Physical Properties of the Purified Cardiac Muscarinic Acetylcholine Receptor†

Gary L. Peterson, Larry C. Rosenbaum, David J. Broderick,‡ and Michael I. Schimerlik*
Department of Biochemistry and Biophysics, Oregon State University, Corvallis, Oregon 97331
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ABSTRACT: The physical properties of the cardiac muscarinic acetylcholine receptor (mAcChR) purified from porcine atria as recently described [Peterson, G. L., Herron, G. S., Yamaki, M., Fullerton, D. S., & Schimerlik, M. I. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 4993–4997] have been examined by D_2O/H_2O sucrose gradient sedimentation and Sephacryl S-300 gel filtration in Triton X-405 and by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). From the sedimentation experiments the partial specific volume and sedimentation constant for the mAcChR–Triton X-405 complex were determined to be 0.813 cm^3/g and 5.30 S, respectively, which lead to an estimate of the molecular weight of the complex of 143 000. Gel filtration in Triton X-405 gave an estimate of the Stokes radius (4.29 nm) and an apparent molecular weight of 116 000. Combination of sedimentation and gel filtration gave an apparent molecular weight of 137 000 and a frictional ratio (f/f_0) of 1.21 for the complex. The partial specific volume of the receptor calculated from composition was 0.717 cm^3/g assuming 26.5% by weight carbohydrate. The amount of bound Triton X-405 was estimated at 1.011 g/g of mAcChR, which gave an apparent molecular weight of 70 900 (sedimentation) or 68 200 (sedimentation plus gel filtration) for the uncomplexed receptor. SDS–PAGE experiments at acrylamide concentrations ranging from 6% *T* [monomer plus bis(acrylamide)] to 17% *T* gave a linear range of apparent molecular weight from 67 600 (6% *T*) to 98 600 (17% *T*), and calibration against the retardation coefficient, K_r, determined from Ferguson plots gave an apparent molecular weight of 89 100 ± 6700. From a newly developed, novel evaluation scheme the anomalous migration of the mAcChR in SDS–PAGE was found to be due to both an excess charge density and an abnormally large shape parameter (K_r), and the true molecular weight of the protein portion of the mAcChR ligand binding polypeptide was estimated to be between 50 000 and 60 000.

The muscarinic acetylcholine receptor (mAcChR)[1] appears to minimally consist of an 80 000-dalton ligand binding protein in brain, heart, and smooth muscle (Birdsall et al., 1979; Venter, 1983), as determined by specific alkylation with the radioactive antagonist [^{3}H]propylbenzilylcholine mustard and SDS–PAGE. These findings agree with radiation target size analysis of membrane-bound receptor (Venter, 1983) and hydrodynamic studies of the detergent-solubilized receptor (Haga, 1980; Berrie et al., 1984a). This protein is also a sialoglycoprotein (Herron & Schimerlik, 1983; Shirakawa et al., 1983), but the extent of glycosylation is not known. We have purified the cardiac mAcChR from porcine atria (Peterson et al., 1984) and found a single ligand binding protein (mLBP) of apparent molecular weight of 78 000 on SDS–PAGE. A second smaller polypeptide of apparent molecular weight of 14 800 was also purified by affinity chromatography, but did not bind ligands. A similar structure has been more recently reported by Haga and Haga (1985) for a purified preparation of porcine brain mAcChR, although a somewhat smaller molecular weight (70 000) was reported for the ligand binding protein, in agreement with that reported by Andrē et al. (1983) for apparently purified, but inactive, mAcChR from calf brain. To date, a full study of the hydrodynamic properties of the purified mAcChR has not been published. In this paper we report on our studies of the hydrodynamic properties of the purified cardiac mAcChR as determined from rigorous analysis by gel chromatography, D_2O/H_2O sucrose density gradient centrifugation, and SDS–PAGE. The amino acid and amino sugar composition of the purified cardiac mAcChR is also reported.

Experimental Procedures

Materials. [^{3}H]-L-QNB (30–40 Ci/mmol) was from New England Nuclear. D_2O (99.8%) was from Aldrich Chemical Co. Acrylamide, *N*,*N*′-methylenebis(acrylamide), and SDS were from Bio-Rad Laboratories. Blue Dextran 2000 was from Pharmacia. Sephacryl S-300, Triton X-405 and the molecular weight markers given in Table I were from Sigma Chemical Co. The marker proteins were examined by SDS–PAGE to verify that a single prominent band of appropriate molecular weight was identifiable. The marker proteins chosen were limited to those for which accurate physical properties were known from sequence data. Additional chemicals were of the highest purity grade available. Nicotinic (n)AcChR was a gift from Drs. B. M. Conti-Tronconi and M. A. Raftery (California Institute of Technology) and consisted of a purified membrane fraction from *Torpedo californica* in which the α, β, γ, and δ chains composed about 90% of the total protein (SDS–PAGE analysis). Purified mAcChR was prepared from

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‡ Present address: Department of Agricultural Chemistry, Oregon State University, Corvallis, OR 97331.

[1] Abbreviations: mAcChR, muscarinic acetylcholine receptor; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; mLBP, muscarinic ligand binding protein; mSCP, small copurifying protein observation in the purified cardiac mAcChR preparation; [^{3}H]-L-QNB, [^{3}H]-L-quinuclidinyl benzilate; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; Tris-HCl, tris(hydroxymethyl)-aminomethane hydrochloride; nAcChR, nicotinic acetylcholine receptor; *N*, number of amino acid residues in the polypeptide chain; % *T*, total acrylamide concentration [monomer plus bis(acrylamide)]; % *C*, bis(acrylamide) concentration as a percent of total acrylamide.

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Table I: Molecular Weight Markers[a]

no.	protein	tissue source	N^b	M_r^c	CHO[d] (wt %)	R_s (nm)	$s_{20,w}$ ($\times 10^{-13}$ s)
1	α_2-macroglobulin	human plasma	1451[e]	160 837[e]	10.15[e]		
2	β-galactosidase	*E. coli*	1021	116 116			15.93[f]
3	phosphorylase *a*	rabbit muscle	841	97 114			
4	transferrin	human	679[g]	75 156[g]	5.54[h]		5.10[i]
5	albumin	bovine serum	582	66 296		3.5[j]	
6	catalase	bovine liver	505	57 471			11.15[k]
7	hexokinase A and B	yeast	477	52 819			
8	immunoglobulin G,H chain	human myeloma	447	48 472	3.44		
9	enolase	yeast	436[l]	46 510[l]			
10	ovalbumin	chicken egg	385	42 807	4.29	3.0[j]	
11	aldolase A	rabbit muscle	363[m]	39 210[m]			7.35[n]
12	alcohol dehydrogenase	yeast	347[o]	36 691[o]		4.6[p]	
13	lactate dehydrogenase	porcine heart	333	36 457			
14	glyceraldehyde-3-P-dehydrogenase	yeast	331	35 549			
15	chymotrypsinogen A	bovine pancreas	245	25 666		2.3[q]	
16	concanavalin A	jack bean	237	25 571			
17	lentil lectin, β chain	*Lens culinaris*	159[r]	17 572[r]			
18	myoglobin	sperm whale muscle	153[s]	17 198[s]			
19	lysozyme	chicken egg white	129[t]	14 314[t]			
20	cytochrome *c*	horse heart	104	11 761		1.7[j]	2.10[u]

[a] This table is an updated and extended version of that presented by Peterson and Hokin (1981). Only the newly updated and extended data are cited in this table. [b] Number of amino acid residues in the polypeptide chain. [c] Molecular weight of the protein portion of the polypeptide chain (monomer) determined from sequence data. [d] Weight percent carbohydrate relative to total weight (protein plus CHO). [e] Sottrup-Jensen et al. (1984). [f] Craven et al. (1965). [g] MacGillivray et al. (1983). [h] Dorland et al. (1977). [i] Roberts et al. (1966). [j] Tanford et al. (1974). [k] Samejima et al. (1962) for the dimer. [l] Chin et al. (1981). [m] Tolan et al. (1984). [n] Taylor and Lowry (1956) for the dimer. [o] Jörnvall (1977). [p] Horiike et al. (1983) for the tetramer. [q] Fish (1975). [r] Foriers et al. (1981). [s] Edmundson (1965). [t] Canfield and Liu (1965). [u] Atlas and Farber (1956).

porcine atria as previously described (Peterson et al., 1984). Buffer A consisted of 25 mM imidazole, 1 mM EDTA, 0.02% NaN_3, 0.1 mM phenylmethanesulfonyl fluoride (added fresh), and 0.35% (w/v) Triton X-405, pH 7.4.

D_2O/H_2O Sucrose Gradient Sedimentation in Triton X-405. Purified mAcChR was preincubated with 20 nM [^{3}H]-L-QNB for 10 h at 5 °C, diluted 10-fold with buffer A, and brought to a final volume of 0.5 mL with a solution of 5 mg/mL each of cytochrome *c*, aldolase, catalase (sucrose gradient purified), transferrin, and β-galactosidase in buffer A. The final samples, containing 30 pmol of mAcChR, were then layered onto 12.6 mL of 5–20% linear sucrose density gradients prepared in buffer A. The D_2O sucrose gradients were prepared in a similar fashion, substituting D_2O as the solvent in buffer A and in the molecular weight marker solution. The gradients were centrifuged at 38 000 rpm and 5 °C for 17.5 h in a Beckman SW-40 rotor in a Beckman Model L5-65 ultracentrifuge. The gradients were fractionated by displacement from the bottom with 50% sucrose in either D_2O or H_2O using an Isco Model 185 density gradient fractionator. Each fraction (0.3 mL) collected was then assayed in terms of specific binding of [^{3}H]-L-QNB as described (Peterson & Schimerlik, 1984). Cytochrome *c*, aldolase, catalase, and transferrin were monitored by A_{280} as the gradients were fractionated or alternatively determined by *o*-phthaldehyde fluorescence assay (Peterson, 1983). β-Galactosidase was assayed according to the method of Hestrin et al. (1955). Sucrose density was measured by refractometry at 20 °C by using a Zeiss refractometer.

Gel Filtration in Triton X-405. A sample of 30 pmol of purified mAcChR to a final volume of 0.5 mL was preincubated with [^{3}H]-L-QNB and diluted with buffer A containing 5 mg/mL each of cytochrome *c*, ovalbumin, bovine serum albumin, yeast alcohol dehydrogenase, and chymotrypsinogen A as described. The sample was chromatographed on a Sephacryl S-300 column (0.6 cm × 40 cm) preequilibrated at 5 °C with buffer A. Fractions of 0.27 mL/tube were collected on a Gilson microfractionator. The void volume (V_0) and the total volume available to solvent (V_T) were determined with Blue Dextran 2000 by measuring the absorbance at 650 nm and with free [^{3}H]-L-QNB by scintillation counting, respectively. The partition coefficient, K_D, was determined by the equation

$$K_D = (V_E - V_0)/(V_T - V_0) \quad (1)$$

where V_E is the elution volume of the marker proteins or receptor. Cytochrome *c*, ovalbumin, bovine serum albumin, and chymotrypsinogen A were determined by the Folin phenol method (Peterson, 1983), and yeast alcohol dehydrogenase activity was assayed according to Racker (1955). Specific binding of [^{3}H]-L-QNB for each fraction was determined as above. Volume measurements were determined by weight to improve accuracy (Tanford et al., 1974).

