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TIME-DEPENDENT EFFECT OF THE PARTITIONING OF ELECTROPLAX MEMBRANES IN AQUEOUS BIPHASIC SYSTEMS USING COUNTER-CURRENT DISTRIBUTION TECHNIQUE AND HEXAETHONIUM–POLYETHYLENE GLYCOL

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SUMMARY

Cholinergic receptor-rich membranes from the electroplax of *Torpedo californica* have been partitioned between the upper phase and the interface of an aqueous two-phase system consisting of water, dextran and polyethylene glycol. The partitioning of membranes containing the cholinergic receptors was influenced by including polyethylene glycol-bound hexaethonium in the upper phase. The partition behaviour was studied by using the two-phase systems for counter-current distributions with a varying number of steps. The membranes showed time-dependent distributions, which is assumed to be due to changes in the internal distribution of membrane constituents and/or formation of larger membrane aggregates. Despite this effect, good purification of the cholinergic membranes could be obtained. The consequences of the time-dependent effect for the partitioning of membranes within aqueous two-phase systems as well as the possibilities to reduce it are discussed.

INTRODUCTION

Membranes rich in cholinergic receptors, from electroplax of Torpedo rays, have in several cases been purified by partitioning between the phases (and the interface) of aqueous two-phase (liquid–liquid) systems[1–5]. The distribution of the membranes within the two-phase systems has been influenced by using either affinity ligands bound to polyethylene glycol (PEG, enriched in the upper phase)[1–4] or PEG sulphonate[5]. By using quaternerized amino ligands such as hexaethonium [Et_3N^+–$(CH_2)_6$–N^+Et_2–, where Et = ethyl] or bis(triethylaminoethyl)resorcinol, a standard preparation of nicotinic cholinergic membranes, obtained by sucrose zonal centrifugation, could be further separated into several fractions of which one had a 3–4 times higher specific activity than the starting material[4]. The fractions were studied by counter-current distribution (CCD), which is a well-established method for the fractionation of thylakoid membranes from spinach chloroplasts[6–9]. To obtain reproducible results with the membranes from electroplax it was, however, necessary to include proteins as stabilizor in the systems[3,4].

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The present work has been carried out to investigate the phenomenon of time-dependent partitioning of preparations of synaptic membranes, which has been observed when membrane mixture was analysed by CCD[10,11]. This time-dependence points to a change of the membranes, which is reflected in their partition behaviour. Because of the simple rules determining the shapes and position of the CCD curve and the dependence of the number of transfers, possible changes of the state of the membranes can be suggested. The aim has also been to investigate whether this time-dependence influences the purification obtained with PEG-bound affinity ligands for cholinergic receptors, *i.e.* affinity partitioning.

MATERIALS AND METHODS

Polymers

PEG was purchased from Union Carbide (New York, NY, U.S.A.) as Carbowax 8000, and had a molecular weight of 7000–9000. Dextran T-500 was purchased from Pharmacia (Uppsala, Sweden) and had a molecular weight of 500 000. Stock solutions of the polymers were prepared and standardized as described elsewhere[6,12].

Hexaethonium-PEG (HE-PEG) was prepared via bromo-PEG according to Johansson *et al.*[4] (non-crosslinked type).

Protein assay

Protein was determined according to Bradford using Coomassie Brilliant Blue G[13] and measured photometrically at 595 nm. Membrane samples were incubated, prior to assay, with 0.5 *M* phosphoric acid at 50°C for 2 h.

Two-phase systems

Phase systems were prepared by weighing out stock solutions of polymers and adding salt, buffer solutions, water and membrane sample to the final weight. Polymer concentrations are given in per cent, weight by weight (%, w/w). For direct partitioning measurements, 5-g systems were used. All partitioning and CCD experiments were carried out at 4°C.

Torpedo membrane preparations

Preparation of electroplax tissue, homogenizing and zonal centrifugation in a sucrose gradient were carried out as described previously[3,14], with the exception that the initially used buffer contained 3 m*M* EDTA, 1 *M* sodium chloride and 10 m*M* sodium phosphate buffer (pH 7.4). The tissue was homogenized in a Sorvall omnimixer for 4 min, in intervals of 30 s, at maximum speed.

Bungarotoxin labelling

I^{125}-α-Bungarotoxin was obtained from New England Nuclear (NEX-126, 80–160 Ci/mmol). The labelled toxin was purified by ion-exchange chromatography on a CM-Sephadex column[15]. Membranes were prelabelled with the toxin, not exceeding 2% of the total number of receptors.

