CHROM. 21 097

PARTITION OF HYDROPHOBIC COMPOUNDS BETWEEN TWO LIQUID PHASES OF SIMILAR HYDROPHOBICITY

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(First received September 7th, 1988; revised manuscript received November 11th, 1988)

SUMMARY

Liquid–liquid two-phase systems have been attained by dissolving pairs of polymers in organic solvents with or without the addition of water. The solvents include dioxane, N,N-dimethylformamide, tetrahydrofuran, dimethyl sulphoxide and acetonitrile. The compositions of the phases have been determined in some cases. Phases (in equilibrium) with nearly the same ratio between the organic solvent and water have been obtained. Hydrophobic compounds have been partitioned within two-phase systems containing dimethylformamide. One of these systems has been used for fractionation of zein (from corn) by counter-current distribution.

INTRODUCTION

Polymeric substances, dissolved together in a solvent, may give rise to more than one liquid phase. This behaviour was observed by Beijerinck[1] and has been a subject of concern to the paint and plastic industry[2,3]. The two liquid phases obtained by using water as a solvent for two polymers were introduced by Albertsson[4] as a tool for fractionation of biomaterial. These aqueous two-phase systems have been useful for the partition of proteins and membrane particles because of the high water content in both phases.

For many biochemical applications it may be valuable to have phases which both, and to the same degree, exhibit a greater hydrophobicity than aqueous systems. If so, it should be possible to partition less water-soluble biochemicals. The present work demonstrates that the replacement, to various degrees, of the water in the aqueous two-phase systems with organic solvents can increase the solubility of hydrophobic compounds in both phases.

MATERIALS AND METHODS

Chemicals

The organic solvents were of analytical grade and were used without further purification. Zein from corn, stearic acid and β-carotene from carrot were obtained from Sigma (St. Louis, MO, U.S.A.). [(1*S*)-*endo*-]-(−)-Borneol, (1*S*)-(−)-β-pinene and β-estradiol were obtained from Aldrich (Steinheim, F.R.G.). Glycerol tripalmitate

was from BDH (Poole, U.K.), cholic acid from Schwartz/Mann (Orangeburg, NY, U.S.A.) and benzoic acid from Baker (Phillipsburgh, NJ, U.S.A.). An extract containing chlorophyll was obtained from spinach leaves by treating them with acetone and filtering. The filtrate was evaporated to dryness and the residue was dissolved in acetone and finally filtered. Before use the acetone was evaporated.

Polymers

Polyethylene glycol (PEG) with a molecular weight, $M_r = 8000$ was obtained from BP Chemicals (Hythe, U.K.). Dextran T500, Ficoll 400 and hydroxypropyl-dextran 500 (with 1.5 mol hydroxypropyl groups per mol glucose) were obtained from Pharmacia (Uppsala, Sweden). Hydroxypropyl-starch (Aquaphase PPT) was obtained from Perstorp Biolytica (Lund, Sweden). Polyvinyl acetate, "medium molecular weight" catalogue No. 18,948-0, was from Aldrich.

Preparation of the two-phase systems

The dry polymers, the solvents and the water were weighed out. In the case of dextran a 20% stock solution was prepared[4]. All concentrations of the polymers and solvents are given in per cent (w/w) of the total system. The mixtures were in most cases heated in a water-bath (50°C) to reduce the time taken to dissolve the polymers. Before equilibration the systems were brought to 22 ± 1°C.

Turbidometric titration

Phase transition points were determined by the addition of solvent mixtures to two-phase systems which were being shaken continuously until the turbidity ceased. Percentage compositions were calculated from the initial and final weights.

Analysis of phase composition

The composition of the phases of a dextran–PEG–dimethylformamide–water two-phase system was analysed by gel chromatography on a Sephacryl S-300 column (25 cm × 1.5 cm) using water as the eluent. The eluate was analysed with a refractive index monitor (Multiref 902B; Optilab, Vällingby, Sweden). Dextran and Ficoll were in some cases analysed by polarimetry[4].

