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Partitioning of proteins and thylakoid membrane vesicles in aqueous two-phase systems with hydrophobically modified dextran

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Abstract

Dextrans were modified with hydrophobic groups, *i.e.* benzoyl and valeryl groups. Benzoyl dextran and valeryl dextran form aqueous two-phase systems with poly(ethylene glycol) (PEG) as well as with dextran. Two-phase systems are also formed between two valeryl dextrans with different degrees of substitution as well as between benzoyl dextran and valeryl dextran. Phase diagrams for aqueous two-phase systems composed of PEG–valeryl dextran, dextran–valeryl dextran, valeryl dextran–valeryl dextran and valeryl dextran–benzoyl dextran have been determined. The effects of these hydrophobic groups on the partitioning of amino acids, proteins and membrane vesicles in aqueous two-phase systems have been studied. In a PEG 8000–dextran T500 aqueous two-phase system containing only phosphate buffer β-galactosidase was partitioned mostly to the top phase. However, by introducing a small amount of benzoyl groups (degree of substitution 0.054) or valeryl groups (degree of substitution 0.12) in the lower phase, the partition coefficient of this enzyme could be decreased by more than 100 times. A similar, but weaker, effect on partitioning was observed for bovine serum albumin, lysozyme, lipase and β-lactoglobulin. The partitioning of thylakoid membrane vesicles was strongly affected by the hydrophobic groups on dextran. The membrane vesicles were partitioned toward the phase containing the hydrophobic groups.

1. Introduction

Aqueous polymer two-phase systems formed of poly(ethylene glycol) (PEG) and dextran are widely used for separation and purification of macromolecules, membranes, cell organelles and cells [1]. In some applications of aqueous two-phase systems, the basic phase forming polymers (PEG and dextran) have been substituted by other polymers, *e.g.* poly(vinyl alcohol), hydroxypropyl starch or ethylhydroxyethyl cellulose [2–4]. In other cases, the basic phase forming polymers have been modified with different groups for special applications. PEG has been used to carry different ligands for affinity partitioning [nicotinamide–adenine dinucleotide (NADH), fatty acids, textile dyes, charged groups] [5–8]. Dextran derivatives have also been used in aqueous two-phase systems (hydroxypropyl-dextran, diethylaminoethyl-dextran, textile dye-dextran) [1,8,9]. In a previous study benzoyl dextran was synthesized and its use in aqueous two-phase systems was investigated [10]. In the present work, dextran has been modified with valeryl groups. The valeryl group was chosen in order to make comparisons with benzoyl dextran, *i.e.* to compare the effects of

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aromatic and aliphatic substitutions. The degree of substitution of valeryl dextran was changed by varying the reaction conditions. Phase diagrams for aqueous two-phase systems composed of PEG–valeryl dextran, dextran–valeryl dextran, valeryl dextran–valeryl dextran and valeryl dextran–benzoyl dextran have been determined.

Aqueous two-phase systems containing hydrophobically modified dextran have new and different properties for partitioning of biological materials compared to the mostly used PEG–dextran systems. The present study is devoted to a systematic investigation into the properties of aqueous two-phase systems containing dextran with covalently bound benzoyl or valeryl groups. The partitioning of amino acids, dipeptides, proteins and thylakoid membrane vesicles was studied in systems with hydrophobically modified dextrans. A series of proteins with differing surface hydrophobicities was studied in order to observe the effect of hydrophobic interactions on protein partitioning. Viscosity measurements were made in order to study the interactions between hydrophobic groups on the dextran backbone.

2. Materials and methods

2.1. Chemicals

Dextran T500 with a mass-average molecular mass of 500 000, was obtained from Pharmacia (Uppsala, Sweden), and PEG 8000 with a number-average molecular mass of 8000, from BP Chemicals (Hythe, UK). Valeryl chloride and triethylamine were obtained from Aldrich-Chemie (Steinheim, Germany). Benzoyl dextrans were synthesized according to our previous work [10]. All other chemicals were of analytical grade.

2.2. Biological materials

Amino acids (L-tryptophan, L-phenylalanine) were obtained from E. Merck (Darmstadt, Germany); L-tyrosine from Becton Dickinson (Orangeburg, NY, USA) and the dipeptides tyrosine–tyrosine and glycine–tryptophan from Sigma (St. Louis, MO, USA). Bovine serum albumin (BSA) was purchased from Boehringer (Mannheim, Germany). The following proteins were obtained from Sigma: β-galactosidase (EC 3.2.1.23), from *Escherichia coli;* lysozyme (EC 3.2.1.17), from chicken egg white; lipase type 1 (EC 3.1.1.3), from wheat germ; β-lactoglobulin A, from bovine milk ; myoglobin, from horse skeletal muscle and cytochrome *c*, from horse heart. Thylakoid membranes, membrane vesicles from grana stack (B3) and membrane vesicles from stroma lamellae (T3) were isolated from spinach leaves according to Albertsson and Yu [11] and Andreasson *et al.* [12].

2.3. Synthesis of valeryl dextran

Valeryl dextrans were obtained by reacting dextran T500 with valeryl chloride in presence of triethylamine. The reactions were carried out in an ice bath. The reaction and purification procedures are the same as described in our earlier paper [10]. The concentration of dextran was determined by polarimetry [1]. The content of valeryl groups on dextran was determined by NMR measurements using ^2H_2O as solvent. The degree of substitution (DS) is defined as the number of valeryl groups per glucose unit.

2.4. Two-phase systems

The two-phase systems were prepared from stock solutions of 40% PEG 8000 [all compositions are given in % (w/w) throughout this paper], 20% dextran T500, 11–12% valeryl dextran and 12–15% benzoyl dextran. The stock solutions were weighed out and mixed together with buffer, salt, biomaterials and water. The systems were equilibrated at room temperature and centrifuged 2 min at 1200 *g* for partitioning of amino acids, dipeptides and proteins. For partitioning of thylakoid membranes, B3 and T3 vesicles, the systems were just mixed and left standing for 20 min at room temperature. Suitable amounts of each phase were withdrawn, diluted 5–20 times and analysed. The partition coefficients (K) of the substances are defined as

the ratio of their respective concentrations in top and bottom phase. All partition coefficients are mean values from two or three repeated experiments.

