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# Hydrodynamic properties of muscarinic acetylcholine receptors solubilized from rat forebrain

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**1** Muscarinic receptors from rat forebrain have been solubilized by Lubrol PX, lysophosphatidylcholine (LPC), digitonin and cholate/1 M sodium chloride. The overall level of solubilization was characterized using receptors prelabelled with an irreversible antagonist. The recovery of non-denatured soluble binding activity was estimated using reversible tritiated antagonists.

**2** All these detergents solubilized 60–85% of the total binding sites. In Lubrol PX most of the receptors were recovered in a denatured form. In the other detergents 30–90% of the solubilized receptors were stable and capable of binding reversible [$^3$H]-antagonists with high affinity.

**3** The hydrodynamic properties of the soluble receptors have been examined by gel filtration and sucrose gradient centrifugation in $H_2O$ and $D_2O$.

**4** The soluble receptors in Lubrol PX, lysophosphatidylcholine and cholate were, in general, heterogeneous as regards their molecular size. Estimates of the molecular weight after correction for bound detergent, varied from 82,000 to 134, 000. Conditions were identified under which the receptor was largely monodisperse, and the estimates of molecular weight agreed with values (ca. 83, 000) from sodium dodecylsulphate (SDS) polyacrylamide gel electrophoresis. The amount of bound detergent could not be calculated for the digitonin-muscarinic receptor complex which had an estimated overall median molecular weight of about 290,000.

**5** It is concluded that a subpopulation of muscarinic receptors from the rat forebrain is capable of existing in a monomeric soluble form and binding ligands. There is also evidence that complexes with other proteins can exist, but their specificity and functional relevance are not known.

## Introduction

When muscarinic receptors from the forebrain, smooth muscle and heart are labelled with irreversible ligands and then solubilized by sodium dodecylsulphate (SDS), they migrate as a single band, $M_r = \sim 83$, 000, on SDS-polyacrylamide gel electrophoresis (Birdsall *et al.*, 1979; Ruess & Lieflander, 1979; Amitai *et al.*, 1982; Venter, 1983). These studies show that the ligand binding component(s) of muscarinic receptors in these tissues have similar or perhaps identical molecular weights. Studies on the hydrodynamic properties of the [$^3$H]-quinuclidinylbenzilate ([$^3$H]-QNB) muscarinic receptor complex in Lubrol PX solution indicate that its molecular weight is very similar to that estimated by SDS gel electrophoresis, suggesting that the receptors may be monomers in this detergent (Haga, 1980a). However, only a small fraction of muscarinic receptor sites was solubilized by this procedure and it is possible that the insoluble or denatured binding sites represent receptor aggregates or specific receptor-protein complexes.

Binding studies have indicated that there are several subclasses of muscarinic receptors (Birdsall & Hulme, 1983) with different affinities for agonists (Birdsall *et al.*, 1978) and a selective antagonist (Hammer *et al.*, 1980). It is possible that such subclasses could result from differences in the interaction of the binding protein with other components in the membrane. It is therefore important to determine whether, by the use of different detergents, it is possible to solubilize complexes of the receptor with other proteins.

In this paper, we show that cholate as well as

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lysophosphatidylcholine (LPC) and digitonin can be used to solubilize muscarinic receptors in the rat forebrain in an active form, and describe the hydrodynamic properties of the receptors solubilized by cholate, LPC, digitonin and Lubrol PX.

## Methods

### *Reagents*

[$^3$H]-propylbenzilylcholine mustard ([$^3$H]-PrBCM, 42 Ci mmol$^{-1}$), [$^3$H]-3-quinuclidinylbenzilate ([$^3$H]-QNB, 32 Ci mmol$^{-1}$) and [$^3$H]-N-methylscopolamine ([$^3$H]-NMS, 10 Ci mmol$^{-1}$ and 53.5 Ci mmol$^{-1}$) were synthesized or obtained from New England Nuclear. Digitonin was purchased from Wako Co. Ltd., Osaka. LPC and crude phosphatidylchline (PC, type IX-E), both from egg yolk, and the calibrating enzymes were purchased from Sigma Co. Ltd. The suspension of PC was prepared as follows. PC (5 mg ml$^{-1}$) was added to the standard buffer (20 mM HEPES pH 7.5, 2 mM $MgCl_2$, 1 mM EDTA and 0.15 M NaCl) supplemented with 0.3% cholate and 1 M NaCl, warmed at about 70°C for several minutes and homogenized with a Polytron PTA 10–35. The suspension was used after passing through a membrane filter (HAWP).

### *Membrane preparation and solubilisation*

Crude membrane preparations from rat forebrain were obtained by homogenization of the tissue in 9 volumes of ice cold 0.32 M sucrose/10 mM sodium phosphate buffer (pH 7.5) with a Potter-Elvehjem homogenizer. Subsequent steps were carried out at 4°C. The homogenate was centrifuged at 1,500 *g* for 5 min and the resulting supernatant at 15,000 *g* for 30 min. The pellet was suspended in 10 mM phosphate buffer (pH 7.5) and centrifuged at 15,000 *g* for 30 min. The final pellet was suspended in the phosphate buffer, rapidly frozen, and stored at −70°C. Freezing was not found to affect the hydrodynamic properties of the receptors.

Muscarinic receptors were solubilized by incubating the crude membrane preparation or [$^3$H]-PrBCM-labelled membranes for 5 min at 30°C with a given detergent in the standard buffer. The concentrations of membrane protein and detergents in the solubilizing medium were, unless indicated otherwise: 1.7–1.8 mg protein ml$^{-1}$, 0.4% Lubrol PX, 0.4% LPC, 2% digitonin and 0.3% cholate-1 M NaCl. The incubated suspension was cooled to 4°C and centrifuged at 140,000 *g* for 30 min. The supernatant was used as solubilized receptor immediately after the preparation or stored at −70°C before use. Protein concentrations of membranes and solubilized preparations were determined according to the Lowry method using bovine serum albumin as a standard.

### *Binding assays*

Incubation of membrane preparations with [$^3$H]-QNB or [$^3$H]-NMS was carried out at 30°C for 30 min in 50 mM Tris-HCl buffer (pH 7.5) and the bound [$^3$H]-QNB or [$^3$H]-NMS was assayed by the microcentrifugation procedure described previously (Hulme *et al.*, 1978). Non-specific binding was defined as the radioactivity bound or entrapped in the pellet when the incubation medium contained 1 μM QNB. The binding to solubilized receptors with labelled ligands was carried out as follows: the solubilized preparations, or the fractions after sucrose density gradient centrifugation (50 to 200 μl) were incubated for 30 min at 30°C with [$^3$H]-QNB or [$^3$H]-NMS (10 nM) in the Tris buffer (total volume 250 μl), and 200 μl of the incubated solution was applied to a small Sephadex G-50 (medium) column (4 × 0.8 cm, 2 ml) which was pre-washed with the Tris buffer. After the incubated solution had been absorbed, 100 μl, followed by 700 μl, of the Tris buffer was applied on the column. The whole eluate (total 1 ml) was collected in a liquid scintillation vial and radioactivity in the vial counted after addition of 10 ml of emulsifier scintillant (Packard 299). When membranes were pretreated with muscarinic ligands and solubilized in their presence, the solubilized preparation (200 μl) was passed through the same Sephadex column and the void volume fraction (400 μl) was collected and used for the binding assay. Assays were usually carried out in duplicate.

