The Acetylcholine Receptor and Its Role in Induction of Experimental Autoimmune Myasthenia Gravis

Thesis by

Antonia R. Claudio

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Abstract

I. Rabbit antibodies were produced against purified acetylcholine receptor (AcChR) and each of the four acetylcholine receptor subunits from Torpedo californica. Using the technique of double immunodiffusion in agar, specific- and cross-reactivities were observed between these antibodies and purified acetylcholine receptor and receptor subunits from Torpedo californica, Torpedo marmorata, Torpedo nobiliana, and Narcine brasiliensis. The specificity of each of the four anti-subunit antibodies was shown, suggesting the lower molecular weight polypeptide chains of the AcChR were not degradation products of the higher molecular weight polypeptide chains. The study also demonstrated conservation of AcChR and AcChR subunit antigenic determinants in the four electric rays investigated.

II. Experimental autoimmune myasthenia gravis (EAMG) has been induced in a wide variety of animals using AcChR purified from a variety of electric organ and muscle sources. Electrophoresis of sodium dodecyl sulfate (SDS) polyacrylamide gels heavily loaded with purified AcChR often reveals the presence of minor contaminants. To test if these contaminants or any other components present in Torpedo californica AcChR preparations could induce EAMG, solubilized T. californica membrane fragments were depleted of AcChR by passage over an α-bungarotoxin resin and then injected into Lewis rats in an attempt to induce EAMG. The results demonstrated that some of the minor contaminants present in purified AcChR preparations were antigenic but EAMG could not be induced with preparations enriched in these contaminants or containing other T. californica non-AcChR components.

III. Antisera prepared in rabbits and Lewis rats against Torpedo californica AcChR (purified and denatured to various degrees) were tested for the ability

to inhibit [^{125}I] α-bungarotoxin (α-BuTx) binding to native and detergent solubilized T. californica AcChR. Similar inhibition studies were performed using antisera and antigen-binding fragments (Fabs) directed against each of the four isolated AcChR subunits. None of these antisera or Fabs could inhibit α-BuTx binding to native AcChR. Antisera and Fabs directed against AcChR could inhibit a maximum of 50% α-BuTx binding to solubilized AcChR. The results using Fabs indicated the inhibition was not due to antibody-mediated aggregation of AcChR molecules. A strong correlation was seen between animals with EAMG and the ability of their antisera to inhibit 50% of α-BuTx binding to AcChRs. The results indicated that particular antigenic determinants on AcChRs could induce EAMG and that these determinants were lost with SDS denaturation of AcChR.

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Introduction

The acetylcholine receptor (AcChR) is a glycoprotein located in excitable membranes which binds the neurotransmitter acetylcholine (AcCh) and effects a permeability change in postsynaptic membranes. Electric organ tissue from the fresh water electric eel Electrophorus and the marine ray Torpedo is evolutionarily derived from muscle but the concentrations of AcChR found in these tissues are very high compared with AcChR concentrations in vertebrate muscle. Electric tissue is thus a convenient source of AcChR for biochemical and immunological studies.

The isolation and characterization of AcChRs from a variety of sources has been greatly aided by the use of certain neurotoxins isolated from the venoms of cobras, sea snakes, and the Formosan banded krait, Bungarus multicinctus (1). These neurotoxins are highly basic polypeptides (pI $\sim$9) of 7000-8000 M_r and have been shown, both by physiological and biochemical studies, to bind specifically to AcChRs and prevent the binding of AcCh. The action of most of the toxins is essentially irreversible and the toxins can be radiolabeled without affecting their specificity or binding properties.

Reports of AcChR isolation from the electric organs of the marine rays Torpedo and Narcine, the eel Electrophorus, and from mammalian muscle have yielded monomeric AcChR molecular weights of 170,000 to 360,000, AcChR subunits ranging from 40,000 to 140,000 M_r, and the number of subunits varying from one to five (for a recent review of AcChRs, see reference 2). Some of this variability is apparently due to differences in the method of purification, and the inclusion of protease inhibitors, and not just species differences (3).

The only polypeptide chain which has thus far been assigned a function is the 40,000 M_r chain which appears to contain the AcCh binding site. This chain is specifically labeled with an affinity alkylating agent MBTA, 4-(N-maleimido) benzyltrimethylammonium iodide (4), a fluorescent decamethonium analogue DAP, bis(3-aminopyridinium)-1,10-decane diiodide (5), α-neurotoxins (4, 6, 7), and the cholinergic agonist bromoacetylcholine (8). Suggestive evidence that the 50,000 and 65,000 M_r polypeptide chains may be AcChR subunits has come from studies in which possible ligand-induced conformational changes in the 40,000 M_r subunit resulted in conformational changes in these two chains (9). The possibility remained that some of the lower molecular weight polypeptide chains seen with purified AcChR preparations could be degradation products of the higher molecular weight chains. Immunological evidence presented in Chapter I and reference 10 suggests that the four chains are distinct. This same conclusion was reached from results of peptide mapping of each of the four polypeptide chains (11). The question of whether the other three chains are functional subunits of the AcChR, however, remains unanswered.

Myasthenia gravis (MG) is a neuromuscular disease characterized by muscle weakness which increases with exertion and improves with rest or with the administration of acetylcholinesterase inhibitors. The incidence of the disease is approximately one in 30,000 and is twice as prevalent in females as it is in males. Abundant evidence indicates that the pathogenesis of MG involves an autoimmune attack directed against AcChRs (for recent reviews on MG and EAMG, see references 12-14). Some of this evidence includes the following observations: 1) muscles from patients with MG have reduced numbers of AcChRs, 2) miniature end-plate potentials are reduced in amplitude although the total amount of AcCh released from nerve

terminals is normal, 3) electron micrographs show degeneration and simplification of postsynaptic membranes, 4) anti-AcChR antibodies are found circulating in sera of ∿90% of MG patients, 5) transient neonatal MG has been reported in children of mothers with MG, 6) thymectomy or immunosuppressive drugs may be beneficial for some MG patients, and, one of the strongest pieces of evidence, 7) the immunization of experimental animals with AcChR isolated from Torpedo, Electrophorus or mammalian sources leads to a disease very similar to that of the human disease. Immunization of mammals with AcChR induces an autoimmune response to skeletal muscle AcChR which impairs neuromuscular transmission, causing weakness and death in many animals. The same physiological and biochemical observations made in human MG have also been found in the induced disease, experimental autoimmune myasthenia gravis (EAMG).

When animals are given a single injection of AcChR in adjuvant, the sequence of events following immunization can be followed. Between 8 and 11 days after injection, some animals may exhibit an acute phase of EAMG where signs of weakness and fatigability are demonstrated and animals may die. During this phase antibodies can be seen bound to postsynaptic membranes, complement is present, and neuromuscular junctions (NMJ) become invaded by mononuclear phagocytic cells resulting in degeneration and simplification of postsynaptic membranes. The acute phase remits and the cellular invasion disappears until 28 to 30 days when a second, chronic phase of EAMG begins which is progressive and fatal. The chronic phase appears to be the best model for human MG. During this phase no cellular invasion of the NMJ occurs. Antibodies bound to AcChRs appear to interfere with neuromuscular transmission directly, induce decreased AcChR content by increased AcChR turnover, and alter postsynaptic membrane morphology by antibody-dependent, complement-mediated focal lysis.

In order to ascribe the destruction of the NMJ to AcChRs labeled with antibodies, it is essential to determine if this is the only component at the NMJ which can do so when it becomes immunogenic. This problem is addressed in Chapter II. In 1978, Lindstrom et al. (15) showed that each of the four isolated AcChR subunits could induce EAMG in Lewis rats if administered in very large doses. These results, together with those presented in Chapter II, may be the strongest evidence suggesting that each of the four T. californica AcChR polypeptide chains is an AcChR subunit or at least is a protein which has been highly conserved throughout evolution.

In Chapter III, an investigation of the possible interference of neuromuscular transmission by antibodies is described. Antisera from patients with MG and animals with EAMG have been shown to inhibit AcCh and α-BuTx binding to various preparations of AcChR. Electrophysiological studies with these antisera have shown reduced amplitudes of miniature end-plate potentials (16, 17). The inhibition of α-BuTx binding to T. californica AcChRs is described in Chapter III. A correlation between animals with EAMG (as opposed to animals with anti-AcChR antibodies but no EAMG) and the ability of their antisera to inhibit α-BuTx binding to AcChR is shown.

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Chapter I

Immunological Comparison of Acetylcholine Receptors and their Subunits from Species of Electric Ray

(Portions of this chapter have been published, see Claudio, T., and Raftery, M. A. [1977], Archives of Biochemistry and Biophysics **181**, 484-489.)

ABBREVIATIONS

AcChR: acetylcholine receptor

α-BuTx: alpha bungarotoxin

AcChR-$[SDS]_n$: AcChR in 0.1% SDS

AcChR-$[SDS]_1$: AcChR containing one molecule of SDS per α-BuTx binding site

M_r: molecular weight

SDS: sodium dodecyl sulfate

TCA: trichloroacetic acid

INTRODUCTION

In recent years, several reports dealing with the isolation and purification of nicotinic acetylcholine receptors have appeared. The sources used have been electroplaque membranes of electric rays and electric eels as well as membranes from mammalian muscle. Acetylcholine receptor (AcChR) has been purified from elasmobranchs such as Narcine entemedor (1), Torpedo californica (2-5), Torpedo marmorata (6-8), Torpedo nobiliana (9) and from the electric eel Electrophorus electricus (10-15). Recently, partial purification of AcChR from rat diaphragm (16) and from denervated cat skeletal muscle (17) have been reported. Disagreement continues as to the number, molecular weights and stoichiometries of AcChR subunits. In this communication, immunological evidence is presented which favors a common subunit composition for AcChR purified from four species of elasmobranch. In addition, it is demonstrated that within the detection limits of the assays employed, the four types of polypeptides present in each purified AcChR do not share common antigenic determinants. This latter finding suggests that the lower molecular weight polypeptide chains are not degradation products of the higher molecular weight chains.

MATERIALS AND METHODS

Torpedo californica was obtained locally, T. marmorata was obtained live from the Biological Station, Arachon, France, N. brasiliensis was obtained live from Turtle Cove Lab., Inc., Port Aransas, Texas, and T. nobiliana, frozen at -90°C, was a gift from Dr. J. Bonaventura. Lyophilized crude venom of Bungarus multicinctus, bovine serum albumin, and Triton X-100 were purchased from Sigma Chemical Co. Electrophoresis grade reagents were obtained from BioRad Labs. Sequanal grade sodium dodecyl sulfate was from Pierce Chemical Co. All buffers used were pH 7.4 and contained 10 mM sodium phosphate and 0.02% sodium azide unless otherwise stated.

Preparation of acetylcholine receptor and its α-bungarotoxin (α-BuTx) complex

AcChR was purified from the electric rays, T. marmorata, T. nobiliana, N. brasiliensis (18) and T. californica by affinity chromatography as described previously (2). Protein concentrations were determined by the method of Lowry (19) using bovine serum albumin as the standard. Specific activities of the AcChR preparations were determined with [^{125}I]α-BuTx (20). The AcChR from T. californica used for rabbit immunizations and immunodiffusion assays had a specific activity of 8.8 nmoles α-BuTx bound per mg of protein. AcChR-α-BuTx complex was prepared by first concentrating purified T. californica AcChR to 1 mg/ml in an Amicon Filtration apparatus using an XM-50 Diaflo membrane and then incubating with saturating amounts of α-BuTx.

Preparation of SDS-treated AcChR

Purified AcChR from T. californica was concentrated to 1 mg/ml, SDS was

added to a final concentration of 1% and the solution was incubated for 1 hour at 37°C. This preparation was dialyzed for 72 hours against 2 liters of buffer containing 0.1% SDS with two buffer changes and is referred to as AcChR-$[SDS]_n$. For preparation of an AcChR sample containing less SDS, AcChR-$[SDS]_n$ was dialyzed for 72 hours against 2 liters of buffer containing 0.1% Triton X-100 with two buffer changes. This material is referred to as AcChR-SDS. Preparation of AcChR-SDS for the third and fourth rabbit challenges used the technique of electrodialysis (21) to remove excess SDS. An apparatus similar to that used for electroelution of subunits from SDS gel strips, described in the following section, was used for electrodialysis. AcChR-$[SDS]_n$ was electrodialyzed against 1 mM Tris pH 7.4 with a constant current of 5 mA per tube for 16 hours. The rate of removal of SDS from receptor samples was established using [^{35}S]lauryl sulfate from New England Nuclear Corp.

Preparation of AcChR subunits

AcChR subunits from all four species of ray were originally obtained by preparative continuous SDS polyacrylamide slab gel electrophoresis using the buffer system described by Fairbanks et al. (22). The gel dimensions were 10 x 13.5 x .6 cm and contained ∿90 ml of gel composed of 6.3% acrylamide. Gels were pre-run at a constant current of 75 mA for 30 minutes before the sample was applied and run at 75 mA. The continuous gel system did not resolve all subunits well, thus subsequent gels used the discontinuous gel system described by Laemmli (23) to separate AcChR subunits. The gel dimensions were the same as with the continuous system except the gel contained 80 ml of separating gel (7.5% acrylamide) and 12 ml of stacking gel (4% acrylamide). Gels were run at

a constant current of 25 mA then 75 mA once the protein had entered the separating gel. Three to five mg of AcChR were applied to either type of gel. Five 2 mm-thick lengthwise strips were cut from the gel then stained and destained in 25% methanol, 10% acetic acid $\pm$ 0.05% Coomassie Brilliant Blue while the remainder of the gel was tightly wrapped and stored frozen at -20°C. The stained gel strips were realigned with the frozen gel and sections corresponding to the subunits were excised, forced through a 3 cc disposable plastic syringe, then eluted from the gel by either incubation with 0.1% SDS or electrophoresis. The incubation was done in 15 ml of 0.1% SDS at 37°C for 10 hours. Eluted protein was separated from acrylamide by filtration over a sintered glass funnel followed by Millipore filtration. Electrophoresis was conducted in 10 cc plastic disposable pipettes inserted into a regular tube gel electrophoresis apparatus. Dialysis tubing was attached to the ends of the pipettes and glass wool plugs put in the tips of the pipettes. Protein was eluted from the gel by electrophoresis at 5 mA per tube for 10-12 hours. SDS was removed by electrodialysis from all subunits used in Ouchterlony immunodiffusion assays and from T. californica subunits used for the third and fourth antigenic challenges. Subunits were concentrated by lyophilization and the purity of each tested on analytical SDS polyacrylamide gels.

Preparation of antisera

New Zealand white female rabbits were injected and boosted with 0.15-0.56 mg of protein emulsified in 0.15-0.5 ml complete Freund's adjuvant. The emulsions were administered subcutaneously at six spots on either side of the lower spine. Rabbits were injected with whole AcChR, individual AcChR subunits, AcChR-$[SDS]_n$, AcChR-$[SDS]_1$, or AcChR-α-BuTx complex. For the studies

described here, 40 ml of blood were obtained from an ear vein 16 days after a third challenge, or 4 days after a fourth challenge. Antisera were fractionated by ammonium sulfate precipitation (0-50% followed by 0-40%). Pellets were dissolved in buffer containing 0.85% NaCl and dialyzed against buffer at 4°C for 3 days. The dialysate was clarified by centrifugation at 27,000 x g for 40 minutes.

Precipitation of AcChR and isolated AcChR subunits

AcChR labeled with [^{125}I]α-BuTx or AcChR subunits labeled with [^{125}I] were precipitated with 4 parts methanol, ethanol, or acetone to one part AcChR solution. The ratio of butanol to AcChR was 10:1 and trichloroacetic acid (TCA) was used at a final concentration of 20%. Solutions were mixed thoroughly, incubated 30 minutes at -20°C, and precipitates collected by centrifugation at 9000 x g for 20 minutes. Pellets were washed once in their respective undiluted solvents or 20% TCA. The effectiveness of the different precipitation methods was tested by following the radioisotope and running SDS polyacrylamide gels of the precipitates. The same precipitations were performed on AcChR containing traces of [^{3}H] Triton X-100 to determine the best method for removing AcChR from Triton X-100.

Preparation of [^{125}I] AcChR

Carrier-free sodium iodide-[^{125}I] at 100 mCi/ml was obtained from Amersham Searle. Biogel P-2, 100-200 mesh was obtained from BioRad Laboratories. A modification of the method described by Greenwood et al. (24) was used to radiolabel purified AcChR. 0.52 mg of acetone precipitated AcChR were resuspended to a concentration of 0.2 mg/ml in 0.5 M sodium phosphate buffer,

1% SDS, pH 7.0. 2.5 μg of Chloramine-T in 0.5 M sodium phosphate buffer were added and mixed followed by 0.25 mCi of [^{125}I] and a 90-second incubation period. After adding 50 μg of tyrosine in 0.5 M buffer, the sample (2.6 ml) was applied to a 20 ml Biogel P-2 column equilibrated in 10 mM sodium phosphate, 0.1% SDS, pH 7.4. [^{125}I]AcChR was separated from [^{125}I]tyrosine by a modification of the centrifuge de-salting method described in (25). The column was poured in a 20 ml disposable syringe and the void volume removed by a 2-minute centrifugation at 2000 rpm in an International Refrigerated Centrifuge Model PR-6. After application of the sample to the column, [^{125}I]AcChR was collected by centrifugation at 500 rpm for 5 minutes followed by 2 minutes at 2000 rpm.

Preparation of [^{125}I]AcChR subunits

[^{125}I]AcChR was prepared as described above. A discontinuous preparative slab gel was prepared as described above and 4.2 mg of AcChR containing trace amounts of [^{125}I]-labeled AcChR were applied and run. At the completion of the electrophoresis, the gel was cut in half; half was stained and destained while the other half was autoradiographed. Subunit bands were excised from both halves and used in studies to determine the yield and length of time required to electro-elute subunits from gel slices.

RESULTS

Treatment of AcChR with α-BuTx or SDS

Based on the specific activity of the AcChR, which was determined with [^{125}I]α-BuTx, unlabeled α-BuTx was added to AcChR to just saturate all the toxin

binding sites. Once AcChR was bound by unlabeled α-BuTx, an aliquot of this complex was assayed with [^{125}I]α-BuTx to check that no free toxin binding sites were available. The results of the assay indicated all sites were occupied with unlabeled α-BuTx. Such unlabeled α-BuTx-AcChR complexes were used as antigens for rabbit immunizations.

[^{125}I]α-BuTx binding to AcChR which had been incubated with 1% SDS for 1 hour at 25°C, 1 hour at 37°C, or 3 minutes at 100°C yielded identical results indicating possibly ∿5% α-BuTx binding activity remaining. No toxin binding activity was detected with isolated 51,000, 60,000 or 64,000 M_r subunits but possibly ∿5% was seen with 41,000 M_r subunit or a mixture of each of the four subunits. AcChR incubated with 1% SDS and then dialyzed against buffer containing 0.1% Triton X-100 regained 20% of its original α-BuTx binding ability.

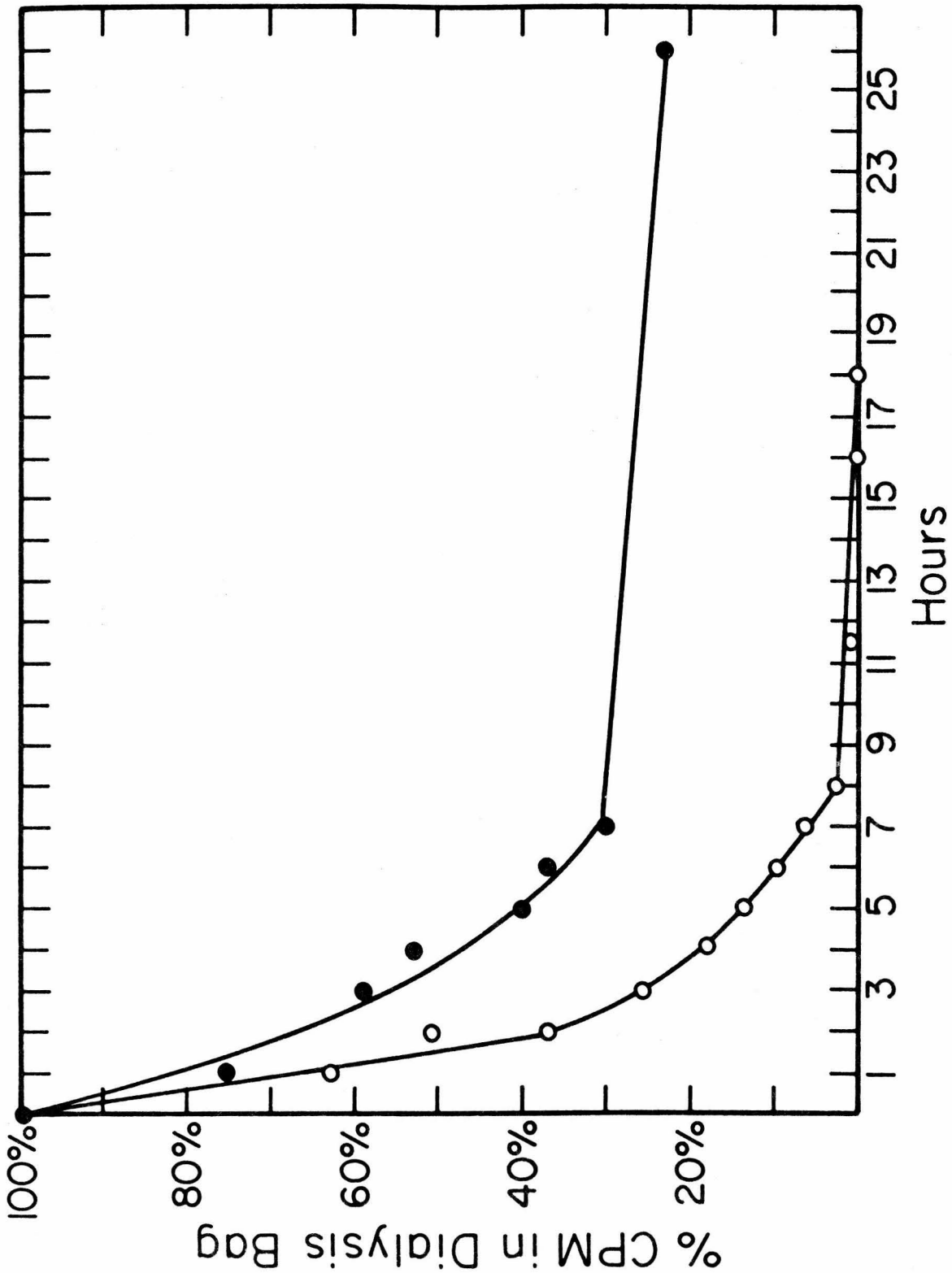
Electrodialysis of SDS

A time course study of [^{35}S]lauryl sulfate electrodialysis from AcChR in 10 mM sodium phosphate, 0.1% SDS, pH 7.4 or a similar buffer containing 0.5% Triton X-100 is shown in Figure 1. After 17 hours of electrodialysis of AcChR-$[SDS]_n$ (AcChR in the presence of 0.1% SDS), the SDS content was reduced to one molecule per 125,000 M_r AcChR. This material is referred to as AcChR-$[SDS]_1$ since it contains one SDS molecule for each α-BuTx binding site. It can be seen that if Triton X-100 is added to the AcChR + SDS dialysis bag, the rate of SDS dialysis is greatly decreased. This is probably due to the formation of large mixed detergent micelles.

