Biochimica et Biophysica Acta, 1029 (1990) 295–302
Elsevier

BBAMEM 75049

Partition of synaptic membranes in aqueous two-phase systems at subzero temperatures by using anti-freeze solvent

Göte Johansson, Monica Joelsson and Björn Olde

Department of Biochemistry, Chemical Center, University of Lund, Lund (Sweden)

(Received 16 May 1990)

Key words: Two-phase system; Subzero partitioning; Synaptic membrane; Acetylcholinesterase; Succinate dehydrogenase

The freezing point of aqueous two-phase (liquid-liquid) systems containing water, dextran and poly(ethylene glycol) has been lowered by including glycerol. Biological membranes, obtained by fragmentation of a crude synaptosomal preparation from calf brain cortex, have been included in the two-phase systems. The effects of temperature and the concentration of glycerol on the partition of the membranes within the systems have been investigated. Considerable stabilisation of the membranes was noticed when they were partitioned at −10 °C compared with 0 °C. The influences of glycerol, ethylene glycol, *N*,*N*-dimethylformamide and tetrahydrofuran on the phase-forming properties of the systems and on enzyme activities are also presented. Possible use of the above systems for studies and separation of biological membranes are discussed.

Introduction

Aqueous two-phase sytems, composed of water, dextran and poly(ethylene glycol) (PEG) have been used for the purification of biological particles by liquid-liquid extraction [1,2]. During the last ten years the introduction of affinity ligands, restricted to one of the liquid phases, has greatly enhanced the selectivity in the partitioning.

In the present work the effects of partial exchange of water for organic (water-soluble) solvents were studied. Aqueous two-phase systems have been used for partition of synaptic and mitochondrial membranes at temperatures down to −10 °C. This sub-zero partition is of interest since it may stabilize the structure of the membranes. Earlier works with synaptic membranes have shown a time-dependent change in the partition which is assumed to be casued by rearrangements of structural elements in the membranes [3,4]. The partition of the enzymes alkaline phosphatase and phosphofructokinase in solvent-containing dextran-PEG systems at sub-zero temperature has been published earlier [5].

Abbreviations: AChE, acetylcholinesterase; LDH, lactate dehydrogenase; SDH, succinate dehydrogenase.

Correspondence: G. Johansson, Department of Biochemistry, Chemical Center, P.O. Box 124, S-221 00 Lund, Sweden.

Materials and Methods

Chemicals

Poly(ethylene glycol) with $M_r = 3000–3800$ (PEG 3400) and with $M_r = 7000–9000$ (PEG 8000) were purchased from Union Carbide, New York, U.S.A. and dextran with $M_r = 40\,000$ (dextran 40) or 500 000 (dextran 5000) from Pharmacia, Uppsala, Sweden. Solvents were of analytical grade. Biochemicals were provided by Sigma, St. Louis, MO. Tresyl chloride (2,2,2-trifluoroethanesulfonyl chloride) was purchased from Fluka (Buchs, Switzerland). Cresyl violet was obtained from Aldrich (Steinheim, F.R.G.). All other chemicals were of analytical reagent grade. Synaptic membranes were prepared from calf brain cortex as described by Hajós [6] with minor modifications. The membranes were passed twice through a Yeda press [7].

Synthesis of ligand-polymers

Cresyl violet was bound to dextran 500 via tresylated dextran [7,8]. A 20% solution of dextran 500 in dimethylsulfoxide containing 3.8% (v/v) triethylamine was slowly mixed with dichloromethane to a final concentration of 16% (v/v). The mixture was cooled on ice and tresyl chloride (0.1 g/g dextran) was added slowly. The reaction was allowed to proceed for 30 min at 0 °C followed by 15 h at 4 °C. The tresyl-dextran was precipitated by the addition of dichloromethane. The obtained precipitate was treated several times with dichloromethane. The polymer was collected and traces of

solvent were removed by flushing the tresyl-dextran with dry nitrogen. The activated dextran (2.5 g) was dissolved in 15 ml of dimethylsulfoxide containing 0.5 g anhydrous solium carbonate and 0.5 g of cresyl violet was added. The reaction took place at room temperature. After 24 h the dye-PEG derivative was precipitated by addition of 50 ml dichloromethane. The precipitate was washed five times with ethanol until the bulk of free dye was removed. The product was dissolved in water and purified by dialysis against hot water (60 °C). The dry polymer was soaked, sequentially, with *N,N*-dimethylformamide, methanol, and ethanol and was then air-dried.

