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Molecular Biology of Synaptic Receptors

Synapses of the central nervous system, electric organ, and muscle contain a proteolipid with receptor properties.

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The chemical theory of synaptic transmission implies that a specific chemical mechanism, which amplifies the electrical signal, is interposed between the presynaptic and postsynaptic components. Such a mechanism involves (i) a chemical substance, the so-called transmitter, which is synthesized and stored in the nerve endings and is liberated at the arrival of the nerve impulse and (ii) a specific receptor with which the transmitter reacts at the postsynaptic membrane. As a consequence of such interaction a change in ionic permeability takes place which may either depolarize (excite) or hyperpolarize (inhibit) the postsynaptic membrane.

In 1904 Elliot suggested that sympathetic nerves acted by liberation of adrenaline (*1*), and in 1906 Dixon proposed that parasympathetic nerves released a substance similar to muscarine. In 1914 Dale strongly supported the chemical theory of synaptic transmission and suggested that a choline ester, probably acetylcholine, was the transmitter of the parasympathetic system. These postulates were finally proven in 1921 by the classical experiments of Loewi on the vagus stimulation to the frog heart. After these investigations, the theory of a chemical transmission was extended to sympathetic ganglia, the myoneural junction, electric organs, and synapses in the central nervous system, where in addition to acetylcholine and noradrenaline, other biogenic amines, such as dopamine and 5-hydroxytryptamine, and the amino acids γ-aminobutyric acid, glutamic acid, and glycine have also been recognized to act as possible transmitters [for this historical background, see Eccles (*2*)]. De Robertis and Bennett (*3*) correlated the storage of the transmitter with the synaptic vesicles that they found in nerve endings. This relationship was finally established by the isolation of this subcellular component and the demonstration that the fraction containing the synaptic vesicles had the highest concentration of biogenic amines (*4*).

On the side of the receptor, the advances were much less significant. In 1909 Langley postulated the possible existence of a receptive substance at the myoneural junction for drugs similar to curare; however, knowledge about the chemical nature of the receptor made little progress, and attempts to isolate the receptor were disappointing (*5*). The high concentration of acetylcholinesterase, present at the postsynaptic membrane of the myoneural junction, and the fact that this enzyme had some properties in common with the receptor suggested that acetylcholinesterase, with its anionic and ester sites, could be a model for the cholinergic receptor (*6*). However, this concept has remained controversial. Some investigators have suggested that the enzyme and the receptor could be part of the same macromolecule and that the anionic active sites could be fully or partially coincident (*7, 8*). On the other hand, autoradiographic studies of mouse diaphragm (*9*) suggested that acetylcholinesterase and the receptor were two different macromolecules. Axelsson and Thesleff and Miledi (*10*) showed that the receptor and acetylcholinesterase had opposite behaviors after denervation; that is, whereas the latter tended to disappear, there was a considerable increase in cholinergic receptors, as studied iontophoretically, with a wider distribution on the sarcolemma of the skeletal muscle. In the electroplax of *Electrophorus electricus*, Karlin (*11*) stressed the differences between the apparent dissociation constant of the complex of receptor and acetylcholine and that of acetylcholinesterase; more recently (*12*) he emphasized the importance of sulfhydryl groups and of a disulfide bond present in the vicinity of the active site of the receptor in the binding of the acetylcholine and other cholinergic drugs. However, according to Changeux *et al.* (*8*) both the acetylcholine receptor and acetylcholinesterase contain disulfide bonds, and Zupančič (*13*) has reported that, the effect of reagents containing sulfhydryl groups or disulfide bonds on the rabbit ileum is rather unspecific.

In denervated muscle it was found that although acetylcholinesterase was inactivated by some proteolytic enzymes, the receptor properties were not affected (*14*). Changeux *et al.* (*8*) observed that the component which accounts for the binding of acetylcholine to the electroplax membranes was resistant to pancreatic lipase, trypsin, and chymotrypsin but that it was digested by pronase. By means of *p*-(trimethylammonium)-benzene diazonium fluoroborate (TDF), a drug which has a cationic head similar to acetylcholine and which covalently bonds with amino acid residues, the electroplax was

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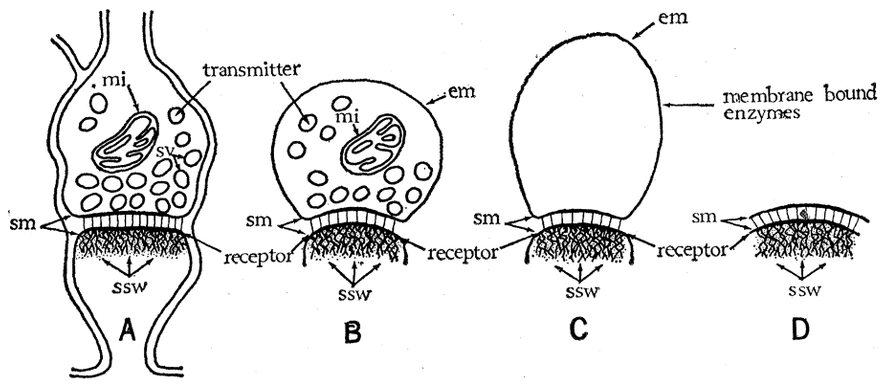

Fig. 1. Diagram of a synaptic region of the cerebral cortex; (A) in situ; (B) after homogenization and gradient centrifugation; (C) after osmotic shock of the isolated nerve ending; and (D) after treatment of (C) with Triton X-100. The systematic dissociation leads to the concentration of the subsynaptic membranes where the receptor is localized; *em*, ending membrane; *mi*, mitochondria; *sm*, synaptic membranes; *ssw*, subsynaptic web; *sv*, synaptic vesicles.

irreversibly inactivated. Because such an inactivation could be protected with *d*-tubocurarine, it was thought that TDF acted on the cholinergic receptor (*15*).

The acetylcholinesterase molecule separated from the electroplax has a total molecular weight of 260,000 and is made up of four polypeptide chains having equal molecular weights (*16*). Changeux *et al.* (*8*) proposed that acetylcholinesterase may contain "catalytic" and "noncatalytic" or "regulatory" subunits and that the latter "might be, or contribute to, the acetylcholine macromolecular receptor" (see below). According to the authors "it is clear that the acetylcholine receptor and acetylcholinesterase are closely related proteins."

