P. M., Boyle, I. T., and DeLuca, H. F. (1973), *J. Biol. Chem. 248*, 6691.
Holick, M. F., Schnoes, H. K., DeLuca, H. F., Gray, R. W., Boyle, I. T., and Suda, T. (1972), *Biochemistry 11*, 4251.
Martin, D. L., and DeLuca, H. F. (1969), *Amer. J. Physiol. 216*, 1351.
Moffett, R. B., and Weisblat, D. I. (1952), *J. Amer. Chem. Soc. 74*, 2183.
Ohtaka, H., Morisaki, M., and Ikekawa, N. (1973), *J. Org. Chem. 33*, 1688.
Suda, T., DeLuca, H. F., and Tanaka, Y. (1970), *J. Nutr. 100*, 1049.
Zinner, G., and Deucker, W. (1961), *Arch. Pharm. (Weinheim) 294*, 370.

Interaction of a Fluorescent Ligand with Membrane-Bound Cholinergic Receptor from *Torpedo marmorata*†

Jonathan B. Cohen‡ and Jean-Pierre Changeux*

ABSTRACT: Studies are presented of the interaction in a physiological ionic environment of the fluorescent probe 1-(5-dimethylaminonaphthalene-1-sulfonamido)propane-3-trimethylammonium iodide (Dns-chol) with membrane fragments rich in cholinergic receptor protein that are purified from the electric organ of *Torpedo marmorata*. Energy transfer permits the preferential excitation of probe molecules interacting with the membrane, and two classes of Dns-chol binding sites are revealed. The effect of cholinergic ligands on Dns-chol fluorescence as well as binding studies with tritiated acetylcholine and tritiated α-toxin from the venom of *Naja nigricollis* show that one of these classes of sites is the physiologically important receptor site of acetylcholine. The second class of Dns-chol sites is associated with spectral emission properties (λ_{max}) that depend upon whether a cholinergic agonist (depolarizing agent) or a cholinergic antagonist (depolarizing blocking agent) is bound to the cholinergic receptor. On the basis of this result, it is concluded that, upon the binding of cholinergic agonists, some change of membrane structure occurs which can be reversed by antagonists and, hence, is associated with the physiological response of the membrane.

It is now well established that the response of an excitable membrane to acetylcholine and to cholinergic agonists involves a selective increase of permeability to cations (Katz, 1966) which is blocked by a class of related compounds, the cholinergic antagonists (Nachmansohn, 1959). Acetylcholine acts as a regulatory ligand controlling membrane permeability. A minimum of two distinct structural elements have, therefore, been postulated: a "receptor" protein which recognizes cholinergic agonists and an "ionophore" which accounts for the selective translocation of ions and which could be part of the receptor protein or constitute a distinct but tightly coupled entity. The hypothesis was proposed that a conformational transition (Nachmansohn, 1959) mediates the interaction between receptor and ionophore (Changeux *et al.*, 1970).

One step toward the analysis of this mechanism was to demonstrate that the permeability response to cholinergic agonists persists *in vitro* in membrane fragments (or microsacs) isolated from the electric organ of *Electrophorus electricus* (Kasai and Changeux, 1971). The amplitude of the response was directly related to the amount of agonist bound to the cholinergic receptor site present in the membrane fragments.

Fluorescent probes have been used extensively to study biological membranes and model systems (Radda and Vanderkooi, 1972; Waggoner and Stryer, 1970), but previous use of fluorescence techniques to study the cholinergic microsacs (Kasai *et al.*, 1969; Wahl *et al.*, 1971) did not provide information of physiological interest. The recent development (Cohen *et al.*, 1972) of a method of preparation from *Torpedo marmorata* electric organ of membrane fragments rich in cholinergic receptor protein makes possible the use of fluorescence techniques to study this particular protein in the membrane environment.

1-(5-Dimethylaminonaphthalene-1-sulfonamido)propane-3-trimethylammonium iodide, (Dns-chol),[1] was introduced by Weber *et al.* (1971) as a fluorescent ligand possessing a high affinity for a proteolipid binding acetylcholine in an organic phase (De Robertis, 1971) and for horse serum cholinesterase in aqueous solution (Mayer and Himel, 1972). We report here studies showing that in *physiological* saline solution Dns-chol interacts strongly with receptor-rich membrane fragments from *Torpedo marmorata*. A strong enhancement of Dns-chol fluorescence occurs upon binding, and the effect can be partially reversed by cholinergic effectors and a snake venom α-toxin in the concentration range expected from the value of their dissociation constants for the cholinergic re-

† From the Departement de Biologie Moléculaire, Institut Pasteur, 75 Paris, France. *Received July 17, 1973*. This work was supported by funds from the Centre National de la Recherche Scientifique, the Délégation Générale à la Recherche Scientifique et Technique, the Collège de France, The Commissariat à l'Energie Atomique, and the National Institutes of Health. A first presentation of this work was given at the fourth annual meeting of the American Society for Neurochemistry (Columbus, Ohio, March 1973).

‡ Recipient of fellowships from the Délégation Générale à la Recherche Scientifique (1972) and the National Eye Institute of the National Institutes of Health (1F02 EY 52,916-01).

[1] Abbreviations used are: Dns-chol, 1-(5-dimethylaminonaphthalene-1-sulfonamido)propane-3-trimethylammonium iodide; physiological saline, 250 mM NaCl–5 mM KCl–4 mM $CaCl_2$–2 mM $MgCl_2$–5 mM sodium phosphate buffer (pH 7.0).

ceptor site. From the analysis of the signal remaining in the presence of an excess of effector, direct evidence is given in favor of some change of membrane structure which occurs upon the binding of cholinergic agonists and which is reversed by different compounds all known to act as potent antagonists.

Materials and Methods

Membrane Fragments. The estimation of the concentration of cholinergic receptor site (Weber *et al.*, 1972) from the binding of [^{3}H]α-toxin from *Naja nigricollis* (Menez *et al.*, 1971) and the isolation of membrane fragments rich in cholinergic receptor (Cohen *et al.*, 1972) have been described. *Fresh* electric tissue (60g) from *Torpedo marmorata* yields 4–6 mg of proteins in fragments which make a band at 1.3 M sucrose. These fragments contain about 1000 nmol of [^{3}H]α-toxin binding sites/g of protein and 100 times less of acetylcholinesterase catalytic sites. The peak of acetylcholinesterase occurs around 0.8 M sucrose. An additional centrifugation step was used to transfer the membranes from concentrated sucrose to a suitable ionic environment. One volume of membranes in 1.3 M sucrose was diluted with one volume of distilled water and centrifuged at 80,000*g* for 75 min; 95% of the supernatant was removed, and the pellet was resuspended in *physiological* saline solution (250 mM NaCl–5 mM KCl–4 mM $CaCl_2$–2 mM $MgCl_2$–5 mM sodium phosphate, pH 7) with a finely tipped Pasteur pipet and ten strokes in a Potter Teflon-glass homogenizer. The final membrane suspensions thus contained 95% of physiological saline solution, 5% sucrose (w/v), and between 0.5 and 2.0 mg of proteins per ml. Spectroscopic experiments were performed on the day of resuspension. The final suspensions were turbid but visually homogeneous. Transmission through 2-mm path length was about 25% at 340 nm and 6% at 280 nm. Proteins were assayed by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Cholinergic Effectors. The iodide salt of Dns-chol used in this work was isolated as a reaction intermediate in the preparation (Weber *et al.*, 1971) of the perchlorate salt of Dns-chol. Stock solutions (10^{-2} M) of this compound were prepared in acetone–water (1:4, v/v). Cholinergic effectors obtained commercially were used without further purification. Concentrated solutions (10^{-2} or 10^{-1} M) were prepared in bidistilled water except for acetylcholine bromide which was prepared in absolute ethanol. When acetylcholine was used, membrane fragments were preincubated 20 min with 10^{-4} M Tetram, a potent acetylcholinesterase inhibitor (Eldefrawi *et al.*, 1971). The purified α-toxin of *N. nigricollis* was a gift of Dr. P. Boquet and the tritiated toxin a gift of Drs. A. Menez, J. Morgat, and P. Fromageot.

