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AFFINITY PARTITIONING OF BIOPOLYMERS AND MEMBRANES IN FICOLL–DEXTRAN AQUEOUS TWO-PHASE SYSTEMS

GÖTE JOHANSSON*, MONICA JOELSSON and BJÖRN OLDE
Department of Biochemistry, Chemical Center, University of Lund, Lund (Sweden)
and
VITHALDAS P. SHANBHAG
Department of Biochemistry, University of Umeå, Umeå (Sweden)
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SUMMARY

The usefulness of an aqueous two-phase system composed of water and the two polymers dextran and Ficoll for affinity partitioning of proteins, nucleic acids, synaptic membranes, and the thylakoid membrane system of chloroplasts was studied. In contrast to the widely used dextran–poly(ethylene glycol) two-phase system the two phases of the present system have very similar properties. The partition coefficients of proteins are close to unity. If polymer-bound ligands are introduced into one phase, ligand-binding biopolymers and particles can be extracted into that phase. Ligands, including fatty acids, diamines, triazine dyes and pharmaceutical substances (with affinity for opiate receptors), have been bound to dextran, Ficoll or poly(ethylene glycol). When used in small amounts, poly(ethylene glycol) partitions into the Ficoll-rich phase. The solubility of proteins in these two-phase systems is higher than in dextran–poly(ethylene glycol) systems, and also the tendency for membranes and cell particles to accumulate at the interface is markedly reduced.

INTRODUCTION

Aqueous two-phase systems composed of dextran and poly(ethylene glycol) (PEG) have found extensive use both for purification and analysis of biomaterials[1–3]. In this system, proteins are usually concentrated in the lower dextran-rich phase. The solubility of proteins in these systems is limited by the presence of PEG[4], which in higher concentrations has been used for precipitation of proteins[5,6]. Membranes and membrane-bound particles[2,7] often favour the upper PEG-rich phase when the system is very close to its critical point, *i.e.* when a minimal dilution of the system transforms it into a homogenous solution. A moderate increase in the concentration of polymers, however, leads to an accumulation of the particles at the interface between the two phases[1,2,7]. Both enzymes[8–13] and membranes enriched in acetylcholine receptors[14,15] have been purified by selective extraction of these materials into the upper phase using biospecific ligands covalently bound to PEG and therefore en-

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riched in the upper phase. In the case of synaptic membranes from brain[16], as well as cholinergic membranes from the electroplax of *Torpedo californica*[17], a time-dependent change in the partition of the material between the upper phase and the interface indicates rearrangements in the membrane structure.

To avoid the above drawback of the dextran–PEG systems, we have tested a two-phase system composed of mainly water, dextran and Ficoll for affinity partitioning purposes. Similar systems have been used by Pestka *et al.*[18] to partition of ribosomes, and by Zaslavsky *et al.*[19,20] to estimate the hydrophobicity of a number of biological molecules. We found partitioning to be influenced by the ligands attached to either dextran or Ficoll, and by the addition of ligand–PEG derivatives.

MATERIALS AND METHODS

Chemicals

Ficoll 70, Ficoll 400, dextran 40 and dextran 500 were purchased from Pharmacia (Uppsala, Sweden). PEG with M_r = 6500–8000 was obtained from Union Carbide (New York, U.S.A.). Ligands were attached to PEG as described elsewhere[15,21–23]. Triazine dyes were bound to dextran and Ficoll using the method described by Johansson and Andersson[24]. All biochemicals were obtained from Sigma (St. Louis, MO, U.S.A.). Salts and buffer substances were of analytical grade.

Proteins

Serum albumin (human) grade V was obtained from Sigma. Spectrin and calmodulin were prepared from bovine brain[25,26]. The latter was radioactivally labelled with ^{14}C according to Dottavio-Martin and Ravel[27]. Protein extract from bakers' yeast was prepared as described before[13].

Nucleic acids

These were purchased from Sigma: RNA from calf liver, prepared by phenol extraction according to Kirkby[28], and high-molecular-weight DNA from calf thymus.

Synaptic membranes from calf brain

The membranes were mainly prepared according to Hajós[29] with minor modifications[16]. Before partition, the membranes (in water) were passed twice through a Yeda press[30].

Thylakoid membranes

These class II chloroplasts were prepared from spinach leaves (*Spinacia oleracea* L.) as described by Andersson *et al.*[30].

