a transition is assumed to be analogous to the well-identified allosteric transitions of regulatory enzymes (references in Monod, Changeux & Jacob, 1963; Monod, Wyman & Changeux, 1965; and Whitehead, 1970) but might present particularities related to the integration of the complex ionophore-

receptor within the membrane framework. The receptor protein would thus be to the selective ionophore what the *regulatory subunit* is to the catalytic subunit of a typical allosteric protein such as aspartate transcarbamylase (Gerhart & Schachman, 1966).

In the resting state, T, the receptor would present a high affinity for antagonists such as *d*-tubo and little or no affinity for agonists such as Deca or Carb; the ionophore would be impermeable to cations. In the excited state, S, the receptor would bind preferentially, and be stabilized by, the agonists; the ionophore would selectively transport cations such as Na^+, K^+ and Ca^{++}. The excitation process is viewed as the displacement by the agonist of the conformational equilibrium from the T to the S state and the effect of the antagonists as a displacement in the reverse direction.

In order to compare theory and experiment, we first need a correlation between the flux of radioactive permeant through the microsac membrane and the "conformation" of the cholinergic protomers present in the membrane. We might reasonably consider that the total flux measured is the sum of three elementary fluxes: the flux through the protomers in the T state with a kinetic constant k_T, the flux through the protomers in the S state with a kinetic constant k_S, and a leak flux insensitive to cholinergic agonists with a kinetic constant k_L. Then, whatever the time course of permeant efflux, exponential or not, we always have:

$$k = \langle t \rangle k_T + \langle s \rangle k_S + k_L$$

with $\langle t \rangle + \langle s \rangle = 1$, where $\langle t \rangle$ and $\langle s \rangle$ are the fraction of cholinergic protomers in the T and S state, respectively. In most of our experiments we measured times for half equilibration, τ and τ_0. These times are always proportional to $1/k$. In addition we always considered as a measure of the response $\frac{\tau_0}{\tau} - 1$. In the framework of the present theory:

$$\frac{\tau_0}{\tau} - 1 = \frac{\langle t \rangle k_T + \langle s \rangle k_S + k_L}{k_T + k_L} - 1 = \langle s \rangle \left(\frac{k_S - k_T}{k_T + k_L} \right) - 1 .$$

In other words, the experimental dose-response curves can be taken as "state functions" (*see* Monod *et al.*, 1965; Changeux & Rubin, 1968; and Buc & Buc, 1968) which represent directly the fraction of protomers which are in the S state. If the T state is largely favored in the membrane at rest, and if the agonist tested binds exclusively to the S state, then the state function is expected to follow very closely the binding function.

The present results are thus consistent with the proposed theory; it is clear, however, that they cannot be taken as proof of this theory. In parti-

cular, it is impossible to assert, on the basis of these results, that only two conformations of the protomer are present and exist prior to the binding of effector. The alternative mechanism of a multiplicity of conformations induced by each different cholinergic ligand cannot be excluded at present. A possible test for a spontaneous equilibrium between a small number of conformations might come from the demonstration of a significant deviation between state function and binding function, in particular, if cooperative interactions are present. Accounting for the fact that the cooperative effects seen in the response curve are rather small ($n_H \sim 2$ for Carb or Deca), such a deviation is not expected to be large and is, at present, beyond the limits of accuracy of our binding experiments.

More direct evidence in favor of the allosteric interactions between ionophore and receptor should come from the direct demonstration of the conformational transitions of the cholinergic protomer in the course of chemical excitation. The results gained recently with microsacs labeled by fluorescent probes look promising in this respect (Kasai, Changeux & Monnerie, 1969; Kasai, Podleski & Changeux, 1970; Wahl, Kasai & Changeux, 1970). Another, but indirect, proof would be offered by the performance of the selective uncoupling of the regulatory interaction between the cholinergic receptor site and the site of ion transport without loss of affinity of the receptor for the agonist and of the ionophore for the permeant ion. This would be analogous to the "desensitization" of a regulatory protein (Changeux, 1961; Gerhart & Pardee, 1962; Monod, Changeux & Jacob, 1963). Additional evidence might be obtained by the study of the purified receptor protein and of the associated ionophore in a reconstituted membrane system. The electric tissue of the gymnote, which is one of the richest sources of these components, should be, again, an excellent material on which to carry out this type of experiment.