Precipitation of AcChR

The results of the precipitation of AcChR-[^{125}I]α-BuTx complexes and

Figure 1. Time course study of [^{35}S]lauryl sulfate electrodialysis. ●, electrodialysis in the presence of 0.5% Triton X-100; o, electrodialysis in the absence of Triton X-100.


% CPM in Dialysis Bag
100%
80%
60%
40%
20%
1
3
5
7
9
11
13
15
17
19
21
23
25
Hours

[^{3}H]-Triton X-100 with different solvents and TCA are listed in Table I. Acetone, butanol, and TCA all precipitated AcChR-toxin complexes equally well. SDS gel electrophoresis of the precipitates confirmed that AcChR was precipitating and not just [^{125}I]α-BuTx. Acetone and methanol were the most effective in not precipitating Triton X-100, however. The method of choice for concentrating AcChR virtually free of Triton X-100 was thus the use of acetone in a 4:1 ratio to AcChR solution.

Time required for electroelution of AcChR subunits and precipitation of AcChR subunits

Compiled in Table II are the times required to electroelute subunits from SDS gel strips which had been either untreated or stained and destained. It can be seen that the 64,000 M_r subunit was the slowest to elute from both stained and non-stained gel strips. Electroelution times of 2-1/2 hours were sufficient to elute 85-90% of the 41,000, 51,000 and 60,000 M_r subunits from untreated gel strips. At least twice as much elution time was required to elute 90% of the 64,000 M_r subunit, however. 85-90% of each of the subunits was eluted from stained gel strips in 12 hours.

Subunits were next dialyzed to free them of SDS and then concentrated by lyophilization in preparation for use as antigens. Because subunits were susceptible to proteolysis during dialysis and because both steps were fairly time consuming, it was desirable to find alternate procedures for preparing subunits.

The electroeluted [^{125}I]-labeled subunits described above from both stained and non-stained gels were each precipitated with acetone, butanol, ethanol, methanol, or TCA as described in the Materials and Methods section. After each

TABLE I

Precipitation of AcChR

Precipitating agent	% AcChR precipitated	% Triton X-100 precipitated
Acetone	100	6
Butanol	100	21
Ethanol	60	20
Methanol	30	0
TCA	100	99

TABLE II

Percentage of [^{125}I]-labeled AcChR subunits electroeluted from SDS gels

		Time of elution		
	Subunit M_r	2-1/2 hr (%)	12 hr (%)	27 hr (%)
Non-stained gel	41,000	89	98	99
	51,000	85	97	98
	60,000	84	98	99
	64,000	41	97	99
Stained gel	41,000		86	93
	51,000		85	93
	60,000		90	98
	64,000		93	98

subunit was precipitated, the precipitate was dissolved in 1% SDS, the solution was clarified by centrifugation, and the supernatants counted in a Beckman 4000 gamma counter and applied to a discontinuous analytical SDS polyacrylamide slab gel and later autoradiographed. The results indicated every solvent and TCA precipitated $\geq$95% of each subunit. In addition, all subunits were equally well resuspended in 1% SDS except the butanol precipitated non-stained 64,000 M_r subunit and butanol precipitated stained 41,000, 51,000, 60,000, and 64,000 M_r subunits. Only $\sim$50% of these subunits could be resuspended in 1% SDS. The autoradiographs of each of these precipitated and resuspended subunits are shown in Figure 2. In all cases, material which did not enter the gel was more prevalent in stained samples than in non-stained samples. Acetone, ethanol and methanol worked equally well for precipitating subunits from electrophoresis buffer and freeing them of Coomassie Brilliant Blue.

Purity of isolated subunits, animal observations, double immunodiffusion results

The techniques of radiolabeling AcChR, adding trace amounts to preparative slab gels and excising bands aligned with the autoradiograph of the gel, had not been worked out at the time of publication of the major portion of this chapter. In addition, the methods of concentrating isolated subunits free of Coomassie Brilliant Blue were also not worked out at that time. Isolated subunits were judged pure based on their Coomassie Brilliant Blue staining gel pattern when rerun on SDS gels. Gels and gel scans of isolated subunits are shown in Figure 3. The molecular weights of the four receptor subunits purified from T. californica were 41,000, 51,000, 60,000 and 64,000 M_r and are referred to as subunits

Figure 2. Autoradiographs of [^{125}I]-labeled AcChR subunits precipitated by various solvents and TCA, resuspended in 1% SDS, and run on 7.5% acrylamide analytical discontinuous SDS polyacrylamide slab gels. (a), acetone; (b), ethanol; (c), butanol; (d), methanol; (e), TCA. In all figures, wells 1-4 are subunits eluted from non-stained SDS gel strips, and wells 5-8 are subunits eluted from stained and destained SDS gel strips. Wells 1 and 5 contained the 41,000 M_r subunit, wells 2 and 6 contained the 51,000 M_r subunit, wells 3 and 7 contained the 60,000 M_r subunit, and wells 4 and 8 contained the 64,000 M_r subunit.

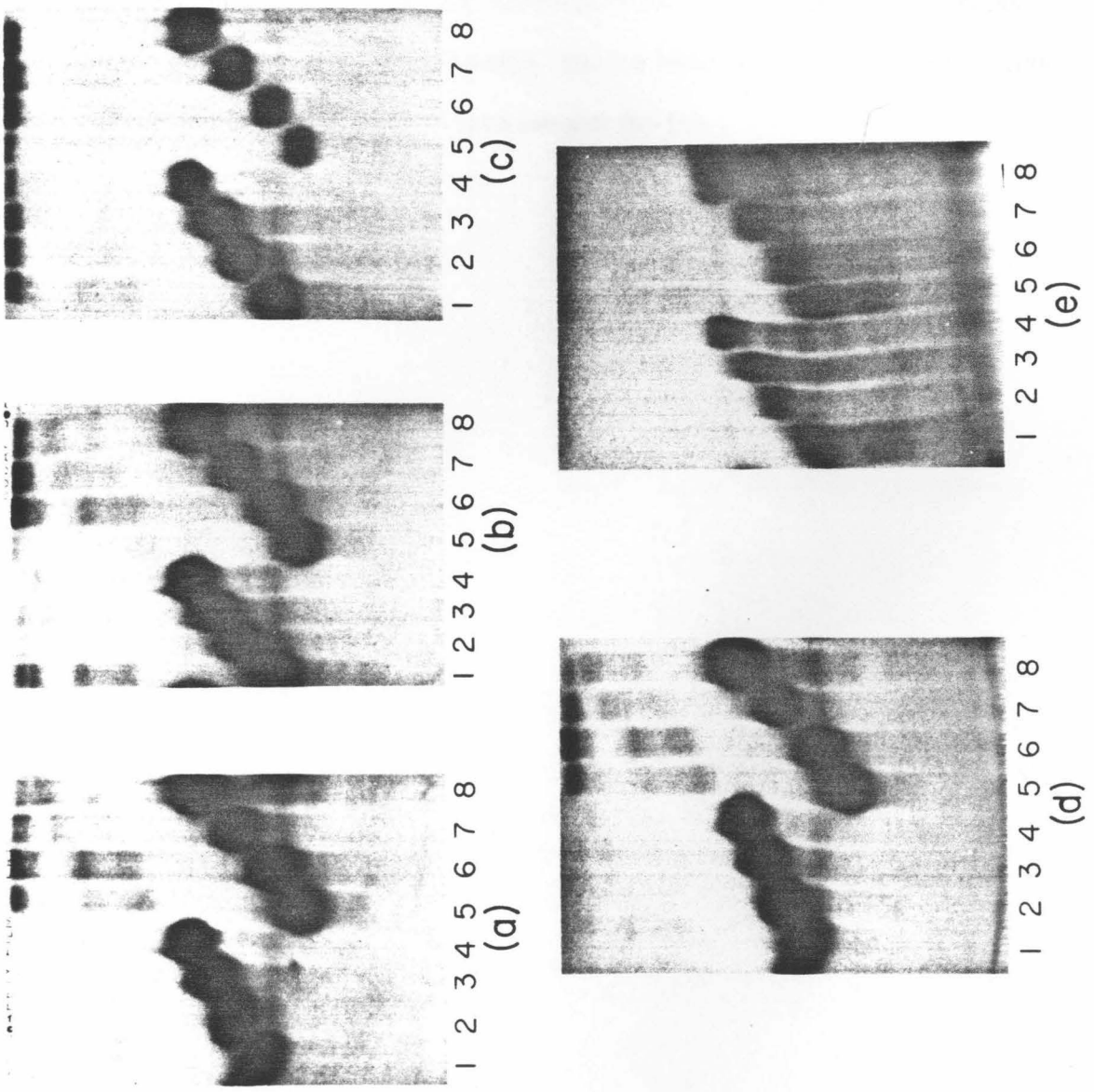
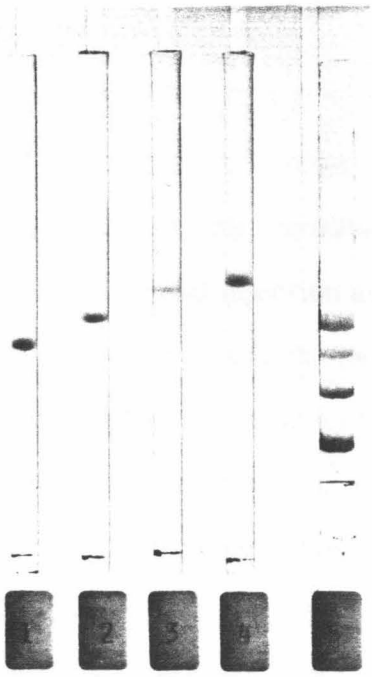
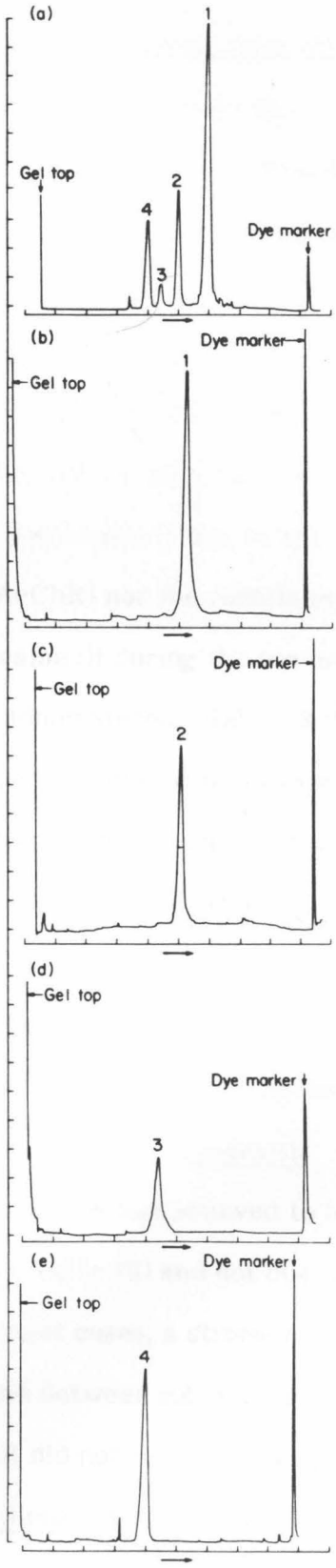

1 2 3 4 5 6 7 8
(a)
1 2 3 4 5 6 7 8
(b)
1 2 3 4 5 6 7 8
(c)
1 2 3 4 5 6 7 8
(d)
1 2 3 4 5 6 7 8
(e)

Figure 3. Stained gels and gel scans of T. californica AcChR. On the left, a section of the 7.5% acrylamide preparative discontinuous SDS gel (No. 5) and each of the eluted subunits 1-4 rerun on 6% acrylamide analytical continuous SDS polyacrylamide gels (Nos. 1-4, respectively). On the right, scans of the preparative slab gel section (a) and of each eluted subunit (b)-(e).


1
2
3
4
5


(a)
Gel top
4
2
1
3
Dye marker
(b)
Dye marker
Gel top
1
(c)
Dye marker
Gel top
2
(d)
Gel top
Dye marker
3
(e)
Dye marker
Gel top
4

1, 2, 3 and 4, respectively. Molecular weights of the AcChR subunits from other rays were very similar to those of T. californica and are listed in Table III.

Two rabbits, one injected with AcChR and the other with AcChR-α-BuTx complex (rabbits 5 and 6), became paralyzed in the hind quarters and were unable to move 21 days after an initial injection. The symptoms were similar to those described by Patrick and Lindstrom (26) as experimental autoimmune myasthenia gravis. These two rabbits were sacrificed on the 22nd day. A second rabbit injected with AcChR-α-BuTx complex (rabbit 9) exhibited normal behavior for 3 months but died 5 days after its third challenge. It is not known if this rabbit developed paralysis. Neither rabbit 8 (injected with AcChR) nor the rabbits injected with purified AcChR subunits (rabbits 1-4) died or became ill during the ten months in which an initial injection and four challenges were administered. Rabbit 8 did die 21 days after its fifth challenge, however. Rabbits 1-4 and four other rabbits injected with isolated subunits (rabbits 11-14) have never demonstrated any signs of muscular weakness. For a complete immunization schedule, see Table IV.

All antibody-antigen interactions were observed by Ouchterlony immunodiffusion assays, the results of which are compiled in Table III. Antibodies to each T. californica receptor subunit reacted with AcChR and with the subunit against which they were made. One exception was seen in the case of N. brasiliensis where the cross-reactivity observed between subunits 3 and 4 was believed to be due to contamination of one subunit with the other (see Table III) and not due to common antigenic determinants between the two. In most cases, a stronger reaction was observed between subunit antibody and subunit than between subunit antibody and receptor. Antibodies to T. californica AcChR did not react with any subunits and did not react with T. nobiliana or N. brasiliensis AcChR. Antibodies

TABLE III

Results of double immunodiffusion assays of antibodies to T. californica AcChR and AcChR subunits reacting with receptor and subunits from four species of electric ray[a]

		Antibodies Against:						
		1 Subunit	2 Subunit	3 Subunit	4 Subunit	AcChR	AcChR-[SDS]$_1$ or AcChR-[SDS]$_n$	Ig[e] Fraction
	Subunit[b]							
T. californica[c]	1	+++	-	-	-	-	+++	-
	2	-	+++	-	-	-	+	-
	3	-	-	+++	-	-	+	-
	4	-	-	-	+++	-	+	-
	AcChR	++	++	++	++	++	+++	-
T. marmorata[c]	1	+++	-	-	-	-		-
	2	-	++	-	-	-		-
	3	-	-	++	-	-		-
	4	-	-	-	++	-		-
	AcChR	++	++	++	+	+	+	-
T. nobiliana[d]	1	+	-	-	-	-		-
	2	-	+	-	-	-		-
	3,4	-	-	+	+	-		-
	AcChR	++	++	++	+	-	+	-
N. brasiliensis[d]	1	+++	-	-	-	-		-
	2	-	+	-	-	-		-
	3	-	-	++	+	-		-
	4	-	-	+	++	-		-
	AcChR	+	+	+	+	-	+	-
AcChR + α-BuTx		+	+	+	+	+	+	
AcChR + SDS		+	+	+	+	+	+	
α-BuTx		-	-	-	-	-		
SDS		-	-	-	-	-	-	

[a] +++ indicates a strong reaction within 24 hr; ++ indicates a weaker reaction within 48 hr; + indicates a weak reaction taking longer than 48 hr.

[b] Molecular weights for subunits 1-4 respectively are: 41,000, 51,000, 60,000, 64,000 daltons for T. californica; 41,000, 51,000, 59,000, 64,000 daltons for T. marmorata; 40,000, 48,000, 60,000, 64,000 daltons for T. nobilana and 43,000, 52,000, 59,000, 64,000 daltons for N. brasiliensis.

[c] T. californica and T. marmorata receptor subunits were prepared on discontinuous SDS polyacrylamide gels.

[d] T. nobiliana and N. brasiliensis receptor subunits were prepared on continuous SDS polyacrylamide gels.

[e] Ig fraction isolated from a non-immunized control rabbit.

TABLE IV

Immunization Schedule

		Injections			Bleedings			
Rabbit #	Antigen	mg	date	notebook	ml	date	notebook	Comments
1	41,000 M_r subunit	.43	7-16-75	TCIII-71	10	9-5-75	TCIII-119	
		.40	8-4-75	TCIII-92	40	11-8-75	TCIII-160	
		.34	8-22-75	TCIII-112	10	4-26-76	TCIII-247	
		.28	10-24-75	TCIII-138	40	4-30-76	TCIII-248	
		.25	4-26-76	TCIII-244	40	7-27-76	TCIV-37	
2	51,000 M_r subunit	.27	7-16-75	TCIII-71	10	9-5-75	TCIII-119	
		.32	8-4-75	TCIII-92	40	11-8-75	TCIII-160	
		.15	8-22-75	TCIII-112	40	4-30-76	TCIII-248	
		.52	10-24-75	TCIII-138	40	7-27-76	TCIV-37	
		.30	4-26-76	TCIII-244				
3	60,000 M_r subunit	.24	7-16-75	TCIII-71	10	9-5-75	TCIII-119	
		.22	8-4-75	TCIII-92	40	11-8-75	TCIII-160	
		.13	8-22-75	TCIII-112	10	4-26-76	TCIII-247	
		.37	10-24-75	TCIII-138	40	4-30-76	TCIII-248	
		.04	4-26-76	TCIII-244	18	6-23-76	TCIII-280	
4	64,000 M_r subunit	.15	7-16-75	TCIII-71	10	9-5-75	TCIII-119	
		.15	8-4-75	TCIII-92	40	11-8-75	TCIII-160	
		.13	8-22-75	TCIII-112	10	4-26-76	TCIII-247	
		.27	10-24-75	TCIII-138	40	4-30-76	TCIII-248	
		.17	4-26-76	TCIII-244	40	7-27-76	TCIV-37	
5	AcChR	.39	7-22-75	TCIII-71	110	8-13-75	TCIII-105	paralysis, exsanguinated
6	AcChR + α-BuTx	.42	7-22-75	TCIII-71	80	8-13-75	TCIII-105	paralysis, exsanguinated
7	AcChR + SDS	.32	7-22-75	TCIII-71	10	9-5-75	TCIII-119	
		.22	8-5-75	TCIII-92	40	11-13-75	TCIII-166	
		.53	8-29-75	TCIII-112	80	4-26-76	TCIII-247	sick for previous 3 weeks, exsanguinated
		.43	10-28-75	TCIII-138				

TABLE IV (continued)

8	AcChR	.4	7-25-75	TCIII-71	10	9-5-75	TCIII-119	
		.59	8-5-75	TCIII-92	40	11-13-75	TCIII-166	
		.56	8-29-75	TCIII-112	40	4-30-76	TCIII-248	
		.48	10-28-75	TCIII-138	35	1-18-77	TCV-94	
		.54	4-26-76	TCIII-244	35	1-27-77	TCV-105	1-30-77 died unobserved but looked healthy previous 2 days
		.5	1-6-77	TCV-79				
9	AcChR + α-BuTx	.4	7-25-75	TCIII-71	10	9-5-75	TCIII-119	
		.56	8-5-75	TCIII-92	-	11-2-75		died unobserved
		.43	8-29-75	TCIII-112				
		.48	10-28-75	TCIII-138				
10	AcChR - SDS	.5	7-16-75	TCIII-71	10	9-5-75	TCIII-119	
		.39	8-5-75	TCIII-92	40	11-13-75	TCIII-166	
		.53	8-29-75	TCIII-112	40	5-3-76	TCIII-251	
		.48	10-28-75	TCIII-138				
		.7	4-29-76	TCIII-244				
11	41,000 M_r subunit	.28	6-29-76	TCIII-284	15	7-22-76	TCIV-26	
		.15	7-14-76	TCIV-17				
12	51,000 M_r subunit	.3	Same as rabbit 11		40	7-22-76	TCIV-26	
		.15						
13	60,000 M_r subunit	.3	Same as rabbit 11		40	7-22-76	TCIV-26	
		.15						
14	64,000 M_r subunit	.3	Same as rabbit 11		40	7-22-76	TCIV-26	
		.15						

made against AcChR-$[SDS]_n$ or AcChR-$[SDS]_1$ reacted with whole AcChR from all four ray species. These antibodies also reacted with T. californica subunits. They reacted strongly with subunit 1, moderately well with subunits 2 and 3 and weakly with subunit 4. Illustrative Ouchterlony plates are shown in Figure 4. In (a-d), examples of the specificity of the subunit antibodies are shown. In (e), receptor is shown to react with AcChR antibodies and each of the four subunit antibodies. A reaction of partial identity can be seen with a spur-like projection extending toward well 1 from well 6. This is to be expected since the center well contained whole receptor, well 6 contained whole receptor antibodies and well 1 contained only antibodies to subunit 1. None of the acetylcholine receptors or subunits reacted with the immunoglobulin fraction isolated from a non-immunized control rabbit. In addition, antibodies made against the AcChR or subunits did not react with α-BuTx or SDS but did react with AcChR-α-BuTx complex and with AcChR-$[SDS]_n$. It should be noted that if substantial amounts of SDS were present in subunit preparations, false precipitation lines were formed (Figure 5). The precipitation lines usually disappeared in 30-40 hours and did not stain with Aniline Blue Black.

DISCUSSION

Rabbits injected with whole AcChR exhibited varying degrees of susceptibility to paralysis. In all cases, the rabbit had produced antibodies against the AcChR as shown by double immunodiffusion assays. Similar types of variability have previously been observed in rabbits, rats and guinea pigs (26-30). In some cases, animals developed paralysis after a single injection, others required 2 or 3 injections and some showed no clinical effects after 4 or 5 injections.

Figure 4. Ouchterlony double immunodiffusion in 1% agar. Immunoprecipitates in (a), (d), and (e) were stained in 0.1% Aniline Blue Black (subsequent studies determined Coomassie Brilliant Blue was a superior stain). Immunoprecipitates in (b) and (c) were visualized with a diffuse light source. Antibodies were to T. californica AcChR and AcChR subunits. In (a), the center well contained antibodies to subunit 1, wells 1-4 contained T. californica subunits 1-4, respectively, and wells 5 and 6 contained T. californica AcChR. In (b), the center well contained antibodies to subunit 2, well 1 contained subunit 1 from T. nobiliana, well 2 contained subunits 3 and 4 from T. nobiliana, wells 3, 4 and 6 contained T. californica AcChR and well 5 contained subunit 2 from T. californica. In (c), the center well contained antibodies to subunit 3, well 1 contained subunit 1 from T. nobiliana, well 2 contained subunit 2 from T. nobiliana, wells 3, 4 and 6 contained T. californica AcChR, and well 5 contained subunit 3 from T. californica. In (d), the center well contained antibodies to subunit 4 and wells 1-6 were the same as in (a). In (e), the center well contained T. californica AcChR, wells 1-4 contained antibodies to subunits 1-4, respectively, well 5 contained the immunoglobulin fraction from a control rabbit and well 6 contained antibodies to whole AcChR.

