Distribution of Proteins in Aqueous Two-Phase Systems Formed by Dextran and Polyethylene Glycol. Influence of Protein Hydrophobicity

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Abstract. Aqueous two-phase systems formed by mixing polyethylene glycol (PEG) and dextran (DXT) were studied. The composition of DXT and PEG phase was established by the size exclusion HPLC method. The concentrations of DXT and PEG in both phases as a function of total concentration of both polymers was fitted by nonlinear regression. This procedure yielded an empirical numerical model (with two independent variables: bulk concentration of DXT and PEG) characterizing the composition of either phase at any applied concentration of the polymers. The distributions of bovine serum albumin (BSA) and immunoglobulin G (IgG) were studied in this system in relation to the composition of both phases. Both proteins were predominantly distributed in the dextran phase. The protein affinity to the respective phase was found to increase with: i) the increasing phase dextran concentration; and ii) the decreasing phase PEG concentration. Chemical modification of primary amino groups of BSA by 2,4,6-trinitrobenzene sulfonic acid (TNBS) raised the affinity of the conjugate to the less polar PEG phase.

Key words: Dextran/Polyethylene glycol — Aqueous two-phase systems — SEC-HPLC — Protein hydrophobicity — Protein modification

Introduction

Aqueous two-phase systems (ATPS) are generally composed of water solutions of two structurally distinct hydrophilic polymers with different polarities. Spontaneous phase separation occurs beyond a critical concentration of these components with each of the two resulting phases getting enriched with respect to one of the components (Albertsson 1970). The partitioning of materials between the top and the bottom phase is enabled by differences in the surface properties of the biomolecules to be separated (Wiegel et al. 1991). Many factors may influence the partitioning of biomolecules in ATPS. These factors may be inherent to the

system itself (such as choice of the system components, molecular weight of the components forming the two-phase systems, concentration of salts in the respective phases, ionic strength, pH etc.) and to the protein to be partitioned (molecular weight, hydrophobicity, charge, conformation etc.; Albertsson 1970). Hydrophobicity of proteins may be considered as an important factor influencing the partitioning of proteins in ATPS. Therefore, partitioning of proteins in ATPS allows to deduce the effective hydrophobicity of proteins (Shanbhag and Axelsson 1975). Also hydrophobicity of proteins can be deduced: i) from the primary structure of proteins (Bigelow 1967); ii) according to interactions of proteins with hydrophobic fluorescent labels (Kato and Nakai 1980); iii) from measurements of interfacial tension between aqueous protein solution and a non polar liquid such as corn oil (Keshavartz and Nakai 1979); iv) using hydrophobic interaction chromatography (Hjertén et al. 1974) or reverse phase HPLC (Asenjo et al. 1994); v) using equilibrium adsorption of proteins on hydrophobized sorbents (Gemeiner et al. 1989; Breier et al. 1994a); vi) by fractional precipitation of proteins using ammonium sulfate (Asenjo et al. 1994). Mutual relations between data on protein hydrophobicity obtained by methods i), ii) and iii) yielded three independent variables and ATPS data have been described recently (Breier et al. 1994b). However, direct correlation was only observed for ATPS data and those obtained by measurement of interfacial tension (method iii). This indicated that application of different methods for estimating protein hydrophobicity may yield different results reflecting different interferences of protein hydrophobicity with other physico-chemical properties of the protein molecules. However, in the case of proteins modified by a hydrophobic reagent i.e., when besides hydrophobicity, other physico-chemical properties of the samples are relatively constant, the measurement in ATPS may more precisely reflect the changes of protein hydrophobicity due to the modification. Registration of changes of calreticulin hydrophobicity induced by its covalent modification with 2,4,6,-trinitrobenzene sulfonic acid (TNBS, Breier and Michalak 1994) may be considered an example.

In the present work we studied the partition of BSA, IgG and BSA modified to different degrees by TNBS. The aim of this study was detailed description of protein partition (in ATPS formed by polyethylene glycol and dextran as the top and the bottom phases) in relation to the composition of both (PEG and DXT) phases and protein hydrophobicity.

Materials and Methods

Materials

Polyethylene glycol (PEG 6000, Mw=6000 g/mol) and dextran 70, (Mw=60.000 g/mol) were obtained from Lachema (Brno, Czech Republic) and Biotika (Slovenská Ľupča, Slovakia), respectively. Bovine serum albumin (BSA, SEVAC, Prague, Czech Republic) and

normal human immunoglobulin G stabilized with glycine (Imuna Šarisske Michalany Slovakia) were extracted by ethanol chloroform 1 3 and lyophilized All other chemicals (analytical grade) were supplied by Sigma (USA) and Lachema (Brno Czech Republic)

Preparation of DX1/PEG aqueous two phase systems

Two phase systems were prepared by mixing DXT (final concentration 1 15% w/v) and PEG (final concentration 2 15% w/v) in 4 ml of 1 mmol l^{-1} Tris-HCl buffered solution pH 8 0 The mixtures were equilibrated to phase separation for 48 hours at room temperature Aliquots of both phases were used to estimate DXT and PEG contents by size exclusion HPLC In the case of protein partition 0 1 ml of protein solution (3 6 mg ml ^{1}) was added to 3 9 ml of ATPS After 48 hours protein concentrations in both phases were determined by the method of Bradford (1976) using the respective equilibrated phases without proteins as blank (Breier and Michalak 1991)