In the mechanism of action of the receptor mentioned at the beginning there are two different steps: (i) binding of released transmitter to specific sites in the receptor and (ii) production of a response which may be detected by physiological, pharmacological, or biochemical methods (*17*). This definition excludes the sites to which the drug may be bound without producing a response (for example, sites of loss, "silent" receptors, or "spare" receptors). It is evident that even if receptors could be separated from the membrane, as a general rule only the first step of the drug receptor interaction could be studied. However, by means of artificial membranes and biophysical methods it is possible to study certain responses of the isolated receptor.

In the last decade several attempts have been made to isolate the cholinergic receptor. Chagas *et al.* (*18*), using electric tissue of fish, separated a protein which precipitated with triethiodide of gallamine. Similarly, soluble proteins which precipitated with *d*-tubocurarine were extracted from electric tissue (*19*) or from medullated fibers. However, the validity of these findings was not confirmed (*20*), and the findings were attributed to unspecific binding by mucopolysaccharides, which are abundant in electric tissue (*5, 21*). A ribonucleoprotein which precipitates with tubocurarine was also isolated from skeletal muscle (*22*). In all of these studies there were two main pitfalls. One was the lack of a structural approach; that is, the soluble proteins that were separated were probably not related to the postsynaptic membrane. The other was the use of high concentrations of the ligand, which produces unspecific binding with anionic groups of various chemical components of the cell.

Our approach to the problem of isolating receptors from central and peripheral synapses differs from that mentioned above and is based on the following premises: (i) the receptor should be a macromolecule essentially located in the subsynaptic membrane; (ii) it should be a protein intimately bound or built into the lipoprotein structure of the membrane; (iii) it should show high affinity for binding the natural endogenous transmitters and a specific competition with drugs that act as agonists or antagonists; and (iv) such macromolecules should be capable of undergoing some kind of reversible change when they react with the transmitter in order to account for the translocation of ions through the membrane and the subsequent bioelectrical response.

Changeux *et al.* (*8*) have postulated the presence in the membrane of an "acetylcholine-protomer" comprising two structural entities, the acetylcholine macromolecular receptor endowed with the capacity of specific recognition for acetylcholine and the acetylcholine-ionophore involved in the selective passage of cations promoted by the acetylcholine binding to the macromolecular receptor. These two coupled functions could be carried out by the same macromolecule, but they could be performed by different polypeptidic or lipoproteic entities.

Table 1. Distribution of acetylcholinesterase (AChE) and binding of *d*-[dimethyl-^{14}C]tubocurarine (DMTC), [5-^{14}C]hydroxytryptamine (5HT), [^{14}C]lysergic acid diethylamine (LSD), [^{14}C]-dibenamine, [^{14}C]propanolol, and [^{3}H]chloropromazine to the subcellular fractions of M_1 on a density gradient. Acetylcholinesterase is expressed as relative specific activity; the binding of drugs is expressed as specific binding ratio (counts per milligram of protein in a fraction divided by counts per milligram of protein in the total particulate). Results of the first two columns correspond to rat cerebral cortex, the others to cat basal ganglia. [From (*26*) and (*27*) and unpublished data.]

Sub-fraction	AChE	DMTC	5HT	LSD	Dibena-mine	Propa-nolol	Chloro-promazine
			Myelin				
M_1 0.8	1.64	2.14	0.38	0.60	0.61	0.69	0.42
			Nerve-ending membranes				
M_1 0.9	3.40	4.16	2.08	1.33	3.06	3.40	2.32
M_1 1.0	3.45	6.88	2.79	2.09	3.14	3.30	3.22
M_1 1.2	1.44	3.00	1.84	1.64	3.42	1.80	2.82
			Mitochondria				
M_1 pellet	0.38	1.60	0.52	0.86	0.82	0.56	0.45

Isolation of Nerve-Ending Membranes and Junctional Complexes

Interest in the isolation and characterization of receptors from the central nervous system started in our laboratory as a consequence of work on cell fractionation of the cerebral cortex and other gray areas of the brain [for a review, see (*23*)]. From the

very beginning, we observed that the isolated nerve endings carried attached to them the subsynaptic membrane where the receptor was expected to be present (*24*) (Fig. 1B). Later on, when the nerve endings were disrupted by osmotic shock and the synaptic vesicles were first separated (*4*), it was observed that the bulk fraction M_1, which sedimented with low gravitational force (20,000*g* for 30 minutes), contained the nerve-ending membranes with the attached subsynaptic component (Fig. 1C). By gradient centrifugation of the M_1 fraction we separated several subfractions containing nerve-ending membranes, myelin, or mitochondria (Table 1). A further step in this dissociation of the synaptic region was obtained by treating the nerve-ending membranes with low concentrations of the nonionic detergent Triton X-100. This procedure "dissolved" most of the limiting membrane leaving intact the so-called junctional complex (*25*) (Fig. 1D). This complex comprises the two synaptic membranes joined together by the intersynaptic filaments and the subsynaptic web, a system of fine filaments implanted on the subsynaptic membrane which penetrates, at various distances, into the postsynaptic cell.

The separation of these morphologically homogeneous fractions of nerve-ending membranes and of junctional complexes provided an excellent material with which to study the high affinity for binding with labeled transmitters and synaptic blocking agents. A simple centrifugation technique based on the exposure of the material to low concentrations of the ligand (10^{-7} to 5×10^{-6} mole/liter) followed by centrifugation and washings was used (*26*).

As shown in Table 1, after binding of subcellular fractions from the cerebral cortex with the cholinergic blocking agent *d*-[dimethyl-^{14}C]tubocurarine, the radioactivity was concentrated in those fractions of nerve-ending membranes (Fig. 1C) which were rich in acetylcholinesterase (M_1 0.9 and M_1 1.0). Similar results were obtained with [methyl-^{14}C]hexamethonium and [^{3}H]alloferine (alcuronium chloride) (*26*). The binding of these cholinergic blocking agents could be interfered with by competition with atropine and acetylcholine. When similar experiments were performed on the isolated junctional complexes (Fig. 1D), the binding of *d*-[dimethyl-^{14}C]tubocurarine and [methyl-^{14}C]hexamethonium was about the same as that in the original nerve-ending membranes, whereas acetylcholinesterase and other membrane-bound enzymes were greatly reduced in the sediment but were found in the supernatant (Table 2). These findings supported the interpretation that the receptor properties are localized in the junctional complexes and probably in the subsynaptic membrane. We also concluded that acetylcholinesterase has a wider distribution in the nerve-ending membrane and probably represents a macromolecular entity different from the receptor.