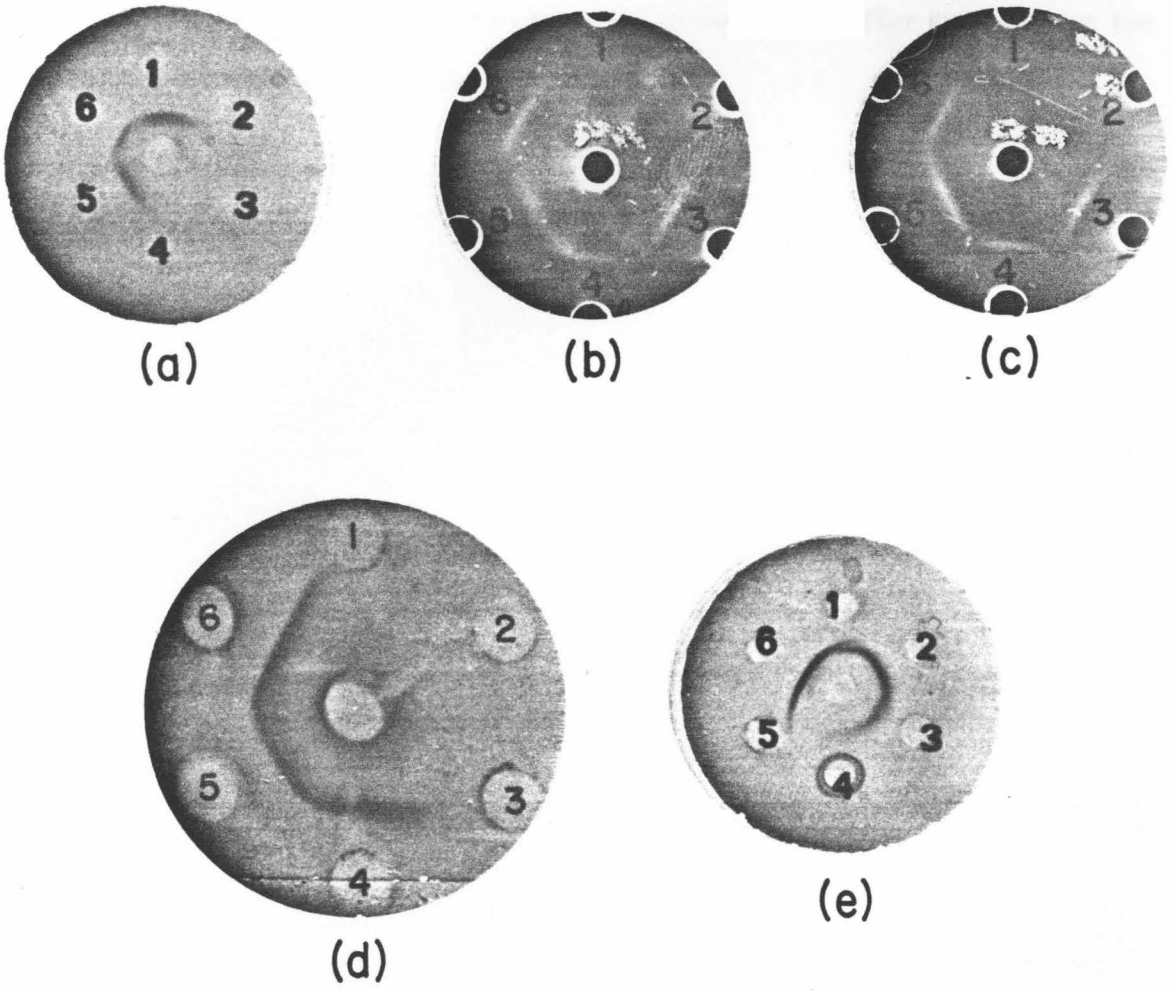
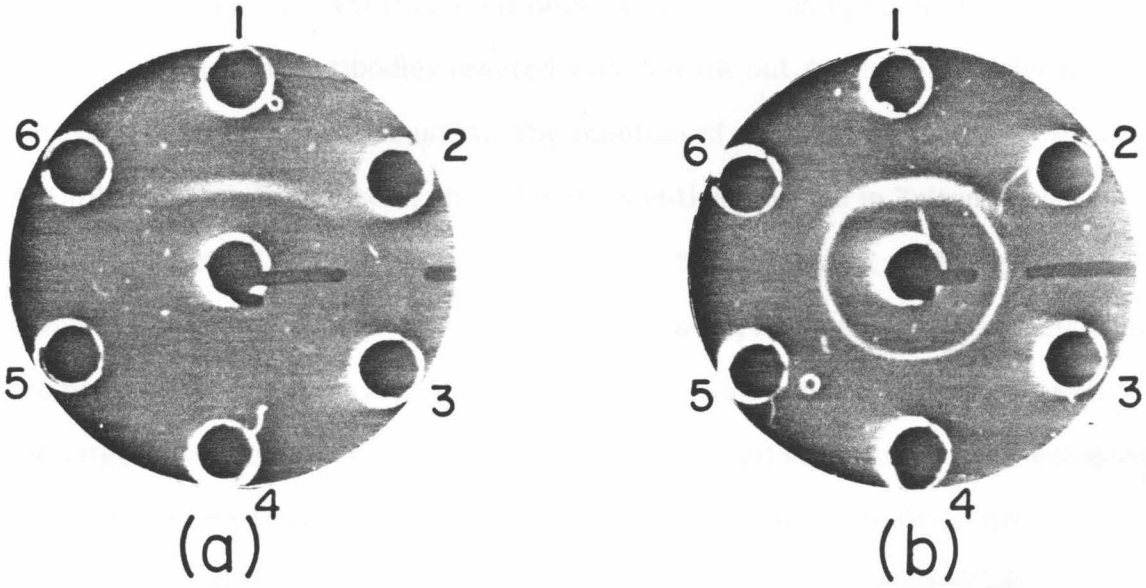

1
2
3
4
5
6
(a)
(b)
(c)
(d)
(e)

Figure 5. Ouchterlony double immunodiffusion in 1% agar. Immunoprecipitates were visualized with a diffuse light source. The center wells contained antibodies to subunit 1, wells 1-4 contained subunits 1-4, respectively, well 5 contained subunit 2 and well 6 contained subunit 3. In (a), the antigens were in a buffer containing 0.1% Triton X-100. In (b), the antigens were in the same buffer as in (a), but the buffer also contained 0.1% SDS.


1
2
3
4
5
6
(a)
1
2
3
4
5
6
(b)

The antisera used in this study appeared to be specific by immunodiffusion assays. This specificity suggests that the lower molecular weight polypeptide chains seen in AcChR subunit gel patterns (Figure 3) are not degradation products of the larger chains. The specific reactions seen between T. californica subunit antibodies and T. marmorata, T. nobiliana and N. brasiliensis subunits demonstrate the presence of common antigenic determinants on similar subunits from these four different species of electric ray. These molecules are similar not only antigenically but also by several of their physical and chemical properties (18).

All subunit antibodies reacted with AcChR but AcChR antibodies did not react with any of the subunits. The reaction of each subunit antibody with AcChR indicates that none of the subunits is entirely buried in Triton X-100-solubilized AcChR. This is in accord with data from chemical analysis of the subunits (5). In addition to determinants on isolated, SDS denatured subunits which are not present on intact receptor, there are probably several determinants on the intact receptor which are not present on individual subunits. Unique receptor determinants might result from subunit-subunit interactions or from differences in conformation of subunits between an isolated, denatured state and as part of the receptor complex. In the intact receptor, the contribution of exposed determinants from any one subunit may be very small such that this population of antibodies would not be seen by the immunodiffusion assay and would result in the observation that AcChR antibodies do not react with individual subunits. Antibodies to AcChR-$[SDS]_1$ or AcChR-$[SDS]_n$ showed a positive response to intact AcChR and also to each subunit although to varying degrees. The strongest responses were between anti-AcChR-[SDS] antibodies and subunit 1, which could be due to the greater number of these subunits in intact AcChR compared with subunits 2, 3 and 4.

In this study, antisera produced against isolated Torpedo californica AcChR subunits were tested for cross-reactivity with AcChR subunits isolated from four species of electric ray. Since the publication of these data, more immunological data has appeared on the subject (31) and peptide maps of AcChR subunits have been reported (32). The results from peptide maps agree with the immunological evidence that the lower molecular weight polypeptide chains are not derived from the higher molecular weight chains. The immunological evidence presented in reference (31) described the inhibition by polypeptide chains of the reaction of antisera to these chains with native T. californica AcChR. Those data show ∿5-7% contamination of 3 of the 4 subunits. Using a technique much more sensitive than Ouchterlony double immunodiffusion in agar, data will be presented in Chapter II showing similar contamination of some of the anti-AcChR subunit antisera. In Chapter III, the ability of these antisera and others to inhibit α-BuTx binding to AcChR will be described.

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Chapter II

Is Experimental Autoimmune Myasthenia Gravis Induced Only by Acetylcholine Receptors?

ABBREVIATIONS

AcChR: acetylcholine receptor

EAMG: experimental autoimmune myasthenia gravis

SDS: sodium dodecyl sulfate

α-BuTx: alpha bungarotoxin

BSA: bovine serum albumin

DEAE: diethylaminoethyl

SACI: Staphylococcus aureus Cowan I strain

Tris: Tris(hydroxymethyl)aminomethane

M_r: molecular weight

MF: membrane fragments

MF + T: membrane fragments + 1% Triton X-100

MFTS: Triton X-100 solubilized membrane fragments; supernatant from 100,000 x g centrifugation for 1 hour

MFTS-AcChR: MFTS depleted of AcChR

INTRODUCTION

Patrick and Lindstrom (1) first demonstrated that purified acetylcholine receptor (AcChR) from Torpedo californica could induce a disease similar to the human autoimmune disease, myasthenia gravis, when injected into rabbits. It has subsequently been shown that the disease, experimental autoimmune myasthenia gravis (EAMG), could be induced in a wide variety of animals using AcChR purified from a variety of electric organ or muscle sources. The receptor used in most studies for the induction of EAMG is usually highly purified although the disease can be induced with crude receptor preparations (T. Claudio, unpublished observations) or Triton extracts of crude preparations (2). Disagreement continues as to the number and apparent molecular weights of the receptor subunits as judged by their migration on sodium dodecyl sulfate (SDS) gel electrophoresis (for a compilation of data on subunit molecular weights, see references 3 and 4). Reports of the number of subunits vary from one to five with apparent molecular weights (M_r) ranging from 40,000 to 140,000. In our laboratory, we regularly see four bands migrating with apparent M_r of 41,000, 51,000, 60,000 and 64,000. Even the most highly purified AcChR preparations, however, can show minor bands at positions other than those of the four subunits when gels are heavily loaded with receptor. Minor bands not considered to be subunits can also be seen on gels heavily loaded with receptor purified from different electric organ or muscle sources (5-14). More references to AcChR preparations can be found in the Introduction to Chapter I. However, AcChR was not applied to many of the gels in sufficient quantities to be able to see minor contaminants.

At the time these studies were begun, it had thus far not been possible to induce EAMG with AcChR denatured in SDS or with isolated subunits (8,14,15).

One report has subsequently appeared in the literature (13) demonstrating that EAMG could be induced with very large amounts of any one of the four receptor subunits. As was pointed out in that paper, however, three of the four subunits were contaminated with small quantities of other subunits. The possibility remained, therefore, that minor contaminants, either those seen on heavily loaded gels or comigrating with subunits, could be responsible for inducing EAMG. If the disease is induced by antibodies binding the AcChR which initiates an indiscriminate destruction of postsynaptic membranes, then it may be possible for any protein located in this region to induce EAMG under similar circumstances. We therefore set out to determine if any other components present in Torpedo membrane fragments could also induce EAMG.

In this study, we have prepared Torpedo californica membrane fragments containing AcChR, solubilized them in Triton X-100, then removed the AcChR by passing the solution over an α-bungarotoxin (α-BuTx) affinity resin. These and other antigens were injected into female Lewis rats in an attempt to determine which components present in Torpedo membrane fragments could induce EAMG.

MATERIALS AND METHODS

Purification of α-bungarotoxin

Bungarus multicinctus (Formosan banded krait) venom was purchased from Miami Serpentarium Labs. α-BuTx was purified from venom by the procedure of Clark et al. (16) with modifications described by Blanchard et al. (17). Briefly, 500 mg of venom were dissolved in 15 ml of 0.1 M ammonium acetate buffer, pH 5.8, and applied to a 2.5 x 48.8 cm Sephadex CM-25 column equilibrated in the same buffer. The various components of the venom were fractionated using

a linear gradient of 600 ml of 0.1 M and 0.25 M ammonium acetate. The α-BuTx peak was pooled (74 ml), diluted with water (100 ml) to reduce the ionic strength and rechromatographed using the same linear gradient except with 700 ml of each of the two buffers.

Coupling of α-BuTx to Sepharose 4B-200

Sepharose 4B-200 was purchased from Pharmacia Fine Chemicals. α-BuTx was coupled to the resin with cyanogen bromide by the method of March et al. (20). 100 g of Sepharose 4B-200 were washed with ∿3 liters of cold distilled water, then 200 ml of 2 M sodium carbonate. A slurry of 100 g of resin plus 100 ml of 2 M sodium carbonate was stirred, then 9.2 g of cyanogen bromide in 20 ml of acetonitrile were added, stirred vigorously for 1.5 minutes, poured onto a sintered glass funnel and washed with 800 ml of each of the following: a) 0.1 M sodium bicarbonate, pH 9.5; b) distilled water; and c) 0.2 M sodium bicarbonate, pH 9.5. 50 mg of α-BuTx plus trace [^{125}I]α-BuTx plus 100 ml of 0.2 M sodium bicarbonate, pH 9.5, were added to 100 g of activated resin. Coupling took place at 4°C for 24 hours with constant shaking. The resin was filtered (filtrate was saved to determine the coupling efficiency) and washed with 3 cycles of 1.5 liters of each of the following: a) 0.1 M sodium acetate, 0.5 M sodium chloride, pH 4; b) 0.1 M sodium bicarbonate, 0.5 M sodium chloride, pH 9.5, and finally equilibrated in 10 mM sodium phosphate, 0.1% Triton X-100, 50 mM sodium chloride, pH 7.4.

To determine the amount of active α-BuTx coupled to the resin, fixed amounts of resin were incubated with increasing amounts of solubilized membrane fragments containing AcChR. Bound AcChR was removed from the solution by centrifugation and aliquots of the supernatant were assayed with [^{125}I]α-BuTx. To correct for nonspecific AcChR binding to the resin or to coupled α-BuTx or

nonspecific pelleting during centrifugation, similar incubations with resin were set up with AcChR saturated with unlabeled α-BuTx plus a trace of [^{125}I]α-BuTx.

Preparation of solubilized membrane fragments containing and depleted of AcChR

Torpedo californica was obtained locally. Crude Triton X-100 solubilized membrane fragments (MFTS) were prepared according to the procedure of Vandlen et al. (21) up to the point of application of the material to the quaternary ammonium affinity resin. MFTS depleted of AcChR (MFTS-AcChR) were prepared by passing MFTS four times over the α-BuTx resin described above. 60 gm of toxin resin were rocked with MFTS for 12 hours at 4°C such that all the receptor present in MFTS should be bound by the resin. MFTS-AcChR were filtered away from the resin on a Buchner funnel. Toxin resin was then washed extensively with 10 mM Na phosphate, 50 mM NaCl, 0.1% Triton X-100, pH 7.4 (wash buffer) followed by 1 M NaCl, 8 M urea, then reequilibrated in wash buffer before MFTS-AcChR were incubated again with the resin. 1 M NaCl did not dissociate AcChR-toxin complexes but $\geq$6 M urea did.

Preparation of purified AcChR

Purified acetylcholine receptor was prepared as described in reference 21. The concentration was 0.06 mg/ml protein as determined by the method of Lowry et al. (22).

Preparation of [^{125}I]α-BuTx

Carrier free sodium iodide-[^{125}I] at 100 mCi/ml was obtained from Amersham Searle. Biogel P-2, 100-200 mesh was obtained from BioRad Laboratories. Diethylaminoethyl (DE52) cellulose was obtained from Whatman Biochemical Ltd. A modification of the method described by Greenwood et al. (23) was used

to radiolabel purified α-BuTx. To 100 μg of α-BuTx (at a concentration of 0.8 μg/ml in 0.5 M sodium phosphate buffer, pH 7.0) was added 1 mCi ^{125}I and 10 μg of 1 mg/ml Chloramine T in the same buffer. After thorough mixing and a 90 second incubation, 100 μg of 1 mg/ml tyrosine in 0.5 M Na phosphate buffer were added, mixed, and incubated 90 seconds. 6 mg of 1% bovine serum albumin (BSA) in 50 mM Na phosphate buffer, pH 7.4, were added and the sample (∿800 μl) applied to a 30 ml P-2 column equilibrated in and eluted with 1% BSA in 50 mM Na phosphate buffer. The pooled material was then passed over a 2 ml DE52 column equilibrated in 50 mM Na phosphate buffer in order to remove any material which might bind nonspecifically to DEAE filter discs in the filter disc assay described under "Quantitation of AcChR in rat muscle." BSA and sodium azide were added to the material passing through the column to final concentrations of 0.1% and 0.02% respectively. Aliquots of this material were frozen at -80°C. The specific activities of [^{125}I]α-BuTx preparations used in these studies were determined as described in reference 17 and varied from 140-210 Ci/mmole. The purity of iodinated products was tested by running the samples on a discontinuous 8-18% exponential gradient SDS gel. The autoradiographs of the dried gels showed a single [^{125}I] component which comigrated with nonradiolabeled α-BuTx.

Immunization schedule

Female Lewis rats aged 6 months weighing between 220 and 280 grams, purchased from Simonson Laboratories Inc., were used in these studies. Antigens were emulsified in an equal volume of Freund's complete adjuvant and administered by subcutaneous injections along the lower back at two week intervals. Animals received a total of 5 or 6 injections and were sacrificed at 78 and 85 days after the initial injection. The antigens injected were as follows:

MFTS: 100 µg protein containing 6.5 µg AcChR. 5 injections total.

MFTS + SDS: same as MFTS but containing 2% SDS. 5 injections total.

MFTS - AcChR: 100 µg protein possibly containing 16.4 ng AcChR. 5 injections total.

AcChR: 10 µg AcChR. 6 injections total.

AcChR + SDS: same as AcChR but containing 2% SDS. 6 injections total.

Control: 10 mM Na phosphate, 50 mM NaCl, 0.1% Triton X-100, pH 7.4. 5 injections total.

Extraction of AcChR from rat muscle

The extraction of AcChR from rat muscle was essentially the same as that described by Lindstrom et al. (24). Sets of one rat from each group were homogenized and assayed before the next set was homogenized. Rats were anesthetized with ether, exsanguinated, then the head, tail, skin and viscera were removed. The carcasses were stored frozen at -80°C until homogenization. All homogenizations and centrifugations were done in the centrifuge tubes to minimize losses due to solution transfers. The buffer used in homogenizations was 10 mM sodium phosphate, 0.1 M NaCl, pH 7.4. Carcasses were homogenized at 4°C with 150 ml of buffer for 30 seconds full speed with a Brinkman Instruments Type PT10/35 Polytron homogenizer. 100 ml of buffer were added and homogenized another 30 seconds. After centrifugation in a GS3 rotor, 9000 rpm for 20 minutes, homogenization and centrifugation were repeated on the pellets. The second pellets were homogenized with 150 ml buffer plus Triton X-100 to a final concentration of 2% for 2 x 30 seconds, full speed, with the Polytron homogenizer. The solutions

were stirred for 1 hour at 4°C then spun 10^5 x g for 1 hour. Floating lipids were removed from supernatants by filtration through glass wool.

Quantitation of AcChR in rat muscle

Whatman DEAE cellulose filter discs (DE81), 2.4 cm diameter, were obtained through VWR Scientific Co. The assay used to quantitate rat AcChR was essentially that described by Schmidt and Raftery (25) with the following modifications: rat MFTS were incubated with excess [^{125}I]α-BuTx for 3 hours, only 50 µl of a solution of rat MFTS plus [^{125}I]α-BuTx could be applied per filter disc, and discs were washed for 30 minutes with three buffer changes in order to lower the nonspecific binding of [^{125}I]α-BuTx. It should be noted that with rat MFTS prepared as described above, the binding of rat AcChR to filter discs was not linear beyond 40 µl of undiluted MFTS. Although the buffers used in the disc assay for rat (10 mM sodium phosphate, 0.1 M NaCl, 2% Triton X-100, pH 7.4) and T. californica (10 mM sodium phosphate, 50 mM NaCl, 0.1% Triton X-100, pH 7.4) were different, both worked equally well in the assay. Filter discs were counted in a Beckman 4000 Gamma counter. The concentration of AcChR per rat is expressed as pmoles of [^{125}I]α-BuTx binding sites per gram carcass weight.

Antibody precipitation assays

Rabbit anti-rat IgG serum was purchased from Cappel Laboratories, Inc. Goat anti-rat IgG was purchased from Antibodies Incorporated. Staphylococcus aureus Cowan I strain (SACI) was prepared as described in reference 26 and was a generous gift of Deborah Dison Hall. Antibody titers against AcChR in rat MFTS or purified Torpedo californica AcChR were determined as described in reference

27 when a second antibody alone was used (either goat or rabbit anti-rat IgG). Otherwise, the titers were determined as follows: assays were done in 1.5 ml polyethylene micro test tubes from Cole Scientific, Inc. Varying amounts of [^{125}I]α-BuTx-rat AcChR complex were incubated with 1 µl of serum for 10 hours at 4°C. 10 µl of rabbit anti-rat IgG serum were added and incubated 20-24 hours at 4°C or 5 hours at 25°C. 400 µl of 5% SACI were added and incubated for 30 minutes at 25°C. The immune precipitates were collected by 5-minute centrifugations in an Eppendorf 3200 Centrifuge and pellets washed once by resuspension in 10 mM Na phosphate, 50 mM NaCl, 0.1% Triton X-100, pH 7.4, followed by a second centrifugation. The pellets were counted in a gamma counter by cutting off the bottoms of the centrifuge tubes. Determination of antibody titers against Torpedo AcChR were the same as with rat AcChR except serum was diluted 20-fold. Antibody titers are given as nmoles of [^{125}I]α-BuTx binding sites precipitated by one liter of serum.

The amount of AcChR isolated in rat MFTS with antibody attached was quantitated by precipitating receptor-[^{125}I]α-BuTx complexes with a second antibody alone or a second antibody followed by SACI. SACI was used because precipitates were larger and more stable than second antibody precipitates alone. Although the use of SACI gave higher background values than second antibodies alone, the values obtained in the two methods were identical once respective backgrounds were subtracted. SACI did not precipitate rat IgG, therefore rabbit anti-rat IgG was added before adding SACI. SACI also did not precipitate the goat IgG used in these experiments.