Calculation of Hydrodynamic Data from Sedimentation Studies. The partial specific volume for the purified mAcChR–[^{3}H]-L-QNB complex in Triton X-405 was calculated by using eq 9 from O'Brien et al. (1978):

$$\bar{\nu}_A = \frac{(1 - \bar{\nu}_B\rho_D{}^B) - a(1 - \bar{\nu}_B\rho_H{}^B)}{\rho_H{}^A(1 - \bar{\nu}_B\rho_D{}^B) - a\rho_D{}^A(1 - \bar{\nu}_B\rho_H{}^B)} \quad (2)$$

where $\bar{\nu}_A$ and $\bar{\nu}_B$ are the partial specific volumes of the unknown and known, respectively; $a = (y_D{}^B y_H{}^A)/(y_H{}^B y_D{}^A)$, the migrational distances (y) traveled from the meniscus by the unknown (A) and known (B) in H_2O (H) and D_2O (D); ρ_H and ρ_D represent the average densities taken at $0.5y$ in the gradient for H_2O and D_2O, respectively. This relation was shown to yield the highest degree of accuracy in estimating $\bar{\nu}_A$ when $\bar{\nu}_A$ and $\bar{\nu}_B$ were unequal (O'Brien et al., 1978). The known molecular marker used was human transferrin ($\bar{\nu}_B = 0.724$).

Equation 14 of O'Brien et al. (1978) was used for calculation of the sedimentation coefficient of the receptor complex in Triton X-405:

$$s_{20,w}{}^A = \frac{y_A(1 - \bar{\nu}_A\rho_{20,w})(1 - \bar{\nu}_B\rho_{T,m}{}^B)}{y_B(1 - \bar{\nu}_B\rho_{20,w})(1 - \bar{\nu}_A\rho_{T,m}{}^A)} s_{20,w}{}^B \quad (3)$$

where $\rho_{20,w}$ is the density of H_2O at 20 °C, $\rho_{T,m}$ is the average density at 5 °C in the H_2O gradient and $\bar{\nu}_A$ was from eq 2. This relation was shown to be highly accurate when compared to a standard of similar $s_{20,w}$ value and essential when $\bar{\nu}_A$ and

$\bar{\nu}_B$ were unequal (O'Brien et al., 1978). Transferrin was again used as the standard ($s_{20,w}$ = 5.10 S; see Table I). The molecular weight was determined from a variation of the Svedberg equation, assuming the receptor is a globular protein (Gibson et al., 1976):

$$\frac{M_A}{M_B} = \left[\frac{s_{20,w}{}^A(1 - \bar{\nu}_B\rho)(\bar{\nu}_B)^{1/3}}{s_{20,w}{}^B(1 - \bar{\nu}_A\rho)(\bar{\nu}_A)^{1/3}} \right]^{3/2} \quad (4)$$

where M_A and M_B are the molecular weights in Triton X-405 of mAcChR and transferrin, respectively; ρ is the density of H_2O at 5 °C, and $\bar{\nu}_A$ and $s_{20,w}{}^A$ are calculated from eq 2 and 3. To correct for the apparent weight contribution and buoyancy effects of the Triton X-405 bound to the mAcChR, the following equation was used to estimate grams of detergent bound per gram of receptor, δ_D (Tanford & Reynolds, 1976):

$$\delta_D = (\bar{\nu}_c - \bar{\nu}_p)/(\bar{\nu}_D - \bar{\nu}_c) \quad (5)$$

where $\bar{\nu}_c$, $\bar{\nu}_p$, and $\bar{\nu}_D$ correspond to the partial specific volumes of the mAcChR–Triton X-405 complex, the uncomplexed receptor and the detergent, respectively. $\bar{\nu}_c$ ($=\bar{\nu}_A$) was calculated from eq 2. $\bar{\nu}_p$ was calculated from compositional analysis. The partial specific volume of Triton X-405 was determined by pycnometry and found to be 0.908 cm^3/g, and the critical micelle concentration (cmc) was estimated by the method of Rosenthal and Koussale (1983) which gave a value of 0.066% (w/v).

The molecular weight for the mAcChR, free from bound detergent (M_p), was calculated by using the relationship (Tanford & Reynolds, 1976):

$$M_c = M_p(1 + \delta_D) \quad (6)$$

where M_c is the molecular weight of the mAcChR–Triton X-405 complex calculated from eq 4 ($=M_A$) and δ_D is from eq 5.

Calculation of Hydrodynamic Data from Gel Filtration. The Stokes radius (R_s) for the mAcChR–[^{3}H]-L-QNB complex chromatographed on the Sephacryl S-300 column in 0.35% Triton X-405 was determined according to the method of Ackers (1967), using the relation:

$$R_s = a_0 + b_0 \operatorname{erf}^{-1}(1 - K_D) \quad (7)$$

where the intercept (a_0) and slope (b_0) are calibration constants for the particular gel used. The $\operatorname{erf}^{-1}(1 - K_D)$ values were obtained from the table given by Fish (1975). The molecular weight of the mAcChR–Triton X-405 complex was calculated according to eq 1 of Siegel and Monty (1966):

$$M_c = \frac{6\pi\eta_{20,w}\mathcal{N}R_s s_{20,w}}{1 - \bar{\nu}\rho_{20,w}} \quad (8)$$

where $\eta_{20,w}$ is the viscosity of H_2O at 20 °C. $s_{20,w}$ is from eq 3, $\bar{\nu}$ is from eq 2, R_s is from eq 7, $\rho_{20,w}$ is the density of H_2O at 20 °C, and $\mathcal{N}$ is Avogadro's number. The molecular weight of the uncomplexed receptor (i.e., corrected for bound detergent) was then calculated according to eq 5 and 6. The frictional coefficient ratio (f/f_0) of the mAcChR was calculated from the relation (Siegel & Monty, 1966):

$$f/f_0 = \frac{R_s}{(3\bar{\nu}M_c/4\pi\mathcal{N})^{1/3}} \quad (9)$$

Sample Preparation for SDS–PAGE. The digitonin/cholate detergent system used to solubilize the mAcChR and to maintain stability throughout purification was found to affect migration of the receptor in SDS–PAGE in a load-dependent fashion. The bulk of this detergent was therefore removed by binding the purified mAcChR to the 3-[(2′-aminobenzhydryl)oxy]tropane–agarose affinity resin (Peterson et al., 1984) and eluting with 2% SDS in 0.25 M NaCl, 0.1 M DTT, and 0.02 M Tris-HCl, pH 6.8. Elution of the denatured mAcChR was verified by SDS–PAGE. All standard proteins (at 2.5 mg/mL) were solubilized in 2.5% SDS, 10 mM DTT, and 20 mM Tris-HCl, pH 6.8. To inactivate any proteolytic activity and to ensure full SDS binding, protein reduction, and proper stacking characteristics, all the samples were heated to 100 °C for 2–3 min and then dialyzed overnight against 0.2% SDS, 1 mM DTT, and 20 mM Tris-HCl, pH 6.8 (Peterson & Hokin, 1981). The samples were then made 7–10% (v/v) in glycerol and 2 mg % in the tracking dye bromophenol blue and stored at −80 °C.

The discontinuous buffer system of Laemmli was used in these studies. Minislab gels measuring 0.8 mm × 80 mm × 80 mm were cast with 4 mm wide sample wells. Protein concentration was adjusted so that all bands were easily detectable and not overstained when coelectrophoresed on one slab gel and stained with silver nitrate according to the method of Wray et al. (1981). The use of silver staining, which was necessary for detection of the mAcChR protein, also allowed for use of low concentrations of marker proteins so that overloading gel effects were avoided. The standards were run in four suitable groupings, and all samples wer run in duplicate lanes on each gel. The leading discontinuity boundary was marked by perforation following completion of the run (single % *T* gels). The stained gels were photographed (3 × 5 format Polaroid) and the negatives scanned at 600 nm to locate the peak staining intensity for each band. All peaks appeared Gaussian and, except for the mAcChR ligand binding polypeptide, were narrow. The scans were made slowly and recorded over a span of about 300 mm to increase measurement accuracy.

Gradient acrylamide SDS–PAGE gels were run by using gradients of 10–17% *T* [total monomer and bis(acrylamide)] at four different bis concentrations (2.60, 3.23, 3.85, and 4.46% *C*). The molecular weight was then determined from a log–log plot of molecular weight vs. % *T* (Lambin, 1978) for the standard proteins. A similar calibration was also made vs. *N*, the number of amino acid residues in the polypeptide chain.

Single acrylamide concentration gels were run at each integer total acrylamide concentration from 5% *T* to 17% *T* at two bis concentrations (2.60 and 3.85% *C*). The migration of each band was compared relative to the migration of the leading discontinuity boundary (R_F). A single solution of acrylamide–bis(acrylamide) was used for each set of gels (5–17% *T* at 2.60% *C* and 5–17% *T* at 3.85% *C*) to minimize errors in gel preparation. The data from these studies were evaluated at each single % *T* by a plot of log M_r (or log *N*) vs. R_F (designed M_rR_F plot) by an iterative least-squares fit to the sigmoidal relation

$$R_F = a/(1 + M_r/c)^m \quad (10)$$

where *a*, *c*, and *m* are regression constants (Peterson & Hokin, 1981) for ≥8% *T*, or by a linear least-squares fit (<8% *T*). The data were also evaluated by Ferguson plots (log R_F vs. % *T*) for each protein using a weighted [$W = 1/(1 + 1/R_F{}^2)$] least-squares method (Rodbard & Chrambach, 1971, 1974) to the relation

$$\log R_F = \log Y_0 - K_R(\%\ T) \quad (11)$$

where the intercept, log Y_0, is a measure of the free electrophoretic mobility and the slope, K_R, is the retardation coefficient for the protein examined. K_R is directly related to M_r

and the data were evaluated by a linear least-squares fit to log M_r vs. log K_R (designated M_rK_R plot) (Peterson & Hokin, 1981).

