A 5-hydroxytryptamine 5-HT₃ receptor binding site has been purified from deoxylcholesterol-solubilized NCB20 cell membranes. Purification (1,700-fold) was achieved in one step by affinity chromatography with L-685,603 immobilized on agarose. The 5-HT₃ selective antagonist [³H]Q ICS 205-930 labeled a single population of receptors in the affinity-purified preparation with a Dₘ of 3.1 ± 0.9 nM/mg protein and Kᵦ of 0.40 ± 0.05 nm (mean ± S.E., n = 3). The rank order of potency for a series of competing compounds confirmed that [³H]Q ICS 205-930 was labeling a 5-HT₃ receptor in the purified preparation, and the inhibition constants for all antagonists were unchanged after purification. The purified 5-HT₃ binding site eluted from a Sepharose 6B gel filtration column in a similar manner to the crude solubilized preparation (Stokes radius of 4.9 nm, apparent molecular size 250,000). Polyacrylamide gel electrophoresis of the affinity-purified receptor showed two broad bands by silver staining, with apparent molecular masses of 54,000 and 38,000. Gel filtration of the affinity-purified material yielded a single peak labeled by [³H]Q ICS 205-930 with an apparent molecular size of 250,000, which was also composed of two bands of 54,000, 38,000, and 36,000, consistent with these being the constituents of the 5-HT₃ receptor.

The neurotransmitter 5-hydroxytryptamine (5-HT) exerts its effects through several distinct receptor types. These have been classified into three major classes: 5-HT₁, 5-HT₂, and 5-HT₃ (Bradley et al., 1988; Peroutka, 1988) and more recently a fourth type, 5-HT₄, has been described (Dumuis et al., 1988; Clarke et al., 1989). All of these, with the exception of the 5-HT₃ receptor, are linked to their functional effects through G proteins (for a review, see Strange, 1988). The 5-HT₃ receptor has recently aroused much interest as a potential therapeutic target for several types of centrally active drugs (for review see Costall et al., 1988). Biochemically, it is distinct from all other 5-HT receptors in that it forms an ion channel (Derkach et al., 1989; Yakel and Jackson, 1988) which has been demonstrated in several cell lines and in primary cultures of neuronal cells to be a cation channel, permeable to Na⁺ and K⁺ (Derkach et al., 1989; Yakel and Jackson, 1988; Lambert et al., 1989). As such it represents a novel receptor, being the first amine neurotransmitter receptor which functions as an ion channel.

Considering the electrophysiological similarity between the 5-HT₃ receptor and the nicotinic acetylcholine receptor (Yakel and Jackson, 1988) it is plausible that the 5-HT₃ receptor also belongs to the superfamily of ligand-gated ion channels. It may therefore be expected to share some amino acid sequence homology with other, better characterized members such as the nicotinic acetylcholine receptor (Lindstrom et al., 1987), the GABA receptor (Schofield et al., 1987), the glycine receptor (Grenningloh et al., 1987), and the kainate receptor (Wada et al., 1989). No structural or sequence information is available yet for the 5-HT₃ receptor. The "well-trodden" path to this information is to purify the receptor and obtain partial amino acid sequence data from which the full primary sequence can be obtained by molecular cloning.

Using the radioligand [³H]Q ICS 205-930 (Watling et al., 1988) to identify the 5-HT₃ receptor we have reported solubilization of this receptor from rat brain (McKernan et al., 1990). However, the specific activity of 5-HT₃ receptors solubilized from rat brain is low and an enriched source of receptor would provide a better starting material for purification. Binding studies have been carried out using receptors from various cell lines (Nejt et al., 1980; Hover and Nejt, 1988) and we have recently reported that the NCB20 cell line expresses 20-30-fold more 5-HT₃ receptor binding sites/mg of protein than rat brain (McKernan et al., 1990b). We have therefore used this cell line as an enriched source of receptor from which to purify the 5-HT₃ receptor binding site.