Two-phase systems

Stock solutions of polymers (20–40% w/w) were weighed out together with buffer, salts and PEG solution (40% w/w) and (after mixing) the sample material. The final compositions of the systems are given at the appropriate points in the text. The systems were equilibrated at either 0 or 23°C and carefully mixed. Centrifugation for 10 min at 1000 *g* was used (at the same temperature) to speed up the separation.

Samples for analysis were withdrawn from the phases, avoiding eventual material present at the interface. When membranes and particles were partitioned, samples were also taken from the mixed system before settling.

Analysis

Protein was determined according to Bradford[31], glucose 6-phosphate dehydrogenase according to Noltmann *et al.*[32] and nucleic acids photometrically at 260 nm using a double-beam Hitachi 100-60 spectrophotometer. ^{14}C activity was determined with a Searle Mark III scintillator using 0.5-ml samples in 5-ml scintillation cocktail containing 2.2 g of Omnifluor (New England Nuclear, Boston, U.S.A.) per litre of toluene–triton X-100 (2:1, v/v). Membranes were determined by measurement of the light scattering taken as the apparent absorbance at 400 or 550 nm using the spectrophotometer mentioned above.

Description of the partition

The partition of proteins and nucleic acids is described by the partition coefficient, K, defined as the ratio between the concentrations in upper and lower phase, respectively. For membranes and particles the partition is given as the percentage of material in upper phase, at the interface and in the lower phase. For each system composition, it is indicated which phase contained the main part of Ficoll (together with eventual small additions of PEG derivatives). The two phases differ only slightly in density and, depending on polymer concentrations and temperature, the dextran-rich phase is either the lighter or the denser phase.

Solubility of proteins

An 0.85 g amount of a 20% polymer solution or phase from a two-phase system was mixed at 23°C with 0.15 g of a solution of rabbit γ-globulin (50 g/l). The mixture was incubated for 15 min, followed by centrifugation for 10–120 min, depending on the viscosity, at 2250 *g*. The protein concentration was determined in the supernatant as described above.

RESULTS

Proteins

Three proteins, human serum albumin, spectrin and calmodulin, have been partitioned in a system containing 11% Ficoll 400, 7% dextran 500 and 0.1% PEG (Table I). In this system Ficoll and PEG were mainly in the lower phase. In the absence of ligand both albumin and calmodulin showed partition coefficients close to unity. Spectrin, on the other hand, showed a high affinity for the dextran-rich phase with $K = 39$. Another cytoskeletal protein, actin, partitioned with $K = 1.1$–1.7 (F-actin) and $K = 1.6$–2.6 (G-actin) depending on the protein concentration.

In contrast to dextran–PEG two-phase systems, the cytoskeletal proteins showed no tendency to precipitate. If PEG (0.1%) carrying certain ligands (Table I) was used, the proteins could in some cases be extracted into the lower phase. Quaternized long-chain α,ω-diamines were especially active in extracting spectrin and calmodulin. The affinity partitioning effect on calmodulin could not be reduced by excluding Ca^{2+} from the system, as was possible for the effect caused by PEG palmitate.

TABLE I

PARTITIONING OF HUMAN SERUM ALBUMIN, SPECTRIN AND CALMODULIN

System: 11% Ficoll 400, 7% dextran 500 and either 0.1 or 0.5% PEG (with or without ligand) at 23°C. Buffers: for albumin, 25 m*M* sodium phosphate buffer (pH 7.0); for spectrin and calmodulin, 28 m*M* HEPES–HCl, 0.28 m*M* magnesium chloride, 1 m*M* calcium chloride, 2.8 m*M* EGTA (pH 7.5). Ficoll and polymer-bound ligand in lower phase.

PEG-bound ligand	*K*			
	Human serum albumin (4 g/l)		*Spectrin (0.15 g/l), 0.1% PEG*	*Calmodulin (3.6 g/l), 0.1% PEG*
	0.1% PEG	*0.5% PEG*		
–	0.92	0.88	39	1.40
Palmitate	0.56	0.43	16.5	0.69
Tetraethonium	0.95	1.01	N.D.*	0.33
Octaethonium	0.88	N.D.	N.D.	0.075
Dodecaethonium	0.89	1.12	0.26	0.044
Tetra(diethonium)	0.91	0.93	20	0.46
Cibracon blue F3G-A	0.46	0.26	16.1	1.79

* N.D. = not determined.