Solubility measurements

The solubility of benzoic acid, in mixtures of organic solvents and water, was measured by the absorbance at 272 nm of the saturated solutions. Cloud points for hydrophobic compounds dissolved in organic solvents (20 g/l) were determined by the dropwise addition of water. For polymers the cloud points were ascertained by the addition of organic solvents to aqueous solutions (10%, w/w).

Partition coefficients

Samples of measured volumes were withdrawn from the phases and diluted in 65% (v/v) dioxane in water. The concentration of the partitioned substance was determined photometrically with an Hitachi 100-60 spectrophotometer. The following wavelengths were used: zein, 277.5 nm; β-carotene, 450 nm; β-estradiol, 280.5 nm; benzoic acid, 272 nm; and chlorophyll a (extracted into acetone from spinach), 665 nm. In some cases the solutes and polymers of the diluted phases (10–20 times) were

separated by gel chromatography (Sephadex LH-20, 50 cm × 1.3 cm) in dimethyl-formamide and the refractive index of the eluate was monitored.

Counter-current distribution

A nineteen-transfer counter-current distribution was carried out manually as described elsewhere[5]. The volumes in each tube were 2.1 and 2.0 ml for the upper and lower phases, respectively. A sample of 25 mg zein per ml was introduced in system number zero. After the completed transfer, 1.6 ml dimethylformamide were added to each tube to disrupt the systems. The protein content was determined according to Bradford[6]. The yellow constituent in zein was measured by the absorbance at 400 nm.

RESULTS AND DISCUSSION

Solubility of hydrophobic compounds and polymers

The solubility of many organic compounds in water is low while they are very soluble in a number of organic solvents. By adding solvents which mix with water the solubility can be gradually increased. Even a moderate addition of solvent may have a marked effect on the solubility, as demonstrated here for benzoic acid, Fig. 1. A number of hydrophobic substances chosen from groups of compounds of
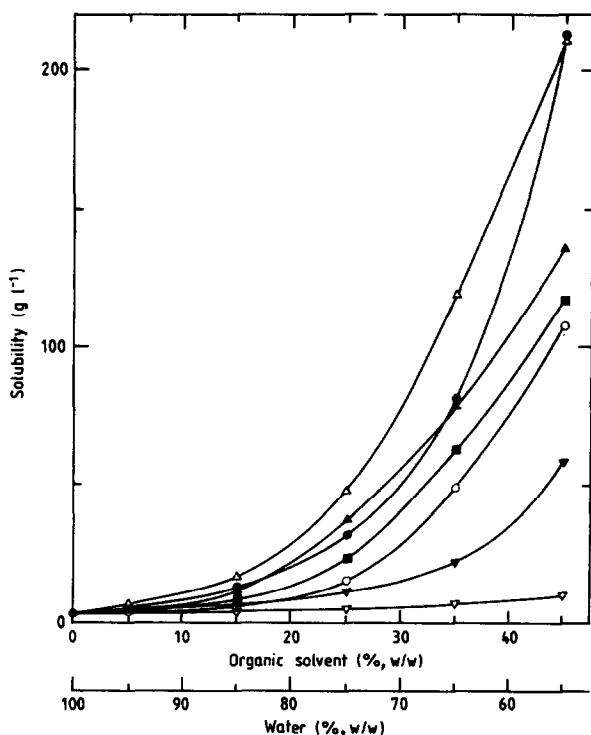

Fig. 1. Solubility of benzoic acid in mixtures of organic solvent and water at 22°C. Solvents: ∇ = ethylene glycol; $\blacktriangledown$ = dimethyl sulphoxide; $\bigcirc$ = ethanol; $\blacksquare$ = dioxane; $\blacktriangle$ = acetonitrile; $\bullet$ = N,N-dimethyl-formamide; $\triangle$ = acetone.