Table 2. Binding of *d*-[dimethyl-^{14}C]tubocurarine (DMTC) and [methyl-^{14}C]hexamethonium (MHM), and acetylcholinesterase activity (AChE) in nerve-ending membranes (subfraction M_1 1.0) of rat cerebral cortex after treatment with Triton X-100. Results are expressed as percentage of the control (Triton divided by control times 100) obtained from the absolute values per milligram of protein and in ratios of DMTC to AChE and MHM to AChE in two experiments [data from (*25*)].

DMTC	MHM	AChE	DMTC/ AChE	MHM/ AChE
150.5	150.0	50.0	3.0	3.0
164.0	179.0	45.5	3.6	3.9

Similar binding studies were carried out with other drugs. The nerve-ending membranes isolated from basal ganglia, hypothalmus, and mesencephalic nuclei of the cat had a high affinity for binding [^{14}C]serotonin and [^{14}C]histamine; for the alpha adrenegic blocking agents [^{14}C]Sy28 (*N*-α-naphthylmethyl *N*-ethyl-β-bromoethylamine) and [^{14}C]-dibenamine; for the beta adrenergic blocking drug [^{14}C]propanolol (*27*); and for some psychotropic and neuroleptic drugs such as [^{14}C]lysergic acid, [^{3}H]-chloropromazine, and [^{3}H]trifluoropromazine (Table 1). [^{14}C]Mescaline did not bind to the membranes, but became slightly concentrated in the synaptic vesicles. This finding indicates that this psychotropic drug may be active presynaptically.

Isolation of Central Receptor Proteolipids

The experiments with cholinergic blocking agents led us to investigate to which of the macromolecular components of the membrane the ligand was bound. The crucial experiment was performed with nerve-ending membranes labeled with *d*-[dimethyl-^{14}C]-tubocurarine (Table 3). When such membranes were extracted with a mixture of chloroform and methanol, the radioactivity was recovered in the organic phase containing the lipids and small amounts of proteolipid protein. Only a few counts were observed in the water phase containing the gangliosides and other water-soluble components and in the protein residue, which comprises 93 to 94 percent of the total protein (*28*). Because it was shown by several methods that the label was in a bound form, the problem consisted of determining with which of the components of the organic phase the drug was associated. Experiments with thin-layer chromatography on silica gel showed that the *d*-[dimethyl-^{14}C]tubocurarine remained at the point of origin, together with the proteolipids; however, the most decisive results were obtained with the use of a special method of column chromatography with Sephadex LH-20 followed by elution with chloroform and a series of mixtures of chloroform and methanol of increasing polarity. The radioactivity appeared together with the last peak of proteolipid protein in the chloroform and methanol (4 : 1 by volume) (*28*). In other experiments the lipid extract was first precipitated with diethyl ether, a treatment which removes all cholesterol, 80 percent of the phospholipids, and 50 percent of the cerebrosides to the supernatant. The residue was redissolved in chloroform and methanol and then passed through the Sephadex

Table 3. Binding of *d*-[dimethyl-^{14}C]tubocurarine by nerve-ending membranes (subfraction M_1 1.0) of the cat cerebral cortex. Effect of extraction with chloroform and methanol (2 : 1) and partition of the extract with water. Results are given in disintegrations per minute per gram of tissue and in percentage of control [data from (*28*)].

Sample	Content	Binding	
		Disintegrations per minute per gram of tissue	Percentage of control
Control pellet	Subfraction M_1 1.0	17,632	100
Residual pellet	Proteins	897	5
Upper phase	Gangliosides	891	5
Lower phase	Lipids and proteolipids	15,424	87

LH-20 column. In this case also, the drug appeared together with the last peak of protein. In this region there were only small amounts of lipid phosphorus represented mainly by phosphatidyl inositol (Fig. 2A). From these results and the controls with free drug, we concluded that, at low concentrations, the *d*-[dimethyl-^{14}C]tubocurarine fixed by the nerve-ending membrane appeared exclusively bound to the proteolipid. Only with higher concentrations of the drug (above 1×10^{-6} mole/liter) did the other proteins of the membrane show unspecific binding. From these experiments and others in which the proteolipid was first extracted and then bound to the drug, it was evident that this special protein of the membrane had the high affinity for binding necessary for it to qualify as "the receptor proteolipid" (*28*).

Proteolipids represent a special type of lipoprotein macromolecules present in biological membranes and are characterized by their hydrophobic properties—that is, their solubility in organic solvents. They were first isolated from white matter in which they constitute an important part of the protein of myelin (*29*), and they have since been found in mitochondria, chloroplasts, microsomes, and various subcellular membranes of the cerebral cortex (*30*). That the receptor proteolipid isolated from the nerve-ending membranes is different from that of myelin was demonstrated by binding experiments with *d*-[dimethyl-^{14}C]tubocurarine which showed that myelin has a much lower binding capacity.

Experiments on nerve-ending membranes from basal ganglia of the brain in which different drugs active in synaptic transmission were used showed that the high affinity for binding was also characteristic of a proteolipid fraction. For example after binding with [5-^{14}C]hydroxytryptamine the membranes were extracted with a system of butanol and water and the bound amine was recovered in the butanol phase. After column chromatography on Sephadex LH-20 with an elution system as indicated above, 80 percent of the radioactivity appeared at the end of the column together with about 14 percent of the proteolipid protein and negligible amounts of lipid phosphorus (*31*). Such results demonstrate that the central receptor for serotonin is also of proteolipid nature. Although less tightly bound, the adrenergic blocking drugs [^{14}C]Sy28, [^{14}C]dibenamine, and [^{14}C]propanolol also showed a high affinity for binding with proteolipids in the nerve-ending membranes of the central nervous system (*27*).

