Organization of Ligand Binding Sites at the Acetylcholine Receptor: A Study with Monoclonal Antibodies†

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ABSTRACT: We have studied 20 monoclonal antibodies directed against both the solubilized and the membrane-bound receptor from *Torpedo marmorata*. We find the following: (i) Six of the antibodies compete with cholinergic ligands for receptor binding and, hence, are directed against the ligand binding regions. (ii) Of these six antibodies, two cross-react with receptor from *Electrophorus electricus*, rat myotubes, and chicken sympathetic ganglia. These two antibodies therefore define a preserved structure within the ligand binding regions. The other four antibodies bind to structures not common between the receptor preparations tested. (iii) From competition binding studies using internally ^{3}H-labeled antibodies, nine nonoverlapping antigenic regions were defined at the surface of the receptor. Three of these regions overlap with the ligand binding regions. Since two of these three regions do not overlap with each other, two structurally distinct ligand binding regions must exist at the receptor. (iv) From competition binding studies with representative cholinergic ligands, the antibodies directed against the ligand binding regions can be subdivided into three groups: one group competes with all ligands tested; the second group competes with all ligands except the bismethonium compounds; the third group competes with all ligands except the bismethonium compounds and tubocurarine. The results are summarized in a model of the organization of ligand binding sites at the receptor: There are two ligand binding regions differing in their antigenic properties. Furthermore, either there exists separate sites for distinct groups of ligands within each of these binding regions or some ligands produce conformational changes of the receptor that reversibly abolish some antigenic sites. In any case, the cholinergic ligands must interact with the receptor by more and/or other structural determinants than are provided by the structure of acetylcholine.

Cholinergic excitation of muscle cells is caused by binding of acetylcholine to its postsynaptic receptor and the consecutive transient gating of the receptor-controlled ion channel. Drugs can interfere with this reaction sequence at different levels (Maelicke et al., 1977b; Heidmann & Changeux, 1978; Karlin, 1980): The agonists and antagonists of acetylcholine compete with the transmitter for receptor binding. This binding is mutually exclusive, which is consistent with binding to identical sites. Local anesthetics affect the gating of the ion channel (Steinbach, 1968) by binding at a site remote from the acetylcholine binding sites. Another independent class of binding sites probably exists for direct-channel blockers (Colquhoun, 1979).

As has been shown by reconstitution studies (Boheim et al., 1981), all of these sites are located at the receptor molecule as it is obtained by affinity chromatography from detergent-solubilized membranes (Rüchel et al., 1981). The topography of sites at the receptor is further complicated by the existence of two classes of agonist sites (Maelicke et al., 1977a; Prinz & Maelicke, 1983), two classes of toxin sites (Maelicke et al., 1977a; Damle & Karlin, 1978), and heterogeneity in the binding of tubocurarine (Neubig & Cohen, 1979). Furthermore, the phenomenon of accelerated dissociation of receptor–toxin complexes in the presence of large concentrations of small ligands (Maelicke et al., 1977a; Maelicke & McConnell, 1978; Kang & Maelicke, 1980) can best be explained by assuming either separate sites for toxin and small ligands or additional allosteric sites for the latter ones. Locally separate ligand binding sites are also indicated from binding studies with antibodies from the serum of myasthenic patients: These antibodies compete with some but not all nicotinic ligands in binding to chicken embryo myogenic cultures (Fulpius et al., 1980).

To understand the regulation of the receptor by its ligands and modulators, a structural and functional characterization of the types of sites is required. As one approach to this problem, we have probed the ligand binding sites of the receptor from *Torpedo marmorata* with a library of monospecific antibodies. Although relatively large in size, antibodies are directed against rather small regions of the antigen (Tzartos et al., 1981). If such antigenic sites are located within or overlapping with the ligand binding site(s), antibody and ligand will compete in binding. Using monospecific antibodies of this kind, we have investigated the following problems: Do all agonists and antagonists of acetylcholine bind to identical sites? If not, are the ligand binding sites separate entities or partially overlapping?

To address these questions, we have developed new assays for both the selection and classification of antibodies directed against the ligand binding sites. The selected antibodies were then employed in competition binding studies with other monospecific antibodies of their kind and with nicotinic ligands. The antibodies competing with nicotinic ligands did not all compete with each other. In addition, the antibodies distinctly differed in their competition pattern with the ligands. Taken together, these results indicate the existence of at least two well-separated regions of ligand binding at the receptor. Furthermore, the nicotinic ligands do not bind to identical sites.

Materials and Methods

Balb/c and C57BL/6 mice were obtained from the Zentralinstitut für Versuchstierzucht, Hannover, Germany. The NS-1 myeloma cell line (secretes κ chains) was kindly provided by Dr. Maureen Howard of the Walter and Eliza Hall Institute, Melbourne, Australia. The X63.Ag8.653 myeloma cell line (nonsecreting) was kindly provided by Dr. M. Ra-

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Poly(ethylene glycol) 4000 (gas chromatography grade) and carbamoylcholine were from Merck, Darmstadt. Aminopterin, hypoxanthine, thymidine, 2,2′-azinobis(3-ethylbenzothiazolinesulfonic acid) (ABTS), *d*-tubocurarine, and decamethonium were from Sigma, München. Hexamethonium was from Fluka, Buchs, Switzerland. All gel electrophoresis materials were from Bio-Rad, München. L-[4,5-$^3H_2$]Leucine (130 Ci/mmol) was purchased from Amersham Buchler, Braunschweig. Peroxidase-labeled rabbit anti-mouse IgG (H + L) was from Nordic Immunological Laboratories, Leuven, Holland.

Acetylcholine receptor from *Torpedo marmorata* and *Electrophorus electricus* was purified as previously described (Rüchel et al., 1981). Receptor-rich membrane fragments from *Torpedo* were prepared according to Fels et al. (1982). The specific activities (in terms of toxin binding sites per mass of protein) were 7000–8000 nmol/g for the affinity-purified receptor and 1500–2000 nmol/g for the membrane-bound receptor. Periodate oxidation of recepor was performed as described by Wonnacot et al. (1980). ^3H- and ^{125}I-labeled α-neurotoxins were prepared as previously described (Maelicke et al., 1977a; Miskin et al., 1978). Cell culture sera and solutions were obtained from GIBCO-Europe, Karlsruhe; Flow Laboratories, Bonn; and Seromed, München. All buffer materials and chemicals were from Merck, Darmstadt.

