Characterization of the Calcium-Binding Sites of the Purified Acetylcholine Receptor and Identification of the Calcium-Binding Subunit†

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ABSTRACT: The acetylcholine receptor isolated from *Torpedo ocellata* binds ~8 mol of a fluorescent lanthanide, terbium, per mol of α-bungarotoxin-binding sites. This process is accompanied by a fluorescence enhancement of 10^4 ($\lambda_{excitation}$ 295 nm; $\lambda_{emission}$ 546 nm) and allows detection of receptor–terbium complexes at μM concentrations. In the presence of calcium two types of terbium-binding sites are revealed, both with dissociation constants (for terbium) in the 18–25 μM range. About 60% of these sites bind calcium with an apparent dissociation constant of 1 mM. Most of the terbium-binding sites are associated with a subunit of the receptor of about 40 000 molecular weight, which we isolated. On the intact molecule, the same subunit also reacts with the affinity label *p*-(*N*-maleimido)-α-benzyl[*trimethyl*-^{3}H]ammonium iodide, which is an analogue of receptor activators. The terbium-binding sites are preserved when the receptor is degraded by trypsin and chymotrypsin to peptides of molecular weight of 8000 and less. These binding sites are, therefore, determined by structural features of the peptide chain rather than by the three-dimensional arrangement of the intact receptor. The affinity for terbium in the subunit and the 8000 mol wt peptides is the same as in the intact molecule. In the subunit and the peptides, all the terbium can be displaced from its binding site by calcium, but the affinity for calcium decreases by a factor of 4 ($K_{Ca} \approx 4$ mM). Acetylcholine does not interact with the terbium-binding sites in the subunits. In the intact acetylcholine receptor, acetylcholine displaces 3 to 6 terbium ions per α-bungarotoxin binding site.

Interactions between acetylcholine or its cogeners and the membrane-bound acetylcholine receptor initiate changes in the permeability of the neural membrane to inorganic ions and thus in the electrical potential. Experiments with nerve axons and single electroplax cells demonstrated that calcium ions strongly influenced the electrical behavior of excitable membranes, presumably by regulating the transport of inorganic ions across them (Frankenhaeuser, 1957; Frankenhaeuser & Hodgkin, 1957; Bartels, 1971).

The purification of the acetylcholine receptor protein in a number of laboratories (e.g., Klett et al., 1973; Biesecker, 1973; Karlin & Cowburn, 1973; Meunier et al., 1974; Chang, 1974; Schmidt & Raftery, 1973), and in particular by a rapid procedure (Eldefrawi & Eldefrawi, 1973) which gives very good yields of a highly purified receptor from *T. ocellata,* has made it possible to investigate the interaction of Ca^{2+} with this protein. These studies indicate that Ca^{2+} competes with acetylcholine and with a fluorescent ligand for binding sites on the receptor protein purified from *Torpedo californica* and *Electrophorus electricus* (Eldefrawi et al., 1975; Martinez-Carrion & Raftery, 1973; Chang & Neumann, 1976). In investigations using receptor-rich membrane fragments from *T. marmorata* an increased affinity of the receptor for acetylcholine receptor ligands in presence of Ca^{2+} was observed (Cohen et al., 1974). We have shown (Rübsamen et al., 1976a) that terbium (Tb^{3+}), which has been employed previously to characterize inorganic ion-binding sites of transferrin (Luk, 1971), trypsin (Epstein et al., 1974), and thermolysin (Bernier et al., 1975), binds to the purified acetylcholine receptor with a large fluorescence enhancement at 546 nm when excited in the 285–295-nm wavelength region. Ca^{2+} competes with Tb^{3+} for a majority of the sites. Acetylcholine receptor activators compete with Ca^{2+}-binding sites while inhibitors do not (Rübsamen et al., 1976a). The results have been confirmed and extended by Change & Neumann (1976).

This paper deals with further characterization of the interaction of acetylcholine receptor ligands with the Tb^{3+}-binding sites, and the purification and preliminary characterization of the subunit which contains the Ca^{2+}-binding sites. A short report of preliminary studies has appeared (Rübsamen et al., 1976b).

Experimental Procedures

Preparation of Acetylcholine Receptor and Assay of α-Bungarotoxin Binding Sites. Acetylcholine receptor protein was purified from the electric organ of the electric ray *T. ocellata* (collected near Alexandria, Egypt) by affinity adsorption on a cobra α-neurotoxin gel, according to a modified method from Eldefrawi & Eldefrawi (1973). Diisopropyl phosphorofluoridate (0.1 μM) was present during homogenization and solubilization of the electric tissue and 1 mM EDTA[1] was used during the entire purification procedure (Eldefrawi et al., 1975) which takes only 1 day. The receptor

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[1] Abbreviations used: $NaDodSO_4$, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; MBTA, *p*-(*N*-maleimido)-α-benzyltrimethylammonium iodide; dimethyl-POPOP, 1,4-bis-2-(4-methylphenyloxazolyl)benzene; PPO, 2,5-diphenyloxazole.

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was subsequently dialyzed against a tenfold volume of 10 mM Pipes (Sigma), 0.03% Triton X-100 (Sigma), pH 6.5, the pH being adjusted with Tris base (Sigma). The external solution was exchanged fives times at intervals of 4 h or more. The sixth exchange was made against the same buffer containing 10 μM $TbCl_3$ (Alfa Ventron). Tb^{3+} was subsequently removed by another three exchanges of the external solution against 2 mM Pipes/Tris buffer (pH 6.5) containing 0.03% Triton X-100. The complete procedure was carried out at 4 °C. Nalgene containers were used to prevent contamination of the preparation with Ca^{2+} or other inorganic ions. Contamination with Ca^{2+} was checked by atomic absorption. Protein concentrations were determined by the method of Lowry et al. (1951) using recrystallized bovine serum albumin as a standard. In addition to the protein concentration, we routinely determined the concentration of α-bungarotoxin binding sites in the preparations by titration with [^{125}I]-α-bungarotoxin prepared as described by Bulger et al. (1977): A 1.3–1.5 μM solution of [^{125}I]-α-bungarotoxin was made in 10 mM Na_2HPO_4, 1% Triton X-100 (pH 7.4). The receptor concentration was adjusted to 0.03–0.04 mg/mL using the same buffer. In small Nalgene test tubes (holding 1 mL total), 0, 1, 2, 3, 5, 10, 20, 25, 35, and 50 μL of the receptor solution were diluted with the phosphate buffer to give a total volume of 125 μL to which was added 5 μL of the [^{125}I]-α-bungarotoxin. The tubes were capped and incubated at 20 °C for 3 h. Free and bound toxin were then separated by applying 100 μL of the incubation mixture, followed by two 50-μL aliquots of the phosphate buffer to a small carboxymethylcellulose column (Whatman CM 52, 1 mL of packed resin in a glass Pasteur pipet) equilibrated with the same buffer (Kohanski et al., 1977). The receptor–toxin complex was eluted with 1 mL of the phosphate buffer.