Hexaethonium-PEG 8000 (*N,N'*-pentaethyl-1,6-diaminohexane-PEG) was synthesized as described by Johansson et al. [9].

Two-phase systems

The systems were prepared from stock solutions of dextran (20% (w/w)) and PEG (40% (w/w)), buffer (200 mM Tris-HCl, 40 mM $MgCl_2$), water and organic solvent. Concentrations are given in percentage weight by weight except for salts which are given in mmol per kg system. Membrane suspension in water-solvent mixture (containing 8–16 mg protein per ml) was added to the systems (adjusted to the same temperature) which were equilibrated by careful mixing for 20 s. The systems were left for 15 min to settle at the same temperature and finally centrifuged for 10 min at $1000 \times g$ (same temperature) to obtain clear phases.

Distribution of membranes

The partition of membranes was determined by analysis of samples of mixed system (total material) and of the phases after settling. The percentual recovery of membranes in the two phases was calculated from the volume of the phases and the amount of the interface was obtained as the difference. The samples were diluted 2–21-times and analyzed for membrane content, via light scattering measurements at 405 or 600 nm (the latter in the case of systems containing hexaethonium-PEG), and enzyme activities.

Volume ratio

The ratio between the volumes of the upper and lower phase was determined by equilibrating the systems (10 g) in calibrated graduated cylinders at given temperatures.

Transition point

The concentrations of the two polymers dextran and PEG at which the systems become homogeneous were determined by turbidometric titration at the actual temperature ±0.2 °C. Known weights of systems were titrated under shaking with a mixture of solvent and water (in the same proportion as in the system) until the two phases just disappeared. The final concentrations of polymers were calculated from the total weight.

Enzyme analysis

Acetylcholinesterase (EC 3.1.1.7) was determined according to Ellman et al. [10] and succinate dehydrogenase (EC 1.3.99.1) as described by Earl and Korner [11]. Lactate dehydrogenase (EC 1.1.1.27) was measured at 340 nm as described by Bergmeyer [12].

Freezing point

The freezing points were determined by freezing the systems in acetone-dry ice mixture and by following the temperature at which the gel-like solid was liquified.

Viscosity

The viscosity was determined with an Ubbelodhe capillary viscometer using 2.5 ml phase. The flow time for water was 9.8 s. The temperature was 22 °C.

Time of phase separation

Two-phase systems with a height of 5 cm were mixed and the time for separation was measured. The separation was completed when the mixing zone was reduced to 2 mm.

Results

The aqueous two-phase systems consist of two liquid phases which both have a high content of water, 75–95% (w/w). The water-soluble polymers, used to separate the water into two phases, do not markedly lower the freezing point of the water. The phases freeze between 0 and −1 °C. In order to partition biological membranes at sub-zero temperatures we have replaced various amounts of the water for (water-soluble) organic solvents. The addition of solvent, besides the lowering of the freezing point, also changes the physical properties of the system. Furthermore, the organic solvent, in some cases, causes denaturation of biological materials.

Effect of solvents on phase formation

The influence of solvent on the phase formation, for systems containing dextran and PEG, in the ratio 1 : 0.681, was determined by the change in transition point (going from two phases to one phase), Table I. While tetrahydrofuran and *N,N*-dimethylformamide markedly affected the transition point already at moderate concentrations the two well-known anti-freezing agents, ethylene glycol and glycerol, could to a high degree replace the water without changing the phase formation.

TABLE I

Effect of solvents on phase formation

Transition points (2 → 1 phase) for system consisting of equal amounts of dextran 500 and PEG 8000 in water and solvent. Standard deviation was ± 0.03 units in the percentage concentration scale. The solvent concentration is given as percentage of the total system. Temperature, 22 °C.

Solvent concentration	Concentration of dextran and poly(ethyleneglycol), respectively at the transition point (% (w/w))			
	0	20	40	60
Tetrahydrofuran	3.76	2.35	[a]	[a]
N,N-Dimethyl-formamide	3.76	2.98	1.90	< 0.1
Ethylene glycol	3.76	3.67	3.49	3.00
Glycerol	3.76	3.80	3.79	3.62

[a] Precipitation of dextran.