Immunization and Cell Fusion. Young female Balb/c or (Balb/c xC57BL/6) F1 mice were immunized with purified receptor by either of two procedures: (i) intravenously followed by a booster after 1 week or (ii) intraperitoneally with complete Freund's adjuvant followed by a booster (intravenous, without adjuvant) after 1 month. Mice received approximately 100 μg of purified receptor or 500 μg of protein in membrane fragments as antigen.

Spleens were removed from the immunized mice 3–4 days after the booster and dissociated into single cells by gentle trituration. A total of 10^8 spleen cells were fused with 2×10^7 myeloma cells (NS-1 or X63.Ag8.653) according to the method of Köhler & Milstein (1975) using 1 mL of 50% poly(ethylene glycol) 4000. The fused cells were distributed among 24-well Costar tissue culture dishes in HAT selective medium (hypoxanthine/aminopterin/thymidine) together with nonimmune Balb/c peritoneal macrophages as feeder layer (Fazekas et al., 1980).

Half the medium was replaced twice weekly. After 2 weeks, aminopterin was omitted from the medium. Supernatants from wells containing growing hybrids were assayed by an enzyme-linked immunosorbent assay (ELISA) (Norcross et al., 1980; Watters & Maelicke, 1982). Positive hybrids were cloned by limiting dilution. They were preserved by freezing in medium containing 30% serum and 10% dimethyl sulfoxide (Me_2SO) and stored in liquid nitrogen.

Purification of Antibodies. Antibody-producing clones were grown in γ-globulin-free medium. The collected supernatant was treated with an equal volume of saturated ammonium sulfate at pH 7.4 and stirred for 2 h at 4 °C. The precipitate was pelleted by centrifugation, taken up in a small volume, and dialyzed against 20 mM sodium phosphate buffer, pH 8.0. The material was chromatographed in the same buffer on a column of DEAE-Affigel blue (Hudson & Hay, 1976), immunoglobulin G (IgG) eluting in the unbound fraction. The fractions were pooled according to the results of ELISA (Watters & Maelicke, 1982), concentrated by ultrafiltration over an Amicon PM30 membrane, and sterilized by filtration through cellulose acetate 0.22-μm filters (Millipore). The resulting preparation was essentially pure IgG as judged by sodium dodecyl sulfate ($NaDodSO_4$) gradient gel electrophoresis in the presence of dithiothreitol. Electrophoresis was performed as previously described (Rüchel et al., 1981). The concentration of antibodies was determined spectrophotometrically by assuming an absorbance of 1.6 for a 1 mg/mL solution at 280 nm and 1-cm path length.

Internal Labeling of Monoclonal Antibodies with [^3H]-Leucine. Hybridoma clones were grown in minimum Eagle's medium (MEM) containing 5% dialyzed fetal calf serum (FCS). Approximately 2×10^6 cells were washed twice with leucine-free MEM (GIBCO) containing 5% dialyzed FCS and suspended in 2 mL of the same medium. Approximately 0.4 mL of [^{3}H]leucine of high specific activity was added and the culture incubated overnight. The supernatant was removed and dialyzed extensively against phosphate-buffered saline (PBS) at 4 °C. This supernatant was used directly in the competition binding studies described below.

Determination of the IgG Subclass of the Antibodies. The cell culture supernatant (1 mL) was mixed with saturated ammonium sulfate (1 mL) for 2 h at 4 °C. The precipitate was dissolved in 100 μL of H_2O, and the IgG subclass was then determined by Ouchterlony double immunodiffusion against anti-mouse IgG subclass specific antisera (Nordic).

Enzyme-Linked Immunosorbent Assay for Anti-acetylcholine Receptor Antibodies. Gilford "Cuvette Paks" were coated with purified *Torpedo* acetylcholine receptor (AcChR) by incubation with a dilute solution of AcChR in 0.1 M $NaHCO_3$, pH 9.0, overnight at 4 °C. Excess antigen was removed by washing twice with Dulbecco's Ca^{2+}–Mg^{2+}-free phosphate-buffered saline containing 0.05% Tween 20 (PBS–Tween). Culture supernatants or serum was then added and incubated for 2 h at 37 °C or overnight at 4 °C. After two additional washes with PBS–Tween, peroxidase labeled anti-mouse IgG (diluted 1:1000 in PBS–Tween) was added and similarly incubated. The plates were then washed 3 times with PBS–Tween and incubated with the substrate solution (0.25 mM ABTS and 0.18 mM H_2O_2 in 0.1 M sodium phosphate, pH 6.8). After an appropriate time, the reaction was stopped with 0.5 volume of 20% sodium dodecyl sulfate ($NaDodSO_4$), and the absorbance at 405 nm was read from a Gilford EIA manual reader.

In other experiments, crude membrane fragments and whole cells were used as antigens in the ELISA test. When cellular antigens (rat myotubes or sympathetic ganglion cells) were used, these were first fixed by incubation for 30 min at room temperature in PBS containing 0.25% glutaraldehyde (Stocker et al., 1979).

Solid Phase Binding Assays. Flexible polyvinyl microtiter plates (Dynatech) were coated with purified acetylcholine receptor or membrane fragments and washed as described above. Various amounts of labeled ligand [[^{3}H]leucine-labeled antibody, [^{3}H]pyridoxamine phosphate labeled α-cobratoxin (Maelicke et al., 1977a), or ^{125}I-labeled α-bungarotoxin (Miskin et al., 1978)] in PBS–Tween in a total volume of 140 μL/well were incubated 1–2 h at 37 °C or overnight at 4 or 20 °C. The wells were then washed extensively with PBS–Tween, cut out with an electrically heated wire, placed individually into counting vials, and counted, in either a γ radiation counter or a liquid scintillation counter. In the latter case, 10 mL of Quickzint (Zinsser, Frankfurt) was added to each vial.

Each antibody was labeled in turn with [^{3}H]leucine to determine competition among the different antibodies. Fifty microliters of labeled supernatant plus 50 μL of unlabeled

culture supernatant from the same or other clones was added to duplicate wells. Bound counts generally ranged from 2000 to 20 000 cpm/well, being reduced to between 800 and 300 cpm/well where there was competition between the labeled and unlabeled antibodies.