Measurement of Tb^{3+} Binding to the Acetylcholine Receptor. All fluorescence measurements were made using a Perkin-Elmer MPF3 spectrofluorimeter equipped with a thermostated holder for a microcell. The excitation wavelength was 295 nm, and the emission spectrum was recorded from 535 to 560 nm. A cutoff filter for wavelengths below 430 nm was used in the emission beam. The slit width was normally 10 nm for both excitation and emission. The sensitivity of the instrument was set at 10 or 30 for titration of the receptor with Tb^{3+} and at 30 or 100 for studies of the displacement of Tb^{3+} by effectors. All experiments were done at 20 °C, in 2 mM Pipes/Tris, 0.03% Triton X-100, pH 6.5. For titration of the receptor with Tb^{3+}, 600 μL of receptor (0.04–0.08 mg/mL) was pipetted into a stoppered 4 × 4 mm microcell and a total of 10 μL of 1 mM and 15 μL of 10 mM $TbCl_3 \cdot 6H_2O$ in the same buffer were added in steps of 0.5–1 μL. Three measurements were made for each concentration of Tb^{3+} used and the solutions were mixed before each measurement by inverting the microcell. The peak heights of the different scans were averaged. All fluorescence readings were normalized by dividing by the reading obtained from a 10^{-3} M solution of $TbCl_3$ in H_2O. The Tb^{3+} concentrations in the stock solutions were checked by titration with EDTA using arsenazo I (Aldrich) as indicator (Woyski & Harris, 1963) and by atomic absorption spectroscopy. For determination of the displacement of Tb^{3+} by chemical mediators, an amount of Tb^{3+} was added that was, by at least a factor of 6, below its dissociation constant, and the solution was incubated for 5–10 min at 20 °C. The effector was then added stepwise and fluorescence was recorded after each addition as described for the titration with Tb^{3+}, except that five measurements were made at each Tb^{3+} concentration.

Determination of the Number of Moles of Tb^{3+} Bound to the Acetylcholine Receptor. Four 1.5-mL solutions of receptor (0.05–0.08 mg of protein/mL) in 2 mM Pipes/Tris buffer, 0.03% Triton (pH 6.5) were dialyzed against a tenfold volume of the same buffer containing various amounts of Tb^{3+} (5–30 μM). After 12 h at 4 °C, the outside solution was exchanged and dialysis continued for another 12 h. Three hundred microliters of the dialyzed receptor solution was titrated with Tb^{3+} using fluorescence measurements. Another 100 μL was used for titration with [^{125}I]-α-bungarotoxin. A total of 900 μL was lyophilized, 300 μL at a time, in a 1-mL quartz vial. Nine hundred microliters of the bath solution was lyophilized in the same way. The lyophilized samples were dried for 6 h at 80 °C and the vials heat-sealed and used for activation analysis (Bowen & Gibbons, 1963). The amount of Tb^{3+} bound at saturation was calculated from a Scatchard (1949) plot of bound Tb^{3+} vs. bound Tb^{3+}/free Tb^{3+} as determined by activation analysis. The dissociation constant for Tb^{3+} obtained by this method was checked with the dissociation constants derived from a plot of the fluorescence obtained at the four Tb^{3+} concentrations used, and from the fluorescence-titration to saturation of each of the four samples.

Equilibrium Dialysis with [^{3}H]Acetylcholine. The acetylcholine receptor solution was incubated with 0.1 mM tetram (*O,O*-diethyl *S*-diethylaminoethyl phosphorothiolate) for 1 h at 20 °C to inhibit esterase activity without affecting acetylcholine binding (Eldefrawi et al., 1971). Aliquots (0.45 mL, 0.05–0.08 mg of protein/mL) were then pipetted into 6-in. long dialysis bags (0.25 in., Union Carbide), and each bag was placed in an Erlenmeyer flask containing 50 mL of 2 mM Pipes/Tris buffer (pH 6.5), 0.03% Triton X-100, and one of a series of concentrations of [^{3}H]acetylcholine (sp act. 49.5 mCi/mmol, from New England Nuclear) and 1 μM tetram. The flasks were then shaken for 16 h at 4 °C. Three 100-μL aliquots were taken from both the bath and the bag and counted in 10 mL of scintillation solution (3.8 L of toluene, 18.05 g of PPO, 1.22 g of dimethyl-POPOP, 150 mL of BBS-3 (Beckman) in a Packard 3380 liquid scintillation spectrometer. The experiment was performed in triplicate.

Synthesis of p-(N-Maleimido)-α-benzyl-[trimethyl-^{3}H]ammonium Iodide ([^{3}H]MBTA). The affinity label was synthesized essentially according to the procedure described by Karlin et al. (1971). The following changes in the published procedure were made.

p-Nitrobenzyldimethylamine (I) was synthesized by a procedure modified from Goss et al. (1926). Sixty-one grams of hexamethylenetetramine (0.44 mol) was dissolved in the smallest quantity of boiling ethanol and about 600 mL of a hot alcoholic solution containing 50 g (0.291 mol) of *p*-nitrobenzyl chloride was added. The quaternary salt, which separated on cooling of the solution overnight at 4 °C, was collected, suspended in about 400 mL of boiling water, and 350 mL of concentrated HCOOH was added. The clear solution was heated for 22 h at 90 °C while stirring constantly. The solution, on an ice bath, was made alkaline with NaOH pellets that were added slowly, while stirring. The solution was extracted five times with 100 mL of ether. The combined ether fractions were dried over $CaCl_2$ and the ether was removed under reduced pressure. The remaining brown oil was transferred into a 50-mL flask and distilled under reduced pressure. The yield was 19.6 g (37%) of a yellow oil (I), bp 148 °C (18 mmHg), and 10.5 g (20%) of a yellow oil bp 120–148 °C (18 mmHg).