TABLE II

Effect of viscosity of phases and time for phase separation

System composition: 7% (w/w) dextran 500, 5% (w/w) PEG 8000 and various amount of organic solvent. Temperature, 22 °C. Standard deviation of the viscosity measurements was within ± 5% of the values. The phase separation was studied with systems of 5 cm height. The time for formation of 50% (v/v) bulk phases was determined. Standard deviation: ± 0.5 min.

Solvent (% of weight of system)	Viscosity (relative to water)		Time for 50% phase separation (min)
	top phase	bottom phase	
None	1.6	19	4
20% Ethylene glycol	2.0	34	7
40% Ethylene glycol	2.7	75	12
20% Glycerol	2.1	34	8
20% Dimethyl-formamide	2.0	63	7

Effect of solvent on the enzyme activity of lactate dehydrogenase

Biologically active substances like enzymes may tolerate low concentrations of organic solvents without loosing their catalyzing effect. At these concentration even a minor activation (5–10%) of the enzymes can be observed. The effect of various concentrations of the four solvents on lactate dehydrogenase can be seen in Fig. 1. Glycerol was found to be the most gentle solvent and it could be used up to a concentration of 80% of the weight of the solution.

Viscosity of phases and time for phase separation

For extraction purposes the use of two-phase systems might be limited by extreme time for phase separation or too high viscosity which can seriously reduce the mixing of the two phases and the mass transfer between them. The solvents do increase both viscosity of the phases and the time for phase separation, Table II, but not to such an extent that the practical use of the systems is limited.

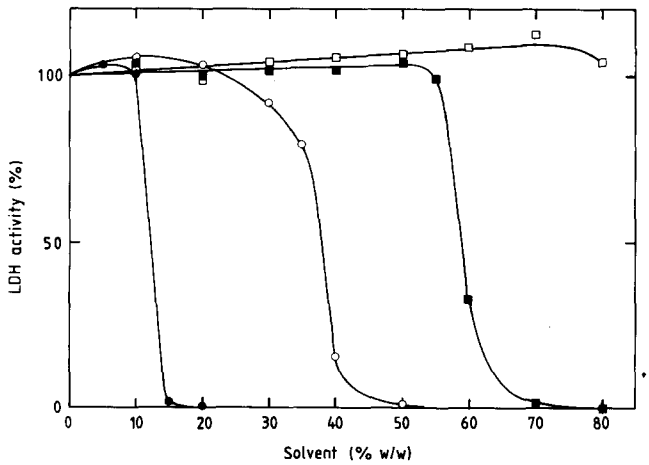

Fig. 1. Effect of organic solvents in water on the enzymatic activity of lactate dehydrogenase (LD). ●, Tetrahydrofuran; ○, *N,N*-dimethyl-formamide; ■, ethylene glycol; and □, glycerol. The solvent mixture contained 25 mM sodium phosphate buffer pH 7.0 and 90 U/ml LDH present in an added muscle extract (2.5%). The enzyme-solvent mixture was incubated one min at 22 °C before activity measurements. Standard deviation: ± 3 percentage units.

Effect of polymer concentration on partition of a soluble enzyme

The inclusion of either ethylene glycol or glycerol in a dextran-PEG two-phase system, Table III, changed only to a low degree the partition of lactate dehydrogenase between the phases. The partition coefficient (equal to the ratio between enzyme activity in upper and lower phase, respectively) varied between 0.06 and 0.18. Dimethylformamide, on the other hand, strongly reduced the partition coefficient. With all three solvents, the partition coefficient of the enzyme was increased 150–900-times by including a PEG-bound affinity ligand for lactate dehydrogenase (Procion yellow HE-3G) [13], Table III.

