Molecular Forms of Acetylcholine Receptor. Effects of Calcium Ions and a Sulfhydryl Reagent on the Occurrence of Oligomers†

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ABSTRACT: Sucrose density gradient centrifugations of crude and purified preparations of acetylcholine receptor from *Torpedo californica* show varying relative amounts of a heavy (H) form (~13 S) and a light (L) form (~9 S). In the present paper the chemical processes responsible for the occurrence of these two forms and the difference in their properties have been analyzed. The findings suggest that the H form is a dimer of the L form linked by an *intermolecular* disulfide bond, through 67 000-dalton subunits. The reduction of this disulfide bond in the H oligomer appears to occur to a variable extent during the homogenization of the tissue and the extraction of the receptor protein. The extent to which reduction occurs is shown to be strongly dependent on the Ca^{2+} concentration. Evidence is presented that *N*-ethylmaleimide, a sulfhydryl alkylating agent, prevents the formation of the L oligomer—resulting in predominantly the H oligomer—when this agent is used in the buffer solutions for the tissue homogenization and detergent extraction. The analysis suggests that the H oligomer is the native form of acetylcholine receptor present in the membrane. An interpretation is proposed wherein the reduction of the H oligomer is accomplished in the membrane by certain postulated protein sulfhydryl groups with unusually low redox potentials. These sulfhydryl groups are presumably in close proximity to the receptor, and their alkylation by *N*-ethylmaleimide is responsible for the preservation of the H oligomer. A comparative analysis of sucrose density gradient centrifugation profiles of acetylcholine receptor maximally labeled with [^{125}I]-α-bungarotoxin and [^{3}H]acetylcholine bound samples at equilibrium concentrations of 4.6, 12, and 35 nM of free [^{3}H]acetylcholine indicates that the H oligomer has a higher affinity for acetylcholine (K_d, $3 \pm 1 \times 10^{-9}$ M) than the L oligomer (K_d, $2 \pm 1 \times 10^{-8}$ M).

The acetylcholine receptor is generally recognized as a key protein involved in the control of ion permeability in excitable membranes (Nachmansohn and Neumann, 1975). The electric tissue of various *Torpedo* species, which are highly specialized for bioelectrogenesis and among the richest sources of acetylcholine receptor, has therefore frequently been used for the isolation and purification of this protein. Although remarkable progress has been made in the study of the purified acetylcholine receptor in recent years, there are marked discrepancies in reports of its basic molecular properties. Among the properties which show unexplained variabilities are: the heterogeneity of molecular forms; the molecular weights of these forms; the binding stoichiometry and binding constant of the biological activator, acetylcholine; and the existence and the extent of cooperativity in the acetylcholine binding process. (For reviews, see: Changeux, 1975; Karlin, 1974; Rang, 1975; Neumann and Bernhardt, 1977.)

There are at least two forms of acetylcholine receptor from *Torpedo* species. Varying relative amounts of high and low molecular weight forms have been observed by sedimentation in sucrose gradients (Miledi et al., 1971; Raftery et al., 1972; Gibson et al., 1976; Karlin et al., 1975; Edelstein et al., 1975). Recently a series of differently sedimenting forms of acetylcholine receptor, labeled with radioactive α-bungarotoxin, a specific irreversible marker for receptor (Lee and Chang, 1966; Changeux et al., 1970), was demonstrated under various conditions and interpreted in terms of a series of oligomers (Gibson et al., 1976). These investigators also determined molecular weights of two major oligomers, corrected for bound detergent, by comparing sedimentation in H_2O and D_2O. They assigned values of molecular weights of 330 000 to a 13.15 ± 1.1 S, heavy oligomer (H), and 190 000 to a 9.1 ± 0.8 S, light oligomer (L).

Important questions arise as to whether one or both of these observed oligomers are "native" membrane components and, if this is the case, what are the differences in their properties and specific functions in the membrane. In the present studies, we have investigated the chemical processes responsible for the occurrence of the H and L forms in detergent extracts of *Torpedo californica* electric organ and the differences in their affinities to acetylcholine.

Materials and Methods

Torpedo californica were obtained live from Pacific Bio-Marine Supply Co., Venice, Calif. When not used immediately, the electric organs were frozen and stored in liquid nitrogen. Lyophilized venom from *Bungarus multicinctus* was supplied by the Miami Serpentarium Laboratory. The nonionic detergent, Renex 30 (polyoxyethylene (12) tridecyl ether), and Flaxedil (gallaminetriethiodide) were generous gifts from Imperial Chemical Industries Ltd., Wilmington, Del., and American Cyanamide, respectively. [*acetyl*-^{3}H]Acetylcholine, 250 mCi/mM, was obtained from Amersham Searle Corp. Sucrose was the "ultra pure" grade from Schwarz/Mann. Tetram (*O,O*-diethyl-*S*-(β-diethylaminoethyl)phosphorothiolate) was synthesized as described previously (Calderbank and Ghosh, 1955).

Extraction of Acetylcholine Receptor. For studies involving the effect of EDTA,[1] NEM, and/or calcium concentrations during the receptor extractions, several parallel experiments were performed with the electric tissue from each *Torpedo*. All buffer solutions were deaerated and contained 5×10^{-5}

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[1] Abbreviations used are: NEM, *N*-ethylmaleimide; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); EDTA, ethylenediaminetetraacetate; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

M phenylmethanesulfonyl fluoride (Sigma), a protease inhibitor, and all procedures were carried out at 4 °C. The compositions of the various stock buffer solutions were as follows—solutions I, II, III, and IV: 5 mM sodium phosphate (pH 7.0, containing 0.1, 0.2, 0.5, or 1 mM $CaCl_2$, respectively), solutions V and VI: 20 mM Hepes (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.0), 2 or 3 mM Ca^{2+}, respectively; solution VII: 5 mM sodium phosphate, 1 mM Ca^{2+}, 5 mM NEM (pH 7.0); solution VIII: 10 mM sodium phosphate, 2 mM disodium ethylenediaminetetracetate (EDTA) (pH 7.0); solution IX: 10 mM sodium phosphate, 2 mM EDTA, 5 mM NEM (pH 7.0). Slices of fresh electric organs (40 to 150 g) were mixed with 2 volumes of appropriate buffer and homogenized in a Waring Blendor three times for 5 s (step 1) and centrifuged at 25 000*g* for 1 h. The pellet was rehomogenized three times for 5 s with 1 initial tissue volume of 1 M NaCl in appropriate buffer (step 2) to extract high salt soluble proteins. The homogenate was then centrifuged as before and this salt extraction process was repeated (step 3). Subsequently the pellet was twice washed by suspension in stock buffer and centrifuged as above (steps 4 and 5). The resulting pellet was then suspended in 1.2% Renex 30 in appropriate stock buffer in the ratio of about 20 mL per 40 g of original tissue and shaken overnight at 4 °C followed by centrifugation at 46 000*g* for 1 h (step 6). The supernatant ("crude acetylcholine receptor") was immediately applied to the affinity column, except for a small portion which was set aside in ice for assay purposes. When liquid-nitrogen-frozen tissue was used, the tissue was pulverized and thawed quickly in the selected buffer solution by homogenization at room temperature. The rest of the procedure was the same as for the fresh tissue preparation.