The relation of log Y_0 to K_R was further evaluated by comparison of the joint 95% confidence envelopes for log Y_0 vs. K_R (designated Y_0K_R plot) of the standard proteins in comparison to the receptor subunit proteins. The methods for calculation and statistical evaluation of these confidence envelopes are given in detail by Rodbard and Chrambach (1974). Briefly, the variance, s_0^2, for the Ferguson plots (eq 11) were pooled to increase reliability:

$$\bar{s}_0^2 = \frac{\sum s_{0i}^2(n_i - 2)}{\sum(n_i - 2)} \quad (12)$$

The 95% confidence envelopes were determined from the F statistic for 2 and $\sum(n - 2)$ df at the mean log $R_F = \bar{Y}$ and mean slope = $\overline{K_R}$ for each Ferguson plot:

$$F = \frac{(\bar{Y} - \alpha)^2\sum w + (\overline{K_R} - \beta)^2[\sum w(\%T - \%\,\bar{T})]}{\bar{s}_0^2} \quad (13)$$

where $\%\,\bar{T}$ is the mean $\%\,T$, β is the "true" slope, and α is the "true" expectation of log R_F given $\%\,T$ values. Each point on the confidence envelope for log R_F vs. K_R were then calculated for a corresponding ellipse in terms of the y intercept (log Y_0) vs. K_R, using the relation

$$\eta_0 = \alpha - \beta(\%\,\bar{T}) \quad (14)$$

The thickness of the ellipse at slope = $\overline{K_R}$ is defined where $\beta = \overline{K_R}$, and thus

$$\eta_0 = \log Y_0(\text{at } \overline{K_R}) = [\bar{Y} \pm (F2\bar{s}_0^2/\sum w)^{1/2}] + \overline{K_R}(\%\,\bar{T}) \quad (15)$$

The ends of the ellipse at $K_{R,max}$ and $K_{R,min}$ = $[\overline{K_R} \pm [F2\bar{s}_0^2/\sum w(\%\,T - \%\,\bar{T})^2]^{1/2}$ are defined where $\alpha = \bar{Y}$, and thus

$$\eta_0 = \log Y_0(\text{at } K_{R,max} \text{ and } K_{R,min}) = \bar{Y} - (\%\,\bar{T})(K_{R,max} \text{ or } K_{R,min}) \quad (16)$$

Compositional Analyses. Amino acid and amino sugar analyses were performed on a preparation of purified atrial mAcChR estimated from silver-stained gels to consist of 96% mLBP (the remainder being mSCP). Samples (0.5–0.7 mL) of this preparation in 0.8% digitonin, 0.16% cholate, 25 mM imidazole, and 1 mM EDTA, pH 7.4, or the detergent buffer only, were dried under vacuum and, for amino acid analysis, hydrolyzed at 110 °C for 22, 48, and 72 h in vacuo in 6 N HCl plus 1% phenol under vapor phase. The amino acids were analyzed by using the PICO-TAG system (Waters Associates) with a 4.5 mm × 250 mm C_{18} IBM column following precolumn derivatization with phenyl isothiocyanate (Heinrikson & Meredith, 1984). For amino sugar analysis the samples were hydrolyzed at 100 °C for 4 h in vacuo in 4 N HCl (Spiro, 1966) and analyzed on a 3 mm × 33 mm DC4A resin (Durrum) column with *o*-phthaladehyde detection (Roth & Hampai, 1973; Benson & Hare, 1975).

Results

D_2O/H_2O Sucrose Gradient Sedimentation. As shown in Figure 1, the purified mAcChR exchanged into Triton X-405 sedimented in sucrose gradients as a single prominent peak with a small faster sedimenting shoulder. Total receptor recovery from the gradient was about 40% even when preincubated with the specific antagonist [^{3}H]-L-QNB. Considerably lower recoveries were experienced without the stabilizing

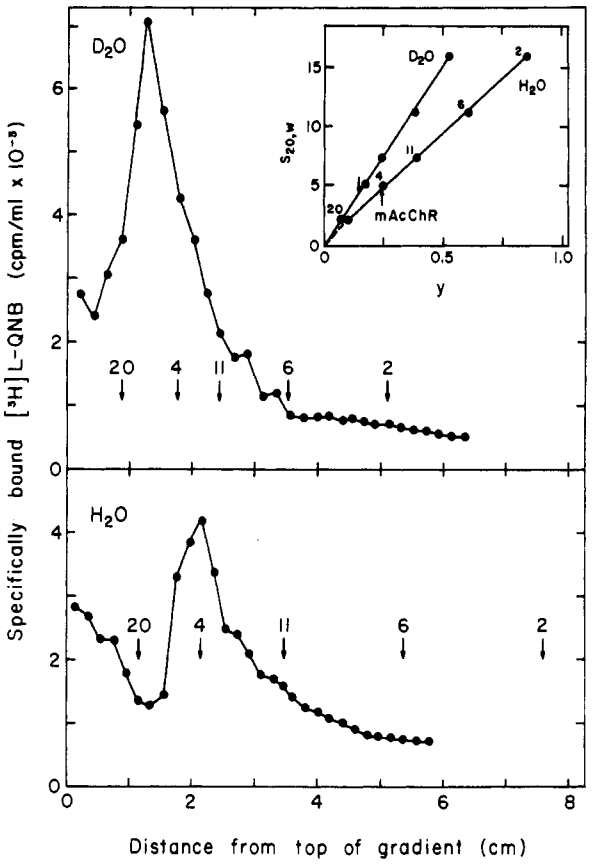

FIGURE 1: Sedimentation of purified mAcChR through sucrose gradients in Triton X-405. The mAcChR complex with [^{3}H]-L-QNB in 0.35% Triton X-405 was sedimented in D_2O and H_2O sucrose gradients as described under Experimental Procedures. The sedimentation positions for the standards are indicated with numbers corresponding to those of Table I. The inset shows the relationship of the sedimentation coefficients to the respective fractional migration distance, y, for the standards. The data were fitted by linear least-squares regression which gave $r^2 = 0.999$ for both D_2O and H_2O. The y intercept was not significantly different from zero for the D_2O fit and just significant ($P \approx 0.05$) for H_2O with $s_{20,w}$ = 0.46 S at y = 0.

preincubation with QNB. Imidazole buffer rather than phosphate buffer was also found to improve stability (up to 2-fold) which agreed with the previously reported behavior of imidazole as a low-affinity mAcChR ligand (Peterson & Schimerlik, 1984). Presumably the mAcChR was simply less stable when exchanged into 0.35% Triton X-405. Detergent exchange was assumed to be virtually complete. Studies with our crude digitonin/cholate extract mAcChR demonstrated no detectable difference in sedimentation characteristics when the receptor was exchanged into Triton X-405 by means of DEAE column chromatography or by simple dilution as above.

The partial specific volume of the purified mAcChR calculated from the sedimentation data of Figure 1 according to eq 2 yielded a value of $\bar{v}_A$ = 0.813 cm^3/g with transferrin as the known standard. This compared to a value of 0.815 cm^3/g obtained for sedimentation of the crude extract receptor preparation. Accuracy of this approach depends on linearity of $s_{20,w}$ on y, the fractional distance migrated, during sedimentation of the standards and on the choice of a standard of similar $s_{20,w}$ value to that of the unknown (O'Brien et al., 1978). Moreover, this approach is essential when the unknown and standard partial specific volume are unequal. The inset to Figure 1 demonstrated that $s_{20,w}$ was linear with respect to y or very nearly so for both the D_2O and H_2O experiments. The sedimentation coefficient for the purified mAcChR cal-

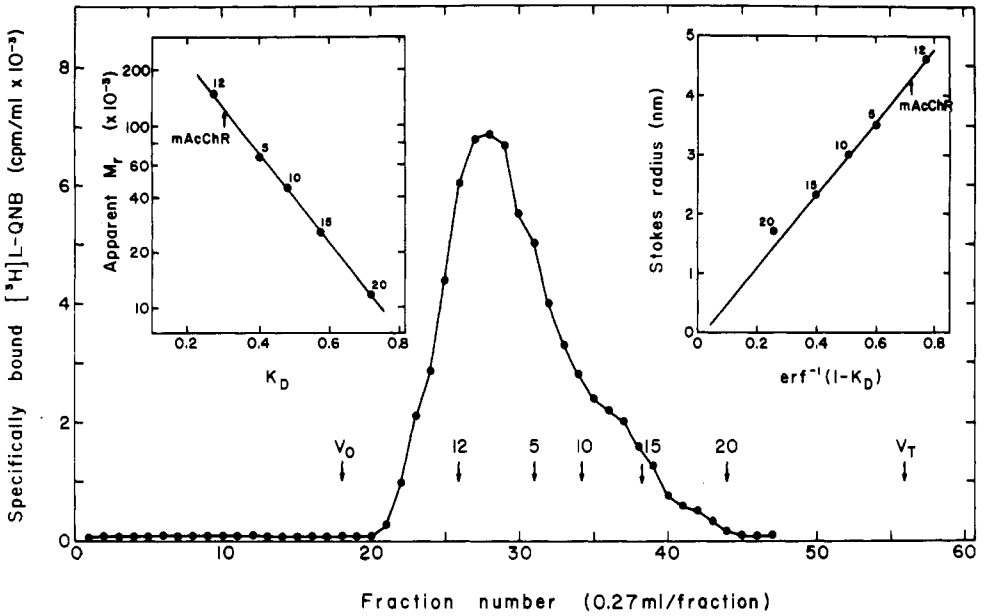

FIGURE 2: Gel filtration of purified mAcChR on Sephacryl S-300 in Triton X-405. The purified mAcChR was preincubated with [^{3}H]-L-QNB and chromatographed in Triton X-405 as described under Experimental Procedures. The receptor was chromatographed together with the indicated standards (numbers correspond to those in Table I) showing their observed elution positions. Identical results were obtained when the receptor was chromatographed separately from the standards. V_0 and V_T represent the elution positions of Blue Dextran 2000 and free [^{3}H]-L-QNB, respectively. The inset on the left represents the calibration of M_r vs. K_D (calculated from eq 1). Linear least-squares regression gave the following relation: $\log M_r = 5.82 - 2.45K_D$, $r^2 = 0.998$. The inset on the right represents the calibration of the Stokes radii on the inverse error function of $1 - K_D$. Least-squares regression, omitting cytochrome *c* (no. 20) from the analysis, yielded the relation $R_s = 6.14\ \mathrm{erf}^{-1}(1 - K_D) - 1.38$, $r^2 = 0.999$. The *y* intercept was not significantly different from zero.

culated according to eq 3 was $s_{20,w}{}^A = 5.30 \times 10^{-13}$ s, which was very close to that of the standard (transferrin $S_{20,w} = 5.10 \times 10^{-13}$ s).

An apparent molecular weight for the mAcChR–Triton X-405 complex was calculated from the sedimentation and partial specific volume measurements according to eq 4 and found to be 143 000. Since the amount of bound detergent could not be measured directly, it was estimated from the partial specific volumes measurements according to eq 5. The accuracy of this method for determining bound detergent depends on the accuracy of the component $\bar{v}$'s. As indicated, accuracy of $\bar{v}_c$ was determined with at least a theoretically high degree of confidence. $\bar{v}_D$ was measured directly. $\bar{v}_p$ was calculated from the composition (Table III) which contained an element of guesswork concerning the amount of covalently attached carbohydrate. When the estimate of $\bar{v}_p = 0.717$ was used, δ_D was calculated from eq 5 as 1.011 g/g, and the apparent molecular weight of the uncomplexed mAcChR was calculated from eq 6 to be 70 900. Thus, 50% of the weight of the receptor–Triton X-405 complex was due to detergent bound at potentially hydrophobic regions of the mAcChR.