**EXPERIMENTAL PROCEDURES**

**Materials**—Diaminodipropylamine-agarse and octyl β-glucoside were from Pierce Chemical Co. Dicyclohexylenediamine was from Fluka. Desalting columns (PD-10) and gel filtration and polyacrylamide gel electrophoresis molecular weight markers were from Pharmacia LKB Biotechnology Inc. Kits for silver staining and protein assay were from Bio-Rad. Drugs were from the following sources: MDL 72222 from Research Biologicals Inc.; quipazine from Miles Laboratories Inc.; BRL 24924 and zacopride from Beechams Pharmaceuticals; B-hydroxytryptamine (5-HT) from Sigma; ketanserin from Janssen; ICS 205-930, Q ICS 205-930, and 2-methyl-5-HT were synthesized in the Chemistry Department at Merck (Harlow, Essex, UK). GR 38032 F was from Glaxo. Triton X-100 and glycerol were from British Drug House. Deoxylcholesterol was from Calbiochem. Soybean L-a-phosphatidylycholine (Type III-S) and all other reagents were from Sigma.

**Synthesis of L-685,603**—L-685,603 was synthesized from L-680,652 in a two step procedure. L-680,652 was dissolved in acetone.
and 1 equivalent of ethyl 4-bromobutyrate was added. After stirring at room temperature overnight, the resulting precipitate was filtered. The quaternary salt was heated in refluxing hydrobromic acid for 5 h and evaporated to dryness. The product (L-685,603) was dissolved in water, filtered, and freeze dried. The purity of this compound was greater than 99.5% as determined by elemental analysis, and the structure was confirmed by mass spectroscopy and NMR. The carboxyl group of L-685,603 was coupled to an agarose bead via a 9-atom hydrophilic spacer, diaminodipropylamine. A peptide bond was formed through a carbodiimide coupling reaction carried out in dimethylformamide (DMF). The scheme of these reactions is shown in Fig. 1.

Diaminodipropylamine-agarose (25 ml) was washed on a scintillator glass funnel (G3) sequentially with 10 volumes of distilled H2O, 10 volumes of 50% DMF, and 20 volumes of 100% DMF. The resin was transferred to a stopped glass measuring cylinder to which was added 20 mg of L-685,603 (dissolved in DMF), 10 ml of 0.5 m dicyclohexylcarbodiimide (DCC, dissolved in DMF by gentle heating) and approximately 2 mg of the catalyst dimethylaminopyridine. The reaction mixture was adjusted to a final volume of 50 ml with DMF. The concentrations of L-685,603 and DCC represented 6% of and a 10-fold excess over the concentration of free amino groups on the spacer arm, respectively. After rotating for 90 min at room temperature, excess L-685,603, DCC, dimethylaminopyridine, and the by-product, dicyclohexyl urea, were removed and the resin was washed with 5 × 5 volumes of DMF. Excess reactive free amine groups were blocked by performing a second carbodiimide coupling reaction with acetic acid. To the derivatized resin was added 10 ml of 0.6 m DCC (freshly dissolved in DMF) 200 µl of glacial acetic acid and 2 ml of dimethylaminopyridine. The volume was adjusted to 50 ml and the mixture was rotated for 17 h at room temperature. After the blocking reaction the resin was washed extensively on a scintillator glass funnel with 50 volumes of DMF, 50 volumes of 50% DMF, and 50 volumes of H2O.

The amount of L-685,603 coupled to the resin was determined by UV spectroscopy. 50 µl of a 50% slurry of resin in H2O was added to 0.95 ml of 50% glycerol. The resuspended slurry was scanned over the wavelength 380–190 nm against a blank of 50% glycerol in a Shimadzu UV-3000 double beam spectrophotometer. The spectrum obtained was very similar to that for unterivatized L-685,603 and the UV absorption of the unreacted agarose beads was negligible. Assuming that the L-685,603 coupled to the agarose has an extinction coefficient the same as that of unreacted L-685,603, the substitution of L-685,603 on the agarose affinity support was 0.6 pmol/ml resin. Affinity resins were also prepared with higher substitutions (3.0 and 4.8 pmol/ml resin) by allowing the initial reaction to continue for 4 and 17 h, respectively. The affinity resin as synthesized here and stored in 100 mM Tris-HCl, 0.5% Triton, 1 mM EDTA, 0.2% sodium azide at pH 7.5 was stable for at least 4 months at 4 °C.