Purification of Acetylcholine Receptor. The affinity chromatography method for the purification of receptor using covalently bound methyl *N*-6-aminocaproyl-6'-aminocaproyl-3-aminopyridinium bromide (Dicaproyl-MP)/Sepharose 4B resin was essentially the same as previously reported (Chang, 1974; Penn et al., 1976) except that the nonionic detergent, Renex 30, was used instead of Triton X-100 and Brij 35. The chromatography buffers had the same composition as those used for the final extraction step but with 0.5% Renex 30, and NEM and phenylmethanesulfonyl fluoride were omitted. The acetylcholine receptor was selectively eluted from the column with 70 μM Flaxedil in 0.1% Renex containing buffer solution.

Radioactive Labeling of α-Bungarotoxin and Binding Assay. α-Bungarotoxin was purified from the snake venom of *Bungarus multicinctus* according to Clark et al. (1972) and Lee et al. (1972). The α fraction was iodinated with carrier-free Na[^{125}I] (New England Nuclear) diluted 250-fold with cold NaI and by the use of Chloramine-T according to the method described by Greenwood and Hunter (1963). After gel exclusion chromatography on Sephadex G-15 (0.05 M ammonium acetate (pH 5.0)), monoiodinated α-bungarotoxin was separated from noniodinated and di- and polyiodinate forms on a CM-52 column by applying a linear gradient of 0.05 M (pH 5.0) to 0.6 M (pH 6.5) ammonium acetate buffer. The [^{125}I]-α-bungarotoxin binding capacities of crude and purified acetylcholine receptor preparations were determined by the DEAE-cellulose filter disk method (DE-81, Whatman) described previously (Chang, 1974). Protein concentrations were estimated either by the method of Lowry et al. (1951) or by the absorbance at 280 nm using an extinction coefficient of $\epsilon_{280nm}^{1\%} = 17$ (Chang, 1974).

Sucrose Gradient Centrifugation. Samples were incubated with [^{125}I]-α-bungarotoxin, using a slight excess of the amount calculated to saturate the acetylcholine receptor as determined by DEAE-81 filter disc assay. The incubated samples were layered on a 5-mL linear gradient of 5 to 20% sucrose in 0.2% Renex 30, 100 mM NaCl, 1 mM $CaCl_2$, and 5 mM sodium phosphate (pH 7.0) (or 20 mM Hepes (pH 7.0)) in cellulose nitrate tubes. They were centrifuged for 5 h at 4 °C in a Beckman SW 50.1 rotor at 50 000 rpm in a Beckman Model L 2-65B ultracentrifuge. Beef liver catalase and *E. coli* β-galactosidase were included as internal markers in selected runs. The gradients were fractionated by collecting 18 drops per fraction through the bottom of the centrifuge tubes (the first two fractions were 65 drops each). Aliquots of 0.1 mL from each fraction were counted in 7 mL of Scintisol (Isolab) in a Searle Mark III liquid scintillation counter.

Reduced acetylcholine receptor was prepared by incubating the receptor sample with 5 mM dithiothreitol for 5 to 12 h at 4 °C, followed by addition of 10 mM NEM to inactivate the dithiothreitol before incubation with [^{125}I]-α-bungarotoxin. For the reoxidation experiment, reduced receptor (L form) was prepared from a purified H predominant sample as above, and this was incubated with 10 mM potassium ferricyanide at pH 7.5 for 12 h at 4 °C. The resulting reaction mixture was dialyzed against 0.1% Renex 30, 100 mM NaCl, 10 mM sodium phosphate buffer (pH 7.0), before addition of [^{125}I]-α-bungarotoxin for the sucrose density gradient sample.

Sucrose gradient centrifugation of the receptor containing [^{3}H]acetylcholine was carried out in the following way. A receptor sample (0.12 nmol of toxin sites) was incubated with 80 μM Tetram (to inhibit acetylcholinesterase) for 1 h and dialyzed overnight against 50 mL of 0.5% Renex 30, 5 mM sodium phosphate (pH 7.0), 1 mM NaCl, and 2 μM Tetram, containing the specified concentration of [^{3}H]acetylcholine. Radioactivities in aliquots of samples from both inside and outside of the dialysis bag were counted and the concentration of [^{3}H]acetylcholine was determined. Sucrose gradients were prepared in the manner described above except that they contained 5 μM Tetram and [^{3}H]acetylcholine at the same concentration as the dialysis medium after equilibrium had been reached. The [^{3}H]acetylcholine bound receptor sample was layered on the gradient and centrifuged as described above.

Equilibrium Dialysis-Binding Assays. Binding of [^{3}H]-acetylcholine to crude and purified acetylcholine receptor preparations was determined by equilibrium dialysis at 4 °C as described previously (Chang and Neumann, 1976). All dialysis samples were incubated in 50–80 μM Tetram for 1 h prior to the dialysis. A 1 mM stock solution of [^{3}H]acetylcholine in distilled water was first prepared on the basis of the quantity indicated by the manufacturer (Amersham/Searle) and then the concentration was precisely determined by the method of Hestrin (1949). A dry sample of acetylcholine bromide (Eastman) recrystallized from isopropyl alcohol was used to prepare eight standard solutions between 0.2 mM and 1.5 mM for the assay.