### *Preparation of [$^3$H]-PrBCM-labelled membranes*

Membrane preparations were incubated in a phosphate buffer (10 mM, pH 7.5) with [$^3$H]-PrBCM that had been cyclised by preincubation for 60 min at room temperature in the same buffer (Burgen *et al.*, 1973). The incubated suspension was centrifuged at 140,000 *g* for 30 min and washed twice with the Tris buffer. The resulting pellet was suspended in the Tris buffer and stored at −70°C. Bound [$^3$H]-PrBCM in solubilized preparations was assayed by suspension in emulsifier scintillant directly or after collecting the void volume fraction from the Sephadex column assay.

Reaction of [$^3$H]-PrBCM with muscarinic receptors exhibited biphasic kinetic characteristics (see Results). Two sets of conditions were used for labelling the membranes so that differences in the solubilization characteristics of the rapidly and slowly labelled components could be investigated. In preparation I, membranes (0.34 mg protein ml$^{-1}$) were incubated

with 3 nM [$^3$H]-PrBCM for 15 min at 4°C, resulting in 0.98 pmol of bound [$^3$H]-PrBCM mg$^{-1}$ protein. The unalkylated receptor binding sites decreased by ~50% to 1.16 pmol mg$^{-1}$ protein from 2.21 pmol mg$^{-1}$ protein. In preparation II, [$^3$H]-PrBCM (5.2 nM) was added to the membranes (0.72 mg protein ml$^{-1}$) when the incubation started and after 80 min (final concentration 10.2 nM), and the mixture was incubated for a further 160 min. The total bound [$^3$H]-PrBCM was 2.38 pmol mg$^{-1}$ protein and the [$^3$H]-QNB binding sites were decreased from 2.17 to 0.41 pmol mg$^{-1}$ protein, indicating that 81% of the binding sites had been alkylated.

*Gel filtration*

An Ultrogel ACA 34 column (1.5 × 26 cm) was equilibrated and eluted at 4°C in the standard buffer supplemented with 0.1% Lubrol PX, 0.2% digitonin or 0.3% cholate-1 M NaCl-0.25% PC, except when described otherwise. When LPC was used as a detergent, a 0.1% solution was stored at 4°C overnight, and any resulting precipitate removed by filtration before use. The column was eluted at a hydrostatic pressure of 30 cm at a rate of about 10 ml h$^{-1}$. The volume of applied samples and each fraction were 1 ml and about 1.6 ml, respectively, unless otherwise stated. The overall recovery of receptor-bound [$^3$H]-PrBCM ranged between 70 and 90% and that of bound [$^3$H]-QNB was 63%.

The calibration of the column was carried out as described by Haga (1980) with the enzymes (plus β-galactosidase, Stokes radius 6.84 nm) used for calibration of the sucrose density gradients. Blue dextran (1 mg ml$^{-1}$) and cyclic AMP (0.1 mM), were used for estimation of excluded and included volumes, $V_o$ and $V_t$ respectively. The Stokes radius of the receptor was calculated from the following equation:

Stokes radius (a) (nm) = $8.92 - 9.03\,(V_e - V_o)/(V_t - V_o)$ where $V_e$ is the elution volume of the receptor.

*Sucrose density gradient centrifugation*

Linear sucrose gradients were made from 5 and 20% sucrose in $H_2O$ or in 90% $D_2O$ – 10% $H_2O$ (5.5 to 6 ml each); the buffer and detergents were the same as those used in gel filtration. The solubilized preparations were mixed with calibrating enzymes (catalase 0.069 mg ml$^{-1}$, fumarase 0.012 mg ml$^{-1}$, lactate dehydrogenase (LDH) 0.031 mg ml$^{-1}$, malate dehydrogenase (MDH) 0.0035 mg ml$^{-1}$ and cytochrome C 0.69 mg ml$^{-1}$), and 0.5 or 1 ml of the mixture was applied to the gradient. The centrifugation was carried out in a Beckman SW 42.1 rotor at 100,000 *g* and 4°C for 19 h, after which 0.5 ml fractions were collected using a peristaltic pump. When [$^3$H]-PrBCM or [$^3$H]-QNB prelabelled components were applied to the gradient 200 μl fractions were assayed by the Sephadex G50 column assay. In the case of unlabelled receptors, 200 μl of each fraction was assayed for [$^3$H]-QNB binding activity as already described. The recovery of bound [$^3$H]-QNB and [$^3$H]-QNB binding activity varied from 50% to 80%. The high recovery of bound [$^3$H]-QNB after the prolonged sucrose density centrifugation reflects the extremely slow off-rate ($t_{\frac{1}{2}} >> 2$ days) at 4°C for [$^3$H]-QNB and [$^3$H]-NMS bound to muscarinic receptors in membranes and in solution (unpublished results).

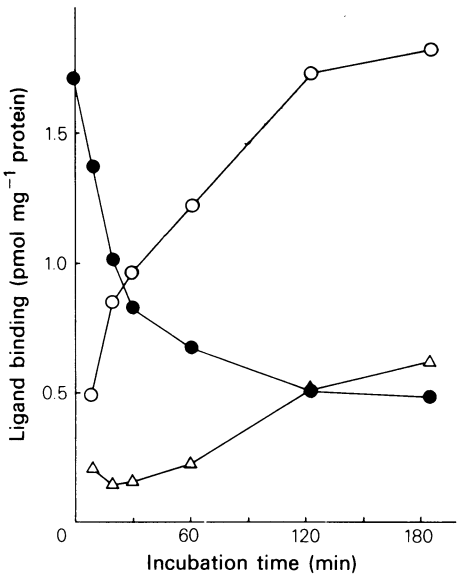


**Figure 1** Time course of [$^3$H]-propylbenzilycholine mustard ([$^3$H]-PrBCM) binding and the accompanying decrease in [$^3$H]-3-quinuclidinylbenzilate ([$^3$H]-QNB) binding sites. Membranes (0.68 mg protein ml$^{-1}$ were incubated with [$^3$H]-PrBCM (12.5 nM) at 4°C. At the time indicated on the abscissa 0.25 ml of the incubated suspension was added to 0.75 ml of 10 mM $Na_2S_2O_3$ in 10 mM phosphate buffer (pH 7.5) to stop further alkylation and the suspension was centrifuged for 5 min in a microfuge at 4°C. The superficially washed pellets were directly assayed for bound radioactivity, or were suspended in 1 ml of [$^3$H]-QNB (4.3 nM) in 50 nM Tris-HCl buffer (pH 7.5) with or without 1 μM QNB. The latter suspensions were incubated for 30 min at 30°C and the specific binding was measured as described in the text. The non-specific binding of [$^3$H]-PrBCM was estimated by preincubation for 30 min with 10 μM QNB and then incubating with [$^3$H]-PrBCM in the presence of 10 μM QNB. Aliquots were centrifuged at the times indicated and the pellets counted. (●) Specific binding of [$^3$H]-QNB; (○) binding of [$^3$H]-PrBCM in the absence of QNB; (△) binding of [$^3$H]-PrBCM in the presence of QNB.

The physical properties and assay methods for the calibrating enzymes have been described in a previous paper (Haga *et al.*, 1977).