Counter-current distribution

A thin-layer CCD apparatus (IRD, Stockholm, Sweden) constructed accord-

ing to Albertsson[6,11,16] was used. With a set of plates containing 60 cavities (0.55-ml volume of the slots in the lower plate), a 0.50-ml aliquot of lower phase and a 0.70-ml aliquot of upper phase was used. With a set containing 120 cavities (0.65-ml volume of lower slots), the same volumes of phases were used as above. The mixing time was 12.5–30 s and the settling time was 4.2–8.3 min. Sample was introduced in the first cavity (No. 0) in a 1.2-ml two-phase system. To obtain a more accurate protein determination, the prelabelled membranes were mixed with non-labelled membranes in the ratio 1:3.5. After the counter-current distribution, 400 μl of water were added to each chamber to obtain homogenous solutions, which were analysed for toxin, using a Beckman gamma-counter, and also for protein.

RESULTS

The partitioning of the membrane-bound receptors within the two-phase aqueous system can easily be followed by labelling a small fraction (<2%) of the receptors with I^{125}-bungarotoxin[4]. Throughout this work, a two-phase system has been used with the composition 4.6% (w/w) dextran–3.8% (w/w) PEG. The system also contained either 15 m*M* sodium phosphate buffer (Figs. 1–3) or 8 m*M* sodium chlorid and 5 m*M* imidazole · HCl (Fig. 4), pH 7.4. Membranes included in this system were found mainly in the lower phase, 90%, based on the partitioning of I^{125} activity measurements, while 9% of the receptors were accumulated at the interface. Consequently, not more than 1% of receptor-containing membranes was present in the upper phase. The partitioning of the membranes can, however, be drastically

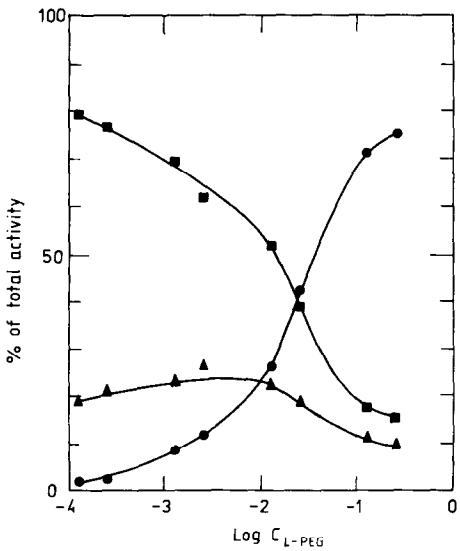

Fig. 1. Distribution of membrane-bound cholinergic receptors as a function of concentration of ligand–PEG. This concentration, $C_{L\text{–}PEG}$, is given in percent of total PEG (*i.e.* PEG + ligand–PEG). The ordinate shows the per cent of total receptor (bungarotoxin binding) in upper phase (●) in lower phase (■) and at the interface (▲). The two-phase system contained 4.6% (w/w) dextran, 3.8% (w/w) total PEG, 15 m*M* sodium chloride and 5 m*M* sodium phosphate buffer (pH 7.4). Temperature, 4°C.

changed by including small amounts of polymer-bound ligands for the receptor[4] in the system. The ligand used here is a bis-quaternary compound, hexaethonium, similar to the well-known agonist for nicotinic cholinergic receptors, hexamethonium. The membranes showed an increased affinity for the upper hase when increasing amounts of ligand were used (Fig. 1). This effect was already noticable when only 0.001% of PEG was replaced by ligand–PEG. Since the total content of the ligand–PEG preparation was 38 g/l and the ligand content of the ligand–PEG preparation was around 19% (monosubstituted), the ligand concentration at this point $9 \cdot 10^{-9}$ *M*, assuming the molecular weight of PEG ot be 8000. This is the overall