For synthesis of p-Aminobenzyldimethylamine (II) (modified from Stedman, 1927), 30 g of I was mixed with 51 g of granulated tin, and 200 mL of concentrated HCl was added slowly with efficient cooling on ice and constant stirring.

The solution was then heated on a water bath for 30 min, cooled, brought to pH 4–5 by addition of Na_2CO_3, and the volume adjusted to about 500 mL. The Sn^{2+} ions were removed from the solution by passing a vigorous stream of H_2S through it for about 30 min at 20 °C while stirring. After another 30 min, the SnS was filtered off by suction, the filtrate was made alkaline with Na_2CO_3, and brought to pH 11 with 1 N NaOH. The solution was extracted with ether, the ether was removed, and the resulting yellow oil was distilled under reduced pressure (yield: 13.6 g (55%) of a colorless oil, bp 137 °C (18 mmHg).

N-(4-α-Diemthylaminobenzyl)maleamic Acid (III) and 4-(N'-maleimido)-N,N-dimethylbenzylamine (IV) were prepared essentially as described by Karlin et al. (1971). Compound IV was found to undergo considerable polymerization within a week, even when stored at 0 °C in the dark. Therefore, before the next step, the sample was dissolved in CCl_4 and the insoluble polymer removed by filtration.

Synthesis of p-(N-maleimido)-α-benzyl[trimethyl-^{3}H]ammonium iodide was modified from Karlin et al. (1971). The vial containing 25 mCi of [^{3}H]CH_3I (300 mCi/mmol, 11.8 mg, from ICN) was heat sealed onto a vacuum line. Compound IV (131 mg) was dissolved in 0.5 mL of dry CH_2Cl_2 and 0.5 mL of dry ether, and the solution transferred to the reaction chamber of the vacuum line which had been flushed with dry argon. The reaction chamber was cooled with liquid nitrogen. After the reaction mixture was well frozen, the pressure in the vacuum line was reduced to 10^{-3} Torr. The connection to the vacuum pump was shut off and the seal of the vial containing the [^{3}H]CH_3I was broken. After 10 min, the reaction chamber was sealed off under vacuum and stored for 6 days at 20 °C in the dark. Then the reddish precipitate was removed and washed four times by suspending it in ether and centrifuging in a Vari Hi-speed centricone. Further purification was achieved by dissolving the precipate in 0.2 mL of CH_2Cl_2 and 0.8 mL of acetonitrile and removing the red contaminant by centrifugation. The yellow solution was then added dropwise to 7 mL of ether with vigorous stirring. The bright yellow precipitate was collected by centrifugation and dried *in vacuo*. The procedure was repeated once more (yield: 15 mg (48%)). The UV spectrum (Karlin et al., 1971) indicated that the substance was pure. Storage of the [^{3}H]MBTA at −196 °C as a dilute solution in acetonitrile and determination of concentration for labeling experiments were as described by Karlin et al. (1971).

Affinity Labeling of the Acetylcholine Receptor. The labeling procedure was a modification of that given by Karlin & Cowburn (1973). Acetylcholine receptor (0.6 mg) was reduced for 10 min at 27 °C with 0.2 mM dithiothreitol in 900 μL of 0.2% Triton X-100, 150 mM NaCl, 19 mM phosphate buffer, 1 mM EDTA, pH 8. The reaction was then slowed by adding 100 μL of 0.53 M phosphate buffer, pH 5.5, in order to lower the pH to about 6.5, and by cooling in ice. The receptor was immediately separated from the dithiothreitol by passing it through a Sephadex G-25 (superfine) column (0.6 cm wide and 20 cm high) which was equilibrated with the same buffer used for reduction except that it was at pH 7. The fractions containing the receptor were pooled and 500-μL samples were labeled at a final concentration of [^{3}H]MBTA (300 mCi/mmol) of 1.07 μM and of 0.2 mg of receptor protein/mL at 25 °C for times varying from 2 to 10 min. The reaction was stopped by adding 100 μL of 12 mM β-mercaptoethanol and placing the vial on ice. Free and bound label were separated using another G-25 column of the same size, which was equilibrated with 0.1 mM phosphate buffer at pH 7.5. The labeled fractions were pooled and dialyzed overnight against 0.1 mM phosphate buffer, pH 7.5, at 4 °C. After this they were dialyzed in Spectrapore No. 1 tubing (Horizon Ecology Co.) for 6 h at room temperature against a tenfold volume of 0.1% $NaDodSO_4$, 0.1 mM phosphate buffer, pH 8. The receptor was lyophilized and then denatured by dissolving it in 50 μL of 2% β-mercaptoethanol, 2% $NaDodSO_4$, 10% sucrose and incubated at 40 °C for 2 h. Samples containing about 30 μg of protein in 15 μL were applied to analytical 6.4% $NaDodSO_4$–polyacrylamide gels (for gels and buffer composition see below), which had been prerun at 1.5 mA/tube for 3 h. The separating run was done at 3 mA/tube. The gels were stained for protein with 7.5% trichloroacetic acid and 0.25% Coomassie Brilliant Blue (Bio-Rad) at 20 °C for 10 h and then destained by diffusion in 7.5% acetic acid at 40 °C for about 1 day with several exchanges of the destaining solution. The gels were scanned at 560 nm in a Gilford spectrophotometer and then cut into slices, 1 mm thick. The slices were placed in scintillation vials, dried for 4–6 h; then 100 μL of H_2O and 500 μL of NCS solubilizer (Amersham) were added. The vials were capped and incubated at 40–60 °C for 24 h. Afterwards 10 mL of scintillation cocktail was added and the samples were counted in a Beckman LS-230 scintillation counter.

