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EFFECT OF DEXTRAN- AND POLY(ETHYLENE GLYCOL)-BOUND PROCION YELLOW HE-3G ON THE PARTITION OF MEMBRANES FROM CALF BRAIN SYNAPTOSOMES WITHIN AN AQUEOUS TWO-PHASE SYSTEM

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SUMMARY

Membranes obtained by lysis and Yeda-press treatment of synaptosomes (nerve endings) from calf brain cortex have been partitioned within the aqueous phases (and the interface between them) of a Ficoll–dextran–poly(ethylene glycol)–water two-phase system. By introducing the dye Procion yellow HE-3G in the upper phase, bound to poly(ethylene glycol), or in the lower phase, bound to dextran, the partition of the membranes could be strongly affected. The influence on the partition was more pronounced when the dye was bound to dextran. By using a number of two-phase systems in a counter-current distribution process, it was shown that the membrane preparation was inhomogeneous and that the fractions obtained differed in their contents of acetylcholinesterase, succinate dehydrogenase and ATPase. The affinity partitioning effect depended strongly on the concentration of polymer-bound dye. An optimum dye concentration was found when Procion yellow HE-3G was bound to poly(ethylene glycol). When the same dye was bound to dextran, the number of dye molecules per dextran molecule influenced the effectiveness of the extraction.

INTRODUCTION

Biological membranes can be fractionated by partition within aqueous two-phase (liquid–liquid) systems obtained by dissolving two polymers, usually dextran and poly(ethylene glycol) (PEG), in water[1]. The partitioning involves both the phases and the interface between them[1,2]. It can be drastically influenced by the addition of various salts[2], by binding of a chemical group (ligand) with affinity for certain membranes to one of the phase-forming polymers[3–5] or by using charged polymers[6,7]. The last two ways of enhancing the effectiveness or the selectivity of the extraction have

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been studied for cholinergic membranes from electroplax of *Torpedo californica*[3–5] and *Torpedo marmorata*[6].

One ligand which recently has been shown to influence the partition of synaptic membranes from cerebral cortex[8] is the reactive dye Procion yellow HE-3G. This type of dye has been widely used in affinity chromatography of proteins[9,10], but also in affinity partitioning of enzymes[11–13].

In the present work the influence of Procion yellow HE-3G (bound to dextran or PEG) on the partition of synaptic membranes (from calf brain cortex) in a Ficoll–dextran–PEG–water two-phase system has been investigated. The membrane heterogeneity has also been studied by using this two-phase system in a counter-current distribution process in an apparatus where phase separation was facilitated by centrifugation[14].

MATERIALS AND METHODS

Dextran T-40 (mol. wt. 40 000), dextran T-500 (mol. wt. 500 000), Ficoll 400 (mol. wt. 400 000) and Ficoll 70 (mol. wt. 70 000) were purchased from Pharmacia (Uppsala). PEG (mol. wt. 6000–7500) was obtained from Union Carbide (New York, NY, U.S.A.) as Carbowax 6000. Procion yellow HE-3G was a gift from Swedish I.C.I. (Göteborg). The test kit for cholesterol assay was purchased from Boehringer (Mannheim). Materials for the biochemical assays were obtained from Sigma (St. Louis, MO, U.S.A.). All salt and buffer substances were of analytical grade.

Polymer-bound Procion yellow

Procion yellow HE-3G was bound to PEG, Ficoll 400 and dextran T-500 in basic aqueous solutions described elsewhere[12,15,16]. The Procion yellow–Ficoll (PrY–Ficoll) contained 0.28 μmol dye per gram. The Procion yellow–PEG (PrY–PEG) had a dye/polymer molar ratio of 1:1, or 150 μmol dye per gram polymer. Procion yellow–dextrans (PrY–dextran) had molar ratios of 1:3 and 3:1, or 0.8 and 7.2 μmol dye per gram polymer, respectively.