Determination of Sulfhydryl Groups. The sulfhydryl group concentrations in both purified and crude detergent extracts of acetylcholine receptor were determined spectrophotometrically by a slight modification of a method previously described (Habeeb, 1972). A purified acetylcholine receptor sample (300 μL, about 0.3 mg) was mixed with 250 μL of 0.1 M Tris-HCl (pH 8.0) buffer solution containing 5 mM EDTA and 4% sodium dodecyl sulfate in 1-cm light path semimicrocuvette. Fifty microliters of 10 mM 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) in 0.1 M Tris-HCl (pH 8.0) was added and the absorbance at 412 nm vs. time was recorded against a blank (without protein) until there was no further increase

in optical density. In the case of crude receptor extracts, 200 μL of sample, 600 μL of buffer solution, and 100 μL of DTNB solution were used. The concentration of sulfhydryl groups was calculated using an extinction coefficient of 13 600 M^{-1} cm^{-1} (Ellman, 1959). For the purified receptor, the sulfhydryl group concentration was expressed relative to the nanomoles of α-bungarotoxin sites.

Polyacrylamide Gel Electrophoresis in Sodium Dodecyl Sulfate. The method was essentially that of Fairbanks et al. (1971). Samples were made 2% sodium dodecyl sulfate and dialyzed against Fairbank sample buffer solution (1% sodium dodecyl sulfate, pH 8.0) overnight at room temperature. They were then incubated for 2 h at 60 °C either with or without 40 mM dithiothreitol. Parallel gels were run using aldolase, ovalbumin, γ-globulin, transferrin, and β-galactosidase for molecular weight markers.

Results

Relationship between H and L Oligomers. Sucrose density gradient centrifugation of detergent extracts of both fresh and liquid-nitrogen-frozen electric tissue always showed two α-bungarotoxin binding components. Catalase and β-galactosidase used as internal standards in sucrose gradient sedimentation suggest that the positions of these two acetylcholine receptor peaks are consistent with the previous assignments of about 9 S and 13 S for the light form, L, and the heavy form, H, respectively (Raftery et al., 1972; Gibson et al., 1976; Karlin et al., 1975). A sedimentation profile of [^{125}I]-α-bungarotoxin labeled acetylcholine receptor in 1% Renex extract of fresh electric tissue is presented in Figure 1a. The relative amounts of H and L forms in this preparation were 69.5% and 30.5%. However, when the same sample was incubated with the disulfide reducing agent dithiothreitol (5 mM) prior to α-bungarotoxin labeling, the amount of H form decreased to 4.5% with a quantitatively corresponding increase in the L form to 95.5%, as shown in Figure 1b. It is noteworthy that such dithiothreitol treatment of the acetylcholine receptor did not change significantly its α-bungarotoxin binding capacity, because this observation stands in contrast to acetylcholine equilibrium binding studies with dithiothreitol-treated receptor which indicated an apparent decrease in high affinity (K_d ~10^{-8} M) acetylcholine binding (Chang and Bock, unpublished results; Eldefrawi et al., 1972).

The treatment of purified reduced receptor, predominantly L form, with the oxidizing agent potassium ferricyanide, resulted in partial reconversion to the H form. However, in addition, higher molecular weight aggregates were formed presumably by nonspecific intermolecular disulfide bonding involving additional SH groups of the receptor.

Effect of Ca^{2+} Concentration and EDTA in Extraction Solution on the Distribution of Oligomeric Forms. Comparative studies of the proportions of H and L forms found after homogenization and detergent extraction at various concentrations of Ca^{2+} and EDTA are summarized in Table I. Increasing concentrations of Ca^{2+} ions in the 0 to 1 mM range resulted in an increase in the proportion of L oligomer to as high as 84%. Ca^{2+} concentration higher than 1 mM resulted in little additional increase in L oligomer; furthermore, the detergent extraction of receptor became inefficient—the yields dropped from 2 ± 0.4 nmol to 0.6 nmol (at 3 mM Ca^{2+}) of α-bungarotoxin sites per g of initial tissue. Although variations among preparations from different torpedos were found, the H/L ratios obtained under identical extraction conditions were qualitatively consistent. This was also true when liquid-nitrogen-frozen tissue was used, although in this case the amount of L oligomer found was generally somewhat higher than from the corresponding fresh tissue.

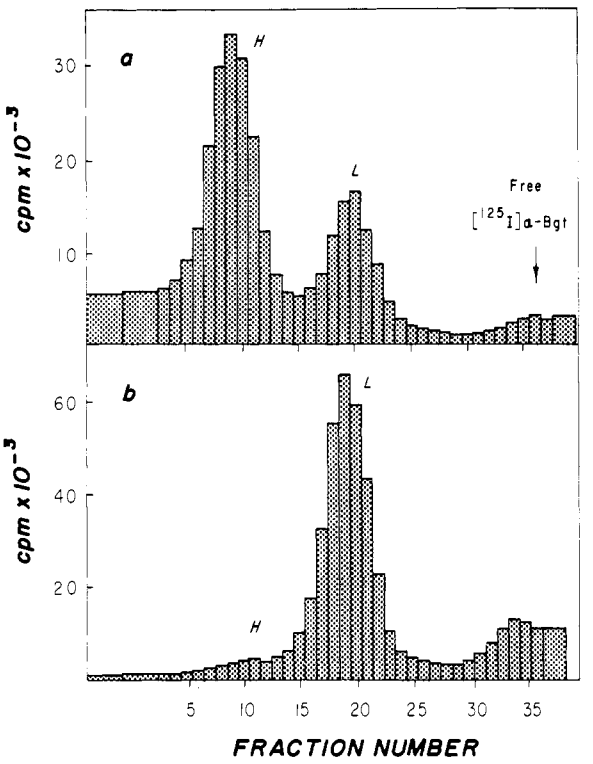

FIGURE 1: Effect of a disulfide reducing agent on acetylcholine receptor oligomers. (a) Sucrose density gradient sedimentation profile of [^{125}I]-α-bungarotoxin labeled crude acetylcholine receptor from fresh *Torpedo* electric tissue extracted with 1% Renex 30 in 0.1 mM Ca^{2+}, 5 mM sodium phosphate, pH 7.0. The 70-μL sample contained 0.14 nmol of toxin sites. (b) The same amount of sample in the same solvent as in a incubated in the presence of 5 mM dithiothreitol for 5 h at 4 °C. Subsequently NEM (10 mM) was added to inactivate dithiothreitol and the same amount of [^{125}I]-α-bungarotoxin was added as in a. The concentration of nonionic detergent in both samples was identical. All gradients were fractionated through the bottom of the centrifuge tubes.