TABLE I

SOLUBILITY OF SOME HYDROPHOBIC COMPOUNDS IN A SERIES OF ORGANIC SOLVENTS AT 22°C

+ = The compound is soluble to a concentration of at least 20 g/l; − = the solubility is less then 20 g/l. Values give the cloud points (in per cent organic solvent of total solvent) when solutions of the compounds (20 g/l in the organic solvent) were titrated with water.

Solute	Solvent							
	Ethylene glycol	Dimethyl-formamide	Tetrahy-drofuran	Aceto-nitrile	Dioxane	Ethanol	Acetone	Dimethyl-sulphoxide
Glycerol tri-palmitate	−	−	+ 75	−	+ 92	−	−	−
Stearic acid	−	+ 92	+ 63	−	+ 82	+ 94	+ 99	−
β-Carotene	−	+ 74	+ 56	+ 58	+ 69	+ 62	+ 63	+ 92
β-Pinene	−	+ 79	+ 61	+ 64	+ 70	+ 61	+ 63	+ 89
β-Estradiol	+ 94	+ 55	+ 33	−	+ 48	+ 55	+ 47	+ 65
Borneol	+ 82	+ 47	+ 34	+ 26	+ 33	+ 30	+ 28	+ 64
Cholic acid	+ 51	+ 23	+ 23	−	+ 23	+ 23	−	+ 39

biochemical interest were tested in eight (water-soluble) solvents, Table I. In several cases 50% or more of the water had to be replaced by the solvent in order to get a reasonable solubility of the hydrophobic solute.

To be able to generate a liquid–liquid two-phase system, with comparable solvent composition in both phases, it is necessary to find two polymers which are soluble in the solvent (or mixture of solvents) but partially exclude each other. Table II shows the solubility of some polymers in a number of solvents. Good solvents for the polymers tested are water, ethylene glycol, glycerol, dimethyl sulphoxide and N,N-dimethylformamide. The last two also have acceptable properties for the hydrophobic substances. Because of the unpleasant properties of dimethyl sulphoxide, dimethylformamide was chosen as the solvent for the systems used to study the partition of hydrophobic substances.

Systems composed of an organic solvent and two polymers

Ficoll 400 and PEG 8000, dissolved together in N,N-dimethylformamide, gave rise to two-phase systems (at 22°C) when the concentration of each polymer exceeded 7.5% (w/w). In these systems the Ficoll was found mainly in the lower phase and the PEG in the upper phase. When PEG was added with hydroxypropyl-dextran 500 (1.5 hydroxypropyl groups per glucose unit), in the same solvent, the concentration of each polymer needed to be higher than 7.1% in order to get two phases. Hydropropyl-dextran is, in contrast to dextran, very soluble in dimethylformamide, Table II.

TABLE II

SOLUBILITY OF SOME POLYMERS IN A SERIES OF SOLVENTS AT 22°C

+ = The polymer is soluble to a concentration of at least 5% (w/w); − = the solubility is less than 5% (w/w). Values give the cloud points (in per cent organic solvent of total solvent) when 10% aqueous solutions of the polymers were titrated with the solvent.

Solvent	Dextran 500	Ficoll 400	Aquaphase	Hydroxypropyl-dextran 500	PEG 8000	Polyvinyl acetate
Water	+	+	+	+	+	−
Glycerol	+	+	+	+	84	−
Ethylene glycol	+	+	+	+	>95	−
Dimethyl sulphoxide	+	+	+	+	+	+
Ethanol	30	61	50	91	+	+
Tetrahydrofuran	30	47	42	59	+	+
Dioxane	46	69	64	80	+	+
Acetonitrile	22	36	31	44	+	+
Dimethylformamide	62	+	+	+	+	+
Acetone	24	46	33	61	+	+

Partition in systems with organic solvents

The partitions of some hydrophobic substances within two-phase systems based on N,N-dimethylformamide are given in Table III. Substances with lower molecular weights showed a somewhat higher affinity for the upper phase with partition coefficients in the range 1–2.5. In the case of an hydrophobic protein, zein from corn, a partition coefficient less than one was observed.

