Journal of Chromatography, 311 (1984) 277—289
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2265

LIQUID—LIQUID EXTRACTION OF MEMBRANES FROM CALF BRAIN USING CONVENTIONAL AND CENTRIFUGAL COUNTER-CURRENT DISTRIBUTION TECHNIQUES

GÖTE JOHANSSON*, HANS-ERIK ÅKERLUND and BJÖRN OLDE

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

(First received April 17th, 1984; revised manuscript received June 26th, 1984)

SUMMARY

Neural membranes isolated from calf brain have been partitioned in aqueous two-phase systems containing dextran and polyethyleneglycol. When the partition was repeated several times, using counter-current distribution technique, the distribution of the membranes between the upper phase and the interface changed in a non-ideal manner and in favour of the interface. By using a centrifugal counter-current distribution device the time for the experiment could be reduced by a factor of 7—8 and the distribution was similar to what could be expected for ideally behaving membranes. The time-dependent change of the membranes is discussed in terms of aggregation and lateral membrane perturbations. Despite this effect a certain fractionation has been achieved as deduced from analysis of cholesterol content, opiate receptor activity and acetylcholinesterase activity along the counter-current distribution row of fractions. Compared to the starting material these activities were enriched some two-fold in certain fractions.

INTRODUCTION

In order to understand the structure and function of a particular membrane it is important to isolate that individual membrane in a pure and native state. This is particularly important for membranes of the highly organized brain tissue. Homogenization of such tissue inevitably gives an extremely complex mixture of membranes. It is therefore desirable that several fractionation methods, working according to different separation principles, are used in sequence. The most common method used so far is some type of centrifugation. Another method of great potential value is partition of the material between the aqueous liquid phases (and interface between them) of aqueous dextran—polyethyleneglycol two-phase systems. This method complements

0378-4347/84/$03.00

centrifugation in that it separates material according to differences in surface properties rather than size and density. The properties and preparation of aqueous two-phase systems have been summarized elsewhere [1—3].

Of the great variety of membranes studied in this way the thylakoid membranes in chloroplasts constitute a considerable part [2, 3], but cholinergic membranes from electroplax of *Torpedo californica* [4—6] and *Torpedo marmorata* [7] have also been purified and examined. Typical for the partition of membranes is that they not only distribute between the two phases but they can also accumulate at the interface between the phases [1, 2]. The fractionation of heterogeneous material can be drastically influenced by addition of various salts [2, 8], charged polymers [7, 9, 10], or polymer-bound groups which bind to the membranes [4, 6]. Especially promising is the technique where specific high-affinity ligands have been bound to polyethyleneglycol [4—6, 11, 12]. To improve the purification obtained in a single partition step, counter-current distribution (CCD) is often used. This is carried out by successively shifting the position of the upper phases of a row of two-phase systems, where the sample has been introduced in the first system of the row. The phase volumes are chosen to maintain the interface, and the material concentrated at it, stationary.

Using the CCD technique it should be possible to resolve membrane preparations that differ in their chemical composition and biochemical activities into various fractions. Even if systems with a rather high degree of specificity are designed, multi-step partition may be necessary to resolve membranes with small variations in composition, e.g. two kinds of receptors bound to otherwise identical membrane fragments. In such cases CCD would offer two interesting features: the quantitative composition of a membrane mixture can be established and at the same time several fractions of increased purity may be isolated. A possible drawback is, however, that the membrane structure may be influenced by the two-phase system, causing, for example, loss of biological activity, formation of large membrane aggregates, or gradual change of the partition between the phases. The fluidity of membranes and the well known fact that polyethyleneglycol causes capping of membrane proteins and induces fusion of cells make it necessary to investigate the stability of membrane structures in aqueous two-phase systems.

In the present work the heterogeneity of a crude preparation of synaptic membranes from brain has been studied by using the CCD technique. The influence of the two-phase systems on the status of the membranes has been analysed by using CCD with different numbers of transfers and by comparing two types of CCD apparatus: the conventional thin-layer CCD device, constructed by Albertsson [13], and a new construction based on centrifugation which allows the CCD process to be run in a considerably shorter time [14]. Short (nine steps) CCD has also been performed by manual transfer of the upper phases along a row of test-tubes.

MATERIALS AND METHODS

Materials

Polyethyleneglycol (PEG), M_r = 3500—4500, was purchased from Union

Carbide (New York, NY, U.S.A.). Dextran T500 (M_r = 500,000) was from Pharmacia (Uppsala, Sweden). Hexaethonium—polyethyleneglycol (HE—PEG) and bis(triethylaminoethyl)resorcinol-NH-azelate polyethyleneglycol (BTR—PEG) were prepared as described by Johansson et al. [6]. The biochemicals and substrates used for enzyme analysis were from Sigma (St. Louis, MO, U.S.A.), except for the set for cholesterol determination, which was obtained from Boehringer (Mannheim, F.R.G.). Tritiated etorphine was purchased from Amersham Radiochemical Centre. Dextrophan and levorphanol were kind gifts from Dr. M. Kanje, Department of Zoophysiology, University of Lund (Lund, Sweden). All salt and buffer substances were of analytical grade.