Membrane preparation

The preparation of synaptosomes from calf brain cortex was performed with a slightly modified version of the method described by López-Pérez *et al.*[17]. Unless stated otherwise, all centrifugation steps were performed in a GSA-rotor with a Sorvall RC-2B refrigerated high-speed centrifuge at 3°C. A 100-g amount of cerebral cortex was minced with a pair of scissors. The tissue was suspended in 500 ml of 0.32 *M* sucrose containing 1 m*M* Tris–HCl buffer, pH 7.4, and homogenized with 20 strokes in a glass Potter-Elvehjem homogeniser 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 buffer, in Sorvall GSA centrifuge flasks; the contents were centrifuged at 20 000 g_{max} for 30 min. The white upper part of the pellet was recovered from 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 homogeniser and passed twice through a Yeda press under 100 atm nitrogen.

Assays

Light scattering was measured at 400 or 500 nm (in the presence of Procion yellow) using an Hitachi 100-60 spectrophotometer. After treating the membranes for 1 h at 50°C with 0.5 *M* phosphoric acid, the protein concentration were determined as described by Bradford[18]. Acetylcholinesterase was determined by the method of Ellman *et al.*[19] and succinate dehydrogenase as described by Earl and Korner[20]. ATPase was determined according to Scharschmidt *et al.*[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 phosphate, pH 7.8 (concentration based on phosphate). A detailed description of the preparation of the systems can be found elsewhere[1,2]. Stock solutions of Ficoll and dextran were treated before use with a mixed ion exchanger (Amberlite MB-1) to remove traces of salt. Single partition experiments were carried out at 0–2°C. The systems containing membranes (4.2–4.4 mg protein/l) were carefully mixed for 30 s and then centrifuged (in the cold) for 10 min at 1000 g_{max}. Small samples were withdrawn for analysis from the mixed system, upper phase and, in some cases, lower phase. The partition of ligand–polymers in systems without membranes was determined from diluted phases by absorbance measurements at 390 nm.

Counter-current distribution (CCD)

A new type of CCD apparatus invented by Åkerlund[14] was used. It accelerated the separation of the phases by use of a centrifugation step at 100 *g*. The apparatus contained 60 chambers arranged in a circle which allowed the upper phases to be transferred in a stepwise manner to the neighbouring lower phases during centrifugation. The apparatus was operated in a cold-room at 4°C. The membranes samples (each 1.3 ml) were included in chambers 0–4 of the CCD machine; the same amounts of the mixed two-phase system (without membranes) were loaded in chambers 5–59.

In some experiments PrY–PEG or PrY–dextran, at concentrations of 0.005–0.1% of the total PEG or 0.001–0.05% of the total dextran, respectively, were included in the systems. The shaking time was 4 min, centrifugation time was 12 min and 55 transfers were applied. After each experiment, the systems were transformed into homogeneous solutions by the addition of 1.3 ml of 5 m*M* Tris phosphate, pH 7.8, per chamber. The fractions, diluted 25 times, were analyzed for light scattering by measuring the apparent absorbance at 400 nm or (if Procion yellow was used) 500 nm. The contents of some chambers were pooled to give main fractions which were concentrated by centrifugation for 120 min at 45 000 g_{max}, after two-fold dilution, and were finally resuspended in 1–2 ml of distilled water. The fractions were analyzed for phosphate (mainly from phospholipids), cholesterol, protein and marker enzymes.

RESULTS

In the aqueous two-phase system both Ficoll and PEG are mainly present in the upper phase and dextran in the lower phase. This is reflected in the partition coefficients of the dye–polymer derivatives. The partition coefficients (ratios of the concentrations of polymer-bound dye in the upper and lower phases) were found to be 4.2 for PrY–Ficoll, 5.4 for PrY–PEG and 0.28 for PrY–dextran.

Partition of synaptosomal membranes as a function of the concentration of PEG-bound Procion yellow

In a two-phase system containing 9.3% Ficoll 70, 10.3% dextran T-40 and 2.3% PEG at pH 7.8, the synaptic membranes were distributed with 42% of the total membranes in the upper phase and the remaining membranes were found at the interface and to some extent in the lower phase. By replacing an increasing amount of the PEG with PrY–PEG, the partition of the membranes into the upper phase first increased, passed through a maximum value (58%) and then decreased (to below 30%) as shown in Fig. 1.