Size exclusion HPLC chromatography

Concentrations of DXT and PEG in both phases were determined by size-exclusion HPLC using a high pressure pump HPP 5001 (Laboratorni pristroje Prague Czech Republic) an eight port switching valve equipped with two 0 1 ml loops (Model PK1 Instrumentation Developing Workshops Czechoslovak Academy of Sciences Prague Czech Republic) two stainless steel columns (250 × 8 mm) packed with Separon HEMA-S 1000 and HEMA-S 100 (mean particle size 10 μm) connected in series (Tessek Prague Czech Republic) and a differential refractometric detector (RIDK 102 Laboratorni pristroje Prague Czech Republic) These analyses were carried out at 25 °C using 1 mmol l^{-1} Tris HCl buffered solution (pH 8 0) as the mobile phase and at a flow rate of 0 7 ml min^{-1}

Chemical modification of BSA by 2 4 6 trinitrobenzene sulfonic acid

Proteins (100 mg) were left to react with 2 4 6 trinitrobenzene sulfonic acid (TNBS 1 and 50 mg) in borate buffer solution (20 ml) under continual control of pH (9 0) After 2 hours the sample was dialyzed two times against bidistilled water The proteins were lyophilized and stored at 4 °C

Data processing

Relationships between the estimated concentrations of DXT or PEG in both phases of the systems and the total amounts of these two components were fitted as a function of two independent variables by nonlinear regression using the SigmaPlot Scientific graphic system version 5 0 (Jandel Corporation 1986 1992)

Results

Description of DXT/PEG aqueous two phase system formation

Formation of DXT/PEG aqueous two-phase systems was studied in the concentration range of both polymers of 2 15% w/v The interval of critical bulk concentrations of DXT and PEG to give rise to a two phase system was established (Fig 1*A*) The formation of ATPS as a function of bulk concentration of both polymers yielded a non-monotonous function Fig 1*A* shows that for intervals of

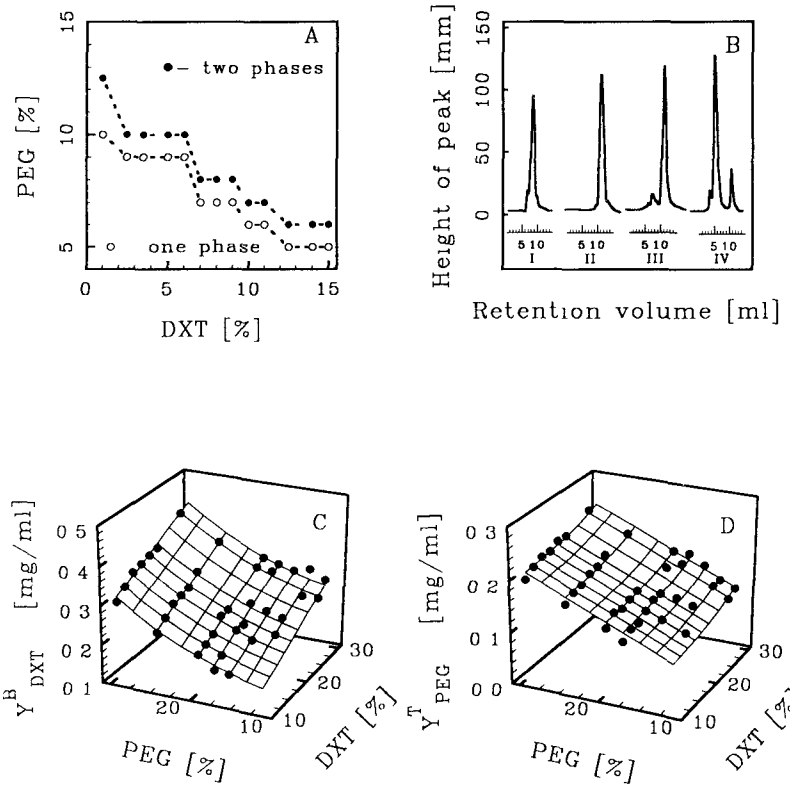

Figure 1. Formation of aqueous two phase system from the DXT and PEG. *A* Interval of critical concentrations of DXT and PEG for the formation of a two-phase system. DXT and PEG (final concentrations 2–15%) were mixed in 1 ml of Tris-HCl buffer (pH 8.0; 1 mmol l^{-1}). The entire mixture was stirred and the phase formation checked after 24 hours. *B* Analysis of phase composition by SEC-HPLC. I – elution profile of pure DXT (7% w/v). II – elution profile of pure PEG 10% (w/v). III – elution profile of top phase and IV – elution profile of bottom phase of ATPS containing 15% (w/v) DXT and 10% (w/v) PEG. *C* Relation between DXT concentration in bottom (i. e. DXT) phase and bulk concentrations of DXT and PEG as two independent variables. Experimental data were fitted according to Equation 1 and plotted in a three coordinate system. Points represent measured data and the mesh represents computed data (Equation 1). *D* Relation between PEG concentration in top (i. e. PEG) phase and bulk concentrations of DXT and PEG as two independent variables. Experimental data were fitted according to Equation 2 and plotted in a three-coordinate system. Points represent measured data and the mesh represents computed data (Equation 2).

DXT concentrations between 1 and 2, 6 and 7, 9 and 10, 11 and 12.5 (each in percentage w/v) ATPS formation depended on the amount of both DXT and PEG

For DXT concentrations outside of the above intervals, formation of a two-phase system was relatively independent of DXT amount, and predominantly depended on PEG amount.