Effect of NEM in the Extraction Solution on the Occurrence of Oligomers. The effects of the addition of a sulfhydryl alkylating agent, NEM, at various steps in tissue processing on the H/L ratio are summarized in the lower half of Table I. When 5 mM NEM is added to all solutions used for homogenization of tissue (steps 1 to 5) and to the 1% detergent extraction (step 6), over 80% H oligomer was consistently obtained (Figure 2a). In contrast, when NEM is added after the initial tissue homogenization step (step 1) or only to the 1% detergent solution (step 6), only about 40% H oligomer was found (Figure 2b). When 5 mM NEM was present in steps 1-6, the addition of either Ca^{2+} or EDTA had no effect on the H/L ratio.

Effects of Purification on the H and L Distribution. After extraction of crude receptor in 1 mM Ca^{2+} (solution IV), subsequent purification in the presence of 1 mM Ca^{2+} resulted in about a 30% increase of the H oligomer. In contrast, when the extraction and the purification were carried out in the presence of 2 mM EDTA (solution VIII), little change in the H/L ratio was noted; roughly equal amounts of H and L oligomers were found (Figure 3b) as had been observed previously (Karlin et al., 1975). Similarly there was no significant change in the H/L ratio during the purification steps when 5 mM NEM was present during the extraction (steps 1-6), either in the presence of Ca^{2+} or EDTA (Figure 3a).

Acetylcholine Binding Properties. Equilibrium acetylcholine

TABLE I: Effect of Ca^{2+}, EDTA, and NEM in Extraction Solutions on the Occurrence of High (H) and Low (L) Molecular Weight Oligomers of Acetylcholine Receptor.[a]

Extraction: Addition to buffer solutions[b]	Soln	Used in steps[c]	Rel peak area[d] % H	Rel peak area[d] % L	No. of expts[e]
0.1 mM Ca^{2+}	I	1-6	68 (66, 69)	32 (31, 34)	2
0.2 mM Ca^{2+}	II	1-6	52	48	1
0.5 mM Ca^{2+}	III	1-6	32	68	1
1 mM Ca^{2+}	IV	1-6	27 (16-37)	73 (63-84)	5
2 mM Ca^{2+}	V	1-6	29	71	1
3 mM Ca^{2+}	VI	1-6	17	86	1
2 mM EDTA	VIII	1-6	56 (53, 58)	45 (42, 47)	2
1 mM Ca^{2+}, 5 mM NEM	VII	1-6	85 (81-87)	15 (14-19)	4
1 mM Ca^{2+} and	IV	1-5			
1 mM Ca^{2+}, 5 mM NEM	VII	6	39 (26-59)	61 (41-74)	3
1 mM Ca^{2+} and	IV	1			
1 mM Ca^{2+}, 5 mM NEM	VII	2-6	43	57	1
2 mM EDTA, 5 mM NEM	IX	1-6	83 (80-86)	17 (14-20)	3

[a] Analyses were carried out by sucrose density gradient centrifugation of [^{125}I]-α-bungarotoxin complex of 1% Renex extracted crude acetylcholine receptor preparations. [b] For composition of solutions, see under Materials and Methods. [c] Extraction steps as described under Materials and Methods. [d] Relative peak areas were estimated by a cut and weigh method. When more than one experiment was involved; average values are given along with ranges in parentheses. [e] Each extraction was from a different Torpedo. A total of five live torpedos and liquid-nitrogen-frozen tissue from two torpedos were used.

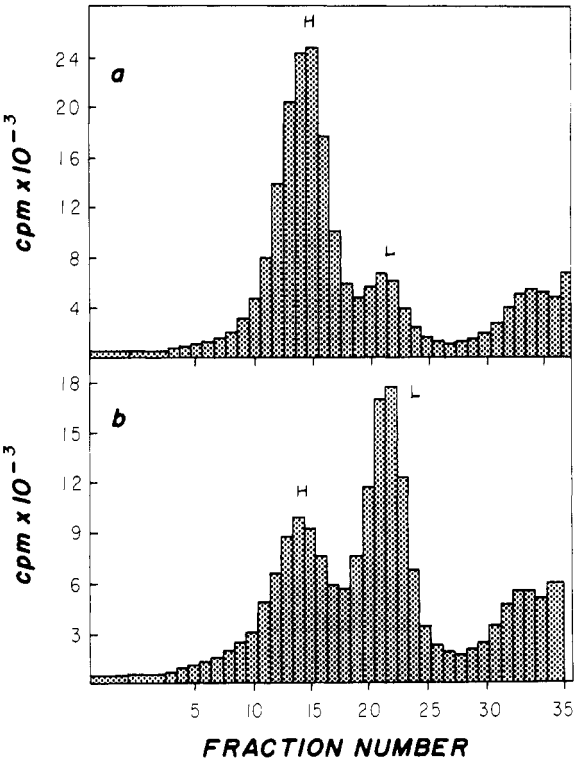

FIGURE 2: Sucrose density gradient sedimentation profile demonstrating effects of NEM during different stages of extraction on the occurrence of acetylcholine receptor oligomers. (a) The extraction solutions from the homogenization of the fresh tissue (step 1) until the detergent extraction (step 6), all contained 1 mM Ca^{2+} and 5 mM NEM (solution VII). See under Materials and Methods. The 30-μL sample of crude acetylcholine receptor extract contained 0.13 nmol of α-bungarotoxin sites. (b) The extraction procedure with tissue from the same *Torpedo* was identical with a except that no NEM was present in step 1 (solution IV). The 30-μL sample of crude 1% Renex 30 extract contained 0.11 nmol of α-bungarotoxin sites.

binding studies with a crude detergent extract (1 mM Ca^{2+}), in which the L oligomer is predominant (about 65%), showed a major dissociation constant of $2 \pm 1 \times 10^{-8}$ M, in good agreement with previous reports (Eldefrawi et al., 1975; O'Brien and Gibson, 1975; Chang and Neumann, 1976). In addition, an upward curvature in the Scatchard plot in the 1.5 to 3 nM acetylcholine range was observed in some experiments. This phenomenon has been interpreted previously as an indication of a positive cooperativity in binding of acetylcholine to the receptor (Eldefrawi and Eldefrawi, 1973b; O'Brien and Gibson, 1975; Gibson, 1976). We found under the same conditions that a plot of [bound acetylcholine]$^{-1}$ vs. [unbound acetylcholine]$^{-1}$ in the acetylcholine concentration range of 1.5-9 nM was linear and clearly associated with an additional binding constant K_d of $3 \pm 1 \times 10^{-9}$ M. The detailed description of these studies is beyond the scope of this paper and will be presented elsewhere (Chang and Bock, manuscript in preparation).