Counter-current distribution of synaptosomal membranes at various concentrations of PrY–PEG

The synaptosomal membranes were studied in a multistep extraction procedure, counter-current distribution (CCD)[14] using the same two-phase system as

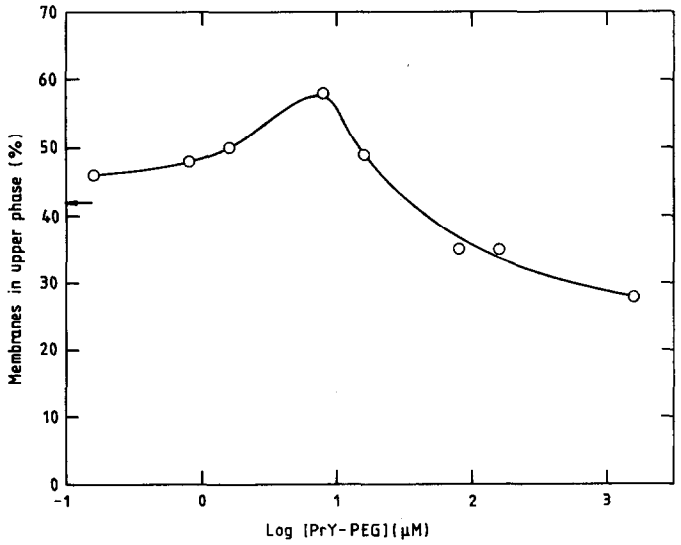

Fig. 1. Effect of the concentration of PEG-bound Procion yellow HE-3G on the partition of synaptosomal membranes. The diagram shows the percentage (of total membranes) recovered in the upper phase as a function of the concentration of PrY–PEG. The arrow indicates the partition of membranes in the absence of PrY–PEG. The membrane concentration was determined by light-scattering measurements. Two-phase system: 9.3% (w/w) Ficoll, mol. wt. 70 000; 10.3% (w/w) dextran, mol. wt. 40 000; 2.3% (w/w) PEG, mol. wt. 6000, including PrY–PEG; and 5 m*M* Tris phosphate buffer (concentration based on phosphate), pH 7.8. Temperature: 3°C.

above, Fig. 2. Two well separated fractions or "peaks" were obtained in the absence of Procion yellow, Fig. 2A. When the system contained 8.0 μM PEG-bound Procion yellow, Fig. 2B, two peaks were still found but the relative proportion of membranes in the fractions had changed in favour of the peak to the right. At higher concentrations of PrY–PEG more and more material was moved into the left peak and at 160 μM Procion yellow, Fig. 2E, the right peak was no longer detectable as a separate fraction. The relative amounts of membranes in the first and second peaks in relation to the concentration of Procion yellow, estimated from the CCD diagrams, are shown in Fig. 3. Pooled fractions were analyzed (Table I).

The dextran–Ficoll–PEG system can be used directly, without ligands, for separation of the synaptic membranes from cortex into two distinct fractions, Fig. 2. No detrimental effects of the system on the membranes, *e.g.*, aggregation, which has sometimes been observed in dextran–PEG systems used for CCD[22], could be detected. The low concentration of PEG, used for more rapid settling of the phases, did not affect the membranes. The succinate dehydrogenase activity, used as a marker for mitochondria fragments, remains in the left fraction showing that these kinds of membranes have minimum affinity for the upper phase. However, the left fraction