Purification of Subunits of the Acetylcholine Receptor. The purified receptor was lyophilized and then denatured by treating about 600 μg with 200 μL of 2% $NaDodSO_4$, 2% β-mercaptoethanol, 10% sucrose, for 2 h at 40 °C. Gel slabs (0.3 × 10.3 × 8.7 cm) were made 12 h before use from the following: 6.4% acrylamide, 0.2% *N,N'*-methylenebis(acrylamide), 0.5% $NaDodSO_4$ (all from Bio-Rad), 0.3 M Tris, 0.24% ammonium persulfate, 0.04% *N,N,N',N'*-tetramethylethylenediamine (Eastman), pH 8.8. The gels were cleaned by a prerun at 15 mA per slab for 3 h using the following buffers: (upper buffer (per liter)) 6.32 g of Tris base, 3.94 g of glycine, 0.3 g of $NaDodSO_4$ (pH 8.9); (lower buffer) the same composition and pH but no $NaDodSO_4$ was added. The separating run was done at 30 mA per slab at 20 °C in the same buffer system. Acetylcholine receptor (600 μg) was applied to each slab. After the run (approximately 3 h), the gels were laid on an ice-cold glass plate and cut into slices 0.45 cm thick. The slices were slid into glass Pasteur pipets, the tips of which had been cut off and fire polished in order to obtain a tube with a faint constriction at one end. Before adding the slice, the constricted ends were plugged with a small amount of fresh gel and soaked after polymerization for 3 h in distilled water. The space between the glass and the gel slice was filled with new gel. After polymerization the outside of the tube was carefully rinsed with distilled water. This was necessary since small poly(acrylamide) gel particles interfered with the protein determination and the Tb^{3+} fluorescence assay. Spectropore No. 1 dialysis tubing (cleaned by treating with ethanol, $NaHCO_3$, and EDTA) was cut into strips, 10 cm long, knotted at one end, filled with 500 μL of lower buffer, and slipped over the constricted end of the pipet so that the end was immersed in the buffer. The bags were tied to the pipet using Parafilm. The protein was recovered from the slices by electrophoresis for 12 h at 3mA/tube at 4 °C. After this, the dialysis tube was taken off the pipets, the upper end was tied with thin nylon thread, and the bags thus formed were suspended in the dialysis buffer. The subunits were dialyzed against a tenfold excess of 2 mM Pipes/Tris buffer containing 0.03% Triton X-100, pH 6.5. The dialysis solution was exchanged nine times at intervals of 4 h or less. The buffer for the sixth exchange contained 10 mM Tb^{3+}. The fractions were stored in Nalgene test tubes at 1 °C.

Analysis for Tb^{3+} Binding and Protein Concentration of the Isolated Subunits. The volume of each protein fraction was

TABLE I: Dissociation Constants of Inorganic Ions from the Isolated Acetylcholine Receptor, Subunit IV, and Acetylcholine Receptor Peptides.[a]

Ion	K_D[b]	% of Tb^{3+}-binding sites that interact with competing ion	$\Delta F_{p(max)}$[e] (mg^{-1})
A. Acetylcholine receptor			
Tb^{3+}	18-25 μM[c]		2-2.3[c]
Ca^{2+}	1 ± 0.4 mM[c]	60	
Mg^{2+}	2 ± 0.4 mM	60	
Na^+	60 mM[d]	100[d]	
K^+	130 mM[d]	100[d]	
B. Sununit IV			
Tb^{3+}	19 ± 1 μM		3-4
Ca^{3+}	4 ± 0.7 mM	100	
C. Acetylcholine receptor peptides			
Tb^{3+}	22 ± 2 μM		2.2
Ca^{2+}	3.9 ± 0.9 mM	100	

[a] The values were determined by fluorescence measurements of Tb^{3+} binding (in absence and presence of other inorganic ions) in 2 mM Pipes/Tris buffer, pH 6.5, 0.03% Triton X-100, 20 °C. [b] The measured equilibrium constants represent averages of a distribution of constants. [c] Obtained in experiments with six different receptor preparations. [d] The experiments with NaCl and KCl were conducted at 50 mM concentrations of these salts. Control experiments with Tris-HCl and choline hydrochloride indicated that an ionic strength of 50 mM or less did not affect the dissociation constant of the receptor-Tb^{3+} complexes significantly. K_{Tb} was found to be 26 ± 0.2 μM in 50 mM Tris-Cl and 32 ± 0.2 μM in 50 mM choline chloride solutions. [e] The normalized fluorescence (see Experimental Procedures) of bound Tb^{3+} per milligram protein at 546 nm obtained at saturation with Tb^{3+}.

adjusted to 1.5 mL. In a microcuvette 100 μL of each fraction was diluted with 100 μL of Pipes buffer. A total of 10 μL of a 1 mM Tb^{3+} solution was added in steps of 3 and 7 μL. Fluorescence was measured after each addition as described above. Protein concentration was determined by the method of Lowry et al. (1951). The values for Tb^{3+} fluorescence (at 50 μM) and for protein concentration were corrected using readings obtained from a blank gel run in parallel that had not been loaded with receptor. Slices cut at the sides of the gels from top to the dye front (Bromphenol Blue) were fixed and stained for protein.

Preparation of Acetylcholine Receptor Peptides. To the purified receptor (0.94 μM α-bungarotoxin binding sites) in 2 mM Pipes/Tris buffer, 0.03% Triton X-100 (pH 6.5), were added 1 μM trypsin and 1 μM chymostrypsin (Worthington). The mixture was left for 48 h at 4 °C; then titration with Tb^{3+} was performed as described for the intact molecule. The data were corrected by subtracting the readings from a Tb^{3+} titration of a sample of the enzymes alone incubated under identical conditions.

Results

Fluorescence data for titration of the acetylcholine receptor with Tb^{3+} were calculated from Scatchard plots of $\Delta F/\Delta F_{max}$ vs. $\Delta F/F_{max}/[Tb^{3+}]$. ΔF is the observed fluorescence intensity in arbitrary units at 546 nm, a peak in the fluorescence spectrum, minus the intensity at 535 nm, a trough in the spectrum. ΔF_{max} is the maximum observed intensity when all ligand-binding sites are occupied. The ΔF_{max} values, normalized to moles of α-bungarotoxin-binding sites, agreed within 15% for six preparations of purified receptor tested. A similarly close agreement between receptor preparations was found for the dissociation constants for Tb^{3+} and Ca^{2+} (Table I). The affinities of other inorganic ions for the purified receptor were determined by titration with Tb^{3+} in the presence of the ion (Rübsamen et al., 1976). This procedure is illustrated in experiments with acetylcholine in Figure 1. Mg^{2+} binds to the Tb^{3+}-binding sites with an affinity similar to Ca^{2+}, and Na^+ and K^+ with affinities 60 to 130 times lower. The number of Tb^{3+} bound per receptor molecule was determined by neutron