Once a crude or purified acetylcholine receptor preparation has been obtained, the addition of 5 or 10 mM NEM did not affect the acetylcholine binding properties. This is in agreement with observations by others (O'Brien and Gibson, 1975). In such preparations both H and L forms are present: in fact the L form is usually the predominant one. When, however, such NEM treatment is instituted from the very first tissue homogenization step—the condition that yields predominantly (over 80%) the H oligomer—then 80% of the acetylcholine binding sites were associated with a dissociation constant of $3 \pm 1 \times 10^{-9}$ M and the additional 20% with a dissociation constant of $2 \pm 1 \times 10^{-8}$ M. The ratio of acetylcholine binding sites to α-bungarotoxin binding sites was approximately 0.5.

Sulfhydryl Group Determinations. In the presence of sodium dodecyl sulfate the reaction of DTNB with crude extracts of acetylcholine receptor preparations showed that there are high concentrations of sulfhydryl containing proteins. In extracts that were approximately 2 μM in α-bungarotoxin binding sites, the sulfhydryl group concentration was 0.25 to 0.3 mM. The ratio of fast reacting SH groups (those which completely react with DTNB within 2 min) to α-bungarotoxin sites appeared to be a function of the Ca^{2+} concentration of the medium. At 0.2, 1, and 3 mM Ca^{2+} concentrations, the ratios were 61, 95, and 128, respectively.

The sulfhydryl contents of the *purified* acetylcholine receptor preparations obtained from various crude extracts of one *Torpedo* electric organ were as follows. The receptor purified in the presence of 1 mM Ca^{2+} had the highest sulfhydryl content, 2.9 nmol of total SH per nmol of α-bungarotoxin site,

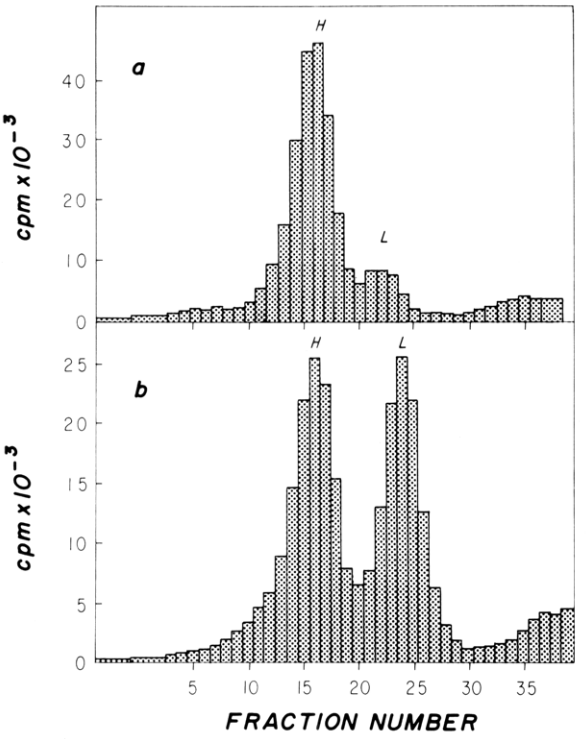

FIGURE 3: Sucrose density gradient centrifugation of [^{125}I]-α-bungarotoxin labeled purified acetylcholine receptor. (a) All extraction steps (1–6) were carried out in the presence of 5 mM NEM, 1 mM Ca^{2+} (solution VII). (b) The extractions, step 1–6, were carried out in the presence of 2 mM EDTA (solution VIII). the 50-μL samples contained 0.13 and 0.17 nmol of α-bungarotoxin sites, respectively.

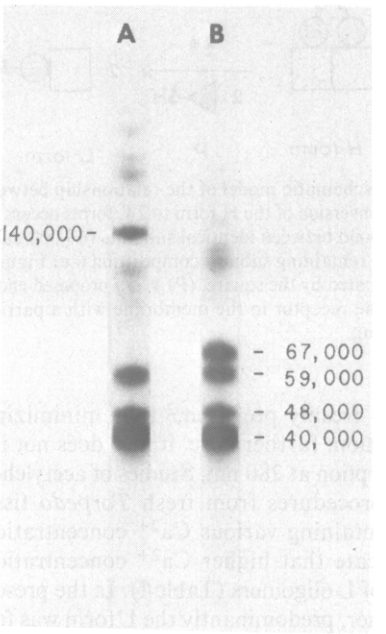

FIGURE 4: Sodium dodecyl sulfate–polyacrylamide gel (5.6% acrylamide) electrophoresis of purified acetylcholine receptor in the absence and presence of disulfide reducing agent. This preparation consist of 80% H oligomer and 20% L oligomer as demonstrated by sucrose density gradient centrifugation. The extraction solutions contained 1% Renex, 5 mM NEM, 1 mM EDTA, 20 μM phenylmethanesulfonyl fluoride, and 15 mM sodium phosphate (pH 7.0). The affinity chromatography buffers did not contain NEM, EDTA, or Ca^{2+}. (A) Nonreduced sample: 20 μg of receptor in 1% sodium dodecyl sulfate, 2 mM EDTA, and 100 mM Tris-HCl (pH 8.0). (B) Parallel sample after disulfide bond reduction in 40 mM dithiothreitol at 60 °C for 1 h in the same medium as A.

equivalent to 34 nmol of SH/mg of protein. Of these, 1.8 nmol reacted rapidly and 1.1 nmol reacted slowly (required up to 30 min) with DTNB. However, the receptor purified from a crude preparation containing 2 mM EDTA showed one less fast reacting SH group per α-bungarotoxin site than the receptor in 1 mM Ca^{2+}. After extraction of the receptor in the presence of 1 mM Ca^{2+} and 5 mM NEM (solution VII), subsequent immediate purification in the presence of Ca^{2+} but the absence of NEM surprisingly resulted in the retention of 2.2 nmol of SH per α-bungarotoxin site, of which 1.1 nmol is a fast reacting sulfhydryl group.