Cell Culture—NCB20 cells were grown in Dulbecco's modified Eagle's medium containing Hepes (25 mM), the antibiotics penicillin (100 IU/ml) and streptomycin (100 µg/ml), glutamine (2 mM), and fetal bovine serum (9%). Cells were grown in flat Falcon 850-ml flasks and were subcultured every 2 or 3 days. Cells were used from passage 39 to 60.

Membrane Preparation—In a typical experiment 10–15 flasks of cells were used. Cells were harvested by scraping and were centrifuged at 1,000 × g for 5 min. The supernatant medium was discarded and the pellet resuspended in 10 mM Tris-HCl, 10 mM EDTA, pH 7.5, at 4 °C. The preparation was homogenized using a Sefar Ultra-Turrax homogenizer (3 × 5-s bursts at setting 5) in 10 mM Tris-HCl, 10 mM EDTA, pH 7.5, at 4 °C containing phenylmethylsulfonyl fluoride (0.1 mM), soybean trypsin inhibitor (10 µg/ml), chymotatin (10 µg/ml), bacitracin (10 µg/ml), and sodium azide (10 µg/ml). Membranes were then centrifuged at 48,000 × g for 20 min at 4 °C. The pellets were washed twice in the same buffer by centrifugation and resuspension and were stored as pellets at −20 °C for up to 4 weeks with no detectable loss in binding activity.

Solubilization of 5-HT3 Receptors—Pellets of frozen NCB20 membranes were resuspended at a ratio of 2 ml of buffer:1 flask of cells. In a typical experiment, membranes derived from 15 flasks of cells were resuspended at 4 °C in 30 ml of the following buffer: 100 mM Tris-HCl, 1 mM EDTA, 0.05% Lubrol, 10% glycerol, pH 7.5, containing the same protease inhibitors as were present during the membrane preparation. Membranes were then solubilized by the dropwise addition of 3 ml of 5% deoxycholate and subsequent homogenization on ice with 3 × 15-s bursts at setting 5 using a Sefar Ultra-Turrax homogenizer.

The mixture was then centrifuged at 100,000 × g for 1 h to yield the deoxycholate-solubilized preparation. 50–55% of the [3H]Q ICS 205-930 binding activity in the membranes was recovered in the deoxycholate-solubilized preparation. Solubilized 5-HT3 receptors from NCB20 cells are not stable in deoxycholate (McKernan et al., 1994) and therefore the solubilized preparation was immediately syringed filtered into buffer of the following composition (Buffer A): 100 mM Tris-HCl, 1 mM EDTA, 10% glycerol, 0.1% Triton X-100, pH 7.5, at 4 °C. Aliquots (2.5 ml) of the soluble preparation were applied to Pharmacia PD-10 columns pre-equilibrated with buffer A and the solubilized receptor was eluted in 3.5 ml, thereby diluting the receptor preparation by 1.4-fold.

Affinity Chromatography on L-685,603 Agarose—1 ml volumes of resin were packed in Bio-Rad polypropylene Econocolumns and washed with 20 column volumes of buffer of composition: 100 mM Tris-HCl, 1 mM EDTA, 10% glycerol, 300 mM KCl, 0.6% Triton X-100, pH 7.5, at 4 °C (Buffer B). The concentrations of KCl and Triton X-100 in the solubilized preparation were adjusted to 300 mM and 0.5%, respectively, to try and prevent nonspecific adsorption of protein to the affinity resin. Solubilized receptor was applied to the affinity resin at a flow rate of approximately 2 ml/h (this was usually carried out overnight). The column was then washed with 60–100 column volumes of Buffer B at a flow rate of 4 ml/h. Immediately prior to biopspecific elution of the receptor, the column was washed with 10 volumes of buffer B which contained reduced amounts of KCl (150 mM) and Triton X-100 (0.25%, Buffer C). [3H]Q ICS 205-930 binding activity was then eluted with 10 ml of the same buffer containing additionally 5 mM L-685,602 or 1 mM guipazine.