Effect of glycerol on synaptic membranes

The glycerol was, because of the above results, chosen as a promising candidate for lowering the freezing-point of systems for partition of biological membranes at sub-zero temperatures. The membranes used were obtained by fragmentation of synaptosomes (from calf brain cortex) and these fragments are a mixture of synaptic plasma membranes (nerve endings) and mitochondrial membranes. Common enzyme markers for these kinds of membranes are acetylcholinesterase and succinate dehydrogenase, respectively [14]. For determination of total amount of membranes the amount of membrane protein can be used or the light scattering

TABLE III

Effect of polymer concentration on the partition coefficient, K, of a soluble enzyme

Lactate dehydrogenase (LDH, 4 $U \cdot ml^{-1}$) was partitioned in a system containing 7% (w/w) dextran 500, 5% (w/w) PEG 8000, 25 mM sodium phosphate buffer (pH 7.0) and various amounts of solvent. The partition was made with or without PEG-bound Procion yellow HE-3G (0.05 mM). Temperature, 0 °C. Standard deviation: 5% of the K value.

Solvent	Concn. of solvent (%)	K_{LDH}	
		without ligand	with ligand
Water	0	0.06	37
Ethylene glycol	10	0.08	27
	20	0.08	25
	30	0.10	27
	40	0.14	25
	45	0.16	24
Glycerol	10	0.06	38
	20	0.09	35
	30	0.11	28
	40	0.18	31
N,*N*-Dimethylformamide	5	0.025	24
	10	0.014	13
	15	0.007	4.8
	20	0.0012	1.1
	25	0.0003	0.16

TABLE IV

Effect of glycerol on synaptic membranes

Activity of acetylcholinesterase (AChE) and succinate dehydrogenase (SDH), light scattering and protein of synaptic membranes which had been incubated in water-glycerol mixtures for various periods of time at 0, −10 and 22 °C. Standard deviation: ±3 percentage units.

Temperature (0 °C)	Concn. of glycerol % (w/w)	Time of incubation (min)	Enzyme activity in percentage relative to water at 0 °C, 5 min	
			AChE	SDH
0	0	125	88	72
	0	1240	69	94
	20	125	99	84
	20	1240	81	108
	40	125	92	79
	40	1240	76	89
	60	125	90	76
	60	1240	57	77
−10	20	1080	95	92
	40	1080	78	80
	60	1080	59	74
22	0	155	107	179
	20	155	117	188
	40	155	106	144
	60	155	90	109

TABLE V

Effect of concentration of glycerol on the partition of membranes

Fragmented mixture of synaptosomes and mitochondria was partitioned in two-phase systems composed of 10.1% (w/w) dextran 40, 6.9% (w/w) poly(ethylene glycol) 3400, 2 mM Tris phosphate buffer (pH 7.8), and various concentrations of glycerol. Membrane content: 1.2 g protein per liter system. Temperature, 0 °C. U, upper phase; I, interface and L, lower phase. Standard deviation: ±3 percentage units.

Glycerol (% (w/w))	Partition								
	Protein			SDH			AChE		
	%U	%I	%L	%U	%I	%L	%U	%I	%L
0	49	34	17	7	74	19	60	28	12
5	38	47	15	10	69	21	58	25	17
10	38	49	13	10	66	24	60	20	20
15	38	49	13	11	65	24	59	23	18
20	48	39	13	11	71	18	62	22	16
25	53	38	9	18	70	12	72	10	18

caused by the membranes. The influence of glycerol on the above membrane properties is shown in Table IV.

Effect of concentration of glycerol on partition of membranes

The partition of total membranes as well as the partitions of membranes containing acetylcholinesterase and succinate dehydrogenase, respectively, were not markedly influenced by glycerol when the concentration was increased up to 25% (w/w) of the system, Table V.

Influence of temperature on partition of membranes

The phase transition occurs at lower concentrations of dextran and PEG when the temperature is decreased, Table VI. By knowing the temperature dependence of this point to the binodal curve a two-phase system with corresponding 'distance' from the transition point can

TABLE VI

Change in the position of the transition point (on the binodal curve) with temperature

The systems contained dextran 40 and PEG 3400 in the proportion 1:0.681 as well as 20% (w/w) glycerol and 2 mM Tris phosphate buffer (pH 7.8). The system froze at −18 °C. Standard deviations: ±0.03 percentage units.