2.5. Assays of amino acids, dipeptides, proteins and thylakoid membrane vesicles

Amino acids (L-tryptophan, L-tyrosine and L-phenylalanine) and dipeptides (tyrosine–tyrosine and glycine–tryptophan) were determined by high-performance liquid chromatographic (HPLC) measurements. A gel filtration column was used to separate the amino acids and dipeptides from polymers. The tryptophan and glycine–tryptophan were detected at 279 nm, tyrosine–tyrosine at 223 nm, phenylalanine at 259 nm and tyrosine at 278 nm. Proteins (BSA, lipase and β-lactoglobulin) were assayed by using Coomassie Brilliant Blue G and measured at 595 nm with BSA as standard [13]. Myoglobin and cytochrome *c* were determined by absorbance at 409 and 408 nm. Phases of the same two-phase systems but without proteins were used as references. β-Galactosidase activity was measured according to Veide *et al.* [14]. Lysozyme activity was measured according to the Worthington Manual [15]. Thylakoid membranes, B3 and T3 vesicles were determined at 680 nm.

2.6. Phase diagrams

Phase diagrams were determined by combining titration and analysis of the composition of top and bottom phases [1]. In the systems composed of PEG and valeryl dextran, the concentration of valeryl dextran was determined by polarimetry and PEG by refractive index. The introduction of valeryl and benzoyl groups had no noticeable effect on the specific rotation of dextran, $[\alpha]_D^{25} = +199°$ ml g^{-1} dm^{-1}. The concentration of valeryl dextran in each phase was determined separately and its contribution was subtracted from the refractive index values. In the system valeryl dextran–benzoyl dextran, the total dextran concentration was measured with polarimetry and the benzoyl dextran was measured by absorbance at 273 nm. The valeryl dextran concentration was obtained by subtracting the benzoyl dextran from the total dextran concentration [10]. In the systems composed of dextran–valeryl dextran and valeryl dextran–valeryl dextran, the binodal curves were determined by titration. The compositions of the phases were not determined in this case.

2.7. Measurement of viscosity

The viscosity of benzoyl dextran and valeryl dextran solutions were measured in both dilute and concentrated systems. In the dilute system the viscosity was determined by the time spent for a certain volume (7.0 ml) of polymer solution to pass through a Ostwald capillary viscometer at constant temperature (25°C). In the concentrated systems the viscosity was measured in a similar way but the viscometer was replaced by a Pasteur capillary pipette. The solution volume was 1.65 ml and temperature 22°C.

3. Results

3.1. Stability of hydrophobically modified dextrans

Dextran T500 modified with benzoyl and valeryl groups have been stored in the cold room (4°C) for long periods. The stability of the hydrophobically modified dextrans was determined by HPLC measurement. For a 13.8% solution of benzoyl dextran (DS = 0.137) stored for one year and a 11.1% valeryl dextran solution (DS = 0.20) stored for four months at 4°C, no low-molecular-mass components were found.

3.2. Viscosities

The viscosities of benzoyl dextran solutions were measured in both dilute systems with a polymer concentration of 0.00225 g/ml at 25°C and in concentrated systems with polymer concentrations of 2–15%. In a dilute dextran solution system, no significant viscosity change was observed when the DS of benzoyl dextran was

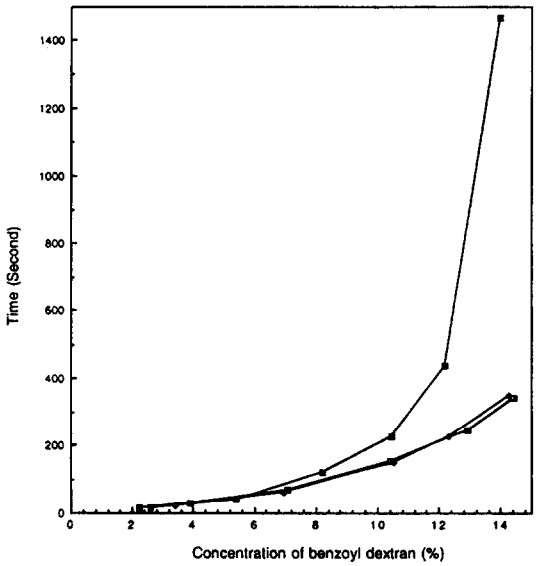


Fig. 1. The influence of polymer concentration on the viscosity of benzoyl dextran. Aqueous solutions (1.65 ml) of dextran T500 (□) and benzoyl dextran with DS of 0.087 (◆) and 0.137 (■) were passed through a Pasteur capillary pipette at room temperature (22°C).

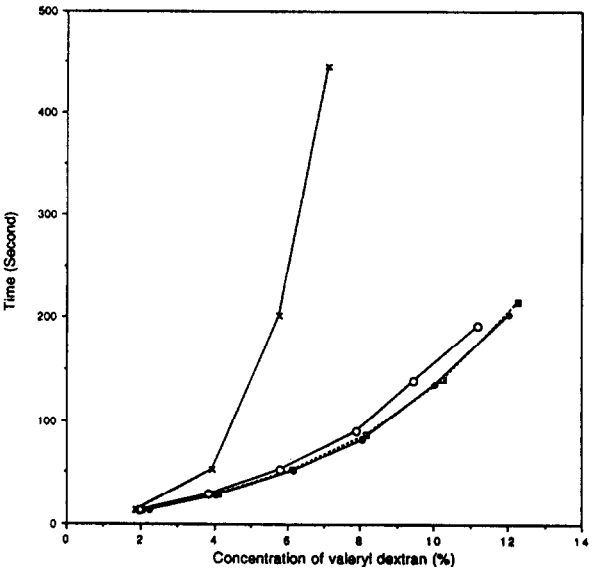


Fig. 2. The influence of polymer concentration on the viscosity of valeryl dextran. Aqueous solutions (1.65 ml) of dextran T500 (□, broken line) and valeryl dextran with DS of 0.025 (●), 0.12 (○) and 0.20 (×) were passed through a Pasteur capillary pipette at room temperature (22°C).

varied between 0 and 0.137. The viscosities of concentrated solutions of benzoyl dextran at room temperature are shown in Fig. 1. The viscosities of valeryl dextran solutions were measured in solutions with polymer concentrations of 1.8–13% at room temperature (22°C). The results are shown in Fig. 2. For benzoyl and valeryl dextran with DS of 0.14 and 0.20, respectively, a sharp increase in solution viscosity is observed when the polymer concentration is increased.

