A MUSCARONE-BINDING MATERIAL IN ELECTROPLAX AND ITS RELATION TO THE ACETYLCHOLINE RECEPTOR, I. CENTRIFUGAL ASSAY

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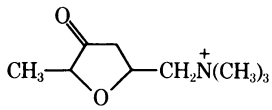
Abstract.—A muscarone-binding material has been found in *Torpedo* electroplax. The material behaves in several ways as one would expect of the binding component of the acetylcholine receptor, especially in its high affinity for muscarone (approximate $K = 7 \times 10^{-6}M$), acetylcholine (approximate $K = 1.1 \times 10^{-6}M$), curare, and nicotine, and its insensitivity to noncholinergic agents; the reversibility of its muscarone binding; and its suitably small amount (about 1.8 nmole/gm). The material appears not to be acetylcholinesterase.

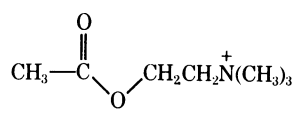
The existence of an entity called "receptive substance" was proposed in 1906 by Langley[1] to account for the effects of certain exogenous and endogenous agents on activity in the nervous system. Although pharmacology and physiology have necessarily assumed the existence of such an entity (now usually called "receptor") during the intervening 60 years, a receptor has never been isolated, nor indeed has it been established that receptor activity exists in subcellular preparations. The two principal claims that such activity had been observed[2, 3] were later withdrawn.[4, 5]

The difficulties encountered to date have raised the possibility that receptor cannot be isolated, because its properties emerge from a complex array within the receptive membrane. We feel that this conclusion is premature and we have continued to search in accordance with the principles of the earlier studies, that is, to seek (in the first place) a macromolecule which can bind transmitter, and whose binding can be blocked by pharmacologically appropriate drugs, and not by inappropriate ones.

The most direct way to look for acetylcholine receptor activity would appear to be to examine acetylcholine binding to tissues. However, acetylcholinesterase is present in a very high amount in all receptor-containing tissues and rapidly hydrolyzes the acetylcholine. The report that muscarone, a close analog of acetylcholine, is not destroyed by acetylcholinesterase, is five times more potent than acetylcholine on muscarinic junctions, and is twice as potent as acetylcholine on nicotinic junctions[6–8] suggested that it was the ideal compound to explore binding to cholinergic receptors of either kind. It should be emphasized that muscarone is therefore entirely unlike the better known compound muscarine as far as specificity is concerned; muscarine is almost exclusively active upon muscarinic receptors.



Muscarone



Acetylcholine

Methods.—Synthesis: We prepared *N*-methyl-H^3-DL-muscarone iodide (referred to below simply as "muscarone") from *nor*-muscarone, generously donated by the Geigy Co. of Switzerland. It was reacted with H^3-methyl iodide in anhydrous benzene by heating under nitrogen for 18 hr at 70°C in a sealed ampoule suspended in an oil bath. After four crystallizations from acetone-ether, the product (colorless needles) was obtained in 80% yield, with a specific activity of 81 mc/mmole. Radiopurity was established by finding a single peak in three different paper chromatographic systems; the *Rf*'s were 0.95 with bottom phase and 0.61 with top phase of *n*-butanol–acetic acid–water, 4:1:5 volume ratio and 0.30 with *n*-butanol saturated with water.

Preparation and assay of tissue: Electric organ was dissected out of male and female *Torpedo marmorata* and *oscellata*, and homogenized in a Servall Omnimixer at 20% in Tris buffer 0.05 *M* containing 0.2 *M* NaCl. The mixture was spun at 27,000 × *g* for 30 min in the cold, and the supernatant discarded. The volume of the viscous precipitate was measured, and portions corresponding to 25 gm original electroplax were pipetted into tubes and stored frozen at −18°C. For use, a tube was thawed, rehomogenized in 10 vol of Tris buffer in a Dounce hand homogenizer, spun as before, and the precipitate suspended in buffer. Further additions, as indicated in the text, were also made in 0.05 *M* buffer, so that the final volume was 3 ml, containing precipitate from 5 gm of electroplax, and muscarone which (except as indicated) was 2.5×10^{-7} *M*. After 60 min at room temperature, the mixture was spun in the cold at 11,000 × *g* for 15 min, and 0.5 ml of the supernatant was added to 10 ml of Bray's counting solution[9] (modified by using dimethyl POPOP in place of POPOP) and counted in the Tri-Carb scintillation counter.