Finally, we wish to emphasize that the pharmacological and electrophysiological properties of the constitutive synapses of the eel electroplax are very similar to those of the typical neuromuscular junction in higher vertebrates. The main conclusions obtained with the electric organ as a biological material might reasonably be extended to other cholinergic synapses and even to chemically excitable membranes in general.

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References

Buc, M. H., Buc, H. 1968. Allosteric interactions between AMP and orthophosphate sites on phosphorylase b from rabbit muscle. *In*: Regulation of Enzyme Activity and Allosteric Interactions. E. Kvamme and A. Pihl, editors. p. 109. Universitetsforlaget, Oslo.

Changeux, J.-P. 1961. The feed-back control mechanism of biosynthetic L-threonine deaminase by L-isoleucine. *Cold Spring Harbor Symp. Quant. Biol.* **26**:313.

– 1966. Responses of acetylcholinesterase from *Torpedo marmorata* to salts and curarizing drugs. *Mol. Pharmacol.* **2**:369.

– 1969. Remarks on the symmetry and cooperative properties of biological membranes. *In*: Symmetry and Function of Biological Systems at the Macromolecular Level. A. Engström and B. Strandberg, editors, *Nobel Symp.*, vol. 11, p. 235. Wiley, Interscience Division, New York.

– Blumenthal, R., Kasai, M., Podleski, T. R. 1970. Conformational transitions in the course of membrane excitation. *In*: Ciba Symposium, Molecular properties of Drug Receptors. R. Porter and M. O'Connor, editors p. 197. Churchill, London.

– Kasai, M., Huchet, M., Meunier, J. C. 1970*a*. Extraction à partir du tissu électrique de gymnote d'une protéine présentant plusieurs propriétés caractéristiques du récepteur physiologique de l'acétylcholine. *Compt. Rend. Acad. Sci. Paris* **270**:2864.

– – Lee, C. Y. 1970*b*. The use of a snake venom toxin to characterize the cholinergic receptor protein. *Proc. Nat. Acad. Sci.* **67**:1241.

– Leuzinger, W., Huchet, M. 1968. Specific binding of acetylcholine to acetylcholinesterase in the presence of eserine. *F.E.B.S. Letters* **2**:77.

– Podleski, T. R. 1968. On the excitability and cooperativity of the electroplax membrane. *Proc. Nat. Acad. Sci.* **59**:944.

– – Kasai, M., Blumenthal, R. 1970. Some molecular aspects of membrane excitation studied with the eel electroplax. *In*: Excitatory Synaptic Mechanisms. P. Andersen and J. Jansen, editors. p. 123. Universitetsforlaget, Oslo.

– – Meunier, J. C. 1969. On some structural analogies between acetylcholinesterase and the macromolecular receptor of acetylcholine. *J. Gen. Physiol.* **54**:225 S.

– Rubin, M. M. 1968. Allosteric interactions in aspartate transcarbamylase. III. Interpretations of experimental data in terms of the model of Monod, Wyman and Changeux. *Biochemistry* **7**:553.

– Thiéry, J., Tung, Y., Kittel, C. 1967. On the cooperativity of biological membranes. *Proc. Nat. Acad. Sci.* **57**:335.

Ehrenstein, G., Lecar, H., Nossal, R. 1970. The nature of the negative resistance in bimolecular lipid membranes containing excitability inducing material. *J. Gen. Physiol.* **55**:119.

Gerhart, J. C., Pardee, A. B. 1962. The enzymology of control by feed-back inhibition. *J. Biol. Chem.* **237**:891.

– Schachman, H. K. 1965. Distinct subunits for the regulation and catalytic activity of aspartate transcarbamylase. *Biochemistry* **4**:1054.

Hille, B. 1970. Ionic channels in nerve membranes. *Prog. Biophys. Mol. Biol.* **21**:1.

Johnson, S. M., Bangham, A. D. 1969. Potassium permeability of single compartment liposomes with and without valinomycin. *Biochim. Biophys. Acta* **193**:82.

Karlin, A. 1967. On the application of a plausible model of allosteric proteins to the receptor for acetylcholine. *J. Theoret. Biol.* **16**:306.