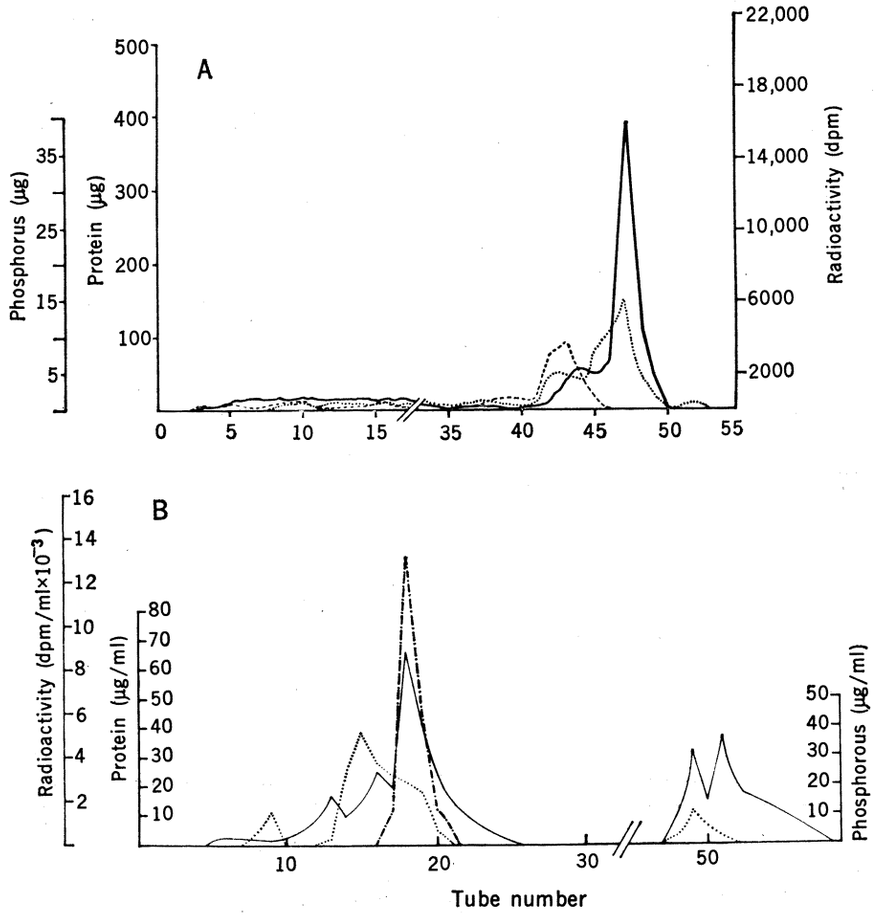

Fig. 2. (A) Chromatographic pattern of a lipid extract precipitated with ether from total particulate of the cerebral cortex of the cat. The precipitate, redissolved in chloroform and methanol (2 : 1 by volume), was passed though a column of Sephadex LH-20 and eluted with the following mixtures of solvents: 80 milliliters of chloroform and 20 milliliters each of chloroform and methanol in the ratios of 15 : 1, 10 : 1, and 6 : 1; and 80 milliliters of chloroform and methanol (4 : 1). Fractions of 4 milliliters per tube were collected. Observe that the *d*-[dimethyl-^{14}C]tubocurarine ([^{14}C]DMTC), which was added to the particulate at a concentration of 1.5×10^{-6} mole/liter appears together with the last peak of protein in chloroform and methanol (4 : 1) (*28*). Key: ——, ^{14}[C]-DMTC; • • • • •, protein; – – – –, phosphorus. (B) Chromatographic pattern obtained from lyophilized electric tissue of *Electrophorus*. The chloroform and methanol (2 : 1) extract was submitted to binding with 5×10^{-7} molar [^{14}C]acetylcholine ([^{14}C]ACh) and then passed through the Sephadex LH-20 column. Observe that the radioactivity appears only in peak 3 of proteolipid protein which is eluted with chloroform (*35*). Key:——, protein; – • – • –, [^{14}C]ACh; • • • • •, phosphorus; dpm, disintegrations per minute.

Isolation of a Receptor Proteolipid from the Electroplax

Our work on the isolation of receptors from the central nervous system had the disadvantage of having to deal with a very complex population of nerve endings involving different chemical transmitters. A tissue with a purely cholinergic innervation is provided by the electric organs of fishes such as *Torpedo* and *Electrophorus*. The so-called electroplax is a large, sheetlike, modified muscle cell, physiologically adapted to the generation of transcellular current. This function is a consequence of the asymmetry of the electroplax, since on one side it is densely innervated by cholinergic terminals whereas on the other it is not innervated and is electrically inexcitable. A single electroplax of *Electrophorus* weighing 30 to 50 milligrams contains some 30,000 to 50,000 synapses. Ehrenpreis *et al.* (*32*) estimated that the re-

ceptor protein present in 1 kilogram of this electric tissue should be of the order of 10 to 20 milligrams.

We have extracted the proteolipids from lyophilized electric organs of *Torpedo* and *Electrophorus* with a mixture of chloroform and methanol (2 : 1) and then studied the high affinity for binding labeled acetylcholine, hexamethonium, and *p*-(trimethylammonium)-benzene diazonium fluoroborate (TDF). After the binding, the lipid extract was passed through the Sephadex LH-20 column. The proteolipid protein was eluted with the chloroform in a sharp peak which contained most of the bound radioactivity; the rest of the protein, which was eluted at the end of the column, showed practically no radioactivity (Fig. 2B). Phospholipids were also eluted with chloroform but the maximum amount of lipid phosphorus appeared slightly earlier than the peak of receptor protein. With the three cholinergic drugs the pattern of elution was very similar, and the free drugs were retained by the column (*33*). From these experiments we concluded that a receptor proteolipid which shows high affinity for binding acetylcholine and cholinergic blocking agents is extracted from the electric tissue.

Knowing the protein content of the receptor peak we could calculate the amount of receptor proteolipid present in the electric tissue. In *Torpedo* we calculated 65 milligrams per kilogram of fresh tissue; in *Electrophorus* the amount was about half that figure (26 milligrams per kilogram of fresh tissue). The higher content in *Torpedo* may be related to a richer innervation and to the fact that bioelectrogenesis only results from postsynaptic potentials whereas in *Electrophorus* spikes are also generated (*34*).

A study of the binding of the proteolipid receptor (peak 3) of *Electrophorus* with concentrations of [^{14}C]acetylcholine ranging from 7×10^{-7} to 5×10^{-5} mole/liter resulted in a saturation curve which did not have the characteristic shape (that is, a rectangular hyperbola) expected for the saturation of a single set of binding sites. The use of both double reciprocal plotting and the Scatchard equation (Fig. 3) suggested that the binding was multiple. Two sets of binding sites, one of high affinity with a dissociation constant K_1 equal to 1×10^{-7} and another of low affinity with K_2 equal to 1×10^{-5}, were demonstrated (*35*). If it is assumed that in the receptor peak there is a homogeneous population of proteolipid molecules with a molecular weight of 40,000 (both assumptions being supported by the electron microscopic observations), it may be postulated that per proteolipid molecule there is one binding site of high affinity and about ten of low affinity (Fig. 3). Since between the two affinity constants there is a 100-fold difference, one may speculate that physiologically the amount of acetylcholine acting on a synapse (*36*) would bind only to the first site, which would be the true receptor site.