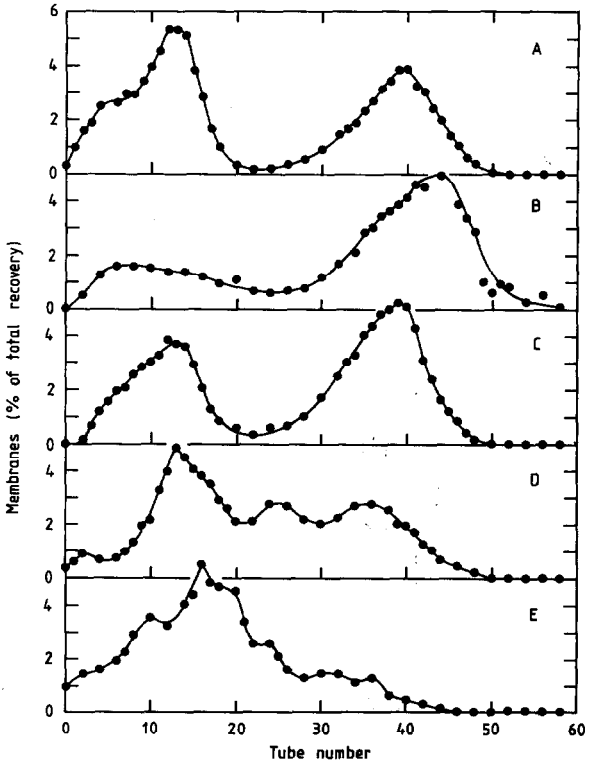

Fig. 2. Centrifugal counter-current distribution (CCD) of synaptosomal membranes. The membrane concentration was determined by light-scattering measurements. System and temperature as in Fig. 1. (A) No PrY–PEG; (B) 8 μM PrY–PEG; (C) 16 μM PrY–PEG; (D) 80 μM PrY–PEG and (E) 160 μM PrY–PEG. Total amount of membranes loaded in chambers 0–4 corresponded to 4.8 mg protein.

TABLE I

DISTRIBUTION OF PROTEIN (Pr), SUCCINATE DEHYDROGENASE (SDH), ACETYLCHOLINESTERASE (AChE) AND ATPase WITHIN THE TWO MAIN PEAKS OF THE COUNTER-CURRENT DISTRIBUTION IN FIG. 2

Fraction	*No PrY–PEG*				*8 μM PrY–PEG*				*16 μM PrY–PEG*			
	% Pr	*% SDH*	*% AChE*	*% ATPase*	*% Pr*	*% SDH*	*% AChE*	*% ATPase*	*% Pr*	*% SDH*	*% AChE*	*% ATPase*
Left part tubes 0–27	60	92	23	50	26	90	4	45	40	89	15	57
Right part tubes 28–55	40	8	77	50	74	10	96	55	60	11	85	43

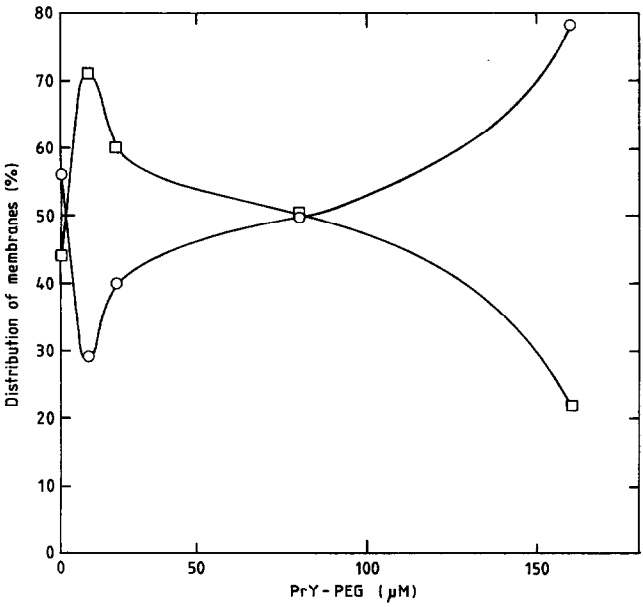

Fig. 3. Distribution of membranes between the two peaks in the CCD experiments (in Fig. 2) in relation to the concentration of PrY–PEG: □—□ right peak; O—O, left peak.

in Fig. 2A also contains other kinds of membranes since the part of the acetylcholinesterase activity present in this fraction can effectively be moved to the right by increasing its affinity for the upper phase by using a moderate concentration of PEG-bound ligand, Table I.

Partition of membranes as a function of dextran-bound Procion yellow

The partition of synaptic membranes was also shown to be influenced by PrY–dextran, which is present in the lower phase. An increase in the concentration of Procion yellow in this phase reduced the amount of membranes in the upper phase, Table II. In the range of dye concentrations studied, 17% of the total material could be transferred into the lower phase plus the interface, corresponding to 2/5 of the membranes originally present in the upper phase. This PrY–dextran had a dye:dextran molar ratio of 3:1. When a PrY–dextran preparation with a molar ratio of 1:3 (mostly containing monosubstituted dextran) was used, Table II, a 10 times higher concentration of dextran-bound Procion yellow was necessary to get the corresponding effect.