Binding of [3H]Q ICS 205-930 to Soluble and Membrane Preparations—Membrane binding was performed in a total volume of 0.5 ml containing 50–100 µg of membrane protein in 5 mM Tris-HCl, 1 mM EDTA, pH 7.5, at 4 °C and 0.10–5 nm [3H]Q ICS 205-930. Nonspecific binding was defined as that not displaced by 10−5 M L-680,659. Binding was carried out for 30 min at 4 °C and the incubations were terminated by filtration through GF/C filters followed by 2 × 3 ml washes with ice-cold 5 mM Tris-HCl, 1 mM EDTA, pH 7.5.

Binding to solubilized receptors was also carried out in a total volume of 0.5 ml, containing a maximum of 50 µl of solubilized receptor preparation as described previously (McKernan et al., 1990). Binding was carried out at 4 °C for 30 min and the incubations were terminated by filtration through GF/B filters which had been pre-soaked for at least 3 h in 1% polyethyleneimine. During the filtration procedure the binding was carried out using [3H]Q ICS 205-930 at a concentration of 0.7 nM. Nonspecific binding was generally less than 10% under these conditions.

Protein Determination—Protein was estimated in membrane and
solubilized preparations using Bio-Rad protein reagent. Estimates of protein content in washes and eluates from the affinity resin were made using a precipitation assay as follows: Protein was precipitated from volumes of up to 1 ml by addition of trichloroacetic acid to a final concentration of 10%. Samples were centrifuged for 5 min at full speed in an Eppendorf microcentrifuge. The supernatant was removed and the pellet washed once with 1 ml of acetone by mixing and recentrifugation. The final pellet was dissolved in 50 μl of 1 M NaOH. 750 μl of H₂O was added followed by 290 μl of Bio-Rad protein reagent. Protein standards of bovine serum albumin were similarly precipitated from the same Triton and salt buffer. Alternatively, proteins were assayed on SDS-polyacrylamide gels by quantitative densitometry using a Quantimet 970 system (Cambridge Instruments). A standard curve was constructed by densitometry of bovine serum albumin and ovalbumin applied over the range 50-200 ng which was linear.

SDS-Polyacrylamide Gel Electrophoresis—Samples of up to 150 μl from various stages of the purification procedure were precipitated by a CHCl₃/MeOH procedure (Wessel and Flugge, 1984). The precipitated protein was resuspended in 20 μl of electrophoresis sample buffer. Samples were analyzed on 9% polyacrylamide mini-gels which were fixed in 40% methanol and 10% acetic acid and stained according to the method of Giulian et al. (1983) using a Bio-Rad silver stain kit.

Gel Exclusion Chromatography—Gel exclusion chromatography was carried out at room temperature on a Superose 6B column (1 × 31 cm) using a Pharmacia fast protein liquid chromatography system. The column was equilibrated with 50-100 ml of buffer containing 10 mM Tris-HCl, 1 mM EDTA, 10% glycerol, 0.2% Lubrol, 500 mM NaCl, pH 7.5, at 4 °C. Receptor was loaded in a volume of 200 μl at 0.5 ml/min. 0.5-ml fractions were collected, and 20-μl volumes were assayed for [³H]IUCS 205-930 binding. The column was calibrated with the following marker proteins (Stokes radius, in nanometers): chymotrypsinogen (2.09), aldolase (4.81), catalase (5.2), ferritin (5.1), thyroglobulin (8.5). The void volume and total column volume were determined using blue dextran and [³H]inositol, respectively.

RESULTS

Preparation of L-685,603-Agarose—Preliminary experiments were conducted to optimize the affinity resin. Four resins were synthesized with increasing amounts of L-685,603 coupled to the side arm. These contained 0 (resin totally blocked with acetic acid), 0.6, 3, and 4.8 μmol of L-685,603/mg of resin and were tested in a batchwise manner for their ability to retain receptor. 1 ml of solubilized receptor preparation was mixed at 4 °C for 1 h with 100 μl of each resin, after which time the resin was allowed to settle under gravity and the protein and receptor remaining in the supernatant were measured (see Fig. 2). All these resins retained receptor from the supernatant. Nonspecific absorption of protein onto the resin increased with higher substitutions of L-685,603. Nonspecific binding of protein was due to binding to the ligand L-685,603, and not to the side arm. Since blocked (derivatized) resin retained very little protein. The resins substituted at a concentration of 3.0 and 4.8 μmol/mg both retained significant (p < 0.05) amounts of protein, whereas the lower substituted (0.6 μmol/mg) resin did not. The lowest substituted resin, however, did retain less receptor (200 fmol/ml) compared with the other two resins (280 fmol/ml).