Staining of gels with antibody

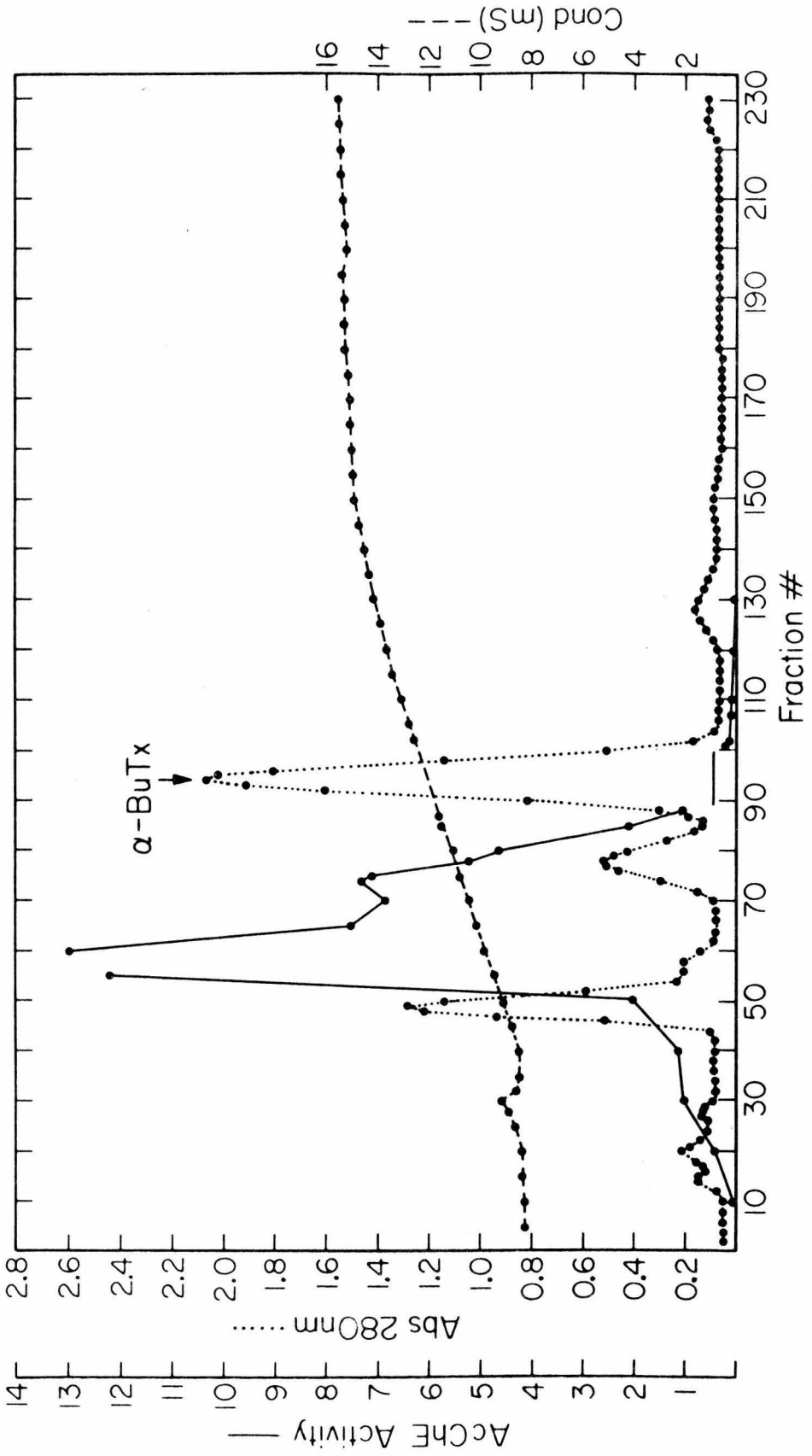
A modification of the procedure described by Burridge (28) was used to analyze the specificity of antisera directed against purified Torpedo AcChR. Discontinuous gels (19) with an exponential gradient of 8-14% acrylamide in the separating gel were used. After electrophoresis, gels were fixed by swirling in 700 ml of 46% methanol, 8% acetic acid for 2 hours then rinsed about 20 hours with 4 changes of buffer A (0.05 M Tris, 0.15 M NaCl, 0.1% sodium azide, pH 7.4) until the pH was between 7.2 and 7.4. Gels were next rocked in buffer A containing 0.25% gelatin about 4 hours before slicing them and placing each section of MFTS, MFTS-AcChR and AcChR in a 16 x 6 x 3.5 cm plastic box. 75 µl of antiserum in 15 ml of buffer A plus gelatin were gently rocked for 16-20 hours, washed 48 hours with 6 changes of buffer A, followed by about 4 hours with buffer A plus gelatin. If rat serum was used, then 50 µl of rabbit anti-rat IgG serum were added then incubated and washed as with the first antibody. Next, 3-5 x 10^6 cpm of [^{125}I]protein A ([^{125}I]protein A had a specific activity of 12 µCi per µg and was a generous gift of Charles M. Rice) were added in 15 ml of buffer A plus gelatin and gently rocked 10-12 hours, followed by 48 hours of washing with 6 changes of buffer A. Gels were stained and destained in 25% methanol, 10% acetic acid $\pm$ 0.05% Coomassie Brilliant Blue, rocked 2-4 hours in 10% acetic acid, 3% glycerol, dried and autoradiographed.

RESULTS

Purification of α-BuTx and coupling to Sepharose 4B-200

Figure 1 shows the fractionation of crude Bungarus multicinctus venom on a Sephadex CM-25 column. Acetylcholinesterase activity trailed off into the α-BuTx peak but could be separated from it with rechromatography. Upon

Figure 1. Profile of Bungarus multicinctus venom fractionation on a Sephadex CM-25 column. Acetylcholinesterase activity is plotted as the change in absorbance at 412 nm per minute per 25 µl of sample.


AcChE Activity ——
Abs 280nm .....
Cond (mS) – – –
Fraction #
α-BuTx

rechromatography, the α-BuTx peak migrated as a single component and was judged pure by this and the following criteria: no acetylcholinesterase activity could be detected using the assay method of Ellman et al. (18), and the purified toxin migrated as a single component on an 8-18% exponential SDS polyacrylamide gel using the discontinuous gel system of Laemmli (19). Rechromatography of the α-BuTx peak is probably unnecessary if the gradient used to fractionate the components consists of 700 ml of each of the two buffers and if acetylcholinesterase activity is monitored.

The coupling efficiency of α-BuTx to Sepharose 4B-200 was 96%, determined by measuring the trace [^{125}I]α-BuTx added to unlabeled α-BuTx at the time of coupling. 15% of this could bind AcChR yielding 9 nmoles of active α-BuTx coupled per gram dry weight of resin.

Effectiveness of removal of AcChR from Torpedo MFTS

After passing MFTS over the α-BuTx affinity resin, the material which did not bind to the resin (MFTS-AcChR) was assayed for its ability to bind [^{125}I]α-BuTx as a first approximation of the amount of AcChR removed. The starting material could bind 21.6 μg [^{125}I]α-BuTx per ml of MFTS. After one passage over the toxin resin, the eluted material contained 1% of the AcChR present in the starting material. After a total of 4 passages over the resin, MFTS-AcChR bound 0.02 μg toxin per ml (0.1% of the AcChR in the starting material). If this were an accurate determination of the total amount of AcChR present and not merely the active AcChR present, then the animals received 16.4 ng AcChR per injection and a total of 82 ng of AcChR before sacrifice. According to the results of Lennon et al. (29), it would appear that more than 140 ng but less than

440 ng of active Electrophorus electricus AcChR was needed to induce EAMG in female Lewis rats.[1]

If in fact only 16.4 ng of AcChR were present in 100 μg of MFTS-AcChR, then the receptor subunit bands should not be visible on an SDS polyacrylamide gel if 100-150 μg of total protein were applied. SDS gels and gel scans of MFTS and MFTS-AcChR are shown in Figures 2 and 3. The scans suggest that all of the subunit with an apparent M_r of 51,000 (band 2) was removed from MFTS-AcChR. However, a band migrating at the 41,000 M_r (band 1) position is still visible. When a gel with 7.5% acrylamide in the separating gel (Figure 3) was run instead of the 8-14% exponential gel (Figure 2), the band migrating at the 41,000 M_r position on the exponential gel resolved into 41,000 and 43,000 M_r components (bands 1a and 1b in Figure 3). It is possible that the AcChR 41,000 M_r subunit is still not totally resolved from other components present in MFTS with this gel system either. The effective removal of the 60,000 and 64,000 M_r species could not be accurately determined by this method. Several bands are present in these regions and none are well resolved from the others as evidenced by the broad band widths and high backgrounds. Because MFTS is treated in no way other than passage over an α-BuTx resin, it seems unlikely that subunits should have dissociated from one another leading to selective removal of some but not all of the subunits. The apparent removal of the 51,000 M_r subunit would imply the same removal of the other subunits. When affinity column purified AcChR was applied to the α-BuTx resin, washed extensively with 10 mM sodium phosphate, 0.1% Triton X-100,

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[1]These values of active AcChR were calculated assuming two α-BuTx binding sites per AcChR and a M_r of 250,000 for the AcChR. In reference 8, however, it appears as though ~1/2 of the AcChR is active giving values of AcChR necessary to induce EAMG of >280 ng but < 880 ng.

Figure 2. 8-14% exponential discontinuous analytical SDS polyacrylamide slab gels and gel scans of MFTS and MFTS-AcChR. MFTS are shown in (a) and MFTS-AcChR are shown in (b). Bands 1-4 correspond to subunits 41,000, 51,000, 60,000 and 64,000 M_r, respectively. Band 5 is a protein migrating with apparent M_r of 90,000. In the figure on the left, band (1a) is the 41,000 M_r subunit and band (1b) is a protein migrating with apparent M_r of 43,000. These two bands were not resolved well when scanned at 550 nm with a slit width of 0.05 mm.

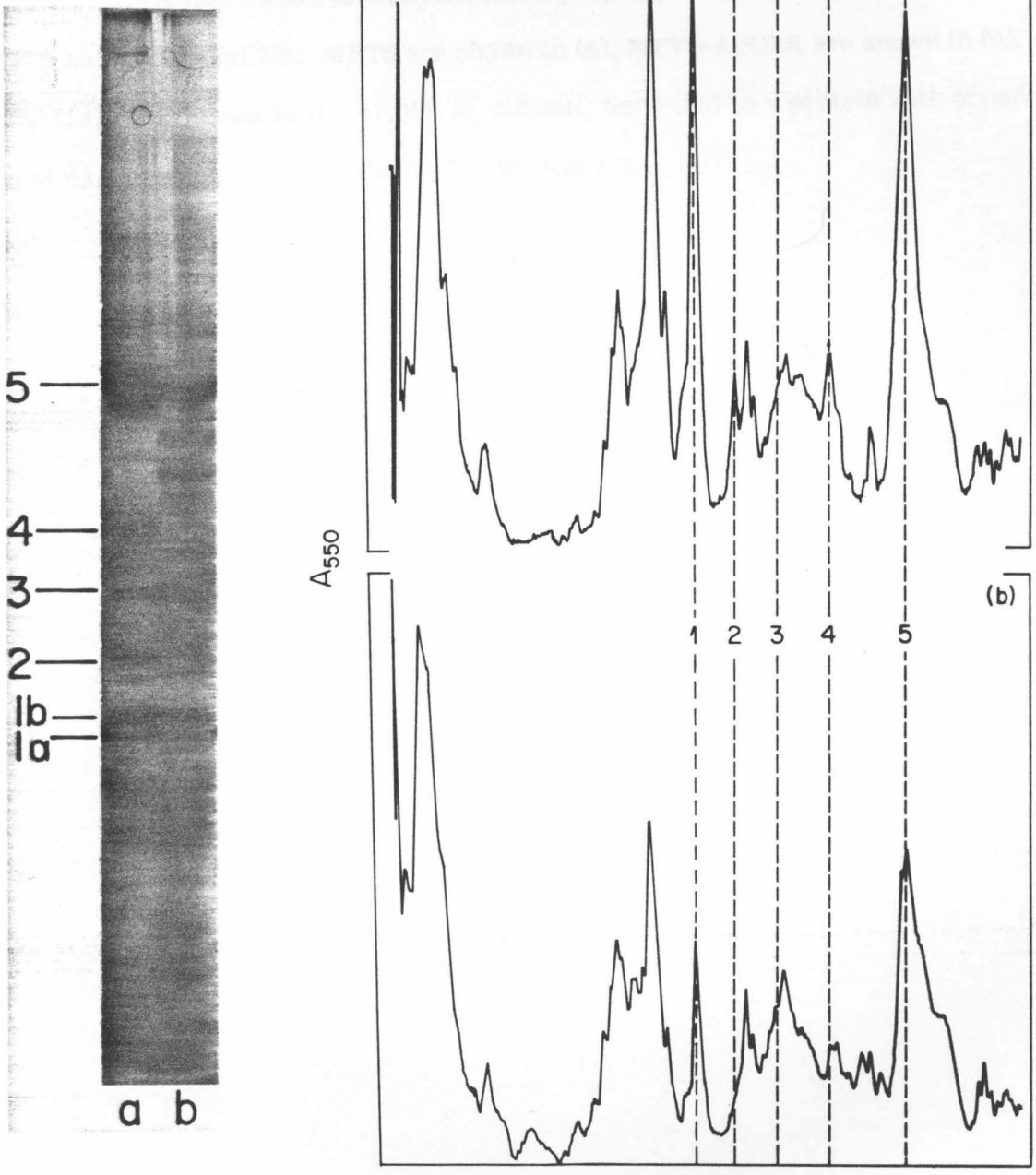
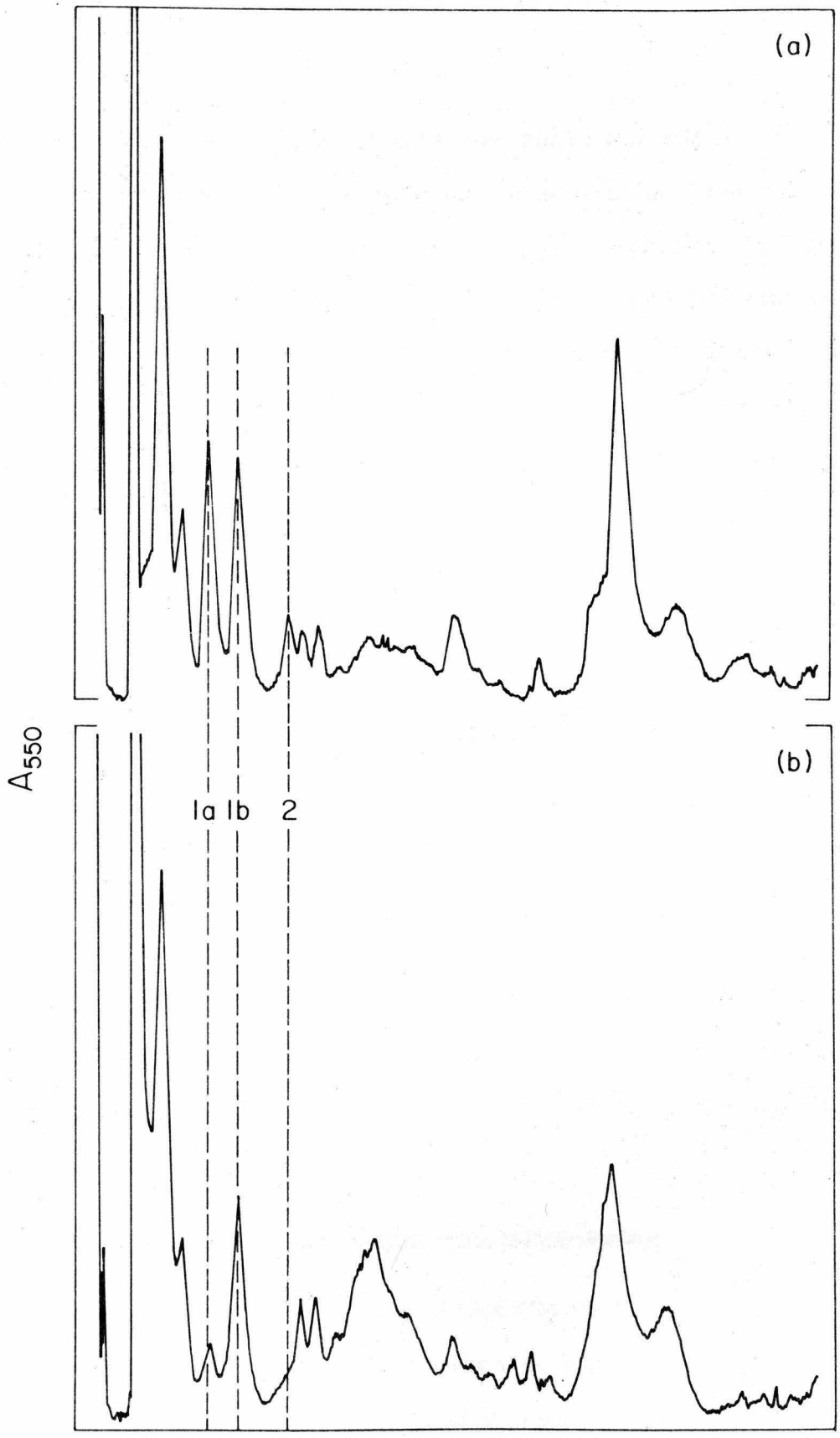

5
4
3
2
Ib
Ia
a b
(a)
(b)
A_{550}
1 2 3 4 5

Figure 3. 7.5% discontinuous analytical SDS polyacrylamide slab gel scans of MFTS and MFTS-AcChR. MFTS are shown in (a), MFTS-AcChR are shown in (b). Band (1a) corresponds to the 41,000 M_r subunit, band (1b) to a protein with apparent M_r of 43,000 and band (2) to the 51,000 M_r subunit.


(a)
(b)
A_{550}
Ia
Ib
2

50 mM NaCl, pH 7.4, followed by 1 M NaCl, then eluted with 8 M urea, all four subunits were present when SDS polyacrylamide gels were run of the eluted material. Quantitation of subunits could not be made because of the presence of material which did not enter the gel, probably caused by the 8 M urea treatment. Even if the gel scans of MFTS-AcChR showed all four subunits had been removed, the sensitivity of Coomassie Brilliant Blue staining of proteins on SDS gels is probably less than the minimum amount of AcChR needed to induce EAMG in rats, especially with multiple injections in Freund's complete adjuvant. In addition, aggregates or degradative fragments of AcChR will not be recognized as such in the presence of the multitude of bands seen with MFTS-AcChR, making absolute determination of the amount of AcChR remaining in these preparations very difficult.

Double immunodiffusion assays were performed in an attempt to determine if antigenic AcChR fragments or aggregates were present in MFTS-AcChR which had not been detected either by [^{125}I]α-BuTx binding or by their staining patterns on SDS polyacrylamide gels. The results showed a weak positive reaction between rabbit anti-AcChR antiserum (a description of this antiserum can be found in reference 14 or Chapter I) and MFTS-AcChR. These results will be discussed in detail under "Double immunodiffusion results" where evidence is presented that purified Torpedo AcChR contains minor contaminants which are also immunogenic.

Effectiveness of removal of only AcChR from MFTS-AcChR

The α-BuTx affinity resin was designed for the dual purposes of completely removing AcChR from MFTS and at the same time not removing any components other than the AcChR. Comparing gels and gel scans of MFTS vs. MFTS-AcChR

(Figures 2 and 3), it can be seen that all components present in MFTS are also present in MFTS-AcChR except possibly some or all of the AcChR subunits. The relative proportions of most of the staining bands have been altered, however, by passage over the toxin resin. Although the staining intensity of several of these components has been reduced (i.e., band 5), none appear to have been removed as effectively as AcChR subunits. In addition, all are represented in greater quantities in MFTS-AcChR than in purified AcChR. In Figure 4, an 8-15% exponential discontinuous SDS gel shows which components were removed from the toxin resin with 1 M NaCl, 2 M urea, and 8 M urea.

Animal observations

At the termination of this experiment, only 3 of 5 rats injected with purified AcChR (rats 46, 47 and 48) and none of the five MFTS rats showed obvious physical signs of EAMG. The affected animals had uncontrollable tremors and would collapse after a brief period of exercise. In addition, these were the only animals which, after gaining weight steadily during the first month of injections, lost weight during the second month. Only rat 46, however, had a significantly lower carcass weight than the average (70% of the average carcass weight).

At an earlier point in these experiments, C57Bl/6 and BALB/c mice purchased from Simonson Laboratories, Inc. were also used. Groups of 5 mice were injected with 50 μg of MFTS, MFTS containing 2% SDS or MFTS-AcChR and a control group over a period of 3 months and received a total of 300 μg of protein. None of these mice demonstrated any observed physical signs of EAMG and all contained their full complement of muscle receptors (Figure 5). Two other groups received a total of 4 μg of AcChR or 4 μg of AcChR + SDS but none of

Figure 4. 8-15% exponential discontinuous analytical SDS polyacrylamide slab gel of material which bound to α-BuTx coupled to Sepharose 4B resin. Different quantities of material were applied to lanes (a) and (b), (c) and (d), and (e)-(h). Lanes (a) and (b) show the material eluted from the resin with a 1 M NaCl wash. Lanes (c) and (d) show the material eluted with 2 M urea. Lanes (e)-(h) show the material eluted with 8 M urea. Lanes (i) and (j) are molecular weight standards (phosphorylase a, 94,000 M_r and two degradation products; bovine serum albumin, 68,000 M_r; pyruvate kinase, 57,000 M_r; heavy chain of rabbit IgG, 50,000 M_r; ovalbumin, 43,000 M_r; D-amino acid oxidase, 37,000 M_r; soybean trypsin inhibitor, 21,000 M_r; β-lactoglobin, 18,400 M_r; α-BuTx, 8000 M_r). Lane (k) is material eluted from the toxin resin with 8 M urea. Lane (l) is detergent solubilized membrane fragments before application to the toxin resin.

