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Effect of some poly(ethylene glycol)-bound and dextran-bound affinity ligands on the partition of synaptic membranes in aqueous two-phase systems

Göte Johansson*, Björn Olde, Monica Joelsson

Department of Biochemistry, Chemical Center, University of Lund, Box 124, S-221 00 Lund, Sweden

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Abstract

Ligands with an apparent affinity for various structural elements on the surface of synaptic membrane fragments have been bound to the polymers poly(ethylene glycol) and dextran. The ligand–polymer derivatives have been included in aqueous two-phase systems composed of water, poly(ethylene glycol) and dextran. The uneven distribution of the polymers resulted in the concentration of the polymer-bound ligand in one of the two phases. The effect of the ligand–polymer on the partition of membranes was studied by using synaptic membranes from calf brain, obtained by standard centrifugation methods. By using ligand-containing two-phase systems for nine-step counter-current distribution of membranes, it was shown that the distribution behaviour of various parts of the membrane preparation could be affected. The distribution was followed by determination of opiate binding, acetylcholinesterase, and total membrane (using protein and light-scattering measurements).

1. Introduction

Brain synaptic membranes have been isolated mainly by centrifugation techniques [1–3]. These preparations are inhomogeneous mixtures of membrane fragments, and it is difficult to isolate membranes containing specific macromolecular structures of great interest, *e.g.* receptors, ion channels and translocators.

The present work deals with the possibility of fractionating synaptic membranes from the brain into subpopulations by partitioning between the two liquid phases (and the interface between them) of aqueous biphasic systems. Other types of membrane, *e.g.* thylakoid membranes from spinach chloroplasts [4,5], have been subfractionated with this method. Because the separation obtained by partitioning depends on the surface properties of the membranes [6], it should be possible to obtain a higher degree of selectivity than with centrifugation, where size and density are the determining parameters. Specific extraction using poly(ethylene glycol)-bound cholinergic ligands, *i.e.* affinity partitioning, in combination with the biphasic systems has already been used for the purification of membrane-bound nicotinic cholinergic receptors from the electroplax of *Torpedo californica* [7,8]. Two examples of affinity extraction of synaptic membranes

* Corresponding author.

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using polymer-bound naloxone and Procion yellow HE-3G, respectively, have been published [9,10]. The general idea behind the affinity partitioning of membranes is to localize an affinity ligand for certain membrane structural elements in one of the phases. In this study, a number of receptor ligands were bound to polymers used in two-phase systems, and the effects of the polymer-bound ligands on the partition of brain synaptic membranes have been tested.

2. Experimental

2.1. Chemicals

Dextran ($M_r = 500\,000$) was obtained from Pharmacia (Uppsala, Sweden). Poly(ethylene glycols) ($M_r = 3000–3700$ and 6000–7500) were purchased from Union Carbide (New York, USA) as Carbowax 4000 and 6000, respectively. [^{3}H]Etorphine (34 Ci/mmol) was obtained from The Radiochemical Centre (Amersham, UK). Morphine hydrochloride (pharmacological grade) was obtained from the university pharmacist, naloxone was donated by Endo (Garden City, NY, USA) and etonitazen was donated by Ciba-Geigy (Basel, Switzerland). Dextrorphan and levorphanol were kind gifts from Dr. Martin Kanje (Dept. of Zoophysiology, University of Lund). The *m*-aminophenyl boronate hemisulphate was purchased from EGA (Steinheim, FRG) and 2,2,2-trifluoroethanesulphonyl chloride (tresyl chloride) and fatty acids from Fluka (Bucks, Switzerland). All other chemicals used were of analytical-reagent grade.

2.2. Membrane preparation

The preparation of synaptic membranes from calf brain was performed mainly as described by Hajós [2]. The method was slightly modified, as described earlier [9], to increase its capacity. The membranes were suspended in 50 m*M* Tris–HCl (pH 7.4) to give a protein concentration of 6 mg/ml. The membrane suspension was stored at −80°C. All centrifugations were performed in GS-3 and SS-34 rotors in a Sorvall RC-2B refrigerated high-speed centrifuge. Before partition the membranes were thawed and pelleted at 45 000 g_{max}. After resuspension of the pellet in water to a protein concentration of 20 mg/ml, the mixture was passed twice through a Yeda-press (Scientific Instruments, Rehovot, Israel) using nitrogen at a pressure of 10 MPa.