Hydrodynamic Properties from Gel Filtration. Figure 2 shows the elution pattern of the purified mAcChR from the Sephacryl S-300 column run in the presence of Triton X-405. The elution profile was relatively symmetrical with the exception of a small lower molecular weight shoulder. A plot of the log M_r for the standards was linear with respect to their partition coefficient, K_D (Figure 2, inset), calculated from the elution volumes according to eq 1, predicting an apparent molecular weight for the mAcChR–Triton X-405 complex of 116 000. Application of eq 6 assuming $\delta_D = 1.011$ as above yielded an apparent molecular weight of 57 800 for the uncomplexed receptor. The Stokes radii for the standards were also linear with respect to K_D ($R_s = 62.1 - 65.3K_D$, $r^2 = 0.975$), which yielded an apparent R_s for the mAcChR–Triton X-405 complex of 4.10 nm. The more rigorous method of Ackers (1967) relating the Stokes radius to the inverse error function of $1 - K_D$ (eq 7) produced a value of 4.29 nm for the Stokes radius of the mAcChR–Triton X-405 complex (Figure 2, inset). Cytochrome *c* was detectabely anomalous in these correlations in agreement with Siegel and Monty (1966), and its elimination improved r^2 from 0.993 to 0.999 [$R_s = 6.14\ \mathrm{erf}^{-1}(1 - K_D) - 1.38$]. The nonlinear calibration curves reported by le Maire et al. (1980) for Sephacryl S-300 were not observed in our studies. The *y* intercept was not statistically significant from 0. The molecular weight of the receptor–detergent complex obtained from eq 8 which included the sedimentation parameters $s_{20,w}$ and $\bar{v}$ for the complex was calculated as 137 000, giving an apparent molecular weight of 68 200 for the uncomplexed receptor from eq 6. A frictional ratio (f/f_0) of 1.21 in comparison to an anhydrous sphere was obtained from eq 9. The mAcChR thus behaved as a globular particle defined as $f/f_0 < 1.25$ (Tanford et al., 1974). Table II summarizes the physical properties of the purified mAcChR obtained from the sedimentation and gel filtration experiments above as well as the SDS–PAGE experiments below.

Composition Analysis. The amino acid and amino sugar content of the purified cardiac mAcChR is given in Table III. Cysteine and tryptophan were not determined, but their approximate contributions were estimated from their average contribution to three other integral membrane proteins; the nAcChR, the Na,K-ATPase, and the sodium channel. The purposes of estimating these residues was to allow for calculation of the partial specific volume of the mAcChR, which would be little affected by minor inaccuracies associated with the estimated values. The carbohydrates which were expected, but not determined, included the common hexoses and sialic acid. Sialic acid was known to be present on the cardiac mAcChR from neuraminidase digestion experiments (Herron & Schimerlik, 1983). The likely contribution of hexoses and sialic acid residues was estimated from their mean contribution to the above-mentioned three proteins relative to that of their amino sugar content. Amino sugars make up a large portion of the carbohydrate content of these proteins (40.1 ± 9.6%

Table II: Summary of Physical Properties of the Purified Cardiac mAcChR

Sedimentation Experiments in Triton X-405		
partial specific volume	$\bar{\nu}_c$[a] (cm³/g)	0.813
	$\bar{\nu}_p$[b] (cm³/g)	0.717[c]
sedimentation constant	$s_{20,w}$ (s)	5.30×10^{-13}
bound Triton X-405	δ_D (g/g)	1.011
molecular weight	M_c[a]	143 000
	M_p[b]	70 900
Gel Filtration Experiments in Triton X-405		
Stokes' radius	R_s (nm)	4.29
molecular weight (gel filtration alone)	M_c[a]	116 000
	M_p[b]	57 800
molecular weight (gel filtration plus sedimentation)	M_c[a]	137 000
	M_p[b]	68 200
frictional ratio	f/f_0	1.21

		SDS–PAGE Experiments			
		gradient gel[d]	single % T[e]	M_rK_R plot[f]	predicted[g]
mLBP	molecular weight, M_r	80 000 ± 2800	83 300 ± 10 400	89 100 ± 6700	50 000–60 000
	no. of residues, N	701 ± 21	729 ± 84	797 ± 60	450–540
mSCP	molecular weight, M_r	13 600 ± 320	14 600 ± 380	14 000 ± 1100	14 300
	no. of residues, N	124 ± 3	131 ± 4	130 ± 10	130

[a] For the receptor–Triton X-405 complex. [b] For the uncomplexed receptor. [c] From Table III. [d] Mean ± SD of the results from the four different cross-linked gels. [e] Mean ± SD of the value obtained from log M_r (or log N) calibration vs. R_F for each single % T at 2.6% C plus 3.85% C. [f] Combined determinations made at 2.6% C and 3.85% C. [g] From evaluation by the scheme shown in Table V (see Discussion).

Table III: Compositional Properties of the Purified Cardiac mAcChR

residue	mol %[a]	nearest integer per M_r 70 000
Ala	7.88 ± 0.05	37
Arg	2.76 ± 0.05	13
Asx	11.16 ± 0.13	53
Cys	(1.90 ± 0.56)	9
Gly	6.84 ± 3.14	32
Glx	9.80 ± 0.76	46
His	1.20 ± 0.02	6
Ile	6.56 ± 0.25	31
Leu	8.59 ± 0.00	41
Lys	3.53 ± 0.04	17
Met	1.44 ± 0.16	7
Phe	3.66 ± 0.16	17
Pro	6.40 ± 0.13	30
Ser	8.95 ± 0.43	42
Thr	5.98 ± 0.22	28
Trp	(2.23 ± 1.17)	11
Tyr	2.74 ± 0.01	13
Val	8.39 ± 0.83	40
	100.01	473
glucosamine	7.76	37
hexoses	(6.59 ± 3.18	31
sialic acid	(4.38 ± 0.80)	21
partial specific volume (cm³/g)	0.733 cm³/g[c] 0.717 cm³/g[d]	
hydrophobicity parameters[b]	$H\Phi_{av}$ = 1137 Z = 0.369	

[a] Amino acid and amino sugar analyses were performed as described under Experimental Procedures. The numbers in parentheses were for residues not determined but estimated from their mean compositional contribution to the nAcChR (Vandlen et al., 1979), the Na,K-ATPase (Kyte, 1972), and the sodium channel (Miller et al., 1983). The contribution of hexoses and sialic acid were estimated relative to the amino sugar content of each of the above proteins, which averaged 40.1 ± 9.6% of the total carbohydrate by weight. [b] Barrantes (1975). [c] Amino acids only. [d] Amino acids plus carbohydrates.

by weight) and thus serve as the best reference point for estimating the remaining components. The partial specific volume of the mAcChR including carbohydrate as calculated from the composition in Table III was 0.717 cm³/g. The amount of carbohydrate on the cardiac mAcChR was estimated at 26.5% of the total by weight. The nearest integer residue content for a protein of total M_r 70 000 (amino acid plus carbohydrate) was calculated from the composition and is given in Table III. The hydrophobicity parameters $H\Phi_{av}$ and Z were calculated according to Barrantes (1975) and are also given in Table III. The specific QNB binding site concentration for the receptor preparation in Table III was 12.4 nmol/mg of protein on the basis of the summation of the total amino acids.

SDS–PAGE Experiments. The gradient acrylamide SDS–PAGE experiments produced a slightly sigmoidal calibration curve of log M_r (or log N) vs. log % T, regardless of the cross-linking concentration. The apparent molecular weight (or N) values of the mAcChR LBP and the SCP were the same within experimental error for all gradient experiments, as were the values for the nAcChR subunits. The average values for molecular weight and N of the mAcChR LBP and SCP from these experiments are shown in Table II. The subunits of the nAcChR were treated in this and the subsequent SDS–PAGE experiments below as unknowns for evaluation purposes. Their sequences, and hence true molecular weight and N, are known. The percent deviations from the true molecular weight and N for the nAcChR subunits by the gradient SDS–PAGE experiments as well as the other SDS–PAGE experiments are summarized in Table IV.

The SDS–PAGE experiments run at single uniform acrylamide concentrations showed a sigmoidal relationship of log M_r (or log N) vs. R_F for gels from 8% T to 17% T at 2.60% C and from 7% T to 17% T at 3.85% C (eq 10). At lower % T gels a linear fit to the data was obtained. The calibration curves appeared very similar to those of a previous study of the Na,K-ATPase subunits [Figure 1 in Peterson & Hokin (1981)]. The coefficients of determinations for the sigmoid and linear least-squares fits of log M_r vs. R_F ranged from 0.985 to 0.998 and were usually better than 0.990. Virtually identical results were obtained when log N was calibrated vs. R_F, with only slight improvement in the data fit. The apparent molecular weight of the mLBP varied as a direct function of % T, irrespective of the cross-linking concentration (2.6 and 3.85% C) as shown in Figure 3. The apparent molecular weight ranged from a low of 69 000 (632 residues) at 7.1% T and 3.85% C to a high of 99 500 (858 residues) at 16.2% T and 3.85% C, with an approximately linear dependence of

Table IV: Predicted Accuracy (as Percent Deviation) in the Apparent Molecular Weight or N for the nAcChR Subunits by SDS-PAGE

subunit	M_r[a]	N	gradient gel	single % T [M_r (% T) plot][b] 6% T	17% T	Ferguson data M_rK_R plot
α	50 116[c]		−14.6	−27.2	−9.0	−1.2
β	53 681[d]		−8.6	−14.9	−0.7	+4.7
γ	56 248[e]		−2.2	−15.2	+5.2	+8.8
δ	57 565[d]		+10.6	+5.9	+15.6	+10.8
α		437[c]	−8.9	−22.9	−4.5	+2.4
β		493[d]	−9.1	−15.3	−2.0	+2.8
γ		489[e]	+1.2	−11.1	+9.0	+11.4
δ		522[d]	+8.0	+4.5	+14.1	+9.0

[a] For the amino acid portion only. [b] Determined from the regression of apparent molecular weight (or N) on % T (as shown in Figure 3). [c] From Noda et al. (1982). [d] From Noda et al. (1983a). [e] From Claudio et al. (1983).