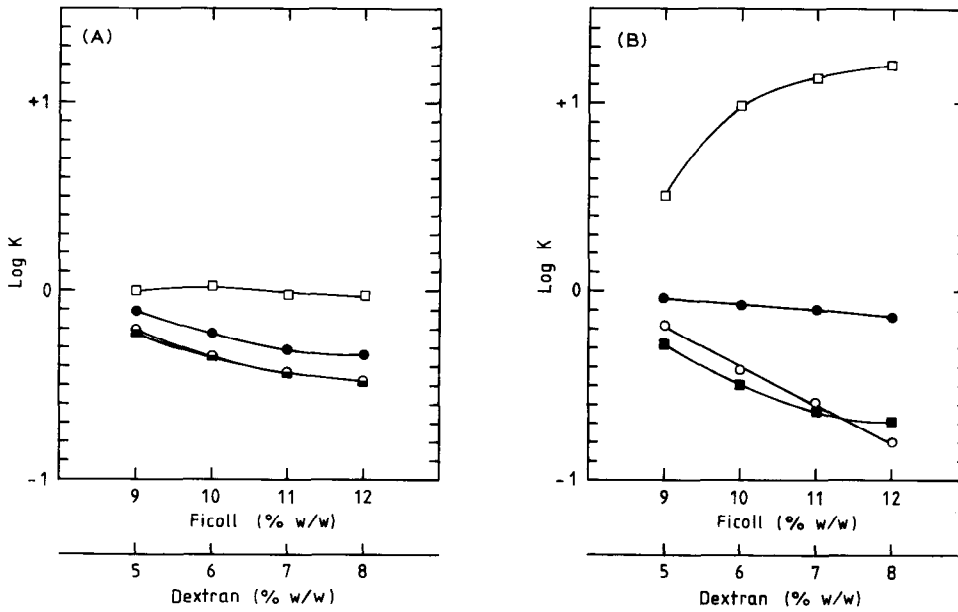

Fig. 1. Partition of a protein extract from yeast using polymer-bound Procion yellow HE-3G (Pr). (A) Partition coefficient, *K*, of total protein; (B) partition coefficient, *K*, of glucose 6-phosphate dehydrogenase. System: Ficoll 400, dextran 500, 0.1% PEG 6000 and 25 m*M* sodium phosphate buffer (pH 7.0). Temperature, 23°C. Data points: ● = no ligand; □ = Pr dextran (0.41 μmol dye per g dextran); ■ = Pr Ficoll (0.28 μmol dye per g Ficoll); ○ = Pr polyethyleneglycol (100% of total PEG, 0.14 mmol dye per g PEG). Ficoll in lower phase.

TABLE II

PARTITIONING OF NUCLEIC ACIDS

System: 11% Ficoll 400, 7% dextran 500 and 0.1% PEG at 23°C. DNA from calf thymus and RNA from calf liver (A). Concentration of nucleic acid in the system corresponds to $A_{1\,cm}^{260}$ = 3.6–3.8. Ficoll in lower phase.

Salt composition	*Nucleic acid*	*Percentage distribution*		*K*
		Top phase	*Bottom phase*	
10 m*M* Sodium phosphate buffer	DNA	98	2	44
(pH 7.0)	RNA	59	41	1.45
10 m*M* Lithium phosphate buffer	DNA	3	97	0.033
(pH 7.0)	RNA	49	51	0.96
10 m*M* Sodium phosphate buffer	DNA	99	0.5	200
(pH 7.0) and 25 m*M* potassium chloride	RNA	71	29	2.5

The total protein present in an extract of bakers' yeast partitioned with an average K value close to unity. The partition was slightly dependent on the concentration of phase-forming polymers (Fig. 1). Glucose 6-phosphate dehydrogenase, present in the extract, was extracted into the upper phase by using Procion yellow HE-3G bound to dextran. The same enzyme could equally well be extracted into the lower phase by binding the same dye either to Ficoll or to PEG.

Nucleic acids

The partitioning of DNA was strongly dependent on the salt composition of the system K = 0.03–200, whereas the partitioning of RNA was only moderately

TABLE III

PARTITIONING OF DNA FROM CALF THYMUS

System: 11% Ficoll 400, 7% dextran 500, 0.125% PEG (with or without ligand) and 27.5 m*M* sodium phosphate buffer (pH 7.0). Temperature, 23°C. Concentration of DNA in the system corresponded to $A_{1\,cm}^{260}$ = 7.6. Ficoll and polymer-bound ligand in lower phase.