TABLE III

PARTITION OF SOME HYDROPHOBIC COMPOUNDS IN TWO-PHASE SYSTEMS WITH N,N-DIMETHYLFORMAMIDE AS THE SOLVENT

The partition coefficient is defined as the ratio between the concentrations of the compound in the upper and lower phases. Composition of system: A, 8.5% Ficoll 400 and 8.5% PEG 8000; B, 8.1% hydroxypropyl-dextran 500 and 8.1% PEG 8000; C, 10% hydroxypropyl-dextran 500 and 10% PEG 8000. Temperature: 22°C. N.D. = Not determined. Values with an asterisk are partition coefficients obtained via gel chromatography of the phases (on Sephadex LH-20 in dimethylformamide) with values within ±10%. All other partition coefficients (±2%) were determined via photometric measurements.

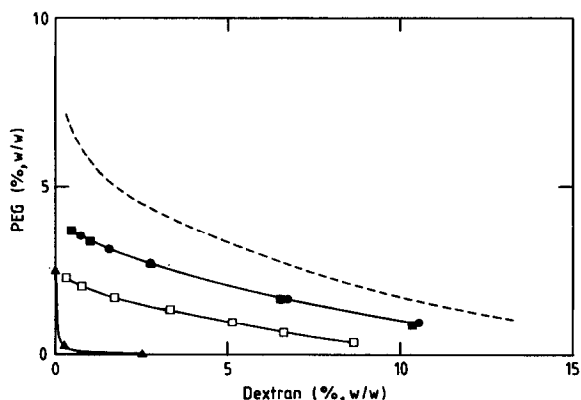| Partitioned compound	Molecular weight	Concentration (g/l)	Partition coefficient in system		
			A	B	C
Benzoic acid	122	2	1.28	1.18	1.35
Borneol	154	10	N.D.	1.3*	1.5*
β-Carotene	537	2	2.09	1.69	2.14
Chlorophyll a	894	0.05	2.08	1.68	2.43
Cholic acid	387	10	N.D.	1.9*	2.3*
β-Estradiol	272	2	1.45	1.26	1.36
β-Pinene	136	10	N.D.	1.5*	1.9*
Stearic acid	284	30	N.D.	1.4*	2.4*
Zein	⩾40 000	10	1.20	0.76	0.54

Fig. 2. Transition (one to two phases) curves for systems composed of dextran 500, PEG 8000, water and organic solvent (25%, w/w of total system). Solvents: ■ = dioxane; ● = N,N-dimethylformamide; □ = tetrahydrofuran; ▲ = acetonitrile. Temperature: 22°C. The broken line indicates the corresponding curve with water as the solvent (data from ref. 4).

Systems containing both organic solvents and water

The influence of solvents on the formation of two liquid phases was studied with a dextran–PEG system. Fig. 2 shows the borderline between the one- and two-phase areas (the binodial curve) when 25% (w/w) of the system consisted of organic solvent. The phase transition occurs at lower and lower concentrations of polymers according to the following order of organic solvents: N,N-dimethylformamide = dioxane < tetrahydrofuran < acetonitrile. The concentration range, in which a solvent can be used, was studied by following the transition points (using equal amounts of the two polymers) at increasing concentrations of organic solvent, Fig. 3. In the case of ethylene glycol up to 60% of the water can be replaced by this solvent with only minor changes in the transition point. Dioxane and N,N-dimethylformamide showed identical behaviour up to 25% (of total system) but deviated at higher concentrations.
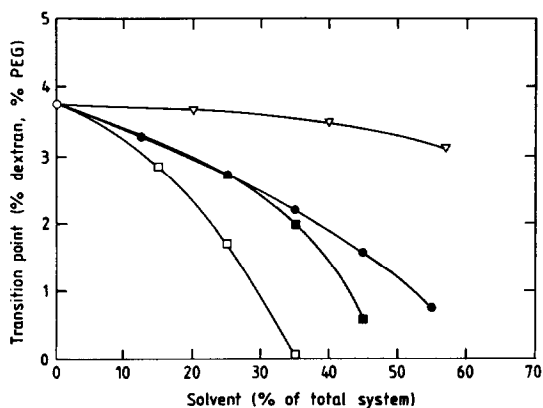