Polyacrylamide Gel Electrophoresis in Sodium Dodecyl Sulfate. Purified acetylcholine receptor, 80% in H oligomer, was subjected to electrophoresis in 1% sodium dodecyl sulfate both in the presence or absence of the disulfide-reducing agent dithiothreitol. The four polypeptide banding pattern in the reduced gel in Figure 4B is similar to that previously reported (Karlin et al., 1975; Raftery et al., 1975). In the absence of disulfide reduction, the lower three polypeptide species were still apparent. However, protein staining of the 67 000-dalton species was missing; instead a higher molecular weight species of about 140 000 daltons became evident (Figure 4A). When receptor having predominantly L oligomer was used, the 140 000-dalton band was not apparent in the absence of the disulfide reducing reagent. However, after disulfide reduction the L form gave a banding pattern similar to the reduced H form.

Differences in Acetylcholine Affinities between H and L Oligomers. When a 1% Renex extract (solution IV) of crude acetylcholine receptor from fresh tissue was labeled with a slight stoichiometric excess of [^{125}I]-α-bungarotoxin, the L oligomer was demonstrated to be the predominant form (63%) by sucrose density gradient centrifugation (Figure 6a). [^{3}H]Acetylcholine binding affinities between H and L oligomers in the same sample were analyzed at three different equilibrium concentrations of free [^{3}H]acetylcholine: 4.6 nM, 12 nM, and 35 nM. The results are presented in Figure 6b–d. It is seen that at 4.6 nM [^{3}H]acetylcholine the binding was predominantly to the H oligomer (Figure 6b); at 12 nM [^{3}H]acetylcholine there was a proportionally larger increase in binding to the L peak than to the H peak (Figure 6c); and at 35 nM [^{3}H]acetylcholine (Figure 6d), binding to the L peak exceeded binding to the H peak.

Discussion

Defining the native state in the study of solubilized membrane-bound proteins such as the acetylcholine receptor is a difficult problem. Two molecular forms of the acetylcholine receptor, H (~13 S) and L (~9 S), are always observed in varying relative amounts in freshly prepared detergent extracts from *Torpedo californica*, as was found also by other investigators (Gibson et al., 1976). We now report that the H oligomer can be converted to the L oligomer by incubating with a disulfide reducing agent at 4 °C while maintaining the same nonionic detergent concentration (Figure 1). This suggests that the H oligomer is a dimer of L oligomers joined by an intermolecular disulfide bond.

Our acetylcholine receptor solubilization method involves homogenization of electric tissue in an appropriate buffer solution (pH 7.0) followed by two 1 M NaCl extractions to remove high salt soluble proteins. Finally, the acetylcholine receptor is extracted from the membrane fragments by 1% Renex 30 in buffer solution. The nonionic detergent Renex 30 (polyoxyethylene (12) tridecyl ether) was chosen for our solubilization because it does not have detectable oxidizing contam-

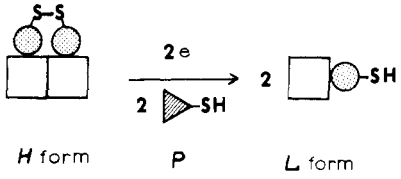

FIGURE 5: A schematic model of the relationship between H and L oligomers. The conversion of the H form to 2 L forms occurs by the reduction of a disulfide bond between identical subunits (67 000 dalton) designated by circles. The remaining subunit composition (see Figure 4) is not specified and designated by the square. (P) ▶ is a proposed endogenous protein located near the receptor in the membrane with a particularly reactive sulfhydryl group.

inants when freshly prepared,[2] thus minimizing sulfhydryl group oxidation; furthermore, it also does not interfere with protein absorption at 280 nm. Studies of acetylcholine receptor extraction procedures from fresh *Torpedo* tissue in buffer solutions containing various Ca^{2+} concentrations or 2 mM EDTA indicate that higher Ca^{2+} concentrations favor the occurrence of L oligomers (Table I). In the presence of 1 mM Ca^{2+} or higher, predominantly the L form was found. If Ca^{2+} is removed by EDTA, equal or higher amounts of H oligomer relative to controls (1 mM Ca^{2+}) are found. These observations might be interpreted as a protection of the acetylcholine receptor in a "native" L state by Ca^{2+} which prevents the formation of aggregates arising by sulfhydryl oxidation. However, in the presence of the sulfhydryl alkylating agent, NEM, a surprising result is obtained which conflicts with this interpretation. If 5 mM NEM and 1 mM Ca^{2+} are in the extraction buffer from the first tissue homogenization (step 1), about 85% of the receptor is found in the H form. In contrast, when NEM is added at later steps, only 40% H form is found. At this Ca^{2+} level a value of 63–84% L is obtained when no alkylating agent is added. These observations suggest that the "native" state of the acetylcholine receptor is the H form. This interpretation can be reconciled with the previously mentioned observations by postulating that, when the membrane is disrupted during the homogenization and extraction without sulfhydryl protection (absence of NEM), a portion of the H oligomers is reduced to generate two L forms. This reduction presumably is accomplished by a certain protein in the membrane that contains sulfhydryl groups both with unusually low redox potentials and with close proximity to the receptor. Titration of sulfhydryl groups in crude 1% detergent extracts demonstrated that the solution is buffered in terms of sulfhydryl groups due to the large number of nonreceptor sulfhydryls: as much as a 0.2–0.3 mM sulfhydryl concentration is present in extracts due to contributions of various other membrane bound proteins. The mechanism by which Ca^{2+} brings about more L form is unclear. We suggest two possible explanations for this result: (a) Ca^{2+} provides a favorable condition for the proposed reduction of the intermolecular disulfide bond; and (b) Ca^{2+} prevents the reoxidation of the L form to reconstitute the original H oligomer.

Frozen electric tissue preparations produced relatively more L forms than those from fresh tissue; and in contrast to fresh tissue extracts, such L forms do not readily convert to the H form during purification. This suggests that the particular SH group participating in oxidative intermolecular disulfide bonding has already been oxidized during purification (perhaps intramolecularly) and thus is no longer available for oxidative intermolecular disulfide bonding. Although a low concentration of Ca^{2+} is desirable during the extraction procedure to obtain a higher yield of total acetylcholine receptor as well as a higher proportion of H oligomers, the absence of Ca^{2+} results in rapid conversion of both high affinity (K_d $\sim 10^{-8}$ M and $\sim 10^{-9}$ M) acetylcholine binding sites to lower affinity (K_d $\sim 10^{-6}$ M). This change does not appear to be related to the H/L interconversion.