Temperature (°C)	System composition	
	% dextran	% PEG
20	9.66	6.58
15	9.58	6.52
10	9.47	6.45
5	9.32	6.35
0	9.10	6.20
−5	8.88	6.05
−10	8.61	5.87
−15	8.30	5.66

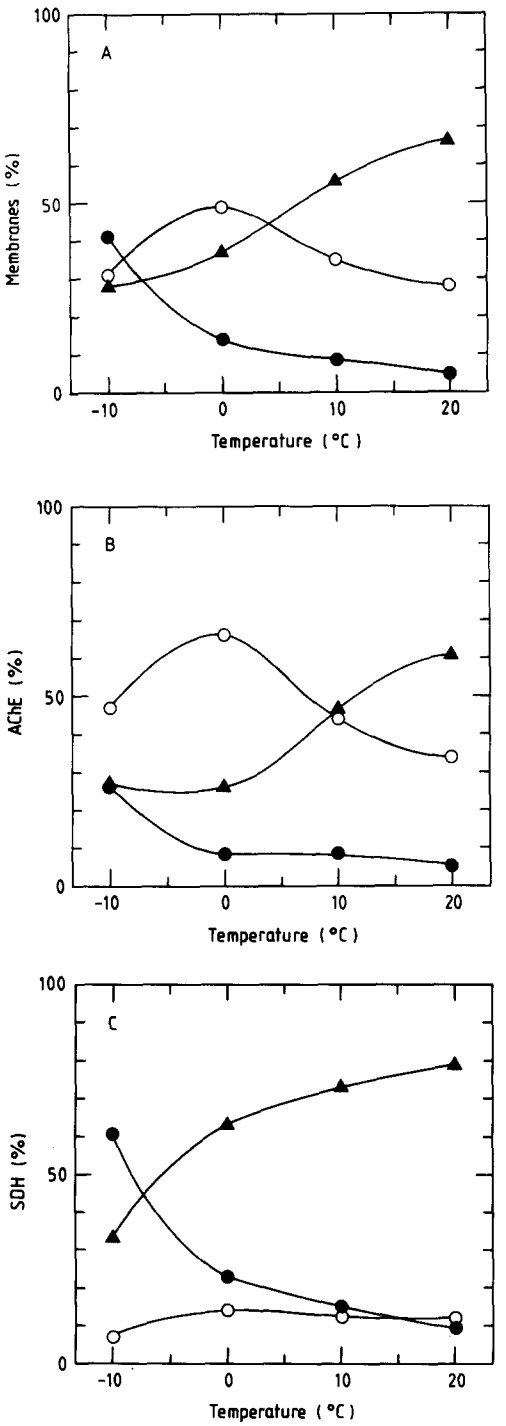

Fig. 2. Influence of temperature on the partition of membranes (fragmented synaptosomes and mitochondria). The polymer composition of the systems were adjusted to the same 'distance' from the transition point. The distribution of total membranes, A, was determined by light scattering measurements. B, distribution of acetylcholinesterase (AChE) and C, distribution of succinate dehydrogenase (SDH). ○, Upper phase; ▲, interface; and ●, lower phase. Standard deviation: ±3 percentage units.

be chosen. The effect of temperature on the partition of membranes in such a series of two-phase systems is shown in Fig. 2.

Effects of ligand-polymers on the partition at various temperatures

The partition of synaptic membranes can be influenced by restricting an affinity ligand to one of the phases [8,9,15]. This is done by covalent attachment of the ligand either to PEG (mainly present in the upper phase) or to dextran (in the lower phase). The effect of the hexaethonium ($(C_2H_5)_3N^+(CH_2)_6N^+(C_2H_5)_2$-) group on the partition of synaptic membranes at two temperatures is shown in Fig. 3. This PEG derivative is known to extract membranes containing cholinergic receptors to the upper phase at low concentrations. Higher concentrations of hexaethonium-PEG, however, cause the membranes to collect at the interface [4,9] (studied in normal water-containing system) and this may be due to lateral rearrangement of the membranes, eventually followed by aggregation. The same phenomenon was also observed when the membranes were partitioned in systems containing 20% glycerol at 0 °C, Figs. 3A–C. It occurred both for synaptic membranes (acetylcholinesterase) and mitochondrial membranes (succinate dehydrogenase). When the partition was carried out at −10 °C, Figs. 3D–F, no increased affinity of the membranes for the interface was observed at high ligand concentration.