Fig. 3. Transition points (from one to two phases) of phases containing dextran 500 and PEG 8000 (in equal weights) as function of the solvent content (in %, w/w of total system). Solvents: ▽ = ethylene glycol; ● = N,N-dimethylformamide; ■ = dioxane; □ = tetrahydrofuran. Temperature: 22°C.

TABLE IV

RATIOS BETWEEN THE VOLUMES OF THE UPPER AND LOWER PHASES FOR SOME
TWO-PHASE SYSTEMS CONTAINING DEXTRAN 500 AND PEG 8000, WITH OR WITHOUT
ORGANIC SOLVENT (25% OF TOTAL SYSTEM)

Temperature: 22°C. Data for the solvent-free system have been obtained from ref. 4.

Solvent	Composition of system (%, w/w)			Volume ratio
	Dextran	PEG	Organic solvent	
Water	8.0	3.0	—	0.5
	5.0	5.0	—	1.7
	3.0	6.0	—	3.8
N,N-Dimethylformamide	8.0	2.0	25	0.5
	5.0	5.0	25	2.6
	3.0	6.0	25	6.0
Dimethyl sulphoxide	8.0	2.0	25	0.04
	5.0	5.0	25	2.0
	3.0	6.0	25	3.9
Dioxane	8.0	2.0	25	0.4
	5.0	5.0	25	2.5
	1.5	5.0	25	8.4
Tetrahydrofuran	8.0	1.5	25	1.1
	5.0	5.0	25	4.0
	1.5	4.0	25	14
Acetonitrile	1.5	0.25	25	16

The upper limit for the inclusion of N,N-dimethylformamide was around 60%. For
dioxane and tetrahydrofuran the limits were 47 and 35%, respectively.

The volume ratios for a number of systems containing 25% organic solvent are
shown in Table IV. The volume of the upper phase increased with the concentration of
PEG which shows that this polymer was enriched in the top phase. The compositions
of the phases may be presented in the form of a phase diagram. Such a diagram is given
for the dextran–PEG–water–dimethylformamide system, Fig. 4. The three-dimen-
sional diagram is divided into two parts by a curved surface, the binodial surface.
Systems which have compositions above this surface give rise to two phases. The
compositions of the two phases are found on the binodial surface. Furthermore, the
points representing the total composition of the system and its phases lie on a straight
line (the tie-line). The ratio between the weights of the phases is equal to the ratio of the
segment lengths of the tie-line (intersected by the point for the total composition). It
was found that the upper phases were somewhat richer in dimethylformamide than the
lower phases, Table V and Fig. 4. On the other hand, the same is true for water. The
relative amount of water and dimethylformamide in the phases did not differ by more
than 2 percentage units.