A schematic model of the relationship between H and L oligomers with a specific subunit involving disulfide bonding is shown in Figure 5. Sodium dodecyl sulfate gel electrophoresis data in Figure 4 appears to support the model. When H form samples are subjected to electrophoresis in the absence of a reducing agent, they show only the three smaller polypeptides and a protein band corresponding to 140 000 daltons. H form samples run after reduction show all four polypeptides and very little 140 000-dalton material. Thus reduction of H form samples appears to generate the 67 000 dalton component from disulfide-linked species of about 140 000 daltons.[3] This 67 000-dalton component thus is proposed to correspond to the circle species in the model Figure 5. The well-characterized disulfide bond (probably intrapolypeptide) near the receptor active site which is affinity alkylatable after reduction is a 40 000-dalton species (Karlin et al., 1975) and therefore appears to bear no relationship to our proposed intersubunit disulfide bond in the H oligomer.

The problems associated with the heterogeneity of acetylcholine affinites of acetylcholine receptor from *Torpedo* have been even more complex than the heterogeneity of molecular forms. Wide ranges of affinities of the receptor for acetylcholine (K_d of $\sim 10^{-9}$ M to $\sim 10^{-6}$ M) from both fresh and frozen electric tissue, which upon purification convert to varying extents to lower affinities (K_d $\sim 10^{-6}$ M), have been reported (Eldefrawi et al., 1972; Eldefrawi and Eldefrawi, 1973a; Moody et al., 1974; Sugiyama and Changeux, 1975; O'Brien and Gibson, 1975; Chang and Neumann, 1976). However, recently the results of [^{3}H]acetylcholine binding properties from various laboratories have converged to the extent that in crude receptor preparations from fresh tissue only high affinity sites (K_d $\sim 10^{-8}$ M) are apparent (see review in Neumann and Bernhardt, 1977). In addition, various bell-shaped curves in the Scatchard plots of acetylcholine bindings at low concentration ranges ($\sim 10^{-9}$ M) were observed in some cases and this phenomenon has been interpreted as positive cooperativity associated with a K_d = $\sim 10^{-8}$ M of acetylcholine binding (Eldefrawi and Eldefrawi, 1973b; O'Brien and Gibson, 1975; Gibson, 1976). Our experiment presented in Figure 6 suggests that in the $\sim 10^{-9}$ M acetylcholine range the observed acetylcholine binding is composed of contributions from different distinct binding sites on the H and L forms which differ in binding affinities and are not conformationally linked. The maximally [^{125}I]-α-bungarotoxin labeled sample in Figure 6a indicates that the L oligomer is predominant (63%). Nevertheless, at 4.6 nM equilibrium concentration of free [^{3}H]-acetylcholine (Figure 6b), the binding of [^{3}H]acetylcholine is predominantly to the H oligomer. At 12 nM equilibrium concentration of free [^{3}H]acetylcholine (Figure 6c), there is a relatively larger increase in binding to the L oligomer, and,

[2] We have recently found that aqueous solutions of some nonionic detergents, including Renex 30, generate detectable SH oxidizing agents, presumably due to formation of peroxides, after storage for a week or longer at room temperature.

[3] After submission of this paper, a recent article by Suárez-Isla and Hucho (1977) came to our attention which supports this model of intermolecular disulfide bonding. These authors found from the study of sodium dodecyl sulfate–polyacrylamide gel electrophoresis of acetylcholine receptor-rich membrane that an isolated 140 000-dalton species, presumably derived from the receptor, is a disulfide-bridged dimer of 68 000 dalton subunits.

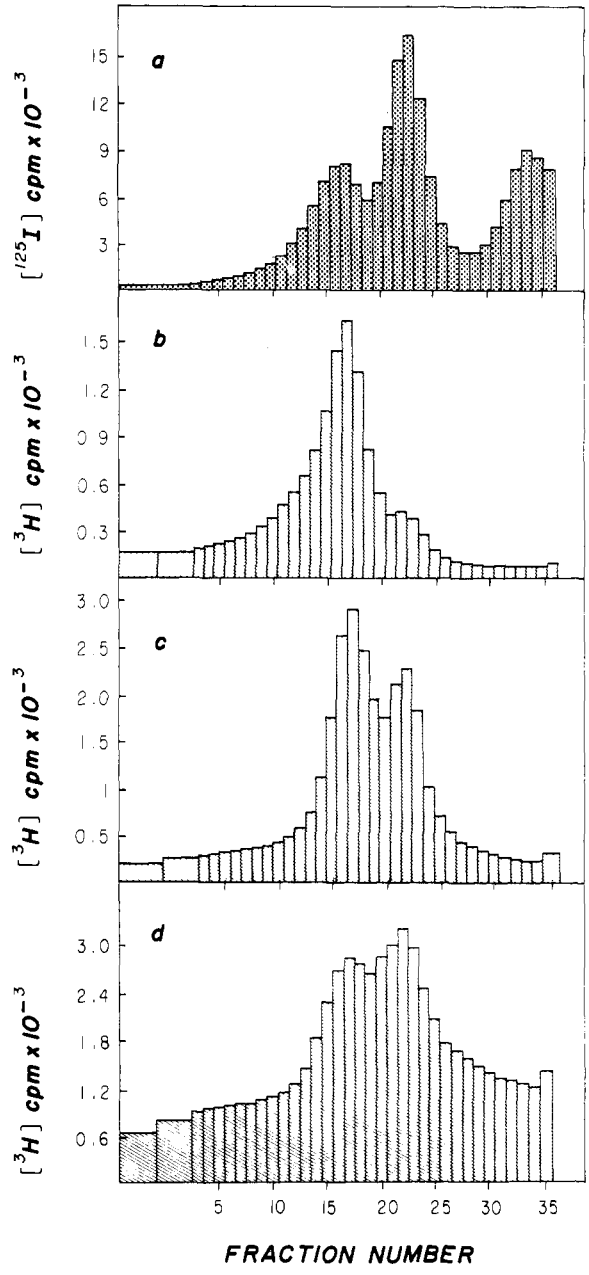

FIGURE 6: Demonstration of differences in acetylcholine affinities between H and L forms of acetylcholine receptor. The same 1% Renex crude receptor preparation from fresh tissue in solution I was used for the following four sucrose density gradient centrifugations. (a) The 30-μL sample was maximally labeled with [^{125}I]-α-bungarotoxin (0.11 nmol). (b) A 100-μL [^{3}H]acetylcholine-bound sample (total, 7500 cpm) at 4.6 nM equilibrium concentration of free [^{3}H]acetylcholine. (c) A 100-μL (19 500 cpm) [^{3}H]acetylcholine-bound sample at 12 nM equilibrium concentration of free [^{3}H]acetylcholine. (d) A 100-μL (29 500 cpm) [^{3}H]acetylcholine-bound sample at 35 nM equilibrium concentration of free [^{3}H]acetylcholine. The latter three sucrose gradient solutions, b, c, and d, contained 5 μM Tetram in addition to 4.6 nM, 12 nM, and 35 nM concentrations of [^{3}H]acetylcholine, respectively. The detailed sample and sucrose gradient preparations were described in Materials and Methods.