Membrane preparation

Synaptic membranes from calf brain cortex were prepared according to the method of Hajós [15] with slight modifications. The synaptosomes were lysed in ice-cold distilled water for 1 h and collected by centrifugation at 45,000 g_{max} for 1 h. The resulting pellet was suspended in water and passed twice through a Yeda press before it was used for partition or CCD.

Two-phase systems

The two-phase systems were made from 40% (w/w) PEG and 20% (w/w) dextran stock solutions. The concentration of polymers in the systems were 5.3% (w/w) dextran and 5.3% (w/w) PEG. All systems contained 5 m*M* potassium phosphate buffer, pH 7.4, and various concentrations of potassium chloride. Detailed descriptions of the preparation of aqueous two-phase systems can be found elsewhere [1, 16].

Counter-current distribution

The two types of CCD apparatus used were the thin-layer machine constructed by Albertsson [1, 13] and the centrifugal machine invented by Åkerlund [14]. Both were built by the Chemical Center Workshop at the University of Lund. The volumes of the two phases in each chamber, settling (or centrifugation) time and shaking time are given in the figure legends. Each set of discs contained 60 chambers (see Fig. 1). The samples were included in the systems of chamber 0-2 (26 transfers) or 0-3 (55 transfers). After the run the systems were transformed to one phase by addition of 0.9 ml of ice-cold water. The obtained fractions (diluted 30—50 times) were analysed with respect to apparent absorbance at 400 nm, caused by light scattering, using a Hitachi 100-60 spectrophotometer with 1-cm cuvettes. Pooled fractions were concentrated by centrifugation 60 min at 45,000 *g* and resuspended in a small volume of 50 m*M* Tris—HCl buffer, pH 6.4 (1—2 ml). These fractions were analysed for acetylcholinesterase [17], cholesterol [18], phosphate [19], and stereospecific opiate binding [20]. Protein was determined according to the method of Bradford [21] after treatment of the membranes for 3 h with 0.5 *M* phosphoric acid at 50°C.

Manual CCD was performed by using 8-g systems in test-tubes as described elsewhere [16]. After each transfer (performed with a Pasteur pipette), leaving all material at the interface with the lower phase, the tubes were mixed by

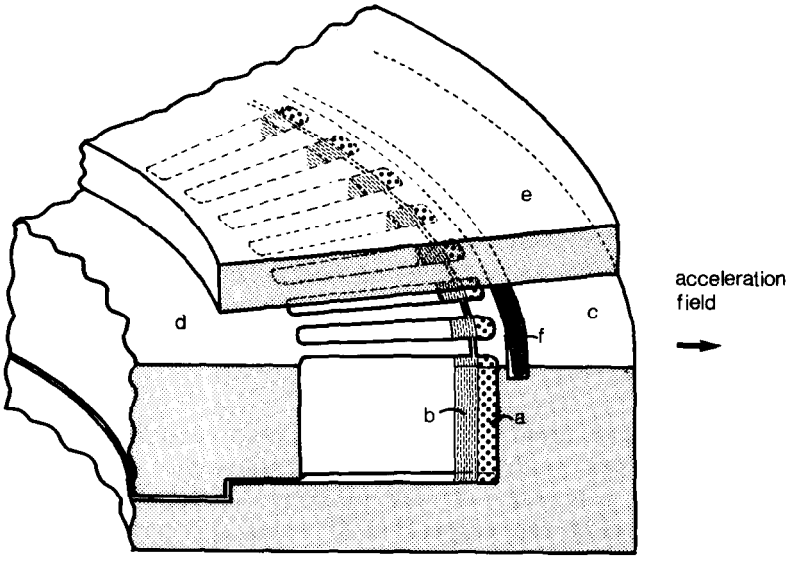


Fig. 1. Section of the separation unit for the centrifugal CCD apparatus. It is composed of four units: c = the outer ring with cavities for the lower phase; d = the inner ring with cavities for the upper phase; e = the lid ring; f = an O-ring for sealing. The position of the two-phase system during centrifugation (b = upper phase; a = lower phase) is shown in the sectioned chamber.