The difference in molecular weights of both polymers enabled quantitative estimation of both polymers in DXT- and PEG-phases by size-exclusion HPLC (Fig. 1*B*). The compositions of both phases, in relation to bulk concentrations of both polymers as two independent variables, were fitted according to the following empirical equations, which were found to satisfactorily describe the process:

$$y^B_{\mathrm{DXT}} = p_1 c_{\mathrm{DXT}} + p_2 \exp(p_3 c_{\mathrm{PEG}}) \quad (1)$$

$$y^T_{\mathrm{PEG}} = P_1 c_{\mathrm{PEG}} + P_2 \exp(P_3 c_{\mathrm{DXT}}) \quad (2)$$

$$y^T_{\mathrm{DXT}} = -q_1 c_{\mathrm{DXT}} + q_2 \exp(-q_3 c_{\mathrm{PEG}}) \quad (3)$$

$$y^B_{\mathrm{PEG}} = -Q_1 c_{\mathrm{PEG}} + Q_2 \exp(-Q_3 c_{\mathrm{DXT}}) \quad (4)$$

where $p_1, p_2, p_3, P_1, P_2, P_3, q_1, q_2, q_3, Q_1, Q_2, Q_3$ represent constants and were computed by nonlinear regression; $y^B_{\mathrm{DXT}}, y^T_{\mathrm{PEG}}, y^T_{\mathrm{DXT}}$ and y^B_{PEG} are equilibrium concentrations of DXT and PEG respectively in the DXT (*B*-bottom) and the PEG (*T*-top) phases.

Nonlinear fitting of the experimental data by these equations provided a good characterization of the respective relations (Fig. 1*C* and *D*). Parameters p_1, p_2, p_3, $P_1, P_2, P_3, q_1, q_2, q_3, Q_1, Q_2, Q_3$ characterized by S.E.M. values are summarized in Table 1. Moreover, experimental data gave the same binodial as those computed

Table 1. Values of parameters in Equations 1–4

	Parameter	Value ±S.E.M. [$\times 10^{-2}$]
Equation 1	p_1	0.634 ± 0.013
	p_2	3.269 ± 0.204
	p_3	6.551 ± 0.188
Equation 2	P_1	0.668 ± 0.005
	P_2	0.217 ± 0.049
	P_3	9.898 ± 0.720
Equation 3	q_1	0.057 ± 0.002
	q_2	17.340 ± 0.292
	q_3	8.390 ± 0.107
Equation 4	Q_1	0.201 ± 0.006
	Q_2	9.218 ± 0.239
	Q_3	1.527 ± 0.075

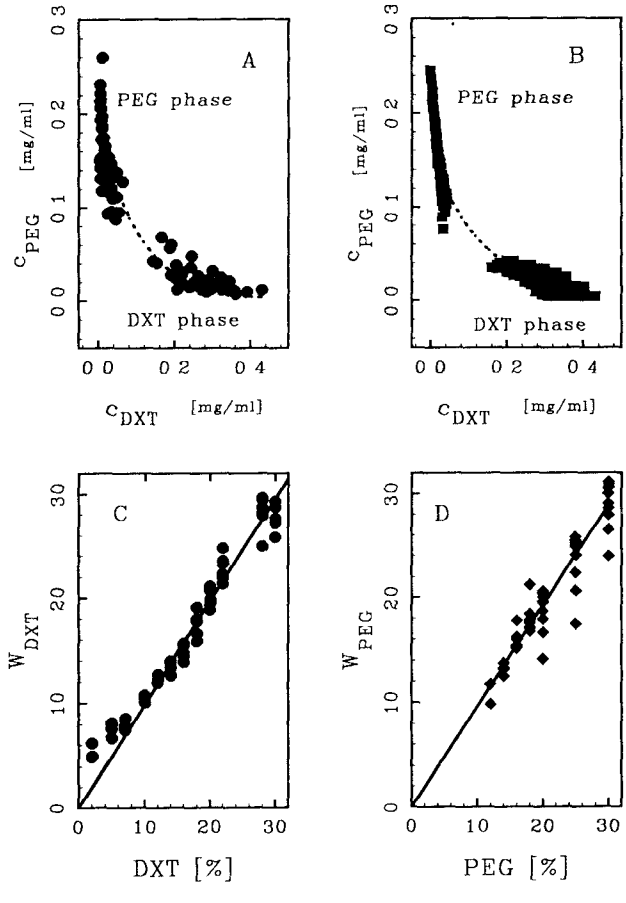

Figure 2. Verification of empirical numerical model for the description of ATPS formation given by Equations 1–4. Binodial curves $y = a \exp(b\,x)$, ($a = 0.189 \pm 0.005$, $b = -9.316 \pm 0.757$) characterizing distributions of both sets of measured data (*A*) and data computed by Equation 1–4 (*B*). Correlation of bulk DXT (*C*) and PEG (*D*) concentrations with the corresponding concentrations given from material balance from Equations 5, 6. Slopes 0.984 ± 0.011 and 0.962 ± 0.011 and correlation coefficients 0.977 and 0.945 were obtained by linear regression of data on *C* and *D*, respectively.