For determination of competition between unlabeled antibodies and ^{125}I-labeled α-bungarotoxin, coated plates were incubated with given concentrations of antibody for 1 h at 37 °C or overnight at 20 °C, after which binding curves for labeled toxin were determined (Maelicke et al., 1977a). From the competition binding curves, the equilibrium dissociation constants of the antibodies were calculated as described (Maelicke et al., 1977a).

Binding Assay with AcChR in Solution. In these studies, the DEAE-cellulose filter disk assay (Maelicke et al., 1977a) was employed. Using constant concentrations of antibody and varying concentrations of ^{3}H-labeled toxin, we determined the inhibition pattern and inhibition constant.

Fidelity and Error Range of the Assays. The employed assays varied considerably in their significance and also depended on whether solubilized receptor, membrane fragments, or whole cells were employed as antigens. The most critical parameter of the ELISA was the "background absorbance" measured in the absence of specific antibody. Background absorbances with solubilized receptor as antigen were 0.1 A_{405} unit or less, with the absorbance readings in the presence of specific antibody extending at least to 0.4 A_{405} unit and often to up to 2 A_{405} units (Figures 1 and 2). With membrane fragments or whole cells as antigens, more variable and often considerably higher background absorbances were measured. These were considerably smaller when the coated cuvettes were incubated with bovine serum albumin or other nonspecific proteins prior to the application of specific antibody. As a further limitation, the antigen concentration in membranes and cells often was considerably lower than in the purified preparations. As a consequence, there may exist more cross-reactivity than we were able to unequivocally establish (Table I).

The binding assays were limited mainly by the accuracy of the determination of antibody concentration. In particular, we do not know whether all monoclonal antibodies of each preparation were fully active, i.e., bind with the same affinity to the antigen supplied. Such loss in activity may be caused by the ammonium sulfate precipitation employed to concentrate the antibodies and the consecutive chromatography. While prepared with the least number of manipulations, this reservation also applies to the internally labeled antibodies. As a consequence, the reported K_D values may have an error margin of 1 order of magnitude. Similarly, we do not put much significance on the obtained total concentrations of antibody binding sites. Therefore, we have deliberately calculated the competition data with α-cobratoxin (Figure 3) and the binding data with ^{3}H-labeled antibodies, assuming the same number of sites for both toxin and antibody. As a consequence, the binding data should be considered qualitative rather than quantitative support of the ELISA data.

Because of the relatively high specific activity of the labeled antibodies and toxins applied, these binding data have good numerical significance. In particular, the characterization of antigenic sites by antibody competition is highly significant.

Results

Selection of Clones. An ELISA test with peroxidase-labeled rabbit anti-mouse IgG was employed for the selection of anti-AcChR antibody producing clones (Figure 1). A positive ELISA test required (i) the presence of antibodies directed against the receptor (Figure 1a) at a concentration of at least 10^{-13} mol/mL of medium (Hudson & Hay, 1976; Watters & Maelicke, 1982) and (ii) the native structure of both the receptor and the antibodies. The latter was shown by the background readings observed when either denatured receptor (boiled) or denatured antibodies (boiled or treated with sodium dodecyl sulfate or dithiothreitol) were employed. With the limitation that only antibodies of the IgG class are detected by the ELISA, the following differences were observed when solubilized or membrane-bound receptor was used as antigen: From two mice immunized with purified *Torpedo* receptor protein, a total of 149 growing hybrids were obtained. Of these, 88 (59%) were positive for antibodies directed against the purified receptor. Of the 88, 64 (73%) were also positive for antibodies directed against *Torpedo* membrane fragments. In contrast, from 110 growing hybrids produced by using *Torpedo* membrane fragments as antigen, 38 made antibodies to *Torpedo* membranes, but only 7 of these (18%) recognized purified receptor as antigen. These findings indicate that (i) most antigenic determinants of the receptor are preserved during solubilization and purification and (ii) the majority of antigenic determinants of membrane fragments do not belong to the receptor moiety.

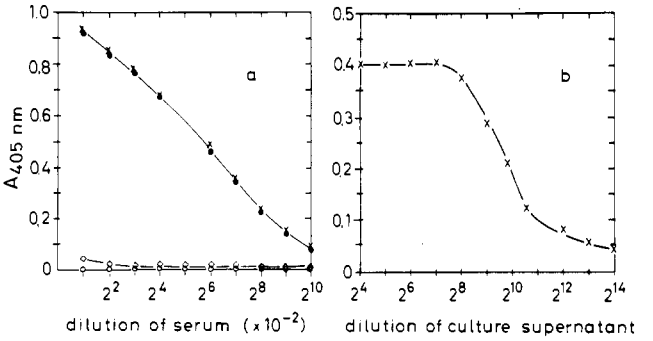

FIGURE 1: (a) Peroxidase-linked immunoassay of rabbit serum. Serial dilutions (1 in 2) of rabbit anti-*Torpedo* AcChR antiserum (● and ×, duplicate measurements), normal rabbit serum (◊), and cuvettes not coated with AcChR (○). (b) Peroxidase-linked immunoassay of cell culture supernatant. Serial dilutions (1 in 2) of culture supernatant from clone 8 (Table I) producing anti-*Torpedo* AcChR antibodies.

Of the clones producing antibodies to determinants existing on both the purified and the membrane-bound receptor, we have selected 20 for further studies. The antibodies secreted by these clones were tested for (i) their IgG subclass, (ii) their possibility of being directed against the ligand binding site(s) or other regions of the receptor, (iii) their affinities of binding to the receptor, and (iv) their competition binding patterns with cholinergic ligands.

Characterization of Monoclonal Antibodies. The IgG subclass of the antibodies was determined by Ouchterlony double immunodiffusioin using cell culture supernatants concentrated by ammonium sulfate precipitation and anti-mouse IgG subclass specific antisera. The results are listed in Table I. The antigenic sites for two antibodies (mAbs13,20) apparently involved portions of the carbohydrate moiety as their binding diminished by about 50% with periodate-treated receptor.