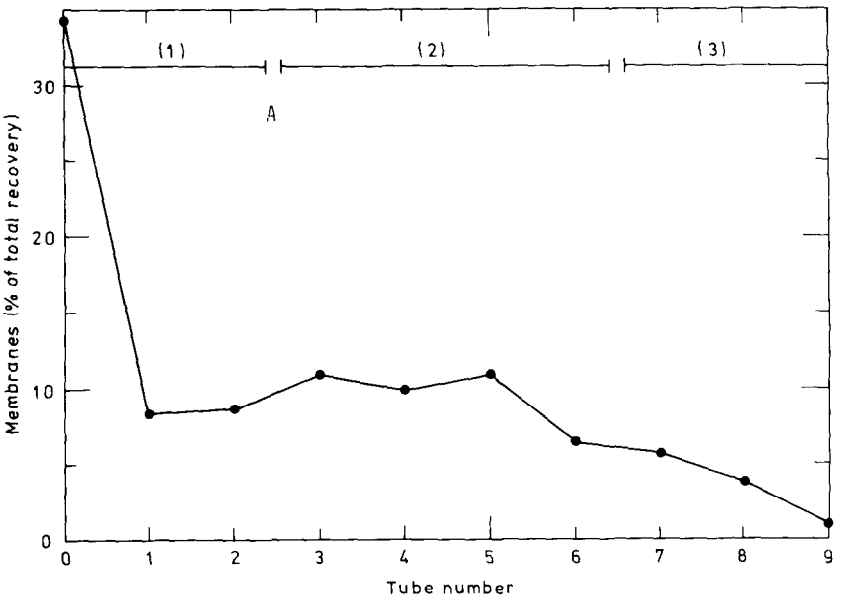
twenty inversions and centrifuged for 5 min at 800 *g*. After nine transfers 4 ml of water were added to break the two-phase systems.

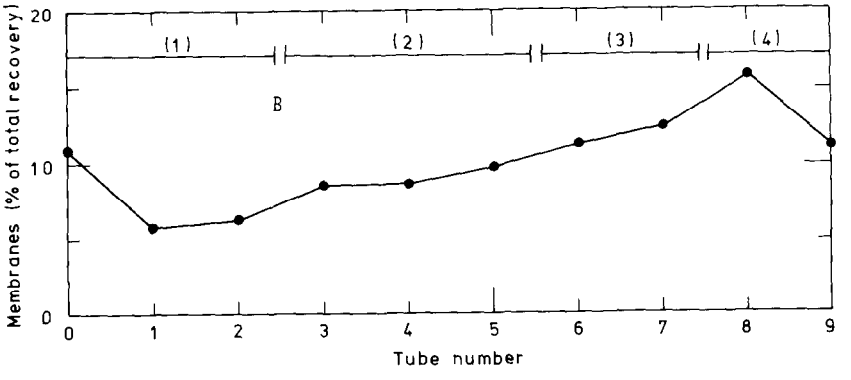
RESULTS

Conventional CCD

Manual CCD with nine transfers (Fig. 2A and B) showed that synaptic membranes partly moved along with the upper phase when the system did not contain any ligand—PEG but only the standard salts, 10 m*M* potassium chloride and 5 m*M* potassium phosphate buffer, pH 7.4. In this experiment (Fig. 2A) the membranes were not pre-extracted and a large fraction remained at the start position, tube No. 0. The membranes in this tube had a marked yellow tint in contrast to the moving material. When HE—PEG was included in the system (Fig. 2B), the material was spread out and more membranes were found in the tubes to the far right. Hexaethonium is a ligand for nicotinic cholinergic receptors [6].

The distribution of membranes in a conventional CCD apparatus is shown in Fig. 2C—E. With 55 transfers and no ligand—PEG the material was found in the left part of the CCD train (Fig. 2C) with a clear tendency to split into two peaks. A corresponding CCD with a HE—PEG-containing system (Fig. 2D) gave rise to a heterogeneous peak which had travelled more to the right. By using HE—PEG but reducing the number of transfers to 26 the peak had travelled relatively more to the right (R_f = 0.57) than with 55 transfers (R_f = 0.41). R_f is defined as the peak position divided by the number of transfers. This relative mobility should be independent of the number of equilibration steps if an ideally behaving substance is partitioned [22].





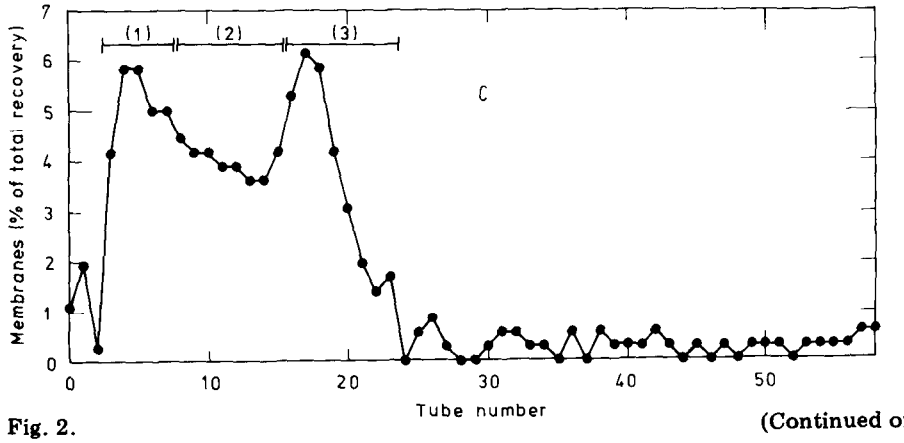


Fig. 2.