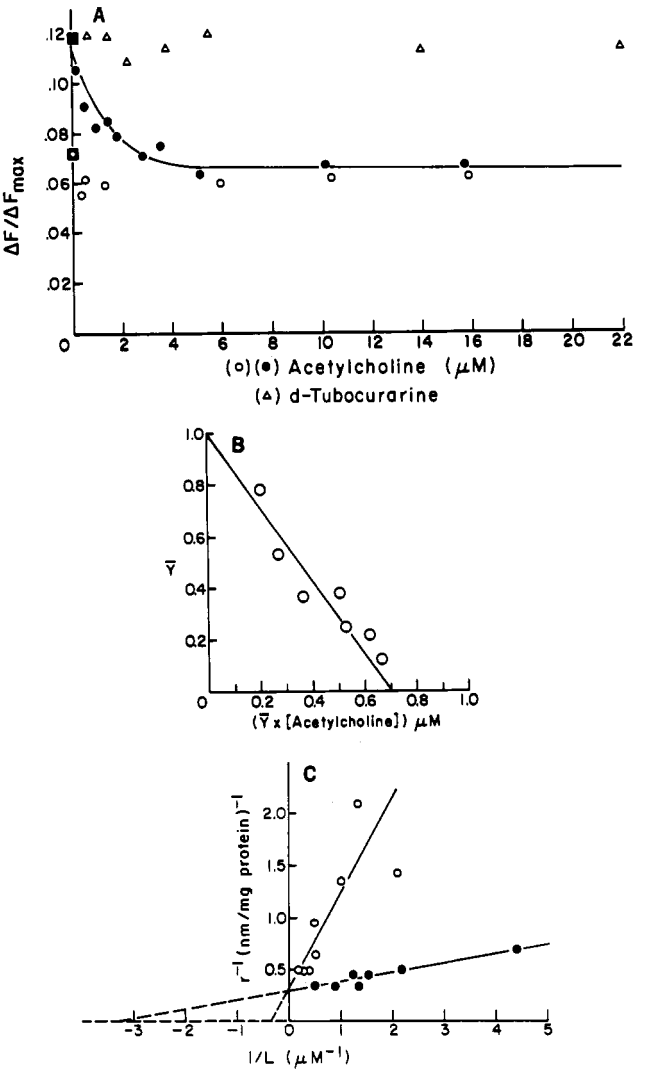

FIGURE 1: Displacement of Tb^{3+} from the purified acetylcholine receptor by acetylcholine (in 2 mM Pipes/Tris buffer (pH 6.5), 0.03% Triton X-100, 20 °C). (A) Displacement of Tb^{3+} by acetylcholine. Acetylcholine receptor (0.6 μM α-bungarotoxin-binding sites), 3.3 μM $TbCl_3$; (●) titration with acetylcholine in absence of $CaCl_2$; (○) titration with acetylcholine in presence of 6 mM $CaCl_2$; (▵) titration with *d*-tubocurarine; (■) receptor-Tb^{3+} complex before addition of ligand; (□) receptor-Tb^{3+} complex in presence of 6 mM $CaCl_2$ before addition of ligand. (B) The decrease in fluorescence signal due to displacement of Tb^{3+} from the receptor by acetylcholine is plotted according to a linear equation, $\bar{Y} = \Delta F_\alpha'/\Delta F_\alpha = 1 - \Delta F_\alpha'/\Delta F_\alpha([L]/K_D)$, where [L] is the molar concentration of acetylcholine and K_D the receptor-acetylcholine dissociation constant. Subtracting the value for ΔF obtained at saturating concentrations of acetylcholine (see A) from the observed values obtained in absence and presence of acetylcholine gives the values of ΔF_α and $\Delta F_\alpha'$, respectively. The equation has been derived previously, and the simple assumptions involved have been stated (Rübsamen et al., 1976a). The reciprocal slope of the graph gives K_D = 0.7 μM. (C) Binding of [^{3}H]-acetylcholine to the acetylcholine receptor (in 2 mM Pipes/Tris buffer, 0.1% Triton X-100 (pH 6.5), 4 °C). r = nmol of ligand bound per mg of protein; L = ligand concentration; (●) in absence of Tb^{3+}; (○) in presence of 50 μM Tb^{3+}. The dissociation constants for Tb^{3+} and acetylcholine, calculated from a least-squares analysis of these data, are: $K_{D(Tb)}$ = 10 ± 4 μM; $K_{D(acetylcholine)}$ = 0.3 ± 0.03 μM.

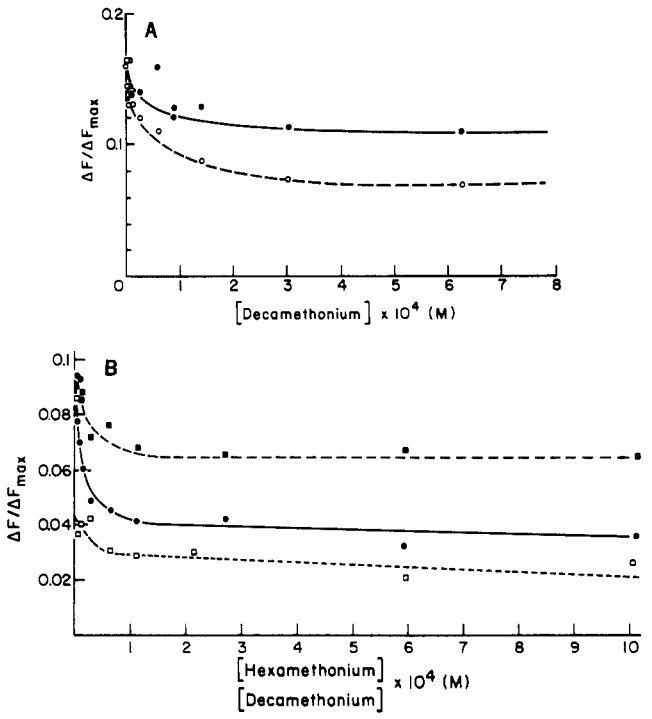

FIGURE 2 (A): Displacement of Tb^{3+} by decamethonium before and after treatment of the acetylcholine receptor with α-bungarotoxin. Acetylcholine receptor (0.6 μM α-bungarotoxin sites), 4.2 μM Tb^{3+}. (□) Acetylcholine receptor, kept at 20 °C for 2 h before titration with decamethonium; (■) acetylcholine receptor, reacted for 2 h at 20 °C with 3 μM Δ-bungarotoxin; (○) receptor titrated with decamethonium; (●) acetylcholine receptor, reacted for 2 h at 20 °C with 3 μM α-bungarotoxin and then titrated with decamethonium. (B) Displacement of Tb^{3+} from acetylcholine receptor by hexamethonium. Acetylcholine receptor (0.6 μM α-bungarotoxin sites), 2.5 μM Tb^{3+}. (■) Titration with hexamethonium; (●) titration with decamethonium; (□) titration with hexamethonium in presence of 6 mM $CaCl_2$.

activation analysis (Bowen & Gibbons, 1963). The average value from five determinations on four different preparations was 5 to 10 mol of Tb^{3+} bound per mol of α-bungarotoxin-binding site. The quantity of receptor required for these measurements and the expense involved in the analysis prevented a more accurate determination. The fluorescence enhancement calculated on the basis of 7 Tb^{3+} bound per receptor site is 1×10^4.