Fluorescence Measurements. Fluorescence spectra were measured at 21° with a FICA differential recording spectrofluorimeter. A 450-W xenon high-pressure lamp (Osram) was the light source, and a rotating mirror illuminated sequentially the sample cell, a rhodamine quantum counter solution, and a reference cell. Excitation spectra were corrected for variations of lamp intensity and excitation monochromator sensitivity, but not for sample absorption. Emission spectra reported here were not corrected for variation of detector photomultiplier spectral sensitivity. Hamatatsu photomultipliers R372UH or R446 were used to detect signal emission and had been preselected for optimum sensitivity around 550 nm. Quartz fluorescence cells (Helma) had 2-mm excitation and 10-mm emission path lengths.

Monochromator slit widths equivalent to 7.5 nm were used for excitation and emission and allowed illumination of a 5 $\times$ 5 mm area of the sample.

In the dual-beam mode, a differential spectrum was recorded between identical samples (0.4 ml) of membrane suspensions except that in one of them the effectors were added. Concentrated stock solutions were used so that less than 5% dilution occurred. Spectra were recorded at 25 nm/min which permitted three different spectra to be recorded during a 15-min exposure of the sample to the light source. Between each spectrum the samples were thoroughly agitated to prevent excessive exposure of a single region of the suspension, and with this precaution it was found that the intrinsic protein fluorescence did not change significantly in the region of Dns-chol emission.

When fluorescence titrations were performed, emission was observed at 550 nm and fluorescence excitation was either at 287 nm (protein excitation) or 340 nm (Dns-chol excitation). The fluorescence intensity as a function of total Dns-chol concentration was determined, and the effect of added nonfluorescent cholinergic effectors studied. A centrifugation assay was used to determine the concentration of free Dns-chol in equilibrium with the membrane fragments; 0.25 ml of a membrane suspension containing Dns-chol was centrifuged at 150,000*g* for 90 min and 0.2 ml of that supernatant was diluted with 0.2 ml of 95% ethanol. Dns-chol concentration was determined from the observed fluorescence intensity at 550 nm (excitation at 340 nm) of the supernatant–ethanol sample by comparison with standard curves made by adding Dns-chol to a supernatant–ethanol blank over concentrations from 2×10^{-7} to 1×10^{-5} M. The supernatant concentration determined in the presence or absence of a cholinergic effector in the original suspension was used in conjunction with the total probe concentration to determine the total quantity of Dns-chol bound to the membranes and the quantity displaced in the presence of effector. When α-toxin was used, standard curves were determined in the presence and absence of α-toxin.

Results

Spectral Evidence for the Interaction of Dns-chol with Membrane Fragments. Excitation and emission fluorescence spectra of Dns-chol in physiological saline solution change markedly in the presence of membrane fragments rich in cholinergic receptor protein (Figure 1). In the excitation spectrum recorded at 550 nm, the band at 340 nm characteristic of Dns-chol in aqueous solution remains, but a new band appears around 280 nm. The emission spectra change as well. For an excitation wavelength of 340 nm, the emission maximum shifts from 560 to 550 nm; a larger shift, to 535 nm, occurs when the excitation wavelength is 287 nm. None of these effects are seen in the absence of membrane fragments; they, therefore, indicate that Dns-chol interacts with the membrane fragments and that an energy transfer between Dns-chol and membrane proteins can occur.

Detailed characterization of the fluorescence properties of Dns-chol in the presence of membrane fragments was hindered by the scattering and intrinsic fluorescence of the membrane suspension. For a typical suspension containing 1 mg of protein/ml and micromolar in α-toxin binding sites, the light intensity detected at 450 nm upon excitation at 287 nm, although smaller than 1% of the peak emission, is still comparable to the intensity (at 540 nm) emitted by a micromolar solution of Dns-chol. The use of differential spectroscopy reduces the

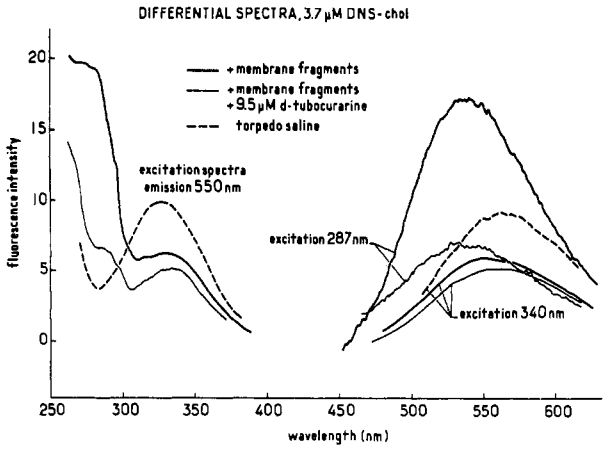


FIGURE 1: Excitation and emission spectra of Dns-chol (3.7 μM) in the presence of receptor-rich membrane fragments (1.1 μM toxin sites, 0.7 mg/ml of protein) in physiological saline solution. Membrane suspensions are in sample and reference cells, Dns-chol in the sample cell. When corrections are made (see Parker, 1968, p 222) for the optical density of the membrane suspensions, the signal at 287 nm is five times that at 340 nm. Spectra recorded after the addition of *d*-tubocurarine chloride to both cells are also shown, as are spectra of Dns-chol in physiological saline soluton.

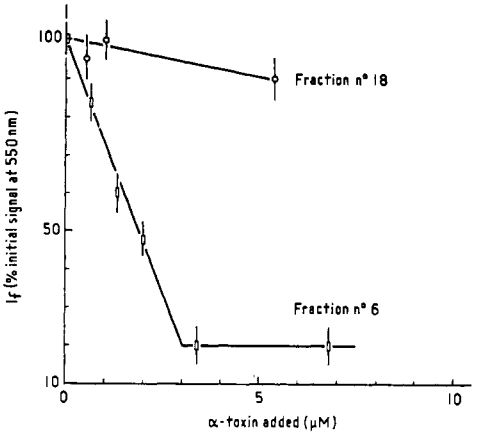


FIGURE 2: Effect of the α-toxin from *N. nigricollis* on the fluorescence of Dns-chol in membrane suspensions in physiological saline. Fraction 6: receptor-rich membrane fragments (0.6 μM α-toxin binding sites, 1.0 mg/ml of protein, 4.4 μM Dns-chol); fraction 18: membranes from peak protein fraction on sucrose gradient (<0.02 μM receptor, 0.9 mg/ml of protein, and 5.0 μM Dns-chol). Intensities are determined at 550 nm for excitation at 287 nm and are expressed as percentage of value observed before addition of toxin. In the absence of α-toxin the fluorescence intensity of fraction 6 was four times larger than that of fraction 18. The abscissa is a measure of apparent, not active, toxin concentration based on the weight of purified toxin in the stock solution. The concentration of *pharmacologically active* toxin which gives the maximum decrease of fluorescence does equal the concentration of [^{3}H]α-toxin binding sites within experimental uncertainty.

detected intrinsic fluorescence by 20-fold, and it becomes possible to obtain spectra of Dns-chol at concentrations as low as 0.5 μM in the presence of a membrane suspension containing less than an estimated 5% of its protein as the cholinergic receptor protein.