Adsorption of receptor to the resin (0.6 μmol/ml) was prevented by preincubation with the 5-HT₃ selective antagonist L-680,652 in a dose-dependent manner (Fig. 2), thus the resin exhibited a biospecific adsorption of receptor. The lowest substituted resin (0.6 μmol/ml) was used in all further experiments, because it retained the least protein and, when used in a column procedure, it retained greater than 90% of the receptor (Fig. 3).

Affinity Chromatography of 5-HT₃ Receptor Binding Sites on L-680,603-Agarose—Fig. 3 shows a typical adsorption and elution profile for affinity chromatography of 5-HT₃ receptor binding sites on L-680,603-agarose. Greater than 90% of the receptor was retained, while less than 1.5% of the applied protein was retained. Extensive washing with buffer B, containing high salt (0.3 M) and high detergent (0.5% Triton), removed loosely bound protein without apparent loss of binding activity. After washing with high salt, the concentration of NaCl and Triton was reduced to 150 mM and 0.25%, respectively. In these fractions no protein was detected by

![Fig. 2. Binding of solubilized 5-HT₃ receptors to L-685,603-agarose. NCB20 cells from five flasks were solubilized as described under "Experimental Procedures." The solubilized preparation was desalted into Buffer A and Triton X-100 and KCl were added to concentrations of 0.5% and 300 mM, respectively. 1-ml aliquots were mixed in a batchwise manner for 1 h at 4 °C with 100 μl of the following resins: 1) diaminodipropylamine-agarose, which had been blocked with acetic acid; 2) L-685,603-agarose (4.8 μmol/ml); 3) L-685,603-agarose (3 μmol/ml); 4) L-685,603-agarose (0.6 μmol/ml); receptor preincubated with 10 μM L-680,652; 6) L-685,603-agarose (0.6 μmol/ml); receptor preincubated with 100 μM L-680,652; 7) solubilized receptor with no additional. Where the solubilized receptor was incubated with resin in the presence of the antagonist L-680,652, this was added to the solubilized preparation 20 min prior to the addition of resin. Where necessary, the antagonist L-680,652 was removed by gel filtration before radioligand binding. In these experiments the soluble preparation contained 0.4 pmol (measured with 0.7 nM [³H]IUCS 205-930) and 0.8 mg of protein/ml. Data shown are the means ± S.D. of triplicate incubations with each resin.](Image)

![Fig. 3. L-685,603-agarose chromatography of solubilized 5-HT₃ receptors from NCB20 cells. Starting material (60 ml of deoxycholate-solubilized material, exchanged into 0.5% Triton buffer containing 0.93 pmol of [³H]IUCS 205-930 binding activity and 1.6 mg protein/ml), was passed through 1 ml of L-685,603-agarose affinity resin at a flow rate of 2.2 ml/h at 4 °C. The column was then washed with approximately 100 ml of buffer B (100 mM Tris-HCl, 1 mM EDTA, 0.5% Triton X-100, 300 mM KCl, 10% glycerol, pH 7.5, at 4 °C) followed by 10 ml of buffer C (100 mM Tris-HCl, 1 mM EDTA, 0.25% Triton X-100, 150 mM KCl, 10% glycerol, pH 7.5, at 4 °C). The receptor was eluted with 10 ml of the latter buffer containing 1 mM quipazine. 2.2-ml fractions were collected during loading and elution of the receptor. 4.4-ml fractions were collected during the washing procedure. Note the change in scale at fraction 4b.](Image)
silver staining of 150-μl aliquots analyzed by gel electrophoresis or by protein assay (detection limit = 300 ng/ml). Protein was eluted from the affinity resin with 1 mM quipazine and binding detected after gel filtration of 100-μl aliquots. Binding activity was eluted with 10 column volumes and this could be achieved in a variety of detergents. Elution buffers containing 25 mM octyl glucoside, 0.6% CHAPS, 0.2% Lubrol, or 0.2% deoxycholate instead of 0.25% Triton X 100 were similarly effective (data not shown).