from the above equations (Fig. 2*A* and *B*). The material balance (W_{DXT}, W_{PEG}) for DXT or PEG in the respective two-phase systems is given from Equations 1–4 as follows:

$$W_{DXT} = [(y^B_{DXT} V^B) + (y^I_{DXT} V^I)]\ 100 \quad (5)$$

$$W_{PEG} = [(y^B_{PEG} V^B) + (y^I_{PEG} V^I)]\ 100 \quad (6)$$

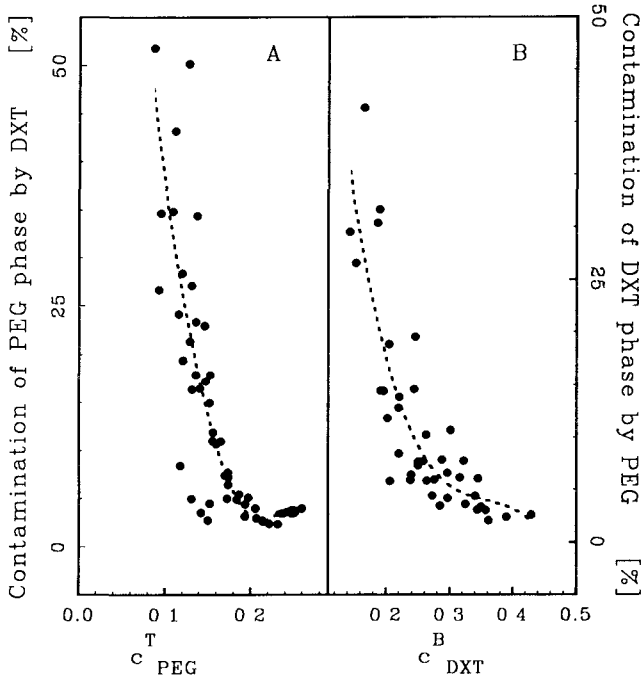

Figure 3. Contamination of PEG phase by DXT (A) as well as DXT phase by PEG (*B*). Data are expressed in percentages of the equilibrium concentration of the minor polymer in relation to the equilibrium concentration of the dominant polymer in the respective phase.

where V^T and V^B represent the equilibrium volume of the top (i. e. PEG) and the bottom (i. e. DXT) phase measured in calibrated test tubes for each composition of the two-phase system applied. When bulk concentrations of DXT and PEG were compared with the corresponding data computed from Equations 5 and 6, a good correlation was observed (Fig. 2*C* and *D*). Cross-contamination of phases (i.e., by PEG in DXT phase or by DXT in PEG phase) was found to decrease exponentially with the equilibrium concentration of the dominant phase polymer (i.e., DXT in DXT phase or PEG in PEG) as documented in Fig. 3.

Relation between protein distribution in a two-phase system and polymer composition of phases

Partitioning of BSA and IgG was studied in two-phase systems formed by mixing proportional ratios of DXT and PEG: DXT/PEG 7/10, 8/8, 10/8, 12.5/12.5, 15/7. Both proteins were found to accumulate in higher concentrations in the bottom (DXT) phase for each composition of the two-phase system. The relationship be-

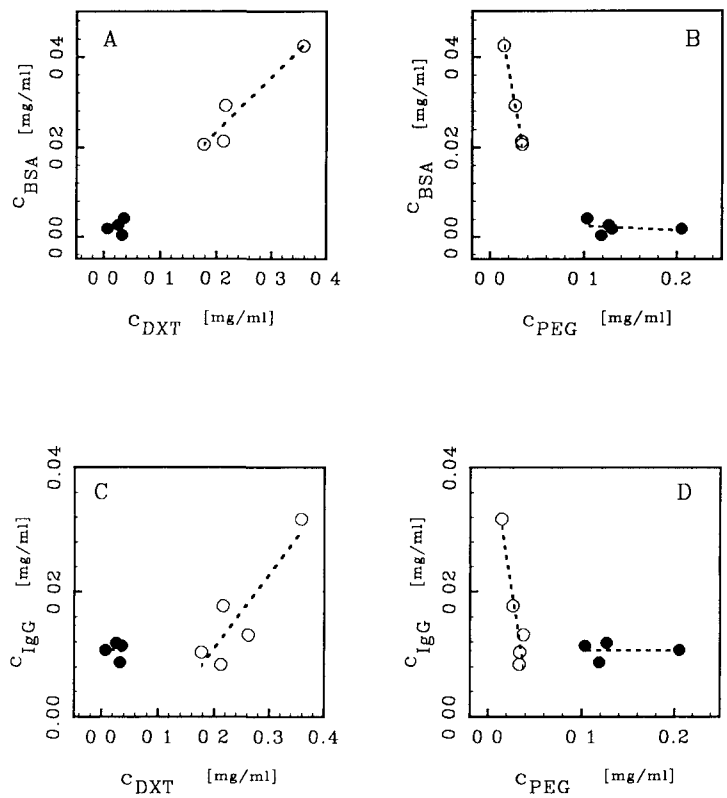

Figure 4. Equilibrium concentration of BSA (*A* and *B*) and IgG (*C* and *D*) in DXT (open symbols) and PEG (full symbols) phase as a function of equilibrium concentrations of DXT (*A* and *C*) and PEG (*B* and *D*) in the respective phase.