## Results

### *Time course of [$^3$H]-PrBCM binding*

Figure 1 shows the time-course of [$^3$H]-PrBCM binding to membrane preparations at 4°C and the accompanying decrease in [$^3$H]-QNB binding activity. The increase in amount of bound [$^3$H]-PrBCM roughly paralleled the decrease in the [$^3$H]-QNB binding sites for the first hour of incubation, indicating that [$^3$H]-PrBCM was bound predominantly to muscarinic receptors. This was confirmed by the observation that [$^3$H]-PrBCM binding was decreased when the membranes were pretreated with non-radioactive QNB (10 $\mu$M) and incubated with [$^3$H]-PrBCM in the presence of QNB (Figure 1). During prolonged incubation, however, total [$^3$H]-PrBCM binding became greater than the decrease in [$^3$H]-QNB binding indicating that [$^3$H]-PrBCM was labelling other components. The rate of non-specific binding of [$^3$H]-PrBCM was slower than that of specific binding, and was more dependent on the concentration of membranes, [$^3$H]-PrBCM, and incubation temperature (data not shown). Lower concentrations of membranes and [$^3$H]-PrBCM and a shorter period of incubation gave a higher ratio of specific to non-specific binding, but at the same time a lower occupancy of muscarinic receptors with [$^3$H]-PrBCM resulted. The two protocols described in this paper resulted in 50% and 80% alkylation of receptor sites with a very high specific: non-specific ratio (> 10:1) in the first preparation (I) and a lower ratio of ~2.5:1 in the second preparation (II).

The binding of [$^3$H]-PrBCM to muscarinic receptors as assessed by the decrease of [$^3$H]-QNB binding activity could not be expressed by first order kinetics and appeared to be composed of at least two components. An analysis of the data in Figure 1, assuming the presence of two kinetic components, suggested that 60% of the receptors were alkylated with a rate constant of $7 \times 10^{-4} s^{-1}$, the remaining receptor population being alkylated at a considerably slower rate ($\sim 3 \times 10^{-5} s^{-1}$).

### *Solubilization of muscarinic receptors*

*Solubilization by Lubrol PX* Figure 2a shows the effects of different concentrations of Lubrol PX on the solubilization of bound [$^3$H]-PrBCM from membranes (preparation I) in which ~50% of the total receptors were labelled with [$^3$H]-PrBCM. About 90% of these sites were solubilized by 0.1–2% Lubrol PX. Using preparation II, in which 80% of the receptors were labelled, only ca. 75% of bound [$^3$H]-PrBCM was solubilized (data not shown). This suggests that the fraction of the muscarinic receptors which were labelled more slowly with [$^3$H]-PrBCM

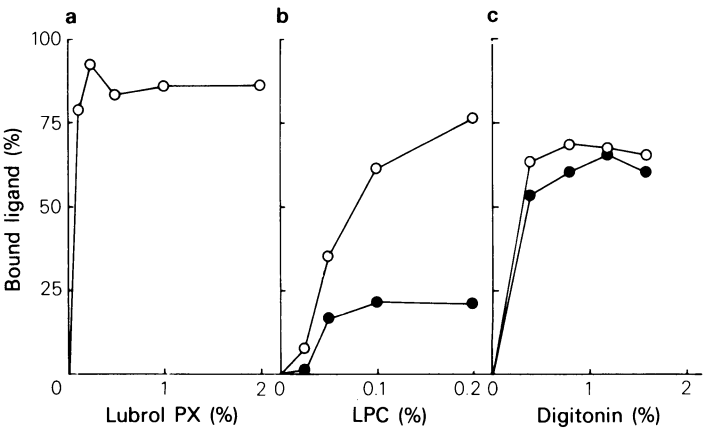


**Figure 2** Concentration dependence of the solubilization of muscarinic receptors by Lubrol PX (a), lysophosphatidylcholine (LPC) (b) and digitonin (c). Mixtures of [$^3$H]-PrBCM-labelled membranes (preparation I, 0.17 mg protein ml$^{-1}$) and non-labelled membranes (1.65 mg protein ml$^{-1}$) were solubilized by different concentrations of detergents as described in the text. One aliquot (0.1 ml) of the soluble preparation was used for assay of the solubilized radioactivity and a second (0.1 ml) for binding assay of the receptor with a saturating concentration of [$^3$H]-NMS (7.8 nM). The 100% values were taken as the amount of [$^3$H]-PrBCM bound to the membranes before solubilization and the binding activity of the membranes before addition of detergents (0.98 pmol mg$^{-1}$ and 1.56 pmol mg$^{-1}$ for the [$^3$H]-PrBCM and specific [$^3$H]-NMS binding, respectively). (○) Solubilized [$^3$H]-PrBCM, (●) solubilized specific [$^3$H]-NMS binding.

and/or non-specifically labelled components were more resistant to solubilization by Lubrol PX. The total yield of muscarinic receptors solubilized by Lubrol PX was estimated to be between 55 and 95%. The range of estimation is wide because it is not known what fraction of the receptors that were not labelled with [$^3$H]-PrBCM could be solubilized and the precise proportion of the solubilized [$^3$H]-PrBCM that was bound to muscarinic receptors.

Membrane preparations solubilized by Lubrol PX only bound labelled muscarinic ligands ([$^3$H]-NMS, [$^3$H]-QNB) when the solubilized receptors were incubated with the labelled ligands in the cold immediately after the preparation. Even when membranes were prelabelled with [$^3$H]-QNB and solubilized with Lubrol PX, only 20–30% of total [$^3$H]-QNB binding sites were solubilized. This complex however, was much more stable than the unliganded soluble receptor. These low yields are considered to be due to inactivation of muscarinic receptors in Lubrol PX and not to inefficient solubilization because at least 55% of prelabelled receptors were solubilized under the same experimental conditions.

*Solubilization by lysophosphatidylcholine* LPC solubilized 20–25% of the muscarinic receptors in an active state as assessed by [$^3$H]-NMS binding (Figure 2b) in agreement with the previous results obtained from porcine caudate nucleus (Haga, 1980b). The yield of bound [$^3$H]-PrBCM solubilized by LPC ranged from 63 to 83% (Figure 2b) and had a tendency to decrease in samples with increased [$^3$H]-PrBCM bound to membranes. In addition to the 20–25% of muscarinic receptors that could be solubilized in the active form, it was calculated that a further 20–60% were solubilized and inactivated.

*Solubilization by digitonin* About 50% of bound [$^3$H]-PrBCM was solubilized by digitonin, irrespective of the amount of [$^3$H]-PrBCM bound to membranes; the values shown in Figure 2c were a little higher than those usually obtained. In contrast to Lubrol PX and LPC, most of the muscarinic receptors solubilized by digitonin were recovered in an active form. Therefore the main reason for the incomplete recovery of the binding activity was incomplete solubilization and not inactivation during solubilization. Most of the activity not solubilized remained in the residual membrane pellet (data not shown).

*Solubilization by cholate* Although more than 50% of bound [$^3$H]-PrBCM could be solubilized by cholate (0.5–2%) only very low levels of active muscarinic receptors were recovered. This is in agreement with previous findings (Hurko, 1978; Hulme *et al.*, 1983). Up to 30% of the receptors could be solubilized in the active form if 1 M NaCl was present with the detergent (Carson, 1982; Hulme *et al.*, 1983). The level of solubilization of activity was a maximum at 0.3–0.5% cholate and decreased at higher detergent concentrations (Figure 3). This decrease could be prevented by preincubation of the membranes with muscarinic ligands e.g. 8 mM carbachol (data not shown) and solubilization in their presence.