With the ELISA test, the cross-reactivity of antibodies with receptor proteins from other sources was determined. For this purpose, the wells were coated with crude membrane fragments or whole cells of the species under study. In the latter case, the cells were fixed with glutaraldehyde prior to the ELISA test. Separate binding experiments with ^{125}I-labeled α-bungarotoxin were employed to ensure sufficiently high receptor

Table I: IgG Subclass, Cross-Reactivity Pattern, and Competition with Neurotoxin Binding of 20 Monospecific Antibodies Directed against Purified and Membrane-Bound *Torpedo* Receptor[a]

hybridoma clone		IgG	cross-reactivity with receptor from			competition
mAb	internal code	subclass	*Electrophorus*	rat	chicken	with α-cobratoxin
1	1-A4-C8	2a				+
2	2-B4-12	2a				+
3	5-6-1-D6	1				+
4	1-C3-C9	3			+	+
5	N-2-F9	2b	+	+	+	+
6	XR-6-G10	2a	+	+	+	+
7	A-1-G11		+			
8	NA-19-E11	1				
9	1-B6-B10	2a				
10	XR-33-E7	1				
11	XR-16-B3	2a		+		
12	XR-64-F11	1				
13	XR-41-G6	1				
14	NB-20-C6	1				
15	NA-23-E4	2a				
16	XR-34-2-C5	1				
17	XR-26-D2	1				
18	XR-46-H8		+	+		
19	1-D2-F10	1				
20	H-5-E9	1				

[a] IgG subclass was determined by Ouchterlony double immunodiffusion, cross-reactivity, and competition with toxin by using the ELISA. Affinity-purified receptor from *Electrophorus electricus* was purified according to Rüchel et al. (1981). Rat myotubes and chicken sympathetic ganglia were obtained according to published procedures (Dvorak et al., 1978; Hamill et al., 1981). Glutaraldehyde-fixed cultures were employed in the ELISA (Stocker et al., 1979).

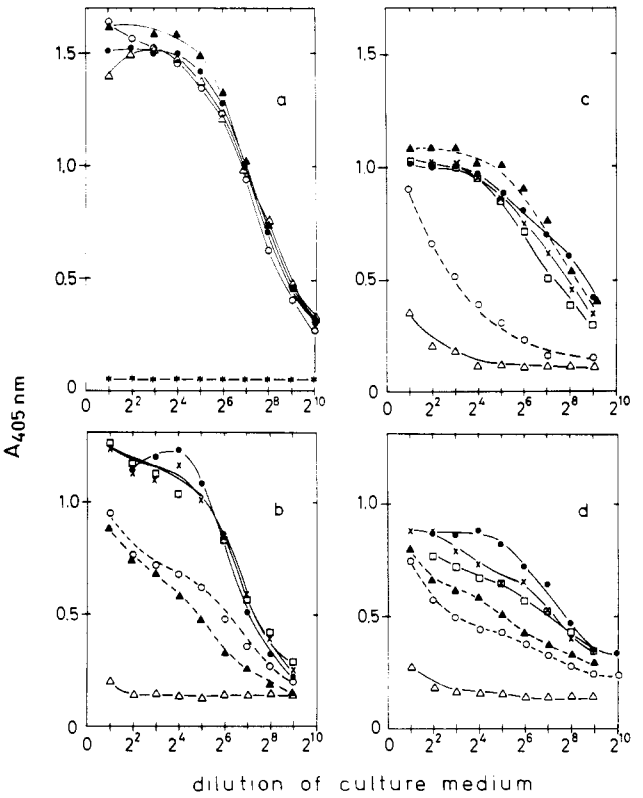

FIGURE 2: ELISA test for four monoclonal antibodies in the presence and absence of competing ligands. Serial dilution curves of hybridoma culture supernatants (a) mAb16, (b) mAb1, (c) mAb6, and (d) mAb5 in the presence or absence of cholinergic ligands. Each curve was obtained by first adding 200 μL of ligand in PBS–Tween to each cuvette of a strip of 10. 200 μL of medium was then added to cuvette 1 and mixed, 200 μL of the contents was transferred to cuvette 2, and so on. (●) Serial dilution in the absence of ligand; (△) α-cobratoxin at initially 1 μM; (○) carbamoylcholine at initially 2 mM; (▲) tubocurarine at initially 1 mM; (×) hexamethonium at initially 10 mM; (□) decamethonium at initially 10 mM; (*) uncoated plates.

densities at the surface of the wells. Except for antibodies with very low affinity, the assay should therefore suffice to establish the basic pattern of cross-reactivities (Table I).

The ELISA test was also employed to distinguish antibodies directed against the ligand binding site(s) from those that were not. For this purpose, serial dilutions of culture supernatants were performed in the presence or absence of cholinergic ligands present at a constant high concentration. As shown in Figure 2a, clone 16 produces an antibody which binds at a site distant to the ligand binding site(s), since the presence of cholinergic ligands including α-cobratoxin does not affect its binding to the immobilized receptor. In contrast, clones 1, 5, and 6 produce antibodies directed against the ligand binding site(s) since antibody binding to the receptor was blocked by α-cobratoxin and small ligands (Figure 2b–d). Of the 20 antibodies studied in this way, 6 (mAbs1–6) fall into this category (Table I).

Two of the six antibodies directed against the ligand binding site(s) of *Torpedo* receptor (mABs5,6) cross-reacted with receptor from other sources. This indicates a preserved structural determinant within the ligand binding region of these nicotinic receptors. mAbs1–4, however, while all competing with toxin and other ligands for receptor binding, showed no cross-reactivity at all with any of the tested receptor preparations. Thus, there exist other structural determinants within the ligand binding region that are not preserved. This is certainly a more complicated pattern of cross-reactivities than was previously suggested (Moshly-Rosen & Fuchs, 1981).

The six antibodies directed against the ligand binding site(s) of *Torpedo* receptor can be subdivided into at least three groups if their competition patterns with the cholinergic ligands are considered (Figure 2, Table II): mAbs2–4 competed with all ligands tested. Binding of mAb1 to the receptor was not inhibited by the bismethonium compounds but was by all other ligands tested. mAb6 did not compete with the bismethonium compounds and tubocurarine but competed with the other ligands (α-cobratoxin, acetylcholine, carbamoylcholine, and succinyldicholine). mAb5 differed only quantitatively from mAb1 in its ligand competition pattern: Its binding to the receptor was only slightly inhibited by the bismethonium compounds.