In studies on the effect of pH, the frozen tube was thawed and homogenized in the Dounce homogenizer with water, spun as usual, then the precipitate suspended in water at the rate of 1.75 ml/5 gm original electroplax. Then 1.75-ml portions were added to 1 ml of acetate-phosphate buffer (sodium acetate 0.15 *M*, Na_2HPO 0.15 *M*) whose pH had been suitably adjusted, the pH of the mixture was measured, and 0.25 ml of 3×10^{-6} *M* H^3-muscarone in water was added.

When various media were explored, water-washing (as in the last paragraph) was used to eliminate Tris buffer; then the precipitate was suspended in the appropriate medium.

Results.—Preliminary studies indicated that there was binding activity in all the precipitates from electroplax homogenates, i.e., that at 1000 × *g*, the precipitate from the 1000 × *g* supernatant spun at 11,500 × *g* and that from the 11,500 × *g* supernatant spun at 100,000 × *g*. These speeds, normally used for mammalian tissues, need have no special validity for electroplax. Nevertheless, the total activity in the high-speed precipitate was relatively small and that in the 11,500 × *g* precipitate was maximal. Thus in a run with 10^{-7} *M* initial muscarone, the binding per gram of original electroplax was 10 pmoles for the low speed, 12 pmoles for the middle, and 1 pmole for the high. As a compromise between convenience and maximal yield, a single spin at 27,000 × *g* was employed from then on.

Different media affected the amount of binding: on incubating with 2.5×10^{-7} *M* muscarone, the pmoles bound per gram of electroplax in duplicate runs were 108 and 104 for water, 40 and 42 for the standard Tris buffer (see *Methods*), and 84 and 82 for Krebs original phosphate mammalian Ringer solution.[10] All the work reported below employed Tris buffer unless otherwise stated.

Binding was rapid, insofar as the somewhat lengthy centrifugal procedure could show. The amounts bound with incubation times of 5 minutes, 30 minutes, and one or two or four hours were virtually identical, the spread being from 48 to 54 pmoles per gram.

The effect of pH was explored. The usual Tris buffer did not permit a wide range of pH, so a wide-ranging system consisting of sodium acetate and disodium phosphate, both at 0.05 M final concentration was prepared. As Figure 1 shows, there was a marked pH-dependence in the range 5–6, but at higher pH there was little loss in activity.

The binding constant for muscarone was obtained by performing experiments at concentrations ranging from 10^{-8} M to 10^{-5} M. The data showed a scattered linear relationship when $1/a$ was plotted against $1/M$, where a is the amount bound per gram of electroplax and M is the concentration of muscarone in the supernatant after equilibrium is achieved. One can readily show from mass-action considerations (e.g., by rearranging equation (15) of Klotz[11]) that if one mole of muscarone binds to one mole of receptor, whose total concentration is R, and the dissociation constant of the muscarone-receptor complex is K:

$$1/a = K/R \cdot (1/M) + (1/R)$$

Consequently the intercept on the $1/a$ axis gives $1/R$, and the intercept on the $1/M$ axis gives $-1/K$.

Figure 2 shows the data. A computerized version of the weighted regression procedure of Wilkinson[12] was used to obtain K and R. The results were that $R = 1.8$ nmoles per gram, $K = 7 \times 10^{-6}$ M. Because of the scatter and the nonlinearity with tissue concentration (see below) these figures are only order-of-magnitude estimates.