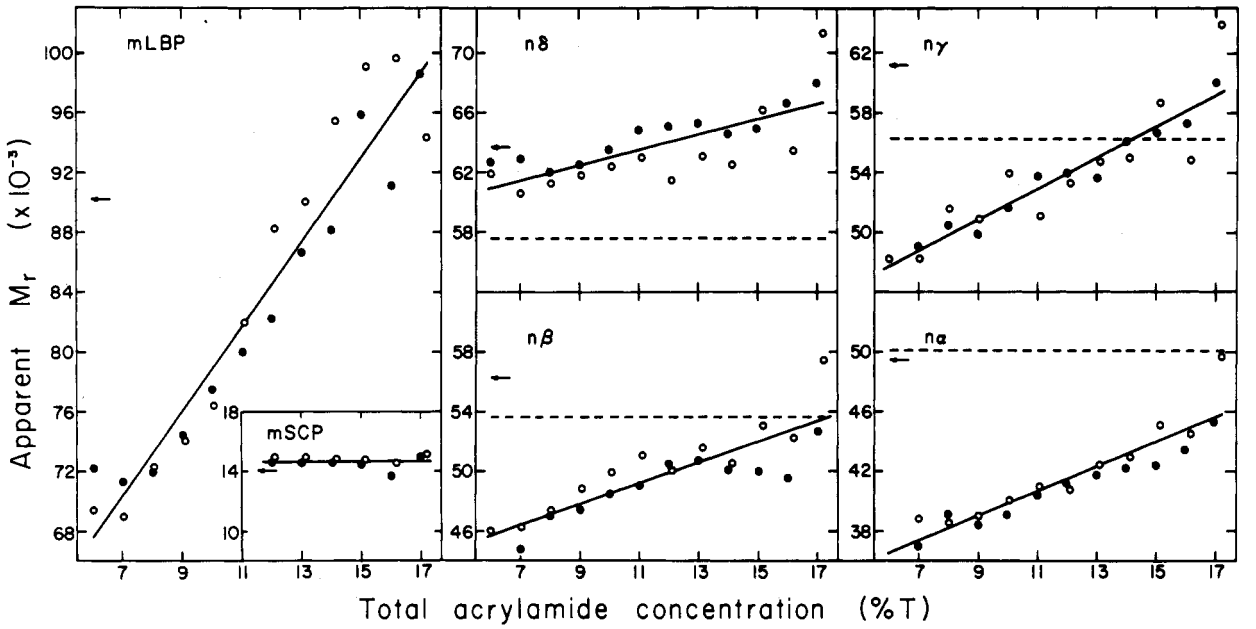

FIGURE 3: M_r(% T) plot of the purified mAcChR and nAcChR proteins. The apparent molecular weight of the unknown proteins was determined from calibration of log M_r vs. R_F (eq 10 or linear equivalent) for the molecular weight marker proteins at single % T at 2.6% C (closed circles) and 3.85% C (open circles) and plotted as a function of % T for each protein. The solid lines were fitted by linear least squares for the combination of 2.6% C and 3.85% C data. The dashed line for the nAcChR subunits represents the true molecular weight from sequence. The arrow indicates the apparent molecular weight as determined from the M_rK_R plot.

molecular weight on % T. The apparent molecular weight of the mSCP was the same over the useful resolving range of 12–17% T (Figure 3). The mean molecular weight and N calculated for the mSCP was 14 600 ± 380 (n = 12) and 131 ± 4, respectively.

The apparent molecular weight of each of the four nAcChR subunits also varied directly in a linear fashion with % T (Figure 3). The slopes were different for each subunit and at least 3-fold less than the slope for the mAcChR LBP. The apparent molecular weights obtained for the nicotinic receptor subunits were an underestimate of the true molecular weight in the case of the α and β subunits, an overestimate in the case of the δ subunit, and of mixed results for the γ subunit. The percent deviations from the true molecular weight for each nAcChR subunit at 6% T and 17% T were calculated from the linear least-squares regression of apparent molecular weight on % T (Figure 3) and are summarized in Table IV.

Ferguson plots (log R_F vs. % T) calculated by weighted least-squares regression to the linear eq 11 gave a coefficient of determination, r^2, > 0.99 for all proteins examined (marker proteins 1–10 and 13–20 in Table I, nAcChR subunits, mLBP, mSCP) with the exception of α_2-macroglobulin for which r^2 was 0.985. Regression of log M_r vs. log K_R (M_rK_R plot) was linear with r^2 = 0.984 for the 2.6% C gel experiment and 0.990 for the 3.85% C gel experiments (using markers 3–10 and 13–18; Table I). For calibration of log N vs. log K_R, r^2 was 0.986 for both cross-linked gel series. The apparent molecular weight and N for the mLBP were calculated as 90 100 ± 7500 and 807 ± 61 for the 2.6% C gel data and 88 100 ± 5800 and 787 ± 59 for the 3.85% C gel data. Corresponding values for mSCP were 13 600 ± 1200 and 126 ± 10 for 2.6% C and 14 400 ± 1000 and 133 ± 10 for 3.85% C. The difference between the two cross-linked gel series were small and not significant for the mAcChR proteins or the nAcChR subunits, and thus, an average was used for the summary in Table II and for evaluation of the percent deviations from the true molecular weight for the nAcChR subunits (Table IV). The M_rK_R plot closely approximated the true molecular weight and N for the α and β nAcChR subunits, but overestimated these parameters for the γ and δ subunits by about 10%.

The joint 95% confidence envelopes for log Y_0 vs. K_R (Y_0K_R plot) of the standards and receptor proteins for the 2.6% C series gels are shown in Figure 4. There was a slight but significant ($P \approx 0.05$) inverse linear relation of log Y_0 vs. K_R over the molecular weight range of most of the standards (15 000–100 000). The nAcChR δ subunit and the mSCP showed log Y_0,K_R values that lay within those for the standard proteins. All of the remaining receptor proteins were found to have log Y_0 values in excess of the standard proteins, indicating that these latter proteins contained excess charge

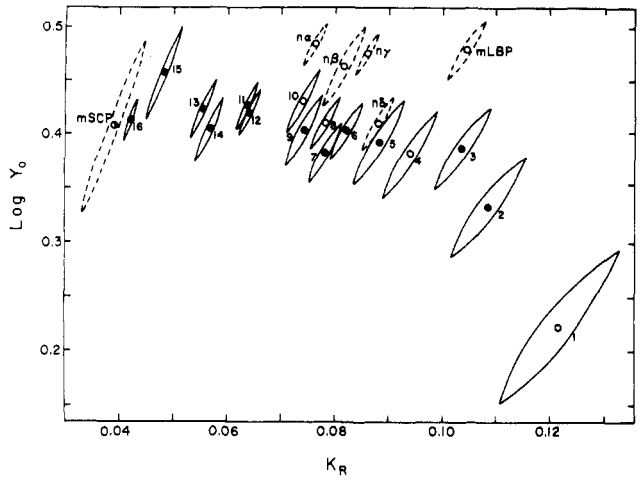

FIGURE 4: Y_0K_R plot for the 2.6% *C* gel series. The joint 95% confidence envelopes for the Ferguson parameters log Y_0 and K_R were calculated according to eq 15 and 16 and plotted as indicated for the marker proteins (numbers correspond to those in Table I) and receptor proteins. The mean log Y_0 and K_R values are indicated by solid circles for nonglycosylated proteins and open circles for glycoproteins, and the envelopes are drawn with solid lines in the case of the marker proteins and broken lines in the case of the receptor proteins. It is not presently known if the mSCP is a glycoprotein.

densities which would cause them to migrate anomalously in SDS–PAGE.

Discussion

Determination of the sedimentation constant by sedimentation velocity experiments in D_2O/H_2O sucrose gradients coupled with an independent estimate of the effective size (Stokes radius) has been an attractive alternative to sedimentation equilibrium for measurement of the molecular weight of limiting amounts of detergent-solubilized membrane proteins (Tanford et al., 1974; Clark, 1975; Fish, 1975, Gibson et al., 1976; O'Brien et al., 1978). Measurement of sedimentation coefficient by rate sedimentation in D_2O/H_2O sucrose gradients and the Stokes radius by gel filtration as performed in these studies implies several underlying assumptions and considerations which could influence the final molecular weight estimate. It is assumed that the amount of detergent bound to the water-soluble marker proteins is negligible and that the amount of detergent bound to the receptor remains the same in both D_2O and H_2O and in the presence of sucrose. Clark (1975) measured the binding of the closely related detergent, Triton X-100, in a number of water-soluble proteins, including most of those used in this study. No binding was observed with a detection limit of about 2% of the weight of the protein. Triton X-100 binding to membrane proteins range from about 20 to 112% (w/w) and is generally slightly lower in D_2O than H_2O (Clark, 1975), which could contribute to a molecular weight estimate that is low by 3–10%. Sucrose may also effect detergent binding to a small degree (Simons et al., 1973), but this is considered negligible in our experiments where binding was estimated from the partial specific volume measurements made from the sucrose gradient experiments (eq 2).

Errors in the estimate of the partial specific volume of the unknown protein have a significant effect on the sedimentation coefficient and hence the molecular weight estimate. Modeling studies by O'Brien et al. (1978) showed that under the conditions examined in this study where the sedimentation coefficient for the mAcChR and the marker protein, transferrin, were very similar (5.3 vs. 5.1) and $\bar{\nu}$ was 0.8 for the unknown (measured 0.813) and 0.7 for the standard (actual value 0.724), the predicted error in the estimate of $s_{20,w}$ was less than 2% by the method employed (eq 2 and 3) compared to up to 30% by other methods. Thus, we believe that our estimate of $\bar{\nu}$ (0.813) and $s_{20,w}$ (5.3 S) for the mAcChR–Triton X-405 to be accurate to within about 5% and contribute to a minimum molecular weight estimate by eq 8. The molecular weight estimate by eq 8 further requires an estimate of the effective molecular size or radius assuming the protein is globular in shape. The Stokes radius is a sufficiently accurate measure of this parameter (Tanford et al., 1974) and was measured by calibration against K_D, using the linear function of R_S vs. $\mathrm{erf}^{-1}(1 - K_D)$ proposed by Ackers (1967), which is independent of assumptions concerning the structure of the gel matrix (Ackers, 1967) or the nature of the solvent system used (Tanford et al., 1974). The gel profile shown in Figure 2 exhibits a trailing edge in the lower molecular weight range. By itself this suggests that the mAcChR interacts with the gel matrix which would invalidate the data obtained from such an elution pattern. However, the apparent molecular weight for the major peak as determined from the elution volume (116 000) is only about 20% lower than that obtained by sedimentation alone (143 000), which suggests that the bulk of the mAcChR was not seriously retarded on the Sephacryl S-300 resin. The apparent molecular weight arrived at by combination of the sedimentation and gel filtration data according to eq 8 (137 000) is very similar to that from sedimentation alone. The frictional ratio determined for the receptor in Triton X-405 was 1.21, which is less than the maximum suggested value of 1.25 descriptive of a globular particle (Tanford et al., 1974), and thus agrees with the underlying assumptions of shape associated with the hydrodynamic studies.