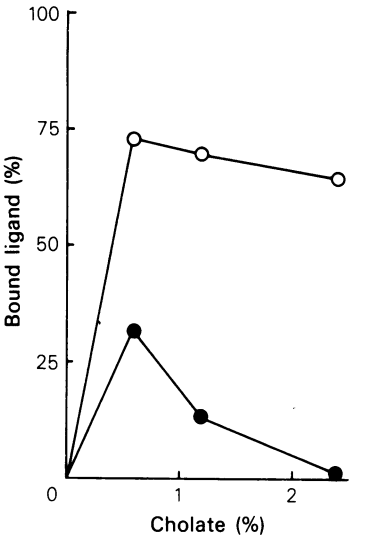


**Figure 3** Solubilization of muscarinic receptors by cholate in the presence of 1 M NaCl.
[$^3$H]-PrBCM-labelled receptors were prepared by incubating membranes (0.68 mg protein ml$^{-1}$) with 4.6 nM [$^3$H]-PrBCM at 4°C for 3 h resulting in 1.55 pmol of bound [$^3$H]-PrBCM per mg protein and in a decrease of [$^3$H]-QNB binding sites from 1.83 to 0.39 pmol per mg protein. Mixtures of the [$^3$H]-PrBCM-labelled membranes (0.39 mg protein ml$^{-1}$) and unlabelled membranes (5.02 mg protein ml$^{-1}$), were incubated with different concentrations of cholate in the presence of 1 M NaCl. The percentages of [$^3$H]-PrBCM bound (○) and [$^3$H]-QNB binding activity (●) that were solubilized were estimated as described in the text and the legend to Figure 2.

*Sucrose density gradient centrifugation and gel filtration of solubilized muscarinic receptors*

*Receptors solubilized by Lubrol PX* Figure 4 shows the density gradient centrifugation of [$^3$H]-PrBCM and [$^3$H]-QNB-labelled components solubilized by 0.4% of Lubrol PX. In agreement with the previous report (Haga, 1980a) the [$^3$H]-QNB labelled receptor migrated as a narrow peak (3.16 *s*, $H_2O$) (Figure 4). However, the [$^3$H]-PrBCM-labelled components sedimented in a broader peak of higher mean *s* value

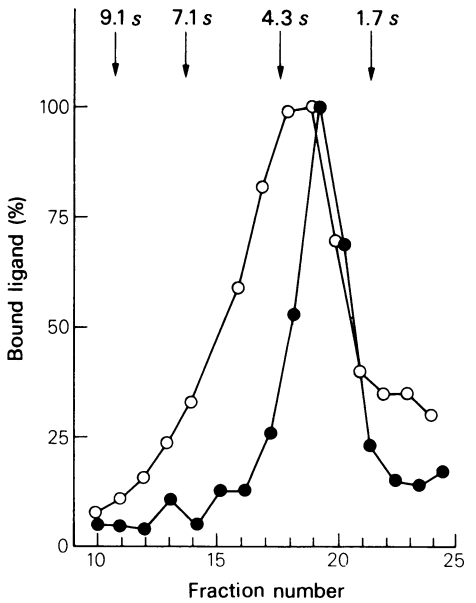


**Figure 4** Sucrose density gradient centrifugation in Lubrol PX. Membrane preparations (1.8 mg ml$^{-1}$) were incubated with [$^3$H]-QNB (1.95 nM) in the standard buffer for 30 min at 30°C. The [$^3$H]-QNB-labelled and [$^3$H]-PrBCM-labelled membranes (preparation II) were solubilized in 0.4% Lubrol PX, and each of the solubilized preparations (0.5 ml) was centrifuged separately in the sucrose gradients ($H_2O$) and assayed as described in the text. The 100% values were 350 fmol ml$^{-1}$ for [$^3$H]-PrBCM and 135 fmol ml$^{-1}$ for specific [$^3$H]-QNB binding. (○) Bound [$^3$H]-PrBCM; (●) bound [$^3$H]-QNB.

(3.9 *s*). The leading edge of the peak corresponds to a much higher sedimentation coefficient than the [$^3$H]-QNB-labelled receptor whereas the trailing edges coincide (Figure 4). These findings suggest that components additional to the monomer form are present in the [$^3$H]-PrBCM labelled soluble preparation. When fraction 15 from the [$^3$H]-PrBCM gradient was re-centrifuged under the same conditions, the radioactivity was present in a peak centred on the same fraction of the gradient, confirming the presence of higher molecular weight species which are not in equilibrium with the receptor monomer.

Similarly, gel filtration of the [$^3$H]-PrBCM-labelled receptor resulted in a broad peak (Figure 5). Re-chromatography of three fractions taken from different positions in the peak yielded distinct elution profiles corresponding to different apparent molecular sizes (Figure 5).

In contrast to the [$^3$H]-PrBCM-labelled components, the [$^3$H]-QNB-labelled receptor migrated as a much sharper peak. Re-chromatography of two fractions gave peaks which coincided with each other and also with the original chromatogram (Figure 6). This

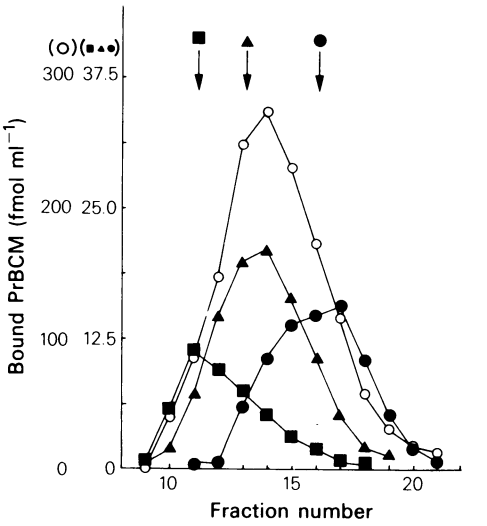


**Figure 5** Gel filtration of [$^3$H]-PrBCM-labelled species in Lubrol PX. [$^3$H]-PrBCM-labelled membranes (preparation II) were prepared and solubilized in 0.4% Lubrol PX as described in the text. One ml of the solubilized preparations was applied on the column of Ultrogel AcA 34 and 1.6 ml fractions (○) collected. Eluates of original chromatography (fractions no. 11 (■), 13 (▲) and 16 (●); 1 ml each), were re-chromatographed on the same column. Blue dextran and cyclic AMP, used for the estimation of the Stokes radius, were eluted as peaks at positions equivalent to fraction numbers 10.4 and 27.7, respectively.

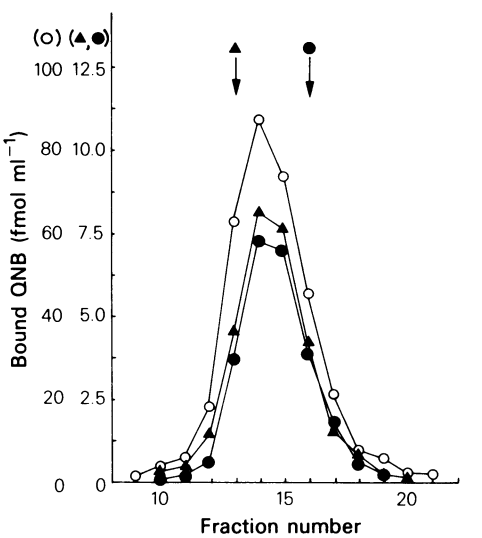


**Figure 6** Gel filtration of [$^3$H]-QNB-labelled components in Lubrol PX. The preparation of [$^3$H]-QNB-labelled components and the procedure for gel filtration are described in legends to Figure 4 and 5. The soluble [$^3$H]-QNB-labelled receptors were gel filtered (○) and two fractions (no. 13 (▲) and 16 (●); 1 ml each) were re-chromatographed on the same column.