That the muscarone binding was reversible was established by the following experiment. After standard incubations with various concentrations of muscarone, and the usual centrifugation, the supernatant was decanted off as completely as possible, its radioactivity measured, and the amount bound to the "first precipitate" calculated. An equal volume of fresh buffer was then added back to the precipitate, which was resuspended and incubated for one hour, then centrifuged again and the second supernatant sampled. Knowing the muscarone in the second supernatant permitted us to calculate (from Fig. 2) the muscarone in the second precipitate, and thus to calculate the percentage of the radioactivity in the first precipitate which distributed itself in the way predicted on grounds of total reversibility. The values found were as follows, in two different runs performed three months apart: with muscarone 2×10^{-6} M, 102 and 133 per cent; with 5×10^{-7} M, 113 and 105 per cent; with $2.5 \times$

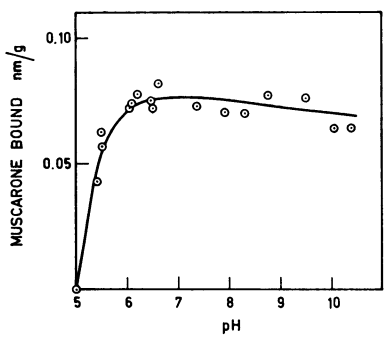


FIG. 1.—Dependence upon pH of muscarone binding to 27,000 × g precipitate.

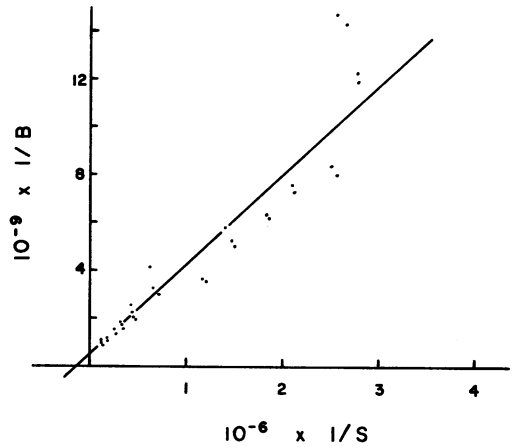


FIG. 2.—Dependence upon muscarone concentration (S) of binding (B) to 27,000 × g precipitate (derived from 5 gm of plax). 12 points at low values of S are omitted because they would be far off scale. The line shown is computed from all points by the Wilkinson method.[12]

10^{-7} M, 83 and 78 per cent. Clearly there is some variation, especially with low counts, but equally clearly the binding of muscarone is substantially reversible.

The dependence of binding upon quantity of tissue preparation was examined (Fig. 3) and found to depart substantially from linearity at both concentrations of muscarone studied.

Some data was gathered on the durability of the preparation. Boiling for three minutes destroyed all activity. The unwashed precipitate from homogenized electroplax could be deep-frozen and stored without loss of activity for at least two weeks. It could also be lyophilized and stored in the cold or at room temperature without loss for a similar period.

The effect of a variety of agents upon muscarone binding was tested by incubating them with 2.5 × 10^{-7} M muscarone in the standard system. As Table 1 shows, blockage of binding was found with all expected agents, that is, those believed to react with cholinergic receptor. In addition, eserine gave mild blockage at the high concentration of 10^{-4} M. This finding conforms with the well-established fact that high concentrations of eserine have a curare-like action, quite unlike the anticurare action (due to acetylcholinesterase inhibition) which is its "normal" effect at low concentrations.[13]

The most important constant desired was the binding constant for acetylcholine. In order to measure this, it was necessary to destroy the acetylcholinesterase in the preparation. As Table 1 shows, eserine is unsuitable because the required high concentration blocks muscarone-binding activity. Fortunately, paraoxon did not block muscarone binding, even at 10^{-4} M, and at this concentration the acetylcholinesterase was virtually completely inhibited as judged by the following test. The paraoxon-treated sample was incubated with muscarone and acetylcholine for various times, the minimum being 20 minutes (i.e., the time between adding muscarone and removing a sample for assay after centrifugation). No progressive loss of acetylcholine-blockage was seen, the per cent blockage being 42 at 20 minutes, 38 at 37, 39 at 54, and 49 at 71 minutes. Clearly the acetylcholinesterase has negligible activity under the conditions of interest.

