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Subfractions of membranes from calf brain synaptosomes obtained and studied by liquid–liquid partitioning

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ABSTRACT

Synaptosomes isolated from calf brain cortex were lysed and fragmented by Yeda press treatment. The obtained membranes have previously been fractionated in a counter-current distribution process using a liquid–liquid two-phase system consisting of water, dextran, Ficoll and poly(ethylene glycol) [*J. Chromatogr.*, 358 (1986) 147]. Using the fact that there are discrete membrane populations, a rapid preparative method for isolation of the two main fractions is presented in the present work, as well as a subfractionation of one of them using liquid–liquid extraction with dextran-bound Procion yellow HE-3G. The content of several membrane constituents, *i.e.* protein, acetylcholinesterase, succinate dehydrogenase and ATPase, as well as opiate binding, were determined for the three fractions. Counter-current distribution of the fractions elucidates their heterogeneity and the effectiveness of the purification.

INTRODUCTION

Biological membranes were fractionated by taking advantage of differences in their partition within (liquid–liquid) aqueous two-phase systems [1]. This was done either by using between one and five extraction steps or by applying a counter-current distribution technique using systems composed of water and the two polymers dextran and poly(ethylene glycol). These techniques have mainly been used for the separation and study of thylakoid membranes [2,3], but membranes of animal origin have also been fractionated in this way. These partitioning studies also include synaptic membranes from *Torpedo californica* [4,5] and *Torpedo marmorata* [6] as well as membranes isolated from brain tissue [7–9]. It has, in a few cases, been shown that membranes with cholinergic [4] or opiate [9] receptors can be effectively extracted into one of the phases by including a suitable receptor ligand, in this phase anchored to the dominating polymer.

While the applications of two-phase partitioning for separation of plant membranes have been without technical problems, work with brain membranes has involved complications [8]. It has been shown that synaptic membranes from calf brain

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change their partition behaviour with time, probably as a result of aggregation or rearrangement of the structural elements of the membranes. This can to some extent be avoided by using systems containing polymers of lower molecular weights and/or addition of stabilizing proteins. In the present work a two-phase system composed of water, dextran, Ficoll and poly(ethylene glycol) was used, which has previously been used for counter-current separation of brain membrane preparations [10]. This system is here shown to separate synaptosome membranes into two classes (without use of affinity ligands). One of these was further fractionated by using a moderate concentration of a dextran-bound dye, Procion yellow HE-3G. The membrane fractions (three in total) were isolated by a simple series of batch extractions.

EXPERIMENTAL

Chemicals

Dextran T-40 (mol. wt. 40 000), dextran 500 (mol. wt . 500 000) and Ficoll 70 (mol. wt. 70 000) were purchased from Pharmacia (Uppsala, Sweden). Poly(ethylene glycol) (PEG, mol. wt. 7000–9000) was obtained from BP Chemicals (Hythe, UK) as Breox 8000. Procion yellow HE-3G was a gift from I.C.I. (Gothenburg, Sweden). Materials for the biochemical assays were obtained from Sigma (St. Louis, MO, USA). All salts and buffer substances were of analytical grade.

Dextran-bound Procion yellow

Procion yellow HE-3G was bound to dextran 500 in basic aqueous solution. This method has been described elsewhere [11–13]. The Procion yellow–dextran (PrY–dextran) contained 7.2 μmol dye per gram of polymer.

