tle or no increase in the ^3H overflow. Thus, although [^3H]adenosine may be taken up by smooth muscle cells as well as by neurons, the increase in overflow after stimulation at 30 pulses per second or application of nicotine cannot be attributed to a release of ^3H from smooth muscle cells as a consequence of change in the muscle tone, but rather to a release of ^3H from neurons. Muscle relaxation and increased overflow of ^3H were also elicited in three taenia coli preparations in which electrical stimulation (0.1 msec, 20 volts, 30 per second for 120 seconds) was applied by two closely positioned platinum rings, through which taenia coli was pulled. Both responses were abolished within 30 minutes of application of tetrodotoxin (2×10^{-7} g/ml). These results are consistent with the evidence that ATP, ADP, and AMP are released during stimulation of Auerbach's plexus, from turkey gizzard, dissected free of smooth muscle (*4*, *8*).

In further experiments, a preparation consisting of perivascular nerve and taenia was used in which the sympathetic supply to the taenia could be selectively stimulated (*7*). In the absence of guanethidine, stimulation of the perivascular sympathetic nerve (2 msec, 15 to 60 volts, 30 per second for 60 to 120 seconds) elicited a reduction in the muscle tone and increased overflow of ^3H in those preparations which were first incubated with [^3H]adenosine. Introduction of guanethidine sulfate (10^{-6} g/ml) abolished both responses to 15- or 30-volt stimulation. With 60 volts, the mechanical response was also abolished, and the ^3H overflow was reduced by guanethidine. From the exclusively adrenergically innervated rabbit aorta previously incubated with [^3H]-adenosine, guanethidine blocked the ^3H overflow following nerve stimulation (*9*). It is not known whether nucleotides are released from cholinergic nerves.

Our results indicate that ATP can be synthesized from adenosine, stored as ATP (but not ADP) in the taenia coli, and released upon activation of both nonadrenergic and adrenergic inhibitory nerves. Although it appears that this property is not unique to the nonadrenergic inhibitory system, it is consistent with the previous evidence that ATP may be the transmitter at this neuroeffector junction (*4*). It remains to be determined what quantitative and other features of ATP release are unique to the nonadrenergic inhibitory neuroeffector system.

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Acetylcholine Binding to Torpedo Electroplax: Relationship to Acetylcholine Receptors

Abstract. *Binding of [^3H]acetylcholine to a particulate fraction of* Torpedo *electroplax was measured by equilibrium dialysis. Two high-affinity sites present on phospholipoproteins bound acetylcholine reversibly, and binding was blocked by nicotinic drugs. Characteristics of this binding suggest that these phospholipoproteins may be acetylcholine receptors.*

Physiologically, one recognizes the acetylcholine receptor (AChR) by the changes in membrane potential or conductance that suitable cholinergic agents cause in the membrane of which AChR is a part. Attempts to isolate AChR require some biochemical index, of which the most favorable appears to be the binding of cholinergic agents having appropriate specificity, affinity, amount, and reversibility. Several previous attempts, in which the binding of *d*-tubocurarine or gallamine triethiodide was used as an index (*1*), were unsuccessful because the high concentrations of ligand or the low ionic strengths of buffers used led to isolation of nonspecific macromolecules. It became apparent that cholinergic ligands also bind to a variety of enzymes, hormones, and mucopolysaccharides (*2*); and the characteristics of their binding are important in distinguishing AChR.

The direct way to identify AChR in vitro is to examine binding of the natural transmitter acetylcholine (ACh). However, acetylcholinesterase (AChE), which hydrolyzes ACh very rapidly, is always present in high concentrations in tissues rich in AChR. Consequently, we started by studying the binding of five cholinergic ligands which are not hydrolyzed by AChE in particulate preparations of electroplaxes from *Torpedo* and *Electrophorus* (*3*), as well as a supernatant fraction of housefly brain centrifuged at 100,000*g* (*4*). Multiple sites for binding cholinergic ligands were revealed in the electroplaxes, and the ones suggested to be on AChR were phospholipoproteins of nicotinic nature (*5*, *6*).