In eight similar experiments, 92.3 ± 4% of applied receptor was bound to the resin and 1.35 ± 0.6% of the applied protein was absorbed. After extensive washing, 44.9 ± 9% of the receptor binding activity was recovered. Binding activity could also be eluted with 1 mM L-680,652 or 1 mM zacopride (data not shown). In three experiments, protein in the peak eluted fraction was measured after trichloroacetic acid precipitation. The Bmax for binding of [3H]Q ICS 205-930 to these three preparations was 1.6, 3.2, and 4.6 pmol/mg protein (3.1 ± 0.9 pmol/mg protein, mean ± S.E., n = 3). A summary of the purification of the 5-HT3 receptor using L-685,603-agarose is shown in Table I. An overall 1700-fold purification of the receptor binding site was achieved by affinity chromatography. In a parallel control experiment in which the solubilized preparation was applied to the affinity resin in the presence of 1 mM quipazine, [3H]Q ICS 205 930 binding was detected in the nonbound fractions and not in the fractions subsequently eluted from the resin by antagonist.

Recovery of [3H]Q ICS 205-930 Binding to Purified Receptor—After elution of the 5-HT3 receptor from the affinity resin it was necessary to remove the antagonist L-680,652 before the receptor could be assayed and its specific activity determined. Since the receptor was relatively pure, it was anticipated that gel filtration might result in a loss of binding activity due to instability of the receptor in the absence of antagonist or phospholipid. To investigate this, and to optimize recovery of receptor binding activity, gel filtration was carried out in the presence of several protein or lipid solutions including nonbound material from the affinity resin (corrected for the small amount of receptor remaining), boiled nonbound material, from which protein had been precipitated and removed, 0.5 mg/ml crude soybean phospholipid, and 10% bovine serum albumin. As demonstrated in Fig. 4, the addition of nonbound material or phospholipid alone allowed for a better recovery of receptor binding, but the stimulation of binding by these solutions was small.

The addition of bovine serum albumin to the receptor eluate either alone, or together with phospholipids, prior to gel filtration did not appear to improve recovery of [3H]Q ICS 205-930 binding. One plausible explanation for these results could be that the ligand employed to elute the receptor, L-680,652, may bind to bovine serum albumin and therefore be carried into the void volume, allowing inhibition of [3H]Q ICS 205,930 binding.

Pharmacological Analysis—Saturation analysis was carried out on the purified receptor (see Fig. 5). In this particular preparation Bmax binding of [3H]Q ICS 205 930 was 4.6 nmol/mg protein, with a Kd of 0.33 nM. The affinity for the purified receptor (0.40 ± 0.05 nM, mean ± S.E., n = 3) was not significantly different from that observed in a crude solubilized receptor (Kd = 0.48 nM, McKernan et al., 1990a). It was important to establish that binding to the affinity purified preparation was characteristic of binding to a 5-HT3 receptor.
site, particularly since the eluting ligand, quipazine, also binds to serotonin uptake sites (Schmidt et al., 1989) and the ligand [3H]Q ICS 205-930 also binds, albeit with a lower affinity, to 5-HT3 receptors (Dumuis et al., 1988). The rank order of potency of a series of antagonists for the [3H]Q ICS 205-930 binding site (zacopride > Q ICS 205-930 > ICS-205930 > quipazine > L-680,652 > GR 38032F > BRL 24924 > MDL 72222) was consistent with binding to a 5-HT3 receptor, and the affinity of most compounds tested was not significantly different from that observed in crude soluble preparations (see Table II). One exception was the agonist 5-HT whose K, in the purified preparation (170 nM) was 4-fold lower than has been observed in a crude soluble 5-HT3 preparation (38 nM, McKernan et al., 1990b).