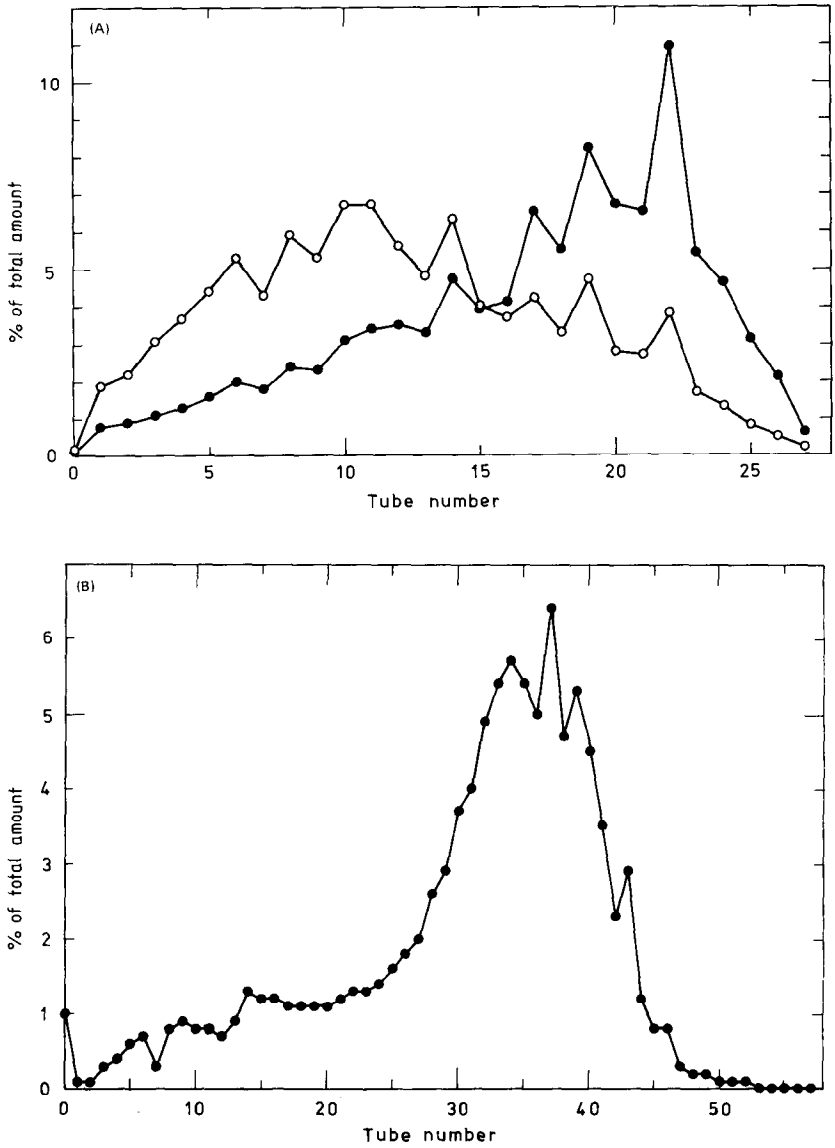

Fig. 2.

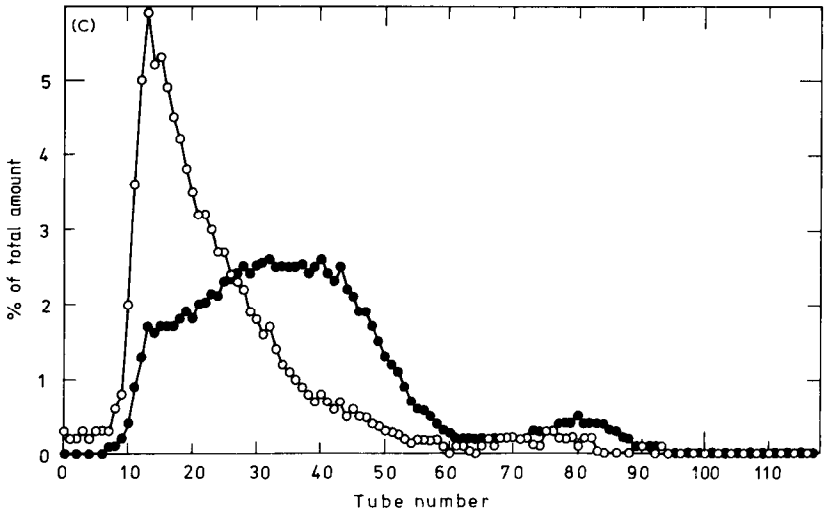

Fig. 2. Counter-current distribution of membranes analysed for protein (○) and cholinergic receptor (toxin binding) (●). Two-phase system as in Fig. 1, containing 0.058% (of total PEG) ligand–PEG. Temperature, 4°C. (A) 28 transfers; (B) 58 transfers, system contained 2 g or bovine serum albumin per litre; (C) 118 transfers. Sample was applied in chamber no. 0. For further technical details, see text.

concentration, while the concentration of ligand in the upper phase is almost twice as high.