Table 4. Effect of 1 molar NaCl on acetylcholinesterase and binding capacity of isolated electroplax membranes. Fractions containing membranes were separated at 0.4 and 1.0 molar sucrose on a gradient, according to the technique of Changeux *et al.* (*37*). Acetylcholinesterase activity is expressed in micromoles per hour per milligram of protein. The binding was performed with [methyl-^{14}C]hexamethonium (MHM) (3×10^{-6} mole/liter), as described by Azcurra and De Robertis (*26*) and was expressed in disintegrations per minute per milligram of protein [data from (*37*)].

Fraction	NaCl (mole/liter)	AChE	MHM
0.4		3500	14,166
0.4	1	353	13,527
1.0		3408	26,316
1.0	1	132	20,000

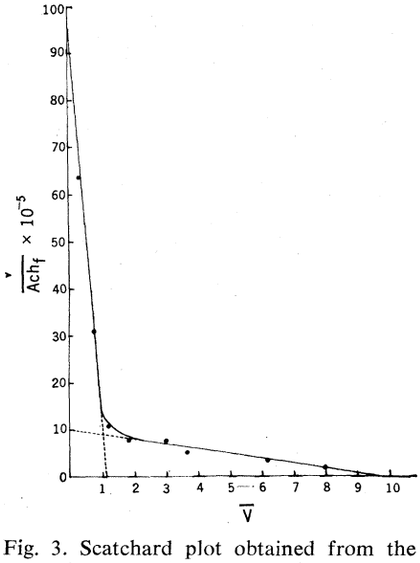

Fig. 3. Scatchard plot obtained from the saturation curve in peak 3 (Fig. 2B) with concentrations of [^{14}C]acetylcholine between 7.0×10^{-7} and 5.0×10^{-5} mole/liter. $\overline{\nu}$ is the average number of moles of acetylcholine bound per mole of proteolipid. [ACh_f] is the concentration of free acetylcholine. At saturation $\overline{\nu}$ equals 10, if a molecular weight of 40,000 is assumed for the proteolipid. Intercepts on the abscissa indicate number of binding sites (n). Intercepts on the ordinate correspond to n/K_{ass}, K_{ass} being the apparent association constant (*35*).

The work on electric tissue received strong support from other experiments in which the membranes of the electroplax were isolated by subcellular fractionation (*37*). In these fractions a parallelism between the acetylcholinesterase activity and the binding of cholinergic drugs was observed. However, when the membranes were incubated with 1 molar NaCl, a treatment which, as demonstrated by Silman and Karlin (*38*), solubilizes most of the acetylcholinesterase, the binding of the cholinergic drugs remained without change (Table 4). These findings were interpreted as conclusive evidence that acetylcholinesterase and the cholinergic receptor are two different macromolecules present in the electroplax membrane but having very different solubility properties. When these membranes from which acetylcholinesterase had been removed were extracted with chloroform and methanol (2 : 1), as in the case of normal membranes or of the intact electroplax, a receptor proteolipid peak having high affinity for binding [^{14}C]acetylcholine was obtained.

Physicochemical Studies on the Interaction of Drugs and Receptors

The receptor proteolipid isolated from the cerebral cortex was studied with physicochemical methods (light scattering, polarization of fluorescence, and electron microscopy) which could give some information about the macromolecular interaction of drug and receptor. The receptor proteolipid has an absorption band at 278 to 280 nanometers which is related to the content of aromatic amino acids. Upon activation at 330 nanometers, there is a fluorescence maximum at 380 nanometers, which is probably due to tryptophan residues (*39*).

It was of great interest to observe that the receptor proteolipid interacted with atropine sulfate dissolved in chloroform and methanol (4 : 1) to give a dramatic increase in light scattering and in polarization of fluorescence (*39*), whereas no changes were observed in the other proteolipid peaks. In both cases the curves of response as a function of dose followed a sigmoid shape characteristic of a cooperative type of reaction (*40*). The Hill

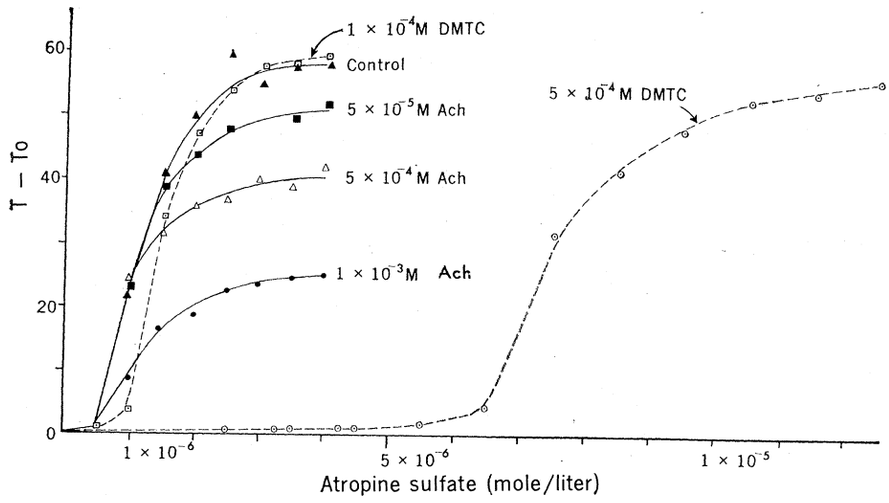

Fig. 4. Changes in light scattering ($T - T_o$) caused by atropine sulfate in the receptor proteolipid peak of the cat cerebral cortex. The light scattering was determined at 90 degrees in an Aminco Bowman spectrofluorometer at a wavelength of 430 nanometers. Both the incident and scattered light were vertically polarized by means of Glann prism polarizers. The cuvette contained 12 milligrams of receptor proteolipid in chloroform and methanol (4 : 1). Observe that in the presence of acetylcholine (ACh) there is a decrease in amplitude of the response. *d*-[Dimethyl-^{14}C]tubocurarine (5×10^{-4} mole/liter), within certain limits, completely inhibits the response produced by atropine sulfate (*39*).

numbers of these curves were, respectively, 3.15 and 3.90 for the light scattering and the polarization of fluorescence (*41*). In the presence of acetylcholine there was a decrease in the amplitude of the light-scattering reaction with a slight reduction in Hill number, whereas with 5×10^{-4} molar dimethyl-*d*-tubocurarine there was a complete blockade, which was surmounted with higher concentrations of atropine sulfate (Fig. 4). At the same time this drug increased considerably the Hill number of the reaction (up to 9) indicating a higher degree of cooperativity. Succinylcholine and hexamethonium also inhibited the reaction but in a way intermediate between that of acetylcholine and dimethyl-*d*-tubocurarine (*39*).