*PEG-bound ligand**		*Percentage distribution*		*K*
		Top phase	*Bottom phase*	
–		93	7	14
$Et_3N(CH_2)_2NEt_2$	Diethonium	94	6	15
$Et_3N(CH_2)_3NEt_2$	Triethonium	70	30	2.3
$Et_3N(CH_2)_4NEt_2$	Tetraethonium	63	37	1.7
$Et_3N(CH_2)_7NEt_2$	Heptaethonium	59	41	1.5
$Et_3N(CH_2)_9NEt_2$	Nonaethonium	64	36	1.8
$Et_3N(CH_2)_{12}NEt_2$	Dodecaethonium	61	39	1.5
$Et_3N[(CH_2)_2NEt_2]_2$	Di(diethonium)	94	6	14
$Et_3N[(CH_2)_2NEt_2]_3$	Tri(diethonium)	77	23	3.3
$Et_3N[(CH_2)_2NEt_2]_4$	Tetra(diethonium)	62	38	1.7

* Et = ethyl.

affected (K = 0.9–2.5) (Table II). The effect of polymer-bound ligands (Table III) was therefore studied under constant salt conditions and with excess of electrolytes (compared with the ligand concentration). RNA partitioned with a K value close to 1.3 and the partition was not effected by any of the ligands tested. High-molecular-weight DNA, on the other hand, showed high affinity for the dextran-rich phase, K > 1. Ligands in the lower phase reduced the partition coefficient of DNA down to a value around unity.

Synaptic membranes

The partitioning of these membranes in the Ficoll–dextran system differed in some important respects from the partitioning in the normally used dextran–PEG system. An important finding was that the membranes accumulated at the interface to a lesser extent in the Ficoll–dextran system (Table IV). In both systems the percentage that collected at the interface increased markedly with increasing concentration of membranes (Table IV).

The dependence of the partitioning on the salt concentration was more pronounced in the Ficoll–dextran system (Fig. 2) where less than 1 mM potassium chloride changed the partitioning almost totally to the lower phase plus the interface. Lithium phosphate, on the other hand, effectively extracted the membranes into the upper phase (not shown).

Ligands that are known to bind to synaptic membranes (Table V) have been coupled to PEG. Two of them, naloxone and naltrexone (binding to opiate receptor sites), are highly potent in extracting the membranes into the Ficoll-rich phase.

Procion yellow HE-3G, a dye known to bind to ATP, NAD and NADP binding sites, was found to be effective (in the polymer-bound state) in extracting the membranes into the dextran-rich phase (Table VI). Only very small amounts of ligand were necessary to affect this change in the partitioning. The same was true for the extraction of membranes into the Ficoll-rich phase with the aid of naltrexone PEG (Table V). Addition of very small amounts of ATP-Mg^{2+} reduced this effect by around one third.

TABLE IV

PARTITIONING OF SYNAPTIC MEMBRANES FROM CALF BRAIN IN TWO SYSTEMS OF DIFFERENT POLYMER COMPOSITIONS

System A: 11% Ficoll 70, 8.5% dextran 40, 2% PEG 6000 and 2.5 mM Tris–phosphate buffer (pH 7.4); Ficoll in upper phase. System B: 8.8% dextran 40, 5.9% PEG 4000, 2.5 mM Tris-phosphate buffer (pH 7.4) and 1 mM potassium chloride. Temperature, 0°C.

Concentration of membranes (grams of protein per litre of system)	*System A*			*System B*		
	In upper phase (%)	*In lower phase (%)*	*At interface (%)*	*In upper phase (%)*	*In lower phase (%)*	*At interface (%)*
0.06	50	35	15	39	25	36
0.10	45	34	21	33	25	42
0.20	33	35	32	29	16	55
0.29	36	34	30	30	15	55
0.39	26	38	36	28	15	57