Membrane preparation

The preparation of synaptosomes from calf brain cortex was performed by a slightly modified version of the method described by López-Pérez *et al.* [14]. Unless otherwise mentioned, all centrifugation steps were performed in a GSA rotor with a Sorvall RC-2B refrigerated high-speed centrifuge at 3°C. A pair of scissors was used to mince 100 g of cerebral cortex. The minced tissue was suspended in 500 ml of 1 m*M* Tris–HCl buffer, pH 7.4, containing 0.32 *M* sucrose, and homogenized by twenty strokes in a glass Potter–Elvehjem homogenizer with a loose-fitting PTFE pestle at 100 rpm. The homogenate was centrifuged at 1000 g_{max} for 10 min. The pellet was discarded and the supernatant was centrifuged at 20 000 g_{max} for 20 min. The resulting pellet was resuspended in 30 ml of 5 m*M* potassium phosphate buffer, pH 7.8, containing 0.32 *M* sorbitol, and was layered over 200 ml of 6% Ficoll 70, in the same medium as above, in Sorvall GSA centrifuge flasks which were centrifuged at 20 000 g_{max} for 30 min. The white upper part of the pellet was recovered, avoiding the brown mitochondrial fraction, and resuspended in 200 ml of 0.32 *M* sucrose followed by centrifugation at 20 000 g_{max} for 30 min. The resulting pellet was resuspended in 175 ml ice-cold water. The membrane suspension was stored at −30°C. Before use the suspension was thawed (30 min), homogenized with a Potter–Elvehjem homogenizer and passed twice through a Yeda press under 100 atm nitrogen pressure.

Assays

Light scattering was measured at 400 or 500 nm (when dye was present) as the apparent absorbance using a Hitachi 100-60 spectrophotometer. After treating the membranes for 1 h at 50°C in 0.5 *M* phosphoric acid, protein concentrations were determined according to Bradford [15]. Acetylcholinesterase was determined by the method of Ellman *et al.* [16], and succinate dehydrogenase as described by Earl and Korner [17]. ATPase was determined according to Scharschmidt *et al.* [18], and stereospecific opiate binding was determined as described by Medzihradsky [19]. Total phosphate was determined according to Ames and Dubin [20] and 2′,3′-cyclic nucleotide-3′-phosphohydrolase according to Sims and Carnegie [21].

Two-phase systems

The basic composition of the two-phase systems used was 10.3% (w/w) Ficoll 70, 9.3% (w/w) dextran T-40, 2.3% (w/w) PEG and 5 m*M* Tris–orthophosphoric acid buffer, pH 7.8 (concentration based on phosphate). The volume ratio (top/bottom) of the phases was 0.54. All partitionings were carried out at 3°C.

Counter-current distribution (CCD)

A special type of CCD apparatus invented by Åkerlund [22] was used. It allowed rapid separation of the two phases by one centrifugation step. The apparatus contained 60 chambers arranged in a circle which allowed the upper phases to be transferred stepwise to the neighbouring lower phases. The volume of the stationary part of each chamber was 0.96 ml. The membrane samples were included in the systems in chambers number 0 and 1 of the CCD machine, 1.3 ml system per chamber. The same amounts of mixed two-phase system (without membranes) were loaded in chambers 2–59. In some experiments PrY–dextran, used at concentrations of 0.028 or 0.111%, was included in the systems. Mixing time was 4 min, centrifugation time was 12 min, and 58 transfers were performed. After the run, the systems were transformed from two phases to one phase by the addition of 1.3 ml of ice-cold water per chamber. The fractions, after ten- to fifty-fold dilution, were analyzed for light scattering measured as the apparent absorbance at 400 nm or (if Procion yellow was present) 500 nm. The contents of chosen sets of chambers were pooled and concentrated by centrifugation for 120 min at 45 000 g_{max} (after two-fold dilution) and resuspended in 1.2–1.5 ml of distilled water. The fractions were analyzed for cholinergic binding, stereospecific opiate binding, acetylcholinesterase, succinate dehydrogenase and ATPase.

Electrophoresis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli [23] was applied with a gradient of 12–20% polyacrylamide. Membrane samples containing 40–60 mg protein were used. The gels were stained with Coomassie brilliant blue and photometrically scanned with an LKB 2202 Ultrascan laser densitometer.

RESULTS

Preparative extraction

The membranes were separated by partition within a two-phase system (Fig. 1) into two main fractions: class A (with affinity for the upper phase) and class B (with affinity for the interface and partly for the lower phase). To remove traces of contaminating membranes of the other class, the upper phase and lower phase (with interfacial material) were 'washed' twice with pure opposite phase.