The changes in light scattering and polarization of fluorescence were interpreted as resulting from an increase in particle size as a result of the association of proteolipid molecules by the atropine sulfate. In fact, with monovalent salts of atropine or with atropine base there was no reaction. The light-scattering phenomenon is not stereospecific since it may be obtained with other bivalent amines such as the sulfates of eserine, amphetamine, dibenzylamine, and strychnine but not with aniline or sodium sulfate. As a working hypothesis it was postulated that in central synapses there is a receptor proteolipid which has group specificity rather than stereospecificity for the various amines. However, the possibility should be considered that once the receptor proteolipid has been separated from the membrane, its stereospecificity is lost.

When the binding of [^{3}H]atropine with the central receptor proteolipid was studied by equilibrium dialysis we found that it bound at very low concentrations (10^{-8} mole/liter) at which there was no change in light scattering. The cooperativity detected in the optical phenomena at the higher concentrations could be accounted for by a kind of chain reaction between proteolipid molecules which had already bound the ligand. Observations with the electron microscope support this interpretation.

The receptor proteolipid of the cerebral cortex is made up of macromolecules about 15 angstroms wide and at least 150 angstroms long which show a tendency to aggregate. Highly purified receptor proteolipid treated with 10^{-8} molar atropine sulfate showed a paracrystalline ordered array of filaments forming tubule-like structures with a 40-angstrom lumen (Fig. 5A); but as the concentration of atropine sulfate was increased, irregular macromolecular aggregates appeared (*42*). These findings demonstrate that the changes in light scattering and polarization of fluorescence when atropine sulfate was added are due to the aggregation of proteolipid molecules. Such an aggregation could be favored by the very low dissociation of the drug in the nonpolar medium in which the reaction is studied.

Electron microscopic studies were made of the receptor proteolipids from the electroplax and from rat diaphragm after addition of acetylcholine or hexamethonium. Paracrystalline arrays of macromolecules were observed when this proteolipid was bound to very small concentrations of the ligands (Fig. 5, B and C). Those obtained with the proteolipid from diaphragm which reacted with methyl-hexamethonium are particularly impressive (Fig. 5C) and indicate not only that there is a rather homogeneous population of macromolecules but also that they are highly reactive to the addition of minute amounts of the ligand. No such changes were observed in the other proteolipid peaks.

At this point it is interesting to emphasize the differences between the receptor proteolipid extracted from central synapses and those obtained from electric tissue and skeletal muscle. Whereas the former are eluted from the Sephadex LH-20 column with chloroform and methanol (4 : 1) and they bind muscarinic cholinergic drugs (atropine), the receptor proteolipids from the electroplax and skeletal muscle are eluted with chloroform and bind only nicotinic blocking agents, showing no light-scattering reaction with atropine sulfate.

Central synapses contain both nicotinic and muscarinic receptors (*43*), whereas the electroplax and the myoneural junction contain only nicotinic cholinergic receptors. Thus, our findings are consistent with the physiological and pharmacological properties of the tissue from which the proteolipid was isolated.

The fact that receptor proteolipids, either from central or peripheral synapses, show macromolecular changes upon addition of very small concentrations of cholinergic drugs, thus producing paracrystalline arrays, may also provide some enlightment on its function within the subsynaptic membrane. It seems possible that in their original localization the receptor proteolipid macromolecules could also undergo conformational changes when they bind to the natural transmitter in situ.

Another point of considerable inter-

est is the hydrophobic behavior of proteolipids. This is accounted for by a special tertiary structure of the polypeptide chain in which nonpolar residues are prevalent at the outer surface of the macromolecule. In proteolipids from myelin a high content of alpha helix has been determined by circular dichroism and optical rotatory dispersion, but the alpha helix is greatly reduced in the water-soluble apoprotein (*44*).

The hydrophobic properties of proteolipids pose very interesting problems in relation to the possible integration of the receptor within the lipoprotein framework of the membrane. Wallach and Zahler (*45*) and Lenard and Singer (*46*) have stressed the importance of hydrophobic proteins and hydrophobic interactions in biological membranes. Models having protein segments which penetrate across the membrane have been produced, and several of these hydrophobic molecules in parallel have been thought to limit pores in the membrane (*45, 46*). The recent work on polypeptide antibiotics and synthetic cyclic polyethers which promote permeability changes in biological and artificial membranes may also bear on this point (*47*). The general feature of these so-called "ionophores" is that they are able to encase the mobile ion (potassium or sodium ions) in hydrophilic groups leaving the hydrophobic residues at the periphery. Because of their hydrophobic properties, ionophores may transverse the lipoidal layers functioning as ionic carriers or, as in the case of polyene antibiotics, may form true pores across the membranes.

Brain proteolipids have tightly bound phospholipids represented mainly by phosphatidyl inositol. This is of particular interest in view of the experiments that demonstrate the increase in turnover of phosphatidyl inositol in brain slices and other tissues under the action of acetylcholine or electrical stimulation (*48*). Recent work on brain slices has demonstrated that the effect of acetylcholine is exclusively on the phosphatidyl inositol that is tightly bound to the receptor proteolipid (*49*). In fact, there is no effect on the unbound phosphatidyl inositol when acetylcholine is added. The action of acetylcholine on the turnover of phosphatidyl inositol is by way of a phospholipid hydrolase that specifically separates the phosphate and inositol moieties, leaving the diglyceride part of the molecule attached to the membrane. We have shown that such an enzyme is also bound to the membrane. According to Durell *et al.* (*50*) this step is followed by a series of enzymatic reactions which lead to the reconstitution of the phosphatidyl inositol molecule. We suggest that the effect of acetylcholine on phosphatidyl inositol may induce changes in the microenvironment of the receptor proteolipid in the synaptic pathways that are repeatedly activated. This effect may subserve more permanent molecular