(Continued on p. 282)

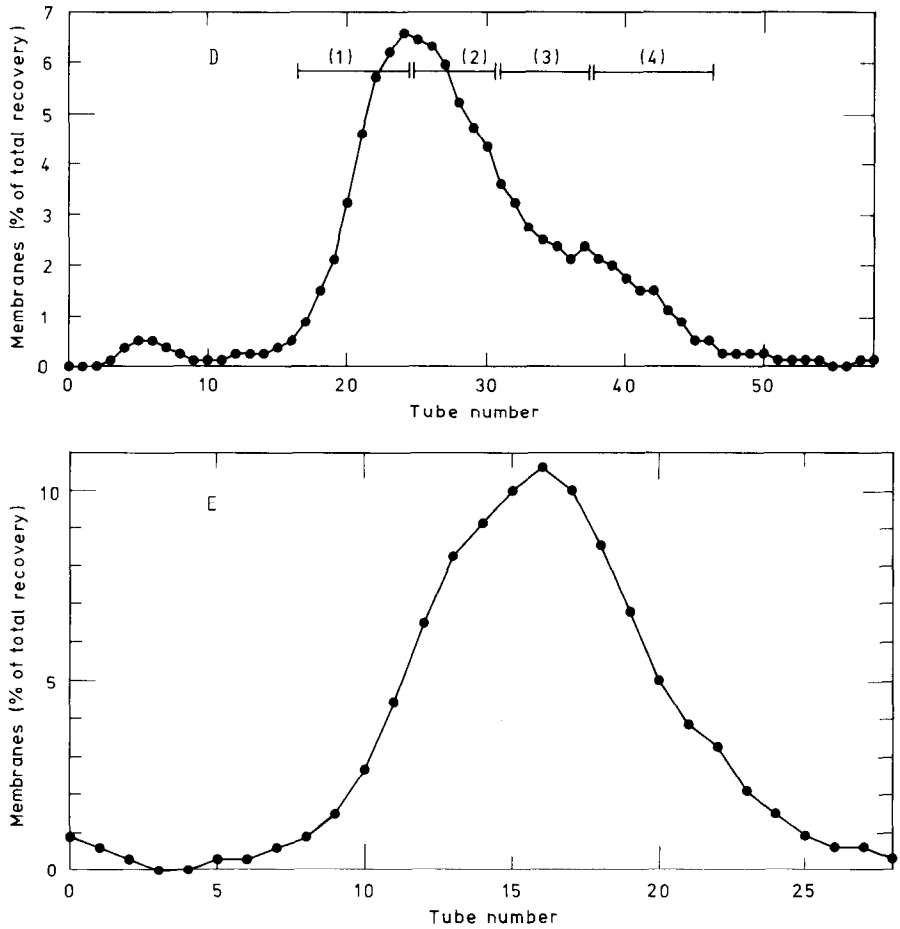


Fig. 2. Conventional CCD, carried out either manually, A and B, or with use of a thin-layer CCD apparatus, C—E. The two-phase systems contained 5.30% (w/w) dextran, 5.30% (w/w) PEG (including ligand—PEG), 5 m*M* potassium phosphate buffer, pH 7.4, and 10 m*M* potassium chloride. Temperature 3°C. The membranes, except in A, were pre-extracted in a system with the same composition as above but with only 5 m*M* potassium chloride, and the upper phase was used together with a pure lower phase as sample system after adjustment of the concentration of potassium chloride. (A) No ligand—PEG, nine transfers; (B) HE—PEG (1% of total PEG), nine transfers; (C) no ligand—PEG, 55 transfers; (D) HE—PEG (1% of total PEG), 55 transfers; and (E) HE—PEG (1% of total PEG), 26 transfers. Mixing time 30 sec and separation time 5 min (manual CCD), or 15 min (thin-layer CCD); 5.6 or 0.7 ml of upper phase (of which 0.35 or 0.19 ml was stationary) and 4.2 or 0.6 ml of lower phase were used for manual and thin-layer CCD, respectively. Pooled fractions are indicated with bars. The ordinate shows percentage in each tube of totally recovered membranes.

Because of the slow settling of the two phases, each transfer cycle required 15 min, corresponding to four transfers per hour. When the number of transfers was increased, the mobility of the main peak decreased (Fig. 2 and Table I).