A dextran derivative, cresyl violet dextran, was found to cause similar 'aggregating' effects on membranes when used for counter-current distribution of synaptic membranes (unpublished results). When used in the glycerol-containing system the dye (present in the lower phase) extracted membranes towards the interface and the lower phase, Figs. 4A–C. In the case of mitochondrial membranes a redistribution between the interface and the lower phase occurred. When lower temperature (−10 °C) was used, less of the membranes remained on the interface and more of them were extracted into the lower phase, Figs. 4D–F. This again points to a stabilizing effect on the membranes when the temperature is lowered to a subzero level.

Discussion

Aqueous two-phase systems have been used a long time for separation and studies of cell components including proteins, nucleic acids, cell organelles and membranes [1,2]. The systems have generally been used at zero to +4 °C and the partitioning of biological materials has been influenced by addition of salts or by using polymer-bound groups. The results presented in this work show that a considerable part of the water can be replaced by (water-soluble) organic substances like glycerol without damaging effects on the two-phase system or its properties concerning partitioning of membranes. The addition of organic solutes widens the possibilities in variation of important properties. These include partitioning below 0 °C, changes in the dielec-

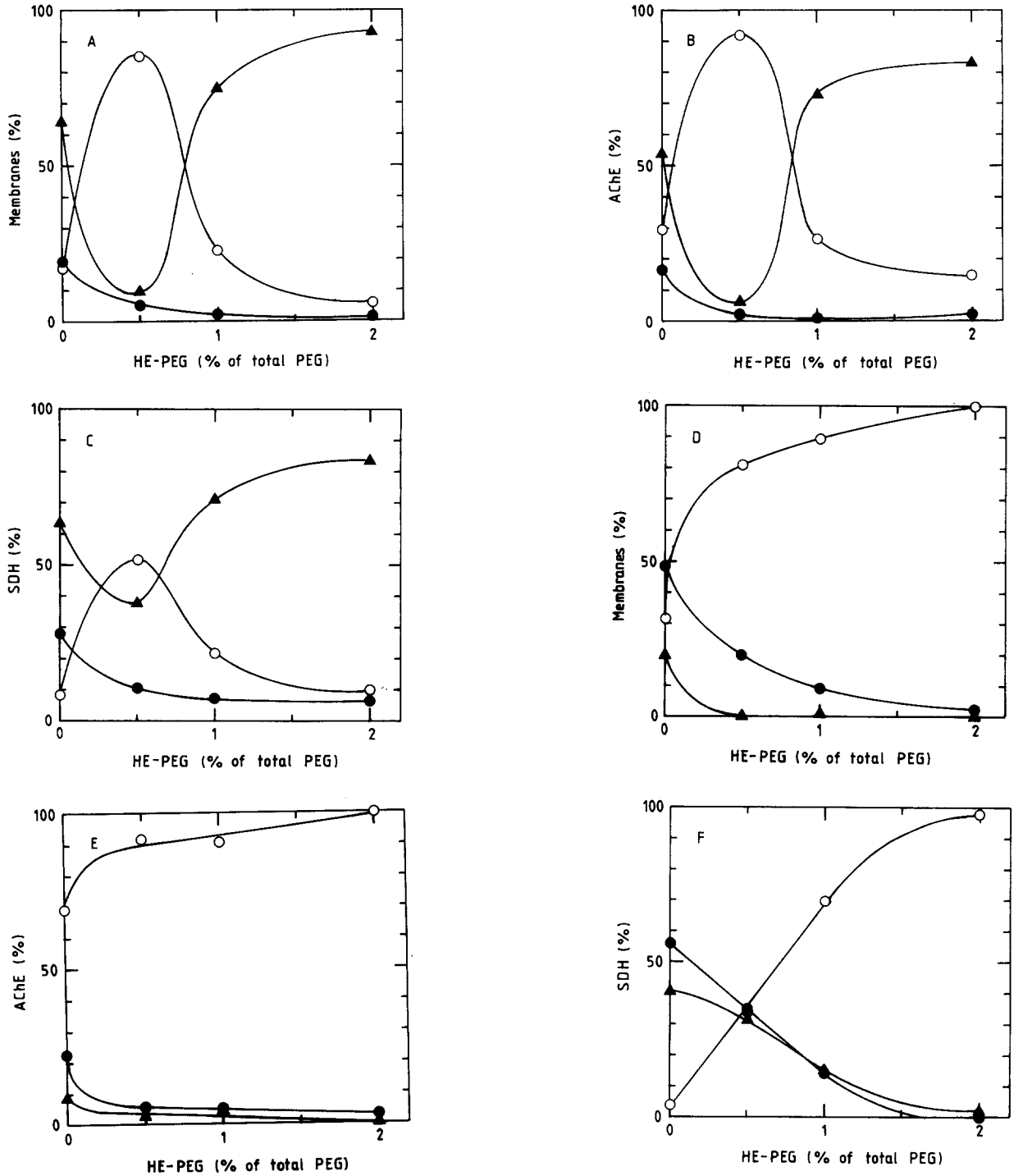

Fig. 3. Extraction of synaptic membranes with polyethyleneglycol-bound affinity ligand, hexaethonium, (HE-PEG). A–C, at 0 °C; D–F, at −10 °C. System composition: 10.1% (w/w) dextran 40, 6.9% (w/w) PEG 3400 at 0 °C and 9.0% (w/w) dextran 40, 6.14% (w/w) PEG 3400 at −10 °C. All systems also contained various amount of crosslinked HE-PEG 8000, 20% (w/w) glycerol and 2 mM Tris phosphate buffer (pH 7.8). Total concentration of membranes corresponded to 1 g protein per liter system. The distribution was determined for light scattering (total membranes), A and D; acetylcholinesterase (AChE), B and E; and succinate dehydrogenase (SDH), C and F. ○, Upper phase; ●, lower phase; and ▲, interface. Standard deviation: ±3 percentage units.

tric constant of the two phases, influence on interaction between (polymer-bound) ligand and membrane receptors and adjustment of association-dissociation equilibria between membrane protein components. The addition of co-solutes increases the possibilities to let the liquid phases mimic the solvent properties in various compartments of the living cell. By using the phase systems at low (subzero) temperature the biological