tween protein concentration in the respective phase as a function of DXT (or PEG) equilibrium phase concentrations (computed from Equations 1–4 using parameters from Table 1.) are shown in Fig. 4. Concentrations of both proteins were found to increase with the increasing DXT concentration, and to decrease with the increasing PEG concentration in DXT phase. These relations could be described by a statistically significant linear regression (correlation coefficients exceeded in each case the value of 0.878, indicating significance at $p < 0.05$, $n = 5$). In contrast, there was only poor correlation between the protein concentration in PEG phase and the polymer phase composition ($p > 0.1$). The protein affinity to the respective phase was evaluated by the partition coefficients $K_{DXT/PEG}$ and $K_{PEG/DXT}$, which represents the ratio of protein concentrations in identified phases. The affinities of IgG and BSA to the respective phase increased with the increasing DXT concentrations in that phase (Fig. 5). In contrast, an increase in PEG concentra-

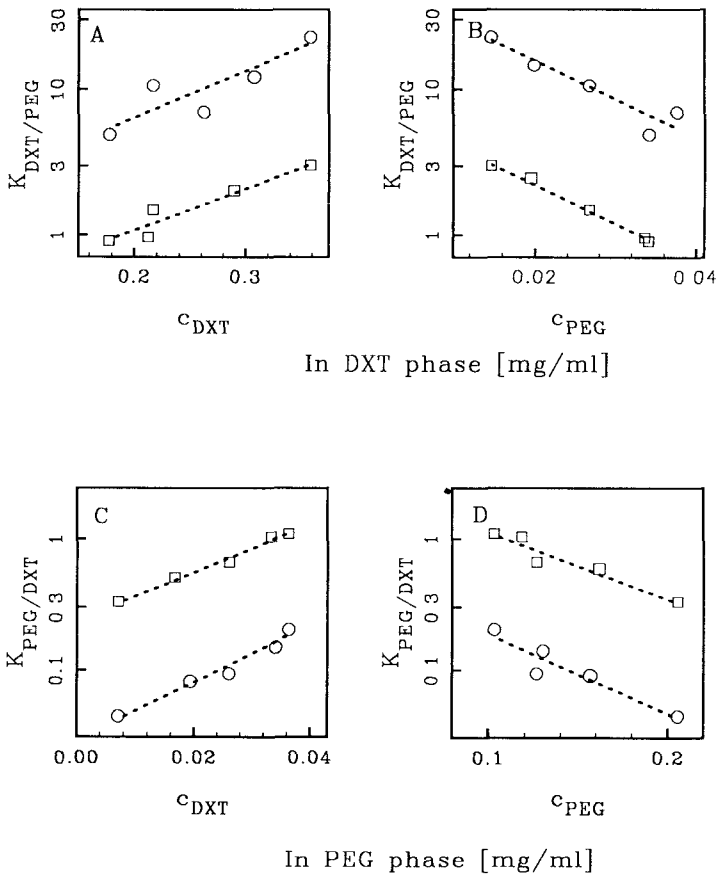

Figure 5. Partition coefficients $K_{PEG/DXT}$ and $K_{DXT/PEG}$ for distributions of BSA (circle) and IgG (squares) in ATPS as a function of equilibrium concentrations of DXT (*A* and *C*) or PEG (*B* and *D*) in DXT (*A* and *B*) or PEG (*C* and *D*) phase.

tion in the phase induced a decrease in affinities of both proteins to the respective phase. These relations gave significant linear correlations ($p < 0.05$) when plotted in semilogarithmic coordinates.

BSA was modified by TNBS to contain a lower degree (0.930 mol) of trinitrobenzene groups per mol of BSA (BSA-TNB1); and a higher degree (8.738 mol) of trinitrobenzene groups per mol of BSA (BSA-TNB10). When the modified BSA preparations were applied to ATPS an increase in $K_{PEG/DXT}$ values and a decrease in $K_{DXT/PEG}$ values were observed (Fig. 6). Nevertheless, the values of $K_{DXT/PEG}$ remained higher than unity and $K_{PEG/DXT}$ remained smaller than unity in all cases. The affinity of BSA-TNB1 to the respective phase was increasing with both