Table II: Competition of Monoclonal Antibodies and Cholinergic Ligands for Receptor Binding[a]

	competing ligand				
mAb	α-cobratoxin	tubo-curarine	hexa-methonium	carbamoylcholine, acetylcholine, or succinyldicholine	deca-methonium
1	+	+	0	+	0
2	+	+	+	+	+
3	+	+	+	+	+
4	+	+	+	+	+
5	+	+	±	+	±
6	+	0	0	+	0

[a] Receptor-coated cuvettes were incubated with competing ligand and monoclonal antibody for 2 h at 37 °C. After two washes, the concentration of receptor-bound monoclonal antibody was determined by the ELISA (see Materials and Methods). Lower absorbance readings, as compared to control incubations in the absence of ligand, were taken as evidence for competition between ligand and antibody for receptor binding. The symbols +, ±, and 0 denote strong, slight, and no competition, respectively.

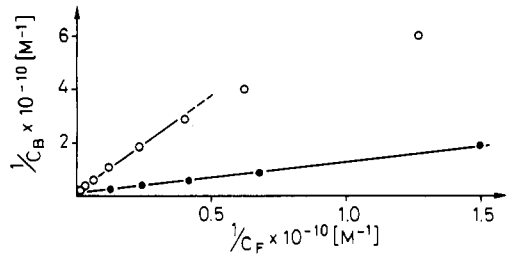

FIGURE 3: Competitive binding of mAb1 and ^{3}H-labeled cobra α-neurotoxin to purified receptor for *Torpedo marmorata*. Competition was measured in solution in 0.1 M NaCl, 0.01 M tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), and 0.01% Tween 80, pH 7.4, by using the DEAE-cellulose filter disk assay (Klett et al., 1973; Maelicke et al., 1977a): incubation time, 200 min; temperature, 22 °C. The specific activity of the ^{3}H-monolabeled toxin was 4.2 Ci/mmol. (●) Toxin binding in the absence of antibody; (○) toxin binding in the presence of 5×10^{-8} M mAb1. The concentration of toxin binding sites (R_0) is 7.1×10^{-10} M, $K_D = 8.6 \times 10^{-10}$ M, and $K_D(app) = 5.2 \times 10^{-9}$ M. If it is assumed that both toxin and mAb1 have the same number of binding sites, the inhibition constant can be calculated according to the equation $K_I = C_I[K_D(app)/K_D^{-1}]^{-1}$ (Maelicke et al., 1977a) with C_I, $K_D(app)$, and K_I denoting the concentration of competing antibody, the apparent K_D for toxin in the presence of antibody, and the inhibition constant for the antibody, respectively. $K_I = 9.9 \times 10^{-9}$ M.

The observed patterns of competition with cholinergic ligands were independent of whether purified receptor or receptor-rich membrane fragments from *Torpedo* were used. The different competition patterns of mAb1, mAbs2–4, and mAb6 were never in doubt while the inhibition of mAb5 by the bismethonium compounds was at the limit of experimental detection.

Binding Studies with Monoclonal Antibodies. The binding studies were performed with purified *Torpedo* receptor (Rüchel et al., 1981) that either was in solution or was immobilized on microtiter plates. The antibodies employed in these studies were obtained in IgG-free medium and were separated from other proteins in the cell supernatant by chromatography on DEAE-Affigel blue (Hudson & Hay, 1976; Gee et al., 1979). The concentration of purified antibodies was estimated spectrophotometrically.

Competition of purified, monospecific antibodies with tritiated α-cobratoxin was studied by means of the DEAE-cellulose filter disk assay (Klett et al., 1973; Maelicke et al., 1977a). As is shown representatively for mAb1 in Figure 3 binding of antibody and toxin was fully competitive. From the apparent K_D value for toxin in the presence of the given concentration of mAb1, an inhibition constant (K_I) of the order of 10^{-8} M was calculated. Due to the deficiencies of the determination of antibody concentration, this value must be considered an upper limit.

Table III: Competition Binding of Monoclonal Antibodies and α-Neurotoxins to Purified *Torpedo* Receptor[a]

	inhibition constant obtained by	
antibody	filter assay	solid phase assay
mAb1	1×10^{-8}	3×10^{-8}
mAb4	7×10^{-8}	
mAb5	8×10^{-10}	4×10^{-9}
mAb6	6×10^{-9}	4×10^{-8}

[a] Binding experiments with receptor in solution were performed by using tritiated α-cobratoxin and the DEAE-cellulose filter disk assay (Maelicke et al., 1977a); those with immobilized receptor were performed by using ^{125}I-labeled α-bungarotoxin and the procedure described under Materials and Methods. Within the limits of experimental error, all binding data were consistent with competition for the same number of sites at the receptor.

More comparable to the conditions of the ELISA, competition binding studies were also performed with receptor-coated microtiter plates (see Materials and Methods). Both for convenience and to obtain sufficient sensitivity, these studies were performed with ^{125}I-labeled α-bungarotoxin. Again, full competition between antibody and toxin for the same number of sites at the receptor was observed. The competition curves differed, however, from the expected linear patterns (Maelicke et al., 1977a) in that the double-reciprocal plots showed considerable curvature. This was probably due to the limited accessibility of the binding site(s) at the immobilized receptor and the resulting nonequilibrium conditions of the assay: (i) The double-reciprocal plots for toxin binding in the absence of competing antibody or ligand showed only little if any curvature; (ii) the presence of tubocurarine did not increase the degree of curvature observed with toxin alone; and (iii) the K_D values for toxin ($K_D = 6.5 \times 10^{-11}$ M) and tubocurarine ($K_I = 1.8 \times 10^{-7}$ M) both measured after 24 h of incubation at 22 °C were consistent with the values obtained with solubilized receptor (Maelicke et al., 1977a; Damle & Karlin, 1978).

Table III summarizes the binding data obtained with receptor in solution and immobilized at the microtiter plates. Although limited by the nonequilibrium conditions of the assay, the data indicate a considerable range of affinities of the monospecific antibodies. Antibody binding to the receptor-coated microtiter plates was consistently of lower affinity than to the receptor in solution.