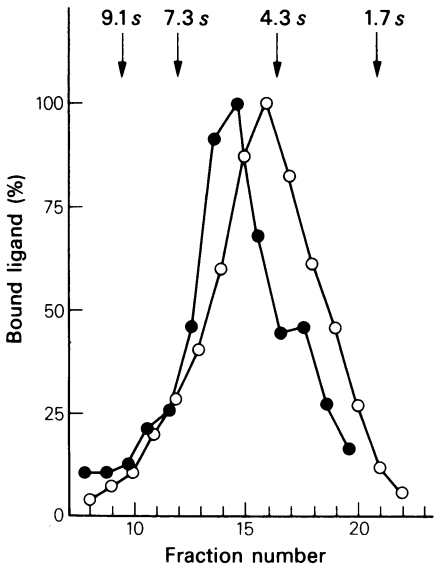


**Figure 7** Sucrose density gradient centrifugation in LPC. [$^3$H]-PrBCM-labelled (preparation II) and unlabelled membranes were solubilized in 0.4% LPC and the solubilized preparations were centrifuged in sucrose gradients as described in the text. The values equivalent to 100% are 426 fmol ml$^{-1}$ for bound [$^3$H]-PrBCM (○) and 103 fmol ml$^{-1}$ for specific [$^3$H]-QNB binding (●). The peak widths for calibrating enzymes at the half maximal activity were 2.9, 2.5 and 3.5 fractions for fumarase, LDH and MDH respectively, and had the same range of values, irrespective of the detergent used. The value for cytochrome C was a little higher and ranged between 3 and 4.5 fractions.

indicates either that the [$^3$H]-QNB-labelled component is homogeneous or that there is rapid equilibrium between several forms.

It seemed possible that the apparent heterogeneity of [$^3$H]-PrBCM-labelled components was due to the non-specific binding (25% of the [$^3$H]-PrBCM bound in preparation II). However when the non-specific binding was examined directly using membrane labelled with [$^3$H]-PrBCM in the presence of 10 μM QNB, very little of the non-specifically bound radioactivity was found in positions with higher sedimentation velocity than the [$^3$H]-QNB-labelled component.

*Receptors solubilized by LPC* Bound [$^3$H]-PrBCM in preparations solubilized by LPC also showed broader peaks than those of the calibrating enzymes, both in sucrose density gradient centrifugation (Figure 7) and particularly on gel filtration (Figure 8), indicating apparent heterogeneity. The peak of [$^3$H]-QNB binding activity in sucrose density gradient centrifugation was a little sharper than that of bound [$^3$H]-

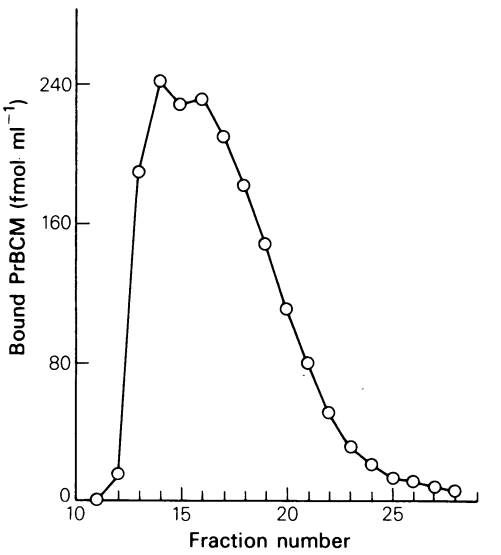


**Figure 8** Gel filtration of [$^3$H]-PrBCM-labelled components in LPC. The preparation of [$^3$H]-PrBCM-labelled membranes (preparation II) and the procedure for gel filtration are described in the text. Blue dextran and cyclic AMP were eluted as peaks at positions equivalent to fractions numbers 13.7 and 36.3 respectively. The volume of each fraction was 1.2 ml in this experiment.

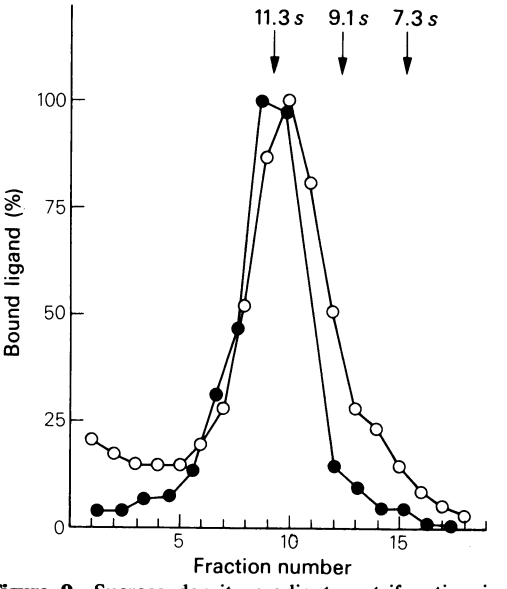


**Figure 9** Sucrose density gradient centrifugation in digitonin. [$^3$H]-PrBCM-labelled membranes (preparation I) and unlabelled membranes were solubilized in 2% digitonin and applied to the sucrose gradients as described in the text. The values equivalent to 100% are 286 fmol ml$^{-1}$ for bound [$^3$H]-PrBCM (○) and 403 fmol ml$^{-1}$ for specific [$^3$H]-QNB binding (●).

PrBCM. However, it was still broader than that expected for a homogeneous component and had a tendency to show a higher sedimentation velocity than that of bound [$^3$H]-PrBCM, in contrast to the preparations solubilized by Lubrol PX.

*Receptors solubilized by digitonin* The [$^3$H]-PrBCM and [$^3$H]-QNB-labelled receptors both sedimented with the same *s* value (11 *s*, Figure 9), which was higher than that found for other detergents but in general agreement with previous estimates (Gorrissen *et al.*, 1978; Hurko, 1978). The width of the peak again suggested heterogeneity and this was confirmed by filtration and re-chromatography of selected fractions (Figure 10).