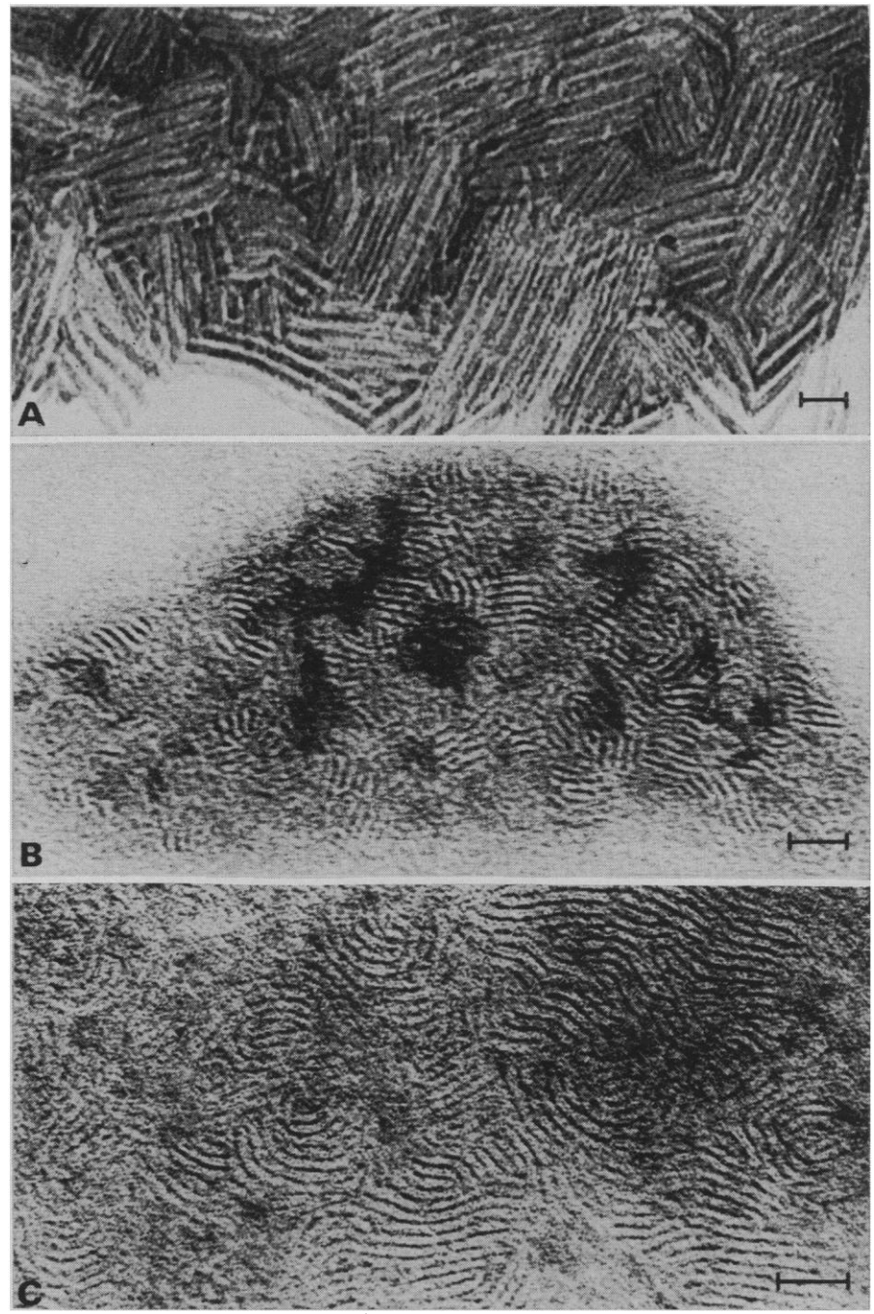

Fig. 5. Electron micrographs of receptor proteolipids interacting with different drugs. The scale indicates a distance of 200 angstroms. (A) Highly purified receptor proteolipid from cat cerebral cortex treated with 10^{-8} molar atropine sulfate. The macromolecules have assumed a paracrystalline array forming packed tubular structures with a lumen of 40 angstroms. The clear lines correspond to the rod-shaped proteolipid molecules [from (*42*)]. (B) Receptor proteolipid from *Electrophorus* (peak 3) with addition of acetylcholine. Piles of rod-shaped structures are observed (× 360,000). (C) Receptor proteolipid from rat diaphragm with addition of hexamethonium. The proteolipid was spread on glutaraldehyde and negatively stained with uranyl acetate. Paracrystalline arrays of filaments make the predominant pattern (× 480,000).

changes of the receptor and may modulate the synaptic function. This could be an excellent mechanism for bringing about longer lasting changes of the synapse, such as those that may occur in learning and memory.

Experiments with [^{3}H]leucine injected intraventricularly were carried out by Lunt (*51*). He followed the decay of the specific radioactivity for weeks in the proteolipids of different membrane fractions and found that the proteolipids of myelin have the well-known, extremely long half-life as did those of mitochondria. On the other hand, the proteolipids from the nerve-ending membrane showed a much shorter half-life (about 15 days), and reached a much higher specific activity. This was considered as another indication that the receptor proteolipid is a specially active molecule in brain.

Of even greater interest are preliminary observations on the turnover of [^{3}H]leucine in the receptor proteolipid of the rat diaphragm. After denervation, coinciding with the period of maximum sensitivity to acetylcholine (*10*), there was an extremely marked increase in turnover of the receptor proteolipid of the denervated hemidiaphragm compared to that of the control hemidiaphragm (*52*).

Model for the Cholinergic Receptor

Figure 6 recapitulates some of the facts and speculations on the macromolecular organization of the receptor. Embedded in the postsynaptic membrane, there are discrete receptor areas in which the receptor proteolipid molecules are located. Because of their hydrophobic surface and elongated shape, the proteolipid molecules may traverse the lipid layers of the membrane from the outer to the inner surface. Such a disposition may be facilitated by the tightly bound lipoid coat, mainly provided in the brain by phosphatidyl inositol. The receptor proteolipid molecules may adopt an oligomeric arrangement, probably tetrameric (*53*). The ionophoric portion of these molecules in parallel may constitute the wall of a pore which is closed at some point in the resting condition so as to stop the passage of ions. Binding of the transmitter molecule to the receptor site of the proteolipid, situated on the outer surface of the membrane, may induce enough reversible change in these macromolecules to permit the translocation of ions through the ionophoric portion of the receptor. If the pore is made of several proteolipid molecules its opening could be easily produced by changes in the degree of interaction between the monomeric units. This change would take place every time that the receptor, with its anionic and stereophilic sites, is occupied by acetylcholine. Such an electrostatic effect would be facilitated by the fact that the proteolipid molecules are held in place by hydrophobic interactions in a highly dielectric medium. Such a reversible physicochemical change occurring at a very fast rate could be the primary phenomenon in synaptic transmission. It remains to be determined if the receptor site and the ionophoric properties of the molecule lie in the same or in different protein subunits as postulated by Changeux *et al.* (*8*). Any explanation of the function of a receptor demands that both the binding of the transmitter and the translocation of ions should be coupled in space and time and should take place within the subsynaptic membrane. Other events taking place in the vicinity of the receptor area would be the splitting of acetylcholine by acetylcholinesterase and the activation of phospholipid hydrolases. The latter will lead to removal of the phosphoinositide moiety of the phosphatidyl inositol, attached to the receptor proteolipid (*49*). Figure 6 emphasizes the molecular bases of these three events that result in the interaction of the transmitter with the receptor area of the synapse.