3.3. Phase diagrams

In PEG–valeryl dextran systems, the valeryl dextrans are enriched in the bottom phase and PEG in the top phase. The phase diagrams for systems composed of PEG 8000 and valeryl dextran with DS values of 0.025, 0.12 and 0.20 at room temperature (22°C) are shown in Figs. 3. For comparison the binodal curve of the PEG 8000–dextran T500 system is also shown in Fig. 3 [1]. Valeryl dextran with a high degree of substitution (DS = 0.20) also forms two-phase systems with dextran, valeryl dextran and benzoyl dextran with low degree of substitution. Valeryl dextran with lower degree of substitution (DS = 0.025) forms two-phase systems with benzoyl dextran with higher degree of substitution (DS = 0.137). The phase diagrams of valeryl dextran (DS = 0.025)–benzoyl dextran (DS = 0.137) and benzoyl dextran (DS = 0.054)–valeryl dextran (DS = 0.20) are shown in Fig. 4. The phase diagrams of dextran–valeryl dextran (DS = 0.20) and valeryl dextran (DS = 0.025)–valeryl dextran (DS = 0.20) are shown in Fig. 5. In these systems the dextran with the highest degree of substitution is always enriched in the bottom phase.

3.4. Partition of amino acids and dipeptides

Amino acids and dipeptides with aromatic groups (L-tyrosine, L-tryptophan, L-phenylalanine, tyrosine–tyrosine and glycine–tryptophan) were partitioned in PEG–valeryl dextran and PEG–benzoyl dextran aqueous two-phase systems. The valeryl dextrans with DS = 0.025, 0.12 and 0.20 were used in PEG–valeryl dextran

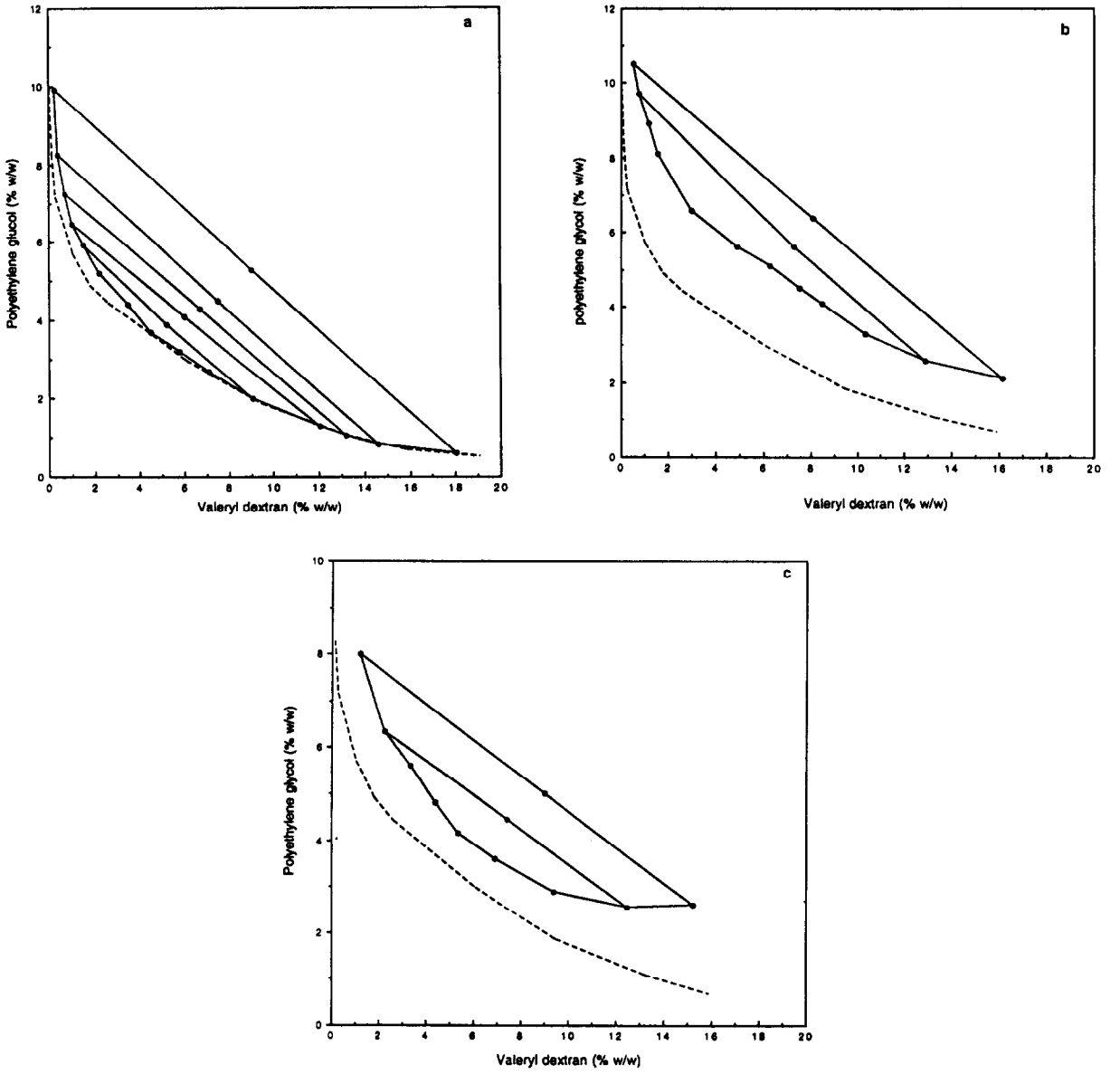
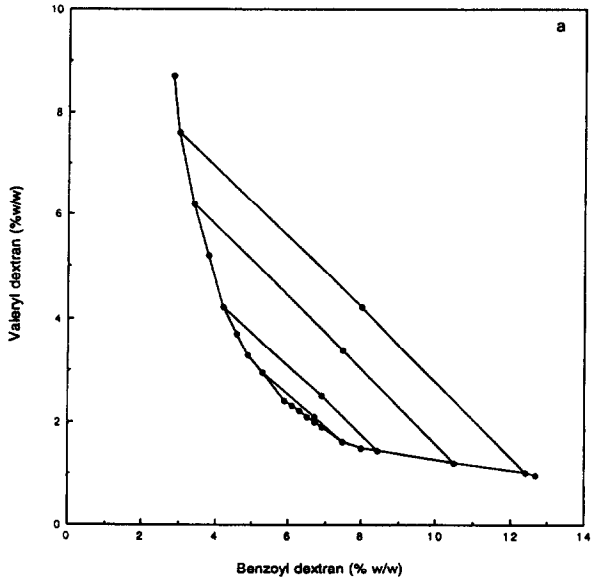