For the experiments with counter-current distribution (CCD), 0.058% of the PEG was replaced by ligand–PEG (log $C_{L\text{-}PEG} = -1.24$). At this concentration of ligand, 60% of the receptors are recovered in the upper phase. In the CCD experiments, a 0.5-ml aliquot of lower phase together with a 0.05-ml (or 0.15 ml at 118 transfers) aliquot of upper phase were loaded in each chamber of the stationary plate of the CCD apparatus (to ensure that the material at the interface did not move). In the mobile plate, 0.65-ml (or 0.55 ml at 118 transfers) aliquots of the upper phase were loaded. Therefore, the overall partition ratio, *i.e.* the ratio between amount of material in the mobile and stationary parts of the system was 1.26 (or 0.88 at 118 transfers). If the receptor-containing membranes had partitioned as a homogeneous fraction, they would theoretically have formed a symmetrical peak with its maximum in tube No. 16 after 28 transfers. With 58 or 118 transfers, the peak should have had its maximum in tube Nos. 32 and 55, respectively. The experimental results when these numbers of transfers were used are shown in Fig. 2.

Three important observations can be made from the outcome of the CCD experiments:

(i) The membranes are not homogeneous. The receptor binding material does not behave like a single component and the total membranes, measured as protein, move slower along the train of CCD tubes than the receptor.

(ii) A very good purification is obtained and the receptor to protein ratio is four times higher in the right part of the CCD train than the original material (Fig. 3).

(iii) When the number of transfers is increased, the material (both total mem-

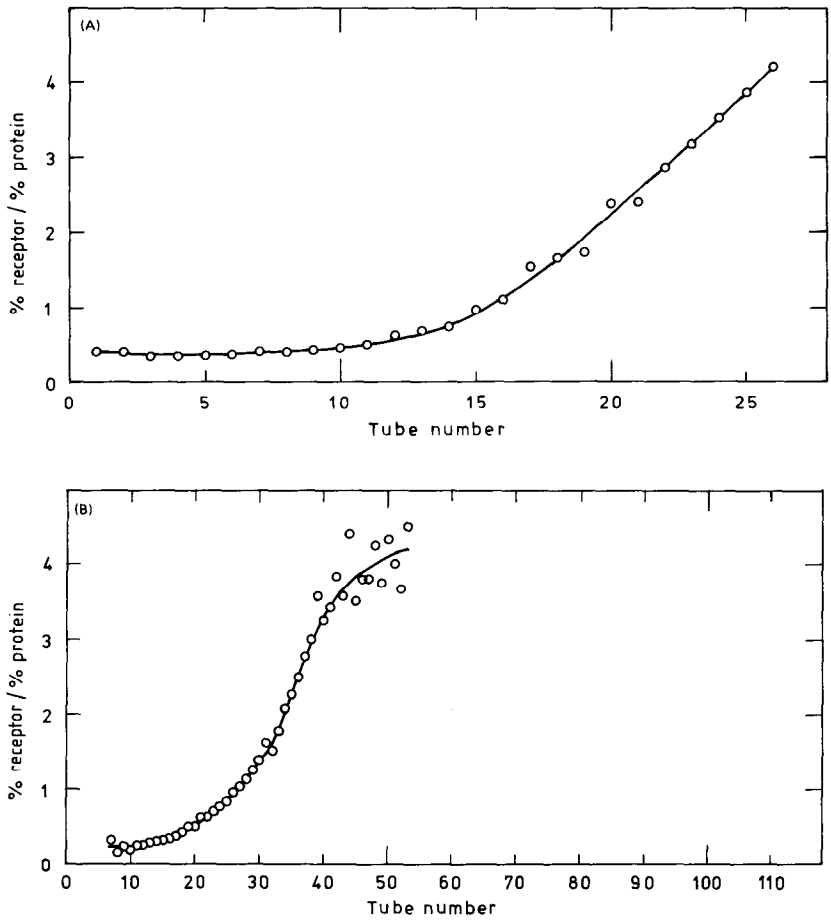

Fig. 3. Ratio between receptor and protein percentages (of total recovery) for each CCD tube, with significant amounts of both. The unit value on the ordinate corresponds to the specific activity of the original material. (A) Corresponds to Fig. 2A; and (B) corresponds to Fig. 2C.

branes and receptor activity) is transferred more and more slowly to the right.