Centrifugal CCD

By applying an acceleration field, the settling time for the phases can be speeded up. The functional unit (Fig. 1), like in the case of the conventional

TABLE I

THE RELATIVE MOBILITY (R_F) OF THE MAIN PEAKS AFTER COUNTER-CURRENT DISTRIBUTION OF SYNAPTIC MEMBRANES USING VARIOUS TIMES, NUMBER OF TRANSFERS, AND PEG-BOUND LIGANDS

The data have been calculated from the experiments in Figs. 2 and 3.

Composition of the two-phase system	Type of CCD	Time (min)	Number of transfers	Peak position (tube number)	R_F
Without ligand	Manual	110	9	4	0.44
	Centrifugal	110	55	24	0.42
				6	0.08
	Thin-layer	842	55	17	0.28
				5	0.06
With HE—PEG (1% of total PEG)	Manual	110	9	8	0.89
	Centrifugal	110	55	46	0.81
	Thin-layer	413	26	16	0.57
	Thin-layer	842	55	24	0.41
With BTR—PEG	Centrifugal	110	55	18	0.30
	Centrifugal	220	110	21	0.18

thin-layer CCD apparatus, comprises two discs of Plexiglass. The centrifugal machine, however, allows the disc system to rotate at relatively high speed. The mixed two-phase systems in the discs will therefore be centrifuged which results in a reduced settling time. The cycling time was 2 min, allowing 30 transfers per hour. Compared with the conventional apparatus the centrifugal CCD is seven to eight times faster. The apparatus has been described in detail elsewhere [14].

The results obtained with the synaptic membrane preparation are shown in Fig. 3, and the relative mobility of the main peak is shown in Table I. Comparison of the results obtained with the two machines shows clearly that the mobility changes with time and that the centrifugal CCD technique makes it possible to run CCD with a large number of transfers ($\simeq$55) without large changes in the partition behaviour. With an even higher number of transfers (Fig. 3D) the retarding phenomenon was also seen with this apparatus. The longer run was performed by continuing the transfers to cover almost two full turns of the circular mobile unit carrying the upper phases. The last 55 transfers seem to have added very little to the mobility of the peak, which means that the material was partitioned exclusively at the interface and bottom phase after 50—60 transfers.

Use of ligand—PEG

The partition of the membranes was found to depend on both the concentration of polymers and the salt composition. The system used here, without any ligand attached to PEG, was chosen so that a large part of the membranes was at the interface or in the lower phase. Adjustment of the partition could be done, from 85% of the membranes in the upper phase to less than 10%, by changing the concentration of potassium chloride from 0 to 20 m*M* (Fig. 4).

The introduction of PEG-bound cholinergic ligands, enriched in the upper phase, changed the partition of the membranes in favour of this phase. This caused a change in the CCD pattern, where part of the material was distributed further to the right (Figs. 2 and 3).