2.3. Opiate receptor assay

The stereospecific opiate binding was measured according to Medzihradsky [11] using the difference in binding of tritiated etorphine in the presence of 0.1 μM dextrorphan and 0.1 μM levorphanol, respectively. The non-specific binding was taken as the binding value in the presence of 0.1 μM levorphanol. The radioactivity was measured by using a Searle Scintillator, Mark III, 6880. The scintillation liquid was a mixture of Omnifluor (New England Nuclear) (4 g/l) and Triton X-100 (Riedel de Haen) (33%) in toluene.

2.4. Acetylcholinesterase assay

Acetylcholinesterase was determined by the method of Ellman *et al.* [12].

2.5. Protein assay

The protein content of the membranes was determined by mixing 25 μl of the membrane suspension with 225 μl of 0.5 *M* H_3PO_4 and keeping the samples at 50°C for 2 h. The samples were mixed with 3 ml of Coomassie Brilliant Blue G solution, and measured at 595 nm according to Bradford [13]. Bovine serum albumin was used as standard.

2.6. Light scattering

This was measured as the apparent absorbance at 400 nm (1-cm path-length) using a Hitachi spectrophotometer 100-60 after diluting the membrane suspension with water to $A_{400} = 0–0.25$.

2.7. Synthesis of polymer derivatives

Membrane ligands were bound to poly-

(ethylene glycol) (PEG) with $M_r = 3000–3700$ (PEG 4000) or 6000–7500 (PEG 6000), and to dextran ($M_r = 500\,000$). Morphine analogues and related compounds were attached to PEG by the azo-coupling recommended by Simon [15]. This method was used to bind morphine, naloxone, levorphanol, dextrophan and etonitazen to *p*-aminophenyl-PEG. The polymer derivatives were checked for purity and degree of substitution by gel permeation chromatography on a 25 × 1.6 cm I.D. column of Sephacryl S-200; the eluates were analysed with an Optilab refractive index monitor and an LKB Uvicord-S monitor set at 280 nm. Prior to use in the partition experiments, the polymers were chromatographed on a 20 × 1.6 cm I.D. Sephadex G-25 Medium column. The polymer peak was collected, and the polymer concentration was determined using a Zeiss immersion refractometer fitted with a thermostatted prism, L1.

Ethonium-PEG

Diamines, $H_2N(CH_2)_xNH_2$, with $x = 2–10$, were bound to PEG via one of the nitrogen atoms by treating the diamines with bromo-PEG ($M_r = 6500$) as described earlier [8]. One gram of each of these derivatives was dissolved in 10 ml of dry acetone, and 100 mg of Na_2CO_3 and 0.5 ml of ethyl iodide were added. The mixtures were heated for 3–4 h at 60°C on a water-bath, and left at room temperature over-night to complete the reaction. The solvent was driven off, the residue dissolved in ethanol, and the solvent evaporated again to remove traces of unchanged ethyl iodide. The product contained 0.35–0.40 mol of diamine per mol of polymer, determined via the nitrogen content.

p-Aminophenyl-PEG (PAP-PEG)

Bromo-PEG ($M_r = 6500$) [14], essentially monosubstituted, reacted with an excess (five-fold) of sodium *p*-nitrophenolate in anhydrous ethanol (24 h reflux). The resulting polymer was recrystallized four times from the same solvent. The *p*-nitrophenyl-PEG so obtained was dissolved in 3 *M* HCl and reduced by stirring the solution with granulated tin (twice the weight of the polymer) for 2 h. The mixture was made alkaline with sodium carbonate, and the PAP-PEG was extracted with chloroform. The chloroform layer was collected and dried with anhydrous sodium sulphate, and (after filtration) the solvent was evaporated. The polymer was recrystallized twice from absolute ethanol. The product contained 0.85 mol of aminophenyl groups per mol PEG, as estimated by acidimetric titration.

Diazotization of PAP-PEG and azo-coupling

To a solution of 1 g of PAP-PEG in 10 ml of 0.5 *M* HCl at 0–4°C, 100 mg of $NaNO_2$ in 1 ml of water were added. After stirring for 20 min, 1.5 ml of ice-cold 10% urea were added to destroy the excess $NaNO_2$. The reaction mixture was then stirred until no more gas (N_2) evolved, which normally took *ca.* 1 h. Then 50–100 mg of the drug (100 mg of morphine or naloxone, 50 mg of levorphanol, dextrorphan or etonitazene), dissolved in 2 ml of 0.25 *M* ice-cold HCl, was added. The pH was adjusted to 7 by addition of solid sodium tetraborate (*ca.* 1.3 g), and the mixture was stirred overnight at 0°C. Next, 10 ml of water (25°C) were added to terminate the reaction. After the addition of solid Na_2SO_4, the polymer formed a liquid phase (containing 60–70% water) which was lighter than the salt solution. This upper phase was mixed with 50 ml of chloroform, and the water was removed by adding anhydrous Na_2SO_4. The solution was filtered and dried again with Na_2SO_4. After filtration the solvent was evaporated. The recovery was 75–80% of the PAP-PEG used. The degree of substitution was estimated by comparing the spectra of the PEG derivatives in the visible and near-UV regions and the spectra of the corresponding ligands coupled by an azo-linkage to aniline: 0.75 mol of morphine, 0.70 mol of naloxone, 0.63 mol of levorphanol and dextrophanol, and 0.48 mol of etonitazen, respectively, per mol of PEG.