TABLE 1. *Blockade of binding of* DL-*muscarone to electroplax fraction.*

Cholinergic Agents*	Final conc. (*M*)	Per cent blockade	Other Agents	Final conc. (*M*)	Per cent blockade
d-Tubocurarine	10^{-6}	25	Tyramine	10^{-4}	12
	10^{-5}	100	Serotonin	10^{-4}	0
Atropine	10^{-6}	0	Norepinephrine	10^{-4}	0
	10^{-5}	20	LSD	10^{-4}	0
Nicotine	10^{-6}	0	Iproniazid	10^{-4}	0
	10^{-5}	54			
Tetraethylammonium	10^{-3}	78	*p*-Hydroxymercuribenzoate	10^{-4}	6
Choline	10^{-3}	33			
Eserine	10^{-5}	0			
	10^{-4}	20			
Paraoxon	10^{-4}	0			
Paraoxon (10^{-4} *M*) plus acetylcholine (10^{-6} *M*)		8			
Paraoxon (10^{-4} *M*) plus acetylcholine (10^{-5} *M*)		87			

* This term is used for agents of any sort whose primary action is upon cholinergic synapses.

The binding constant for acetylcholine to the preparation was measured by incubating various concentrations of acetylcholine, along with a fixed concentration of muscarone, in a preparation previously treated with 10^{-4} paraoxon. Acetylcholine binding is, in this approach, manifested as an inhibition of muscarone binding. Assuming 1 mole of acetylcholine bound per mole of receptor, with binding constant K_t,

$$K_t = \frac{A\ (Rt - Rmi - Ra)}{Ra} \tag{1}$$

where Rt is total receptor, Rmi is receptor-muscarone complex in this inhibited preparation, Ra is receptor-acetylcholine complex, and A is the final concentration of free acetylcholine, that is, after equilibrium is reached. Equation (1) is solved for Ra, and the solution inserted in the equation that describes the extent of muscarone binding, whose affinity is given by K:

$$K = \frac{Mi\ (Rt - Rmi - Ra)}{Rmi}, \tag{2}$$

where Mi is the final free muscarone concentration in this inhibited preparation. Following the above insertion, one finds that the reciprocal fraction of total receptor which is bound to muscarone is given by:

$$\frac{Rt}{Rm} = \frac{K}{Mi} \cdot \frac{K_t + A}{K_t} + 1. \tag{3}$$

In a parallel way, one finds that the corresponding fraction in the absence of acetylcholine ("uninhibited") where Rmu is the new value for receptor-muscarone complex is:

$$\frac{Rt}{Rmu} = \frac{K}{Mu} + 1, \tag{4}$$

combining (3) and (4):

$$K_i = A \Big/ \left\{ \frac{Mi}{K} \left[\frac{Rmu}{Rmi} \left(\frac{K}{Mu} + 1 \right) - 1 \right] - 1 \right\}. \tag{5}$$

Unfortunately the value of A is substantially different from the initial acetylcholine concentration, Ao, and requires separate computation. Since for the muscarone (in the presence of acetylcholine)

$$K = Mi\ Rfi/Rmi, \tag{6}$$

then one may calculate Rfi (the concentration of free receptor) because the experiments give direct measurement of Mi (which is the muscarone in the supernatant) and an indirect measure of Rmi (which is equal to "moles of muscarone bound"). Consequently one may obtain A from:

$$A = Ao - Ra = Ao - (Rt - Rmi - Rfi), \tag{7}$$

where Rt is the total receptor concentration, obtained from the experiment of Figure 2.

Using equations (5) and (7), one may calculate the binding constant for acetylcholine to the preparation for each acetylcholine concentration at which the experiment is performed. Figure 4 shows per cent blockade, by various concentrations of acetylcholine, of the binding of 2.5×10^{-7} M muscarone. From the 12 points of Figure 4 were calculated 12 values of K_i, the binding constant

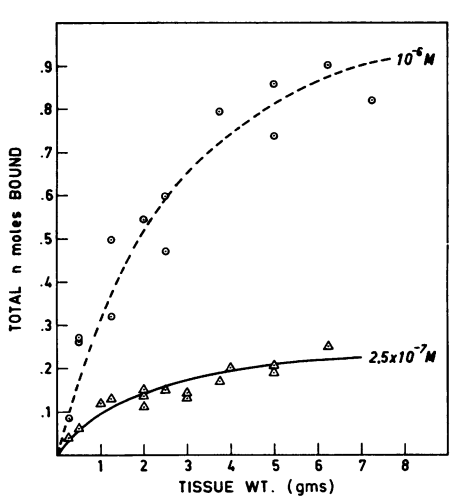


FIG. 3.—Dependence of muscarone binding to 27,000 × g precipitate upon weight of plax from which that precipitate was derived. Two concentrations of muscarone were used as shown.