Fig. 3. Phase diagrams for the system PEG 8000–valeryl dextran at room temperature (22°C). The DS of valeryl dextran was (a) 0.025; (b) 0.12 and (c) 0.20. The dashed line shows the binodial curve for the system PEG 8000–dextran T500 at 20°C, the data are from Albertsson [1].

systems and benzoyl dextrans with DS = 0.054, 0.087 and 0.137 were used in PEG–benzoyl dextran systems. In both PEG–valeryl dextran and PEG–benzoyl dextran systems, the system compositions were: 6% PEG 8000, 6% valeryl dextran (or benzoyl dextran) without addition of buffer or salts. The partitionings were carried out at room temperature. The partition coefficients are all close to 1 which shows that the amino acids and dipeptides partition evenly between the top and bottom phases in these systems.



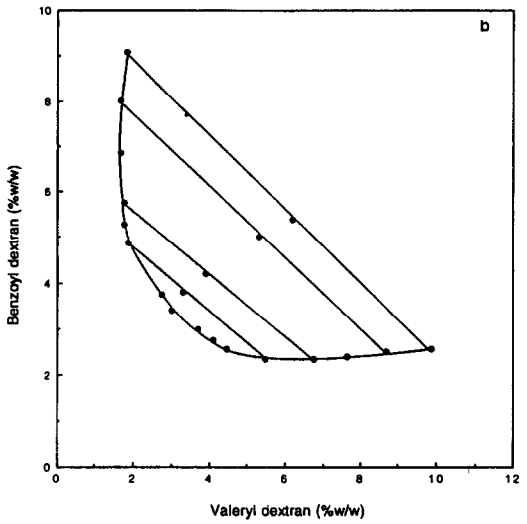


Fig. 4. Phase diagrams for the system benzoyl dextran–valeryl dextran. (a) Valeryl dextran DS = 0.025–benzoyl dextran DS = 0.137. (b) Benzoyl dextran DS = 0.054–valeryl dextran DS = 0.20. The temperature was 22°C.

3.5. Partition of proteins

The proteins β-galactosidase, BSA, lysozyme, lipase-1, β-lactoglobulin A, cytochrome *c* and myoglobin were partitioned in aqueous two-phase systems containing benzoyl dextrans and

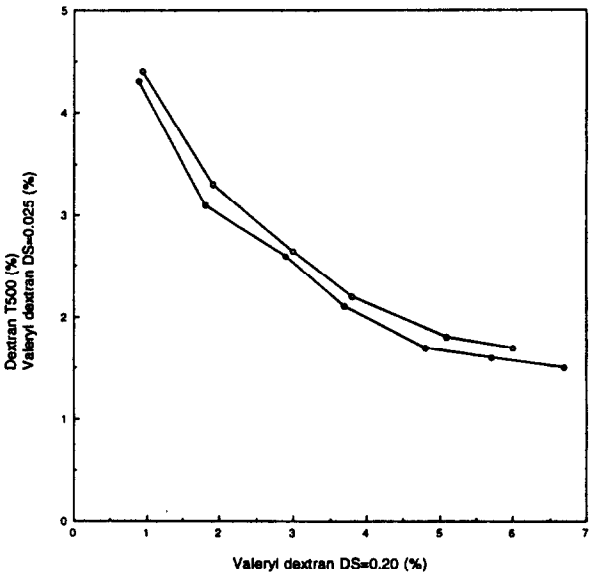


Fig. 5. Phase diagrams for the system dextran T500–valeryl dextran DS = 0.2 (●) and the system valeryl dextran DS = 0.025–valeryl dextran DS = 0.20 (○). The temperature was 22°C.

valeryl dextrans. Tables 1–7 show the partition coefficients of the proteins in PEG–benzoyl dextran, PEG–valeryl dextran systems and in the same systems containing 100 m*M* sodium chloride. All systems contained 10 m*M* sodium phosphate buffer, pH 7.4 for β-galactosidase and

Table 1
Partition coefficient of β-galactosidase in PEG–benzoyl dextran and PEG–valeryl dextran aqueous two-phase system

	K	*K* (with 100 m*M* NaCl)
DS of benzoyl dextran		
0	72	0.095
0.054	0.12	0.0096
0.087	0.026	0.012
0.137	0.022	0.013
DS of valeryl dextran		
0.025	59	0.030
0.12	0.47	0.0079
0.20	0.63	0.0059

The system composition was: 6.0% PEG 8000, 6.0% benzoyl dextran (or valeryl dextran), 10 m*M* sodium phosphate buffer, pH 7.4, without or with 100 m*M* NaCl. Room temperature (22°C).

Table 2
Partition coefficient of bovine serum albumin in PEG–valeryl dextran aqueous two-phase system

DS of valeryl dextran	K	K (with 100 mM NaCl)
0	0.35	0.10
0.025	0.26	0.082
0.12	0.16	0.072
0.20	0.59	0.12

The system composition was: 6% PEG 8000, 6% valeryl dextran, 10 mM sodium phosphate buffer, pH 7.0, without or with 100 mM NaCl. Room temperature (22°C).

pH 7.0 for rest of proteins. For β-galactosidase (Table 1) a strong decrease in the partition coefficient is observed when the DS of benzoyl and valeryl dextran is increased.

Table 2 shows the partition coefficient of BSA in systems composed of PEG and valeryl dextran. The partitioning properties of this protein in PEG–benzoyl dextran systems were shown in an earlier work [10]. The partition coefficients of lipase-1, lysozyme and β-lactoglobulin A are shown in Tables 3, 4 and 5. For these four proteins the same tendency is observed. The K value is first decreased with increasing DS but at high DS the K value is again increased.