Figure 1A shows that acetylcholine displaces about 50% of the Tb^{3+} bound to the receptor (●) when the Tb^{3+} concentration is less than K_{Tb}, the receptor–terbium dissociation constant. When some of the bound Tb^{3+} was first displaced by 6 mM Ca^{2+}, and acetylcholine was subsequently added, acetylcholine did not displace an additional amount of Tb^{3+} (○). This indicates that acetylcholine displaces Tb^{3+} from the Ca^{2+}-binding sites. A quantitative analysis of the displacement of Tb^{3+} by acetylcholine (Figure 1B) gave a dissociation constant for the receptor–acetylcholine complex of 0.7 ± 0.1 μM. At 20 °C a value of 0.3 μM for the same constant is determined by equilibrium dialysis at 4 °C (Figure 1C (●)). In the equilibrium dialysis experiments 50 μM Tb^{3+} (○) acted as a competitive inhibitor. The dissociation constant for the Tb^{3+}–receptor complex obtained from this experiment, 10 ± 4 μM, is in agreement with the dissociation constant determined by direct fluorescence titration (Table I).

Figure 2 shows that the decamethonium (○) displaced about 50% of the Tb^{3+} bound to the receptor. The solid points are the results of an experiment in which the receptor was first reacted for 2 h with a threefold excess of α-bungarotoxin at 20 °C.

TABLE II: Apparent Molecular Weights[a] and Relative Amounts of the Subunits of the Acetylcholine Receptor.

Subunit no.	Mol wt	% of total protein	% of total fluorescence[b]	% of total [^{3}H]MBTA[c] bound
I	81 000	8	5	~10
II	61 000	26	7	~5
III	50 000	21	5	~5
IV	40 000	35	83	~80

[a] The apparent molecular weights were determined by comparison with the standard proteins ovalbumin, bovine serum albumin, and phosphorylase A (molecular weights: 43 000, 68 000, and 100 000 (Weber et al., 1972)). They are average values from determinations on five different preparations. [b] ΔF_p, the normalized fluorescence (see Experimental Procedures) per milligram protein on addition of 50 μM Tb^{3+} to the subunit solution was determined. (ΔF_p × mg of protein/subunit) gives the fluorescence yield per subunit. [c] *p*-(*N*-Maleimido)-α-benzyl[*trimethyl*-^{3}H]ammonium iodide, activator analogue.

Under these conditions, all the α-bungarotoxin binding sites had reacted as checked by titration of the receptor–toxin complex with [^{125}I]-α-bungarotoxin (Kohanski et al., 1977). We have shown previously (Rübsamen et al., 1976a) that α-bungarotoxin does not interfere with Tb^{3+} binding. In the presence of α-bungarotoxin, decamethonium still displaced Tb^{3+} from the receptor–toxin complex (●), although only about one-half the amount which was displaced from the untreated receptor (○). Similar results were obtained when carbamoylcholine displaced Tb^{3+} from the receptor–toxin complex.

The data in Figure 2B indicate that hexamethonium, an inhibitor of receptor-mediated processes, displaces from the receptor about one-half the amount of Tb^{3+} which is displaced by the activators, acetylcholine, decamethonium, or carbamoylcholine. In the experiment shown in Figure 1A, we have demonstrated that acetylcholine does not displace Tb^{3+} from the receptor in presence of 6 mM $CaCl_2$, and that the amount of Tb^{3+} displaced by 6–8 mM Ca^{2+} is essentially the same as that displaced by saturating concentrations of acetylcholine (Figure 1A). In contrast to this, the data in Figure 2B indicate that hexamethonium displaces the same amount of Tb^{3+} in the presence and absence of 6 mM Ca^{2+}. This suggests that hexamethonium displaces Tb^{3+} mostly from sites which do not interact with Ca^{2+}.

To determine which subunits of the acetylcholine receptor carry the binding sites for Ca^{2+}, we isolated the subunits and checked for Ca^{2+} and Tb^{3+} binding. The distribution of protein and Tb^{3+} fluorescence after separation of the subunits on a $NaDodSO_4$ gel is shown in Table II. Assuming that the Tb^{3+}-binding sites in the various subunits contribute equally to the total fluorescence yield, 80% of these sites are associated with subunit IV. The maximum fluorescence per mg of protein, $\Delta F_{p(max)}$ determined from Scatchard (1949) plots, is 3.8 ± 0.2 (mg^{-1}) for subunit IV in this preparation. Subunit IV obtained from nine different receptor preparations gave $\Delta F_{p(max)}$ values 1.5 to 2.0 times higher than the values obtained with native receptor preparations for which $\Delta F_{p(max)}$ was about 2 (mg^{-1}). When 50 μM Tb^{3+} was used in experiments with the other three subunits, the ΔF_p values were consistently less than 0.5 and in two out of nine preparations no fluorescence was observed. Five of the nine preparations investigated gave a similar protein distribution (Table II), and in four preparations almost 50% of the protein was found in subunit IV. The apparent molecular weights of the subunits, as calculated (Weber et al.,

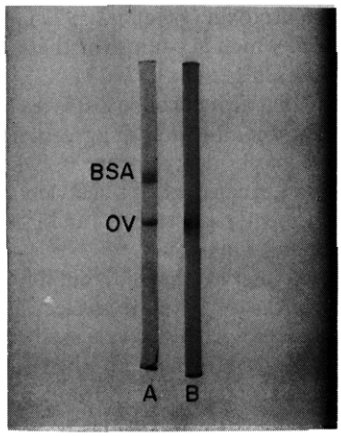

FIGURE 3: Analytical 6.4% $NaDodSO_4$–polyacrylamide gel electrophoresis of subunit IV of the acetylcholine receptor. About 25 μg of the subunit IV was lyophilized and denatured again by the addition of 30 μL of the denaturation mixture as described in the text. Otherwise the subunit reaggregated into larger species. This solution was applied to the gel and standard proteins were run in parallel. (A) Bovine serum albumin (BSA) and ovalbumin (OV); (B) subunit IV.