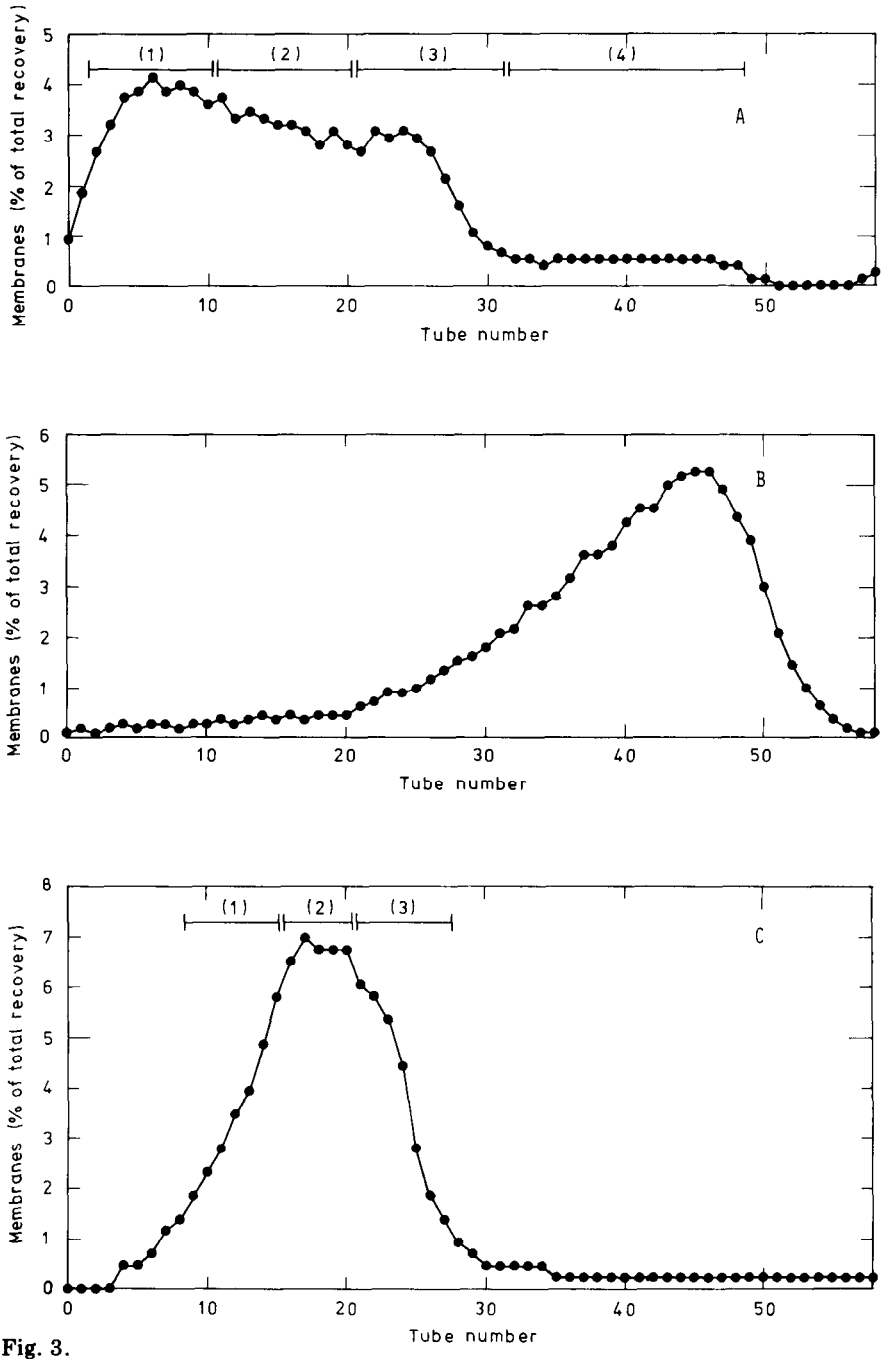


Fig. 3.

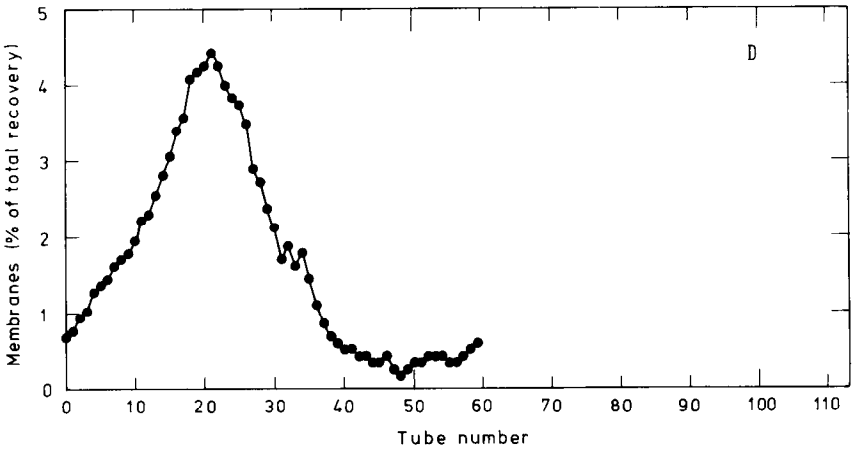


Fig. 3. Centrifugal CCD with the same two-phase system as in Fig. 2. (A) No ligand—PEG, 55 transfers; (B) HE—PEG (1% of total PEG), 55 transfers; (C) BTR—PEG (3% of total PEG), 55 transfers; and (D) BTR—PEG (3% of total PEG), 110 transfers by using two turns with the 60-chamber unit. Mixing time 30 sec and separation time 72 sec; 0.97 ml of upper phase (0.18 ml stationary) and 0.78 ml lower phase.

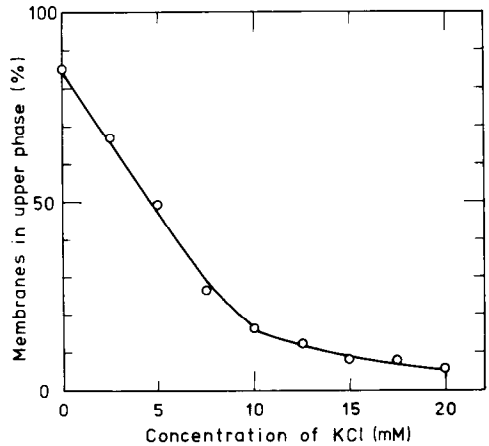


Fig. 4. Effect of potassium chloride on the partition of synaptic membranes (measured as protein content) in the same system as in Fig. 2, except for the concentration of potassium chloride. The membranes corresponded to 0.18 mg of protein per ml of phase system.