Partition of membranes in systems containing polymer-bound Procion yellow in both phases

In systems containing 8 μM PEG-bound Procion yellow the effect of increasing amounts of PrY–dextran on the partition of membranes was studied, Table II. The dextran-bound Procion yellow was able to remove 14% of the total material from the upper phase to the interface and eventually to the lower phase. The largest con-

TABLE II

EFFECT OF PrY–DEXTRAN ON THE PARTITION OF SYNAPTOSOMAL MEMBRANES

The dye:dextran molar ratio (m.r.) of the PrY–dextran was either 1 or 3. System composition as in Fig. 1 with or without 8 μM PrY–PEG. Temperature: 3°C.

Concentration of dextran-bound Procion yellow (μM)	*Per cent of total membrane in upper phase*		
	Systems without PrY–PEG		*Systems with 8 μM PrY–PEG*
	m.r. = 3	*m.r. = 1*	*m.r. = 3*
0	42	42	84
0.5	38	42	78
1	34	41	74
2	30	40	74
5	29	39	72
10	25	37	70

centration of dextran-bound Procion yellow, 10 μM, did not reduce the percentage of membranes in the upper phase to the value obtained without PEG-bound ligand.

Partition of pre-extracted membranes as a function of dextran-bound Procion yellow

The type of membranes with affinity for the upper phase (in a system without ligand) and therefore found in the right part of the CCD diagram, Fig. 2A, were isolated by preparative partition into the upper phase of the same system followed by two washings with pure lower phases. These membranes were found to be more strongly affected in their partition than the total membrane population (Table III). Twenty per cent of the pre-extracted membranes were extracted from the upper phase to the interface (+ lower phase) at 2 μM dextran-bound Procion yellow; only 12% of the original membranes were extracted at this dye concentration, Table II. In the

TABLE III

EFFECT OF PrY–DEXTRAN ON THE PARTITION OF PRE-EXTRACTED MEMBRANES (WITH AFFINITY FOR THE UPPER PHASE)

System as in Fig. 1 but without any PrY–PEG. The molar ratio dye:dextran of the PrY–dextran was 3:1. Temperature: 3°C.

Concentration of dextran-bound Procion yellow (μM)	*Per cent of material/activity recovered in upper phase*		
	Membrane	*Acetylcholinesterase*	*Succinate dehydrogenase*
0	94	88	37
1	80	77	11
2	74	70	0
10	63	60	0
20	55	53	0
51	34	33	0
102	14	17	0

range of dye concentrations studied, 80% of the pre-extracted membranes could be extracted from the upper phase. The activity of acetylcholinesterase and of the total membrane were equally affected in their partition, indicating an homogeneous distribution of the enzyme over the pre-extracted membranes. On the other hand, the remaining succinate dehydrogenase activity was effectively removed from the upper phase at a very low concentration of PrY–dextran.

Counter-current distribution of pre-extracted membranes with PrY–dextran

CCD of the pre-extracted membrane fraction (with affinity for the upper phase) is shown in Fig. 4A. The addition of PrY–dextran to the systems causes a redistribution of the membranes, Fig. 4B and C. At moderate concentrations, 2 μM Procion yellow, three fractions appear and at higher concentrations, 8 μM Procion yellow, practically all material has been moved to a position to the left in the diagram, indicating strong affinity of the material for the lower phase and/or interface. Analytical data for the material in the respective peaks are given in Table IV. At 2 μM PrY–dextran, succinate dehydrogenase is strongly concentrated in the left peak, while the esterase and ATPase are more evenly distributed over the peak. Minor differences in the distribution of the last two enzymes can, however, be seen.