Recently, we also found that several organophosphates, when added at a concentration of 10^{-4} mole/liter, irreversibly inhibited all the AChE present in *Torpedo* electroplax (as judged by the inability to detect any hydrolysis of ACh after dialysis); but the organophosphates did not interfere with the binding of muscarone, nicotine, or acetylcholine (*7*). The degree of inhibition of AChE and blockade of binding varied among the organophosphates and cholinergic tissues tested. Only at higher concentrations did organophosphates reversibly block binding to macromolecules suggested to be AChR. These results were in good agreement with those reported for the physiological effect of organophosphates on AChR of the monocellular preparation of the electric eel (*Electrophorus electricus*) electroplax (*8*).

Being able to phosphorylate the esteratic site of AChE without affecting AChR activity enabled us to proceed and study binding of [^3H]ACh (specific activity 50 mc/mmole; New England Nuclear) to the particulate preparation of *Torpedo* electroplax. The label was on the acetate, so that binding would be observed only if all AChE was inhibited and ACh was intact, because we found the acetate anion did not bind. Binding of choline could be mistaken for binding of ACh, as has happened in an earlier study (*9*).

We measured binding by equilibrium dialysis (at 4°C for 16 hours) in 100

volumes of a modified Krebs Ringer solution (*4*), *p*H 7.4 and ionic strength 0.2, containing various concentrations of [^{3}H]ACh (10^{-9} to 3×10^{-6} mole/liter). Radioactivity in equal samples of the dialysis bag in excess of that in the bath after dialysis represented the amount of ACh bound. Details of the procedure and the preparation of the lyophilized pellet (12,000*g*) of *Torpedo* electroplax (9.3 mg of protein per gram of electroplax, and used at 0.5 g of electroplax per milliliter) have been described (*3, 4*). The organophosphate Tetram (*O,O*-diethyl *S*-diethylaminoethyl phosphorothiolate) was added (at 10^{-4} mole/liter) to the electroplax preparation for 20 minutes before the start of dialysis, as well as to the Ringer solution; AChE was thus totally inhibited throughout the experiment.

When the amount of ACh bound was plotted as a function of its concentration, saturation occurred below 10^{-6} mole/liter. This finding contrasts with a report that in the presence of $10^{-5}M$ eserine binding of ACh to a particulate preparation of *Electrophorus* electroplax, as well as to its purified AChE, did not reach saturation at concentrations up to $2 \times 10^{-5}M$ (*10*). This may be due to the low ionic strength of the dialysis medium, in which case excess ACh inside the dialysis tubing would result largely from Donnan equilibrium rather than from true binding. We found that $10^{-5}M$ eserine partially blocked the binding of ACh to *Torpedo* electroplax, and we could not detect any significant binding of ACh at 5×10^{-8} mole/liter to purified AChE of bovine erythrocytes (Winthrop) at a concentration giving equivalent enzymic activity to that in the electroplax used. At concentrations of ACh above 10^{-6} mole/liter, we found that the amount bound decreased, a probable result of excess transmitter inhibition; this phenomenon is being studied (*11*) especially in relation to desensitization (*12*).

A Scatchard plot (Fig. 1) demonstrated two slopes, indicating the presence of two binding affinities. Computer analysis of this data based on the iterative analysis of the Scatchard equation [whereby contribution of binding of one site to the other is eliminated (*13*)] indicated a binding site of high affinity ($K_1 = 8 \times 10^{-9}$ mole/liter) at 0.1 nmole per gram of electroplax, and one of lower affinity ($K_2 = 6.8 \times 10^{-8}$ mole/liter) at 0.83 nmole per gram of electroplax. These concentrations are much lower than the value of about 160 nmole per gram of *Torpedo* electroplax calculated from the binding of hexamethonium. This value was obtained by gel filtration in Sephadex LH-20 of a proteolipid extract of the *Torpedo* electroplax in chloroform and methanol, and was suggested to represent AChR (*14*). De Robertis *et al.* (*15*) have detected two sites for binding ACh in a proteolipid extracted from *Electrophorus* electroplax; they concluded that the high-affinity site may be AChR. Although this agrees with our detection of a single site in *Electrophorus* electroplax which binds both muscarone and nicotine (*6, 16*) and which we suggested to be AChR, our calculation of its concentration is smaller (about 0.03 nmole per gram of electroplax) than theirs (4.8 nmole per gram of electroplax). They did not measure reversibility of binding of ligands whose physiological action is reversible, which we consider an important criterion for identification of AChR in vitro. They also measured binding in an organic phase, where the polarity of the microenvironment around the active site would be altered from the normal physiological condition, and this should greatly affect binding properties (*17*). In another study (*18*) on *Torpedo* electroplax, in which α-bungarotoxin was used as an irreversible affinity label for AChR, the concentration of binding sites (calculated to be 1.1 nmole per gram of electroplax) was similar to the total binding sites we had previously found for muscarone and nicotine (*3, 5*) and to the ones for ACh (0.93 nmole per gram of electroplax).