Tresyl-PEG

PEG was also activated by introducing a tresyl group, which is more easily replaced by nucleophilic reagents, *e.g.* amines, than the bromo group. The tresylation is based on the method published by Crossland *et al.* [16] and Nilsson and Mosbach [17]: 10 g of PEG ($M_r = 6000–$

7500) were dissolved in 25 ml of dichloromethane containing 420 μl of pyridine. The solution was cooled on ice, and 650 mg of 2,2,2-trifluoroethanesulphonyl chloride were added. The reaction mixture was brought slowly to room temperature (30 min on ice, 1 h at 4°C), to control the reaction rate, and was subsequently stirred for 3 h. The solvent was then removed, and the product was recrystallized from 100 ml of ethanol containing 50 m*M* HCl, followed by two recrystallizations from 150 ml of ethanol and 150 ml of methanol, respectively. The recovery was 93%. The tresyl-PEG was used for further synthesis, as described below. The degree of substitution was not determined.

Tresyl-dextran

First, 10 g of dextran ($M_r = 500\,000$) were suspended in 50 ml of pyridine. The slurry was cooled on ice, and 500 mg of 2,2,2-trifluoroethanesulphonyl chloride were added. The reaction mixture was slowly brought to room temperature (as above) and stirred overnight. The product was collected by suction filtration and washed with several volumes of dry acetone. The recovery was 99%. Tresyl-dextran was used for the synthesis of aminophenyl boronate dextran and Azure A dextran, as described below.

Naloxone-amido-PEG

This derivative was prepared from tresyl-PEG, as described previously [9]. The degree of substitution was estimated photometrically to be 0.6 mol of naloxone per mol of PEG.

Etonitazene-amido-PEG

Etonitazeneamine was prepared by the reduction of etonitazene with sodium dithionite in water at pH 2, as described by Gabor and Leader [18]. A 100-mg amount of the amine was dissolved in 50 ml of methanol containing 1 g of bromo-PEG ($M_r = 6500$) [14], 400 mg of Na_2CO_3, and a small crystal of KI. The reaction mixture was heated on a water-bath at 80°C for 1.5 weeks. The solvent was removed by evaporation. The degree of substitution was determined photometrically to be 0.30–0.35 mol per mol of PEG.

m-Aminophenyl boronate PEG

m-Aminophenyl boronate (150 mg, free base) was dissolved in 25 ml of methanol containing 2.5 g of tresyl-PEG and 500 mg of Na_2CO_3. The reaction mixture was stirred overnight. The product was crystallized at 3°C and collected and washed on a Büchner funnel. The recovery was 60%. The degree of substitution was determined by UV photometry to be 0.4–0.5 mol of aminophenylboronate per mol of PEG.

m-Aminophenyl boronate dextran

m-Aminophenyl boronate (150 mg) was dissolved in 25 ml of 0.1 *M* sodium phosphate buffer (pH 8) containing 2 g of tresyl-dextran, and the mixture was stirred overnight. The product was precipitated by the addition of 75 ml of ethanol. The dextran derivative was further purified by being precipitated two or three times from aqueous solution by the addition of ethanol. The recovery was 92%. The degree of substitution was not determined.

Toluidine Blue PEG

A 30-g amount of carboxymethyl-PEG ($M_r = 5000$), prepared as described previously [19], was dissolved in 150 ml of toluene. Traces of water were removed by azeotropic distillation. The solution was allowed to cool to 50°C, 2.5 ml of triethylamine were added, and then 1 ml of $SOCl_2$ in 20 ml of toluene was added during 5 min with stirring. The mixture was refluxed for 1 h while a stream of nitrogen was passed through the solution. The solution was filtered and evaporated. The residue was dissolved in 150 ml of toluene, and 3.3 ml of triethylamine and 760 mg of toluidine blue O (free base) dissolved in 100 ml of toluene were added during 5 min. The solution was kept at 60°C for 10 min, filtered and evaporated. The polymer was recrystallized 10 times from anhydrous methanol. The degree of substitution was determined photometrically to be 0.15 mol of dye per mol of PEG.