Snake venom α-toxins bind with a high affinity and specificity to the cholinergic receptor protein (for review, see Hall, 1972), and, therefore, their use assists in the analysis of the nature of the Dns-chol fluorescence. In Figure 2 is shown the effect of the α-toxin from *N. nigricollis* on the signal observed with two fractions from the same sucrose gradient having different contents in the cholinergic receptor site. Although the two fractions have approximately the same protein concentration, the fluorescence intensity for a given concentration of Dns-chol with the receptor-rich fraction (6) is about four times larger than that measured with the other fraction (18). In the presence of an excess of α-toxin, the signal given by fraction 6 decreases markedly becoming similar in magnitude to that of fraction 18 which does not change upon addition of α-toxin. Controls demonstrated that there was no interaction between Dns-chol and α-toxin in the physiological saline solution. These results suggest that a significant part of the fluorescent signal seen with the receptor-rich fraction is due to the interaction of Dns-chol with the cholinergic receptor site.

Figure 1 shows that a known cholinergic antagonist, *d*-tubocurarine, has, as expected, the same effect as the α-toxin. The change in fluorescence intensity varies with the wavelength of excitation. *d*-Tubocurarine reduces the emitted signal by 70% when the wavelength of excitation is 287 nm while the signal decreases by only 15% at 340 nm. The interaction of Dns-chol with the cholinergic receptor site can, therefore, be most conveniently followed by energy transfer. Most measurements reported in the subsequent part of this work have been done under these conditions.

Characterization of Dns-chol Binding Sites. FLUORESCENCE SPECTRA. The properties of the sites with which Dns-chol interacts in receptor-rich membrane fragments were first studied by varying Dns-chol concentration. Figure 3A shows the change of fluorescence intensity at 550 nm as the *total* amount of Dns-chol added to a membrane suspension varies between 0.3 and 50 μM. This change depends upon the wavelength of excitation and the presence of a cholinergic effector. For 287-nm excitation, the Dns-chol fluorescence intensity increases initially much more slowly in the presence of α-toxin (5 μM) or acetylcholine (50 μM) than in their absence, but at high Dns-chol concentrations, the two curves have similar slopes. In the presence of 5 μM Dns-chol, acetylcholine diminishes the signal to 30% of the value found in the absence of effector; in the presence of 50 μM Dns-chol, the reduction is only to 48%. Only a small difference (10%) was noticed between the residual intensities measured in the presence of α-toxin or in the presence of acetylcholine. The addition of a cholinergic effector after the α-toxin causes no further decrease of Dns-chol fluorescence (less than 15% change). Acetylcholine and α-toxin therefore act on the same class of Dns-chol sites. A spectroscopic dissociation constant, K_{fluo}, of Dns-chol from these sites was estimated from the decrease in the Dns-chol fluorescence observed in the presence of cholinergic effector.

Figure 3B presents a double-reciprocal plot of the data obtained with different membrane preparations. These plots are linear in the region from 2 to 50 μM, and give a spectroscopic dissociation constant, K_{fluo}, which ranges from 15 to 25 μM.

The residual fluorescence intensity observed in the presence of an excess of cholinergic ligand or α-toxin consists of two components. For excitation at 287 nm, the blue shift of the emission spectrum and the nonlinear variation of fluorescence intensity with total Dns-chol concentration indicates binding to the membrane fragments (Figure 3A). In addition, a contribution of the free Dns-chol fluorescence to the residual intensity is expected and becomes significant at low protein concentration.

MEASUREMENT OF Dns-chol BINDING BY CENTRIFUGATION. In order to relate the observed fluorescence signal with actual binding of Dns-chol, the relative amounts of Dns-chol bound to the membrane fragments, displaced by a cholinergic effec-

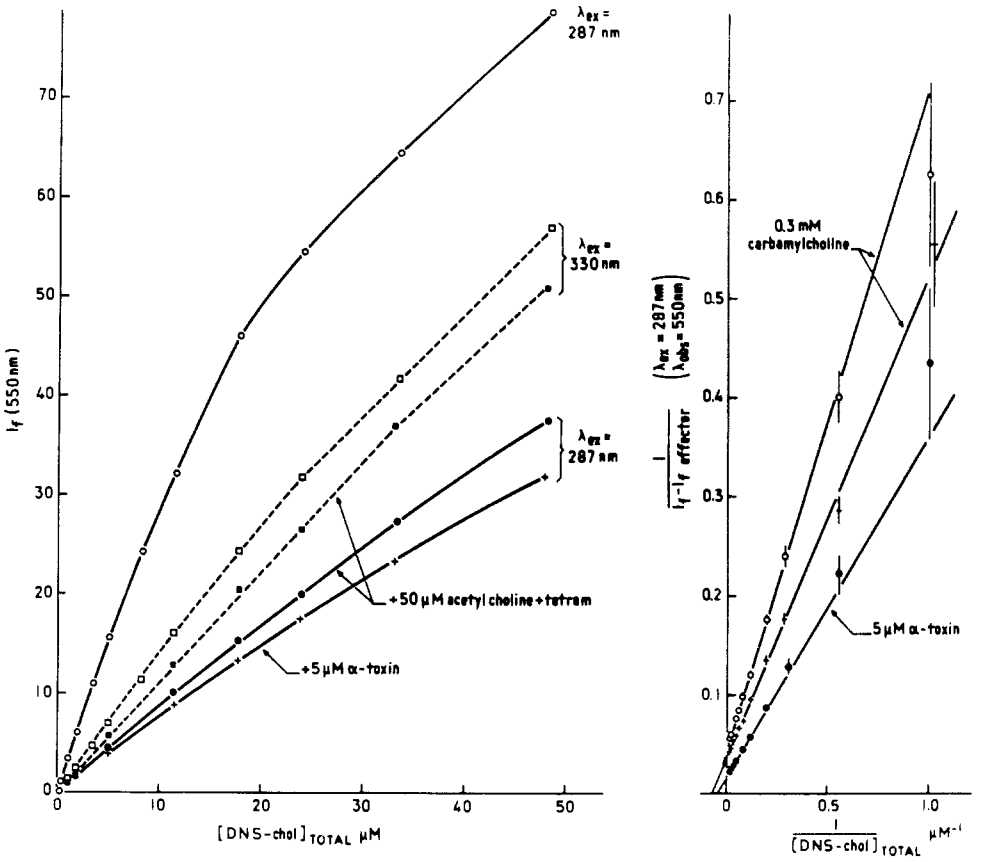


FIGURE 3: (A) Effect of acetylcholine and α-toxin on the Dns-chol fluorescence in a receptor-rich membrane suspension. Experimental conditions: sample and reference cells contain membrane suspensions (0.9 μM in toxin sites; 0.6 mg/ml of protein) in physiological saline. The indicated concentrations of acetylcholine bromide or α-toxin were present in both cells prior to the addition of Dns-chol to the sample cell, and exceed the concentrations necessary to cause the maximal change of Dns-chol fluorescence. (B) Double-reciprocal plot of maximal Dns-chol fluorescence decrease caused by cholinergic effector *vs.* total Dns-chol concentration. Data from part A (●) where the effector is α-toxin is compared with data from another preparation of membrane fragments. In the latter case the signal decrease in the presence of carbamylcholine chloride is presented in two different dilutions of membrane fragments in physiological saline: 0.7 μM in toxin sites and 1.0 mg/ml of protein (+), and 0.35 μM in toxin sites, 0.5 mg/ml of protein (○).