A favourable system would be one in which the ratio between water and organic
solvent can be varied within all possible values. It would then be possible to adjust the
solvating properties according to the actual mixture of substances to be partitioned.
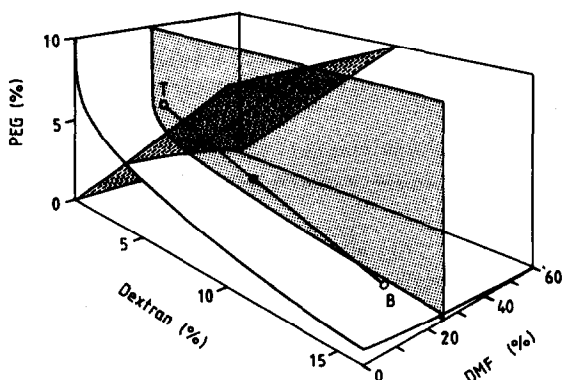
Fig. 4. Phase diagram for the system dextran 500, PEG 8000, water and N,N-dimethylformamide (DMF) at 22°C. The binodial surface is cut by two planes: one where the concentrations of PEG and dextran are equal, the other with constant concentration of DMF (25%, w/w). The total composition of a system (6.9% dextran, 2.7% PEG and 25% DMF) in the latter plane is marked with ●. The composition of the top phase (T) lies behind the plane. The composition of the lower phase (B) is located in front of the same plane. These three points fall on a straight line.

Possible pairs of polymers are Ficoll and PEG as well as hydroxypropyl-dextran and PEG. Both combinations gave two-phase systems in both water and dimethyl-formamide. The behaviour of the systems when they contained both solvents (in various proportions) can be seen in Table VI. Mixtures of the two solvents enhanced the miscibility of the polymers (in solution), and increasing concentrations were necessary to obtain two phases. At certain proportions of solvents no phase separation was obtained even at high polymer concentrations (20% of each).

Partition in systems containing both solvent and water
        The partition of benzoic acid is affected only slightly when the concentration of dimethylformamide is raised from 0 to 45% in a dextran–PEG–water system, Table VII. This is due to the simultaneous and almost equal increase of the hydrophobicity of both phases.

TABLE V

COMPOSITION OF PHASES (IN %, w/w) OF SYSTEMS CONTAINING DEXTRAN 500, PEG 8000, WATER AND N,N-DIMETHYLFORMAMIDE (DMF) AT 22°C

Total system				Upper phase				Lower phase			
Dextran	*PEG*	*Water*	*DMF*	*Dextran*	*PEG*	*Water*	*DMF*	*Dextran*	*PEG*	*Water*	*DMF*
6.7	4.0	89.3	—	0.5	6.8	92.7	—	12.6	1.1	86.3	—
6.5	3.4	77.6	12.5	0.2	5.9	81.2	12.7	13.1	1.1	74.0	11.8
6.9	2.7	65.4	25.0	0.2	4.8	68.7	26.3	14.0	0.8	61.5	23.7
7.5	2.2	55.3	35.0	0.2	4.1	56.7	39.0	15.6	0.3	51.2	32.9
9.3	1.6	44.1	45.0	0.1	3.2	45.5	51.2	18.4	0.3	39.5	41.8

TABLE VI

TRANSITION POINTS (FROM ONE TO TWO PHASES) AT EQUAL CONCENTRATIONS OF
FICOLL 400 AND PEG 8000, OR HYDROXYPROPYL-DEXTRAN 500 (HP-DEXTRAN) AND PEG
8000, RESPECTIVELY, IN MIXTURES OF WATER AND N,N-DIMETHYLFORMAMIDE (DMF)

Temperature: 22°C. Values are in % (w/w)

Solvent mixture		System composition at phase transition point				
Water	DMF	Ficoll	HP-dextran	PEG	Water	DMF
100	0	7.7	—	7.7	84.6	0
90	10	8.5	—	8.5	74.7	8.3
80	20	9.3	—	9.3	65.1	16.3
70	30	11.4	—	11.4	54.0	23.2
60	40	16.0	—	16.0	40.8	27.2
50	50	>20	—	>20	<30	<30
30	70	16	—	16	20	28
20	80	10.6	—	10.6	15.8	63.0
10	90	8.0	—	8.0	8.4	75.6
0	100	7.5	—	7.5	0	85.0
100	0	—	14.8	14.8	70.4	0
90	10	—	17.2	17.2	59.0	6.6
80	20	—	>20	>20	<48	<12
40	60	—	>20	>20	<36	<24
30	70	—	15.3	15.3	20.8	48.6
20	80	—	9.9	9.9	16.0	64.2
10	90	—	7.5	7.5	8.5	76.5
0	100	—	7.1	7.1	0	85.8