– 1969. Chemical modification of the activity of the acetylcholine receptor. *J. Gen. Physiol.* **54**:245 S.

Karlin, A., Prives, J., Deal, W., Winnik, M. 1970. Counting acetylcholine receptors in the electroplax. *In*: Ciba Symposium, Molecular Properties of Drug Receptors. R. Porter and M. O'Connor, editors. p. 247. J. and A. Churchill, London.

Kasai, M., Changeux, J.-P., Monnerie, L. 1969. *In vitro* interaction of 1-anilino-8-naphthalene sulfonate with excitable membranes isolated from the electric organ of *Electrophorus electricus*. *Biochem. Biophys. Res. Commun.* **36**:420.

– Podleski, T. R., Changeux, J.-P. 1970. Some structural properties of excitable membranes labelled by fluorescent probes. *F.E.B.S. Letters* **13**:7.

Kennedy, E. 1969. Studies on the lactose transport system in *Escherichia coli*. *J. Gen. Physiol.* **54**:91 S.

Kremzner, L., Wilson, I. B. 1964. A partial characterization of acetylcholinesterase. *Biochemistry* **3**:1902.

Lee, C. Y., Chang, C. C. 1966. Modes of action of purified toxins from Elapid venoms on neuromuscular transmission. *Mem. Inst. Butantan. Simp. Internac.* **33**:555.

Leuzinger, W., Baker, A. 1967. Acetylcholinesterase. I. Large scale purification, homogeneity, amino acid analysis. *Proc. Nat. Acad. Sci.* **57**:446.

– – Cauvin, E. 1968. Acetylcholinesterase. II. Crystallization, absorption spectra, isoionic point. *Proc. Nat. Acad. Sci.* **59**:620.

– Goldberg, M., Cauvin, E. 1969. Molecular properties of acetylcholinesterase. *J. Mol. Biol.* **40**:217.

Massoulié, J., Riéger, F. 1969. L'acétylcholinestérase des organes électriques de poissons (torpille et gymnote); complexes membranaires. *Europ. J. Biochem.* **11**:441.

Meunier, J.-C., Huchet, M., Boquet, P., Changeux, J.-P. 1971. Séparation de la protéine réceptrice de l'acétylcholine et de l'acétylcholinestérase. *Compt. Rend. Acad. Sci. (Paris)* **272**:117 D.

Monod, J., Changeux, J.-P., Jacob, J. 1963. Allosteric proteins and cellular control systems. *J. Mol. Biol.* **6**:306.

– Wyman, J., Changeux, J.-P. 1965. On the nature of allosteric transitions: A plausible model. *J. Mol. Biol.* **12**:88.

Moore, J. W., Narahashi, T., Shaw, T. I. 1967. An upper limit to the number of sodium channels in nerve membrane. *J. Physiol.* **188**:99.

Mueller, P., Rudin, D. O. 1968. Resting and action potentials in experimental bimolecular lipid membranes. *J. Theoret. Biol.* **18**:222.

– – 1968. Action potentials induced in bimolecular lipid membranes. *Nature* **217**:713.

Nachmansohn, D. 1959. Chemical and Molecular Basis of Nerve Activity. Academic Press, New York.

– 1969. Proteins of excitable membranes. *J. Gen. Physiol.* **54**:187 S.

O'Brien, R. D., Gilmour, L. P. 1969. A muscarone-binding material in electroplax and its relation to the acetylcholine receptor. I. Centrifugal assay. *Proc. Nat. Acad. Sci.* **63**:496.

– – Eldefrawi, M. E. 1970. A muscarone-binding material in electroplax and its relation to the acetylcholine receptor. II. Dialysis assay. *Proc. Nat. Acad. Sci.* **65**:438.

Post, R., Sen, A. K., Rosenthal, A. S. 1965. A phosphorylated intermediate in adenosine triphosphate dependent sodium and potassium transport across kidney membranes. *J. Mol. Chem.* **240**:1437.

Wahl, P., Kasai, M., Changeux, J.-P. 1971. A study on the motion of proteins in excitable membrane fragments by a nanosecond fluorescence polarization spectroscopy. *Europ. J. Biochem.* **18**:332.

Whitehead, E. 1970. The regulation of enzyme activity and allosteric transition. *Prog. Biophys. Mol. Biol.* **21**:321.