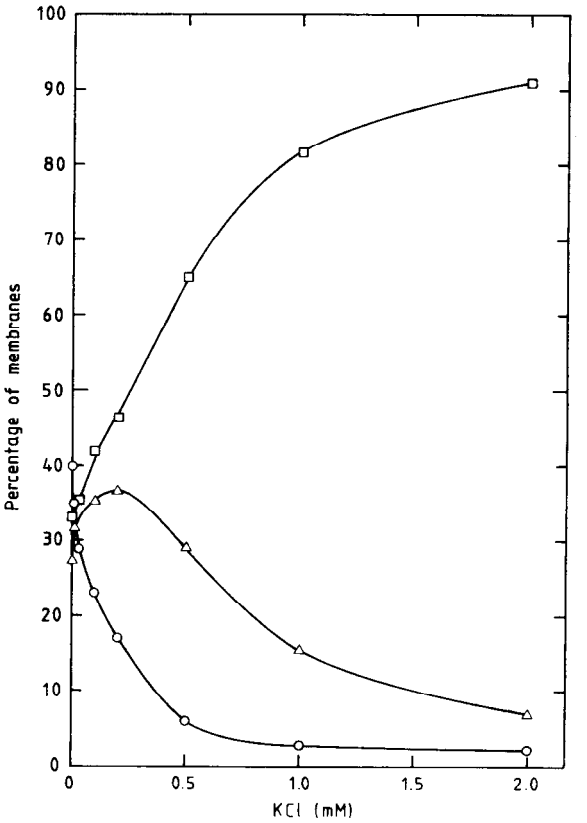

Fig. 2. Effect of potassium chloride on the partitioning of synaptic membranes. System: 11% Ficoll 70, 8.5% dextran 40, 2% PEG 6000, 2.5 m*M* Tris-phosphate buffer (pH 7.4) and various amounts of potassium chloride. Membrane concentration: 0.2 g protein per litre. Temperature, 0°C. Data points: ○ = upper phase; □ = lower phase; △ = interface. Ficoll in upper phase.

TABLE V

EFFECT OF LIGAND–PEG DERIVATIVES ON PARTITIONING OF SYNAPTIC MEMBRANES FROM CALF BRAIN

Systems: 11% Ficoll 70, 8.5% dextran 40, 2% PEG 6000, 2.5 m*M* Tris–phosphate buffer (pH 7.4), and 0–0.05% ligand–PEG 6000. Temperature, 0°C. Membrane concentration of protein, 0.2 g/l. Ficoll and polymer-bound ligand in upper phase.

PEG-bound ligand	*Amount of material (%)*		
	In top phase	*In bottom phase*	*At interface*
–	45	23	32
Naltrexone (0.006%)	62	24	14
Naltrexone (0.012%)	74	17	9
Naltrexone (0.025%)	88	10	2
Naltrexone (0.050%)	94	5	1
Naloxone (0.050%)	95	4	1

TABLE VI

EFFECT OF DEXTRAN-BOUND PROCION YELLOW HE-3G (Pr Dx), PRESENT IN THE UPPER PHASE, ON PARTITIONING OF SYNAPTIC MEMBRANES FROM CALF BRAIN

Systems as in Table V. Temperature, 23°C. Concentration of membranes: 0.2 g per litre of system. Light scattering measured at 550 nm. Ficoll in lower phase.

Pr Dx (% of total system)	*Amount of material (%)*		
	In top phase	*In bottom phase*	*At interface*
–	10	90	–
0.09	24	9	67
0.36	52	4	44
0.72	58	5	37

Thylakoid membrane system (broken or class II chloroplasts)

In Ficoll–dextran systems without ligands (Table VII), these large membrane systems partitioned into the lower (Ficoll-rich) phase. They were extracted into the upper (dextran-rich) phase by Procion yellow HE-3G dextran, but even a relatively high concentration of potassium chloride had only a minimal effect on the partition. The effectiveness of the ligand could be strongly increased by lowering the temperature to 0°C. Under these conditions the thylakoid systems were completely in the upper phase at concentrations of ligand dextran higher than 0.3% (of total system).

Solubility of proteins

The solubility of γ-globulin depends of the kind of polymer present in the solution (Table VIII). The best solubility was found with Ficoll. Also when Ficoll–dextran and dextran–PEG systems were compared (Table VIII), the former showed better solvation properties.

TABLE VII

PARTITIONING OF THYLAKOID MEMBRANES (CLASS II) FROM SPINACH CHLOROPLASTS

System: 11% Ficoll 400, 7% dextran 500, 0.1% PEG 6000 and 27.5 m*M* sodium phosphate buffer (pH 7.0). Temperature, 23°C. The apparent absorbance at 550 nm due to the membranes was 0.6/cm. Ficoll in lower phase. Pr Dx = Procion yellow HE-3G dextran 500 (in upper phase).

Included substance	*Amount of material (%)*		
	In top phase	*In bottom phase*	*At interface*
–	3	97	–
0.09% Pr Dx	18	42	40
0.36% Pr Dx	44	6	50
0.72% Pr Dx	72	7	21
50 m*M* Potassium chloride	4	92	4

TABLE VIII

SOLUBILITY OF RABBIT γ-GLOBULIN IN POLYMER SOLUTIONS AND IN THE PHASES OF TWO-PHASE SYSTEMS

All solutions contained 50 m*M* potassium acetate buffer (pH 5.0). Temperature, 23°C. System A: 11% Ficoll 400, 7% dextran 500 and 0.1% PEG 6000, Ficoll in lower phase. System B: 7% dextran 500 and 5% PEG 6000; 0.85 g of phase plus 0.15 g of γ-globulin solution (50 g/l).