Biochemical Characterization of the Purified Receptor—Aliquots (150 μl) of the fractions eluted from the affinity resin were precipitated and subjected to polyacrylamide gel electrophoresis. Two major bands were always present with apparent molecular masses of 38,000 and 54,000 and the band at 54,000 was always more intensely stained than the smaller band. Both bands were diffuse, but were present in proportion to the amount of [3H]Q ICS 205-930 binding, as shown in Fig. 6. In a parallel control experiment in which the soluble preparation was applied to the resin in the presence of 1 mM quipazine, and consequently the receptor was not retained, the two bands of M, 38,000 and 54,000 were not detectable in the eluted fractions. In two our of seven experiments (including that shown in Fig. 6) a third band was present at a higher molecular mass (~115,000). This was not observed in all gels and may be due to aggregation of lower molecular weight species or alternatively might identify a polypeptide which copurifies with the receptor.

The amount of protein present in the 54- and 38-kDa bands was quantified by densitometry using a Quantimet 970 system. The combined amount of protein present in the two bands (precipitated from 150 μl of the peak eluted fraction) ranged from 210 to 360 ng (three experiments). Using these measures of protein the specific activity of the purified preparation is 5.6 ± 0.4 nmol/mg protein. The relative intensity of staining of the two bands was 2.3 ± 0.3:1 (54:38-kDa, n = 3).

Gel filtration of the affinity purified receptor (200 μl from the peak fraction) on Sepharose 6B was carried out in 0.2% Lubrol. The peak of Q ICS 205,930 binding was eluted with an apparent Stokes radius of 4.9 nm as shown in Fig. 7. This is similar to that observed for crude solubilized 5-HT3 receptor, which has been calculated to have a relative molecular mass of 249,000 (McKernan et al., 1990b). After gel filtration,

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<td><strong>Inhibition constants for purified 5-HT3 receptors</strong></td>
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<td>Values shown are the means of two or three determinations on separate preparations of purified receptor performed in duplicate. There was no evidence of low Hill slopes with any of the compounds tested.</td>
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<td><strong>Compound</strong></td>
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FIG. 6. Polypeptide composition of purified 5-HT3 receptor. SDS-polyacrylamide gel electrophoresis of crude solubilized preparation from NCB 20 cells (1 μl) (A), nonbound fraction from L-685,603-agarose affinity resin (1 μl) (B), fractions eluted from the affinity resin with 1 mM quipazine (~50–500 ng of protein) (C–G). H, molecular mass standards; phosphorylase b (94,000), bovine serum albumin (67,000), ovalbumin (43,000), and carbonic anhydrase (30,000). The gel was silver-stained as described by Giulian et al. (1983). 150 μl from each fraction was precipitated using the CHCl<sub>3</sub>/MeOH procedure and taken up in 20 μl of gel loading buffer. Q ICS 205-930 binding activity in each lane is shown in the lower panel.

FIG. 7. Gel filtration of affinity purified 5-HT3 receptor. 200 μl of the peak fraction eluted from L-685,603-agarose containing 2 pmol of [3H]Q ICS 205-930 binding was applied to a Superose 6B column as described under “Experimental Procedures.” The column was calibrated with the marker proteins (from left): thyroglobulin, ferritin, catalase, aldolase, and chymotrypsinogen A. Fractions from the column were combined and concentrated to 150 μl and then precipitated with CHCl<sub>3</sub>/MeOH and subjected to polyacrylamide gel electrophoresis. A, elution volumes 9–11 ml; B, elution volumes 11–13 ml; C, elution volumes 13–16 ml; D, elution volumes 16–19 ml.

The eluted fractions were pooled into four major fractions, concentrated, precipitated, and subjected to polyacrylamide gel electrophoresis. Two bands (54 and 38 kDa) were detected only in the fraction corresponding to 250 kDa and not in the
fraction which would contain species of lower molecular masses. In two experiments, the combined amount of protein present in these two bands was 250 and 320 ng from which the specific activity after gel filtration was calculated as 6.3 and 5.9 nmol/mg.

**DISCUSSION**

This paper describes the purification of a 5-HT₃ receptor from deoxycholate-solubilized NCB20 cells. Purification was facilitated by the use of NCB20 cells which provide an enriched source of 5-HT₃ receptors, expressing 30–40 times more receptor/mg of protein than rat brain. 5-HT₃ receptors solubilized from rat brain are also retained by the L-685,603 affinity resin in a biopspecific manner and therefore this resin may also be suitable for purification of 5-HT₃ receptors from rat brain or other tissues.