at 35 nM [^{3}H]acetylcholine (Figure 6d), an even larger increase in the L peak. Thus the [^{3}H]acetylcholine binding profile in Figure 6d approaches that of the [^{125}I]-α-bungarotoxin–receptor complex profile of Figure 6a. Our acetylcholine binding studies with predominantly H or L forms suggest that there is an order of magnitude difference in the acetylcholine affinity between H and L: the H form has a K_d of $3 \pm 1 \times 10^{-9}$ M and the L form has a K_d of $2 \pm 1 \times 10^{-8}$ M (Chang and Bock, 1977). If one calculates the degree of binding of [^{3}H]-acetylcholine to these two forms assuming a K_d of 3×10^{-9} M and 2×10^{-8} M for H and L, respectively, 60.5% of the total sites on the H oligomer and 18.7% of those on the L oligomers should have been in a bound state at 4.6 nM equilibrium concentration of free [^{3}H]acetylcholine. On the other hand, at 12 nM and 35 nM acetylcholine concentration, the binding should have increased to 80.0% of the total sites of H and 37.5% for L and 92.1% for H and 63.6% for L, respectively. These estimates agree well with the sedimentation profiles in Figure 6. Therefore we conclude that the heterogeneity of the molecular forms in the acetylcholine receptor from *Torpedo* is also associated with the heterogeneity of acetylcholine affinities: K_d of $\sim 10^{-9}$ M and $\sim 10^{-8}$ M for H and L forms, respectively.

Since our purified acetylcholine receptor binds 10 to 12 nmol of [^{125}I]-α-bungarotoxin per mg of protein, the equivalent weight of acetylcholine receptor which binds 1 mol of α-bungarotoxin would be 80 000 to 100 000. However, equilibrium binding studies of [^{3}H]acetylcholine to the receptor using carefully standardized [^{3}H]acetylcholine concentrations (Hestrin, 1949) consistently indicate 1.0 α-bungarotoxin binding site to a maximum of 0.5 acetylcholine binding site when freshly solubilized AcChR exhibited only high affinities (K_d $\sim 10^{-9}$ M and $\sim 10^{-8}$ M). This suggests the equivalent weight of acetylcholine receptor which binds 1 mol of acetylcholine is 160 000 to 200 000. This corresponds well with the recently determined L form molecular weight of 190 000 (Gibson et al., 1976). Consequently the H form, a dimer of the L, appears to consist of four α-bungarotoxin binding sites and two acetylcholine binding sites. Positive or negative cooperativities have been reported in acetylcholine binding to AcChR from *Torpedo* species (Eldefrawi and Eldefrawi, 1974; O'Brien and Gibson, 1975; Gibson, 1976). Because cooperativity requires the conformational linkage of at least two binding sites (Monod et al., 1965; Koshland and Neet, 1968), the H form, not the L form, is the likely candidate for cooperative acetylcholine binding.

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Synthesis of Two Classes of Small RNA Species in Vivo and in Vitro†

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ABSTRACT: A set of low molecular weight RNA species has been described in mammalian cells. These RNAs are localized primarily in the nucleus and are not involved in protein synthesis (Zieve and Penman, 1976). In the HeLa cell these small RNA species are divisible into two distinct families that have different methods of formation: the class I small RNAs which may be formed by a polymerase type I activity and the class III small RNAs which are clearly synthesized by RNA polymerase III. The class III small RNAs include tRNA, 5S RNA, and species K and L. They are synthesized in HeLa cell nuclei incubated in vitro where their synthesis is unaffected by low levels of α-amanitin but inhibited by high levels of α-amanitin which indicates that they are products of RNA polymerase III. In vivo, the synthesis of the class III small RNAs is insensitive to several inhibitors and to alterations in cellular metabolism that severely inhibit the synthesis of other types of cellular RNA including the class I small RNAs. The class I small RNAs include the nuclear species A, C, D, F, and H. They are not made in detectable amounts in vitro in isolated nuclei which prevents a direct determination of the RNA polymerase responsible for their synthesis. In contrast to the class III small RNA species, their synthesis in vivo is sensitive to inhibitors and to alterations in cellular metabolism which also inhibit the transcription of the 45S rRNA precursor, suggesting that they are transcribed by RNA polymerase I. The small RNA species of *Drosophila melanogaster* cells also form two distinct families, one whose synthesis is sensitive to α-amanitin and high temperature and one which is resistant.

The low molecular weight RNA species, in eukaryotic cells, have been described by several authors (Weinberg and Penman, 1968; Hodnett and Busch, 1968; Goldstein and Ko, 1974; Zieve and Penman, 1976). In mammals, these RNA molecules form a distinct class of at least eight well-defined species that range in size from 4 to 7 S. They have been shown to be relatively abundant and stable (Weinberg and Penman, 1969; Frederiksen et al., 1974). These RNAs can be found associated with different cellular fractions including cytoplasmic membranes (species L) (Zieve and Penman, 1976), the nucleolus (species A) (Weinberg and Penman, 1968; Prestayko et al., 1971) and with nuclear ribonucleoprotein particles containing hnRNA (Deimel et al., 1977; Zieve and Penman, in preparation). They appear to be tightly bound to specific subcellular structures suggesting that they may play a structural role in some cell organelles. The 5S RNA and tRNA are known to be transcribed by RNA polymerase III (McReynolds and Penman, 1974; Weinmann and Roeder, 1974; Marzluff et al., 1974); however, little is known so far about the biosynthesis

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