Azure A dextran

Azure A (200 mg) was dissolved in 25 ml of 0.1 *M* sodium phosphate buffer (pH 8.0) containing 3 g of tresyl-dextran. The reaction mix-

ture was stirred overnight. The product was precipitated with 75 ml of ethanol, and the precipitate was dissolved in 25 ml of water and repeatedly precipitated until no more dye was found in the washing liquid. The recovery was 45%. The product contained 0.15 mmol of dye per gram of dextran, as estimated from the visible spectra.

PEG esters of fatty acids

PEG ($M_r = 6500$) was esterified with fatty acids containing 4 (butyric acid), 6 (caproic acid), 8 (caprylic acid), 10 (capric acid), 12 (lauric acid), 13 (decanoic acid), 14 (myristic acid) and 16 (palmitic acid) carbon atoms. To do this, 15 mmol of the acid was dissolved in 20 mmol of freshly distilled thionyl chloride, and the solution was refluxed for 2 h. Excess thionyl chloride was removed by vacuum distillation (100°C, 12 mmHg), and the remaining acyl chloride was dissolved in 10 ml of toluene. A solution of 100 g of PEG in 600 ml of toluene was dried by azeotropic distillation (100 ml of toluene plus water were distilled off), and 10 mmol of triethylamine were added. The acyl chloride solution was then added dropwise to this mixture under stirring, and the mixture was then refluxed for 30 min. When the temperature had dropped below 35°C, the precipitated salt was removed by filtration and the polymer crystallized at 3°C. The polymer was recovered by suction filtration and recrystallized twice in the cold from 500 ml of absolute ethanol. Traces of solvent were finally removed by vacuum evaporation at 100°C. The degree of substitution was calculated from the amount of hydroxide consumed in the saponification of the PEG esters, as determined by titration with acid: 0.8–0.9 mol of fatty acid was bound per mol of PEG.

Dextran palmitate

A 10-g amount of dextran ($M_r = 500\,000$) was washed with 250 ml of dry acetone and suspended in 100 ml of chloroform and 3 ml of triethylamine. Then 10 mmol of palmitoyl chloride, prepared as described above, was added, and the mixture was stirred for 5 h at 50°C. The solid dextran palmitate was collected by suction filtration, washed with 250 ml of acetone and 250 ml of ethanol, and dried. The degree of substitution was determined by acid saponification, extraction of the palmitic acid with chloroform, and (after removal of the solvent) applying the residue dissolved in dimethylformamide to a column of Sephadex LH-20 (40 × 1.3 cm I.D.) equilibrated with the same solvent. The column was attached to a refractive index monitor (Multiref 902B; optilab, Vällingby, Sweden). The refractive index peak in the position for palmitic acid was compared with the peaks of known amounts of the same acid. The degree of substitution was 0.4–0.5 mmol of palmitic acid per gram of dextran palmitate.

Dextran deoxycholate

A 10-g amount of solid dextran ($M_r = 500\,000$) was suspended in 30 ml of dry acetone and stirred for 1 h. The solvent was removed by suction filtration, and the dextran was suspended in 30 ml of toluene, and 1.2 g of deoxycholic acid and 3 g of dicyclohexylcarbodiimide were dissolved in the mixture, which was stirred at room temperature overnight. The derivatized dextran was collected by suction filtration and washed sequentially with 100 ml of toluene, 100 ml of absolute ethanol and 250 ml of acetone. The degree of substitution was not determined.

2.8. Two-phase systems

Systems were prepared from 20% (standardized) dextran and 40% PEG solutions as described previously [6,20]. All polymer concentrations are given as percentages (w/w). Ligand–polymers were added in 1–5% solutions, and their concentrations in the systems are given as a percentage of the total amount of the parent polymer. Salt and buffer stock solutions were 10–25 times the final concentration. The systems, with space left for the membrane suspension, were weighed in centrifuge tubes and cooled to 0°C in ice water. The membranes were added, after pelleting and resuspension in distilled water: 2 mg of protein per gram of system in the case of single partition; 5 mg of protein per gram of system for the initial two-phase

system used in counter-current distribution. The systems were mixed by 20 inversions of the vessels, and the phases were separated by centrifugation for 7 min (10 g systems) at 800 *g*. By sampling from the mixed system and from the upper phase, after centrifugation (settling), the distribution of membranes was determined by light scattering or by protein analysis. To determine opiate binding, the phases were collected and diluted six-fold with 50 m*M* Tris–HCl buffer. The membranes were pelleted by centrifugation for 30 min at 45 000 *g*.