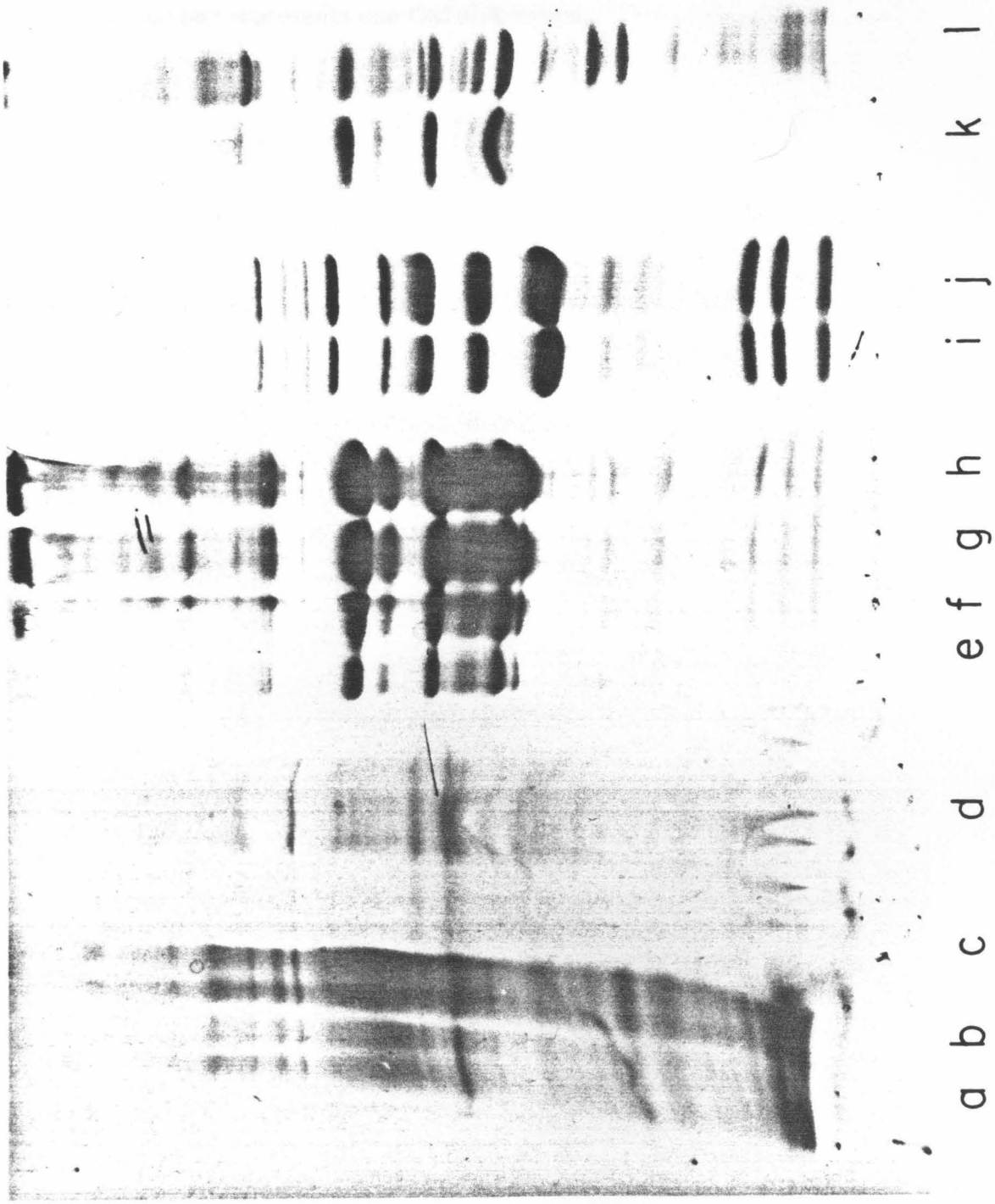
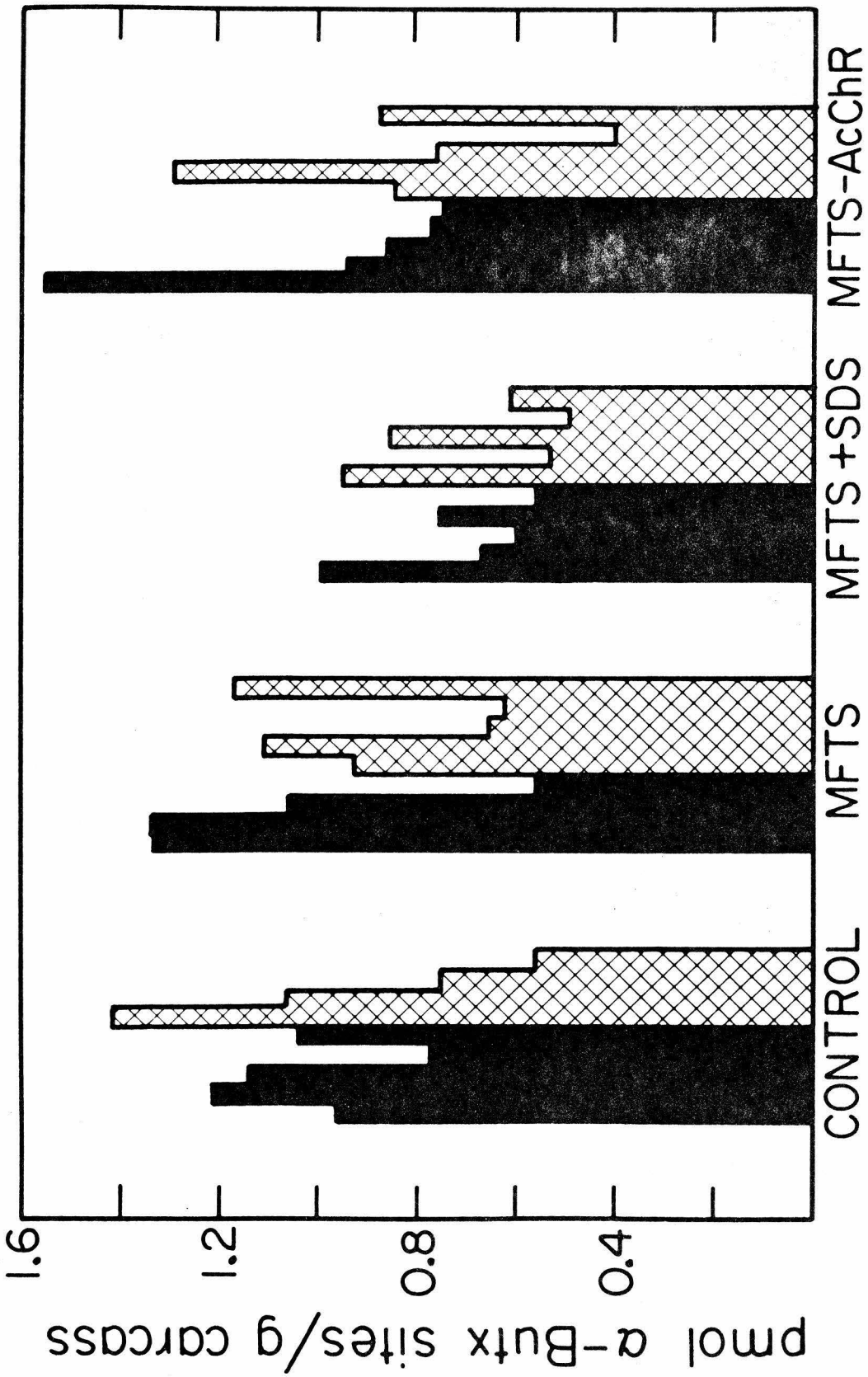

a b c d e f g h i j k l

Figure 5. Quantitation of the number of AcChRs extracted from C57Bl/6 and BALB/c mouse skeletal muscles. Each solid bar represents one BALB/c mouse. Each hatched bar represents one C57Bl/6 mouse.


pmol α-Butx sites/g carcass
1.6
1.2
0.8
0.4
CONTROL
MFTS
MFTS+SDS
MFTS-AcChR

these animals appeared to have any physical signs of EAMG and were terminated after one month.

In addition, two rabbits were injected with membrane fragments (MF), two with membrane fragments solubilized in 1% Triton X-100 (MF + T) and one with MFTS-AcChR. A complete immunization schedule for these rabbits can be found in Table I. Each rabbit injected with MF $\pm$ Triton X-100 received a total of 1 mg of protein containing a total of 424 μg of AcChR. All four animals developed paralysis 5-15 days after their first challenge. The rabbit injected with MFTS-AcChR received a total of 2.8 mg of protein over a one-month period but showed no signs of paralysis during this period or during the next two months.

Quantitation of rat muscle AcChR

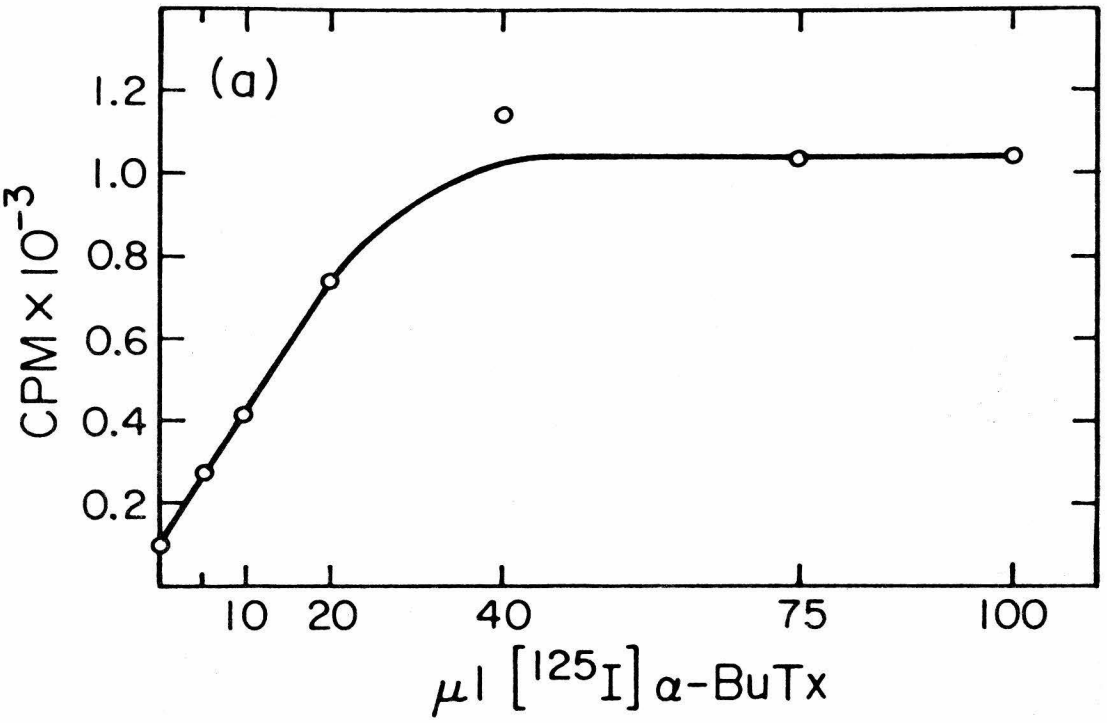
In order to use reduced numbers of muscle AcChRs as the criterion for induction of EAMG, the validity of the filter disc assay in this system and the AcChR extraction procedure had to be shown. The binding of [^{125}I]α-BuTx to rat MFTS was saturable giving rise to an hyperbolic binding curve when fixed amounts of MFTS were incubated with increasing amounts of [^{125}I]α-BuTx (Figure 6a). When all the specific toxin binding sites were bound by unlabeled α-BuTx, no further binding of [^{125}I]α-BuTx was seen with increasing amounts of MFTS-α-BuTx complex above the background value (Figure 6b).

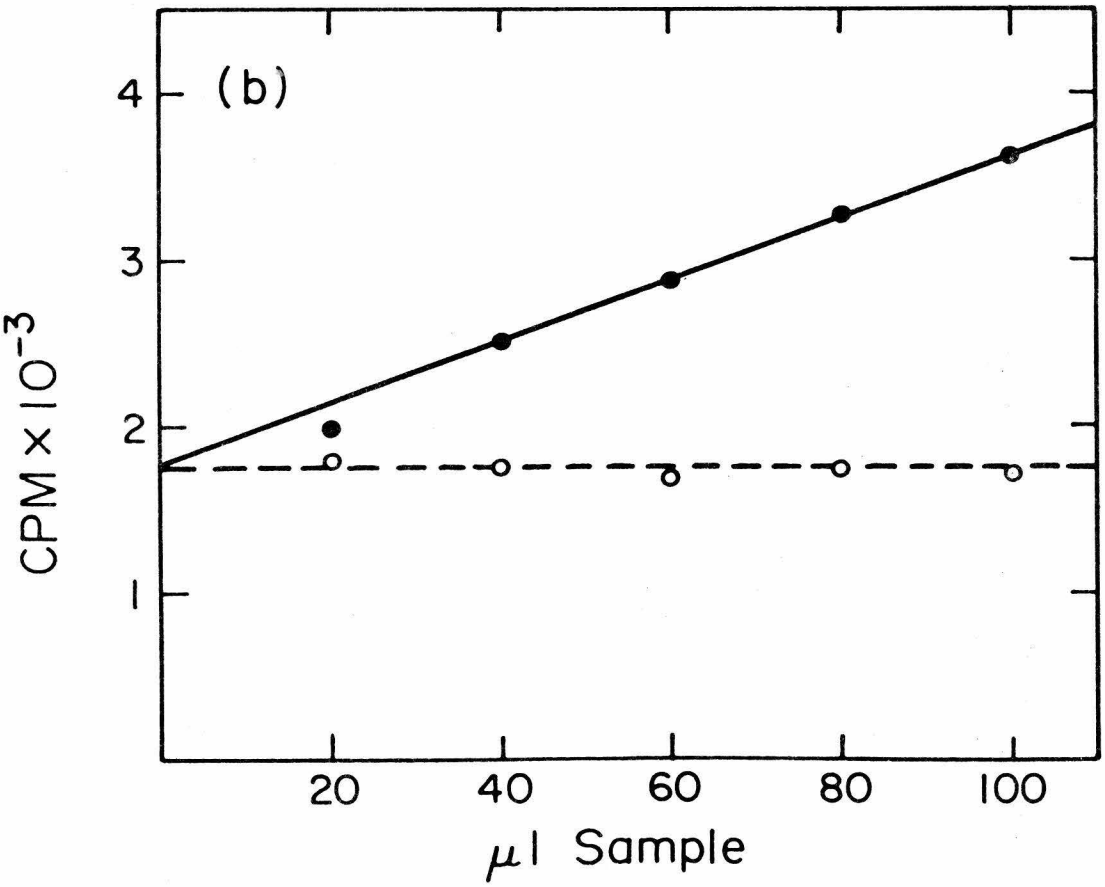
Freezing carcasses at -80°C had no effect on the amount of AcChR which could be extracted from muscle tissue. There was a possibility, however, that receptors crosslinked by antibodies might not be solubilized by Triton X-100 during the extraction procedure and would pellet during the 100,000 x g centrifugation. Animals might appear to have reduced numbers of receptors when only a reduced

TABLE I

Immunization Schedule

		Injections			Bleedings			
Rabbit #	Antigen	mg	date	notebook	ml	date	notebook	Comments
15	MF	.5	5-10-77	TCVI-55	10	5-10-77	TCVI-55	pre-bled
		.5	5-27-77	TCVI-56	100	6-7-77	TCVI-81	paralysis, exsanguinate
16	MF	Same as rabbit 15			10	5-10-77	TCVI-55	pre-bled
					85	6-11-77	TCVI-86	paralysis, exsanguinate
17	MF + 1% Triton X-100	Same as rabbit 15			10	5-10-77	TCVI-55	pre-bled
					100	6-11-77	TCVI-86	paralysis, exsanguinate
18	MF + 1% Triton X-100	Same as rabbit 15			6	5-10-77	TCVI-55	pre-bled
					125	6-1-77	TCVI-75	paralysis, exsanguinate
19	AcChR	.5	5-27-77	TCVI-68	40	5-27-77	TCVI-68	pre-bled
		.5	6-14-77	TCVI-68	100	6-22-77	TCVI-99	paralysis, exsanguinate
20	AcChR	.5	5-27-77	TCVI-68	10	5-27-77	TCVI-68	pre-bled
		.5	6-14-77	TCVI-68	40	7-5-77	TCVI-128	
		.5	7-5-77	TCVI-128	40	7-21-77	TCVI-149	
		1.0	8-22-77	TCVII-57	30	7-29-77	TCVII-8	
		.5	5-11-78	CDSVI-75	40	9-8-77	TCVII-64	
		.5	8-15-78	TCXIII-4	40	5-18-78	CDSVI-75	
					40	5-26-78	CDSVI-75	
					30	6-8-78	CDSVI-75	
					40	8-23-78	TCXIII-4	
21	MFTS-AcChR	.4	7-29-77	TCVII-9	40	8-28-77	TCVII-61	
		.6	8-14-77	TCVII-32	40	9-8-77	TCVII-64	
		1.8	8-28-77	TCVII-61				


(a)
1.2
1.0
0.8
0.6
0.4
0.2
CPM X 10⁻³
10
20
40
75
100
μl [125I] α-BuTx


(b)
4
3
2
1
CPM X 10⁻³
20
40
60
80
100
μl Sample

number was being extracted. With rabbit α-T. californica AcChR antiserum, 70-75% of Torpedo AcChRs could be precipitated with primary antibodies alone without the use of a second antibody or SACI. If a second antibody or SACI was added, 100% of the AcChR could be precipitated. Similar studies with rat α-T. californica AcChR antisera gave very different results. It was not possible to precipitate rat AcChRs by centrifugation at 100,000 x g for 1 hour without the use of a second antibody.

Figure 7 and Table II show that rats injected with purified AcChR or solubilized membrane fragments containing AcChR had 30% the number of receptors as compared with control, AcChR + SDS, or MFTS + SDS rats. MFTS-AcChR rats had 70% the number of receptors as compared with controls. This decrease in AcChR content could be the result of minor contaminants of AcChR still present in MFTS-AcChR or a nonreceptor component present in Torpedo MFTS which could invoke destruction of the postsynaptic membrane leading to nonspecific degradation of acetylcholine receptors. If the former is true, there should be measurable serum titers against Torpedo receptor in the rats injected with MFTS-AcChR. If the latter is true, there should be no measurable serum titers against Torpedo receptor in this group of rats.

Serum titers against Torpedo and rat AcChR

Using 30% of the total number of extractable receptors from muscle as the criterion for induction of EAMG, it was shown that MFTS as antigen did induce EAMG, AcChR + SDS did not, and the results of MFTS-AcChR were ambiguous. Looking at the antibody titer results against rat AcChR shown in Table II, it is difficult to interpret the significance of the differences observed

Figure 7. Quantitation of the number of AcChRs extracted from Lewis rat muscles. Each bar represents one rat.

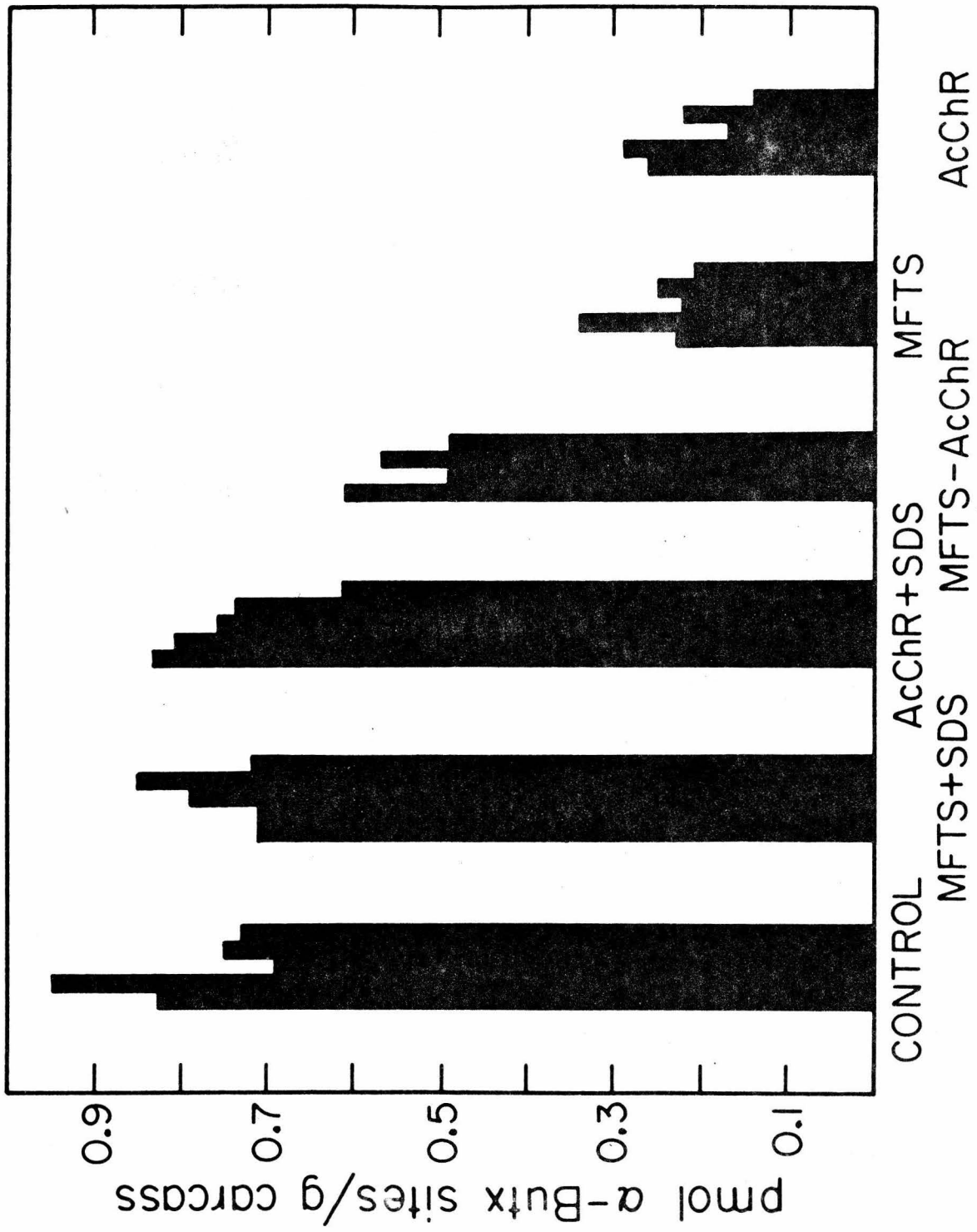

pmol α-Butx sites/g carcass
0.9
0.7
0.5
0.3
0.1
CONTROL
MFTS+SDS
AcChR+SDS
MFTS-AcChR
MFTS
AcChR

TABLE II

Quantitation of the amount of rat muscle AcChR extracted, the amount of antibody-bound AcChR, and serum titers against Torpedo californica and rat AcChR

Antigen injected	Rat #	Extracted muscle AcChR: pmol α-BuTx sites/rat	Extracted muscle AcChR: pmol α-BuTx sites per gram carcass	Extracted muscle AcChR: Antibody-bound AcChR % of total	Serum anti-AcChR titer, nM: Anti-Torpedo	Serum anti-AcChR titer, nM: Anti-rat
Buffer	41	100.2	.82	0.7	4	0
control	42	109.3	.95	0	0	1.2
	43	89.0	.69	0.6	6	0
	44	93.6	.75	0.3	5	0.4
	45	98.7	.73	0	5	0.4
MFTS	31	102.2	.71	1	9	4
+	32	97.8	.71	0	0	0
SDS	33	96.0	.79	0.2	2	0
	34	98.0	.85	0.4	6	0.3
	35	97.0	.72	0.1	0	0.9
AcChR	11	106.8	.83	1.9	230	3.2
+	12	94.6	.81	0	241	2.7
SDS	13	95.3	.76	0.1	177	4.4
	14	93.5	.74	2.1	590	2.2
	15	92.7	.62	0	8	0.1
MFTS-AcChR	36	65.1	.61	6.3	50	0.6
	38	57.1	.49	0.2	106	4.2
	39	75.5	.57	0.3	70	7.0
	40	61.4	.49	8.7	203	5.4
MFTS	26	30.4	.23	37.7	996	14.9
	27	43.2	.34	17	627	11
	28	27.3	.22	15	710	12.8
	29	34.8	.25	5.4	490	10.4
	30	24.1	.21	19.2	736	10.4
AcChR	46	22.9	.26	61.9	2609	57.2
	47	31.4	.29	67	1864	62.8
	48	20.9	.17	44	1676	36.8
	49	32.0	.22	18	739	10.6
	50	17.7	.14	63.6	1900	24.8

	Average picomoles α-BuTx sites per gram carcass	% of control	Average antibody-bound AcChR % of total	Average serum anti-AcChR titer, nM: Anti-Torpedo	Average serum anti-AcChR titer, nM: Anti-rat
Control	.79 ± .10	100	0.32	4 ± 2	0.4 ± 0.5
MFTS + SDS	.76 ± .06	96	0.34	3 ± 4	1.0 ± 1.7
AcChR + SDS	.75 ± .08	95	0.82	249 ± 212	2.5 ± 1.5
MFTS-AcChR	.54 ± .06	68	3.9	107 ± 68	4.3 ± 2.7
MFTS	.25 ± .05	32	18.9	712 ± 186	11.9 ± 1.9
AcChR	.22 ± .06	28	50.9	1758 ± 671	38.4 ± 21.8

among titers of these three groups because of the small sample size and large standard deviations. Although there may be a correlation between anti-rat AcChR antibody titer and induction of EAMG, none can be seen between anti-Torpedo AcChR titers and induction of EAMG as has been reported earlier (29). Although animals injected with AcChR + SDS had a higher anti-Torpedo AcChR titer than those injected with MFTS-AcChR, the AcChR + SDS group had no reduction in the number of their muscle receptors. What is clear is that the MFTS-AcChR group did have a titer against Torpedo AcChR which was significantly higher than the control group. This strongly suggests receptor contamination present in the MFTS-AcChR antigen.