Table 3
Partition coefficient of lipase-1 in PEG–benzoyl dextran and PEG–valeryl dextran aqueous two-phase system

	K	K (with 100 mM NaCl)
DS of benzoyl dextran		
0	0.51	0.39
0.054	0.28	0.30
0.087	0.23	0.23
0.137	0.92	0.55
DS of valeryl dextran		
0.025	0.51	0.37
0.12	0.31	0.28
0.20	1.13	1.22

The system composition was: 6% PEG 8000, 6% benzoyl dextran (or valeryl dextran), 10 mM sodium phosphate buffer, pH 7.0, without or with 100 mM NaCl. Room temprature (22°C).

Table 4
Partition coefficient of lysozyme in PEG–benzoyl dextran and PEG–valeryl dextran aqueous two-phase system

	K	K (with 100 mM NaCl)
DS of benzoyl dextran		
0	0.29	2.06
0.054	0.087	0.052
0.087	0.18	0.50
0.137	0.16	0.45
DS of valeryl dextran		
0.025	0.41	1.81
0.12	0.25	0.71
0.20	0.38	1.11

The system composition was: 6% PEG 8000, 6% benzoyl dextran (or valeryl dextran), 10 mM sodium phosphate buffer, pH 7.0, without or with 100 mM NaCl. Room temperature (22°C).

Table 6 shows the partition coefficient of cytochrome c and Table 7 shows the partition coefficient of myoglobin. The partition coefficient of these two proteins is increased when the degree of substitution of hydrophobic groups is increased.

For all proteins a change in the K value is observed when comparing partitioning in equiva-

Table 5
Partition coefficient of β-lactoglobulin A in PEG–benzoyl dextran and PEG–valeryl dextran aqueous two-phase system

	K	K (with 100 mM NaCl)
DS of benzoyl dextran		
0	1.11	0.29
0.054	0.36	0.075
0.087	0.36	0.12
0.137	0.58	0.044
DS of valeryl dextran		
0.025	0.96	0.22
0.12	0.39	0.16
0.20	2.67	0.039

The system composition was: 6% PEG 8000, 6% benzoyl dextran (or valeryl dextran), 10 mM sodium phosphate buffer, pH 7.0, without or with 100 mM NaCl. Room temperature (22°C).

Table 6
Partition coefficient of cytochrome *c* in PEG–benzoyl dextran and PEG–valeryl dextran aqueous two-phase system

	K	*K* (with 100 m*M* NaCl)
DS of benzoyl dextran		
0	0.19	0.42
0.054	0.16	0.36
0.087	0.34	0.54
0.137	0.48	0.67
DS of valeryl dextran		
0.025	0.20	0.43
0.12	0.35	0.54
0.20	0.52	0.70

The system composition was: 6.0% PEG 8000, 6.0% benzoyl dextran (or valeryl dextran), 10 m*M* sodium phosphate buffer, pH 7.0, without or with 100 m*M* NaCl. Room temperature (22°C).

lent systems containing only a 10 m*M* sodium phosphate buffer and a buffer plus 100 m*M* sodium chloride. This is due to the electrical potential difference generated between the phases by the salts [16].

Table 8 shows the molecular masses, isoelectric points and surface hydrophobicities of the proteins studied. The table also shows the maximum difference in log *K* between the systems

Table 7
Partition coefficient of myoglobin in PEG–benzoyl dextran and PEG–valeryl dextran aqueous two-phase system

	K	*K* (with 100 m*M* NaCl)
DS of benzoyl dextran		
0	0.34	0.36
0.054	0.32	0.29
0.087	0.35	0.35
0.137	0.48	0.49
DS of valeryl dextran		
0.025	0.30	0.33
0.12	0.39	0.38
0.20	0.54	0.47

The system composition was: 6.0% PEG 8000, 6.0% benzoyl dextran (or valeryl dextran), 10 m*M* sodium phosphate buffer, pH 7.0, without or with 100 m*M* NaCl. Room temperature (22°C).

PEG–dextran (Dx) and PEG–benzoyl dextran (BzDx) [or PEG–valeryl dextran (VaDx)] (Eqs. 1 and 2), either without or with 100 mM NaCl.

$$\Delta\log K_{(BzDx)} = \log K_{(PEG-Dx)} - \log K_{(PEG-BzDx)} \quad (1)$$

$$\Delta\log K_{(VaDx)} = \log K_{(PEG-Dx)} - \log K_{(PEG-VaDx)} \quad (2)$$

3.6. Partition of thylakoid membrane vesicles

Whole thylakoid membranes, grana (B3) and stroma lamellae (T3) vesicles were partitioned in aqueous two-phase systems composed of 5% PEG 8000, 5% (dextran T500 + valeryl dextran), 40 m*M* boric acid–tris(hydroxymethyl)aminomethane buffer (H_3BO_3-Tris), pH 7.83. Whole thylakoid membranes as well as B3 and T3 vesicles were almost exclusively partitioned to the top phase in systems without valeryl groups (100% for whole thylakoid membranes, 90% for B3 and 85% for T3 vesicles). By introducing a

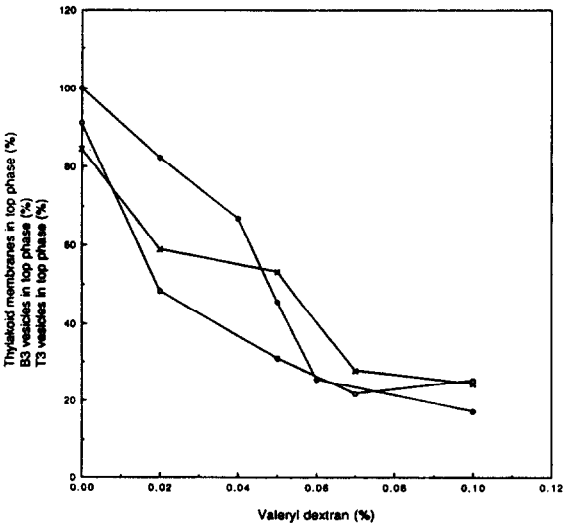


Fig. 6. The influence of valeryl dextran on the partitioning of thylakoid membranes (●), grana (B3) (○) and stroma lamellae (T3) (×) vesicles between the top phase and the interface. The system composition was: 5% PEG 8000, 5% (dextran T500 + valeryl dextran DS = 0.12), 40 m*M* H_3BO_3-Tris, pH 7.83, room temperature (22°C). The percentage of valeryl dextran is the amount of dextran replaced by valeryl dextran.