2.9. Counter-current distribution

This was carried out manually using ten two-phase systems (8 g each) and nine transfers, as described previously [21]. The first system in the row (number 0) contained initially the membrane sample corresponding to 5 mg of protein per gram of system. After each transfer of upper phases, the systems were mixed and centrifuged as described above. After the distributions, the two phases were eliminated by the addition of 50 m*M* Tris–HCl buffer (50% of the volume of the system). The homogenous liquids were analysed.

3. Results

The partition of the synaptic membranes was complicated by the fact that the membranes in the upper phase had a tendency to sediment during the time necessary for the phases to separate [22]. The problem was partly solved by using a low concentration of membranes and a short, standardized separation time. A more successful way was to re-homogenize the membranes (suspended in water) by passing them twice through a Yeda press using a pressure of 10 MPa. This treatment reduced the light scattering of the suspension by 25% and the preparation showed reproducible partition behaviour and no detectable sedimentation. In the two-phase system used throughout this work, containing 5.2% (w/w) dextran (M_r = 500 000), 5.2% (w/w) PEG (M_r = 3000–3700) and 5 m*M* potassium phosphate (pH 7.4) at 0°C, the membranes were found mainly in the upper phase (85%). By using increasing amounts of KCl in the system, less and less material was partitioned into the upper phase: 64% at 5 m*M* KCl, 24% at 10 m*M* KCl and 10% at 15 m*M* KCl, respectively.

3.1. Counter-current distribution

The homogeneity of the membranes treated in the Yeda press was studied by repeated partitioning of the membranes, *i.e.* counter-current distribution (CCD) according to Craig and Post [23]. The material was initially partitioned in system number 0, and the upper phase from this system was then equilibrated stepwise with pure lower phases numbers 1–9, with the transferred upper phase being constantly replaced with a pure upper phase. The membranes accumulating at the interface were not transferred.

Fig. 1A shows the CCD of the membranes in a two-phase system containing 5 m*M* KCl. The protein and light-scattering measurements showed that some membranes had a very high affinity for the upper phase (the peak to the right) while others stayed at the interface and/or

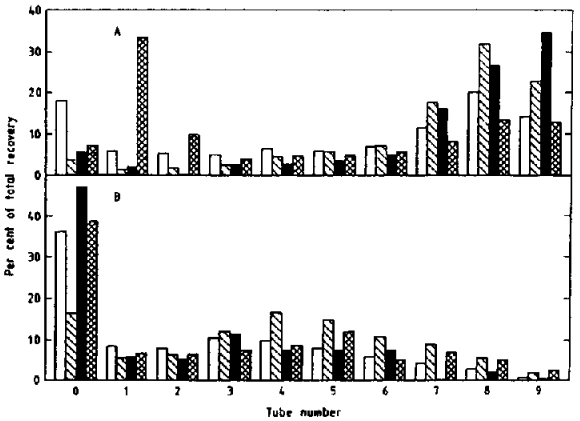

Fig. 1. Counter-current distribution with nine transfers of Yeda-press-treated membranes, corresponding to 5 g of protein per gram of system (25% w/w of system consists of the membrane suspension) in system number 0, in a two-phase system containing 5.2% (w/w) dextran (M_r = 500 000), 5.2% (w/w) PEG (M_r) = 3000–3700), 5 m*M* potassium phosphate buffer (pH 7.4) and 5 or 10 m*M* KCl at 0°C. (□) protein; (▨) acetylcholinesterase; (■) stereospecific opiate binding; (▩) non-specific opiate binding. (A) 5 m*M* KCl; (B) 10 m*M* KCl.

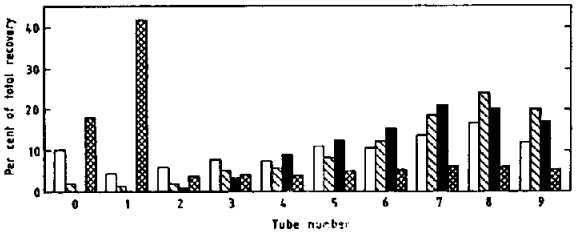

Fig. 2. Counter-current distribution as in Fig. 1, but with 15 m*M* KCl and 1% of the PEG in the form of hexaethonium-PEG.

in the lower phase (the peak to the left). Both acetylcholinesterase and stereospecific opiate binding were found mainly associated with the right-hand protein peak. When the concentration of KCl was increased to 10 m*M* (Fig. 1B), the peaks containing specific opiate binding and esterase activity were shifted to the left of the tube series, and a large portion of both activities remained in tube number one.