Single partition

The partition of membranes within the system was measured as a function of time (Fig. 5). Differences in partition behaviour were found when the system was continuously and gently mixed compared with systems mixed only before sampling. Over a longer period of time 7—20% of the material in the upper phase was decreased by 7—16% units. The decrease in partition, without ligand—PEG, was excluded from this phase. The opposite was true for a system containing HE—PEG where mixing caused decreasing affinity of the material for the upper phase.

TABLE II

PROPERTIES OF THE POOLED FRACTIONS FROM THE CCD EXPERIMENTS IN FIGS. 2 AND 3

The fraction numbers given for the pooled fractions are marked in the corresponding figures.

Figure No.	Ligand—PEG	Fraction	Light scattering (percentage of total)	Protein (percentage of total)	Membrane constituents and activities relative to protein (percentage of total/percentage of total protein)			
					Acetyl-cholin-esterase	Stereo-specific opiate binding	Cholesterol	Phosphate
2A	—	1	51	58	0.5	0.4	—*	—
		2	38	34	1.6	2.0	—	—
		3	11	8	2.1	0.8	—	—
2B	HE—PEG	1	23	21	0.3	0.06	—	—
		2	27	26	0.7	0.9	—	—
		3	23	24	1.3	1.5	—	—
		4	27	29	1.6	1.3	—	—
2C	—	1	26	21	1.0	0.8	—	1.2
		2	32	36	0.9	0.9	—	1.2
		3	22	39	1.1	1.3	—	0.8
2D	HE—PEG	1	33	32	0.8	0.6	0.8	0.9
		2	35	40	0.9	1.2	0.9	0.9
		3	20	20	1.2	1.0	1.4	1.2
		4	12	9	1.4	1.6	1.2	1.3
3A	—	1	33	33	0.7	0.5	—	0.9
		2	37	38	0.9	1.2	—	1.0
		3	26	27	1.4	1.3	—	1.1
		4	4	2	1.8	1.1	—	1.7
3C	BTR—PEG	1	30	18	0.9	0.07	0.6	—
		2	37	40	0.9	1.1	1.1	—
		3	30	39	1.2	1.4	1.2	—

*— = not determined.

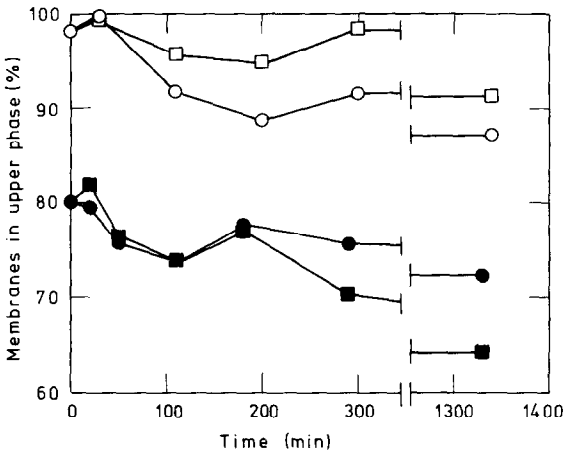


Fig. 5. Incubation of synaptic membranes in two-phase systems. Composition of systems as in Fig. 2 but with 5 m*M* potassium chloride. The membranes were pre-extracted in a system of the same composition using only material in the upper phase. The incubation was carried out at 3°C using resting (□, ■) and continuously mixed (○, ●) systems, either without ligand—PEG (filled symbols) or with HE—PEG, 1% of total PEG (open symbols). The concentration of membranes was measured as the light scattering at 400 nm. Total concentration of membranes corresponded to 0.25 mg of protein per ml.

DISCUSSION

Preparations of synaptic membranes obtained by centrifugation are not homogeneous. Both the basic composition and the size of the membrane fragments from the synaptic regions may vary considerably. Also outer membranes from non-synaptic regions, as well as membranes from other cells (glial cells) and cell organelles, are present to various degrees. The countercurrent distribution is an attractive way to purify synaptic membranes further. By using specific ligands bound to PEG it had earlier been shown that a high selectivity can be achieved in the extraction of synaptic membranes from electroplax [6]. The ligand, concentrated in the upper PEG-rich phase, causes an increased affinity of membranes with ligand-binding sites for this phase. In the case of brain tissue the receptor densities are much lower, and the preparations of synaptic membranes are much more complex.

The results of CCD of the crude synaptic membrane preparation from calf brain (Table II) show that even if the material could not be split up into discrete fractions (or peaks), the composition along the CCD train was not constant but changed gradually. Membranes enriched in certain activities may therefore be isolated by liquid—liquid extraction using these systems. A general trend was that opiate binding, acetylcholinesterase (marker for synaptic membranes), phosphate (mainly from phospholipids) and cholesterol were all enriched in the material distributed to the right. This shows that membranes from the nerve endings have a higher affinity for the upper phase than the bulk material. While a positive ligand effect was seen when the PEG-bound hexaethonium was used, the CCD experiments did not follow the quantitative rules for this kind of distribution. Another cholinergic ligand, BTR, did not show the expected effect when bound to PEG but decreased the mobility of the material.