Table 8
Molecular masses, isoelectric points, surface hydrophobicities and Δlog K of proteins

Protein	M_r	pI	Surface hydrophobicity	ΔLog K^a (no salt)	ΔLog K^b (100 mM NaCl)
β-Galactosidase	465 400 [17,18]	4.6 [19]	–	3.51 2.18	0.86 (BzDx) 1.21 (VaDx)
BSA	69 000 [20]	5.0 [20]	10.33[c]	0.40[d] 0.34	0.19[d] (BzDx) 0.14 (VaDx)
Lipase-1	66 000 [20][e]	5.1 [20][e]	–	0.35 0.22	0.23 (BzDx) 0.14 (VaDx)
Lysozyme	13 900 [22]	11 [23]	7.49[c]	0.25 0.06	0.66 (BzDx) 0.46 (VaDx)
β-Lactoglobulin	35 000 [20]	5.1 [20]	4.41[c]	0.49 0.45	0.82 (BzDx) 0.87 (VaDx)
Myoglobin	17 500 [20]	7.1 [20]	2.99[c]	−0.15 −0.20	−0.13 (BzDx) −0.12 (VaDx)
Cytochrome c	13 000 [22]	9.4 [20]	0[f]	−0.40 −0.44	−0.20 (BzDx) −0.22 (VaDx)

[a] ΔLog K is the maximum log K difference between the systems PEG–Dx and PEG–BzDx or PEG–VaDx without salt.
[b] ΔLog K is the maximum log K difference between the systems PEG–Dx and PEG–BzDx or PEG–VaDx with 100 mM NaCl.
[c] Retention coefficient in hydrophobic interaction chromatogrophy on butylepoxy-Sepharose [24].
[d] Data from ref. 10.
[e] Value for enzyme from guinea pig pancreas.
[f] Determined by hydrophobic partition using fatty acids bound to PEG [25].

small amount of valeryl groups, *e.g.* when 0.1% of dextran was replaced by valeryl dextran (DS = 0.12) the three types of membranes were transferred from the top phase to the interface. The results are shown in Fig. 6. The systems composed of 5% PEG 8000, 5% (dextran + benzoyl dextran) and 15 mM H_3BO_3-Tris, pH 7.83 were also used for partitioning of these thylakoid membranes, B3 and T3 vesicles. The results were qualitatively similar to what was obtained with valeryl dextran (Fig. 7).

The aqueous two-phase systems composed of 6% PEG 8000, 6% (dextran T500 + valeryl dextran) and 10 mM sodium phosphate buffer, pH 7.4, were used to partition the thylakoid membranes, B3 and T3 vesicles from the interface to the bottom phase with increased polymer concentration (Fig. 8). In this system the thylakoid membranes, B3 and T3 vesicles were partitioned to the interface in the absence of valeryl groups on dextran (> 80% for all of them). When 20% of dextran was replaced by valeryl dextran (DS = 0.12), the material was partitioned to the bottom phase (> 80%) (Fig. 8).

4. Discussion

4.1. Hydrophobic association of benzoyl dextran and valeryl dextran

Dextran is a relatively hydrophilic polymer. But benzoyl dextran and valeryl dextran are more hydrophobic compared with dextran and show hydrophobic association in aqueous solutions. The hydrophobicity depends on the degree of substitution. The determination of polymer solution viscosity, phase diagrams, and partition

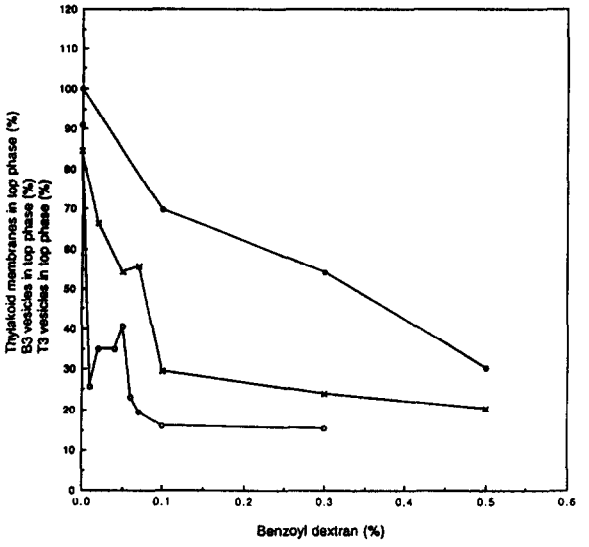


Fig. 7. The influence of benzoyl dextran on the partitioning of thylakoid membranes (●), grana (B3) (○) and stroma lamellae (T3) (×) vesicles between the top phase and the interface. The system composition was: 5% PEG 8000, 5% (dextran T500 + benzoyl dextran DS = 0.087), 15 m*M* H_3BO_3-Tris, pH 7.83, room temperature (22°C). The percentage of benzoyl dextran is the amount of dextran replaced by benzoyl dextran.

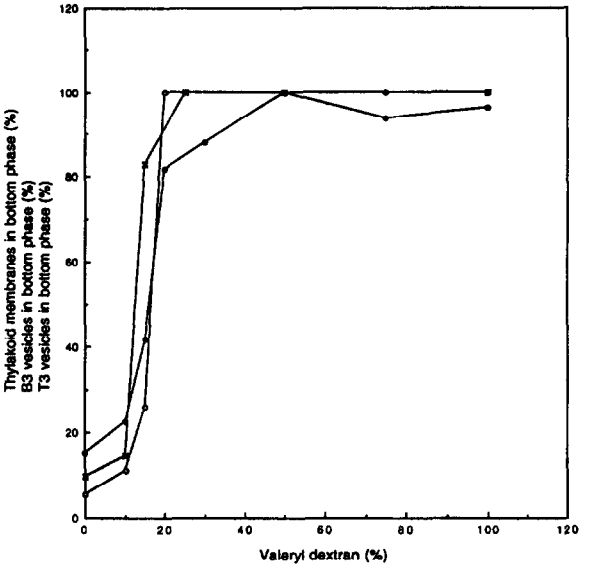


Fig. 8. The influence of valeryl dextran on the partitioning of thylakoid membranes (●), grana (B3) (○) and stroma lamellae (T3) (×) vesicles between the interface and bottom phase. The system composition was: 6% PEG 8000, 6% (dextran T500 + valeryl dextran DS = 0.12), 10 m*M* sodium phosphate buffer, pH 7.4, room temperature (22°C). The percentage of valeryl dextran is the amount of dextran replaced by valeryl dextran.

coefficients of proteins show that the hydrophobic association is increased with increasing of degree of substitution.