Many neurotransmitter receptors have now been purified (for review see Ref. 8). In all cases G-protein-linked receptors have been found to be composed of only one subunit, whereas members of the superfamily of ligand-gated ion channels such as the brain nicotinic acetylcholine receptor (Whiting and Lindstrom, 1986), the glycine receptor (Pfeiffer et al., 1982), and the GABA, receptor (Sigel et al., 1983) are all heterooligomers between 250,000 and 300,000 in size. This makes interpretation of polyacrylamide gel electrophoresis analysis of the proteins present after affinity purification more complex since it may not be easy to determine which bands constitute the receptor. For example, studies of the glycine receptor have demonstrated that the receptor consists of two proteins of M₁₃8,000 and 58,000, but a third protein of M, 83,000 copurifies with the receptor, but does not itself form part of the channel (Langosch et al., 1988).

In the experiments presented here, two major proteins were identified (M₁38,000 and 54,000). At present, we favor the view that the two proteins are separate and distinct species because a range of protease inhibitors were included during membrane preparation and receptor solubilization, and the ratio of the two bands after silver staining appears to be reasonably consistent (2.3 ± 0.3, 54:38-kDa, n = 3). It is possible that as the bands detected by silver staining of the polyacrylamide gel are both broad and indistinct they may be composed of more than one species. This may arise from heterogeneity in post-translational processing, particularly glycosylation, since the solubilized 5-HT₃ receptor binds avidly to lentil lectin and wheat germ agglutinin-lectin (data not shown) or, as has been observed for the GABAₐ receptor, distinct subunits may migrate closely on polyacrylamide gels.

The mean specific activity of the purified based on protein assay of the affinity-purified material receptor was 3.1 nmol/mg protein (highest value 4.6 nmol/mg protein). The theoretical specific activity for a protein of relative molecular mass of 250,000 would be 4 nmol/mg protein assuming that there is one binding site/molecule. It might then be concluded that the receptor is at least 75% pure. However, electrophysiological experiments for most ligand-gated ion channels demonstrate that there are two binding sites per molecule. To date there is no information available as to how many binding sites for agonist or for [³H]Q ICS 205-930 there are for each 5-HT₃ receptor molecule. If there are two then the affinity purified preparation would be at least 40–60% pure. Examination of the polypeptides present on a silver-stained polyacrylamide gel both before and after gel filtration of the affinity-purified material shows essentially the same pattern. Calculation of the specific activity of the receptor based on the quantity of protein detected by densitometry gives values before gel filtration (5.6 ± 0.4 nmol/mg) and after gel filtration (5.9, 6.3 nmol/mg) which are the same, within the limits of experimental error. These two measures may overestimate the specific activity of the purified receptor because they do not allow for the small but significant amount of aggregated material which remains at the origin and does not enter the polyacrylamide gel (up to 25% of the loaded protein). Additionally, calculation of the specific activity by this method is also subject to errors derived from the non-uniform binding of silver to different proteins. Nevertheless, given the calculated specific activities and the lack of other detectable polypeptides, the results presented are consistent with the 5-HT₃ receptor being composed of the two polypeptides of apparent size 54 and 38 kDa. In summary, we have purified the 5-HT₃ receptor from NCB20 cells by affinity chromatography using L-685,603-agarose. Purification to apparent homogeneity is achieved in one step and yields a protein molecule of molecular size of 250 kDa which appears to be comprised of two polypeptides of 54 and 38 kDa. Cloning, sequencing, and expression studies are now required to establish this definitively.

**Acknowledgments**—We would like to thank Drs. Paul Whiting and F. Anne Stephenson for helpful advice during these studies. We are also indebted to Jill Gillard for maintenance of NCB20 cells and preparation of cell membranes, and Roy Hammons, Eleanor Brawn, and Sue Burton for preparation of the diagrams and manuscript, respectively. We thank Dr. M. Rigby for quantitative densitometry of the silver-stained polyacrylamide gels.

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