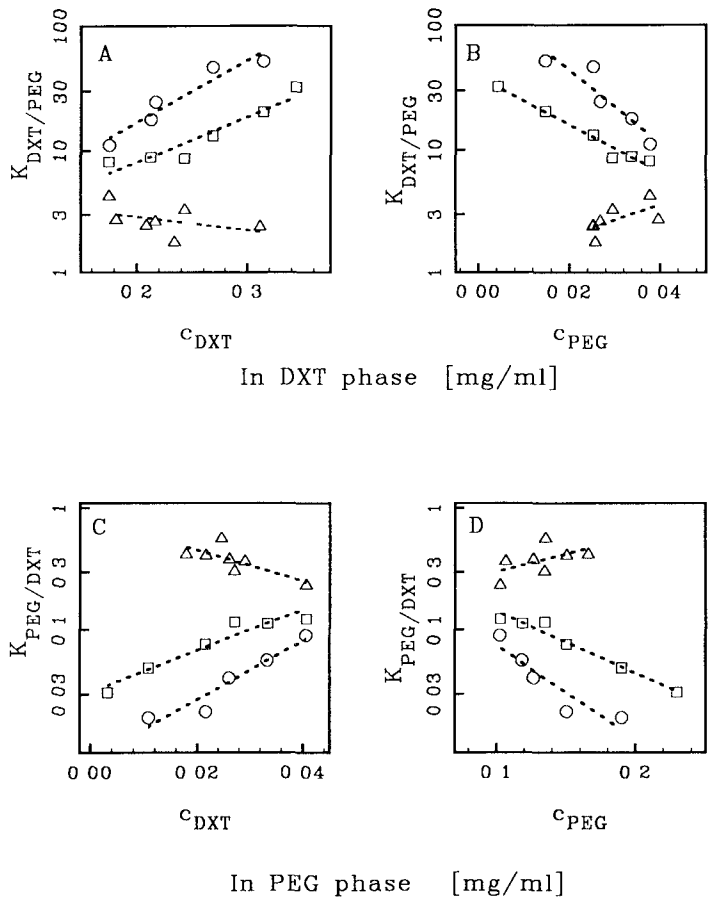

Figure 6. Partition coefficients $K_{PEG/DXT}$ and $K_{DXT/PEG}$ for distributions of non-modified BSA (circle) BSA TNB1 (squares) and BSA-TNB10 (triangles) in ATPS as a function of equilibrium concentration of DXT (*A* and *C*) or PEG (*B* and *D*) in DXT (*A* and *B*) or PEG (*C* and *D*) phase. Proteins modification was carried using the method described in Materials and Methods and yielded 0.930 and 8.738 moles of trinitrobenzene groups per mol of BSA for BSA TNB1 and BSA-TNB10 respectively.

the increasing phase DXT and the decreasing phase PEG concentration whereas BSA TNB10 showed opposite behavior.

Discussion

Polyethylene glycol and dextran represent suitable nonionic polymers forming the top phase with a lower polarity and the bottom phase with a higher polarity, respec-

tively. Theoretical and practical aspects describing this system have been given by Albertson et al. (1970), Brooks et al. (1985) and Bamberger et al. (1985). However, the description of physico-chemical principles of the ATPS formation remains rather empirical. Critical bulk concentrations of DXT and PEG at which phase separation could be observed do not give monotonous function (Fig. 1*A*). We have no explanation for this fact, but any simple equilibrium behavior of DXT and PEG could be expected. Equilibrium concentration of DXT in DXT phase (estimated by HPLC methods, see Fig. 1*B*) was found to increase linearly with the bulk DXT concentration and exponentially with bulk concentration of PEG (Fig. 1*C*). Similarly, equilibrium concentration of PEG in PEG phase was found to increase linearly with the bulk PEG concentration, and exponentially with the bulk concentration of DXT (Fig. 1*D*). This enabled to express the dependencies between equilibrium concentrations of DXT and PEG in DXT and PEG phases by Equations 1 and 2 as a function of bulk concentrations of both polymers. Validity of these equations for equilibrium concentrations of both polymers in their phases was verified by nonlinear regression using bulk concentrations of PEG and DXT as two independent variables (Fig. 1*C*, *D*). Equations 3 and 4 describing equilibrium DXT and PEG concentrations in PEG and DXT phase were deduced from Equations 1 and 2, and were similarly verified by nonlinear regression (not shown). Statistical relevance of parameters $p_1, p_2, p_3, P_1, P_2, P_3, q_1, q_2, q_3, Q_1, Q_2, Q_3$ (see Table 1) indicated a good fit of Equations 1–4 to experimental data. It should be noted that Equations (1–4) represent a fully empirical numerical model to describe the composition of both phases at any applied bulk concentration of PEG and DXT. We do not yet have a physico-chemical explanation for this model, but it is suitable for the description of PEG/DXT ATPS. The validity of this model could also be documented by the fact that data computed from Equations 1–4 gave identical binodial curve as experimental data (Fig. 2*A*, *B*). Moreover, when materials balance of PEG and DXT were computed from Equations 5 and 6 (derived from equations 1–4) the obtained data gave satisfactory agreement with the real amounts of PEG and DXT in ATPS (Fig. 2*C*, *D*).

DXT phase is considered to be more polar and PEG phase less polar in PEG/DXT ATPS (Bamberger et al. 1985). However, it should be stressed that both phases consist from more than one polymer being to some extent contaminated by the other polymer. Thus, polarity of the respective phase is not only determined by the dominant polymer (i.e., DXT for DXT phase or PEG for PEG phase) but also by a minor polymer (i.e., PEG for DXT phase or DXT for PEG phase). Contamination of phases by the minor polymer was found to be negligible (less than 10%) for high bulk concentrations of the dominant polymer. For low bulk concentrations of the dominant polymer considerable contamination of phase by the minor polymer (up to 50%) could be observed (Fig. 3). This indicated that

for studying protein distribution in ATPS, concentrations of both polymers in both phases should be considered.

High viscosity of both phases induced several inaccuracies in the phase separation from ATPS. This may be reflected in a higher probability of diverse errors in estimating PEG and DXT equilibrium concentrations in both phases. Replacement of measured equilibrium phase concentrations of PEG and DXT by data computed from Equations 1–4 (since this model has a sufficient statistical relevance, see above) in constructing the dependencies between protein distribution and phase equilibrium concentrations of both polymers should diminish the errors in the respective estimation.