Table 1. Blockade of the binding of ACh (5×10^{-8} mole/liter) by cholinergic and noncholinergic drugs (10^{-7} mole/liter). Negative values indicate an increase in binding in the presence of a drug. Values in parentheses are not significant as judged by the *t*-test.

Drug	Blockade of ACh binding (%)
Decamethonium	24
d-Tubocurarine	35
Pilocarpine	(−1)
Atropine	(−2)
γ-Aminobutyrate	(−2)
Serotonin	(−7)
Norepinephrine	(−7)

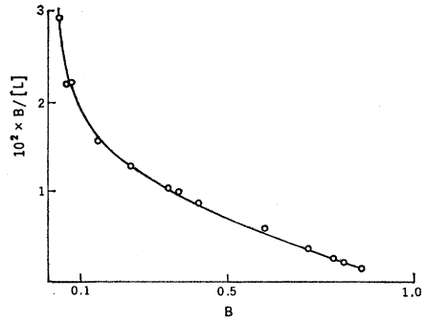

Fig. 1. Scatchard plot of binding of ACh to a particulate fraction of *Torpedo* electroplax ([L], ACh concentration in nanomoles per liter; B, amount of ACh bound in nanomoles per gram of electroplax). Each point represents the average of two experiments, three samples each.

Comparison of binding constants of agonists determined in vitro with those calculated from experiments in vivo should be made with caution. Most responses measured in vivo reflect the combined effect of affinity for the ligand and its ability to depolarize. Because barriers to permeability exist, the actual concentration of ligand at the active site is unknown; therefore many assumptions are made in the calculations. The dissociation constants of ACh reported above are lower than the value of 2.08×10^{-6} mole/liter calculated for ACh in physiological experiments on rabbit stomach muscle (*19*). We had found differences in the same direction and of similar magnitude between dissociation constants in vivo and in vitro for other cholinergic ligands from electroplaxes (*5, 6*). Also in AChE of *Electrophorus* electroplax, dissociation constants of several drugs in vitro were 140 to 510 times lower than ones determined in vivo (*20*). An interesting point is whether disruption of membrane structure favors conformations of receptor with higher affinities. Although these low dissociation constants are thermodynamically feasible (*21*), they are calculated from concentrations, and only after the pure receptor is obtained and the kinetics of ACh binding are studied will the true constants be known.

We found binding of ACh to be totally reversible when we redialyzed in Ringer free of ACh. In the presence of concentrations of other ligands two times higher, only nicotinic ligands were able to compete with ACh and block its binding (Table 1). This blockade was observed at both ACh binding sites. The failure of atropine and pilocarpine to compete with ACh agrees with the nicotinic nature of AChR of the electroplax, whose ontogeny and pharmacology are similar to those of skeletal muscle. When the electroplax homogenate was first treated with trypsin, chymotrypsin, or phospholipase C (1 mg/ml), binding of ACh (5×10^{-8} mole/liter) was reduced by 40, 48, and 66 percent, respectively; this finding suggests that the macromolecules binding ACh are phospholipoproteins.

We had found this same *Torpedo* preparation to have two binding sites for the agonists, muscarone and nicotine; the concentration of the site with highest affinity ranged between 0.06 to 0.08 nmole per gram of electroplax, and the one with lower affinity ranged between 0.4 to 0.8 nmole per gram of electroplax (*5*). Binding activity at both sites was partially destroyed by the above three enzymes. It is apparent that the concentrations of these sites and their enzyme sensitivity are similar for muscarone, nicotine, and acetylcholine. Binding in each case is antagonized by nonradioactive nicotinic ligands, it is reversible and of high affinity, and (at identical concentrations) it is not found with AChE. It is therefore suggested that the binding is to AChR.