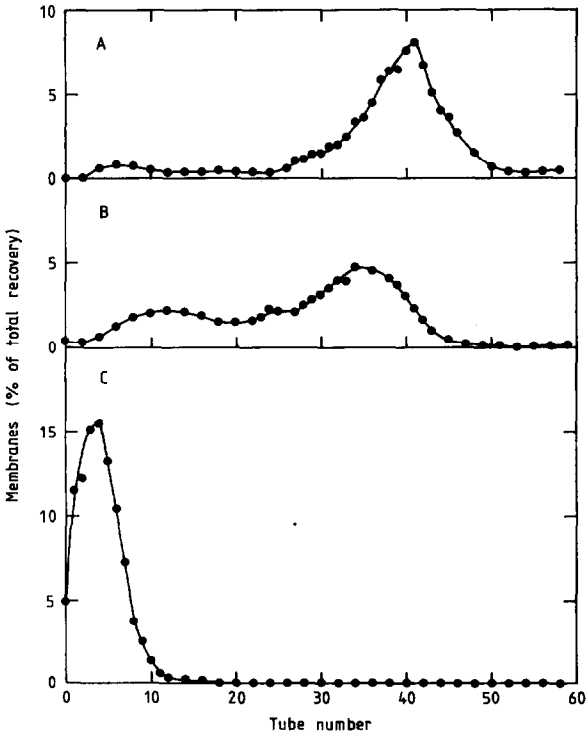

Fig. 4. Effect of PrY–dextran on CCD of pre-extracted membranes (with affinity for the upper phase). System as in Fig. 1 but without PrY–PEG. (A) Without PrY–dextran; (B) 2 μM dextran-bound Procion yellow and (C) 8 μM dextran-bound Procion yellow. The PrY–dextran contained three dye molecules per dextran molecule.

TABLE IV

DISTRIBUTION OF PROTEIN (Pr), SUCCINATE DEHYDROGENASE (SDH), ACETYLCHOLINESTERASE (AChE) AND ATPase WITHIN POOLED FRACTIONS OF THE COUNTER-CURRENT DISTRIBUTION IN FIG. 5

Fraction	*No PrY–dextran*				*2 μM dextran-bound Procion yellow*				*8 μM dextran-bound Procion yellow*			
	% Pr	*% SDH*	*% AChE*	*% ATPase*	*% Pr*	*% SDH*	*% AChE*	*% ATPase*	*% Pr*	*% SDH*	*% AChE*	*% ATPase*
Tubes 3–19	13	63	3	30	30	92	35	30	100	100	100	100
Tubes 20–29					18	8	23	18	–	–	–	
Tubes 30–50	87	37	97	70	52	0	42	52	–	–	–	

DISCUSSION

By use of liquid–liquid extraction, biological membranes can be fractionated according to their surface properties. This fractionation therefore complements the methods based on centrifugation which discriminate according to density and hydrodynamic properties. The aqueous two-phase systems provide two "compartments" (phases) which constitute a mild milieu for biological membranes. The system used in this work, with dextran and Ficoll as the main phase-forming polymers, is in some respects superior to the traditionally used dextran–PEG system[8]. One drawback of the system is the long time for phase separation, due to the similar densities of the phases. The separation time can, however, be reduced to 8–12 min by low-speed centrifugation. The membranes are partitioned between the upper phase, the interface and the lower phase[1,2]. The fact that the phase-forming polymers are mostly concentrated in one phase, dextran in the lower and Ficoll and PEG in the upper phase, also offers the possibility to anchor ligands, with affinity for certain membrane structures, to one of the phases via covalent binding to a polymer. The affinity partitioning of synaptic membranes has been investigated here by using polymer-bound Procion yellow HE-3G.

This dye is able to extract part of the membranes both to the upper phase (when bound to PEG) and to the lower phase (when bound to dextran). Which are the structures on the membranes responsible for the binding of Procion yellow? It is well known that this type of dye, in general, shows affinity for nucleotide binding sites[9,10]. Several membrane constituents have such sites; enzymes with either ATP, NADP or NAD sites are present in the membranes are ATPases, kinases and dehydrogenases. However, nucleotides, when added to the systems, did not compete with the polymer-bound dyes, indicating that the binding sites are less probably of the nucleotide type. The tests were carried out with 0.1 m*M* NAD, NADP, AMP, ATP ($+Mg^{2+}$), ITP ($+Mg^{2+}$) and GTP ($+Mg^{2+}$) at 5 μM polymer-bound Procion yellow (data not shown). Similar dyes have been used for staining of neural tissues[23,24].

The fact that PrY–PEG at over 10–80 μM (Figs. 1 and 2) is less effective and at over 160 μM even reduces the amount of material in the upper phase (compared with a system without ligand) is most probably due to alterations of the membranes. One possibility is that the PrY–PEG causes capping of the proteins due to the fluidity of the membranes. Distinct regions with pronounced affinity for either the upper or the lower phase may be generated on the membrane surface. Such "polarized" particles would be concentrated at the interface where a membrane particle can be partly in both phases. The membranes can also be accumulated at the interface if they form large aggregates which, in turn, may be the result of polarization of the membrane constituents. PrY–dextran did not show the corresponding effect. This difference between the ligand-carrying polymers may be due partly to their molecular weights, and partly to their relative similarity to structures on the surface of the membranes, *e.g.*, polysaccharides.