Antibody binding to the receptor was also measured directly by employing internally labeled antibodies. In contrast to binding studies with Fab fragments (Claudio & Raftery, 1980), the DEAE-cellulose filter disk assay was not applicable. At the pH of the reaction (pH 7.4), both receptor and antibody are bound to the disks, and, thus, separation of free and bound antibody cannot be achieved. The binding studies were

Table IV: Competitive Binding of Monoclonal Antibodies to Receptor-Coated Microtiter Plates[a]

^{3}H-labeled mAb	unlabeled mAb																	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	19
1	+	+	+	+	0	+				/								
2	+	+	+	+	0	+												
3	+	+	+	+	0	+				/								
4	+	+	+	+	0	+												
5	0	0	0	0	+	+												
6	/	/	/	/	/	+												
7							+	+	/									
9									+	+			/					
11											+		/					/
12												+		+				
13						/			/	/	/		+			/		
15								/			/				+	/		
19									/	/							+	+

[a] [^{3}H] Leucine-labeled culture supernatant from one clone was allowed to bind to receptor-coated microtiter plates in the presence of unlabeled culture supernatant from the same or another clone. +, /, and 0 denote full competition, partial competition, and no effect, respectively. Partial inhibition may be the result of differences in affinities, differences in the concentration of the antibodies in the medium, or partial overlapping of antigenic regions. The top left-hand corner shows the competition data for the anti ligand binding site antibodies.

therefore limited to the solid phase assay.

Because of the small amounts of internally labeled antibody available, purification followed by spectroscopic determination of the concentration of antibody was not attempted. Instead, the specific activity of the labeled antibodies was estimated from a binding experiment to immobilized receptor having a known concentration of toxin binding sites. Assuming the same number of sites for antibody and toxin, we then calculated the specific activity of the antibody. Representative binding data for mAb1 and mAb5 are shown in Figure 4. These direct binding studies are complicated by considerable nonspecific binding of the antibodies to the plates. Provided sufficient incubation time was used (24 h at 22 °C or 6 h at 37 °C), and the proper corrections were employed for nonspecific binding (Figure 4), linear double-reciprocal plots and K_D values in the nanomolar range were obtained.

Competition between Monoclonal Antibodies for Antigenic Sites. Competitive binding of different monospecific antibodies to the receptor can be studied by using the solid phase assay and internally ^{3}H-labeled antibodies. If each antibody is labeled in turn and competed with other unlabeled antibodies, these studies may eventually lead to a topographic map of the antigenic sites at the receptor. Two antibodies can be assumed to bind to nonoverlapping sites if the binding of one is not inhibited by the other. Partial or complete inhibition of one antibody by the other implies that both antibodies bind to overlapping or identical antigenic determinants, but the extent of overlap cannot be determined in these experiments.

We have performed such studies with the six antibodies which bind competitively with α-neurotoxins (mAbs1–6). The results are summarized in Table IV. Three separate antigenic sites were defined. mAbs1–4 and mAb6 bound to overlapping antigenic regions; mAb5 bound to a different one which overlapped appreciably only with the region for mAb6. The results summarized in Table IV were further substantiated by applying mixtures of two labeled antibodies in the binding experiments. At saturating concentrations for each antibody, no additional binding was observed for the pairs mAb1–mAb6 and mAb5–mAb6, while some additivity was observed for the pair mAb1–mAb5.

Similar studies were performed with the antibodies mAbs7–20 enabling us to distinguish a total of nine overlapping immunogenic regions. Antibodies 7 and 8, 9 and 10, 12 and 14, and 17 and 19 form pairs of antibodies which compete strongly with one another and define four separate antigenic regions. The other regions are defined by mAbs1–4, mAb5, mAb11, mAb13, and mAb15. Antibody 10 binds adjacent to the ligand binding site since it inhibits binding of antibodies 1 and 3 to the receptor without competing directly with cho-

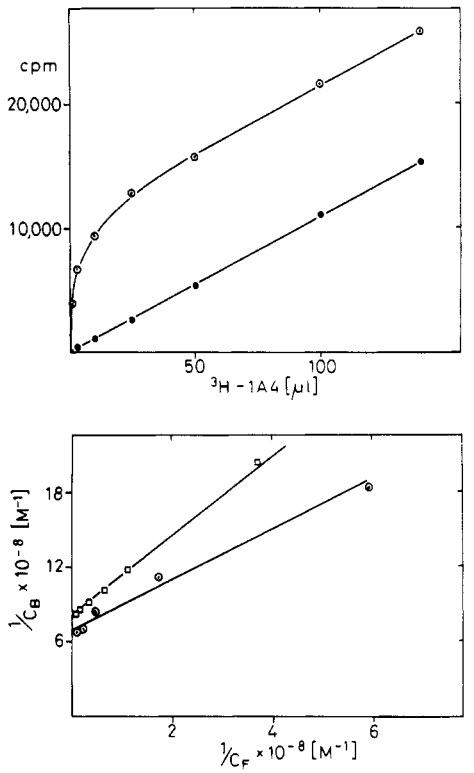

FIGURE 4: Binding of monoclonal antibodies to immobilized, purified receptor from *Torpedo marmorata*. Various amounts of [^{3}H]-leucine-labeled antibody in a total volume of 140 μL were incubated overnight at 20 °C in microtiter plate wells coated with purified *Torpedo* receptor. After six washes, the wells were separated and counted in a liquid scintillation counter. Specific activities were the following: ^{3}H-labeled mAb1, 4.5 Ci/mmol; ^{3}H-labeled mAb5, 5.5 Ci/mmol; ^{3}H-labeled mAb6, 2.1 Ci/mmol. (Top) Binding of mAb1 to microtiter plates coated with receptor (⊙) and with denatured (boiled) receptor (●). By subtraction of the two curves, the amount of specific binding was calculated. (Bottom) Double-reciprocal plots of the binding of ^{3}H-labeled mAb1 (⊙) and mAb5 (□) to the immobilized receptor. In both cases, specific binding was determined as shown above, and the double-reciprocal plot was constructed. The concentration of antibody binding sites at the receptor (R_0) was 1.25 × 10^{-9} M; equilibrium dissociation constants were K_D(mAb1) = 2.9 × 10^{-9} M and K_D(mAb5) = 3.9 × 10^{-9} M.

linergic ligands. Similarly, binding of antibody 13 to the receptor is not affected by ligands but is partially inhibited by mAb6.