Double immunodiffusion results

The results of Ouchterlony immunodiffusion assays are summarized in Table III and representative plates of each of the antisera are shown in Figure 8. Several pieces of evidence suggest that the immunoprecipitation lines seen between anti-AcChR + SDS antisera and MFTS or MFTS-AcChR are not due to AcChR present in the antigens being complexed with anti-AcChR antibodies, but rather, non-AcChR components present in these antigens being recognized by antibodies directed against them. The positions of the immunoprecipitation lines are all much closer to the antigen wells than are precipitation lines formed between anti-AcChR or anti-MFTS antisera and AcChR (compare plate a with plates d and f). There is also no correlation between the anti-Torpedo AcChR titer of the members of the AcChR + SDS group and a positive reaction with the MFTS antigen (compare rats 11 and 13). In addition, if one correlates anti-AcChR titers and Ouchterlony results seen with anti-MFTS and anti-AcChR antisera with AcChR,

TABLE III

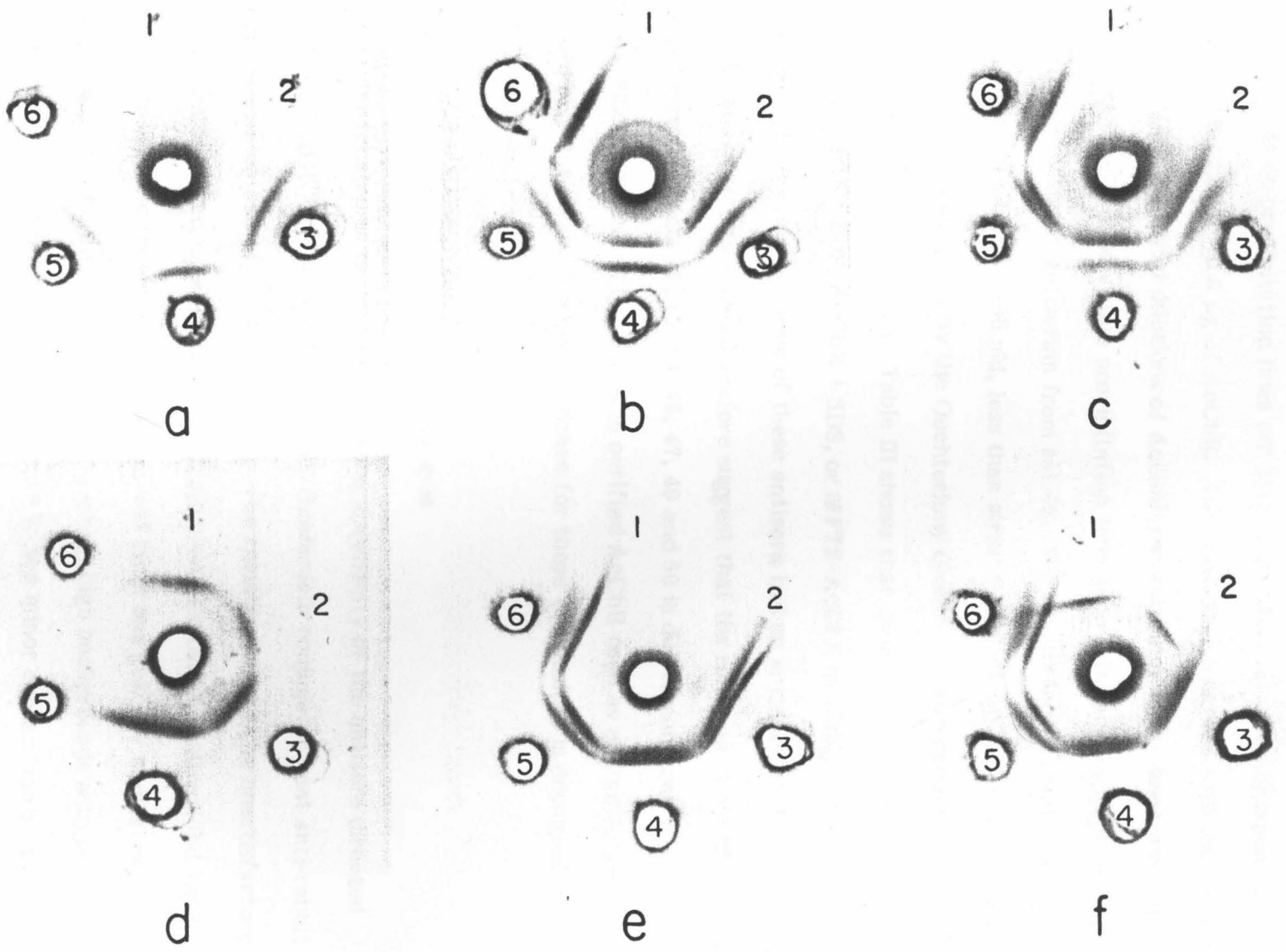
Results of double immunodiffusion assays[a] of antisera to AcChR ± SDS, MFTS ± SDS and MFTS-AcChR reacting against AcChR, MFTS and MFTS-AcChR[b]

Antisera against:	Rat number	Antigens used in Ouchterlony wells: AcChR	MFTS	MFTS-AcChR
Buffer control	41	-	-	-
	42	-	-	-
	43	-	-	-
	44	-	-	-
	45	-	-	-
MFTS + SDS	31	-	++	++
	32	-	++	++
	33	-	++	++
	34	-	+	+
	35	-	+	+
AcChR + SDS	11	-	-	-
	12	-	±	±
	13	-	+	+
	14	-	+	+
	15	-	-	-
MFTS-AcChR	36	-	++	++
	38	-	++	++
	39	± ?	++	+
	40	± ?	++	++
MFTS	26	+	++	++
	27	+	++	++
	28	+	++	++
	29	±	++	++
	30	+	++	++
AcChR	46	+	+	±
	47	+	+	±
	48	±	+	-
	49	-	±±	±
	50	+	++	±

[a] 1% agar in borate buffered saline, stained with Coomassie Brilliant Blue.

[b] ++ indicates more than one stained immunoprecipitation band; + indicates one stained band; ± indicates a band barely visible in a photograph; - indicates no reaction.

Figure 8. Ouchterlony double immunodiffusion plates of various rat antisera against Torpedo californica MFTS, MFTS-AcChR and affinity column purified AcChR. Wells 1 and 2 contained 0.6 μg of AcChR. Wells 3 and 4 contained 20.8 μg of MFTS protein of which 1.4 μg were AcChR. Wells 5 and 6 contained 8.3 μg of MFTS-AcChR protein. The center wells contained 4 μl of rat antisera with the following specificities: (a) rat 14, anti-AcChR + SDS; (b) rat 31, anti-MFTS + SDS; (c) rat 40, anti-MFTS-AcChR; (d) rat 46, anti-AcChR; (e) rat 38, anti-MFTS-AcChR; (f) rat 27, anti-MFTS.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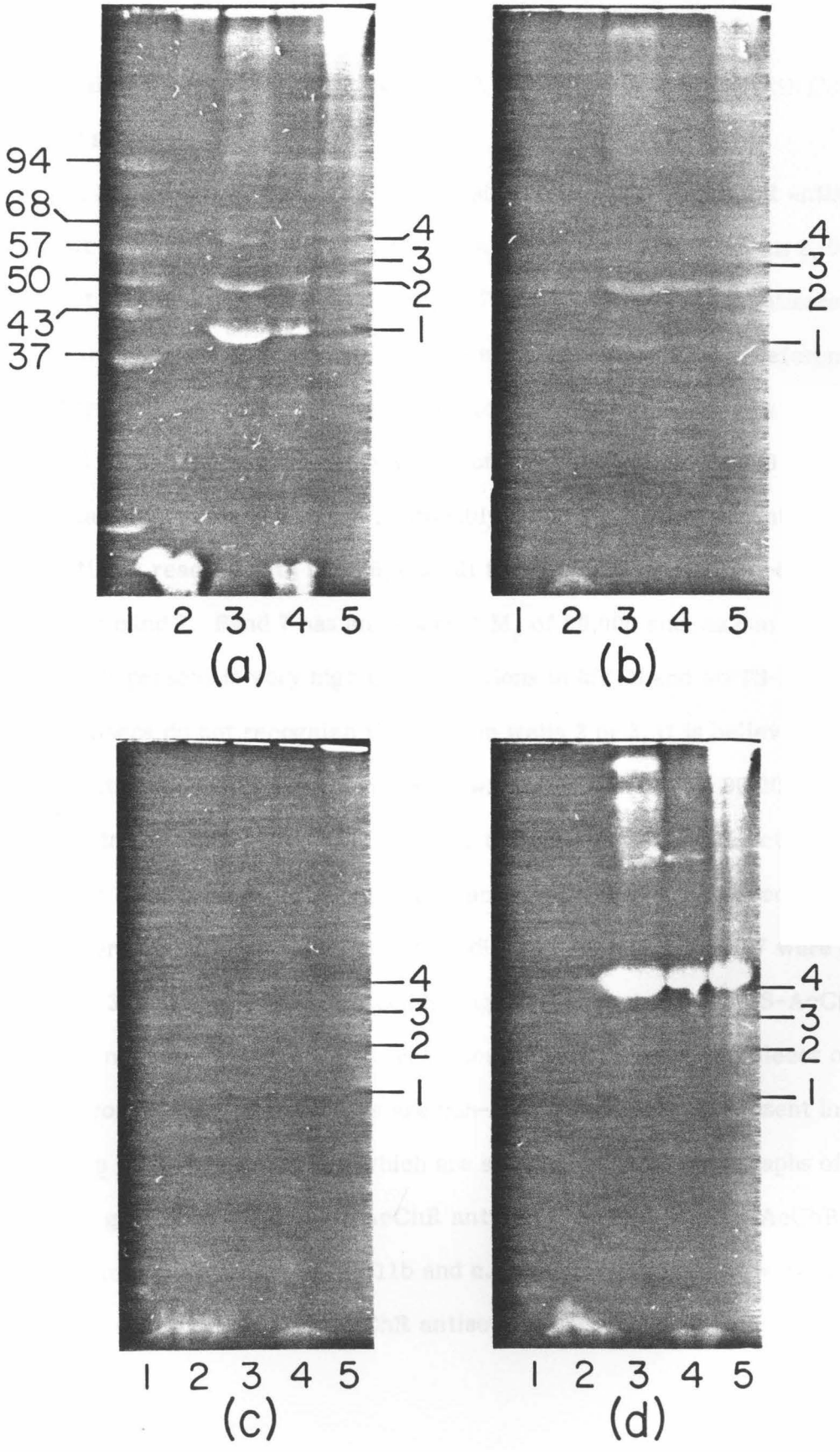
it appears that no precipitation lines are seen with titers less than or equal to about 700 nM against ~0.6 μg of AcChR. This observation agrees with the results obtained by making serial dilutions of AcChR and determining the least amount of receptor which forms visible precipitation lines (upon staining with Coomassie Brilliant Blue) with the antiserum from rat 46. The results (not shown) show that with an antibody titer of 2600 nM, less than about 0.2 μg of AcChR will not form an immunoprecipitation line by the Ouchterlony double immunoprecipitation technique used in these studies. Table III shows that none of the antisera from the control, MFTS + SDS, AcChR + SDS, or MFTS-AcChR (possibly only 2 of the 4) reacted with AcChR and none of these antisera had an anti-AcChR titer $\geq$700 nM. All of the above results would therefore suggest that the reaction of MFTS-AcChR with antisera from rats 12, 13, 14, 46, 47, 49 and 50 is due to nonreceptor components which were not only present in purified AcChR used as an immunogen, but were also antigenic. Further evidence for these conclusions is discussed in the following section.

Antibody staining of SDS polyacrylamide gels

Initial studies to try to analyze the specificity of the antisera directed against purified T. californica AcChR used fluorescein conjugated goat anti-rabbit IgG antiserum instead of [^{125}I] protein A. The results proved to be unsatisfactory for two reasons. Fluorescence decayed quickly with exposure to ultraviolet light making it possible to obtain only a few pictures from one gel. UV excitation of fixed proteins caused them to phosphoresce giving high backgrounds with long exposure times. This fact made it impossible to see minor contaminants. Illustrative examples of this technique are shown in Figure 9. Using a second antibody or

Figure 9. Antibody staining of 8-15% exponential discontinuous analytical SDS polyacrylamide slab gels with fluorescein conjugated goat anti-rabbit IgG. Lane 1 contained molecular weight standards described in the legend to Figure 4. Lane 2 contained MFTS-AcChR, lane 3 contained affinity column purified AcChR, lane 4 contained MFTS and lane 5 contained AcChR eluted from toxin resin with 8 M urea. AcChR subunits of 41,000, 51,000, 60,000 and 64,000 M_r are marked 1-4, respectively. Rabbit antisera of the following specificities were added before the addition of the fluorescein conjugated goat anti-rabbit IgG: anti-41,000 M_r subunit in (a); anti-51,000 M_r subunit in (b); anti-60,000 M_r subunit in (c); anti-64,000 M_r subunit in (d).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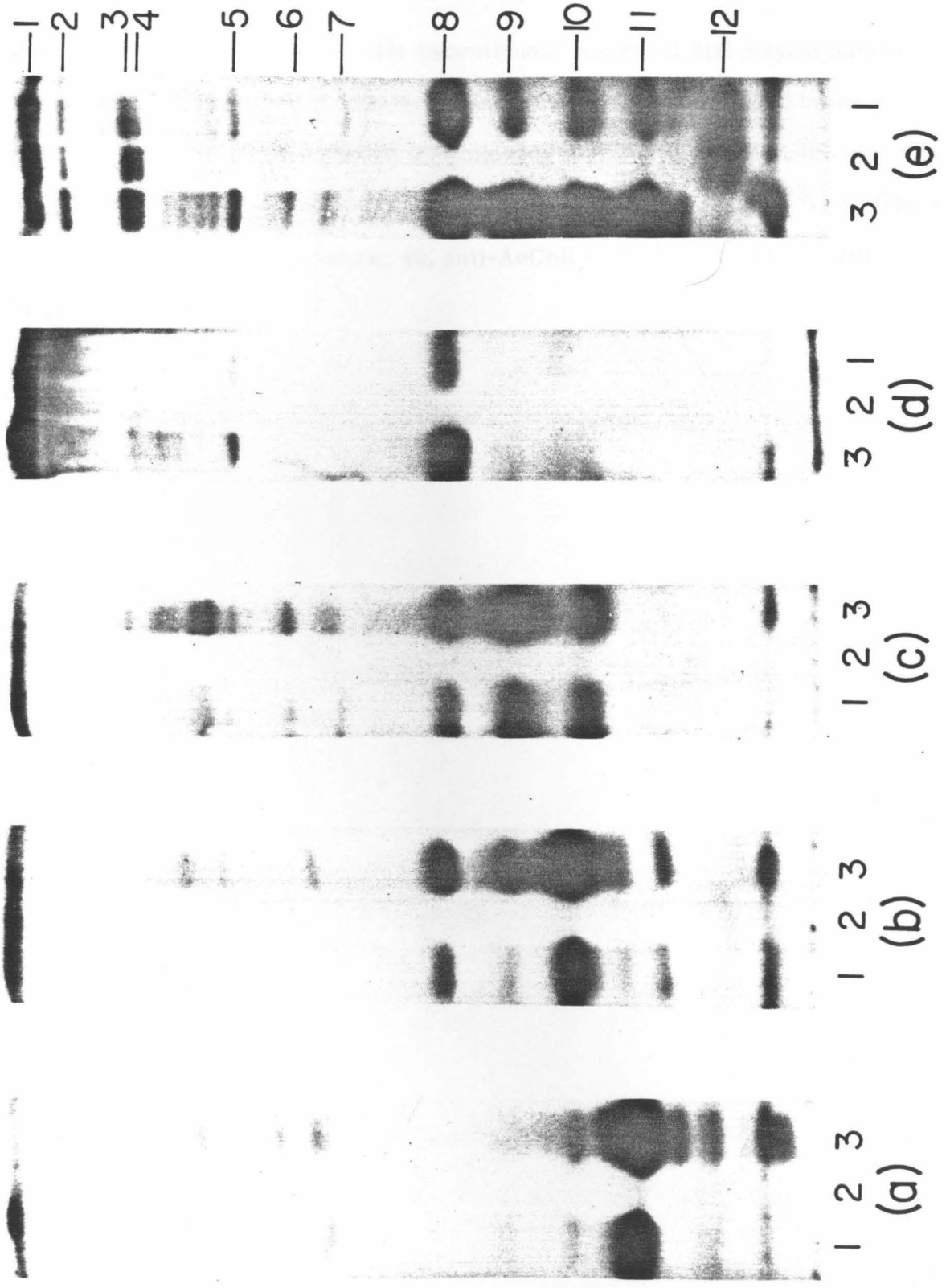
protein A labeled with [^{125}I] eliminated both the difficulties found with fluorescein conjugated second antibodies.

In Figure 10e, it can be seen that rabbit anti-Torpedo AcChR antiserum reacted not only with the 4 bands identified as receptor subunits (bands 8, 9, 10 and 11), but also with other bands marked 1-7 and 12. When rabbit antisera to the individual subunits (preparation of these antisera is described in reference 14 and Chapter I) were used in similar experiments, they reacted with their specific subunit and subunits with which they were contaminated. Anti-64,000 M_r subunit antisera reacted with band 5 which is probably a 64,000 M_r dimer. Anti-60,000 M_r subunit antisera reacted with band 6, and all the antisera except anti-64,000 M_r reacted with band 7. Band 7 has an apparent M_r of 90,000 and, as can be seen in Figure 2, is present in very high concentrations in MFTS and MFTS-AcChR. Because antisera do not recognize this band in wells 2 or 3, it is believed that small amounts of subunits have comigrated with this peak but no 90,000 M_r contaminant exists in the purified AcChR. None of the subunit antisera reacted with bands 1-4 or 12. These results imply that bands 1-4 and 12 are not receptor subunit aggregates or degradative fragments. In addition, bands 1, 3, 4 and 7 were recognized by rat 39 antisera to MFTS-AcChR (Figure 11a), but anti-MFTS-AcChR antisera did not recognize any of the receptor subunits. These two pieces of evidence strongly suggest that there are non-AcChR components present in purified Torpedo AcChR preparations which are antigenic. Autoradiographs of antibody stained SDS gels using rat 46 anti-AcChR antiserum and rat 14 anti-AcChR + SDS antiserum are shown in Figure 11b and c. The results obtained were similar to those found with rabbit anti-AcChR antiserum.