Estimation of the molecular weight of the mAcChR alone requires knowledge of the amount of bound detergent. Accurate estimates of the component specific volumes is required to determine this value according to eq 5. As indicated, the partial specific volume of the receptor–detergent complex was determined with a reasonable degree of confidence. The partial specific volume of the Triton X-405 (0.908 cm^3/g) was the same as that reported for the closely related detergent Triton X-100 (Tanford et al., 1974). The partial specific volume of the uncomplexed mAcChR was calculated from the amino acid and partially estimated carbohydrate composition (Table III). On the basis of the analysis of other integral membrane sialoglycoproteins, the undetermined residues expected to be present in significant quantities (viz., hexoses and sialic acid) may comprise from 50 to 70% of the total carbohydrate (Kyte, 1972; Vandlen et al., 1979; Miller et al., 1983). The estimated contribution of these residues to the mAcChR (Table III) leads to a total carbohydrate content estimated at 26.5% by weight. This figure is consistent with the behavior of the mLBP in SDS–PAGE. The mLBP migrates as a broad diffuse band similar to that observed for the Na,K-ATPase β subunits (Perrone et al., 1975) and Na channel (Miller et al., 1983) which contain 25–30% by weight carbohydrate. In addition the anomalous behavior of the mAcChR in SDS–PAGE in this study cannot be accounted for by abnormal charge density alone but must contain a large contribution of abnormal shape, probably due to a high (20–30%) carbohydrate content (see discussion below). Therefore, we believe the values given in Table III for the estimated portion of the mAcChR carbohydrate content are sufficiently accurate to allow for calculation of the partial specific volume of the receptor protein within 5% error. The amount of lipid bound to the receptor should also be taken into account (Tanford et al., 1974);

however, the small amounts of receptor available did not permit lipid analysis after exchange into Triton X-405. The amount of lipid in the mAcChR–Triton X-405 complex is expected to be insignificant (Clark, 1975). Moreover, any small amount of lipid bound would have a $\bar{v}$ similar to or slightly higher than that of Triton X-405 (Tanford et al., 1974) and would be estimated as detergent with negligible consequences.

The hydrodynamic properties of the mAcChR have previously been examined in crude preparations from rat brain by Haga (1980) and latter by Berrie et al. (1984a). The $s_{20,w}$ values reported for the rat brain mAcChR were lower (3.2–3.8 S) for membranes solubilized in Lubrol PX or cholate/1 M NaCl–phosphatidylcholine, higher (10.8 S) for digitonin, and similar (4.96 S) for lysophosphatidylcholine in relation to our value of 5.3 S. In all the detergents tested, these authors reported higher apparent Stokes radii and frictional ratios than our values. These findings lead to considerable variation in the apparent molecular weight of the protein–detergent complex, which, as pointed out by these authors, suggest molecular weight heterogeneity, particularly with regard to gel filtration. The consistency in the apparent molecular weight of the purified cardiac mAcChR between the sedimentation and gel filtration experiments suggests that a relatively monodisperse system was measured in our studies. The molecular weight of the purified cardiac mAcChR is thus believed to be reliably estimated at 68 000–71 000, including carbohydrate.

The amino acid composition of the porcine atrial mAcChR (Table III) is similar to but not identical with that of the porcine brain (Haga & Haga, 1985). The comparative indexes D (=0.0570), DI (=8.93), or $S\Delta Q$ (=32.5) (Cornish-Bowden, 1983) give only a weak indication that the two proteins are related. This may reflect inherent difficulties in agreement between different laboratories attempting rigorous chemical analysis of limiting amounts of glycoprotein. However, if the two compositions are equivalently accurate, then the comparative indexes suggest that a significant amount of real sequence differences exist between the brain and heart mAcChRs. This is particularly interesting since the brain mAcChR is composed chiefly of the M1 subtype, whereas the heart mAcChR is composed chiefly of the M2 subtype (Hammer et al., 1980), which is a pharmacological distinction currently based on sensitivity to the antagonist pirenzepine. The values for the hydrophobicity parameters $H\Phi_{av}$ and Z (Table III) are consistent with the integral membrane protein classification of the mAcChR. The heart mAcChR was found to be only slightly more hydrophobic than the brain mAcChR (Z_{heart} = 0.369; Z_{brain} = 0.339). In comparison to other proteins the mAcChR is intermediate in hydrophobic character between the Na channel and the nAcChR (Miller et al., 1983), both of which apparently contain numerous transmembrane sequences (Claudio et al., 1983; Noda et al., 1983b, 1984).

The molecular weight estimates of the mAcChR by sedimentation and gel filtration, which include the carbohydrate portion of the molecule, are lower than the estimates obtained by SDS–PAGE, which ideally only measures the protein portion or chain length of the molecule (Reynolds & Tanford, 1970a). The Ferguson plot data, however, indicate that the receptor migrates anomalously in SDS–PAGE, which is due at least in part to an excess charge density presumably from excess SDS binding relative to that of the standards. The diffuse banding pattern of the mAcChR on SDS–PAGE suggests that it contains sufficient carbohydrate such that abnormal shape may also contribute to anomalous migration. No comprehensive evaluation method currently exists for predicting the effects of abnormal charge and shape on the apparent molecular weight. Segrest and Jackson (1972) suggested an empirical method for estimating the amount of carbohydrate on sialoglycoproteins which migrate anomalously by correcting the apparent molecular weight at high % T by the percent carbohydrate determined by the difference in molecular weight between 5% T and 7.5% T. This method did not work, however, for asialoglycoproteins. These authors reported lower than normal SDS to protein binding ratios for glycoproteins, which do not agree with the findings of others (Pitt-Rivers & Impiombato, 1968; Reynolds & Tanford, 1970b), and may be due to the presence of urea in the SDS–PAGE system of Segrest and Jackson. Some membrane glycoproteins apparently bind less than normal amounts of SDS (Banker & Coltman, 1972; Frank & Rodbard, 1975) whereas others bind more than normal (Peterson & Hokin, 1981; Miller et al., 1983; this report). The Ferguson parameters and in particular the Y_0K_R plot (Figure 4) can be used to detect anomalous migration behavior due to abnormal charge, in which case molecular weight calibration vs. K_R may be more accurate (Table IV; Banker & Cotman, 1972). However, this analysis cannot be used alone to validate the use of SDS–PAGE or to suggest that the molecular weight determined from the M_rK_R plot is necessarily more accurate. Frank and Rodbard (1975) assumed that this was the case for their SDS–PAGE studies of rhodopsin, preferring the M_rK_R determined value of 29 500 to the average molecular weight from several single % T gels (~37 500). The true molecular weight for rhodopsin by sequence data has been found to be about 39 000 (Hargrave et al., 1983). Clearly, a more comprehensive evaluation method would be beneficial.

We found that data derived from the Y_0K_R plot, M_r(% T) plot and M_rK_R plot could be evaluated in concert to predict with reasonable confidence when abnormal shape as well as charge properties was attributable to a given protein, approximately what the relative contributions of these parameters were, and in which relative direction the true molecular weight would lie. This was accomplished partially on theoretical bases and partially on emperical modeling studies and was verified by analysis of the nAcChR subunits.

Theoretically the free electrophoretic mobility, log Y_0, is an estimate of the relative net charge on a protein, whereas the retardation coefficient, K_R, can be regarded as a measure or index of shape. Most reduced proteins bind 1.4 g of SDS/g of protein (Pitt-Rivers & Impiombato, 1968; Reynolds & Tanford, 1970b) and hence should have the same charge density regardless of size. Since K_R is a direct function of molecular weight, it should thus be independent of log Y_0 and the Y_0K_R plot should have zero slope. This situation is approached in our gel system (Figure 4) as well as others (Neville, 1971; Banker & Cotman, 1972; Frank & Rodbard, 1975), over the molecular weight range of 15 000–100 000. Relatively large changes in K_R have very small or zero effects on log Y_0. Abnormal charge densities due to abnormal SDS binding can thus be relatively assessed by the percent deviation in the measured log Y_0 for a given protein (with measured K_R) in comparison to the mean log Y_0 of the standards at the same K_R determined from the Y_0K_R plot. Logically, a decrease in normal charge density would result in decreased mobility and overestimation of molecular weight, whereas increased charge density would result in increased mobility and underestimation of molecular weight. SDS–protein complexes form rodlike structures whose apparent size is little effected by changes in the amount of bound SDS (Reynolds & Tanford, 1970a). Significant inherent shape changes could conceivably derive

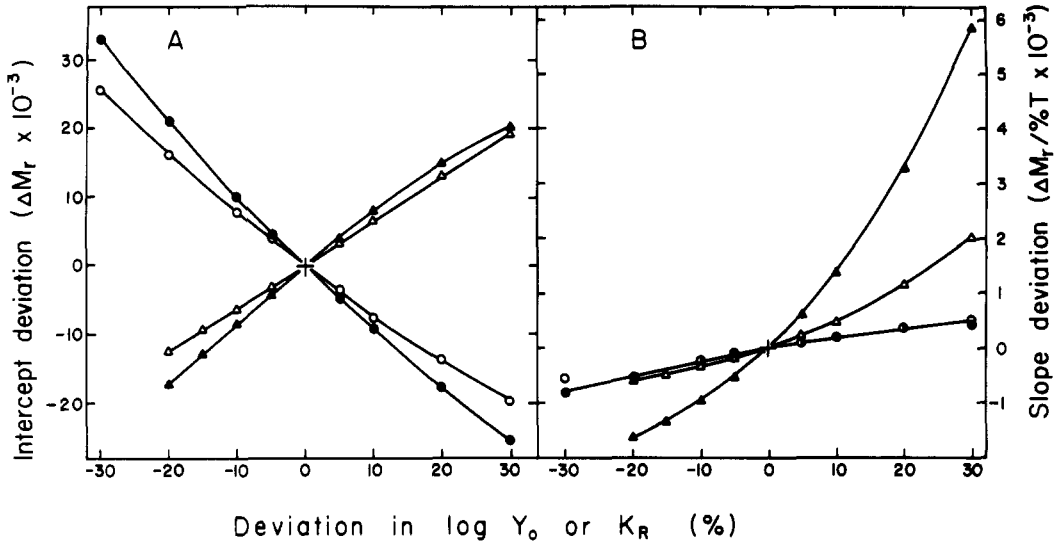

FIGURE 5: Predicted effects of deviations in log Y_0 and K_R on the intercept (A) and slope (B) of the M_r(% T) plot. Deviations in the observed values of log Y_0 (circles) of K_R (triangles) for catalase (open symbols) and for phosphorylase a (closed symbols) were used to recalculate R_F according to eq 11 and then to recalculate molecular weight from the new R_F according to eq 10 for each single % T, and the resulting molecular weight values were plotted as a function of % T. Deviations in the intercept and slope of the linear regression line of the M_r(% T) plot are shown normalized to an ideal zero-effect control.

from covalently attached branched structures such as carbohydrates.