Discussion

We have established a small library of monospecific antibodies to the acetylcholine receptor from *Torpedo marmorata* (Gomez et al., 1979; Moshly-Rosen et al., 1979; James et al., 1980; Tzartos et al., 1981) and report here on their initial characterization. Because we selected only antibodies showing positive ELISA with both the solubilized and the membrane-bound receptor, the antibodies studied are all directed against regions protruding from the plasma membrane and preserved in their structure during solubilization and purification. By employing an ELISA test instead of the commonly used precipitation assay with labeled toxin–receptor complexes, we also detected and characterized antibodies directed against the ligand binding site(s). The regional specificities and affinities of binding of the antibodies were studied by direct binding assays with internally labeled antibodies and by competition with cholinergic ligands.

As previously reported by others (Lennon et al., 1980; Gullick et al., 1981; Moshly-Rosen & Fuchs, 1981), only a few antibodies cross-reacted with receptor from sources other than *Torpedo* (Table I). Expanding these earlier results, we report here on four antibodies (mAsb1–4) specific for the ligand binding region that do not cross-react with any of the other receptor preparations tested. It is particularly noteworthy that antibodies mAb5 and mAb6 cross-react not only with eel receptor and rat myoballs but also with sympathetic ganglion cells from the chick. This reinforces the observation by Patrick & Stallcup (1977) that there exist antigenic similarities between the acetylcholine receptors from muscle and ganglion.

Monoclonal antibodies are monospecific, thereby practically abolishing the chances of extensive cross-linking of antigen molecules that may cause immunoprecipitation. They can therefore be employed in true equilibrium binding studies. Together with their broad range of specificities, they form a group of marker molecules as advantageous as the snake neurotoxins for biochemical studies of the receptor protein. As shown here (Figures 3 and 4, Table III), the antibodies vary considerably in their affinity for the receptor with the majority of the K_D values in the nanomolar concentration range.

Competitive binding between mAbs was employed to define the antigenic regions of the antibodies (Tzartos & Lindstrom, 1980; Tzartos et al., 1981). These studies define several separate antigenic regions for the 20 hybridoma supernatants tested (Table IV). Three of these regions overlap with the ligand binding site(s) at the receptor (Tables I and IV).

The competition patterns for antibody and ligand binding to the receptor deserve more detailed comment: Binding of each of the antibodies mAbs1–6 to the receptor is completely blocked by toxin (Figure 3) while only some of the antibodies of this group are competitive between themselves (Table IV). Moreover, only some of the antibodies (mAbs2–4) compete with all cholinergic ligands tested; the others compete efficiently with only some and compete little or not at all with the other cholinergic ligands tested. For full appreciation of this situation, the relative sizes of the molecules involved must be considered: A bound antibody covers an area of about 10 nm^2, and this constitutes an antigenic region as defined by Tzartos et al. (1981). The toxin molecule is considerably smaller in size (Walkinshaw et al., 1980), covering at most one-third of the area that can be occupied by an antibody. From the structure–activity relationships of cholinergic ligands

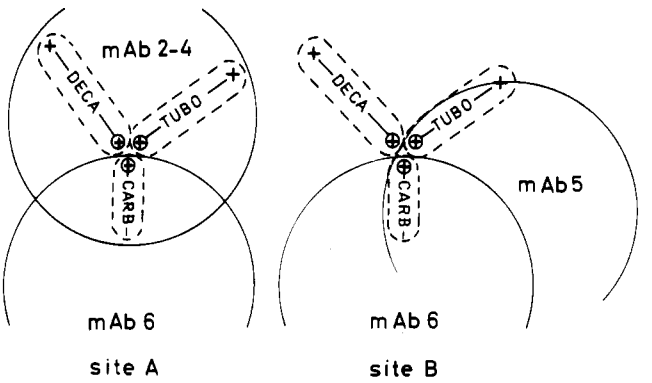

FIGURE 5: Schematic model for the competition between monoclonal antibodies and cholinergic ligands. The model assumes two ligand binding regions (site A and site B) each with three subsites defined by the ligands carbamoylcholine (carb), decamethonium (deca), and tubocurarine (tubo). Antibodies mAbs2–4 only bind to site A, mAb5 only binds to site B, and mAb6 binds to both sites. α-Cobratoxin also binds to both sites. For the purpose of simplicity, the model does not consider mAb1, which binds to site A in a fashion comparable to the binding of mAb5 to site B.

(Beers & Reich, 1970), the size of their binding region can be estimated to less than one-tenth of that of α-cobratoxin. We are therefore faced with the apparent contradiction that two of the larger molecules (mAbs1–4 and mAb5) do not compete with each other while they both do compete with the smaller α-cobratoxin.

This apparent contradiction can be explained by assuming two separate ligand binding sites both of which are recognized by the toxin but only one by mAbs1–4 and the other by mAb5. If this line of argument is continued, mAb6 which competes with both mAbs1–4 and mAb5 must then recognize antigenic determinants existing in both ligand binding sites. Furthermore, because mAb6 can completely prevent the binding of the other five antibodies to the receptor while its binding is only partially inhibited by the other five (Table IV), the two ligand binding sites probably are further apart than the diameter of an antigenic region.

The assumption of two separate binding sites for cholinergic ligands still does not suffice to also explain why antibodies 1 and 6 only compete with some but not all cholinergic ligands tested (Table II). With the assumption of competition being the result of local overlapping of sites, these findings suggest that the ligand binding sites are not single structures. As a minimal requirement, separate sites for groups of cholinergic ligands must be assumed. Furthermore, because of the overlapping competition pattern of the antibodies, these sites must be close to each other. With these assumptions, the competition data can be interpreted as follows. (i) In each cholinergic binding region, there exist three types of subsites for small ligands: acetylcholine, carbamoylcholine, and succinyldicholine bind to subsite 1, the bismethonium compounds bind to subsite 2, and tubocurarine binds to subsite 3. The subsites constitute one single ligand binding site because (a) the small ligands bind mutually exclusively to this site and (b) α-cobratoxin binds mutually exclusively with the small ligands (Maelicke et al., 1977a) and the antibodies mAbs1–6 (Table II) to this site. (ii) There exist two structurally distinct ligand binding regions. Antibodies mAbs1–4 bind to site A, mAb5 binds to site B, and mAb6 binds to both sites (Table IV). (iii) The two ligand binding regions are further apart than the diameter of an antigenic region. This is schematically summarized in Figure 5.