The extraction processes were carried out in 50-ml centrifuge tubes with screw caps using systems of 20 g. Concentrated polymer solutions (40% PEG, 40% Ficoll and 32% dextran) were weighed out together with buffer and water to either 20 g (system to give phases for washing) or to 13.5 g (for the first partition step), leaving 6.5 g for the membrane suspension. After equilibration to 3°C the 'washing' systems were mixed and allowed to settle, while Yeda press-treated membranes (corresponding to 29 mg protein) in 6.5 g of suspension were added to the concentrated (13.5 g) system. This was carefully and gently equilibrated by turning the closed tubes upside down twenty times. The system was centrifuged for 15 min at 3000 g_{max} and 3°C. The upper phase was recovered, avoiding material from the interface (by leaving 1 mm height of the upper phase in the tube), and transferred to another tube to which was also added fresh lower phase to the same total volume as the original system. The membrane-containing lower phase (plus interfacial material) was likewise combined

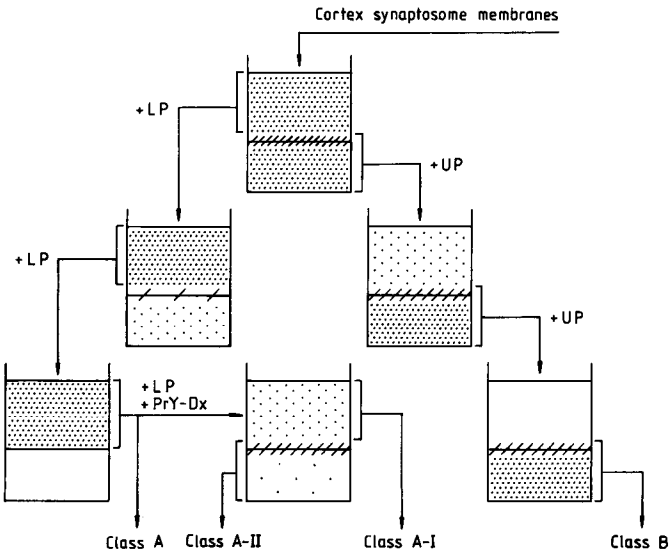

Fig. 1. Flow scheme for the preparative isolation procedure of synaptosomal membrane fractions. UP = pure upper phase; LP = pure lower phase; and PrY–Dx = Procion yellow HE-3G–dextran 500. The two-phase system (20 g) used contained 9.3% (w/w) dextran T-40, 10.3% (w/w) Ficoll 70, 2.3% poly(ethylene glycol) and 5 m*M* Tris–orthophosphoric acid buffer, pH 7.8 (concentration based on phosphate). The concentration of PrY–dextran in the lower phase was 0.056% (4 μM dye), corresponding to 0.028% (2 μM dye) in the complete system. Membrane concentration in the first system corresponded to 1.5 mg protein per ml. Temperature: 3°C.

with pure upper phase. The two tubes were mixed and centrifuged as above. These washings of the phases were repeated once more. The membranes in the upper phase (fraction A) and the lower phase together with the interface (fraction B) of the other system (see Fig. 1) were recovered and each diluted three times with ice-cold water. The membranes were precipitated by centrifugation (45 000 g_{max} for 2 h) and resuspended in a few milliliters of 5 m*M* Tris–orthophosphoric acid, pH 7.0, for analysis. Alternatively (see below) the upper phase was directly used in a further fractionation with a dextran-bound ligand. The membranes showed good purity when analyzed by counter-current distribution (Fig. 2). The fractions A and B (Fig. 1) differed markedly in contents of enzymes and opiate receptor binding (Table I).

The class A membranes were further fractionated by partitioning in systems containing PrY–dextran. Increasing concentration of the dye, greatly restricted to the bottom phase, lowered successively the percentage of membranes in the upper phase. A preparative separation was done (Fig. 1) using the same protocol as above but including dextran-bound dye (2 μM) at a concentration such that 40% of the class A membranes remained in the upper phase. The two obtained subpopulations, class A-I (in upper phase) and class A-II (at interface plus lower phase), showed different behaviour when analyzed by CCD using a two-phase system containing 2 μM dex-