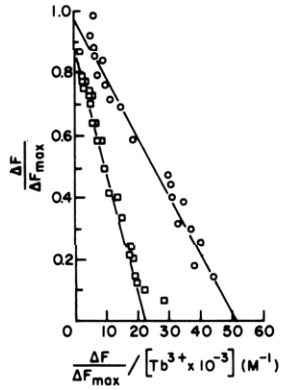

FIGURE 4: Titration of the acetylcholine receptor peptides produced by enzymic hydrolysis (2 mM Pipes/Tris buffer (pH 6.5), 0.03% Triton X-100, 20 °C) with Tb^{3+} in presence and absence of Ca^{2+}. The receptor had 0.9 μM α-bungarotoxin-binding sites before enzyme hydrolysis. The fluorescence measurements at 546 nm were corrected by subtracting the fluorescence obtained at the same Tb^{3+} concentration from a solution of the enzymes used for hydrolysis (1 μM trypsin and 1 μM chymotrypsin). (○) No Ca^{2+} added; (□) 4.3 mM Ca^{2+} added. The dissociation constants calculated from the data are: $K_{Tb} = 22 \pm 0.2$ μM; in presence of 4.3 mM Ca^{2+}; $K_{Tb}' = 51 \pm 0.2$ μM. The dissociation constant for Ca^{2+}, derived from these data, is 4 ± 1 mM.

1972) by comparison with the calibration proteins, phosphorylase A, bovine serum albumin, and ovalbumin, are also given in Table II.

The subunit which carries the binding sites for activators was identified by labeling the receptor before denaturation with the affinity label and activator analogue *p*-(*N*-maleimido)-α-benzyl[*trimethyl*-^{3}H]ammonium iodide, developed by Karlin et al. (1971). Almost 80% of the radioactivity was associated with subunit IV (Table II). Attempts to decrease the labeling of the other subunits by either reducing the reaction time or lowering the concentration of label, were unsuccessful, since the overall labeling pattern remained the same and only the total amount of label incorporated decreased. The total amount incorporated under the conditions used was 3 nmol/g receptor protein. This value is similar to the value reported by Weill et al. (1974) but does not reflect the total number of binding sites measured by α-bungarotoxin. This subunit was isolated for further characterization; an analytical 6.4% $NaDodSO_4$–polyacrylamide gel electrophoresis is shown in Figure 3.

The Tb^{3+}-binding isotherms of acetylcholine receptor peptides in absence and presence of 5 mM Ca^{2+} are shown in Figure 4. The peptides were produced by enzymic hydrolysis of the receptor with trypsin and chymotrypsin to peptides of molecular weight of about 8000 or less as determined by polyacrylamide gel electrophoresis. The fluorescence of bound Tb^{3+} per mg of protein at 546 nm measured at saturation with Tb^{3+}, $\Delta F_{p(max)}$, was 2.2. The same value was obtained with the native receptor prior to enzymic hydrolysis, indicating that Tb^{3+}-binding sites had not been lost in the receptor peptides. The data are plotted in the form of a Scatchard (1949) plot in which $\Delta F/\Delta F_{max}$ gives the fraction of Tb^{3+} sites occupied. As can be seen the data are consistent with independent Tb^{3+}-binding sites in absence of Ca^{2+} (○). The apparent dissociation constant of these sites is 22 ± 0.2 μM. In presence of 4.3 mM Ca^{2+} (□) the apparent dissociation constant for Tb^{3+} increases as is indicated by the increases slope of the line, $K_{Tb}' = 51 \pm 0.2$ μM. Assuming that Ca^{2+} interacts competitively with the Tb^{3+}-binding sites, the apparent dissociation constant for Ca^{2+} from these sites is 3.9 ± 0.9 mM. This is about four times higher than the value obtained in experiments with intact receptor, but is the same as found for the isolated subunit IV (Table I).

Discussion

In our experiments, activators of receptor-mediated processes displaced Tb^{3+} in 80% of more than 30 different receptor preparations investigated. We found that several factors contributed to "inactive" preparations. The time taken to isolate the receptor was critical. The procedure used (Eldefrawi & Eldefrawi, 1973) now requires only 1 day for isolation and purification of the protein and 2 days for exchange of buffers (see the Experimental Procedure). The ability of activators to displace Tb^{3+} lasted for a few more days, when the freshly prepared receptor was stored at 1 °C. The moles of acetylcholine bound per mole of receptor, from 10 to 12 nmol per mg of receptor protein, decreased correspondingly with time. The α-bungarotoxin-binding sites, between 10 and 12 nmol per mg of receptor protein, were usually stable for up to 3 weeks. The decrease in the ability of activators to displace Tb^{3+} also strongly depended on the state of aggregation of the receptor. Concentrations of less than 0.7 μM α-bungarotoxin sites and the presence of 0.03% Triton X-100 are required to prevent aggregation and to obtain displacement of Tb^{3+} by activators (acetylcholine, carbamoylcholine, etc.). A Triton X-100 concentration of 0.03% was chosen since this was the lowest concentration at which a uniform population of 330 000 molecular weight particles was obtained (Edelstein et al., 1975). Higher concentrations of Triton were unfavorable because of light absorption (0.03% Triton gives 0.01 OD at 295 nm) and because increasing the amount of Triton increased the dissociation constants for Tb^{3+} ($K_{Tb} = 62 \pm 1$ μM in 0.1% Triton X-100 solution).

At present we have only an estimate of the moles of Tb^{3+}-binding sites per mole of acetylcholine binding sites and do not know the stoichiometry between Tb^{3+}- and Ca^{2+}-binding sites. This means that Ca^{2+}-binding sites may exist (Eldefrawi et al., 1975) which do not interact with Tb^{3+} and about which we have no information from the present studies. The Tb^{3+}-binding sites are preserved when the receptor is dissociated into subunits by $NaDodSO_4$ or when the receptor is degraded by trypsin and chymotrypsin to peptides of molecular weight of 8000 or less. This suggests that these binding sites are determined by structural features of the peptide chain rather than by the three-dimensional arrangement of the intact receptor.