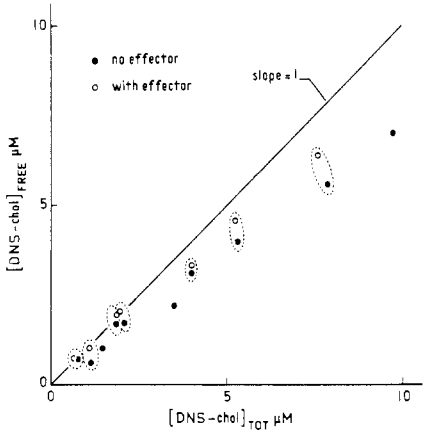


FIGURE 4: Concentrations of free Dns-chol in equilibrium with membrane fragments in physiological saline. Membrane suspensions are incubated with known total Dns-chol concentrations in the absence (●) or presence (○) of α-toxin or carbamylcholine chloride. Dns-chol remaining in the supernatant after ultracentrifugation is determined by a fluorescence assay (see Methods). α-Toxin (7 μM) is the effector except for carbamylcholine chloride (12 μM) at $(Dns\text{-}chol)_{tot}$ = 4.0 μM. The α-toxin concentration exceeds that value required for the maximal Dns-chol displacement. The results are from six different preparations varying in toxin sites from 0.7 to 2.3 μM and in specific activity from 700 to 1700 nmol per g of protein. Paired symbols indicate the same preparation. Precision is ±0.05 μM for concentrations less than 3 μM, increasing to ±0.2 μM by 10 μM.

tor (α-toxin or carbamylcholine), and free in solution were first determined by centrifugation (see Methods). Figure 4 shows these results. In a series of six experiments with four different preparations of membrane fragments, we never observed more Dns-chol displaced by the α-toxin than there are α-toxin binding sites. For total Dns-chol concentrations lower than 2.0 μM and membrane suspensions which were micromolar in toxin sites, essentially all bound Dns-chol could be displaced by the α-toxin. Above 2.0 μM the amount of bound Dns-chol which was no longer displaced by the α-toxin became important; in the presence of 20 μM Dns-chol, less than 1 of the 6 μM bound was displaced by the α-toxin. Nevertheless, under these conditions, total fluorescence intensity decreased by more than 50% upon addition of cholinergic effector (287-nm excitation). These results confirm that at least two distinct classes of Dns-chol binding sites are present on the membrane fragments; they further show that the efficiency of transfer from protein to Dns-chol is greater for the toxin-sensitive binding sites.

EFFECT OF Dns-chol ON THE BINDING OF [^{3}H]α-TOXIN AND [^{3}H]ACETYLCHOLINE. Additional information about the interaction between Dns-chol and the cholinergic receptor protein was found from binding studies with radioactive ligands specific for the cholinergic receptor site. A Millipore filtration assay has been developed by Weber *et al.* (1972) to study the kinetics of binding of [^{3}H]α-toxin to membrane fragments. They have studied the association kinetics in the presence of

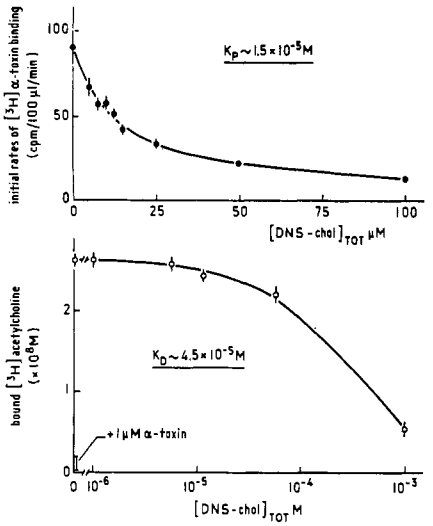


FIGURE 5: (A, top) Initial rate of [^{3}H]α-toxin binding to membrane fragments as a function of Dns-chol concentration. A Millipore filtration assay is used to determine the kinetics of [^{3}H]α-toxin binding. Membrane fragments (5×10^{-9} M toxin sites) are incubated 10 min in physiological saline in the presence of the indicated concentration of Dns-chol prior to the addition of [^{3}H]α-toxin (1 nM). Solid curve calculated for $K_p = 1.5 \times 10^{-5}$ M. (B, bottom) Displacement of [^{3}H]acetylcholine chloride from membrane fragments by Dns-chol. Membrane fragments (3.9×10^{-8} M toxin sites) in physiological saline are preincubated 20 min with Tetram (10^{-4} M) prior to the addition of [*N-methyl*-^{3}H]acetylcholine chloride (120 Ci/mol; 4×10^{-8} M). The free acetylcholine in equilibrium with the membrane fragments is determined from the supernatant radioactivity after ultracentrifugation, and the bound acetylcholine is determined from the difference between free and total acetylcholine. All the bound acetylcholine is displaced by 10^{-6} M α-toxin. The solid curve is calculated from K_D(Ach) = 0.8×10^{-8} M, K_D(Dns-chol) = 4.5×10^{-5} M.

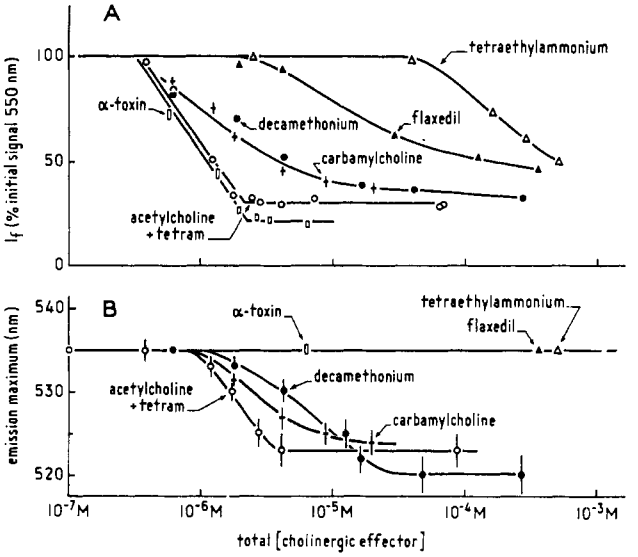


FIGURE 6: (A) Concentration dependence of the effect of cholinergic ligands on the fluorescence of a suspension of receptor-rich membrane fragments containing Dns-chol in physiological saline solution. The intensities determined at 550 nm are expressed as percentages of the intensity observed in the absence of cholinergic effectors. Excitation was 287 nm. Intensities were determined by differential spectroscopy and represent probe fluorescence only. Experimental conditions: α-toxin (0.5 μM toxin sites, 3.7 μM Dns-chol); acetylcholine bromide in the presence of 10^{-4} M Tetram as acetylcholinesterase inhibitor (0.5 μM toxin sites, 3.7 μM Dns-chol); carbamylcholine chloride (0.7 μM toxin sites, 24.2 μM Dns-chol; decamethonium bromide (1.4 μM toxin sites, 3.7 μM Dns-chol); Flaxedil ((gallamine triethiodide) 1.1 μM toxin sites, 3.7 μM Dns-chol); tetraethylammonium chloride (0.5 μM toxin sites, 3.3 μM Dns-chol). (B) The wavelength of maximum emission of Dns-chol as a function of the total concentration of added cholinergic effectors. Conditions as in part A.

a variety of cholinergic ligands and have found that the protection constant characteristic of a ligand's ability to decrease the initial rate of α-toxin binding reflects a competitive interaction between cholinergic ligand and α-toxin at the cholinergic receptor site. Figure 5A illustrates that Dns-chol decreases the initial rate of [^{3}H]α-toxin binding. The protection constant estimated from these data is K_p = 15 ($\pm$4) μM.

The equilibrium binding of [^{3}H]acetylcholine was followed by a centrifugation assay (Kasai and Changeux, 1971). Figure 5B shows an experiment where all the bound acetylcholine was displaced by α-toxin and therefore was associated with the cholinergic receptor site. Under these conditions, Dns-chol displaces acetylcholine as well. Weber and Changeux (1973) have found a dissociation constant for acetylcholine binding to *Torpedo* membrane fragments K_{Ach} = 8 nM. Assuming competitive binding between Dns-chol and acetylcholine, the dissociation constant for Dns-chol calculated from the displacement of acetylcholine is K_D = 45 ($\pm$20) μM. The solid curve of Figure 5B is calculated from that value.

COMPARISON OF SPECTROSCOPIC AND BINDING RESULTS. It can be seen in Table I that the spectroscopic constant estimated from Dns-chol fluorescence which is sensitive to α-toxin is in good agreement with the dissociation constants measured by displacement of radioactive cholinergic ligands. A dissociation constant for Dns-chol can also be estimated from the centrifugation experiment. Since the amount of Dns-chol displaced by the α-toxin was always a small fraction of the total Dns-chol bound in the presence of 20 μM total Dns-chol, it was not possible to determine directly the total number of sites from which Dns-chol is displaced by the α-toxin. However, if one assumes that this number is equal to the number of the toxin sites, then one calculates dissociation constants from the experiments of Figure 4 that vary between 2 and 12 μM, a variability that only emphasizes the imprecision of the estimates.