Counter-current distribution

A water-free system, based on dimethylformamide, was used for multistep
partition analyses of the protein zein from corn, Fig. 5. This system was chosen since
zein does not dissolve in water or in organic solvents containing more than 50% water.
The protein profile shows that zein has at least two components and that the yellow
pigment, probably carotenoids, follows one of them. Zein has been shown to consist of
two main protein components of equal magnitude[7].

TABLE VII

PARTITION OF BENZOIC ACID IN SYSTEMS CONTAINING DEXTRAN 500, PEG 8000, WATER
AND N,N-DIMETHYLFORMAMIDE (DMF)

Concentration of benzoic acid was 2 g l⁻¹. Temperature: 22°C.

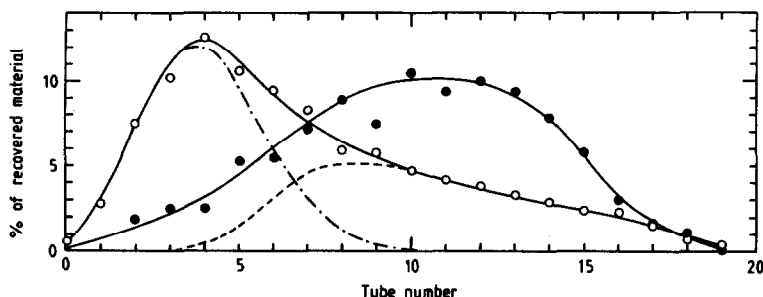| System composition (%, w/w)				Partition coefficient of benzoic acid
Dextran	PEG	Water	DMF	
6.7	4.0	89.3	0	1.27
6.9	2.7	65.4	25	1.29
7.5	2.15	55.35	35	1.31
9.3	1.6	44.1	45	1.42

Fig. 5. Counter-current distribution of zein from corn using a system containing 9% hydroxypropyl-dextran 500, 9% PEG 8000 and 82% N,N-dimethylformamide (DMF). Recovered protein, ○; yellow component, ●. Number of transfers: 19. Temperature: 22°C. The phases were disrupted by the addition of 1.6 ml of DMF. The sample, loaded in tube number zero, contained 100 mg protein. A theoretical curve is included for the left peak, — · — · —, as well as the difference between the experimental protein curve and the theoretical one, — — —.

General remarks

The two-phase systems studied here have comparably low separatory capacity since the substances tested do not partition particularly in favour of one phase or the other. When used in multistep procedure, *e.g.*, counter-current distribution or liquid–liquid chromatography[8] developed for the aqueous two-phase systems, good separation can be foreseen. The new type of two-phase systems may, however, be further improved for specific extractions by introducing chemical groups (ligands) which form strong complexes with the compounds to be extracted. A ligand can be concentrated in mainly one phase by covalently binding to the corresponding polymer. This method of extraction through complex formation in one phase has been applied successfully to proteins in aqueous polymer two-phase systems[4,5]. The increased hydrophobicity is also of interest when the systems are used as enzyme reactors[9,10] (with the enzyme kept in one phase) and the substrate or product has low solubility in water.

CONCLUSIONS

Water-soluble solvents can be included in polymer–polymer (liquid–liquid) two-phase systems and this makes them useful for the partitioning of hydrophobic solutes. The experiments show that liquid–liquid two-phase systems can be attained with great variation in the hydrophobicity but that this property is nearly the same in both phases. Such systems allow the partition of solutes of more hydrophobic character than the usual water–polymer systems do.

ACKNOWLEDGEMENT

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