Polymer solution	*Solubility*	
	g/l	*%*
–	0.63	100
15% PEG 6000	0.038	6
15% PEG 4000	0.045	7
15% Dextran 500	0.29	40–45
15% Dextran 40	0.26	41
15% Ficoll 400	0.36	57
15% Ficoll 70	0.36	57
System A: upper phase	0.34	53
lower phase	0.38	61
System B: upper phase	0.23	37
lower phase	0.30	48

DISCUSSION

Aqueous two-phase (liquid–liquid) systems have been used in biochemical and medical research for 25 years, both for separation and for analytical purposes. Systems containing dextran and PEG have been preferentially used, because of the rapid separation of the two phases after mixing and their relatively low viscosities[1]. The drawbacks are the negative influence of PEG on the solubility of proteins[4–6], and the possible rearrangement of the membrane structure[22], as well as the high price of fractionated dextran. The cost factor makes these systems less attractive for use in large-scale processes. It is therefore of general interest to study the properties of alternative two-phase systems. Of special interest is the usefulness of such systems for affinity partitioning; a kind of selective extraction achieved by covalent attachment of (more or less) specific affinity ligands to one of the phase-forming polymers.

The results show that both biopolymers and biomembranes partition more equally between the phases in Ficoll–dextran than in dextran–PEG. The partitioning can be influenced in both systems either by addition of salt or by using suitable polymer-bound ligands. In the dextran–PEG system the latter polymer has usually been used as the ligand carrier, since the partitioned material is normally found in the lower dextran-rich phase (or at the interface). It has been shown in this work that biomaterial in the Ficoll–dextran system can successfully be extracted into either of the two phases by binding the ligand to the respective polymers. Also, the ligand–PEG derivatives, of which a large number have been described[33,34], can be used for affinity partitioning in the same system since they are concentrated in the Ficoll-rich phase. The fact that the affinity partitioning effect is more pronounced when the

ligand is bound to dextran (Fig. 1) can be explained by the more extreme partitioning of dextran between the phases[1].

For the oligoethonium series (Table III), an effect on the partitioning of DNA is observed when the ligand is triethonium or longer. In the oligo(diethonium) series (Table III), tri(diethonium) or longer ligand is required to get the corresponding effect. Thus, the effectiveness of the ligand obviously depends on the spacing between the nitrogen atoms. Calculations based on bond length of C–C (1.54 Å) and C–N (1.39 Å) and bond angles (108–112°) indicate that the minimal spacing required is 5 Å for oligoethonium and 11 Å for oligodiethonium. These distances match the stretch between neighbouring, 5–6 Å, or every third, 11–12 Å, phosphate group, respectively, in double-stranded DNA, as estimated from models of the DNA helix, *e.g.* in ref. 35. This indicates the importance of spacing between atoms of the ligand responsible for the interaction with a macromolecule or, conversely, yields a measure of the spacing between the atoms in the macromolecule responsible for the interaction.

The positive effect of triazine dyes, used as polymer-bound ligands, in affecting the partition of membranes and large membrane systems (class II chloroplasts) with ATP binding sites, has been shown for the first time in this work. Previous attempts to demonstrate this kind of affinity partitioning have either led to failure, or else only moderate effects have been seen very close to the critical point of the system. Affinity partitioning by using specific ligands will most probably play an important role in future processes for the isolation of membranes and organelles with specific binding sites. Besides the specificity, this extraction method also has the advantage that it can easily be scaled-up[36].

The Ficoll–dextran system is more favourable than the corresponding dextran–PEG system in a number of ways. The solubility of protein is higher (Table VIII), less material collects at the interface when particles are partitioned (Table IV), and this might also reduce the negative effect noticed for synaptic membranes[16]. Since PEG is known to cause protein capping in membranes, the reduced concentration of this polymer could possibly enhance the properties of the two-phase systems when used for membrane partitioning.

One disadvantage of the Ficoll–dextran system is that both polymers are expensive to use on a technical scale. The search for cheaper polymers with properties similar to these of dextran and Ficoll is therefore of great importance.

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