All these results are consistent with the interpretation that the changes of Dns-chol fluorescence intensity observed upon addition of α-toxin or acetylcholine are caused by the physical displacement of Dns-chol from a binding site common for the α-toxin and acetylcholine, the cholinergic receptor site.

Specificity of the Effects of Cholinergic Ligands on the Dns-chol Fluorescence Intensity. In order to further characterize the Dns-chol site which is sensitive to the presence of cho-

TABLE I: Apparent and Real Dissociation Constants of Dns-chol for the α-Toxin-Sensitive Sites Present in *Torpedo* Membrane Fragments.

Experiment	Dissociation Constant (μM)
Decrease of Dns-chol fluorescence in the presence of cholinergic effector: K_{fluo}	15–25
Concentration of Dns-chol reducing rate of [^{3}H]α-toxin binding by 50%: K_p (Millipore filtration assay)	15 ($\pm$4)
Displacement of [^{3}H]acetylcholine: K_D (centrifugation assay)	45 ($\pm$20)

TABLE II: Compared Interaction of Cholinergic Ligands Characteristic of the Nicotinic Receptor with *Torpedo* Membrane Fragments Followed by Fluorometry and Protection against [^{3}H]α-Toxin Binding.

Effector (No. of Expts)	Conditions: α-Toxin Sites (μM)	Conditions: Total Dns-chol (μM)	Conditions: Effector Concn for Half-Max. Fluorescence Decrease (μM)	K_P (μM)[a] Protection *vs.* [^{3}H]α-Toxin Binding
Carbamylcholine (6)	0.1–1.3	3–20	0.8–1.8	0.5 ± 0.2
Decamethonium (2)	0.9, 1.4	3	0.9, 2.2 ± 0.2	0.7 ± 0.1*
Nicotine (2)	0.5, 0.6	3.3, 6.6	2.7, 1.5 ± 0.3	0.8 ± 0.1
Phenyltrimethylammonium (1)	0.6	6.6	1.5 ± 0.2	
d-Tubocurarine[b] (2)	0.1, 1.1	8.2, 3.7	1–2	0.2 ± 0.1*
Gallamine (5)	0.5–1.2	3.3–6.6	5–20	8
Hexamethonium (1)	1.4	3.6	80 ± 10	40 ± 10
Choline (3)	0.5–1.1	3.4	200–400	60
Tetraethylammonium (2)	0.5, 0.6	3.3, 6.6	200 ± 50, 270 ± 30	

[a] Values from Weber and Changeux (1973); values indicated by * are from equilibrium studies. [b] See text.

linergic effectors, the effect of a wide variety of cholinergic ligands on the fluorescence of Dns-chol bound to membrane fragments was explored (Figure 6 and Table II). All of them cause a decrease of fluorescence intensity which, except for *d*-tubocurarine, reaches a plateau, different from zero, at large effector concentration. The approach to the plateau varies with the ligand considered. For α-toxin and acetylcholine (in the presence of Tetram, an acetylcholinesterase inhibitor), the fluorescence decreases linearly with the total amount of effector added until the plateau is reached. For the other compounds, *d*-tubocurarine excepted, the curve resembles a hyperbola and can be characterized by the effector concentration causing the half-maximal response. With *d*-tubocurarine, the decrease of fluorescence intensity shows a concentration dependence similar to that observed with decamethonium or carbamylcholine as long as *d*-tubocurarine concentrations are lower than 20 μM. Above that concentration the fluorescence intensity continues to decrease almost linearly (an additional 20% in the presence of 200 μM *d*-tubocurarine). The concentration given in Table II for its half-maximal effect was calculated by taking as a value for the plateau fluorescence intensity that found for the other cholinergic effectors with the same preparation.

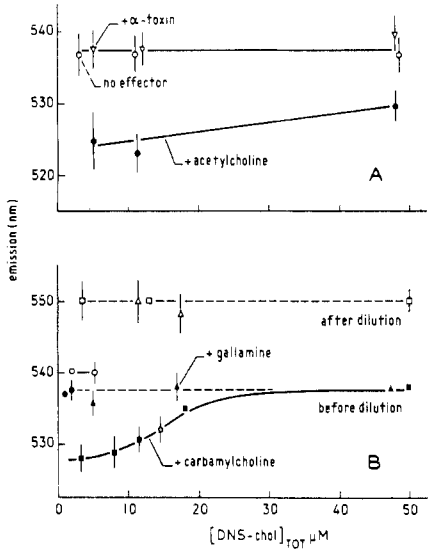


FIGURE 7: Wavelength of maximum emission of Dns-chol in the presence of cholinergic effectors as a function of Dns-chol concentration. (A) The experimental conditions are those of Figure 3A; excitation at 287 nm. (B) Emission maxima observed for a different membrane preparation at different dilutions: solid symbols (0.5 μM toxin sites, 0.7 mg/ml of protein); open symbols (0.1 μM toxin sites, 0.2 mg/ml of protein). Identical membrane suspensions in physiological saline were in sample and reference cells and Dns-chol was added to the sample cell (●, ○). Membrane suspensions were preincubated with carbamylcholine chloride (0.3 mM, ■, □) before the addition of Dns-chol. Gallamine triethiodide (1 mM, ▲, △) was added to membrane suspension containing 5.0 μM in Dns-chol in the sample cell, and then increasing concentrations of Dns-chol were added.

For a given preparation of membrane fragments, the amplitude of the maximal decrease of fluorescence intensity is a function of the concentration of Dns-chol present; for all effectors other than *d*-tubocurarine, the plateau values agree within 10%. At a single Dns-chol concentration the fractional decrease of the observed signal in the presence of cholinergic effectors varies with the concentration of α-toxin binding site and the specific activity of the preparation. As expected for a competitive displacement between effectors and Dns-chol, for a given membrane preparation, the effector concentration which reduces the fluorescence response by half, in general, increases with Dns-chol concentration.

All the results obtained with a wide variety of cholinergic effectors strengthen the view that the fluorescence sensitive to the presence of cholinergic effectors is associated with Dns-chol bound to the cholinergic receptor site.

Spectral Shifts of Dns-chol Fluorescence Emission Associated with the Presence of Cholinergic Agonists. In the course of these studies on the effect of cholinergic ligands on Dns-chol fluorescence, it was noticed that for some compounds, but not for the others, the wavelength for maximal emission shifts slightly to the blue. This is seen in Figure 7A where the wavelengths of maximum emission taken from the experiment of Figure 3A are presented as a function of Dns-chol concentration. For 287-nm excitation and Dns-chol concentration between 1 and 50 μM, the emission maximum in the absence of cholinergic effectors occurs near 537 nm. In the presence of α-toxin, the signal, though reduced in intensity, possesses the same emission maximum. However, in the presence of acetylcholine the emission spectrum is blue shifted by about 15 nm at low Dns-chol concentrations, and the magnitude of the

TABLE III: Maximal Shifts of Dns-chol Emission Maximum Which Accompany the Decrease of Fluorescence Intensity Caused by Cholinergic Ligands.[a,b]

Blue Shift (Occurrences/Expts)	Δλ (nm)	No Shift (Occurrences/Expts)	Δλ (nm)
Acetylcholine (2/2)	15 ± 3	Toxin (4/5)	2 ± 3
Carbamylcholine (7/7)	15 ± 3		
Decamethonium (2/2)	15 ± 3	Gallamine (4/4)	1 ± 3
Nicotine (3/3)	16 ± 4	Tetraethylammonium (3/3)	2 ± 3
Phenyltrimethylammonium (1/1)	17 ± 3		
Choline (2/2)	15 ± 3		