*Receptors solubilized by cholate* On sucrose density gradient centrifugation, the [$^3$H]-PrBCM-labelled receptor solubilized by 0.3% cholate/1 M NaCl exhibited a distribution which was even broader than that found in Lubrol PX, LPC or digitonin (Figure 11). Similarly, a broad distribution was found both for this preparation and the [$^3$H]-QNB prelabelled receptor on gel filtration (Figure 12). These profiles were sharpened by the presence of phosphatidylcholine (PC) in the detergent (Figures 11 and 13). The sedimentation coefficients were greatly decreased (Figure 11) whereas the mean Stokes radius was

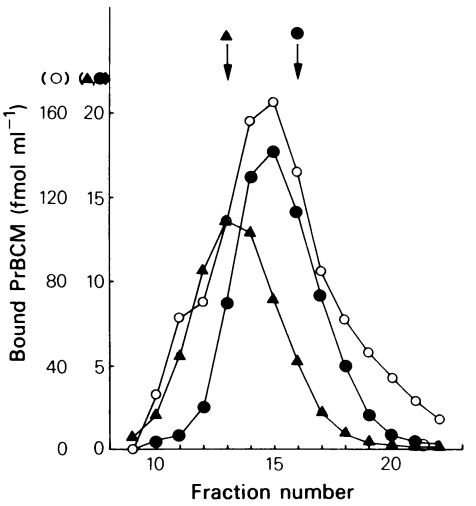


**Figure 10** Gel filtration of [$^3$H]-PrBCM-labelled species in digitonin. [$^3$H]-PrBCM-labelled membranes (preparation II) were solubilized in 2% digitonin and applied to the column as described in the text. After gel filtration (○) two fractions (no. 13 (▲) and 16 (●); 1 ml each) were re-chromatographed on the same column. Blue dextran and cyclic AMP were eluted at positions equivalent to fraction numbers 10.7 and 27.9, respectively.

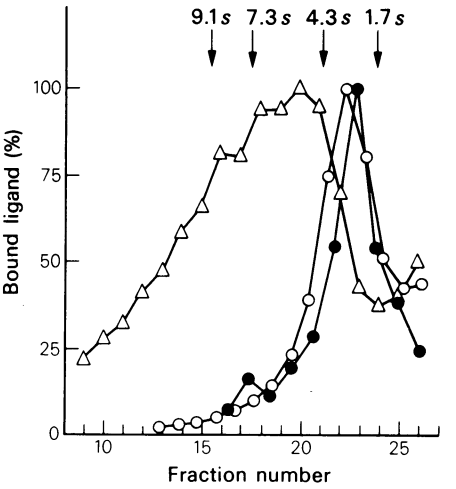


**Figure 11** Sucrose density gradient centrifugation in cholate. A portion of [$^3$H]-PrBCM-labelled membranes (preparation I, 1.7 mg protein ml$^{-1}$) was solubilized with 0.3% cholate-1 M NaCl and 0.5 ml applied to sucrose gradients containing 0.3% cholate-1 M NaCl. Another portion of these labelled membranes (preparation I, 2.4 mg ml$^{-1}$) was solubilized as above, mixed with the same volume of 0.5%PC in 0.3% cholate-1 M NaCl in the standard buffer and 1 ml applied to the sucrose gradients containing 0.3% cholate-1 M NaCl-0.25% PC. Unlabelled membranes (2.4 mg ml$^{-1}$) were also solubilized by 0.3% cholate-1 M NaCl supplemented with PC (0.25%) and applied to gradients containing cholate-NaCl/PC. The values equivalent to 100% are 79 fmol ml$^{-1}$ for [$^3$H]-PrBCM in the absence of PC, 236 fmol ml$^{-1}$ for [$^3$H]-PrBCM in the presence of PC, and 190 fmol ml$^{-1}$ for specific [$^3$H]-QNB binding. (△) Bound [$^3$H]-PrBCM in the absence of PC; (○) bound [$^3$H]-PrBCM in the presence of PC; (●) specific [$^3$H]-QNB binding in the presence of PC.

relatively unchanged (Figure 13). Similar values were found for both the [$^3$H]-PrBCM and [$^3$H]-QNB-labelled receptors. Re-chromatograms of fractions from gel filtration in the presence of PC suggested that the heterogeneity had been largely suppressed but not totally eliminated (Figure 14).

*Hydrodynamic parameters of solubilized receptors* We have estimated hydrodynamic parameters such as Stokes radius, sedimentation coefficients and hence apparent molecular weights from the behaviour of peaks of bound [$^3$H]-PrBCM on gel filtration and sucrose density gradient centrifugation. These values are summarized in Table 1. Although they are an ensemble of the various components they are assumed to reflect primarily the properties of the major component.

The apparent sedimentation coefficients were

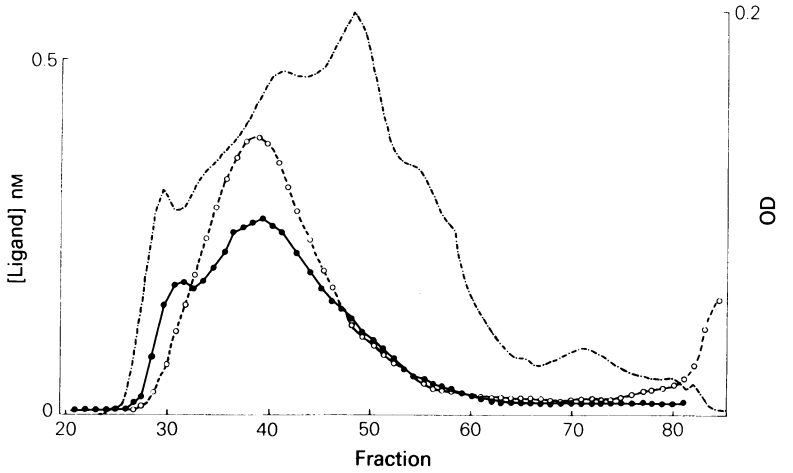


**Figure 12** Gel filtration of [$^3$H]-PrBCM-labelled and [$^3$H]-QNB-labelled rat brain muscarinic receptors in cholate/NaCl. [$^3$H]-PrBCM-labelled membranes (5 mg protein ml$^{-1}$, specific: non specific binding = 5.9:1) were solubilized at 0° in 50 mM Tris, pH 7.5 by the sequential addition with stirring of 1 mM EDTA (5 min), 1 M NaCl (5 min) and 0.3% sodium cholate (30 min). After centrifugation (100,000 g, 60 min) the supernatant (15 ml) was applied to a 370 ml Sephacryl S-300 column equilibrated with cholate/NaCl. Fractions (5 ml) were collected at a flow rate of 0.2 ml min$^{-1}$ and assayed for radioactivity. In a separate experiment, unlabelled membranes at the same concentration were solubilized as above, and the supernatant incubated with [$^3$H]-QNB for 18 h at 0°C. A Sephadex G50 gel filtration assay showed 4.5 nM bound [$^3$H]-QNB. The supernatant (9.5 ml) was gel filtered as above. Recovery of bound [$^3$H]-QNB was 66%. Fraction 30 corresponded to the excluded volume and fraction 80 to the included volume. (●——●) Bound [$^3$H]-PrBCM; (○-----○) specifically bound [$^3$H]-QNB; (-·-·-) optical density.