The effects of abnormal log Y_0 and K_R on migration were modeled with the Ferguson relation by calculating R_F using eq 11 for a specific deviation in log Y_0 or K_R or both at each acrylamide concentration examined. This was done for two standard proteins expected to bracket the mAcChR ligand binding protein, viz., catalase at M_r 57 471 and phosphorylase a at M_r 97 114. Given each newly calculated R_F, the apparent molecular weight was then calculated by using the previously standardized equation relating R_F to log M_r (eq 10) for each single % T gel. These latter calibrations define the normal condition such that a plot of molecular weight vs. % T is ideally a straight horizontal line passing through the true molecular weight. Deviations from ideal log Y_0 and K_R for a given protein result in anomalous migration (R_F) and hence M_r. Such effects are dependent on % T [a direct measure of the gel sieving properties (Chrambach & Rodbard, 1971)] because of the progressively larger influence of the K_R(% T) factor on R_F as % T increases (eq 11).

The effects of deviations in log Y_0 and K_R on the M_r(% T) plot included changes in the slope of the curve (i.e., nonzero slope) and departure of the intercept (M_r at 0 % T) from the true molecular weight. All the M_r(% T) plots modeled exhibited linear functions of M_r on % T, with somewhat more scatter than that shown in Figure 3 for molecular weight determined from the measured R_F's. Because of artifically induced nonideal behavior observed by using the recalculated R_F's rather than the measured R_F's, the effects of changes in log Y_0 and K_R were determined relative to the zero effect model to arrive at normalized intercept and slope changes in the M_r(% T) plot. These modeled effects are shown in Figure 5 for the intercept (panel A) and the slope (panel B). The charge parameter log Y_0 has an inverse relation with the apparent molecular weight in the absence of sieving effects (i.e., at the intercept, 0% T, of the M_r(% T) plot), whereas deviations in K_R have a direct effect on the apparent molecular weight. The effects of deviations in log Y_0 and K_R on the intercept are approximately equal in magnitude, nearly linear in effect, and only slightly dependent on molecular weight due to the slight dependence of log Y_0 on K_R (Figure 4). Both log Y_0 and K_R have a direct effect on the slope of the M_r(% T) plot (Figure 5A). By comparison the effects of log Y_0 deviations are small, independent of molecular weight, and linear, whereas the effects of K_R deviations are large, molecular weight dependent, and apparently exponential. The slope deviations resulting from the combined effects of deviations in log Y_0 and K_R were found to be additive of their independent effects. Intercept effects were found to be less precisely additive but were approximately additive when expressed as percent of intercept change relative to the control model.

An example of the behavior of the M_r(% T) plot for catalase modeled separately for deviations in log Y_0 and K_R is shown in Figure 6. The lines have been extrapolated to intercept the normalized zero effect condition at the true molecular weight (dashed lines in Figure 6). The observed intersection points fell within categorical ranges of % T, which allowed for predictive information on the type of abnormal deviations observed, either charge or shape. Additional predictive information was found when the molecular weight determined from the M_rK_R plot was modeled for deviations in K_R (theoretically, log Y_0 should make no contribution). The arrows in the right panel of Figure 6 depict the M_rK_R determined molecular weight relative to the corresponding M_r(% T) plot. Models combinding the effects of log Y_0 and K_R produced lines whose intersection with the true molecular weight and the M_rK_R determined molecular weight could be predicted on the basis of the independent contributions of log Y_0 and K_R.

The observations and predictions on the Y_0K_R plot, M_r(% T) plot, and M_rK_R plot are summarized in Table V for each of nine possible cases for variations in log Y_0 and K_R representing the various conditions of normal or abnormal charge density and shape of SDS–protein complexes during electrophoresis through Laemmli (1970) polyacrylamide gel systems. Data from the gradient SDS–PAGE experiment are not useful in this analysis. The gradient system gives only a single curve which is sensitive to both charge and shape anomalies (Poduslo & Rodbard, 1980) and represents only part of the M_r(% T) plot data. The observations in Table V were sufficient in most cases to detect the presence of abnormal charge or shape or both in an unknown protein and hence evaluate the validity of SDS–PAGE systems in providing reliable molecular weight data and also to predict with some confidence both the relative extent of the abnormal Ferguson parameter(s) and where the

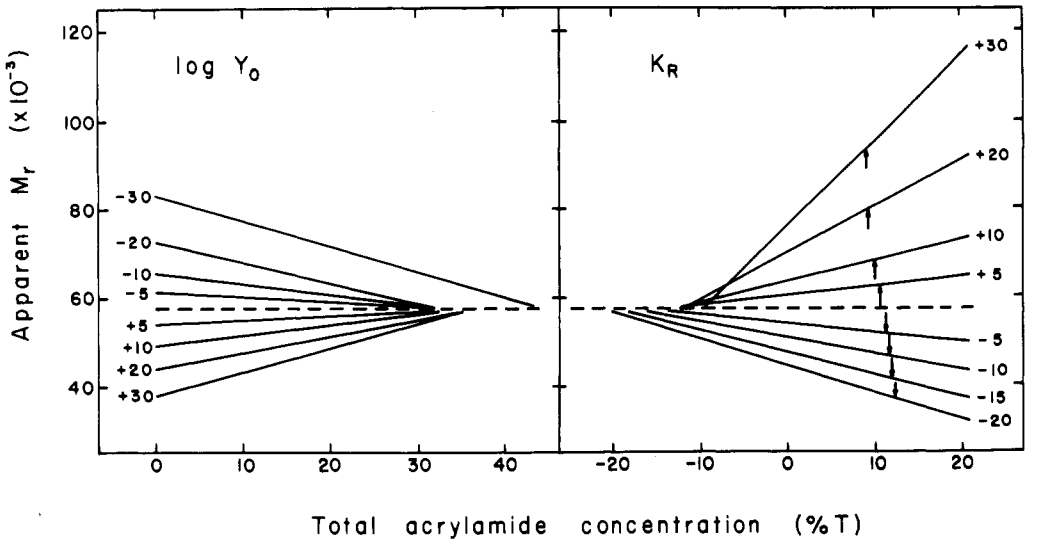

FIGURE 6: Predicted apparent molecular weight of catalase as a function of % T for modeled deviations in log Y_0 and K_R. The M_r(% T) plots for deviations in log Y_0 (left panel) and K_R (right panel) were modeled and normalized to an ideal zero-effect control as described in Figure 5. The linear regression lines and their extrapolated intersection points with the true molecular weight (dashed line) for catalase and the intercept at 0% T are shown. The numbers referring to the regression lines indicate the modeled percent deviations in log Y_0 or K_R. The apparent molecular weight determined from the M_rK_R plot intersects the regression line at the % T indicated by the arrows shown (right panel) for the corresponding deviations in K_R. Ideally, deviations in log Y_0 have no effect on the molecular weight determined from the M_rK_R plot, and the intersections with the regression lines in this case are the same as for the true molecular weight (left panel).

Table V: Observations and Predictions of SDS–PAGE Data Relative to Normal and Abnormal Ferguson Parameters for Charge Density (log Y_0) and Shape (K_R)[a]

	classification		observations				predictions for apparent M_r relative to true M_r		
			Y_0K_R plot	M_r(% T) plot	M_rK_R plot relative to M_r (% T) plot		M_r (% T) plot		M_rK_R plot
case	log Y_0	K_R	log Y_0[b]	slope	M_r	intersect[c]	M_r estimate	intersect[c]	M_r estimate
1	normal	normal	same	zero	same		same		same
2	high	normal	high	positive	larger	35–45	low	35–45	same
3	low	normal	low	negative	smaller	35–45	high	35–45	same
4	normal	high	same	positive	near $\bar{X}$	7–10	high	(–)3–(–)15	high
5	normal	low	same	negative	near $\bar{X}$	8–11	low	(–)8–(–)20	low
6	high	high	high	positive	larger	5–35	mixed	0–17	high
7	low	high	low	(positive)[d]	smaller	(<5)[d]	high	(<0)[d]	high
8	high	low	high	(positive)[e]	larger	(>35)[e]	low	(>45)[e]	low
9	low	low	low	negative	smaller	>17	mixed	(–)17–35	low

[a] Characteristics are for the ideal situation modeled from catalase and phosphorylase a as per Figures 5 and 6. [b] The position of log Y_0 relative to regression line for log Y_0 vs. K_R of standards. [c] The % T where the regression of line of molecular weight vs. % T would intersect the M_rK_R plot determined molecular weight (column 7) or the true molecular weight (column 9). [d] In case 7 K_R effects are more likely to dominate. If log Y_0 dominated, the M_r (% T) slope would be negative and shallow, and intersect the M_rK_R determined molecular weight at >100% T and the true molecular weight at even higher % T. [e] In case 8 log Y_0 effects are more likely to dominate. If K_R dominated, the M_r (% T) slope would be negative and intersect the M_rK_R determined molecular weight at <0% T and the true molecular weight at <(–)20% T.

Table VI: Evaluation of SDS–PAGE Data for the AcChR Proteins According to the Scheme in Table V

	observations							predictions	
				M_r (% T) plot		M_rK_R plot relative to M_r (% T) plot			apparent M_r relative to true M_r
	Y_0K_R plot log Y_0 % deviations			slope (ΔM_r/% T)	intercept ($M_r \times 10^{-3}$)	M_r	intersect (% T)	case	M_r (% T) plot ($M_r \times 10^{-3}$)[a]	M_rK_R plot ($M_r \times 10^{-3}$)[a]
proteins	2.6%[c] (%)	3.85%[c] (%)	mean (%)						
$n\alpha$	18.6	19.9	19.2	830	31.5	high	21.4	2, 6	low (>46)	close (≤50)
$n\beta$	15.2	13.4	14.3	690	41.6	high	21.0	6	mixed (>46)	high (<56)
$n\gamma$	19.2	15.0	17.1	1040	41.4	high	19.8	6	mixed (>50)	high (<61)
$n\delta$	3.0	0.9	2.0	550	57.9	near $\bar{X}$	13.6	4	high (<58)	high (<64)
mLBP	23.1	20.0	21.6	2820	50.6	near $\bar{X}$	14.0	6	mixed (<68, >50)	high (<80)
mSCP	–4.5	–1.1	–2.8	6	14.6	same		1	same (=14.6)	same (=14.6)

[a] The value in parentheses represents the estimate of the true molecular weight to the predictive limit of the analysis scheme.

true molecular weight might lie.

The scheme arrived at and shown in Table V was verified by application of the data from the nAcChR subunits for which the true molecular weight is known from sequence data (Table IV), and the results are shown in Table VI. The α, β, and γ subunits were classified as either case 2 or case 6, the difference being whether K_R made a significant contribution to the anomalous behavior. We could not conclude from the experimental data whether $n\alpha$ behavior corresponded to either case 2 or case 6, but the experimental data for $n\beta$ and in particular $n\gamma$ relative to $n\alpha$ suggest that K_R anomalies are more pronounced, and thus case 6 clearly applied to the $n\beta$ and $n\gamma$ subunits. The $n\delta$ subunit has a small (considerable negligible) contribution due to charge and thus fits case 4

classification. The apparent molecular weight based on these experimental results as fit to the scheme of Table V correctly predicted the direction of the true molecular weight in each nAcChR subunit.