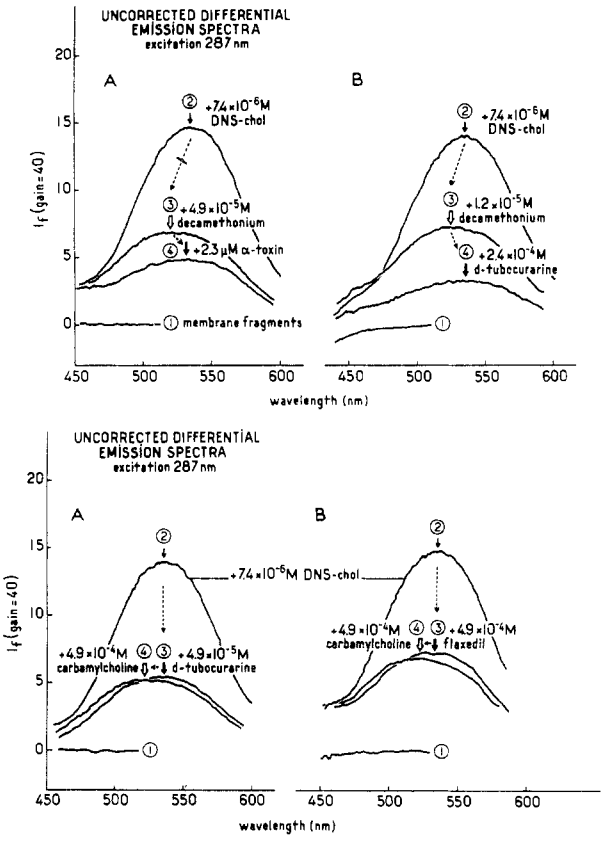
[a] Experiments performed on seven different preparations of membrane fragments; Dns-chol varied from 3 to 6 μM; proteins from 0.5 to 1.5 mg per ml; [^{3}H]α-toxin sites from 0.5 to 1.2 μM. For some experiments cholinergic effectors were added prior to the addition of Dns-chol (as in Figure 3A), while in others the effector was added after the Dns-chol (as in Figure 6B). [b] The pharmacology of these products has been characterized for the *Electrophorus* electroplax. Carbamylcholine and decamethonium are agonists (Nachmansohn, 1959) as are nicotine (Bartels and Podleski, 1964) and phenyltrimethylammonium (Changeux and Podleski, 1968). Choline also causes small depolarizations (Webb and Mautner, 1966). Gallamine (Nachmansohn, 1959) and tetraethylammonium (Podleski, 1968) are antagonists along with the α-toxin (Changeux *et al.*, 1971). The α-toxin of *Bungarus multilinctus* is a depolarizing blocking agent for *Torpedo* electric tissue (Miledi *et al.*, 1971).

shift decreases with increasing Dns-chol concentration. In general, the observed wavelengths of maximum emission depend upon protein, receptor, and Dns-chol concentration. With decreasing receptor concentration, the wavelength characteristics approach the 560-nm emission maximum observed for the free Dns-chol. This is shown in Figure 7B where Dns-chol emission maxima are plotted as a function of Dns-chol concentration in the presence of two cholinergic effectors and at two different protein concentrations. For the concentrated membrane suspension carbamylcholine as acetylcholine gives rise to a blue shift, while in the presence of gallamine no spectral shift occurs. For the diluted suspension, the emission maximum of the 5 μM solution of Dns-chol in the absence of effector occurs at 540 nm; however, in the presence of either carbamylcholine or gallamine, the emission maximum is near 550 nm. These results emphasize that, as expected, the contribution of *free* Dns-chol to the emission spectrum becomes dominant for low concentrations of membrane fragments.

In order to characterize the structural features of the cholinergic ligands responsible for the presence of a shift, a series of effectors were tested. Interestingly, for a given preparation, some compounds cause the blue shift, others do not. Figure 6B presents the emission wavelength observed during the titration of the Dns-chol signal by various cholinergic effectors (Figure 6A). When the shift occurs, it becomes evident in the range of concentrations that cause the decrease of fluorescence intensity. Furthermore, the wavelength shift reaches a maximum value of about 15 nm at effector concentrations similar to those causing the maximum decrease of fluorescence intensity. Table III summarizes the maximum shifts observed for a variety of cholinergic effectors. Wavelength shifts are consistently observed with six effectors; three effectors cause no shift. *d*-Tubocurarine (omitted from Table III) gave variable results; in two of four experiments, a shift occurred. The emission spectra are inherently broad, possessing a half-width at half-intensity of 45 nm in the presence or absence of cholinergic effectors. Under these circumstances, it is not possible to distinguish between different maximal effects of the various compounds that cause the shift. Nevertheless, it is immediately striking that among the compounds causing the shift are found classical agonists of the nicotinic receptor, *e.g.*, acetylcholine and nicotine, while the compounds which did not cause the shift include potent antagonists, *e.g.*, α-toxin and gallamine.

Figure 8 shows that partial titration by decamethonium is accompanied by a shift that the subsequent addition of an excess of α-toxin completely reverses. On the other hand, addition of an excess of carbamylcholine after gallamine is followed by the appearance of a shift with little change of fluorescence intensity (Figure 9). The shift caused by cholinergic effectors in the residual fluorescence spectrum of Dns-chol is, therefore, *reversible*.

Fluorescence Studies with T. marmorata Membrane Fragments Which Do Not Contain α-Toxin Binding Sites. A series of control studies have been made with membrane fragments possessing a low concentration of toxin binding sites. These fragments are associated with the peak of proteins in the sucrose density gradient and in these fragments acetylcholinesterase concentration exceeds that of receptor (Cohen *et al.*, 1972). As noted in Figure 2, the α-toxin does not decrease Dns-chol fluorescence intensity with these fragments. Furthermore, when 10^{-4} M Tetram, an acetylcholinesterase inhibitor, is added to a suspension containing 6 μM Dns-chol, Dns-chol fluorescence intensity decreases by 17 (±4)%. On the other hand, Tetram has no effect on Dns-chol fluorescence with receptor-rich membrane fragments at a comparable protein concentration. Although micromolar concentrations of α-toxin have no effect on Dns-chol fluorescence, carbamylcholine and decamethonium both cause decreases of about 17%. That decrease is not observed if the membranes have been preincubated with Tetram. The concentration of carbamylcholine necessary to produce the half-maximal decrease is 5 (±3) × 10^{-5} M. This concentration is more than an order of magnitude greater than the concentration of carbamylcholine necessary to displace Dns-chol from receptor-rich membrane fragments. These results are those expected for an interaction between Dns-chol and the catalytic site of acetylcholinesterase. With these membrane fragments, cholinergic agonists never cause a shift of the Dns-chol fluorescence maximum (287-nm excitation). Both in the absence of cholinergic effector and in the presence of α-toxin, decamethonium or carbamylcholine, the emission maximum remains 535 (±4) nm for 7 μM Dns-chol, although the fluorescence signals are of about the same intensity as those observed for the receptor-rich fragments in the



FIGURES 8 AND 9: Differential fluorescence emission spectra of membrane suspensions containing Dns-chol and cholinergic ligands. Excitation at 287 nm. Suspensions in physiological saline containing 0.2 μM toxin sites and 0.5 mg/ml of proteins. For A and B, 1 indicates the base line observed for identical suspensions in sample and reference cells; for curves 2, 3, and 4, the sample cell contains 7.4 μM Dns-chol. In spectrum A of Figure 8 (upper) decamethonium bromide was added to the sample cell and then α-toxin. In spectrum B decamethonium bromide has been added followed by *d*-tubocurarine chloride. In spectrum A of Figure 9 (lower) d-tubocurarine chloride was added and then carbamylcholine chloride. In spectrum B gallamine triethiodide (Flaxedil) has been added and then carbamylcholine chloride.

presence of excess effector. This result then reinforces the claim that the spectral shifts observed with the receptor-rich fragments are associated with the presence of the receptor protein.