The viscosity measurements of benzoyl dextrans with DS of 0.053, 0.087 and 0.137 at low concentrations (0.00225 g/ml and less) show that this concentration is low enough to eliminate interactions between polymer molecules. The viscosities of the benzoyl dextrans are very close to normal dextran.

The viscosities of benzoyl and valeryl dextrans in concentrated systems show association between the polymers at high degrees of substitution. The viscosity of benzoyl dextran with DS = 0.137 is increased at concentrations above 6% compared with dextran and benzoyl dextran with lower DS (0.087). This phenomenon shows the hydrophobic interactions between benzoyl dextran molecules and the interactions are increased with increasing polymer concentrations. The hydrophobic association of several dextran molecules makes them behave like a larger molecule. At lower DS or lower polymer concentrations the hydrophobic interactions are too weak to affect the viscosity. The hydrophobic interactions between valeryl dextran polymers with DS = 0.2 are stronger than the interactions for benzoyl dextran with DS = 0.137 as judged from the strong increase in viscosity at 6% concentration for valeryl dextran compared to 12% for benzoyl dextran.

4.2. *Phase diagrams*

The binodal curve of PEG–valeryl dextran (DS = 0.025) is very close to the binodal curve of PEG–dextran T500 but at slightly higher polymer concentrations. When valeryl dextran with DS 0.12 is used, the binodal curve is moved to higher concentrations. This reflects the increased compatability between PEG and valeryl dextrans. When the DS of valeryl dextran is increased to 0.20 the binodal curve is moved to

lower concentrations compared with valeryl dextran with DS = 0.12 (see Fig. 3b and c). This could be due to the higher DS of the valeryl dextran which gives stronger hydrophobic interactions between the valeryl groups on the dextran backbones. This interaction makes the valeryl dextran like a pseudo-higher-molecular-mass polymer. Generally, the higher the molecular mass is, the lower polymer concentration is needed to achieve phase separation [1].

Valeryl dextran with a high degree of substitution (DS = 0.20) can form a two-phase system with benzoyl dextran having a low degree of substitution (DS = 0.054). Conversely, benzoyl dextran with a high DS (0.137) can form a two-phase system with a valeryl dextran which has a low DS (0.025) (see Fig. 4). In these two systems the valeryl dextran and benzoyl dextran with low degree of substitution phase separate in the same way as normal dextran. The valeryl dextran with a high DS (0.20) cannot phase separate with benzoyl dextran which has a high DS (0.137). To form a two-phase system the difference in the degree of substitution of hydrophobic groups between the two phase forming polymers has to be large enough.

4.3. Partitioning of amino acids, dipeptides and proteins

Amino acids and dipeptides with aromatic groups (L-tryptophan, L-tyrosine, L-phenylalanine, tyrosine–tyrosine and glycine–tryptophan) were partitioned in aqueous two-phase systems containing benzoyl dextrans and valeryl dextrans. The partition coefficients of these hydrophobic amino acids and dipeptides are close to 1 which indicates that the amino acids and dipeptides are too small to be affected by the hydrophobic groups on dextran.

The partitioning of proteins is affected by many factors, mainly the composition of the two-phase system and the protein properties [1,3]. In this work, seven proteins (β-galactosidase, BSA, lipase, lysozyme, β-lactoglobulin, cytochrome *c* and myoglobin) were partitioned in PEG–benzoyl dextran and PEG–valeryl dextran systems. The protein properties are collected in Table 8. The $\Delta\log K$ values in Table 8 show the maximum effect of the hydrophobic groups on protein partitioning.

When proteins are partitioned in PEG–benzoyl dextran and PEG–valeryl dextran systems two opposite effects are observed. For β-galactosidase, BSA, lipase, lysozyme and β-lactoglobulin a decrease in the K value is observed when the DS is increased. For cytochrome *c* and myoglobin the K value is increased with increasing DS. The partitioning behaviour of the first group of proteins can be explained by hydrophobic interactions with the benzoyl or valeryl groups.

The K value for all proteins is affected when the salt composition is changed from 10 mM sodium phosphate by addition of 100 mM NaCl to the phase system. This effect has been thoroughly studied in PEG–dextran systems [1,16,26] and can be explained by the electrical potential difference generated by the salts. Phosphate ions have a tendency to partition to the dextran phase [26]. Electroneutrality forces the counterions to partition in the same direction, but the net effect is the generation of an electrical potential difference across the interface with a net negative charge in the bottom phase. The partitioning of proteins will then depend on the protein net charge. A protein with a high negative charge (*e.g.* β-galactosidase at pH 7.4) will partition towards the top (PEG) phase in a system containing 10 mM sodium phosphate. For NaCl there is a weak tendency for Na^+ to partition to the dextran phase and a weak tendency for Cl^- to partition to the PEG phase [16]. When 100 mM NaCl is added to the system containing 10 mM sodium phosphate the NaCl salt will be dominating in the system, and the electrical potential difference will be of opposite sign relative to the potential generated by sodium phosphate. In accordance, β-galactosidase (at pH 7.4) will partition towards the bottom (dextran) phase when NaCl is the dominating salt.