Figure 10. Antibody staining of 7.5% discontinuous analytical SDS polyacrylamide slab gels with [^{125}I] protein A. Lane 1 contained 83 μg of MFTS protein of which 6 μg were AcChR. Lane 2 contained 73 μg of MFTS-AcChR protein. Lane 3 contained ∿14 μg AcChR. Rabbit antisera of the following specificities were incubated with gel strips before the addition of [^{125}I] protein A: anti-41,000 M_r subunit in (a); anti-51,000 M_r subunit in (b); anti-60,000 M_r subunit in (c); anti-64,000 M_r subunit in (d); anti-AcChR in (e). AcChR subunits were marked as follows: 8 = 64,000 M_r; 9 = 60,000 M_r; 10 = 51,000 M_r; 11 = 41,000 M_r.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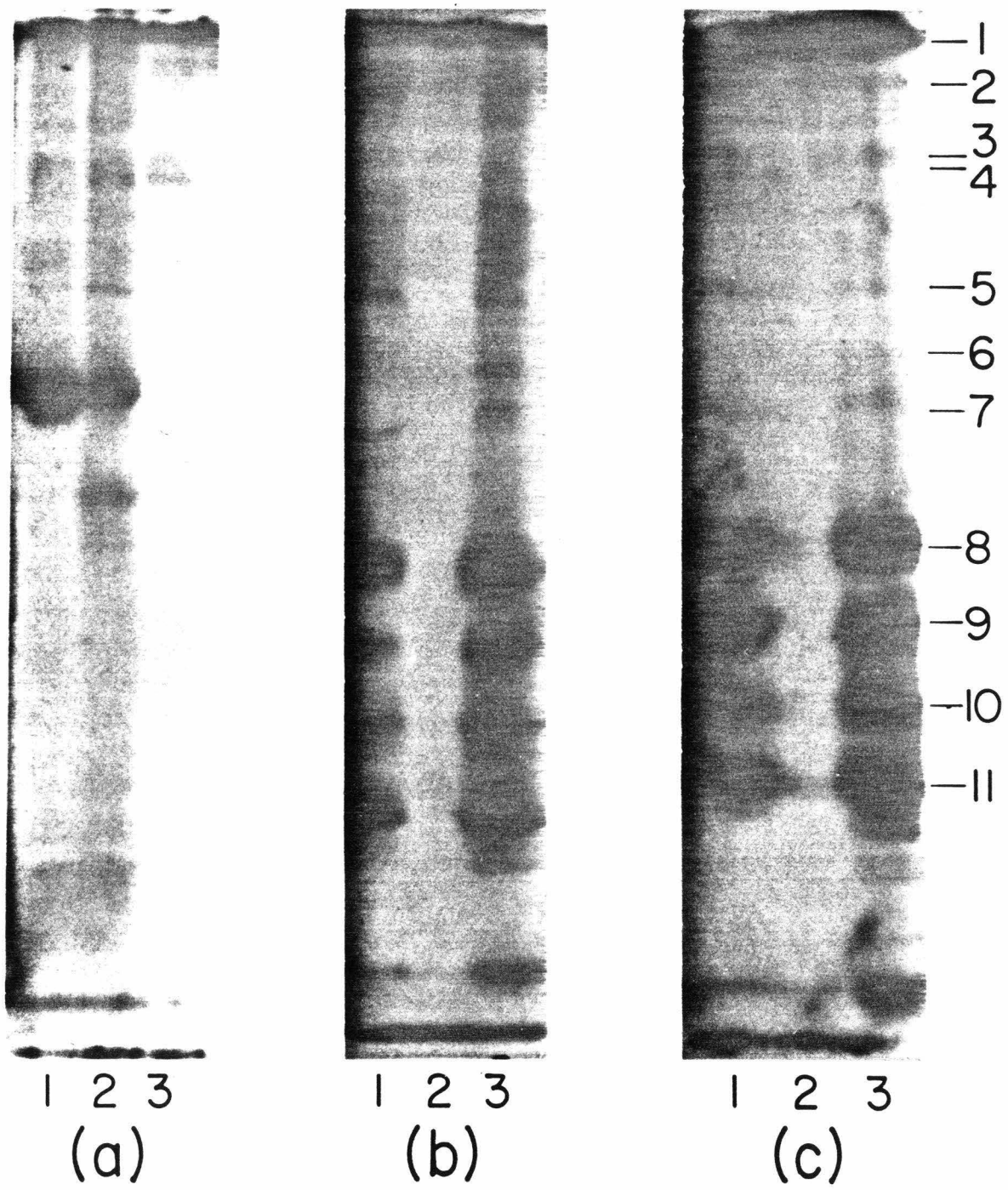
(a)

Figure 11. Antibody staining of 7.5% discontinuous analytical SDS polyacrylamide slab gels with [^{125}I] protein A. Lanes 1-3 and bands 8-11 are described in the legend to Figure 10. Rat antisera of the following specificities were incubated with gel strips before the addition of rabbit anti-rat IgG followed by [^{125}I] protein A: rat 39, anti-MFTS-AcChR in (a); rat 46, anti-AcChR in (b); rat 14, anti-AcChR + SDS in (c).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(a)
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(b)
1 2 3
(c)

DISCUSSION

SDS polyacrylamide gels heavily loaded with purified AcChR preparations in our laboratory and other laboratories can show minor contaminants present in addition to bands considered to be AcChR subunits. Antibody staining of SDS gels shows some of these contaminants are also antigenic. To test to see if these components or any other components present in Triton X-100 solubilized Torpedo membrane fragments could induce EAMG, we tried to selectively remove AcChR from MFTS with an α-BuTx affinity resin. An anti-AcChR antibody affinity column was not used in conjunction with the α-BuTx resin in case the immunogenic non-AcChR components present in purified AcChR were removed from MFTS.

The amount of active [^{125}I]α-BuTx binding AcChR remaining in MFTS-AcChR was 16.4 ng per 100 μg protein. Rats were injected with a total of 82 ng of active AcChR in MFTS-AcChR which, according to Lennon et al. (29), should not be enough to induce EAMG. Coomassie Brilliant Blue and antibody staining of SDS polyacrylamide gels and double immunodiffusion assays were performed in an attempt to determine if inactive AcChR or antigenic AcChR fragments were present in MFTS-AcChR. Although the results of these experiments would indicate that receptor was effectively removed, it is not clear that any of them is sensitive enough to detect the minimum amount of AcChR which is needed to induce EAMG in rats. At sacrifice, however, MFTS-AcChR rats contained only 70% of the number of receptors as compared with control, AcChR + SDS, or MFTS + SDS rats while AcChR and MFTS rats only contained 30%. The explanation for the decreased number of receptors in the MFTS-AcChR group could be found by looking at the specificity of the antisera in these animals. If there had been no antibody titer to rat or Torpedo AcChR in the MFTS-AcChR group

of rats but there had been a decreased number of their muscle receptors, then this would indicate that non-AcChR components present in Torpedo MFTS could induce an antibody-mediated degradation of postsynaptic membranes. Or, if antibody titers to rat AcChR were greater than or equal to titers against Torpedo AcChR, then a situation similar to that reported in reference 24, where syngeneic rat AcChR was used to induce EAMG, would be suggested. Our results, however, showed neither situation was realized in this study. Anti-MFTS-AcChR antibody titers to Torpedo AcChR were measurable, and their titers against rat AcChR were less than 4% of their titers against Torpedo AcChR. Both results strongly suggest AcChR contamination in MFTS-AcChR.

While these experiments were in progress, Lindstrom et al. (13) demonstrated that individual Torpedo californica AcChR subunits (the same 4 seen in our laboratory [30]) could induce EAMG in female Lewis rats if administered in very large amounts. A total of 200 or 250 μg of any Torpedo AcChR subunit would induce EAMG but, as reported in reference 8, 200 μg of Electrophorus electricus AcChR denatured in SDS would not. That individual receptor subunits can induce EAMG, decreases the likelihood that minor contaminants in purified AcChR preparations were responsible for inducing the disease. But as was discussed in that paper, subunits isolated off of preparative SDS polyacrylamide gels were slightly contaminated with other subunits. A different approach, that of selectively removing only AcChR from MFTS, was taken in this study to determine if the AcChR was the only protein present in solubilized Torpedo californica membrane fragments which could induce EAMG. Although not all of the receptor was removed from MFTS-AcChR, scans of SDS gels indicated no other Coomassie Blue staining material was removed as effectively as AcChR. Even though MFTS-

AcChR were greatly enriched in the contaminants present in purified AcChR and presumably any components which might comigrate with AcChR subunits, the animals injected with this antigen had only a partial case of EAMG.

The results of this study suggest that no Torpedo californica components other than the AcChR can induce EAMG, and that the partial induction of EAMG seen in MFTS-AcChR rats was due to AcChR contamination present in the MFTS-AcChR antigen.

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Chapter III

A Correlation between the Presence of Experimental Autoimmune Myasthenia Gravis in Lewis Rats and New Zealand White Rabbits and the Ability of their Antisera to Inhibit α-Bungarotoxin Binding to Acetylcholine Receptors

ABBREVIATIONS

EAMG: experimental autoimmune myasthenia gravis

AcCh: acetylcholine

AcChR: acetylcholine receptor

α-BuTx: α-bungarotoxin

Fabs: antigen-binding fragments of antibody molecules

MF: membrane fragments

MF + T: membrane fragments + 1% Triton X-100

MFTS: membrane fragments solubilized in Triton X-100; supernatant from 100,000 x g centrifugation for 1 hour

MFTS-AcChR: MFTS depleted of AcChR

SACI: Staphylococcus aureus Cowan I strain

M_r: molecular weight

INTRODUCTION

Several recent reviews have been written on the subject of experimental autoimmune myasthenia gravis (EAMG) (1-3). The primary lesion in EAMG is believed to be loss of acetylcholine receptors (AcChRs) rather than blockade of acetylcholine binding sites, although partial inhibition of AcChR activity may be very important in situations where a reduced number of AcChRs already exist.

Several experiments have been performed in which anti-AcChR antiserum was tested for its ability to inhibit α-bungarotoxin (α-BuTx) or acetylcholine (AcCh) binding to AcChRs. Conflicting results have been obtained using both native membrane bound AcChR preparations and detergent solubilized AcChR preparations to inhibit [^{125}I]α-BuTx or [^{3}H]AcCh from binding to AcChR. Anti-Electrophorus electricus AcChR antisera have been shown to inhibit carbamylcholine-induced depolarizations of isolated eel electroplaques (4). By comparing the immunoprecipitation curves of [^{3}H]acetyl receptor with those obtained when [^{3}H]acetyl receptor was preincubated with unlabeled α-BuTx, 6-15% of the antibody activity has been seen directed against the toxin site in some cases (4) but none of the activity was seen directed against the toxin site in other cases (5). Reports of inhibition of [^{125}I]α-BuTx binding to native membrane bound AcChR have varied from 0-100% (5-9) while reports of inhibition to detergent solubilized AcChR have varied from 25-60% (6-8, 10). Similar variability has been reported for inhibition of [^{3}H]AcCh binding to native AcChR, 19-96% inhibition (7), and to detergent solubilized AcChR, 100% inhibition (11).

In this study, antisera prepared in rabbits and Lewis rats against various forms of Torpedo californica AcChR (AcChR ± SDS, MFTS ± SDS, MFTS-AcChR,

AcChR ± α-BuTx, MF ± T) were tested for the ability to inhibit [^{125}I]α-BuTx binding to native and detergent solubilized AcChR. Similar inhibition studies were performed using antisera directed against each of the four isolated AcChR subunits. To determine if any inhibition seen was due to nonspecific antibody-mediated aggregation of AcChR molecules, antigen-binding fragments (Fabs) were prepared from the immunoglobulin fractions of antisera directed against AcChR and each of the AcChR subunits and tested for the ability to inhibit α-BuTx binding to AcChR.

MATERIALS AND METHODS

Preparation of antisera

The preparation of rabbit antisera directed against Torpedo californica AcChR ± SDS, AcChR + α-BuTx, and isolated AcChR subunits is described in reference 12 and Chapter I. The preparation of rat antisera directed against AcChR ± SDS, MFTS ± SDS and MFTS-AcChR and rabbit antisera to AcChR and MFTS-AcChR are described in Chapter II.

Preparation of antigen-binding fragments (Fabs)

Fabs were prepared by enzymatic cleavage with papain according to the procedure described by Porter (13) with a few modifications described by Putnam et al. (14). To the immunoglobulin fraction of antisera was added 1 M sodium acetate buffer, pH 5.5, to a final concentration of 0.1 M, ethylenediamine tetraacetic acid to a final concentration of 2 mM, cysteine to a final concentration of 10 mM and mercuripapain (purchased from Sigma Chemical Co.) at an enzyme to substrate ratio of 1:100. The mixture was incubated at 37° for 5 hours before

the reaction was stopped by the addition of p-hydroxy mercuribenzoate at a final concentration of 1 mM. The solution was dialyzed against 10 mM sodium acetate, pH 7.5, applied to a 215 ml CM-cellulose column equilibrated in the same buffer. 160 ml of 10 mM sodium acetate were applied before starting a linear gradient from 10 mM to 0.5 M sodium acetate, pH 5.5, 400 ml of each. Eluted peaks were pooled and dialyzed against 10 mM sodium phosphate, 0.02% sodium azide, pH 7.4. Protein concentration was determined by the method of Lowry et al. (15). Ionic strength was meaured with a Radiometer Copenhagen Conductivity meter CDM3.

DEAE affinity chromatography

3 ml DE52 columns equilibrated in 10 mM sodium phosphate, 0.1% Triton X-100, 50 mM NaCl, pH 7.4, were used to separate AcChR-[^{125}I]α-BuTx-antibody complexes from unbound [^{125}I]α-BuTx. AcChR-[^{125}I]α-BuTx-antibody complexes were eluted from the column with a 0.3 M NaCl wash.

Inhibition of [^{125}I]α-BuTx binding to AcChR

The preparation of [^{125}I]α-BuTx was described in Chapter II. The specific activity of the [^{125}I]α-BuTx used in these assays was 211 Ci/mmole. The preparations of affinity column purified T. californica AcChR, membrane fragments containing AcChR (MF) and Triton X-100 solubilized membrane fragments (MFTS) have also been described. The DEAE filter disc assay used was essentially the same as the one described in reference 16. Antibody or Fab incubations with AcChR and [^{125}I]α-BuTx were performed in 1.5 ml polyethylene micro test tubes from Cole Scientific, Inc. MFTS were incubated with antibody or Fabs followed by the addition of [^{125}I]α-BuTx, or, MFTS were incubated with [^{125}I]α-BuTx followed

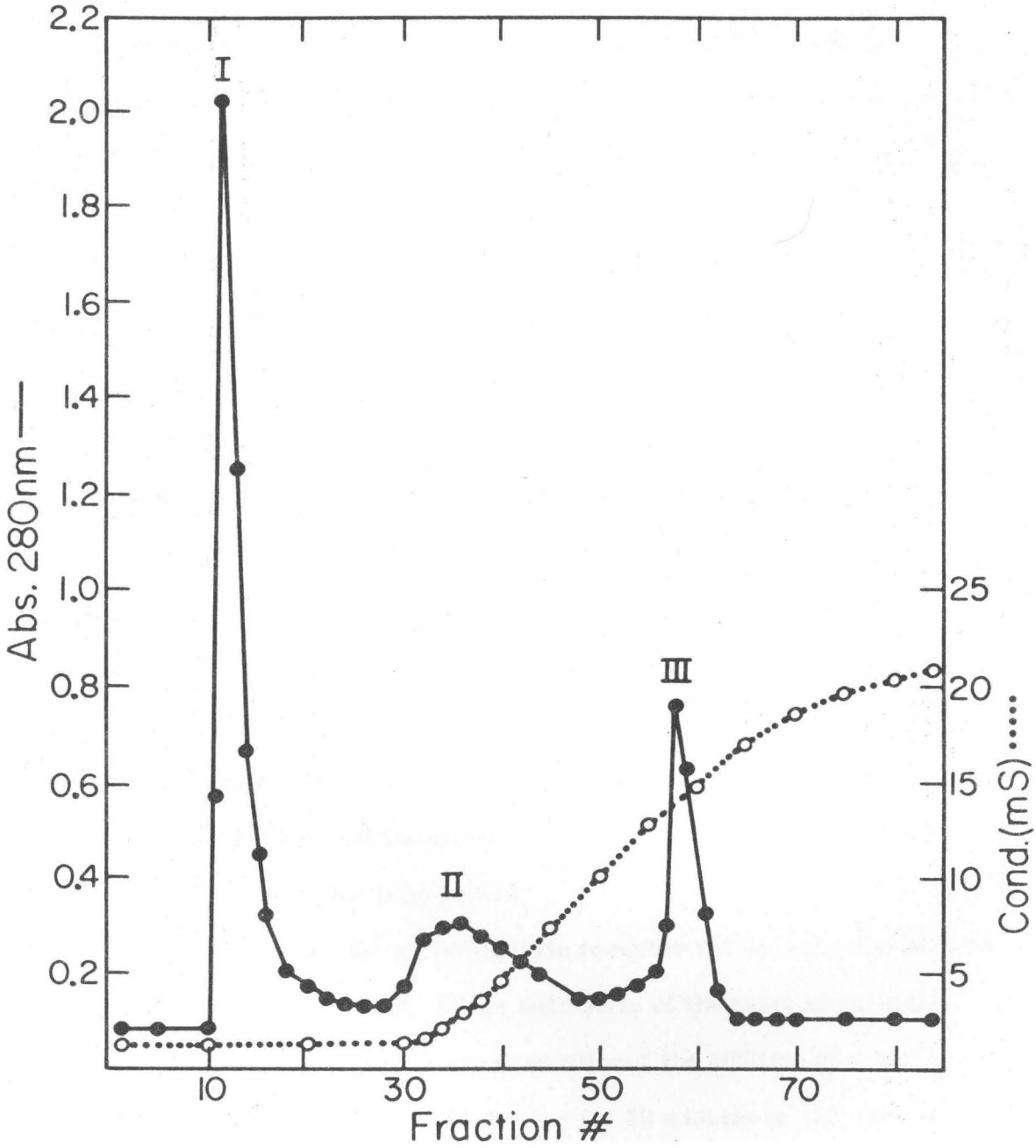
by the addition of antibody or Fabs. 100 µl of solution were pipeted onto DEAE cellulose filter discs (DE81, 2.4 cm diameter obtained through VWR Scientific Co.) and washed in 10 mM sodium phosphate, 0.1% Triton X-100, 50 mM NaCl, pH 7.4, and counted in a Beckman 4000 gamma counter. To calculate the percent inhibition of [^{125}I]α-BuTx binding to AcChR due to antibodies or Fabs, the number of cpm on a filter disc obtained by incubating AcChR with antibody or Fabs first, was divided by the number obtained when AcChR was incubated with [^{125}I]α-BuTx first. Centrifugations were performed in an Eppendorf 3200 Centrifuge (12,000 x g), or a Beckman Airfuge (100,000 x g).

RESULTS

Preparation of Fabs from immunoglobulins

A profile of the papain digestion of one of the antisera immunoglobulin fractions is shown in Figure 1. The profile was essentially identical to those shown in references (13) and (14) and the three peaks eluted at the same ionic strength as the corresponding peaks described in reference (14). Fabs (peak 1) were tested for their ability to bind antigen but not aggregate it. All of the Fabs (except those prepared from the immunoglobulin fraction of a non-immunized control rabbit) bound AcChR labeled with [^{125}I]α-BuTx but did not precipitate this receptor-toxin complex with a 100,000 x g centrifugation for 1 hour. The addition of Staphylococcus aureus Cowan I strain (SACI) also did not precipitate the complex. The addition of a second antibody (goat α-rabbit IgG or sheep α-rabbit IgG) did precipitate the complex by centrifugation at 12,000 x g for 30 minutes.

Figure 1. Profile of the fractionation of Fabs I and II and Fc on a CM-cellulose column after papain digestion of immunoglobulins.


2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
Abs. 280nm
I
II
III
25
20
15
10
5
Cond.(mS)
10
30
50
70
Fraction #

Conditions of the filter disc assay

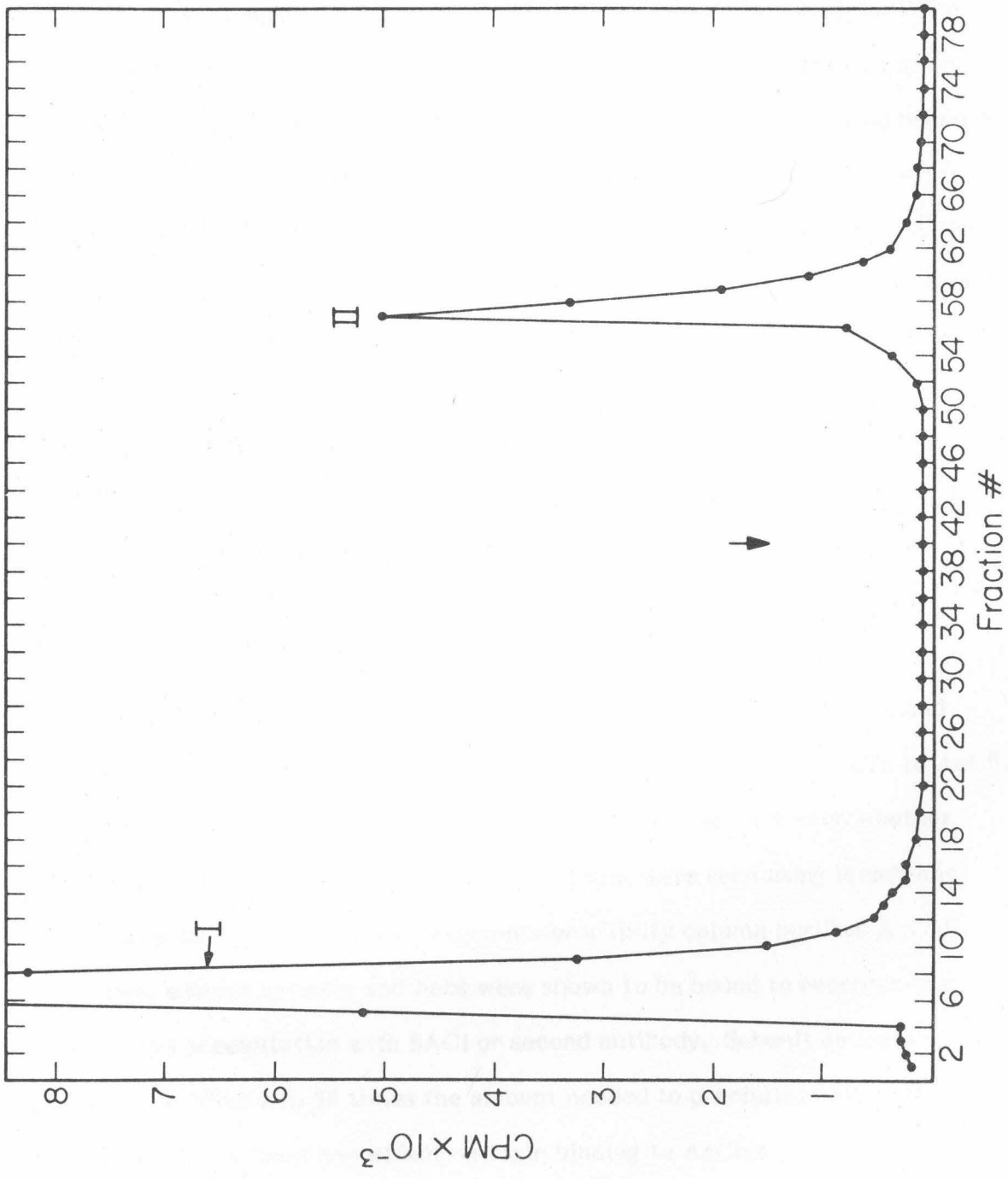
The length of time needed for antibody, Fabs and [^{125}I]α-BuTx to bind to AcChR was established as follows: Antibody or Fabs were incubated with AcChR at 25°C or 4°C for 1, 2, 4 or 24 hours. [^{125}I]α-BuTx was then added at each of these time points and incubated for 1, 2, 4 or 24 hours. Reciprocal experiments were also performed: [^{125}I]α-BuTx was added first followed by antibody or Fabs. The results showed that a one-hour incubation was sufficient for maximum binding of antibody, Fabs or toxin no matter which component was added first. In order to have pseudo-first-order kinetics with respect to toxin binding, however, $\geq$8-fold excess of toxin to toxin sites was needed. An 8-, 10- or 20-fold excess of toxin did not have any effect on the binding of Fabs to AcChR or to AcChR-toxin complex. If receptor-Fab-toxin complexes were allowed to incubate for long periods of time, toxin apparently slowly bound to sites previously unoccupied by toxin. If α-BuTx was added to AcChR before the addition of Fabs, there was no change in the total amount of toxin bound over a 53-hour period. If toxin was added after preincubation with Fabs, the amount of toxin binding slowly increased from 50% to 77% over the 53-hour time period.

Although Fabs would not precipitate receptor-toxin complexes without the addition of a second antibody, intact antibodies of the same specificities would precipitate receptor-toxin complexes without the addition of a second antibody or SACI. A centrifugation at 12,000 x g for 30 minutes or 100,000 x g for 1 hour of antibody-receptor-toxin complexes would precipitate 70-77% of the receptor present. The addition of a second antibody and centrifugation at 12,000 x g for 30 minutes or the addition of SACI and centrifugation at 12,000 x g for 5 minutes would precipitate 100% of the receptor present. For studies of antibody inhibition

of α-BuTx binding to AcChR, it was therefore critical that receptor-toxin-antibody solutions were thoroughly mixed before removing an aliquot for the filter disc assay. Samples were mixed on a Scientific Products Vortex Genie mixer just prior to assay.