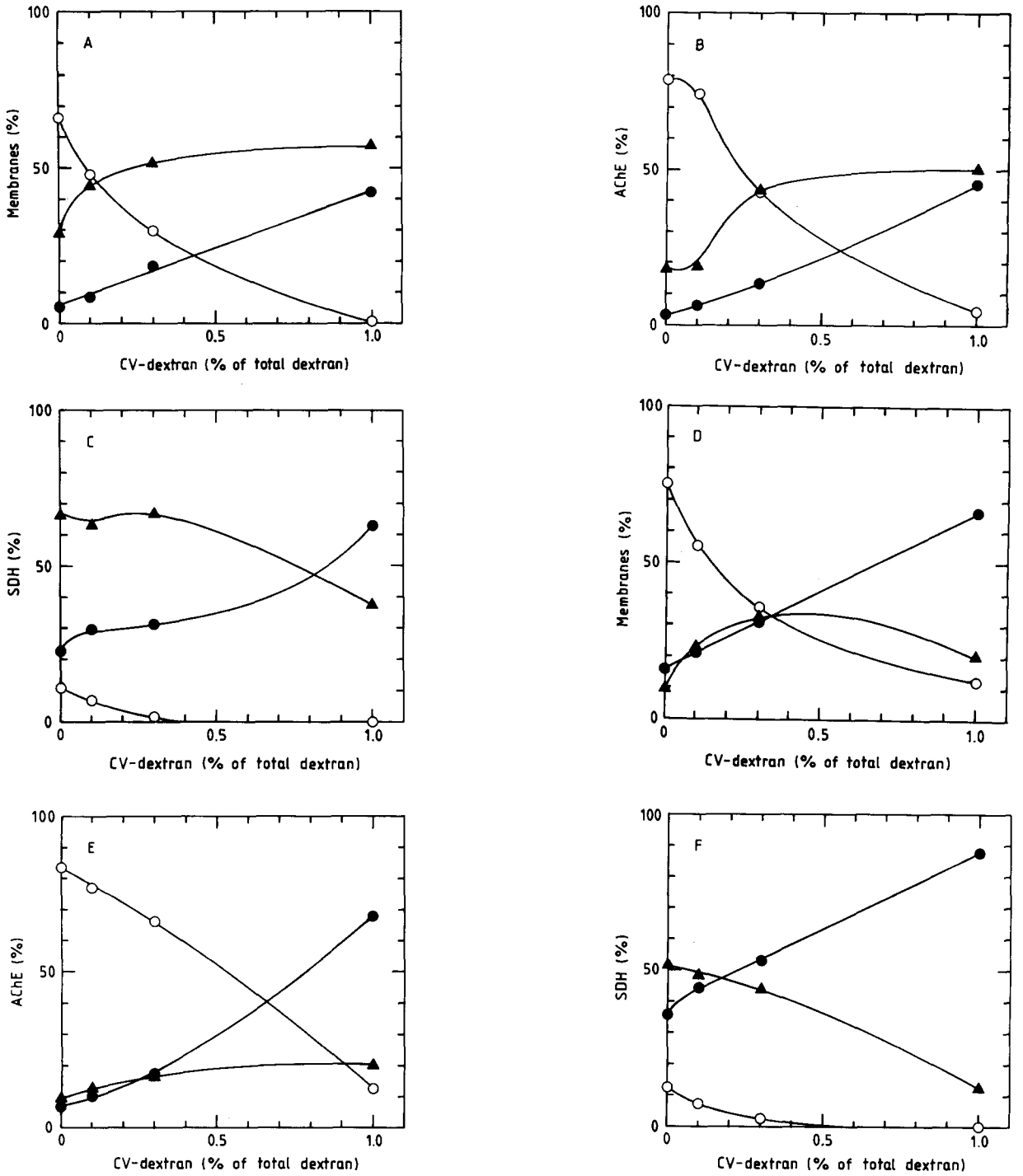

Fig. 4. Extraction of synaptic membranes with dextran-bound dye ligand, cresyl violet (CV-dextran), A–C, at 0 °C; D–F, at −10 °C. System compositions: as in Fig. 3 but with 0.1% of the PEG in form of HE-PEG and with various amount of CV-dextran 500. The distribution was determined for light scattering (total membranes), A and D; acetylcholinesterase (AChE), B and E; and succinate dehydrogenase (SDH), C and F. Symbols as in Fig. 3. Standard deviation: ±3 percentage units.

membranes might also be kept in 'frozen' conformational states which could even be separated from each other by partitioning.

Acknowledgement

Financial support from NFR, the Swedish Natural Science Research Council, is acknowledged.

References

1 Albertsson, P.-Å. (1986) Partition of Cell Particles and Macromolecules, 3rd Edn., Wiley, New York.
2 Walter, H., Brooks, D.E. and Fisher, D. (1985) Partitioning in Aqueous Two-Phase Systems: Theory; Methods; Uses; and Applications to Biotechnology, Academic Press, Orlando.