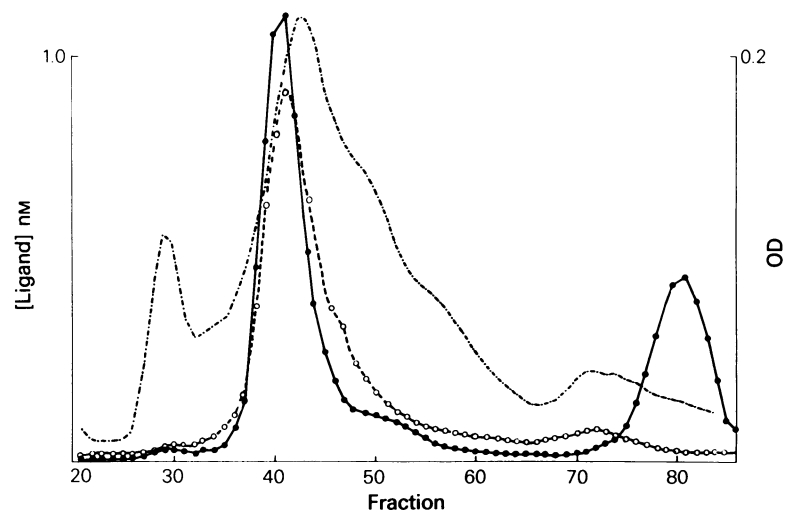


**Figure 13** Gel filtration of [$^3$H]-PrBCM-labelled and [$^3$H]-QNB-labelled rat brain muscarinic receptors in cholate/NaCl/PC. Solubilization of [$^3$H]-PrBCM-labelled and unlabelled membranes was carried out as in Figure 12. The supernatants (5 ml) were diluted 1:1 in 5 mg ml$^{-1}$ egg phosphatidylcholine in cholate/NaCl and applied directly to the S300 column (370 ml bed volume equilibrated with cholate/NaCl/PC) in the case of the [$^3$H]-PrBCM labelled preparation (●——●), or incubated for 18 h with 10 nM [$^3$H]-QNB (○------○) before gel filtration as described in Figure 12. Recovery of bound [$^3$H]-QNB was 74%. (—·—·—) Optical density.

**Table 1** Hydrodynamic properties of [$^3$H]-propylbenzilylcholine mustard ([$^3$H]-PrBCM) or [$^3$H]-3-quinuclidinylbenzilate ([$^3$H]-QNB)-labelled receptors solubilized by several detergents

| | | *Lubrol PX* | | *LPC* | *Cholate/1 M NaCl/PC* | *Digitonin* |
|---|---|---|---|---|---|---|
| | | *[$^3$H]-QNB* | *[$^3$H]-PrBCM* | *[$^3$H]-PrBCM* | *[$^3$H]-PrBCM* | *[$^3$H]-PrBCM* |
| Apparent sedimentation co-efficient ($s_{20,w}$) ($s$) | $H_2O$ | 3.16 | 3.85 ± 0.03 | 4.75 ± 0.07 | 3.46 ± 0.01 | 10.88 ± 0.03 |
| | $D_2O$ | 2.23 | 3.46 ± 0.12 | 2.86 ± 0.05 | 2.29 ± 0.19 | 10.94 ± 0.07 |
| $s_{20,w}$ ($s$) | | 3.24 | 3.88 ± 0.05 | 4.96 ± 0.29 | 3.78 ± 0.14 | 10.76 ± 0.17 |
| Partial specific volume (v) (ml g$^{-1}$) | | 0.835 | 0.786 ± 0.005 | 0.840 ± 0.002 | 0.808 ± 0.002 | 0.710 ± 0.019 |
| Stokes radius (a) (nm) | | 6.92 | 7.01 | 8.29 | 7.05 | 6.83 |
| Molecular weight ($M_r$) (× 10$^3$) | | 154 | 144 | 291 | 157 | 288 |
| Frictional ratio (f:f$_O$)[a] | | 1.87 | 1.97 | 1.81 | 1.91 | 1.58 |
| Bound detergent (g g$^{-1}$ protein) | | 0.81 (1.03–0.61) | 0.30 (0.44–0.15) | 1.16 (1.44–0.89) | 0.42[b] (0.56–0.28) | [c] |
| $M_r$ of protein portion (× 10$^3$) | | 85(76–95) | 111(100–125) | 134 (119–154) | 82(75–89)[b] | [c] |

[a] Not corrected for hydration

[b] This value includes both phosphatidylcholine (PC) and cholate assuming that they are bound to the protein in the proportions in which they were added and that the composite partial specific volume is the weighted mean of the two components (Tanford & Reynolds, 1976; Hanski *et al.*, 1981).

[c] Could not be estimated because the v value of digitonin (0.74) is close to the v value of the complex.

Apparent sedimentation coefficients were estimated using calibration curves (Figure 15). The average of triplicate determinations (± s.d.) are shown. Values of $s_{20,w}$ and v were calculated from the centrifugation data in $H_2O$ and $D_2O$, and the value of Stokes radius (a) estimated as described in the text. The values of $M_r$ and f:f$_O$ were calculated from $s_{20,w}$, v and Stokes radius without assuming solvation (Haga *et al.*, 1977; Haga, 1980). The bound detergent and $M_r$ of the protein in the complex were calculated using the assumption that the observed v represents the average of values for protein of 0.735 ml g$^{-1}$ (or 0.71 and 0.76 ml g$^{-1}$ for the values in parentheses) and detergents; the v values of detergents were taken as 0.958 (Lubrol PX), 0.93 (LPC) and 0.98 ml g$^{-1}$ (PC) (Tanford & Reynolds, 1976) and 0.738 (digitonin, Hubbard, 1954) and 0.75 ml g$^{-1}$ (cholate, Hanski *et al.*, 1981).

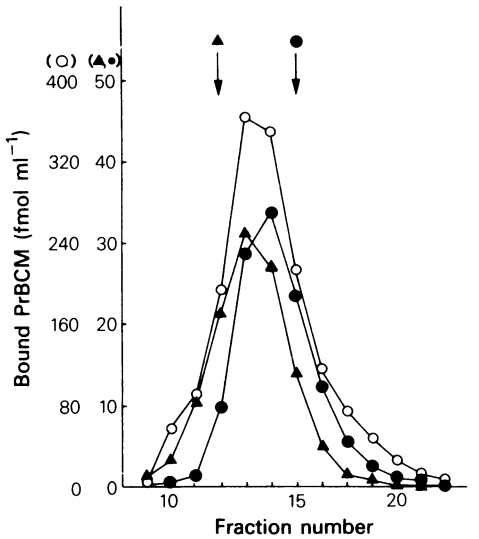


**Figure 14** Re-chromatography of [$^3$H]-PrBCM-labelled species in cholate/NaCl/PC. [$^3$H]-PrBCM-labelled membranes (preparation I, 3.4 mg protein ml$^{-1}$) were solubilized by 0.3% cholate-1 M NaCl, mixed with the same volume of 0.5% PC/cholate/NaCl and applied to AcA-34 Ultrogel column which was equilibrated with 0.3% cholate-1 M NaCl-0.25% PC (see Methods). Eluates of original gel filtration (○) (fractions no. 12 (▲) and 15 (●), 1 ml each) were re-chromatographed on the same column. Blue dextran and cyclic AMP were eluted at positions equivalent to fractions 10.0 and 26.9, respectively.