Subunit IV and peptides of molecular weight of 8000 or less contain most if not all the Tb^{3+}- and Ca^{2+}-binding sites. It is interesting to note that, while in the intact receptor only about 60% of the Tb^{3+}-binding sites interact with Ca^{2+} (Rübsamen et al., 1976a), all the Tb^{3+}-binding sites detected in the subunit and the receptor peptides interact with Ca^{2+}. Presumably, the Tb^{3+}-binding sites which do not interact with Ca^{2+} depend on the three-dimensional structure of the protein or are located on other subunits.

Except for a single report which suggests that the acetylcholine receptor of electric organs is made up of only one kind of subunit (Klett et al., 1973), several others find two major subunits plus one or more minor ones (Hucho & Changeux, 1973; Carroll et al., 1973; Chang, 1974; Lindstrom & Patrick, 1974; Meunier et al., 1974; Michaelson et al., 1974; Weill et al., 1974; Eldefrawi et al., 1975). The 4 subunits of *Torpedo* receptor are immunologically distinct (Claudio & Raftery, 1977; Lindstrom et al., 1978). The variability observed in the number and size of the subunits may be a result of differences in the purity of the receptor and the content of detergent or in the denaturation treatment. Proteolysis that may occur during purification of the receptor may reduce the molecular weight of the smallest subunit (Patrick et al., 1975). In addition, two different methods of $NaDodSO_4$ electrophoresis may produce different estimates of the molecular weight of the subunits (Meunier et al., 1974). Nonetheless, of the two major subunits observed, the one with the smaller molecular weight (30 000-45 000) is the only one found to contain binding sites for both the affinity label, [^{3}H]MBTA, (Weill et al., 1974; Meunier et al., 1974), as well as the inhibitor, ^{3}H-labeled cobra α-toxin (Meunier et al., 1974; Hucho et al., 1976). Our present findings indicate that this is the same subunit which also contains the sites that bind both Tb^{3+} and Ca^{2+}.

Prothrombin also contains Ca^{2+}-binding sites which have a K_D for Ca^{2+} in the mM range and which are formed by a specific amino acid contained in the polypeptide chain rather than by the three-dimensional structure of the intact protein. The Ca^{2+}-binding sites are believed to consist mainly of γ-carboxylglutamic acid residues (Magnusson et al., 1974; Nelsestuen et al., 1975; Stenflo, 1974) and the isolated peptides still bind Ca^{2+}, although to a lesser extent than does the protein (Nelsestuen et al., 1975). Interestingly, the spectral properties of carbonyl complexes of Tb^{3+} (Bernier et al., 1975) are very similar to those found in the receptor.

Activators displace Tb^{3+} from only those sites which also interact with Ca^{2+}, while inhibitors do not. These results are consistent with data obtained with membrane preparations containing acetylcholine receptor which indicated at least two types of ligand binding sites on the receptor, one for activators and one for inhibitors: (i) In the irreversible and specific reaction of α-bungarotoxin with the membrane-bound receptor, *d*-tubocurarine was found to be a competitive inhibitor, and carbamoylcholine and decamethonium were found to be noncompetitive inhibitors (Bulger & Hess, 1977; Hess et al., 1975a,b). In the reversible binding of acetylcholine to the receptor, nicotine was a competitive inhibitor, whereas the inhibitor *d*-tubocurarine was noncompetitive (Eldefrawi, 1974). (ii) In measurements of the acetylcholine receptor-mediated efflux of $^{22}Na^+$ from electroplax membrane vesicles (Hess et al., 1975a,b), *d*-tubocurarine was found to be a noncompetitive inhibitor of both decamethonium- and carbamoylcholine-induced flux (Hess et al., 1976). The observation in this study that decamethonium can displace about 50% of the displaceable Tb^{3+} from the receptor-α-bungarotoxin complex is in agreement with previous experiments which indicated that decamethonium can still occupy 50% of its binding sites in presence of α-bungarotoxin (Fu et al., 1977). A simple model has been developed which accounts for these and other data (Hess et al., 1975a; Bulger et al., 1977).

It is noteworthy that acetylcholine displaces 3 to 6 Tb^{3+} ions per α-bungarotoxin binding site, in agreement with the displacement of Ca^{2+} ions by acetylcholine (Chang & Neumann, 1976). The dissociation constants for acetylcholine determined from Tb^{3+} displacement measurements and [^{14}C]acetylcholine binding are similar.

We have recently observed that Tb^{3+} inhibited acetylcholine receptor-mediated efflux of $^{22}Na^+$ from electroplax membrane vesicles when carbamoylcholine was used as an activator (Lipkowitz, Coombs, & Hess, unpublished). A minimum interpretation of the data, therefore, is that Ca^{2+} regulates receptor-mediated changes in the permeability of neural membranes to inorganic ions by competing for the activator-binding site. Other explanations would be that Ca^{2+} stabilizes a receptor conformation which does not permit transfer of inorganic ions through the membrane or that Ca^{2+} closes ion channels as suggested by Nachmansohn and Neumann (1975). Which, if any, of these suggestions is correct is an interesting question for future research. Our experiments show that fluorescent lanthanides can be important tools in such studies, and in the investigation of the structural features of the peptide chain which is responsible for the Ca^{2+}-binding sites.

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Yeast Inner Histones and the Evolutionary Conservation of Histone–Histone Interactions†

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ABSTRACT: The inner histones of the yeast, *Saccharomyces cerevisiae,* have been isolated and identified by their amino acid compositions. H4 appears to be close to its calf and pea counterparts. H2a, H2b, and H3 have diverged. The isolation of the histones was accomplished by consecutive slab-gel fractionation, and a number of novel features of the method are described. These appear to be generally useful for preparing many types of protein. The binding pattern of the yeast inner histones is identical to the binding pattern for calf and for pea histones. Data on interspecies complexing indicate that the surfaces across which the histones interact are very highly conserved.

Studies on the histones of *Saccharomyces cerevisiae,* the common baker's yeast, began more than 10 years ago (Tonino and Rozijn, 1966). Since that time, evidence has accumulated indicating that yeast contains four inner histones (Wintersberger et al., 1973; Franco et al., 1974; Moll and Wintersberger, 1976; Brandt and von Holt, 1976; Thomas and Furber, 1976; Nelson et al., 1977). However, the presence of H1 in yeast has not yet been demonstrated. While partial fractionations of the yeast histones have been achieved (Franco et al., 1974; Brandt and von Holt, 1976), a complete fractionation has not yet been reported.

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