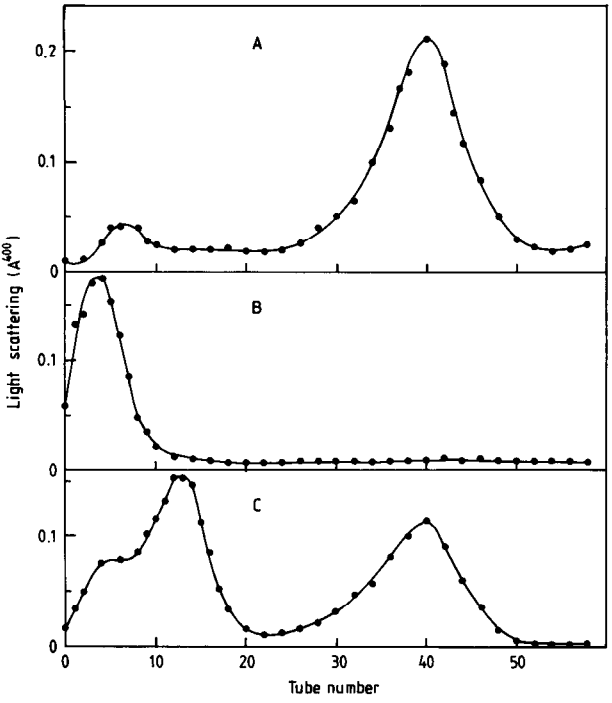

Fig. 2. Counter-current distribution of class A membranes (A), of class B membranes (B), and of original synaptosomal membranes after Yeda press treatment (C), using 58 transfers. The membranes, corresponding to 2–2.5 mg protein, were originally applied in chambers 0 and 1. System as in Fig. 1. For technical details see text. Membrane concentration was measured after dilution as apparent absorbance at 400 nm caused by light scattering. Temperature: 3°C.

TABLE I

ANALYTICAL DATA OF THE ORIGINAL MEMBRANE PREPARATION AND THE FOUR MEMBRANE FRACTIONS A, B, A-I AND A-II SHOWN IN FIG. 1

Specific activities are given relative to protein measured according to Bradford [15].

Fraction	Protein (mg)	Acetyl cholinesterase ($x10^3$) (U/mg)	Succinate dehydrogenase ($x10^3$) (U/mg)	ATPase ($x10^3$) (U/mg)	Specific opiate binding ($x10^5$) (nmol/mg)
Original	29	66	21	225	2.1
A	9.5	81	1.2	271	3.4
B	4.9	3.2	76	312	1.4
A-I	2.8	91	–	397	4.2
A-II	2.8	34	3.7	457	1.7

tran-bound dye (Fig. 3). Analytical data of the subclasses are shown in Table I. The distribution of the various activities within the CCD train in the presence of different concentrations of PrY–dextran (0, 2 and 8 μM dye) (Fig. 4) shows that a moderate concentration of dye in the lower phase gave rise to a further fractionation. With only 2 μM PrY–dextran all the (membrane-bound) succinate dehydrogenase was extracted from the upper phase (recovered in the left peak, Fig. 4B). This shows that membranes of mitochondrial origin are effectively extracted. With 8 μM dye all membranes appear in a single peak to the left in the CCD diagram (Fig. 4C).

Polypeptide pattern

SDS-PAGE of the membrane fractions showed marked differences in the polypeptide pattern (Fig. 5). Some polypeptides dominate in either class A (Fig. 5B) or

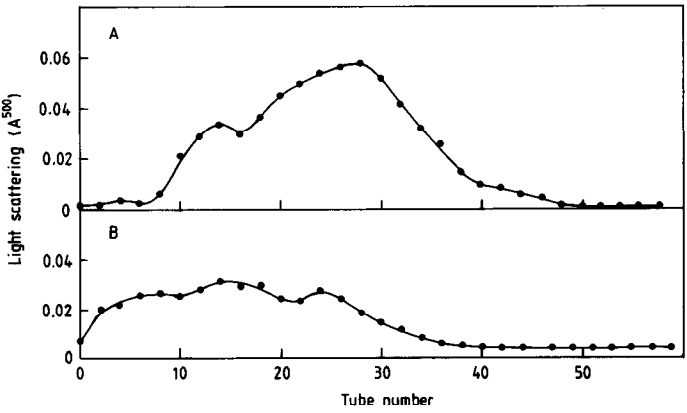

Fig. 3. Counter-current distribution of class A-I membranes (A) and class A-II membranes (B). The membranes (corresponding to 2–2.5 mg protein) were originally included in systems of chambers 0 and 1. The two-phase system contained 2 μM dextran-bound Procion yellow HE-3G. The system composition was otherwise as in Fig. 1. Membrane concentration was measured after dilution as apparent absorbance at 500 nm. Temperature: 3°C.