Both IgG and BSA were found to be in higher concentrations in DXT i.e., the more polar phase, and in lower concentrations in PEG i.e., the less polar phase (Fig. 4). Similarly, lower amounts of some proteins in PEG phase compared to DXT phase were found irrespective from whether ATPS was formed of DXT and non-modified PEG or of DXT and PEG linked with palmitate (Keshavartz and Nakai 1979). In a previous paper (Breier et al. 1984b) out of six proteins studied only chymotrypsin showed a higher affinity to the PEG than DXT phase. Asenjo et al. (1994) described partition of several proteins in ATPS formed of PEG and phosphate buffer containing NaCl. BSA in this case was distributed in the more polar phase, i. e. the phase formed by phosphate buffer. Affinities of BSA and IgG to the DXT phase strongly depend on phase composition. An increase of polarity of the DXT phase, i.e. an increase of equilibrium DXT concentration in DXT phase, results in a proportional increase in equilibrium concentrations of both proteins in DXT phase. In contrast, a decrease of polarity of the DXT phase induced by an increase of equilibrium concentration of PEG in DXT phase yielded a proportional decrease of equilibrium concentrations of both proteins in DXT phase. On the other hand, equilibrium concentrations of both proteins in PEG phase were found to be relatively independent of equilibrium concentrations of PEG or DXT in PEG phase. All the above facts indicated that polarity (composition) of DXT phase represents a dominant characteristics of ATPS important for the final distributions of BSA and IgG.

The affinities of both proteins to DXT or PEG phase could be deduced from the partition coefficients $K_{PEG/DXT}$ ($K_{DXT/PEG}$) that represent the ratio of equilibrium concentrations of both proteins in the respective phase. Compared to BSA, IgG showed a higher affinity to the less polar PEG phase (i.e. higher $K_{PEG/DXT}$ value, see Fig. 5) indicating that IgG has a greater hydrophobicity than BSA. This is in agreement with the hydrophobic parameters computed from the protein primary structure of both proteins (Bigelow 1967). Moreover, IgG interacts more strongly than BSA with immobilized 3-phenoxy-2-hydroxypropyl groups linked with bead cellulose (Gemeiner et al. 1989; Breier et al. 1994a). In contrast, BSA has higher retention coefficients as IgG in hydrophobic interaction chromatography

using both butylepoxy-Sepharose and hexylepoxy-Sepharose (Keshavarz and Nakai 1979). However, retention of proteins in hydrophobic interaction chromatography could be influenced by several factors, namely those concerning the diversity of diffusion of proteins with different molecular weight to the inner volume of the hydrophobized matrices. Thus retention of proteins in this case could not exactly reflect their effective hydrophobicity. On the other hand, when effective hydrophobicities of proteins are estimated using equilibrium adsorption on hydrophobized matrices as in our previous papers (Gemeiner et al. 1989; Breier et al. 1994a), diffusion could be expected to have less influence due to equilibrium condition. The lack of correlation between effective hydrophobicities measured by chromatography (hydrophobic interaction FPLC on Phenyl-Sepharose columns and reverse phase HPLC on C18 columns) and in ATPS formed by PEG and phosphate buffer solution (Asenjo et al. 1994) may be discussed from this point of view.

The affinities of both proteins to the respective phase (deduced from $K_{PEG/DXT}$ and $K_{DXT/PEG}$) increased with the increasing equilibrium concentration of DXT and with the decreasing of equilibrium concentration of PEG in this phase. These relations gave significant correlations in semilogarithmic plot (Fig. 5). Thus, it could be expected that affinities of both proteins to the respective phase would grow exponentially with the increasing phase polarity.

In our previous paper (Breier et al. 1994b), quantitative relation between $K_{PEG/DXT}$ (obtained for several proteins in ATPS formed by DXT and PEG) as a measure of effective hydrophobicity of proteins, and other measures of protein hydrophobicity were obtained. However, the proteins tested did not only differ in the distribution of non polar aminoacid residues, i.e. in hydrophobicity, but also in other physico-chemical properties such as net charge, charge distribution molar mass etc. This diversity of proteins properties other than hydrophobicity introduced problems into the exact description of the relationship between hydrophobicity of a protein and its partition in ATPS. Chemical modification to different degree of one defined protein by a hydrophobic agent yielded a more homogeneous group in which single members predominantly differred in hydrophobicity. Changes of hydrophobicity of calreticulin due to modification by TNBS were found to alter its distribution in ATPS formed by PEG and DXT (Breier and Michalak 1994). Thus modification of protein by TNBS probably represents a simple method for intentionally regulated alteration of protein hydrophobicity. Modification of BSA by TNBS induced a decrease of $K_{PEG/DXT}$ and an increase of $K_{DXT/PEG}$ proportional to the degree of BSA modification (Fig. 6). This behavior indicated that modification of BSA by TNBS decreases its affinity to the more polar phase (DXT) and increase of its affinity to the less polar phase. However, the $K_{PEG/DXT}$ values < 1.00 and/or $K_{DXT/PEG}$ values > 1.00 obtained for any measurement (Fig. 6) indicate that both BSA-TNB1 and BSA-TNB10 still have higher affinities to the more polar DXT phase than to the less polar PEG phase. The slopes of dependen-

cies between $K_{PEG/DXT}$ and/or $K_{DXT/PEG}$ and equilibrium DXT concentrations in DXT and/or PEG phase shown in Fig. 6*A*, *C* decreased proportionally with the increasing degree of BSA modification by TNBS (Table 2). Moreover, while the slopes obtained for non-modified BSA and BSA-TNB1 reach positive values the slope obtained for BSA-TNB10 was found to reach negative value. On the other hand, precisely opposite behavior was found for the effect of BSA modification on the dependencies between $K_{PEG/DXT}$ and/or $K_{DXT/PEG}$ and equilibrium PEG concentration in DXT and/or PEG phase shown in Fig. 6*B*, *D*. It should be noted that, in contrast to the results obtained for non-modified BSA and BSA-TNB1, the affinity of BSA-TNB10 to the respective phase was influenced negatively by the increase of its polarity via an increase of DXT equilibrium concentration and/or a decrease of PEG equilibrium concentration.