The overall distribution ratio, G, for the receptor activity has been calculated through an approximate G value for each tube, i, using the relation $G_i = i/(n-i)$, where n is the number of transfers[17]. The total amount of material in the upper phases, M_u, is then equal to

$$\sum_{i=0}^{n} G_i P_i/(1+G_i)$$

where P_i is the percentage of material in tube i. The total (average) G is equal to $M_u/(100-M_u)$. The calculated total G values are 1.51, 1.14 and 0.45 for 28, 58 and 118 transfers, respectively. The first two values agree fairly well with the value (1.26) calculated above from the single partitioning experiments, but it is much lower when

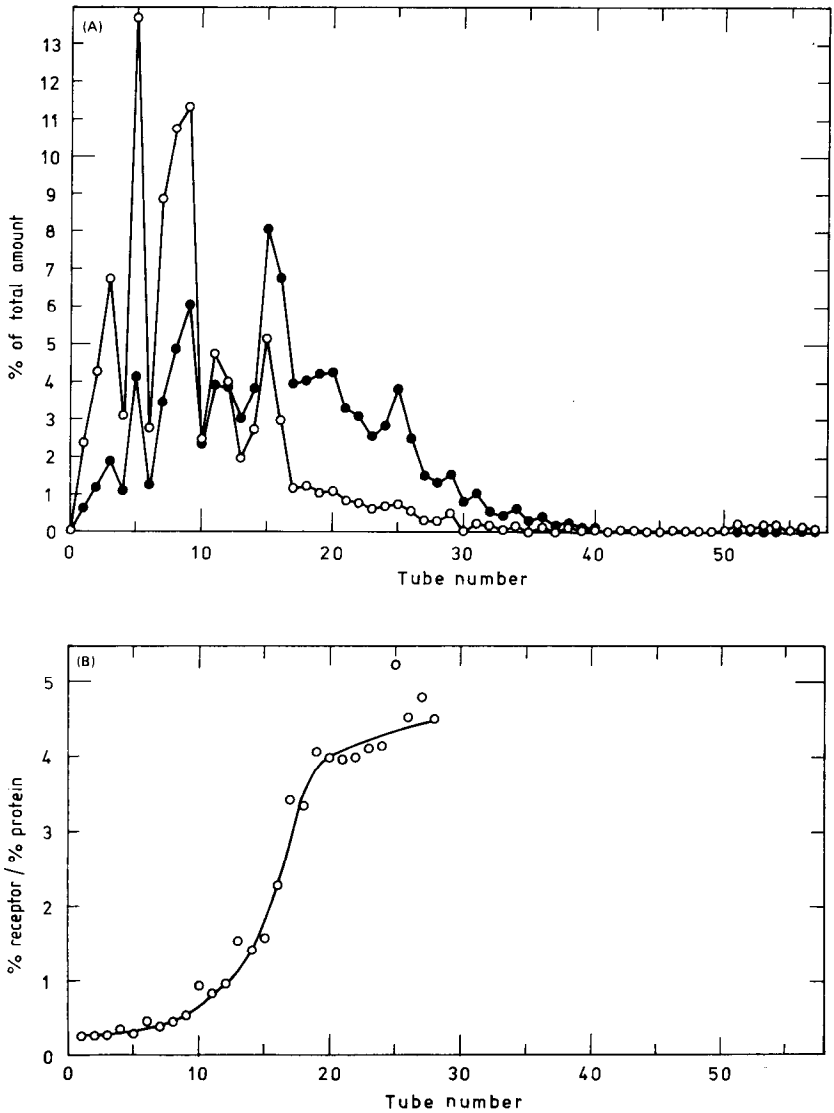

Fig. 4. (A) CCD as in Fig. 2B but using a system containing 0.1% ligand–PEG, 8 m*M* sodium chloride, 5 m*M* (based on Cl) imidazole · HCl (pH 7.4) and no phosphate. (B) Corresponding ratio between receptor and protein percentages.

118 transfers were used (calculated value 0.88). The distribution curves also show a surprising scatter of measured data along the tubes, which points to the presence of large aggregates. On the other hand, the ratio of protein to receptor changes smoothly with tube number. In an attempt to check the effect of other ions on this "scattering", a CCD experiment was carried out using imidazole · HCl instead of phosphate buffer (Fig. 4). In this case, even more pronounced "scattering" was observed, but the purification profile (Fig. 4) was also in this case very smooth and gave the same

TABLE I

EFFECT OF INCUBATION IN 0.9% PEG OR 0.9% (PEG + HE-PEG, 9:1) ON LABELLED MEMBRANES

The membranes were incubated for 21 h during gentle mixing at 0°C. The membranes were then partitioned in a system containing 4.6% (w/w) dextran, 3.5% (w/w) PEG, 15 m*M* sodium chloride and sodium phosphate buffer (pH 7.4). The systems also contained 17.5 mg of HE-PEG per litre. The partitioning was carried out at 0°C.