Discussion

These studies have been undertaken to apply fluorescent techniques to the study of the cholinergic receptor protein in its membrane environment. The development of a preparation of receptor-rich membranes from *Torpedo* electric tissue renders accessible micromolar solutions of α-toxin binding sites. The proteins from these membrane fragments possess an intrinsic fluorescence spectrum typical of proteins containing tryptophan. No change ($\leq 2\%$), however, of this intrinsic protein fluorescence was detected upon addition of carbamylcholine, and extrinsic fluorescence was therefore used.[2]

Dns-chol contains a dansyl moiety making it fluorescent and a trimethylammonium residue creating a structural analogy with known cholinergic effectors. Added to a suspension of membrane fragments containing large amounts of cholinergic receptor protein, its spectral characteristics change. The wavelength of the maximal emission shifts to the blue and excitation by energy transfer from membrane proteins takes place. Dns-chol binds to, or in the close vicinity of, membrane proteins. The α-toxin from *N. nigricollis* venom constitutes an excellent marker of the cholinergic receptor site and is used to identify the various membrane sites to which Dns-chol binds. A large fraction of the fluorescent signal disappears in the presence of α-toxin and is interpreted as the displacement by the α-toxin of Dns-chol bound to the cholinergic receptor site. Direct measurement of Dns-chol binding by ultracentrifugation in conjunction with competition experiments using a variety of cholinergic ligands further supports this interpretation.

As noted in Table I, Dns-chol protects against α-toxin binding, displaces acetylcholine, and is displaced by α-toxin and carbamylcholine. Furthermore, the concentration of carbamylcholine, 2.5 (±1) μM, displacing half the bound Dns-chol measured by the centrifugation assay, is very close to the value causing half-maximal fluorescence decrease. The values of the Dns-chol dissociation constants estimated by the different methods used are in sufficient agreement to lead us to conclude that the decrease of Dns-chol fluorescence observed in the presence of cholinergic effectors is due to the competitive displacement of Dns-chol bound to the cholinergic receptor site. This conclusion is further supported by the observation that the effective concentrations of a large variety of cholinergic ligands determined by fluorescence and the dissociation constants from direct binding are in excellent agreement. The titration of the Dns-chol signal by the cholinergic effectors establishes that all effectors cause essentially similar maximal response, but are effective within different concentration ranges. If the change in fluorescence intensity observed upon addition of cholinergic effectors were linearly proportional to the change of Dns-chol concentration bound to the fragments, it would be possible to estimate a dissociation constant for each cholinergic effector. However, these calculations are not feasible under conditions where the concentration of receptor sites is not very different from the total concentration of cholinergic effector or Dns-chol since the distribution between free and bound compounds becomes significant. For the first five compounds of Table II, the concentrations which give half-maximum decrease of fluorescence intensity lie between 1 and 2 μM, values that are no more than a factor of two larger than the dissociation constants from direct binding, except for *d*-tubocurarine. In this last case the spectroscopic properties of the ligand (adsorption 280 nm, emission near 350 nm) create complications in the measurements. Table II omits the concentrations for α-toxin and acetylcholine, the two compounds that were characterized by a linear titration curve.

These linear curves suggest that the two compounds possess dissociation constants for the receptor that are much smaller than the receptor concentration (micromolar) or the Dns-chol dissociation constant. Under these circumstances, the concentration of α-toxin necessary for the maximum fluorescence decrease is close to the [^{3}H]α-toxin site concentration. The linear titration curves observed for acetylcholine and α-toxin are in agreement with the small dissociation constants found by Weber and Changeux (1973), K_D(Ach) = 0.8×10^{-8} M and K_D (α-toxin) $\sim 10^{-11}$ M.

A nonnegligible fraction of Dns-chol added to a suspen-

[2] Spectroscopic studies in progress on detergent solutions of the purified receptor protein from *Electrophorus* show a 5–10% decrease of intrinsic fluorescence intensity in the presence of cholinergic agonists (acetylcholine, decamethonium, carbamylcholine). In the presence of antagonists, either no change occurs (hexamethonium, tetraethylammonium) or the fluorescence intensity increases (gallamine).

sion of membrane fragments binds to sites distinct from the toxin-sensitive ones. Analysis of the spectrum seen in the presence of an excess of cholinergic effector shows that a 15-nm blue shift occurs with some of the cholinergic ligands used. The spectral properties of Dns-chol bound to the secondary sites appear sensitive to the nature of the compound bound to the cholinergic receptor sites. Data on the pharmacology of *Torpedo* electroplax are limited to a few compounds; acetylcholine and carbamylcholine are agonists and *d*-tubocurarine is an antagonist (Bennett *et al.*, 1961). Extension of the information obtained with *Electrophorus* electroplax to *Torpedo* suggests that all the compounds (*d*-tubocurarine excepted, for technical reasons) which cause the blue shift act as agonists, all the others are antagonists. Choline is not considered to be an agonist but it is an effector of low affinity that does cause a partial depolarization of the eel electroplaque (Webb and Mautner, 1966). The spectral shift, therefore, seems characteristic of the agonistic character of the cholinergic ligand. In addition, antagonists reverse the shift caused by agonists.

The wavelength shifts are observed for both aromatic (nicotine, phenyltrimethylammonium) and nonaromatic compounds, while other aromatic (gallamine) and nonaromatic compounds cause no shift. Furthermore, decamethonium and carbamylcholine cause a wavelength shift in the presence of the receptor-rich membrane fragments but do not produce that effect for the *Torpedo* membrane fragments rich in acetylcholinesterase though they do affect the Dns-chol fluorescence intensity. These facts support the view that the wavelength shifts observed are due to an interaction between Dns-chol and the membrane fragments containing the cholinergic receptor but at a site distinct from the cholinergic receptor site. It is interesting to note that Dns-chol bears structural similarities to local anesthetics such as dibucaine. Both of them are aromatic tertiary amines; it is therefore possible that this second class of Dns-chol sites may be related to the site of action of local anesthetics.

We interpret the decrease of Dns-chol fluorescence intensity caused by the cholinergic effectors on the basis of the physical displacement of Dns-chol from the cholinergic receptor site. The emission shift that develops in the course of the displacement is clearly a property of the Dns-chol molecules which remain bound to the membrane in the presence of an excess of cholinergic effector. The observed emission spectrum of the Dns-chol in the presence of membrane fragments might be interpreted as representing the combined emission from three classes of Dns-chol molecules: (1) Dns-chol remaining bound to the cholinergic receptor site; its emission wavelength is further assumed to be independent of the occupancy of the cholinergic receptor sites; (2) Dns-chol bound to a second class of sites distinct from the cholinergic sites but whose emission wavelength is sensitive to the nature of the cholinergic effector bound to the receptor site; (3) free Dns-chol. Prior to the addition of cholinergic effector, Dns-chol bound to the receptor dominates the emission spectrum, even under conditions where only a small fraction of the Dns-chol interacts with the membrane, because those molecules bound to the receptor are most efficiently excited by energy transfer. Then when a sufficient portion of that population has been displaced, the spectral properties of the molecules bound to the second class of sites become apparent. Finally, in the presence of excess cholinergic effector, the emission spectrum includes contributions from the second class of sites and from the free Dns-chol.

The fluorescence of Dns-chol in solvents of low polarity is characterized by an increase in quantum yield and a blue shift of the emission maximum. The observed emission spectra result from the excitation by energy transfer from the membrane proteins, so the factors that must be important in determining the emission properties include an effective polarizability and viscosity of the probe environment, and the environment of the probe must include proteins and lipids as well as solvent molecules. In any case, the presence of the shift can be taken as a first, but still preliminary, evidence for a structural change characteristic of the agonist character of a cholinergic ligand, and, therefore, associated with the physiological response of the membrane.