Table 2. Relations between slopes of dependencies in Fig. 6 and the amounts of trinitrophenyl groups linked with BSA molecule

	Slopes* $[mg/ml]^{-1}$				Bound TNP** [mol/mol]
	A	*B*	*C*	*D*	
BSA	4.97	-29.53	23.92	-7.66	0
BSA-TNB1	3.64	-18.98	16.66	-5.00	0.930
BSA-TNB10	-1.09	12.27	12.46	2.84	8.738

*Slopes of dependencies shown in Fig. 6: *A*, *B*, *C* and *D* represent the slopes obtained from panel *A*, *B*, *C* and *D* respectively.
** Molar ratios of trinitrophenyl groups linked with BSA.

The results presented here indicate that protein distribution in ATPS could exactly reflect in changes in protein hydrophobicity. Polarity of the respective phase is positively influenced by an increase of equilibrium phase DXT concentration and a decrease of equilibrium phase PEG concentration. Both these concentrations in both phases could be regulated by intentionally chosen bulk concentrations of both polymers in ATPS. Phase composition could be predicted from bulk concentrations of both polymers by Equations 1–4. These represent another possibility to alter the polarity of both phases in addition to modification of DXT (Gemeiner et al. 1991) or PEG (Shanbhag and Axelsson 1975) by hydrophobic ligand.

References

Albertsson P.-A. (1970): Partition of cells particles and macromolecules in polymer two-phase systems. Adv. Protein Chem. **24**, 309—341

Albertsson P.-A., Saskawa A., Walter H. (1970): Cross partition and isoelectric point of proteins. Nature **228**, 1329—1330

Asenjo J. A., Schmidt A. S., Hachen F., Andrews B. A. (1994): Model for predicting the partition behavior of proteins in aqueous two-phase systems. J. Chromatogr. A, **668,** 47—54

Bamberger S., Brooks D. E., Sharp K. A., Van Alstine J. M., Webber T. J. (1985): Preparation of phase systems and measurement of their physicochemical properties. In: Partition in Aqueous Two-Phase Systems (Eds. H. Walter, D. E. Brooks, D. Fisher), pp. 86—130, Academic Press Inc. Orlando

Bigelow Ch. C. (1967): On the average hydrophobicity of proteins and the relation between it and protein structure. J. Theor. Biol. **16,** 187—211

Bradford M. M. (1976): A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72,** 248—254

Breier A., Michalak M. (1994): 2,4,6-trinitrobenzene sulfonic acid modification of the carboxy-terminal region (C-domain) of calreticulin. Mol. Cell. Biochem. **130,** 19—28

Breier A., Gemeiner P., Hagarová D. (1994 a): Partition mechanism of protein adsorption onto bead 2-hydroxy-3-phenoxypropyl cellulose. Role of external surface of cellulose particles. Chem. Papers **48,** 141—145

Breier A., Hagarová D., Gemeiner P., Ziegelhöffer A. (1994 b): Estimation of the effective hydrophobicity of proteins: Re-evaluation of methods. Biotechnol. Techniques **8,** 915—920

Brooks D. E., Sharp K. A., Fisher D. (1985): Theoretical aspects of partitioning. In: Partition in Aqueous Two-Phase Systems (Eds. H. Walter, D. E. Brooks, D. Fisher), pp. 11—84, Academic Press Inc. Orlando

Gemeiner P., Hrabárová E., Zacharová M., Breier A., Beneš M. J. (1989): Partition mechanism of adsorption and the absence of displacement phenomena in the zonal analytical chromatography of proteins on bead 2-hydroxy-3-phenoxypropyl cellulose. Collect. Czech. Chem. Commun. **54,** 2375—2385

Gemeiner P., Kochjarová H., Horváthová M., Breier A. (1991): Hydrophobic partitioning of proteins in a two-phase aqueous system of poly (oxyethylene)-dextran alternatively derivatized by 2-hydroxy-3-phenoxypropyl group. Collect. Czech. Chem. Commun., **56,** 1270—1278

Hjertén S., Rosengren J., Pahlman S. (1974): Hydrophobic interaction chromatography. The synthesis and the use of some alkyl and aryl derivatives of agarose. J. Chromatogr. **101,** 281—288

Kato A., Nakai S. (1980): Hydrophobicity determined by a fluorescence probe method and its correlation with surface properties of proteins. Biochim. Biophys. Acta **624,** 13—20

Keshavartz E., Nakai S. (1979): The relationship between hydrophobicity and interfacial tension of proteins. Biochim. Biophys. Acta **576,** 269—279

Shanbhag V. P., Axelsson C. G. (1975): Hydrophobic interaction determined by partition in aqueous two-phase systems. Partition of proteins in systems containing fatty-acid esters of polyethylene glycol. Eur. J. Biochem. **60,** 17—22

Wiegel D., Richter O., Arnhold J., Arnold K. (1991): Partition of serum lipoproteins in a polyethylene glycol/dextran two-phase system. Biomed. Biochim. Acta **50,** 207—212

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