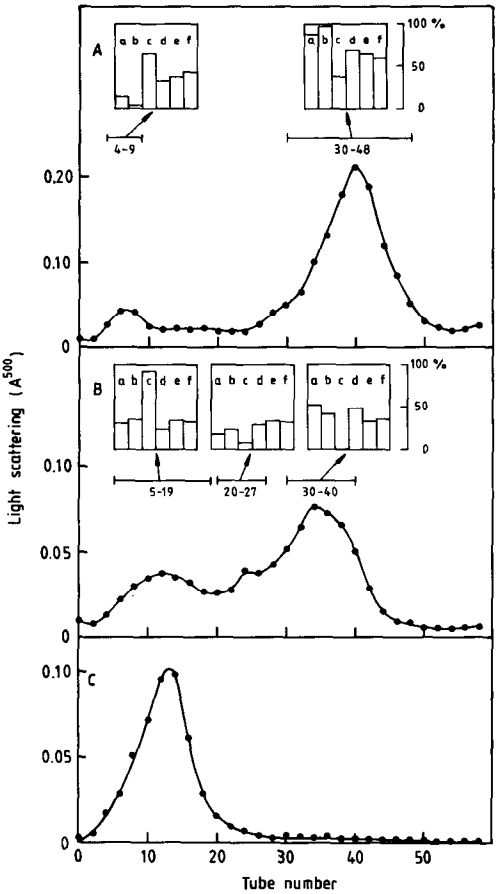

Fig. 4. Effect of dextran-bound Procion yellow HE-3G on the partitioning of class A membranes demonstrated by counter-current distribution. The composition of the two-phase system was the same as in Fig. 1. The system was supplemented by (A) nothing, (B) 0.028% (w/w) PrY–dextran (2 μM dextran-bound dye) and (C) 0.111% (w/w) PrY–dextran (8 μM dextran-bound dye). In (A) and (B) the pooled fractions, indicated by horizontal bars and fraction numbers, were concentrated and washed by centrifugation and analyzed for (a) protein, (b) acetylcholinesterase, (c) succinate dehydrogenase (mitochondrial marker), (d) ATPase, (e) phosphate, and (f) 2',3'-cyclic nucleotide-3'-phosphohydrolase (myelin marker). The percentage distributions of a–f are shown inset. Temperature: 3°C.

class B (Fig. 5C). Within the subclasses A-I (Fig. 5D) and A-II (Fig. 5E), smaller but significant differences can be seen.

DISCUSSION

The membranes obtained by fragmentation of synaptosomes are of several kinds. Some parts may be non-synaptosomal contaminations, *e.g.* various plasma membranes and membranes originating from extrasynaptosomal mitochondria. The pronounced differences in the membrane fragments in the partition within the two-phase system have been demonstrated by CCD (Fig. 2), with two main fractions.