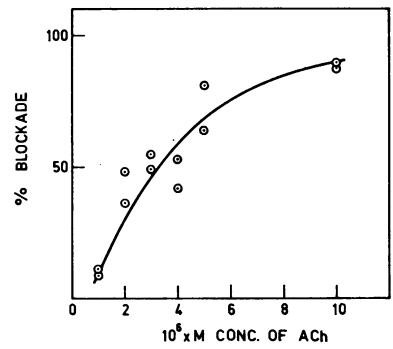


FIG. 4.—Blockade by acetylcholine of muscarone binding to 27,000 × g precipitate from 5 gm of plax.

for acetylcholine. The mean K_i was 1.1×10^{-6} M, and the standard error was 0.3×10^{-6} M.

Discussion.—Does muscarone binding reflect receptor activity?: We have unequivocally shown a suitable affinity for the principal agents of interest, especially muscarone, acetylcholine and tubocurarine. There is an appropriate response to drugs, especially with respect to the strong blockade by curare. In particular, this is the first case of a strong binding of acetylcholine to any macromolecule *in vitro*.

Whether there is an appropriate amount of "receptor" in the tissue is hard to say because of lack of facts about electroplax. For brain tissue, our feasibility calculations, which had to employ very rough estimates indeed, led us to an estimate of 18 nmoles per gm, as follows: about 10^7 neurones per rat brain[14] which weighs about 2 gm, and of which about 15 per cent are cholinergic (based on data for cat, rabbit, and monkey cortex[15] including nicotinic and muscarinic varieties); an average of 1500 synapses per neurone[16] an estimate of 10^7 receptors per synapse (based on curare binding to end-plates[7]). Thus $10^7 \times 0.5 \times 0.15 \times 1500 \times 10^7 = 1.1 \times 10^{16}$ per gm, or 18 nmoles per gm. It seems plausible that the electroplax values should be within 100-fold of the brain values, and in fact we find values of 1.8 nmoles per gm. By contrast, Chagas[4] found that gallamine, a curare-like compound, was bound to cat muscle and to various electroplax fractions at about 10^5 nmoles per gm, an effect which (in the case of electroplax) is probably caused by mucopolysaccharides, as he suggested.

The reversibility of muscarone binding is an important piece of evidence in favor of the identity of muscarone binding and receptor activity. Several researchers[4, 17] have examined bound radioactive curare or gallamine which cannot be removed by dialysis. In our opinion, such binding cannot offer an index of receptor activity, because curare acts reversibly upon receptors.

Undoubtedly the least satisfactory aspect to date concerns localization of the muscarone-binding activity. The present techniques do not permit us (for instance) to be assured that we are dealing with a synaptic species, let alone a postsynaptic one. Rather than attempting to develop a system to isolate postsynaptic membranes from electroplax, we will seek purification of our binding macromolecule, and then use fluorescent or autoradiographic antibody techniques to examine localization.

Muscarone binding and acetylcholinesterase: There have been speculations on the possible identity of acetylcholinesterase and acetylcholine receptor.[18] Our data suggest that the muscarone binding which we are examining is not at the active site of acetylcholinesterase, because of the extreme differences in pH profile and response to eserine and paraoxon and because[19] muscarone binds to acetylcholinesterase with $K = 2.5 \times 10^{-4}$, about 100 times less strongly than to our material.

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gratefully acknowledged from the Guggenheim Foundation and from a U.S. Public Health Service grant GM0780. The following compounds were generously donated: LSD (Sandoz) and Iproniazid (Hoffman-La Roche).

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