In the next CCD experiment (Fig. 2), 15 m*M* KCl was used, which should have caused practically all the membranes to appear to the left of the diagram. This effect was, however, opposed by including PEG-bound hexaethonium in the system. Hexaethonium is known to bind to cholinergic receptors, and it clearly increased the affinity of some membrane fragments for the upper phase. The very broad peaks, to the right, of both protein and opiate binding activity indicated a beginning of fractionation of the synaptic membranes into subpopulations. In all three of these CCDs, the non-specific opiate binding capacity was mainly in the left part of the diagram, especially when hexaethonium-PEG was used.

3.2. Single partition of membranes using polymer-bound ligands

The effects of various ligand–PEGs on the partition of membranes are summarized in Table 1. Except for aminophenyl boronate all the ligands used increased the affinity of the membranes for the upper phase, in which the ligand-PEG were concentrated. Notable were the very strong effect of morphine (azo-coupled) and Etonitazen (amido-linked), as well as the differences observed in the effects of the two stereoisomeric substances levorphanol and dextrophane. A strong effect was also found when quaternized diamines were bound to PEG ("ethonium-PEGs"), as indicated in Table 2.

Table 1
Effect of PEG-bound ligands on the partition of synaptic membranes

PEG-bound ligand	Concentration of ligand PEG (% of total PEG)	Membranes in the upper phase (% of total)
–	0	11
m-Aminophenyl boronate	5	10
Morphine (azo)	5	95
Naloxone (amide)	5	13
Naloxone (azo)	3.7	24
Levorphanol (azo)	3.6	34
Dextrorphan (azo)	3.5	20
Etonitazene (azo)	3.7	21
Etonitazene (amide)	4.2	78
Toluidine blue	5	43

The membranes were pre-extracted and passed through a Yeda press. The two-phase systems contained 5.2% dextran, 5.2% total PEG (including 0–5% ligand-PEG), 5 m*M* potassium phosphate buffer (pH 7.5), 15 m*M* KCl, and membranes corresponding to 2 mg of protein per gram of system (10% of the system consists of the membrane suspension). The temperature was 0°C. The partition was determined as the percentage of membrane (measured as protein) recovered from the upper phase. Maximum deviation, ±3% units.

Table 2
Affinity partitioning of synaptic membranes with ethonium-PEG: Influence of the ethonium chain-length

Chain length of the ethonium ligands (number of Carbon atoms	Per cent material in the upper phase	
	Stereospecific opiate binding	Protein
2	12	9
3	22	11
4	27	10
5	32	24
7	41	20
8	32	30
9	30	24
10	53	43
12	71	43

The membrane preparation used in this experiment was the crude synaptic preparation and it was not subjected to Yeda-press treatment prior to use. The membranes were partitioned in 8-g systems, containing 5.2% (w/w) dextran T 500, 5.2% (w/w) PEG 4000, 5 m*M* potassium phosphate buffer (pH 7.4), 15 m*M* KCl, and membranes corresponding to 2 mg of protein per gram of system (10% of the system consists of the membrane suspension). Also, 2% of the total PEG content was substituted for ligand-PEG. The temperature was 0°C. The total material was measured as the protein content. Maximum deviation, ±3% units.

The degree of extraction depended on the number of carbon atoms between the two nitrogen atoms, and a maximum appeared at 5–7 carbon atoms. At higher numbers (>9) the effect was again stronger.

The effect of aliphatic chains attached to PEG was studied by binding fatty acids of various lengths to the polymer via ester linkages. The PEG esters are located mainly in the upper phase. The partition of the membranes (Table 3) into the upper phase was markedly increased when the fatty acid contained more than 12 carbon atoms.

The binding of ligands to dextran (which is mainly in the lower phase) was also found to affect the partition of the membranes (Table 4). Whereas phenylboronate did not show any effect, deoxycholate and palmitate partly extracted the membranes into the lower phase. The thiazine dye Azure A had no effect on the partition, in contrast to a similar dye, toluidine blue O, which was effective when bound to PEG (Table 1).