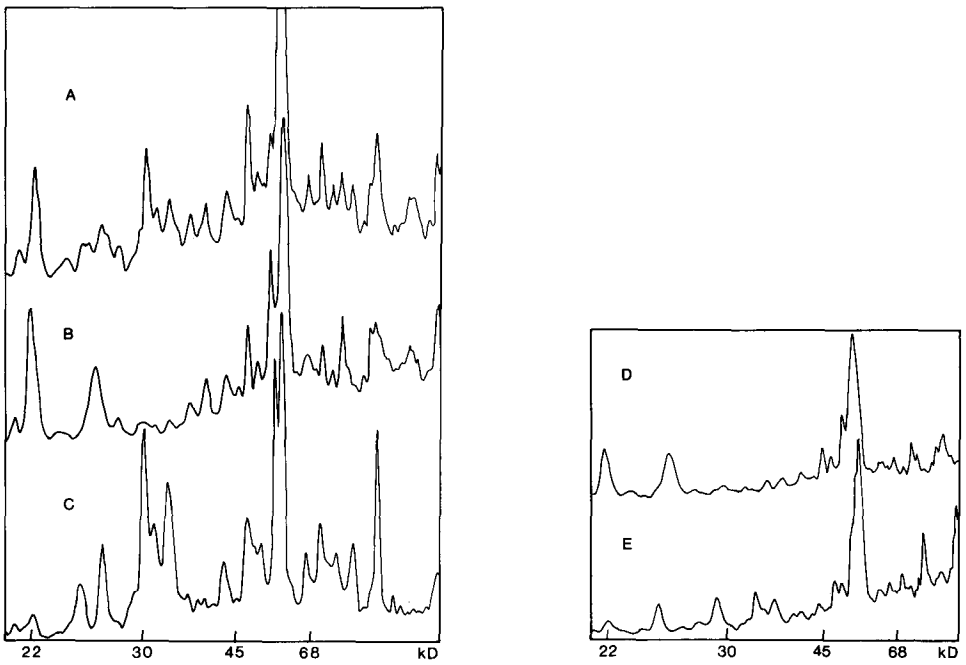

Fig. 5. Scannings of the gels after SDS–PAGE and staining with Coomassie brilliant blue. (A) Original membranes after Yeda press treatment. (B) Class A membranes. (C) Class B membranes. (D) Class A-I membranes. (E) Class A-II membranes. The positions of standard proteins are indicated by their molecular weights in kilodalton (kD).

By using the two-phase system for preparative purposes, larger amounts of purified synaptic membranes (class A), can rapidly be obtained within 30 min. The effectiveness of the extraction (Table I) is shown by the low content of mitochondrial membranes (as measured by succinate dehydrogenase activity) in fraction A (reduced by a factor of 18). On the other hand, acetylcholin esterase and stereospecific opiate binding in this fraction were increased 1.2–1.6 times. Class A membranes contained less myelin, as measured by the activity of 2′,3′-cyclic nucleotide-3′-phosphohydrolase, than the starting material.

The synaptic membranes (class A) are in no way homogeneous, but they contain a variety of membrane fragments differing in composition and properties such as types and concentration of a number of receptors. The synaptosomes from which the membranes are prepared are assumed to consist mainly of presynaptic membranes, but pieces of attached post-synaptic membranes may also occur [14]. By introducing a dextran-bound dye with affinity for membranes, Procion yellow HE-3G, into the lower phase, the class A membranes can be further divided in two subfractions. The nature of the binding sites is unclear, but probably the dye molecule resembles one or more natural ligands, *e.g.* nucleotides. The fact that Procion yellow HE-3G even at very low concentrations (a few micromoles) extracts considerable amounts of membranes points to a strong and probably specific interaction (Fig. 3). The finding that nearly all class A membranes are extracted at high concentration of the dye ligand

indicates that the binding sites are present on most membrane fragments but differ in density or binding strength.

The two fractions class A-I and class A-II are not distinct populations, but the clear differences in polypeptide pattern (Fig. 5D and E) and enzyme activities as well as relative affinity for opiate binding (Table I) show that a simple and quick extraction with a phase containing dye–ligand can also give valuable enrichment of certain types of membranes.

The use of dextran-bound ligands, here illustrated by Procion yellow HE-3G, would allow membranes to be selectively extracted. By using other kinds of ligands (bound to dextran) with specific affinity for various receptors or specific proteins, the membranes enriched in this component could probably selectively be extracted into the lower phase. Affinity partitioning of this kind offers a wide range of possibilities in both choice of ligands and their concentrations. Also, the combination of two ligands in opposite phases and the use of multiple extraction techniques, *e.g.* CCD, would strongly enhance the fractionation power offered by the partition within aqueous two-phase systems. The batch extraction can easily be scaled up to allow the isolation of quantities large enough for detailed characterization of the membranes.

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