The partition coefficient for β-galactosidase (M_r 465 400, pI 4.6) [17–19] was decreased more than 100 times by introducing benzoyl groups (K from 72 to 0.022) or valeryl groups (K from 72 to

0.63). The addition of sodium chloride also decreased the partition coefficient. In the system PEG–valeryl dextran (DS = 0.2) and 1.0 *M* NaCl, no enzyme activity was found in the top phase. Therefore, this enzyme could be completely transferred from top phase to bottom phase by adjusting the polymer hydrophobicity, the salt composition and concentration. This strong change of the partition coefficient may be due to the hydrophobic interactions between hydrophobic groups (benzoyl and valeryl) on the dextran backbone and hydrophobic groups on the protein surface [27,28]. In 1.0 *M* NaCl the hydrophobic interactions are increased. For β-galactosidase from *E. coli* the ratio of tryptophan to all amino acids is unusually high, 3%, compared to 1% for an average *E. coli* protein [17,18,29]. There are 39 tryptophans in each subunit giving a total of 156 tryptophans in *E. coli* β-galactosidase [17]. Tryptophan is a very hydrophobic amino acid. Exposed tryptophans on protein surfaces have been shown to direct the partitioning of proteins to the PEG phase in PEG–salt systems [30]. The much stronger effect on β-galactosidase partitioning observed with benzoyl dextran compared with valeryl dextran (Table 1, systems without salt), could be due to aromatic "stacking" interactions with exposed tryptophans. The Δlog *K* values (Table 8) show that the effect of the hydrophobic groups on the partitioning are much stronger for β-galactosidase than for the rest of the proteins studied.

For BSA the partition coefficient was decreased from 0.35 to 0.16 by increasing the DS of valeryl dextran from 0 to 0.12. A similar effect was observed with benzoyl dextran [10]. The partition coefficients of lipase, lysozyme and β-lactoglobulin in the systems of PEG–benzoyl dextran and PEG–valeryl dextran show the same tendency as for BSA. This can be explained by interactions between benzoyl and valeryl groups on dextran and hydrophobic sites or surfaces on the proteins. The proteins BSA and β-lactoglobulin are known to bind hydrophobic compounds. This has been demonstrated by affinity partitioning using PEG esterified with fatty acids [25]. From Table 8 can be seen that BSA, lysozyme and β-lactoglobulin are relatively hydrophobic proteins as determined by hydrophobic interaction chromatography [24]. Lipase has hydrophobic sites for binding of lipids [21]. The Δlog *K* values in Table 8 are relatively similar for the four proteins in this group. The values are significantly smaller than the corresponding values for β-galactosidase. This can be due to the much larger molecular mass of β-galactosidase or that specific interactions (*e.g.* with tryptophans) are involved with this protein.

The partitioning of cytochrome *c* and myoglobin is affected in a different way by the hydrophobic groups on the dextran. Both proteins are relatively less hydrophobic compared with the proteins in the previous group (Table 8). In both PEG–benzoyl dextran and PEG–valeryl dextran systems the partition coefficient of cytochrome *c* and myoglobin is increased with increasing DS. As an effect of increased hydrophilicity the proteins may experience a repulsive force from the hydrophobic groups.

The increased hydrophobic interactions between the benzoyl or valeryl dextrans with increased DS will also lead to increased *K* values for proteins. The increased interactions lead to association of the dextran polymers as observed in the viscosity measurements (Figs. 1 and 2). This association will result in an exclusion of the protein molecules from the bottom phase, *i.e.* the *K* value will increase much in the same way as when the molecular mass of dextran is increased [1,31]. Increased *K* values are also observed at high DS of hydrophobic groups for BSA, lipase, lysozyme and β-lactoglobulin.

For cytochrome *c* the addition of 100 m*M* NaCl leads to increasing *K* values in all systems studied, but this salt has a very small effect on the partition coefficient of myoglobin (Tables 6 and 7). Cytochrome *c* has a p*I* of 9.4 and myoglobin 7.1 [20]. At pH 7 cytochrome *c* is positively charged and will be partitioned to the bottom phase in a system with sodium phosphate due to the electrical potential difference. The *K* value for cytochrome *c* is increased with addition of NaCl which is in accordance with the reversal of the potential by this salt. The partitioning of myoglobin is affected very little by the change in salt composition which agrees with that the

partitioning is performed close to the p*I* of the protein (7.1). The change of salt composition has an even stronger effect on lysozyme compared with cytochrome *c*. This is in accordance with the higher positive charge of lysozyme due to its higher p*I*.

4.4. Partitioning of thylakoid membrane vesicles

The effect of hydrophobic groups bound to dextran on the partitioning of thylakoid membranes, grana (B3) and stroma lamellae (T3) vesicles is very strong (Fig. 6). In the absence of valeryl groups the three types of membranes are partitioned mostly to the top phase. More than 70% of these materials are transferred to the interface by replacing only 0.1% of the total dextran with valeryl dextran, DS = 0.12. Benzoyl groups show a similar strong effect on these materials (Fig. 7). By replacing 0.01–0.02% of dextran with benzoyl dextran significant separation between grana (B3) and stroma lamellae (T3) vesicles was achieved. This is not surprising since they are of opposite sidedness [32]. At 0.1% there is a considerable separation between whole thylakoid membranes and stroma lamellae vesicles. It is of interest that the two latter are separated although both are right-side out. However, the stroma lamellae vesicles are much smaller in size and their membranes are more curved than the flat membranes of the whole thylakoids, which may explain the difference in affinity for the benzoyl dextran phase. It is also possible with the hydrophobic groups to change the partitioning of thylakoid membranes, grana and stroma lamellae vesicles from the interface to the bottom phase (Fig. 8). The fact that thylakoid membrane vesicles are always partitioned to the phase containing the hydrophobic groups reflects the interactions between hydrophobic groups in the membrane fractions and the benzoyl (or valeryl) groups. The strong effect of dextran bound hydrophobic groups may be used to increase the specificity of PEG–dextran systems in the area of membrane separations, *e.g.* the separation of thylakoid membranes and plasma membranes (see ref. 10).

5. Conclusions

Dextran can be modified with aromatic and aliphatic groups. The hydrophobically modified dextran (benzoyl dextran and valeryl dextran) can offer a relatively more hydrophobic bottom phase compared to dextran in two-phase systems with PEG. These systems provide a special environment to study the hydrophobic interactions between proteins, membranes and hydrophobic groups on the dextrans. These hydrophobic groups show strong interaction with some proteins, like β-galactosidase, and thylakoid membrane vesicles. By changing the degree of substitution of benzoyl or valeryl dextrans, salt composition and concentration, the partitioning of proteins with hydrophobic sites can be influenced. Thylakoid membrane vesicles could be almost totally collected in the hydrophobic phase or at the interface.

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7. References

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