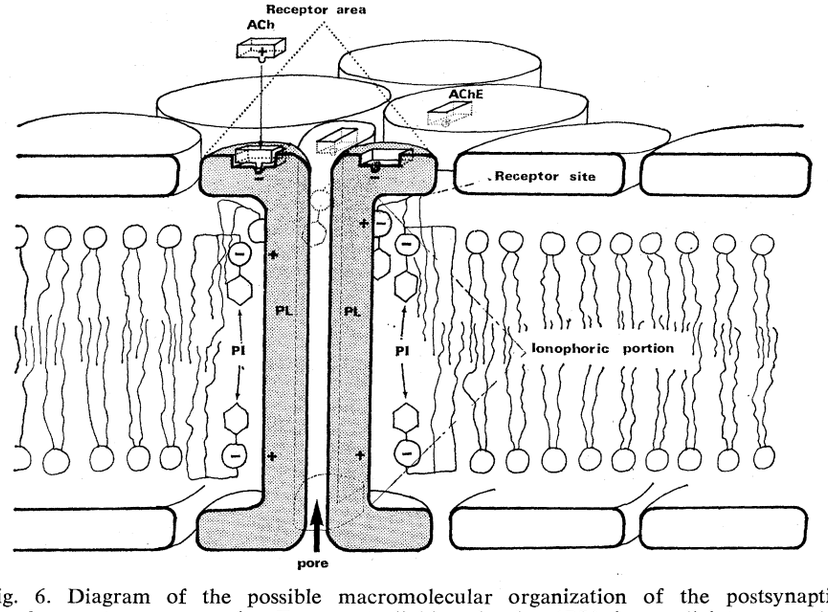

Fig. 6. Diagram of the possible macromolecular organization of the postsynaptic membrane at a receptor site. Four proteolipid molecules (*PL*) in parallel traverse the membrane and with their ionophoric portions constitute the wall of a pore. A receptor site for acetylcholine (*ACh*) is indicated at the outer end of the proteolipid. A receptor area is constituted by the neighboring receptor sites and may form discrete patches on the membrane surface. The presence of acetylcholinesterase (*AChE*) as a separate macromolecule is indicated. The site on the enzyme at which acetylcholine is bound is shown. The diagram also emphasizes the attachment of phosphatidyl inositol molecules (*PI*) to the receptor proteolipid. Such an attachment would be facilitated by charges in the phosphate and in the proteolipid. The turnover of the phosphoinositide moiety of the phosphatidyl inositol would be mediated by a phospholipid hydrolase which may be activated by acetylcholine. (For further details see description in the text.)

Search for a Receptor Response

As mentioned in the introduction, there are two main steps in the mechanism of action of receptors. Everything that has been said in this article pertains to the first step, that of the high affinity for binding with the transmitter. However, some recent experiments done in our laboratory (*54*) may represent a starting point to uncover the second step, the eliciting of a response in the membrane.

Bilayered (black) lipidic membranes of about 1 square millimeter separating two solutions containing 0.1 molar NaCl and 0.05 molar tris(hydroxymethyl)aminomethane buffer were made. Into such membranes the receptor proteolipid of *Electrophorus* was in-

corporated in the proportion (protein to lipid) of approximately 1 to 10,000. By applying different voltages across the membrane we measured the conductivity. The proteolipid produced a considerable decrease in resistance of the membrane, thus increasing its conductivity. This property was common to both the receptor (peak 3) and to a nonreceptor proteolipid (peak 1) obtained from the electroplax of *Electrophorus* (Fig. 2B). However, only the membrane with the receptor proteolipid reacted to the addition of acetylcholine by a considerable and transient increase in conductivity. Such a change is blocked by *d*-tubocurarine.

The surface fine structure of these artificial membranes was studied under the electron microscope. Coinciding with the transient change in conductance there was a transient structural modification at the macromolecular level. The membranes activated with acetylcholine showed a more uneven or corrugated appearance and the presence of dense osmiophilic spots about 20 angstroms in diameter with a heavily stained center. Apparently such spots disappeared and the surface fine structure reverted to the control condition after the end of the acetylcholine effect, upon the vanishing of the conductance change. This and other correlations led up to the hypothesis that the dense spot may actually represent the opening of the potential pore, that is, of the ionophoric portion of the postulated receptor (Fig. 6).

Although we are just at the beginning of a new chapter in the study of the receptor proteolipid the biophysical results so far obtained suggest that a receptor-like response may be produced in these artificial membranes. The bearing that these findings may have on the physiological response of the receptor remains open.

Summary

A special proteolipid (a hydrophobic protein) has been extracted and purified from nerve-ending membranes and total particulate matter of gray areas of the central nervous system. Such a proteolipid shows a high affinity for binding *d*-tubocurarine, serotonin, and atropine and has been called receptor proteolipid. The interaction of this proteolipid with atropine sulfate was studied with light scattering and polarization of fluorescence. The changes observed, which follow a cooperative type of curve, were attributed to the aggregation of the proteolipid macromolecules. Such a phenomenon was then observed under the electron microscope.

A receptor proteolipid having a high affinity for binding acetylcholine, hexamethonium, and other cholinergic drugs was isolated and purified from electric tissue of fishes and from electroplax membranes. Such a proteolipid was also extracted from membranes from which acetylcholinesterase had been removed, and it was concluded that this enzyme and the receptor proteolipid are two different macromolecules. A high affinity binding site with a dissociation constant of K_1 equal to 10^{-7} and about ten sites with K_2 equal to 10^{-5} were recognized in the receptor proteolipid.

Under the electron microscope the receptor proteolipid of brain appears as a rod-shaped macromolecule which may assume paracrystalline arrays with 10^{-8} molar atropine sulfate. Similarly the receptor proteolipid from electric tissue and from skeletal muscle may form paracrystalline arrays under the action of acetylcholine and hexamethonium.

A model of the cholinergic receptor based on the properties of the proteolipid is presented. Preliminary work indicates the possibility of obtaining a biophysical response to acetylcholine when the receptor proteolipid is embedded in artificial bilayered lipid membranes.

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