The validity of the scheme in Table V could also be verified from literature data. In previous studies with the Na,K-ATPase subunits (Peterson & Hokin, 1981) the β subunits of four species behaved according to either case 4 or case 6 (with dominant K_R influence), correctly indicating the molecular weight was overestimated in all measurements. The data of Frank and Rodbard (1975) for rhodopsin best fits case 9. This suggests that the molecular weight from the M_rK_R plot was underestimated and the true molecular weight probably lay in the direction of the low % T values, which is confirmed from sequence data (Hargrave et al., 1983).

For a thorough SDS–PAGE analysis of an unknown protein, it is recommended that near 10 different acrylamide concentration gels be run with 10 or more reliable standards and that the data be analyzed on the Y_0K_R, M_r(% T), and M_rK_R plots according to the scheme in Table V. This scheme allows for considerable confidence in validating SDS–PAGE molecular weight data and in indicating the direction of the true molecular weight when abnormal Ferguson parameter(s) lead to anomalous migration. In our experience the success of this technique depends on rigorous control of sample preparation, gel preparation, and mathematical analysis. In a previous report (Peterson et al., 1984) we found a high molecular weight of 95 800 for the mLBP by gradient SDS–PAGE and subsequently found this to be due to a load-dependent effect of the detergent buffer. Improper gel polymerization can also have serious effects on migration (Rodbard & Chrambach, 1974), and attempts to fit calibration curves of log M_r vs. R_F to a straight line (rather than sigmoidal curves) with few standards can introduce serious errors which obliterate the true behavior of M_r(% T) plots.

The ligand binding component of the mAcChR showed a log Y_0 value only slightly in excess of those of the nicotinic AcChR subunits (Table VI). However, the slope of the M_r(% T) plot was 3.4-fold in excess of $n\alpha$. This suggests that K_R is also abnormal for this polypeptide and perhaps of considerable magnitude (10–30%). The molecular weight determined from the M_rK_R plot lies within the experimental range of the M_r(% T) plot, confirming, according to the model, the dominant character of K_R deviations over log Y_0 deviations in the behavior of the mLBP in SDS–PAGE. These results suggest that the true molecular weight probably lies below the molecular weight estimated at 6% T (i.e., <68,000), but greater than that at the 0% T intercept (i.e., >50 000). It would be reasonable to assume that the shape parameter would be in error about 20–30% from the observed slope for a protein of about M_r 50 000–60 000 (Figure 5B). This is consistent with a relative large carbohydrate content (15–30%) as suggested by the diffuse banding appearance of the mLBP and compositional data (Table III). The sedimentation and gel filtration data give an apparent molecular weight of 68 000–71 000 for the mAcChR including carbohydrate. Assuming a carbohydrate content of 26.5%, the molecular weight of the amino acid portion of the molecule would be 50 000–52 000 in agreement with that arrived at by the SDS–PAGE data and analysis of the abnormal Ferguson parameters.

It is clear that conventional SDS–PAGE overestimates the molecular weight of the mAcChR and that the generally accepted value of near 80 000 is in error. Lower estimates near 70 000 have been reported for the brain mAcChR (Andrē et al., 1983; Dadi & Morris, 1984; Haga & Haga, 1985); however, this is likely to still represent an overestimate of the protein portion of the molecule. The agreement in the molecular weight of the functional mAcChR by sedimentation and gel filtration and of the denatured mLBP by SDS–PAGE leave little room for the addition of a second subunit (viz., mSCP). In the early purified porcine atria mAcChR preparations we reported a near 1:1 stoichiometric amount of mLBP and mSCP. Our experience since that time has continued to show the presence of the mSCP in the purified preparations, but more often at relatively low staining intensity of the mSCP (<5% of mLBP intensity). The presence of a small protein of molecular weight 14 000 has been very recently reported in the purified porcine brain mAcChR (Haga & Haga, 1985), and also at less than equimolar amounts. However, quantitation on the basis of silver staining or autoradiography of iodinated protein can be highly problematical in terms of reproducibility and accuracy particularly for a low molecular weight protein at marginally detectable quantities that could suffer losses during fixation and staining. Thus, it is unlikely that an early resolution of the role of this small protein in the function of the mAcChR will be found.

The specific QNB binding site concentration for the purified mAcChR preparation analyzed for total amino acid content (Table III) was calculated as 12.4 nmol/mg of protein, which is precisely in agreement with our previous findings (Peterson et al., 1984) and that reported by Haga and Haga (1985). This leads to an estimate of one binding site per M_r ~80 000. The molecular weight estimated from the above hydrodynamic properties, however, is 50 000–60 000 for the protein portion of the molecule. Either contaminating proteins make up a larger portion of the purified preparations than is detected by the staining procedures, or not all of the receptor molecules are binding QNB. The latter case is more likely since although only active receptor should be recovered from the affinity column, the preparation must be dialyzed for 2 days to remove the eluting ligand, and some receptor inactivation may occur during this period. Also, the binding site concentration is generally determined by QNB saturation at high site concentration and not by Scatchard analysis, which would tend to give underestimates of the true site concentration.

The mSCP may bind to the mLBP to produce the higher apparent molecular weight shoulder fractions observed in the sedimentation studies (Figure 1). These fractions are higher in molecular weight by about 35 000–40 000. We have also observed a similar higher molecular weight (120 000) mAcChR shoulder region in SDS–PAGE of [^{3}H]propylbenzilylcholine mustard labeled receptor (G. L. Peterson and M. I. Schimerlik, unpublished observations). Others have also reported mAcChR fractions in the M_r 120 000 region by SDS–PAGE (Dati & Morris, 1984; Hootman et al., 1985) and by sucrose gradient sedimentation (Berrie et al., 1984b). Such fractions need not represent a dimer of the mLBP as suggested by Dadi and Morris (1984). In our preparation of the mAcChR for SDS–PAGE studies, lyophilization of the purified receptor exchanged into SDS sample buffer exhibited a small amount of polymer formation. Two polymer bands were observed, both diffuse in migratory behavior and of sufficient quantity that they could only be assigned as polymers of the mLBP, and best corresponded in molecular weight to that of dimer and trimer. The apparent molecular weight of the dimer was larger than 160 000 and had a very steep slope in the M_r(% T) plot as would be expected. The putative trimer was consistent in migration with a trimer interpretation, but extrapolation of the calibration curves to this range of molecular weight were improper. The bands of monomer and

putative dimer and trimer were symmetrical in shape at all gel concentrations with no evidence of multiple components. Because of the very dilute nature of the proteins in the sedimentation shoulder regions, we have not at present been able to directly assess the polypeptide composition. The presence of an agonist increases the amount of receptor in this "shoulder" molecular weight range (~120 000), as well as promote the presence of a third receptor peak corresponding in molecular weight to a dimer in sucrose gradients (unpublished data). Experiments are in progress to characterize these changes and to assess the composition of these peaks, and will be the subject of a subsequent report.

In conclusion, rigorous analysis by D_2O/H_2O sucrose gradient sedimentation and Sephacryl S-300 gel filtration of a monodisperse preparation of purified and active mAcChR in Triton X-405 have led to an estimated molecular weight of 68 000–71 000, including carbohydrate. Composition studies suggested the presence of about 26% carbohydrate in the purified mAcChR, giving an estimated molecular weight of 50 000–53 000 for the protein portion of the receptor. The denatured mLBP migrated anomalously on SDS–PAGE, which was found to be due to both abnormal charge and abnormal shape characteristics. Development of a novel scheme for evaluating the effects of the abnormal Ferguson parameters in SDS–PAGE permitted refining the molecular weight estimate to 50 000–60 000 for the protein portion of the denatured mLBP. Thus, the simplest form of the active mAcChR appears to consist of a single molecule of mLBP.

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Glucocorticoids Decrease the Synthesis of Type I Procollagen mRNAs†

Debra Cockayne, Kenneth M. Sterling, Jr.,‡ Susan Shull, Keith P. Mintz, Sharon Illeyne, and Kenneth R. Cutroneo*

Department of Biochemistry, College of Medicine, University of Vermont, Burlington, Vermont 05405

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ABSTRACT: Glucocorticoids selectively decrease procollagen synthesis in animal and human skin fibroblasts. β-Actin content and β-actin mRNA are not affected by glucocorticoid treatment of chick skin fibroblasts. The inhibitory effect of glucocorticoids on procollagen synthesis is associated with a decrease in total cellular type I procollagen mRNAs in chick skin fibroblasts. These effects of dexamethasone are receptor mediated as determined by pretreatment with the glucocorticoid antagonists progesterone and RU-486 and with the agonist β-dihydrocortisol. Dexamethasone has a small but significant inhibitory effect on cell growth of chick skin fibroblasts. The ability of this corticosteroid to decrease the steady-state levels of type I procollagen mRNAs in nuclei, cytoplasm, and polysomes varies. The largest decrease of type I procollagen mRNAs is observed in the nuclear and cytoplasmic subcellular fractions 24 h after dexamethasone treatment. Type I procollagen hnRNAs are also decreased as determined by Northern blot analysis of total nuclear RNA. The synthesis of total cellular type I procollagen mRNAs is reversibly decreased by dexamethasone treatment. In addition the synthesis of total nuclear type I procollagen mRNA sequences is decreased at 2, 4, and 24 h following the addition of radioactive nucleoside and dexamethasone to cell cultures. Although the synthesis of pro α1(I) and pro α2(I) mRNAs is decreased in dexmethasone-treated chick skin fibroblasts, the degradation of the total cellular procollagen mRNAs is not altered while the degradation of total cellular RNA is stabilized. These data indicate that the dexamethasone-mediated decrease of procollagen synthesis in embryonic chick skin fibroblasts results from the regulation of procollagen gene expression.

Collagen metabolism is markedly altered by glucocorticoids (Cutroneo et al., 1986). Both natural and synthetic glucocorticoids decrease collagen synthesis in various connective tissues including skin (Smith & Allison, 1965; Uitto et al., 1972; Newman & Cutroneo, 1978; Robey, 1979), granuloma (Nakagawa et al., 1971; Wehr et al., 1976; Kruse et al., 1978), and bone (Uitto & Mustakallio, 1971; Rokowski et al., 1981; Oikarinen & Ryhanyen, 1981; Canalis, 1983).

Topical application of high doses of synthetic glucocorticoids to skin results in skin atrophy. Glucocorticoid treatment decreases collagen content of skin which in turn results in a

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* Author to whom correspondence and reprint requests should be addressed.
‡ Present address: Departments of Pediatrics and Biochemistry, Mount Sinai School of Medicine, New York, NY 10029.

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