The effectiveness of the extraction, using the same concentration of dye, depends on the number of ligand molecules per polymer molecule. As demonstrated in Table II, dextran carrying several ligands acts more effectively. This can be compared with the well known chelating effects for metal ions obtained when several ligands are included in one and the same molecule.

The above findings demonstrate that membranes from lysed synaptosomes can be effectively fractionated by partition within an aqueous two-phase system and that the distribution of the various classes of membranes can be affected by including Procion yellow HE-3G in either the upper or lower phase. Too high a concentration of ligand, especially when bound to PEG, may alter the structure of the membranes in such a way that they collect at the interface, eventually in an aggregated form. By using more selective ligands, *e.g.*, for specific receptor sites, the above system will most probably be a valuable tool for selective membrane fractionation. The possibility of very effective separation of two kinds of membranes each carrying one kind of binding site by using two corresponding ligands in opposite phases should be an interesting development of the method.

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REFERENCES

1 P.-Å. Albertsson, *Partition of Cell Particles and Macromolecules*, Almqvist & 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.
3 S.-D. Flanagan, P. Taylor and S. H. Barondes, *Nature (London)*, 254 (1975) 441.
4 S. D. Flanagan, S. H. Barondes and P. Taylor, *J. Biol. Chem.*, 251 (1976) 858.
5 G. Johansson, R. Gysin and S. D. Flanagan, *J. Biol. Chem.*, 256 (1981) 9126.
6 A. Hartman and E. Heilbronn, *Biochim. Biophys. Acta*, 513 (1978) 382.
7 G. Johansson, in E. Reid (Editor), *Methodological Developments in Biochemistry, 2. Preparative Techniques*, Longman, London, 1973, pp. 155–162.
8 G. Johansson, M. Joelsson, B. Olde and V. P. Shanbhag, *J. Chromatogr.*, 331 (1985) 11.
9 P. D. G. Dean and D. H. Watson, *J. Chromatogr.*, 165 (1979) 301.
10 G. Kopperschläger, H. J. Böhme and E. Hofmann, *Adv. Biochem. Eng.*, 25 (1982) 101.
11 K. H. Kroner, A. Cordes, A. Schelper, M. Morr, A. F. Bückmann and M.-R. Kula, in T. C. J. Gribnau, J. Visser and R. J. F. Nivard (Editors), *Affinity Chromatography and Related Techniques*, Elsevier, Amsterdam, 1982, p. 491.
12 G. Johansson, G. Kopperschläger and P.-Å. Albertsson, *Eur. J. Biochem.*, 131 (1983) 589.
13 G. Kopperschläger, G. Lorenz and E. Usbeck, *J. Chromatogr.*, 259 (1983) 97.
14 H.-E. Åkerlund, *J. Biochem. Biophys. Methods*, 9 (1984) 133.
15 G. Johansson and M. Andersson, *J. Chromatogr.*, 303 (1984) 39.
16 G. Johansson and M. Joelsson, *Biotechnol. Bioeng.*, 27 (1985) 621.
17 M. J. López-Pérez, G. Paris and C. Larsson, *Biochim. Biophys. Acta*, 635 (1981) 359.
18 M. M. Bradford, *Anal. Biochem*, 72 (1976) 248.
19 G. L. Ellman, K. D. Courtney, V. Andres and R. M. Featherstone, *Biochem. Pharmacol.*, 7 (1961) 88.
20 D. Earl and A. Korner, *Biochem. J.*, 94 (1965) 721.
21 B. F. Scharschmidt, E. B. Keeffe, N. M. Blankenship and R. K. Ockner, *J. Lab. Clin. Med.*, 93 (1979) 790.
22 G. Johansson, H.-E. Åkerlund and B. Olde, *J. Chromatogr.*, 311 (1984) 277.
23 A. O. W. Stretton and E. A. Kravitz, *Science (Washington, D.C.)*, 162 (1968) 132.
24 N. Suzuki, *Brain Res.*, 311 (1984) 181.