On the basis of the characteristics of binding of muscarone and nicotine at both sites, we suggested that either the two sites are on the same macromolecule and are noninteracting or show negative cooperativity, or each site is carried by a different macromolecule, and the two could bear a relationship like that of isozymes to each other (*5*). By analogy, the same may be true for ACh. There have been several suggestions concerning the allosteric nature of AChR (*22*) and its existence in two conformations in vivo (*23*). Most proposals of protein conformational changes assume that the conformations are in rapid equilibrium; thus we would detect only an average of the two affinities under our experimental conditions. Consequently, it is a remote possibility that the two affinities for ACh presently detected or for other agonists represent the affinities of a single site in macromolecules existing in two conformations (*24*). Further identification and characterization awaits isolation of the binding macromolecules from this heterogeneous mixture.

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21. If it is assumed that the ACh molecule binds to the active site of the receptor by electrostatic (5 to 10 kcal), hydrogen (2 to 5 kcal), and hydrophobic (0.5 to 0.7 kcal) bonds, the average free energy supplied in the reaction would be 10.6 kcal/mole. Such energy is well within that required to give the high binding affinities observed at 4°C (—9.1 and —10.3 kcal/mole for the low and high affinities, respectively).
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24. *Note added in proof*: According to a newly proposed model [N. Laiken and G. Nemethy, *Biochemistry* **10**, 2101 (1971)], "flexible ligands cannot be expected to bind in accordance with the single-class model of the theory of multiple equilibria." The new model proposes that findings such as those described herein may reflect the binding of different ligand configurations to identical sites. As these sites are successively filled, subsequent ligand molecules ar restricted in the number of multifunctional attachments which they can form, and so bind with lesser affinity. This model offers another alternative for explaining the two binding affinities observed here.
25. We thank Dr. R. D. O'Brien for his encouragement and constructive criticism, Dr. T. Podleski for his helpful comments on this manuscript, and Dr. H. Howland for devising the computer program used. This research was supported by PHS grants NS 09144 and GM 07804 and training grant ES 98.

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Ribosome-Catalyzed Polyester Formation

Abstract. *Deamination of phenylalanyl–transfer RNA with nitrous acid yields the α-hydroxyacyl analog, phenyllactyl–transfer RNA. When this is incubated in a protein-synthesizing system directed by polyuridylic acid, it yields an acid-precipitable, alkali-labile polyester of phenyllactic acid.*

Peptidyl transferase is the enzyme in the ribosome which catalyzes the formation of peptide bonds during protein synthesis, and it is responsible for the biosynthesis of all proteins. In a study of the reactivity of this ribosomal enzyme we have demonstrated (*1*) that it can also catalyze the formation of ester bonds (*2*). Thus, for example, the ribosome can form an ester link between *N*-formylmethionine and the α-hydroxy analog of puromycin (*1*). We also have shown that ribosomes are able to insert α-hydroxy acids into protein through the use of α-hydroxyacyl-tRNA which is formed from aminoacyl-tRNA by treatment with nitrous acid (*3*). Hydroxy acids have been incorporated into a fragment of bacteriophage coat protein in an in vitro system with the use of viral RNA as a messenger for protein synthesis. A study of the hexapeptide fragment formed in this system showed that the ribosome would accept deaminated phenylalanyl- or alanyl-tRNA and incorporate them into the correct position in the polypeptide sequence, as determined by the position of the appropriate codon in the messenger RNA, with formation of an ester linkage in place of the normal peptide bond. However, this left open

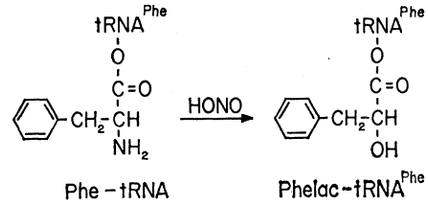

Fig. 1. Conversion of phenylalanyl-tRNA to phenyllactyl-tRNA. [^{14}C]Phelac-tRNA was prepared from [^{14}C]Phe-tRNA by nitrous acid deamination; 3 mg of [^{14}C]Phe-tRNA (*3*) was incubated in 0.25*M* sodium acetate, 0.01*M* magnesium acetate, 1*M* $NaNO_2$, at 24°C for 30 minutes on a Radiometer *p*H-stat maintained at *p*H 4.3 with 25 percent acetic acid. The RNA was then precipitated and washed with ethanol at −20°C. Analysis after alkaline hydrolysis revealed a residue of <0.1 to 0.5 percent phenylalanine. Some of the radioactivity (30 to 40 percent) did not migrate with Phelac, probably because of rearrangement products (*10*). As shown by Carbon (*11*), these conditions result in minimal alteration of the tRNA. These conditions are milder than those used by Herve and Chapeville (*8*) for the same conversion.