Table 3
Affinity partitioning of synaptic membranes with fatty acid-PEG: Influence of the fatty acid chain length

Chain length of the fatty-acid ligands (number of carbon atoms)	Per cent material in the upper phase, measured as the protein content
–	10
4	9
6	11
8	10
10	8
12	11
14	44
16	34

The membrane preparation used in this experiment was the crude synaptic preparation and it was not subjected to Yeda-press treatment prior to use. The membranes were partitioned in 8-g systems, containing 5.2% (w/w) dextran T 500, 5.2% (w/w) PEG 4000, 5 m*M* potassium phosphate buffer (pH 7.4), 15 m*M* KCl, and membranes corresponding to 2 mg of protein per gram of system (10% of the system consists of the membrane suspension). Also, 10% of the total PEG content was substituted for ligand-PEG. The temperature was 0°C. The total material was measured as the protein content. Maximum deviation, ±3% units.

Table 4
Effect of dextran-bound ligands on the partition of synaptic membranes

Dextran-bound ligand	Membranes in upper phase (% of total)
–	64
Deoxycholate	48
Phenyl boronate	64
Azure A	62
Palmitate	40

The two-phase systems contained 5.2% (total) dextran, including 1/20 of ligand-dextran, 5.2% PEG, 5 m*M* potassium phosphate buffer (pH 7.5), 5 m*M* KCl, and membranes corresponding to 2 mg of protein per gram of system (10% of the system consists of the membrane suspension). The temperature was 0°C. The partition was determined as the percentage of membrane (measured as protein) in the upper phase. Maximum deviation, ±3% units.

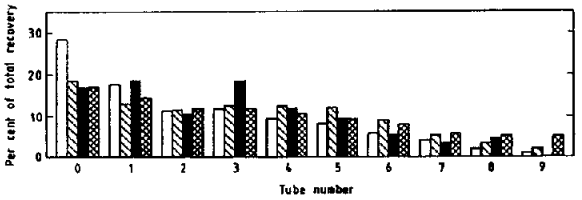

Fig. 3. Counter-current distribution as in Fig. 1, but with 15 m*M* KCl and 5% of total PEG as PEG tridecanoate.

3.3. CCD with ligand-PEG

Fig. 3 shows a CCD with a system containing PEG tridecanoate (C_{13} fatty acid) and 15 m*M* KCl. The material that was moved to the right had a higher light-scattering/protein ratio as well as more esterase activity per protein than the stationary material. The same was true for the non-specific opiate binding. The specific opiate binding/protein ratio on the other hand, showed a maximum around tube 3. In this experiment and in the following CCDs the membranes had, besides being treated with the Yeda press, been extracted into the upper phase of a system containing 5 m*M* KCl. This should, according to the CCD analysis (Fig. 1A), have removed a large part of non-synaptic membranes and material with non-specific opiate binding.

When toluidine blue O was used as PEG-bound ligand (Fig. 4A), the esterase activity showed a higher affinity for the upper phase than the membrane as a whole. The non-specific opiate binding material was moved even more to the right in the diagram. Unfortunately, measurement of the specific opiate binding failed in this experiment. The dye-PEG derivative bound very strongly to the membranes and was not removed, even by intensive washing with buffer indicating a non-reversible binding. This CCD pattern had a close resemblance to the one obtained with a ligand with high affinity for opiate receptors, etonitazene (Fig. 4B). In this case, the specific opiate binding assay also did not work.

The effect of PEG-bound morphine, at high concentration, is shown in Fig. 4C. All membranes were affected in their partition and all the measured parameters had maxima in tubes 4 and

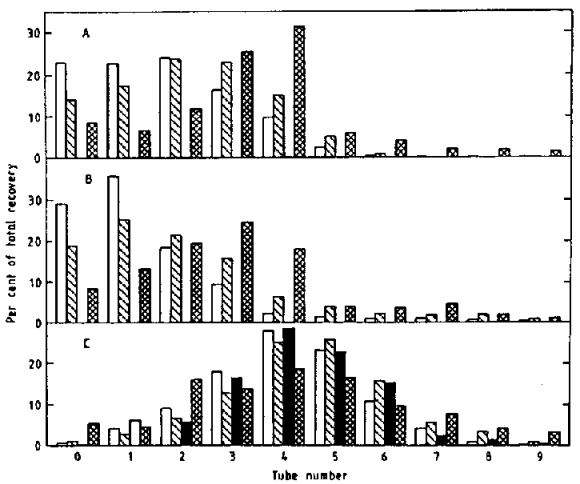

Fig. 4. Counter-current distribution as in Fig. 1, but with 15 m*M* KCl and (A) toluidine blue PEG (4% of total PEG), (B) Etonitazene amide PEG (1.5% of total PEG), or (C) morphine PEG (3% of total PEG). The membranes (initially corresponding to 5 g of protein per gram of system) were pre-extracted in a system with a low concentration of KCl (5 m*M*).