The model is satisfying for the following reasons: (i) The partial inhibition of mAb6 by mAbs1–5 (Table IV) is ex-

plained. The resulting scheme is consistent with the assumption of one ligand binding site per α-subunit (Reiter et al., 1972; Sobel et al., 1977; Moore & Raftery, 1979) and the known organization of subunits within the receptor monomer (Wise et al., 1981; Kistler et al., 1982; Holtzman et al., 1982). (ii) The antigenic determinants for mAbs1–5 do not necessarily have to be wholly within the ligand binding sites or a single receptor subunit. Thus, the different determinants for mAb1 and mAbs2–4 may result from the different environments of the two α-subunits (Wise et al., 1981; Holtzman et al., 1982). (iii) Differences in the properties of the ligand binding sites have been observed previously (Maelicke et al., 1977a; Damle & Karlin, 1978; Neubig & Cohen, 1979; Prinz & Maelicke, 1983). (iv) The competition pattern of mAb1 resembles that of a myasthenic serum studied with chicken embryo myogenic cultures (Fulpius et al., 1980). (v) The model may also relate to the phenomenon of accelerated dissociation of toxin–receptor complexes (Maelicke et al., 1977a; Kang & Maelicke, 1980): The existence of subsites for different groups of cholinergic ligands may explain why additional binding sites apparently become available at a very high concentration of ligand (Maelicke & McConnell, 1978; Kang & Maelicke, 1980). With these occupied, a new and faster route of toxin dissociation is then opened.

The most challenging aspect of the model concerns the nature of the proposed "subsites" for cholinergic ligands. If a purely occupational model is assumed, these are indeed separate sites in the sense that they do not overlap (Figure 5). In fact, they could be as far apart as is permitted by the size of the binding regions of α-cobratoxin and of the antibodies that bind to the same ligand binding site (site A or site B of Figure 5). Both regions are rather large in size (Tzartos et al., 1981; Martin et al., 1983). On the basis of previous binding studies with the acetylcholine receptor (Maelicke et al., 1977a,b; Heidmann & Changeux, 1978; Kang & Maelicke, 1980; Karlin, 1980; Fels et al., 1982), however, fully separate sites for groups of cholinergic ligands appear rather unlikely. In contrast, our findings strongly imply that the receptor assumes different conformational states depending on the nature of the ligand (or antibody) bound. Thus, binding of acetylcholine modifies the structural organization of the receptor in such a way that the antigenic sites for mAbs1–6 are not accessible or not existent anymore. Other ligands, e.g., tubocurarine or the bismethonium compounds, affect the conformation of the receptor differently with some antigenic sites (notably those for mAb1 and mAb6) remaining available. Similar conformational rearrangements of the receptor are also indicated by the accelerated dissociation of toxin–receptor complexes in the presence of cholinergic ligands (Maelicke et al., 1977a; Maelicke & McConnell, 1978; Kang & Maelicke, 1980) and were considered in a reaction model for this process (Kang & Maelicke, 1980). The proposed conformational changes must not be large in size because the rearrangement of one of two amino acid side groups may already suffice to significantly alter or fully inactivate the recognition process for particular ligands (cholinergic ligands, toxins, and antibodies). Such ligand-induced states of the receptor would make ligand recognition an individual rather than a general process.

As one implication of such a mechanism of ligand recognition, some cholinergic ligands must bind to the receptor by other and/or more determinants than are defined by the structure of the natural transmitter. Accordingly, the various ligands of the receptor should not be considered as mere copies of acetylcholine with the structure of the natural transmitter forming the "active site". This may also be the underlying reason for the fact that agonists of acetylcholine vary in their efficacies and that many ligands have "mixed" or "additional" effects.

Acknowledgments

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Registry No. Tubocurarine, 57-94-3; hexamethonium, 60-26-4; carbamoylcholine, 462-58-8; decamethonium, 156-74-1.

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A Fluorescent Aminolipid from a Green Photosynthetic Bacterium†

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ABSTRACT: Kenyon and Gray [Kenyon, C. N., & Gray, A. M. (1974) *J. Bacteriol. 120*, 131–138] first reported the presence of phosphate-free aminolipids in green sulfur bacteria, and we now present a preliminary chemical characterization of the aminolipid isolated from *Chlorobium limicola* f. *thiosulfatophilum*. A major component of our membrane preparations, this lipid contains no phosphorus, glycerol, sugar, ornithine, or lysine. Ultraviolet absorption and fluorescence spectra indicate that the amino moiety of the lipid is an aromatic heterocyclic compound. Infrared spectra indicate that the lipid is a secondary or tertiary amide, and gas chromatographic analysis of the hydrolyzed lipid shows that for each 1100 g of lipid, 1 mol of myristic acid ($C_{14:0}$) is linked in an amide bond. Acid hydrolysis of the lipid yields two fluorescent substances, A (ninhydrin positive) and B (negative), in addition to myristic acid. Proton nuclear magnetic resonance (NMR) studies indicate that substance A contains a butyl group attached to a conjugated ring carbon, two equivalent ethyl groups attached to one or two nitrogen atoms, and two downfield protons (8.4 ppm), perhaps attached to a ring carbon adjacent to a ring nitrogen as in adenine. Substance B also appears to contain a butyl group (not attached to a conjugated ring) as well as two vinyl protons (7.4 and 7.7 ppm) across a ring double bond. *Chlorobium* aminolipid appears to be a new type of lipid, enriched in our membrane preparations from green sulfur bacteria.

In green sulfur bacteria (Chlorobiaceae) roughly half the inner surface of the cytoplasmic membrane is covered with green patches composed of chlorosomes (40 × 70 nm to 100 × 260 nm) and associated base plates (Staehelin et al., 1980; Olson, 1980). Photosynthetic reaction centers inside the membrane are concentrated under the patches. The chlorosomes contain bacteriochlorophyll c, d, or e, which traps the incoming light and transfers the energy to the bacteriochlorophyll a in the reaction centers via the bacteriochlorophyll a in the base plate (Olson, 1980). The reaction center complexes are hydrophobic protein complexes embedded in the membrane, but the base plate consists of hydrophilic bacteriochlorophyll a–proteins arranged in a two-dimensional crystal between the membrane and the chlorosome. These proteins are presumably bound to the membrane by hydrogen bonds, because they can be removed from membranes and/or reaction center complexes by the use of guanidine hydrochloride or arginine hydrochloride but not by the use of high salt or the nonionic detergent Triton X-100 (Olson, 1980). However, the nature of the linking groups on the inside surface of the

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