Acknowledgments

We thank Mrs. O. Stoven for the synthesis of Dns-chol, M. Weber and A. Brisson for assistance with the radioactive ligand assays, and Drs. H. Buc and L. Monnerie for their help with the maintenance of the fluorescence apparatus.

References

Bartels, E., and Podleski, T. R. (1964), *Biochim. Biophys. Acta 79*, 511.

Bennett, M. V. L., Wurtzel, M., and Grundfest, H. (1961), *J. Gen. Physiol. 44*, 757.

Changeux, J.-P., Blumenthal, R., Kasai, M., and Podleski, T. R. (1970), *in* Molecular Properties of Drug Receptors, Porter, R., Ed., London, Churchill, p 197.

Changeux, J. P., Meunier, J. C., and Huchet, M. (1971), *Mol. Pharmacol. 7*, 538.

Changeux, J.-P., and Podleski, T. R. (1968), *Proc. Nat. Acad. Sci. U. S. 59*, 944.

Cohen, J. B., Weber, M., Huchet, M., and Changeux, J.-P. (1972), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 26*, 43.

De Robertis, E. (1971), *Science 171*, 963.

Eldefrawi, M. E., Britten, A. G., and O'Brien, R. D. (1971), *Pestic. Biochem. Physiol. 1*, 101.

Hall, Z. W. (1972), *Annu. Rev. Biochem. 41*, 925.

Kasai, M., and Changeux, J.-P. (1971), *J. Membrane Biol. 6*, 1.

Kasai, M., Changeux, J.-P., and Monnerie, L. (1969), *Biochem. Biophys. Res. Commun. 30*, 420.

Katz, B. (1966), Nerve, Muscle and Synapse, New York, N. Y., McGraw-Hill.

Lowry, O., Rosebrough, N., Farr, A., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.

Mayer, R., and Himel, C. (1972), *Biochemistry 11*, 2082.

Menez, A., Morgat, J., Fromageot, P., Ronseray, A., Boquet, P., and Changeux, J.-P. (1971), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 17*, 333.

Miledi, R., Molinoff, P., and Potter, L. (1971), *Nature (London) 229*, 554.

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

Parker, C. A. (1968), Photoluminescence of Solutions, Amsterdam, Elsevier.

Podleski, T. R. (1967), *Proc. Nat. Acad. Sci. U. S. 58*, 268.

Radda, G. K., and Vanderkooi, J. (1972), *Biochim. Biophys. Acta 265*, 509.

Waggoner, A., and Stryer, L. (1970), *Proc. Nat. Acad. Sci. U. S. 67*, 579.

Wahl, P., Kasai, M., and Changeux, J.-P. (1971), *Eur. J. Biochem. 18*, 332.

Webb, G., and Mautner, H. G. (1966), *Biochem. Pharmacol. 15*, 2105.

Weber, G., Borris, D., De Robertis, E., Barrantes, F., La

Torre, J., and De Carlin, M. (1971), *Mol. Pharmacol. 7*, 530.
Weber, M., Menez, A., Fromageot, P., Boquet, P., and Changeux, J.-P. (1972), *C. R. Acad. Sci., Ser. D, 274*, 1575.
Weber, M., and Changeux, J.-P. (1973) *Mol. Pharmacol.* (in press).

Surface Membrane Glycopeptides Correlated with Tumorigenesis†

Mary Catherine Glick,*,‡ Zelig Rabinowitz, and Leo Sachs

ABSTRACT: Glycopeptides were removed by trypsin from the surface membranes of clones of (1) hamster embryo cells transformed after treatment with the chemical carcinogen, dimethylnitrosamine, (2) variants of these cells with suppression of the transformed phenotype, and (3) revertants of these variant cells back to the transformed state. The glycopeptides were examined by gel filtration after Pronase digestion. In all cases, the gel filtration profiles of the fucose-containing glycopeptides were similar to those obtained from secondary hamster embryo cells. In contrast, the profiles derived from the tumors formed after inoculation into animals of all of these cell lines showed the appearance of a specific group of glycopeptides which was not found in the original cells. Other properties characteristic of the transformed phenotype which were examined were not consistent with either the appearance of these glycopeptides or with tumor cells. These results suggest a correlation between the surface membrane glycopeptides and tumor formation.

Glycoproteins from surface membranes of virus transformed cells have been compared to those obtained from control cells by a number of investigators (Wu *et al.*, 1969; Meezan *et al.*, 1969; Buck *et al.*, 1970; Sakiyama and Burge, 1972; Warren *et al.*, 1972a). The appearance of specific glycopeptides was demonstrated by chromatography on Sephadex G-50 (Buck *et al.*, 1970) or DEAE-cellulose (Glick, 1971), following transformation by RNA or DNA viruses (Buck *et al.*, 1971). None of these reports has been concerned with the relationship of these glycopeptides to tumorigenesis.

The interaction of carcinogens with cells leads in some cases to their malignant transformation and results in the hereditary expression of properties which include the ability to form tumors *in vivo* and to multiply *in vitro* under conditions which inhibit the multiplication of nontransformed cells. Once the transformed properties have been hereditarily expressed, they can again be hereditarily suppressed. That is, cells hereditarily transformed by viral (Pollack *et al.*, 1968; Rabinowitz and Sachs, 1968, 1972) or nonviral carcinogens (Rabinowitz and Sachs, 1970; Hitotsumachi *et al.*, 1972) can produce variants with a suppression of the properties of transformed cells. Variants from hamster embryo cells transformed after treatment with chemical carcinogens acquire, like normal cells (Hayflick, 1965), a limited life span *in vitro* (Rabinowitz and Sachs, 1970). Some of the variants subsequently escape from the limited life span and again revert to the transformed state.

In the present study we have examined the surface glycopeptides of the following cell types: (1) hamster embryo cells transformed after treatment with the chemical carcinogen, dimethylnitrosamine (DMNA),[1] (2) variants of the cells with suppression of the malignant transformed properties, (3) revertants of these variant cells back to the transformed state, and (4) cells derived from tumors of all of these cell types. The properties of these cells make it possible to examine the glycoproteins of the surface membranes in relation to the formation of tumors. This study describes the appearances of specific glycopeptides in all of the tumor cells examined.

Materials and Methods

Description of Cells and Cell Culture. Minced whole embryos from Syrian golden hamsters were the source of secondary hamster embryo cells. Transformed cells were obtained after treatment of the hamster embryo cells with DMNA and subsequent subculture until a cell line was established (Huberman *et al.*, 1968). In the present experiments, a cell line D (Rabinowitz and Sachs, 1970) was used and will be designated "DMNA cells." Variants of these cells were produced as described by induction of variant formation at low cell density (Hitotsumachi *et al.*, 1972). For this induction, transformed cells were seeded at 2000 cells/50-mm petri dish, in Eagle's medium with a fourfold concentration of amino acids and vitamins and 10% fetal calf serum, and the cultures were incubated at 37° for 2 days and at 24° for 4 days. The cells were then seeded on X-irradiated (4000 R) rat embryo feeder layers in 35-mm petri dishes at a dilution of one cell per plate, and variants in plates with single colonies were isolated. The variants which are reported in this study are variants 11 and 13. Subsequent subculturing of these variants produced cells which either had a limited life span or reverted back to the malignant transformed state (Rabinowitz and

† From the Department of Genetics, Weizmann Institute of Science, Rehovot, Israel. *Received August 10, 1973.* Supported by Contract No. NC1-69-2014 with the Virus Cancer Program of the National Cancer Institute, the National Institutes of Health, and American Cancer Society Grants PRA-68 and BC-109.

‡ Present address: Division of Biochemical Development and Molecular Diseases, Children's Hospital of Philadelphia, Philadelphia, Pa. 19146.

[1] Abbreviations used are: DMNA, dimethylnitrosamine; TBS, 0.15 M NaCl–0.02 M Tris-HCl (pH 7.5).