5, showing that the ligand has other (non-specific) binding sites than just the opiate receptors. With morphine, etonitazen and toluidine blue, the extraction of material into the upper phase was changed during the CCD run. In the first partition (in tube 0) very little material was extracted but, after a few transfers of upper phase, larger amounts of membranes were leaving the interface.

4. Discussion

The crude preparation of synaptic membranes must be assumed to contain several kinds of membrane fragment. This heterogeneity was easily demonstrated by a few extraction steps, *e.g.* using the CCD technique (Fig. 1). The material with affinity for the interface contained only a small part of the specific opiate binding and esterase activity, and could be assumed to consist of mitochondrial membranes. This material had a yellow colour, whereas the material moving with the upper phase (at a low concentration of KCl) was white. The marked shift

between the specific opiate binding and esterase curves showed that the membrane fragments with affinity for the upper phase were partitioned via different mechanisms, but might be separated with this technique if a larger number of transfers was used. When the most distal systems after CCD were compared, *i.e.* systems 0 and 9, the degree of separation was striking. These systems might be seen as phases from a single partition that had been washed nine times with the opposite phase: For example, in Fig. 1A the esterase activity per protein unit was eight times as high in tube 9 as in tube 0.

By using a similar system containing sorbitol, López-Pérez *et al.* [24] were able to isolate mitochondria from synaptosomes. The use of hexaethonium-PEG should make it possible to extract preferentially membranes with cholinergic receptors into the upper phase. It was also found that the esterase activity (which is a general plasma membrane with certain preference for cholinergic synaptic membranes) was extracted, and to a somewhat higher degree than the specific opiate receptor (Fig. 2). The ratio esterase/stereospecific opiate binding increases from tube 4 to tube 9 by a factor of 2. Here also, a CCD with larger number of transfers would be necessary to show whether these two activities could be totally separated or if some membrane fragments contain both properties.

When hydrophobic groups bound to PEG are used, the material with the higher lipid content may be assumed to be extracted into the upper phase. A fatty acid containing 13 carbon atoms was used (bound to PEG) because the hydrophobic part is just long enough to interact with the membranes without dipping too deep into the lipid double layer. After CCD (Fig. 2) the light-scattering/protein ratio increased with the tube number. This indicates that the more lipid-rich membranes had been extracted into the upper phase. The material responsible for non-specific opiate binding also increased in the same way, showing that it has a rather hydrophobic character. The CCD also indicates that the opiate receptors are located in less lipid-rich membranes than the acetylcholine esterase.

The thiazine dyes are known to bind to cerebroside sulphate, which in turn might be associated with the opiate receptor [25]. The CCD (Fig. 4A) with such dye bound to PEG should extract specific, as well as non-specific, opiate-binding material together with other material containing cerebroside sulphate. This is also found for the non-specific opiate binding. The negative results of measurements of specific binding might be due to a strong binding of dye close to these sites on the membranes.

An attempt to extract the membranes according to their contents of various sugar residues, using phenyl boronate bound to either PEG or dextran, failed completely. This is probably due to the fact that one of the phase-forming polymers (dextran) was a polysaccharide. To use this approach, two-phase systems composed of non-sugar polymers have to be tried.

In the case of the specific ligands morphine and Etonitazen, as well as toluidine blue, the membranes started to be extracted only when they had been in contact with two to four fresh upper phases, indicating that the membranes extracted the ligand-PEG from the upper phase, and they did not leave the interface until the densities of bound PEG exceed a certain value. This was clearly visible in the case of dye-PEG. Despite intensive washing of the membranes after CCD, the specific opiate binding could not be determined in the case of etonitazen-PEG. Whether this was due to very strong binding of the ligand or to other technical problems is under investigation. Morphine showed a surprisingly non-specific extraction (Fig. 4C) and this can be explained by both the rather hydrophobic character of morphine itself and the fact that it is linked to PEG via a phenylazo group, which will heighten this hydrophobicity. So far, the most promising ligand for selective extraction seems to be etonitazen (Fig. 4B).

Since the ligands are directed in their effects towards different structural elements of the membranes, such as charge lipid layer, cerebroside sulphate, opiate and cholinergic receptors, the extraction can be carried out along several affinity dimensions. The effectiveness of the extraction depends both on the relative amount of the actual membrane element and on

the binding strength, and can be described by a numerical value along this affinity dimension. All these dimensions form a multidimensional space in which each membrane fragment has its unique position. Systems with various ligands in combination with CCD with a large number of transfers could be used to determine the distribution of membrane fragments according to a chosen set of membrane properties.

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