3 Flanagan, S.D., Johansson, G., Yost, B., Ito, Y. and Surtherland, I.A. (1984) J. Liquid Chromatogr. 7, 385–402.

4 Johansson, G. (1986) J. Chromatogr. 361, 131–140.
5 Johansson, G. and Kopperschläger, G. (1987) J. Chromatogr. 388, 295–305.
6 Hajos, P. (1975) Brain Res. 93, 485–489.
7 Nilsson, K. and Mosbach, K. (1981) Biochem. Biophys. Res. Commun. 102, 449–457.
8 Olde, B. and Johansson, G. (1985) Neuroscience 15, 1247–1253.
9 Johansson, G., Gysin, R. and Flanagan, S.D. (1981) J. Biol. Chem. 256, 9126–9135.
10 Ellman, G.L., Courtney, K.D., Andres, V. and Featherstone, R.M. (1961) Biochem. Pharmac. 7, 88–95.
11 Earl, D. and Korner, A. (1965) Biochem. J. 94, 721–734.
12 Bergmeyer, H.U. (1970) Methoden der enzymatischen Analyse, Vol. 1, 2nd Edn., pp. 441–442, Verlag Chemie, Weinheim.
13 Johansson, G. and Joelsson, M. (1986) Applied Biochem. Biotechnol. 13, 15–27.
14 Whittaker, V.P. (1983) in Handbook of Neurochemistry (Lajtha, A., ed.), 2nd Edn., pp. 2–35.
15 Johansson, G., Åkerlund, H.-E. and Olde, B. (1984) J. Chromatogr. 311, 277–289.