The mobility was generally reduced when the time for the experiment was longer. This time-dependent effect influenced in a negative way the possibilities of obtaining a high resolution by using a large number of steps in CCD. Time alone was, however, not sufficient to cause the increased affinity of the membranes for the interface relative to the upper phase, as was shown by incubating the membranes for longer times in the two-phase systems. A possible explanation for the change in partition is that the membrane status changes when fragments of different kinds begin to separate from each other during the CCD run. A partial purification may facilitate membrane aggregation as well as lateral changes within the membranes. An increased affinity for the interface can be expected if some of the membrane components, e.g. certain proteins, are collected in a small region of the membrane fragment (capping). The fragment can be pictured as composed of two parts, one with affinity for the upper phase and one for the lower phase. Such a particle will have a strong affinity for the interface where it can orientate its respective parts towards the two phases. Capping of membrane proteins can be induced by PEG which leads to fusion of cells when these are treated with the polymer [23]. However, the concentration of PEG required for cell fusion [24] is normally much higher than that necessary to obtain two-phase systems with dextran. The changes of the partition may also be due to formation of (small) aggregates of the fragments. It is a general tendency that large particles have higher affinity for the interface than small ones. Aggregation may also reduce the exposed parts of the membranes responsible for favourable interaction with PEG.

The possibility to decrease the time for phase settling by centrifugation, thereby allowing larger numbers of transfers per time unit, works well and no tendency of the membranes to sediment at the relatively low centrifugal force (100 *g*) has been observed.

ACKNOWLEDGEMENTS

We wish to thank Miss Monica Andersson and Miss Sophie Bingsmark for skilful technical assistance. This work has been supported by grants from the NFR — Swedish Natural Science Research Council.

REFERENCES

1 P.-Å. Albertsson, Partition of Cell Particles and Macromolecules, Almqvist and Wiksell, Stockholm, and Wiley, New York, 2nd ed., 1971.
2 P.-Å. Albertsson, B. Andersson, C. Larsson and H.-E. Åkerlund, Methods Biochem. Anal., 28 (1981) 115—150.
3 B. Andersson and H.-E. Åkerlund, Biochim. Biophys. Acta, 503 (1978) 462—472.
4 S.D. Flanagan, P. Taylor and S.H. Barondes, Nature, 254 (1975) 441—443.
5 S.D. Flanagan, S.H. Barondes and P. Taylor, J. Biol. Chem., 251 (1976) 858—865.
6 G. Johansson, R. Gysin and S.D. Flanagan, J. Biol. Chem., 256 (1981) 9126—9135.
7 A. Hartman and E. Heilbronn, Biochim. Biophys. Acta, 513 (1978) 382—394.
8 G. Johansson, Acta Chem. Scand. Sect. B, 28 (1974) 873—882.
9 G. Johansson, in E. Reid (Editor), Methodological Developments in Biochemistry, 2. Preparative Techniques, Longman, London, 1973, pp. 155—162.
10 G. Johansson, Mol. Cell. Biochem., 4 (1974) 169—180.
11 G. Takerkart, E. Segard and M. Monsigny, FEBS Lett., 42 (1974) 218—220.

12 G. Kopperschläger and G. Johansson, Anal. Biochem., 124 (1982) 117—124.
13 P.-Å. Albertsson, Anal. Biochem., 11 (1965) 121—125.
14 H.-E. Åkerlund, J. Biochem. Biophys. Methods, 9 (1984) 133—141.
15 F. Hajős, Brain Res., 108 (1976) 485—489.
16 E. Eriksson and G. Johansson, in E. Reid (Editor), Cell Populations, Ellis Horwood, Chichester, 1979, pp. 81—90.
17 G.L. Ellman, K.D. Courtney, V. Andres and R.M. Featherstone, Biochem. Pharmacol., 246 (1971) 4694—4698.
18 P. Ott, Y. Binggeli and U. Brodbeck, Biochim. Biophys. Acta, 685 (1982) 211—213.
19 B.N. Ames and D.T. Dubin, J. Biol. Chem., 235 (1960) 769—775.
20 F. Medzihradsky, Brain Res., 108 (1976) 212—219.
21 M.M. Bradford, Anal. Biochem., 72 (1976) 248—254.
22 C.J.O.R. Morris and P. Morris, Separation Methods in Biochemistry, Pitman, London, 1964, pp. 559—619.
23 J.M. Robinson, D.S. Roos, R.L. Davidson and M.J. Karnovsky, J. Cell Sci., 40 (1979) 63—75.
24 R.L. Davidson and P.S. Gerald, Methods Cell Biol., 15 (1977) 325—338.