Treatment of the membranes	*Percent of receptor in the upper phase*	
	System mixed by 20 inversions of the test-tube and allowed to settle for 1 h	*Strongly mixed by a Vortex mixer for 4 s every 5 min for 30 min, followed by 1 h of settling*
Stored for 24 h in ice after labelling (control)	68	51
Incubated with PEG for 21 h followed by 4 h of rest	64	49
Incubated with PEG for 21 h followed by 12 h of rest and kept in the two-phase system for 12 h	41	42
Incubated with HE–PEG + PEG for 21 h followed by 4 h of rest	35	35
Incubated with HE–PEG + PEG for 21 h, 12 h of rest, 12 h in two-phase system	20	25

purification factor as in the experiments above. The chloride has, however, the general effect of reducing the distribution ratio[11,12] and the material was therefore retained more than in the case of phosphate buffer.

Changes in the distribution ratio of the membrane-bound receptors were also observed by single partitioning after various treatments of the membranes (Table I). Strong aggetation of the systems before settling, as well as incubation with ligand–PEG before partitioning, influenced the distribution. Also, by keeping the membranes in the system for a longer period, 12 h, the amount of material in the upper phase was reduced after redistribution. This points to a change in the properties of the membranes with time (in the two-phase system) in such a way that their affinity for the interface or the lower phase increases.

DISCUSSION

Counter current distribution is in most cases an excellent way to separate substances by liquid–liquid extraction, while also minor differences in the distribution between two phases can be useful. As for normal chromatographic processes, the travelling distance of a zone (peak) of a homogeneous substance in CCD is proportional to the numbers of transfers, n, while the width of the peak only increases with

$\sqrt{n}$. Here, the presented CCD experiments of cholinergic membranes from Torpedo electroplax do not follow this fundamental behaviour. Instead, the relative travelling distance (i_{max}/n) of the receptor decreases with n. The change in "mobility" is most probably due to a time-dependent change of the membranes, which successively reduces their affinity for the upper phase. This effect may be caused by the contact of membranes with the polymers, especially the ligand–PEG, or by the presence of the interface between the two phases. The results obtained by incubating the membranes with PEG, ligand–PEG or the two-phase system (Table I) favours this idea, as well as the effect of vigorous shaking of membrane-containing two-phase systems. Similar effects have been observed when synaptic membrane fractions obtained from calf brain were analysed by CCD[11]. With this kind of membrane, the changes in distribution were reduced by using phase-forming polymers with lower molecular weight, by using a dextran–Ficoll system with only low concentration of PEG, or by addition of a stabilizing protein to the system[18]. A much clearer separation of the Torpedo membranes was also achieved by addition of serum albumin to the dextran–PEG system[4,10] and by using more rapid CCD processes[10].

The assumed changes of the membranes would mainly be of two kinds: either a polarization within the membrane fragments, making it asymmetrical in certain components such as proteins or lipids, which may be oriented over the interface like a "chemical" dipole; or a formation of large aggregates, which have a tendency to accumulate at the interface between the phases. Formation of extremely large aggregates could also explain why the CCD profile is saw-tooth shaped (especially pronounced in Fig. 4a) instead of the normal smooth appearence. Visible aggregation was, however, not observed.

Even when the material slows down in transferring mobility, the purification is good with around 30% of the receptors with a 2–4 times increase in specific activity. This means that the purification was already obtained after 20–30 transfers and that further transfers did not improve the resolution. A plausible explanation for the observed decrease in mobility is that the membranes began either to aggregate or to be polarized by the CCD process when they had reached a certain degree of purity.

Despite the good purification obtained, the change in partitioning behaviour of the membranes is an unwanted phenomenon, which strongly reduces the resolving capability of multistep procedures. The full resolving power might be necessary when more complex mixtures of membrane fragments are to be fractionated. The main routes for avoiding these negative effects should be (i) use of shorter times for each cycle in the CCD[10,11], (ii) use of polymers with less effect on the membranes[19,20], (iii) use of stabilizing additives in the system, *i.e.* soluble proteins[4,18], or (iv) use of CCD at sub-zero temperatures to freeze the membranes in their native state.

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