Two other questions concerning the validity of the use of the filter disc assay were: 1) How much serum could be applied per filter disc before receptor-toxin complexes were inhibited from binding? and 2) Because the filter discs are washed in a pH 7.4 buffer and IgG does not bind DEAE at this pH, would receptor-toxin-antibody complexes not bind to filter discs? It was determined, using serum from a control rat, that 33 µl of serum inhibited ∿25% of the receptor-toxin complex from binding to a filter disc. The first test to determine if antibody bound to receptor inhibited receptor binding to filter discs, was to add [^{125}I]α-BuTx to AcChR before adding antibodies. With increasing amounts of antiserum, no inhibition of receptor binding to filter discs occurred until concentrations of serum were reached at which binding sites on filter discs for receptor were nonspecifically blocked by serum factors. To rule out the possibility that a particular fraction of antibodies was prevented from binding to receptor when α-BuTx was added first, and that these antibodies when allowed to bind receptor prevented receptor from binding DEAE filter discs, DEAE columns were run on receptor-toxin-antibody/Fab complexes and assayed. Details of the columns are described under Materials and Methods. Complexes (either with antibody or toxin bound first) were applied to columns and washed with 3 column volumes of buffer before eluting receptor complexes with sodium chloride. A typical elution profile is shown in Figure 2. No peaks of radioactivity were seen except the free, unbound [^{125}I]α-BuTx peak (peak I) and the receptor complex peak (peak II) which was eluted with high

Figure 2. DEAE affinity column profile of MFTS-[^{125}I]α-BuTx/Ab/Fab complexes in the presence of excess [^{125}I]α-BuTx. Peak I represents free, unbound [^{125}I]α-BuTx which does not bind DEAE. The arrow above fraction 40 shows where 0.3 M NaCl was applied to the column in order to elute MFTS-toxin, MFTS-toxin-Ab or MFTS-toxin-Fab complexes (peak II) from the column.


CPM x 10⁻³
Fraction #
I
II

salt. The area under the receptor complex peak was greater when toxin was added to receptor first compared with the area under the peak when antibody or Fabs were added first. This result agrees with those obtained by the filter disc assay but a corresponding increase in the free toxin peak could not be measured because of the large excess of toxin added to the incubation mixture. However, when the free toxin peak was pooled and SACI added to the material, SACI did not precipitate any radioactive material from this peak, demonstrating that no IgG bound to receptor-toxin complexes was present in this peak. This result, plus the result that 100% of receptor-toxin-antibody complexes could be precipitated with the addition of SACI when toxin was added before antibody, strongly suggest that the DEAE filter disc assay is an appropriate assay for determining if antibodies or Fabs can inhibit [^{125}I]α-BuTx binding to AcChR.

Inhibition of [^{125}I]α-BuTx binding to AcChR

Antisera directed against isolated T. californica AcChR subunits and Fabs prepared from these antisera had no effect on the binding of α-BuTx to AcChR. No differences in the total amount of [^{125}I]α-BuTx binding were seen whether toxin or Ab/Fab was added first, and no differences were seen using membrane fragments, solubilized membrane fragments or affinity column purified AcChR. In all cases, subunit antisera and Fabs were shown to be bound to receptor-toxin complexes by precipitation with SACI or second antibody. Subunit antisera were tested in quantities 5 to 20 times the amount needed to precipitate all receptor-toxin complexes without any effect on toxin binding to AcChR.

Anti-AcChR antisera from three rabbits and one rat all failed to inhibit toxin binding to T. californica membrane fragments containing AcChR, as did

anti-AcChR Fabs prepared from a rabbit other than those just mentioned. However, when solubilized membrane fragments or affinity column purified AcChR were used, anti-AcChR antisera or Fabs could inhibit up to 50% of [^{125}I]α-BuTx binding to AcChR. Figure 3 is a plot of the effects of adding increasing amounts of anti-AcChR Fabs on toxin binding to AcChR. By titering the Fabs, it could be shown that 100% of the receptor was bound with a 150 µl addition of Fabs. ∿50% inhibition of toxin binding was seen with 200 µl of Fabs and no further inhibition was obtained with the addition of twice as many Fabs. With anti-AcChR antisera, a 50-fold excess of antisera over the minimum amount needed to inhibit 50% of toxin binding did not lead to an increase in the amount of inhibition obtained (Figure 4).

The antisera from 11 different rabbits immunized with various forms of AcChR (AcChR ± SDS and AcChR + α-BuTx) were tested for the ability to inhibit α-BuTx binding to solubilized T. californica membrane fragments containing AcChR. Including multiple bleedings from some rabbits, a total of 19 sera were tested. The results are summarized in Table I. Complete immunization schedules can be found in Chapters I and II and Table II of this chapter. The average percent toxin inhibition from the antisera of the eight rabbits injected with purified AcChR was 49% ± 13.3%. The average percent inhibition from the two rabbits injected with AcChR denatured in SDS was only 7% ± 3.9%. Antisera from the one rabbit injected with AcChR + α-BuTx could only inhibit 17% of toxin binding to AcChR. The receptor preparation used for injection into this rabbit (rabbit 6) was the same AcChR preparation used for injection into rabbit 5 except receptor was not complexed with α-BuTx when injected into rabbit 5. As can be seen in Table I, antiserum from rabbit 5 inhibited toxin binding to AcChR by 42%. Determining

Figure 3. Inhibition of [^{125}I]α-BuTx binding to MFTS by rabbit anti-AcChR Fabs. When increasing amounts of anti-AcChR Fabs were added to MFTS labeled with [^{125}I]α-BuTx, the line drawn through open circles (o) was obtained. When increasing amounts of anti-AcChR Fabs were added to MFTS before the addition of [^{125}I]α-BuTx, the line drawn through closed circles (●) was obtained.

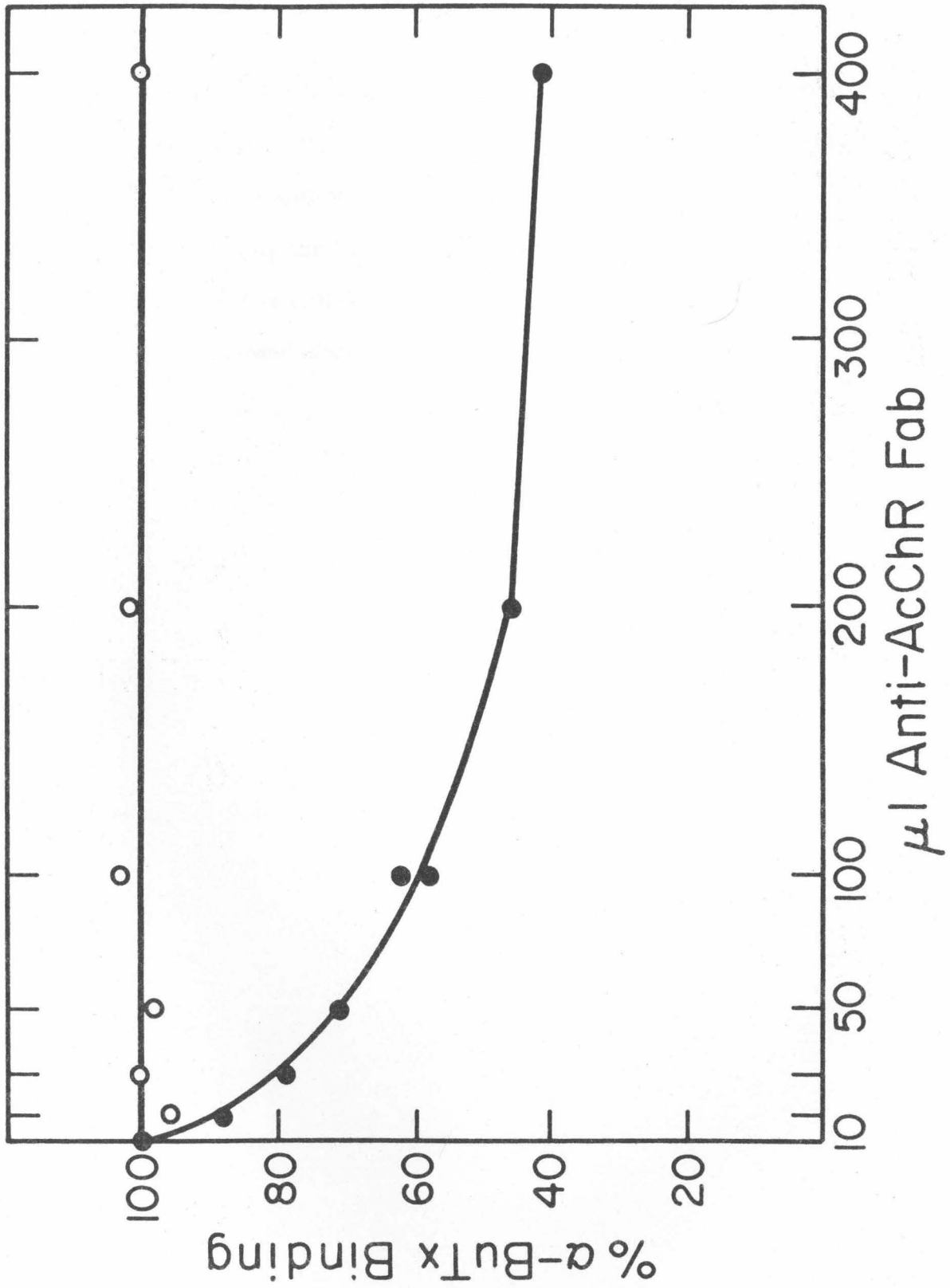

% α-BuTx Binding
100
80
60
40
20
10
50
100
200
300
400
μl Anti-AcChR Fab

Figure 4. Inhibition of [^{125}I]α-BuTx binding to MFTS by rat antisera directed against AcChR ± SDS and MFTS ± SDS. Incubations of MFTS with five anti-AcChR + SDS antisera and five anti-MFTS + SDS antisera before the addition of [^{125}I]α-BuTx are represented by open squares (▣). Incubations of MFTS with five anti-AcChR antisera and five anti-MFTS antisera before the addition of [^{125}I]α-BuTx are represented by closed circles (●).

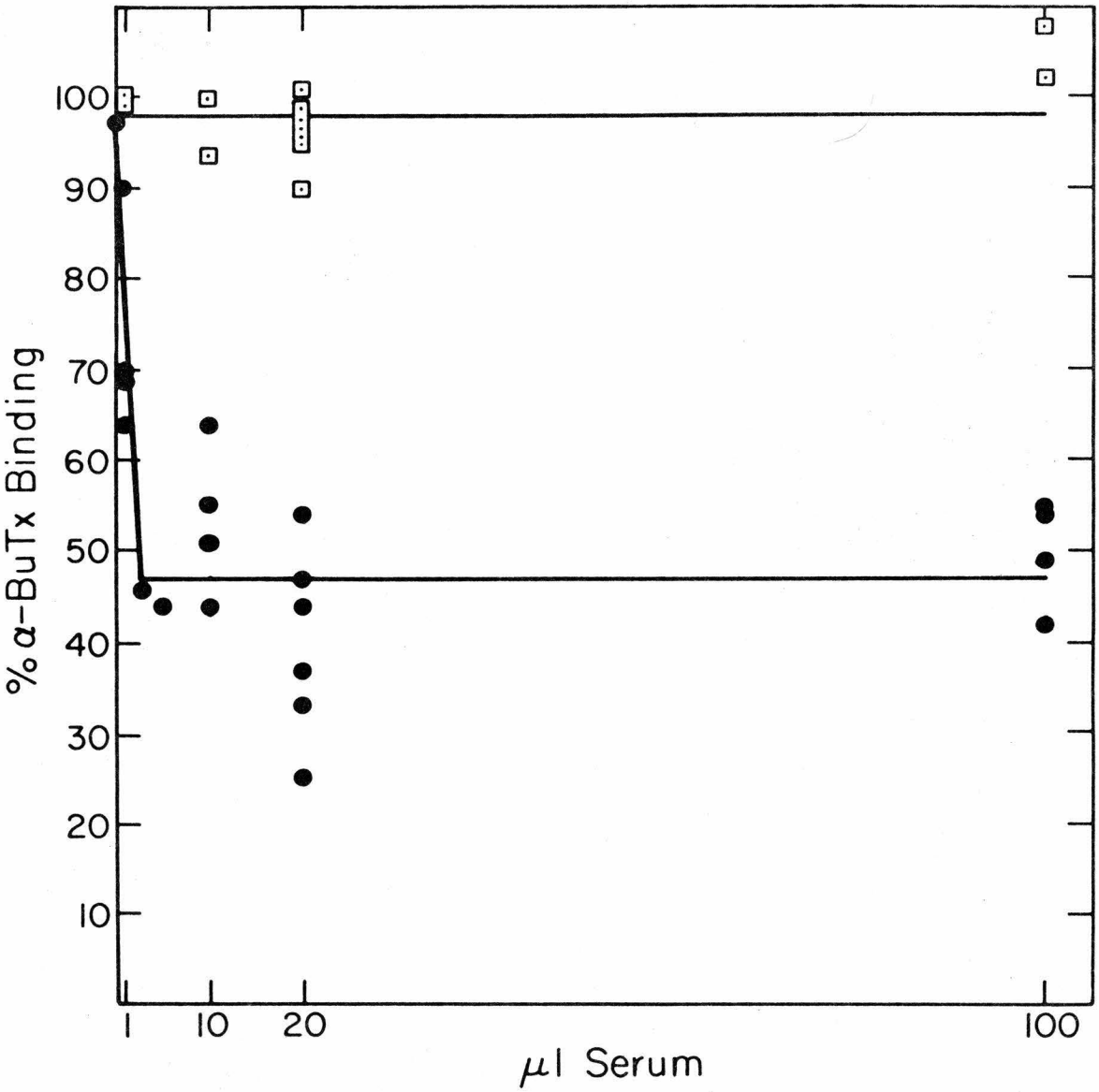

% α-BuTx Binding
100
90
80
70
60
50
40
30
20
10
1
10
20
100
μl Serum

TABLE I

% Inhibition of [^{125}I]αBuTx binding to MFTS by various rabbit antisera

Rabbit No.	Antigen	% Inhibition of α-BuTx Binding (Avg.)		Notebook	Comments
5	AcChR	42	(57±8.6)	TCIII-105	paralysis in 22 days
22	"	50		VD-38	paralysis in 19 days
23	"	62		CHI-103	paralysis in 32 days
24	"	60		CHI-110-2	paralysis in 23 days
25	"	54		CHI-110-4	paralysis in 23 days
8	"	62		TCV-105	died, unobserved 21 days after a 6th injection
19	"	70		TCVI-99 (serum)	paralysis in 26 days
19	"	59		TCVI-99 (Ig)	paralysis in 26 days
20	"	34	(35±5.7)	CDSVI-75B	no paralysis during 2 years
20	"	39		TCVI-128	"
20	"	26		TCVII-8	"
20	"	38		TCVI-149	"
20	"	40		TCVII-64	"
10	AcChR - SDS	10	(6±5)	TCIII-251	no paralysis during 9 months
10	"	3		TCIII-166	"
7	AcChR + SDS	11	(8±4.2)	TCIII-247	no paralysis during 3 months
7	"	5		TCIII-166	"
6	AcChR + α-BuTx	17		TCIII-105	paralysis in 22 days

TABLE II

Immunization Schedule

Rabbit #	Antigen	Injections			Bleedings			Comments
		mg	date	notebook	ml	date	notebook	
22	AcChR	1	2-10-75	VC-149		3-1-75	VD-38	paralysis
		0.5	2-23-75	VC-149				
23	AcChR	1	2-6-76	CHI-74	~120	3-10-76	CHI-103	paralysis
		1	2-17-76	CHI-74				
		0.5	3-5-76	CHI-75				
24	AcChR	0.5	4-5-76	CHI-110-2	~100	4-28-76	CHI-110-2	paralysis
		0.5	4-19-76	CHI-110-2				
25	AcChR	0.5	4-5-76	CHI-110-4	~60	4-28-76	CHI-110-4	paralysis
		0.5	4-19-76	CHI-110-4				

the percent toxin inhibition with antiserum from rabbit 6 (AcChR + α-BuTx) was complicated by the fact that the animal made antibodies to α-BuTx in addition to AcChR. Identical values for the amount of receptor-toxin complex bound to filter discs were obtained from the other 18 antisera tested when [^{125}I]α-BuTx was added to MFTS before antiserum. For the rabbit injected with AcChR + α-BuTx, however, a value 1.8 times the average of the other antisera was obtained. In all cases, saturating amounts of antisera were present and all of the receptor-toxin complexes could be precipitated.

An interesting observation was seen with antisera from rabbit 20. This animal had been injected 6 times over a period of 15 months with different AcChR preparations in Freund's Complete Adjuvant, Freund's Incomplete Adjuvant or no adjuvant. Although the animal would become irritable approximately 5 days after an injection, it has never developed paralysis. The antisera from five bleedings were tested for the ability to inhibit toxin binding to AcChR. All antisera did inhibit toxin binding but the percent inhibition was consistently lower than the average of the other anti-AcChR antisera. The average for rabbit 20 was 35% $\pm$ 5.7% inhibition compared to 57% $\pm$ 8.6% inhibition for the others.

The antisera from 29 Lewis rats immunized with AcChR $\pm$ SDS, MFTS $\pm$ SDS, MFTS -AcChR and buffer were tested for the ability to inhibit α-BuTx binding to solubilized membrane fragments. The results are summarized in Table III. Total muscle AcChRs were extracted and quantitated in each animal. An animal was judged to have EAMG if it had $\sim$30% the number of muscle AcChRs compared with control rat muscle AcChRs (see Chapter II). The antisera from 15 out of 15 rats which did not have EAMG could inhibit [^{125}I]α-BuTx binding to AcChR by only 2.7% $\pm$ 2.8%. The antisera from 10 out of 10 rats which did have EAMG

TABLE III

% Inhibition of [^{125}I]α-BuTx binding to MFTS by various rat antisera

Animal No.	Antigen	% Inhibition of α-BuTx Binding	Average % Inhibition	Comments[a]
41	Buffer control	0-1	1 ± 0.9	No EAMG
42		0		"
43		2		"
44		0		"
45		2		"
11	AcChR + SDS	5	3 ± 2.6	No EAMG
12		0		"
13		4		"
14		0-6		"
15		4		"
31	MFTS + SDS	5	4 ± 3.7	No EAMG
32		0		"
33		10		"
34		4		"
35		3		"
36	MFTS - AcChR	0	1 ± 1.8	± EAMG
38		0-2		"
39		4		"
40		0		"
46	AcChR	56-58	54 ± 6.0	EAMG
47		67		"
48		46		"
49		45-51		"
50		56		"
26	MFTS	46-49	55 ± 11.7	EAMG
27		75		"
28		63		"
29		45		"
30		53		"

[a]EAMG as determined by quantitation of the number of muscle AcChRs extractable from each animal. No EAMG indicates all AcChRs present; ± EAMG indicates 70% present; EAMG indicates 30% present.

inhibited [^{125}I]α-BuTx binding to AcChR by 55% ± 9%. The antisera from the four rats injected with MFTS-AcChR could not inhibit toxin binding to AcChR but they only contained 70% the number of muscle AcChRs as compared with control rat muscle AcChRs. Saturating amounts of antisera (enough to precipitate all receptor-toxin complexes) were not present in all tests. Saturating amounts were present in antisera from the AcChR, MFTS, and AcChR + SDS groups. Although saturating amounts were present in the AcChR + SDS group, none of these antisera could inhibit toxin binding to AcChR.

DISCUSSION

Antisera prepared against Torpedo californica acetylcholine receptor subunits or Fabs prepared from the immunoglobulin fraction of these antisera could not prevent [^{125}I]α-BuTx from binding to T. californica membrane fragments containing AcChR, Triton X-100 solubilized membrane fragments or affinity column purified AcChR. Anti-AcChR antisera and anti-AcChR Fabs could inhibit toxin binding to solubilized membrane fragments or purified AcChR to the extent of ∿50% but had no effect on non-solubilized membrane fragments. Quantities of anti-AcChR and anti-AcChR subunit antisera, and anti-AcChR and anti-41,000 M_r subunit Fabs were used such that all receptor-toxin complexes could be precipitated with the addition of SACI or a second antibody. The maximum amount of toxin inhibition which could be achieved, however, was only ∿50%. A 50-fold excess of antibody over the minimum amount needed to inhibit 50% toxin binding did not increase the percent inhibition. The result that anti-AcChR Fabs could inhibit the same amount of toxin binding to AcChR as anti-AcChR antibodies demonstrated that the inhibition of toxin binding was not due to antibody-mediated

aggregation of receptor molecules. Antibodies directed against AcChR could inhibit toxin binding although antibodies directed against isolated subunits or AcChR denatured in SDS, which all bound solubilized AcChR, could not inhibit toxin binding. Specific antigenic sites must therefore exist on AcChR molecules which, when bound by antibodies, can inhibit 50% of the toxin binding to AcChRs.

An average value of 49% toxin inhibition was seen with antisera from eight rabbits injected with T. californica AcChR. The average from two rabbits injected with AcChR + SDS was only 7% inhibition. The average percent toxin inhibition seen with antisera from 10 Lewis rats injected with AcChR or MFTS was 55%. But an average of only 3% inhibition was seen with antisera from 10 rats injected with AcChR + SDS or MFTS + SDS. A striking correlation was seen between rats with experimental autoimmune myasthenia gravis and the ability of their antisera to inhibit toxin binding to AcChR. The antisera from 10 out of 10 rats with EAMG inhibited toxin binding to AcChR while the antisera from 15 out of 15 rats which did not have EAMG did not inhibit toxin binding. A similar correlation probably also applies to rabbits. Although such a correlation exists, the physiological significance of the phenomenon is unclear. It should be noted that inhibition of toxin binding to non-solubilized membrane fragments could not be achieved with any antisera.

Objections with demonstrating impairment of [^{125}I]α-BuTx binding to solubilized AcChR have been two-fold (3). One objection has been that receptors may become aggregated by antibodies which results in impaired access of toxin. A second objection has been that any effect due to antibodies directed at intracellular determinants would be without effect in vivo. The first objection has been overcome by the results of the Fab experiments described in this chapter.

The second objection may still be a valid one. However, the results that anti-AcChR + SDS antiserum cannot inhibit toxin binding but anti-AcChR antiserum can, plus the correlation of these data to animals with EAMG, is suggestive evidence that the population of antibodies causing this inhibition may have some physiological significance.

This study therefore indicates that particular antigenic determinants on AcChRs can induce EAMG, and that these determinants are lost with SDS denaturation of AcChR. It will be important in future research to ascertain which determinants these are, and which are altered by denaturation. Further studies of AcChR-α-BuTx complex used as the immunogen can be expected to lead to critical insight into the nature of autoimmune myasthenia gravis.

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