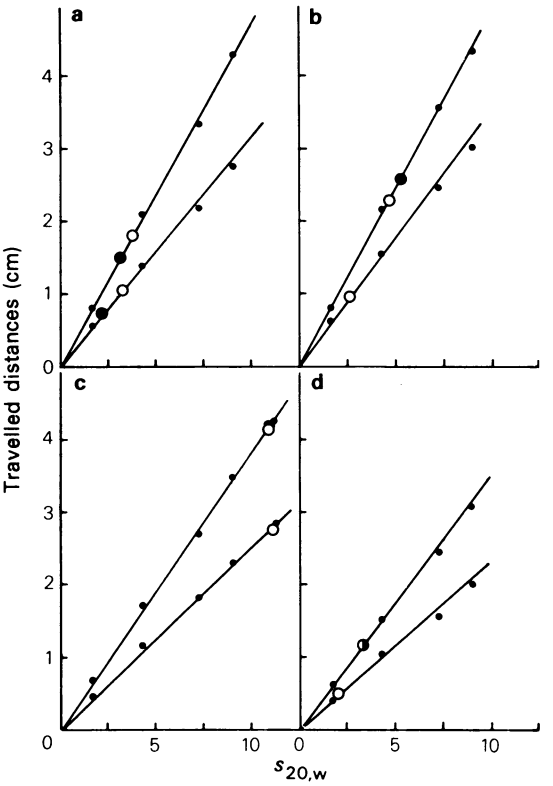


**Figure 15** Calibrating curves for sucrose density gradient centrifugation in $H_2O$ and $D_2O$. Distances travelled by calibrating enzymes were plotted against their known sedimentation coefficients ($s_{20,w}$). The calibrating enzymes were cytochrome C (1.71 *s*, malate dehydrogenase (MDH) (4.32 *s*), Lactate dehydrogenase (LDH) (7.3 *s*), fumarase (9.09 *s*), and catalase (11.3 *s*). The centrifugation was carried out at 100,000 $g_{av}$ (as described in the text for 19 h except for the centrifugation in digitonin, where the centrifugation time was 15 h). The steeper line in each of the calibration plots refers to centrifugation in $H_2O$; the shallower to centrifugation in $D_2O$. (○) Bound [$^3$H]-PrBCM; (●) bound [$^3$H]-QNB or specific [$^3$H]-QNB binding activity. (a) Lubrol PX, (b) lysophophatidylcholine, (c) digitonin, (d) cholate/1 M NaCl. There was no evidence of dissociation of the calibrating enzymes into monomers in these detergents.

higher in $H_2O$ than in $D_2O$ for the preparations solubilized by Lubrol PX, LPC and cholate-NaCl-PC (Figure 15 and Table 1). The values of partial specific volumes, (v, ml g$^{-1}$ calculated using the method of Clark (1975) are higher (0.79–0.84) than those of most proteins (0.71–0.76). These deviations are consistent with the presence of bound detergent and/or phospholipid, since Lubrol PX, LPC and PC have much higher partial specific volumes (Tanford & Reynolds, 1976) than proteins. Amounts of bound detergent were estimated (assuming the partial specific volume of protein to be 0.735 ml g$^{-1}$) to range from 0.3 to 1.3 g g$^{-1}$ protein (Table 1).

In contrast, digitonin solubilized preparations gave identical *s* values in $H_2O$ and $D_2O$ and a partial specific volume of 0.71 which is similar to that of most proteins and digitonin (Hubbard, 1954). This indicates that muscarinic receptors in digitonin probably do not bind to a detectable amount of endogenous phospholipid. As a consequence, the amount of bound digitonin could not be calculated.

Apparent molecular weights of the detergent receptor complexes were calculated by combining data from sucrose density gradient centrifugations in $H_2O$ and $D_2O$ with data from gel filtration (Edelstein & Schachman, 1967; Haga *et al.*, 1977; Haga, 1980a). It is of interest that the Stokes radius is smallest for the digitonin complex which has a higher apparent molecular weight whereas the [$^3$H]-PrBCM-labelled receptor in LPC has the highest Stokes radius and an equally high molecular weight. These differences presumably reflect differences in the structure of the receptor-detergent complexes. Apparent molecular weights of the protein components were calculated from the overall molecular weights and the estimate

of bound detergent. The value obtained for [$^3$H]-QNB-labelled receptor in Lubrol PX was 85,000 in agreement with the previous results (Haga, 1980a). The mean values obtained for [$^3$H]-PrBCM-labelled components solubilized by Lubrol PX and LPC were 111,000 and 134,000, respectively. The value for [$^3$H]-PrBCM labelled components solubilized by cholate-NaCl-PC can be estimated to be 82,000 assuming that cholate and PC are bound in proportion to their concentrations. This estimate would suggest that the main component of bound [$^3$H]-PrBCM in cholate/NaCl/PC is the muscarinic receptor monomer. These estimation procedures could not be used to calculate the contribution of bound digitonin to the overall molecular weight of the digitonin receptor-complex ($M_r$ 290,000).

## Discussion

Our data show that muscarinic receptors can be solubilized by a variety of detergents but in no case was it possible to solubilize all the receptor binding sites. We have examined whether this was due to a rapid denaturation of the receptor after solubilization or incomplete solubilization of the binding sites. This was accomplished by comparing data from receptors irreversibly labelled before solubilization with those from receptors labelled with reversible antagonists after solubilization. We have shown previously that [$^3$H]-PrBCM and reversible ligands such as [$^3$H]-QNB label the same site (Hulme *et al.*, 1978). About 60–85% of the total binding sites could be solubilized by the detergents examined. Lubrol PX behaved as a denaturing detergent whereas digitonin produced little, if any, rapid denaturation. Lysophosphatidylcholine exhibited intermediate properties. The addition of 1 M NaCl to 0.3% cholate changed the detergent system from being denaturing to non-denaturing, and furthermore enhanced receptor solubilization. In addition to the well-known ability of antagonists to protect receptors against denaturation, we have shown that binding of an agonist to the receptor stabilized the receptor to the action of cholate (unpublished results).

We have identified two sets of conditions in which the labelled receptor is essentially homogeneous (Lubrol PX-[$^3$H]-QNB-receptor complex; cholate/NaCl/PC) with molecular weights which agree with those estimated by SDS-polyacrylamide gel electrophoresis (Birdsall *et al.*, 1979; Ruess & Lieflander, 1979; Amitai *et al.*, 1982; Venter, 1983). In general, however, the soluble receptor-detergent complexes exhibited heterogeneity which was manifest both in gel filtration and sedimentation studies. This was particularly noticeable with the [$^3$H]-PrBCM pre-labelled receptor and could be largely attributed to the existence of higher molecular weight species. In Lubrol PX there was evidence that the high molecular weight species bind less detergent than the monomers, suggesting the existence of hydrophobic interactions between the proteins. Because of the heterogeneity, the estimates of the mean molecular weights of the complexes varied from 140,000–290,000 and 95,000–130,000 after subtraction of the estimated contribution from bound detergent. Such correction was not possible in the case of the digitonin-receptor complex where the average molecular weight of 290,000 was in fact similar to that of a rhodopsin-digitonin complex which contained only one protein molecule of $M_r$ 42,000 (Hubbard, 1954).

Our results suggest that in all these detergent systems a substantial fraction of the receptors are monomeric and capable of binding ligands, and that there are in addition complexes which may be specific or non-specific in origin. There is evidence of specific complexes from target size analysis (Uchida *et al.*, 1982), photo affinity labelling (Avissar *et al.*, 1983), and binding studies (Birdsall & Hulme, 1983 and references therein). In the accompanying paper we describe the solubilization and characterization of a specific muscarinic receptor-guanine nucleotide binding protein complex from the rat myocardium (Berrie *et al.*, 1984). The evidence is, however, that the structural integrity of the muscarinic binding site is much less dependent on protein interactions than that of, for example, the nicotinic acetylcholine receptor from Torpedo.

It should be noted that the binding properties of muscarinic receptors in the cerebral cortex are complex and there appear to be receptor subclasses (Birdsall *et al.*, 1978; Hammer *et al.*, 1980; Birdsall & Hulme, 1983). The possible contribution of these subclasses to the observed heterogeneity of the soluble receptor may be explored by the use of selective ligands.

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