



# Analysis of partitioning of organic compounds and proteins in aqueous polyethylene glycol-sodium sulfate aqueous two-phase systems in terms of solute–solvent interactions



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## ABSTRACT

Partition behavior of nine small organic compounds and six proteins was examined in poly(ethylene glycol)-8000-sodium sulfate aqueous two-phase systems containing 0.5 M osmolyte (sorbitol, sucrose, trehalose, TMAO) and poly(ethylene glycol)-10000-sodium sulfate system, all in 0.01 M sodium phosphate buffer, pH 6.8. The differences between the solvent properties of the coexisting phases (solvent dipolarity/polarizability, hydrogen bond donor acidity, and hydrogen bond acceptor basicity) were characterized with solvatochromic dyes using the solvatochromic comparison method. Differences between the electrostatic properties of the phases were determined by analysis of partitioning of sodium salts of dinitrophenylated (DNP-) amino acids with aliphatic alkyl side-chain. It was found out that the partition coefficient of all compounds examined (including proteins) may be described in terms of solute–solvent interactions. The results obtained in the study show that solute–solvent interactions of nonionic organic compounds and proteins in polyethylene glycol-sodium sulfate aqueous two-phase system differ from those in polyethylene glycol-dextran system.

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## 1. Introduction

Aqueous two-phase systems (ATPS) are formed in mixtures of two (or more) water-soluble polymers, such as dextran and Ficoll, or a single polymer and specific salt, e.g., polyethylene glycol (PEG) and sodium sulfate, in water above certain critical concentrations. Two immiscible aqueous phases are formed in the mixtures. Solutes from small organic compounds to proteins and nucleic acids distribute unevenly between the phases of an ATPS and may be separated. ATPS formed by polyethylene glycol (PEG) and inorganic salt, such as sodium sulfate, phosphate or citrate, are commonly used for separation of proteins and nucleic acids due to their low cost, good operational characteristics (low viscosity of the phases, high settling speed) and easy scale-up [1–18]. Extraction in ATPS

has been demonstrated as an efficient method for large scale recovery and purification of proteins [1–13] and nucleic acids [14,15] as well as various other materials. Design of optimal extraction conditions for any target product remains currently an empirical process, and high throughput methods for screening different separation conditions have been developed [16–18]. For rational design of the optimal separation conditions it is important to understand the mechanisms of solute distribution in polymer-salt ATPS at the molecular level.

We reported [19–21] previously that different salt additives (NaCl, NaH<sub>2</sub>PO<sub>4</sub>, NaClO<sub>4</sub>, NaSCN) at the concentrations from 0.027 M up to ca. 1.9 M affect partition behavior of small organic compounds in PEG-sodium sulfate ATPS according to the salt effects on the water structure. Despite broad biotechnological and pharmacological applications of this approach, the molecular mechanism of solute partitioning in PEG-salt ATPS remains unclear, however.

It has been established [22–29] that solute partitioning in two-polymer ATPS is governed by the solute–solvent interactions in

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the coexisting phases. Partition coefficient of a solute in an ATPS is defined as the ratio of the solute concentration in the top phase to the solute concentration in the bottom phase and therefore may be described as [23–28]:

$$\log K = S_s \Delta\pi^* + B_s \Delta\alpha + A_s \Delta\beta + C_s c \quad (1)$$

where  $K$  is the solute partition coefficient;  $\Delta\pi^*$ ,  $\Delta\alpha$ ,  $\Delta\beta$  and  $c$  are the differences between the solvent properties of the top and bottom phases (solvent dipolarity/polarizability, hydrogen-bond donor acidity, hydrogen-bond acceptor basicity, and electrostatic interactions, respectively);  $S_s$ ,  $B_s$ ,  $A_s$ , and  $C_s$  are constants (solute-specific coefficients) that describe the complementary interactions of the solute with the solvent media in the coexisting phases; the subscript 's' designates the solute.

The differences between the solvent dipolarity/polarizability,  $\Delta\pi^*$ , hydrogen-bond donor acidity,  $\Delta\alpha$ , hydrogen-bond acceptor basicity,  $\Delta\beta$ , may be quantified using a set of solvatochromic dyes [23–29] (see below). The difference between the electrostatic properties of the phases may be determined from the analysis of the partition coefficients of a homologous series of sodium salts of dinitrophenylated (DNP-) amino acids with aliphatic alkyl side-chains [22–28] (see below). It has been shown that for a given compound (including proteins) the solute-specific coefficients may be determined by multiple linear regression analysis of the partition coefficients of the compound in multiple two polymer ATPSs formed by different polymers but with the same ionic composition [23–28]. It was also shown [23,26] that the partition coefficients of compounds with pre-determined solute specific coefficients in new ATPS with established solvent properties of the phases could be predicted with the 90–95% accuracy.

Huddlestone et al. [30,31] examined the solvent properties of the coexisting phases in PEG-2000- $K_3PO_4$  and PEG-2000- $(NH_4)_2SO_4$  ATPSs and found negligible differences between the solvent dipolarity/polarizability,  $\Delta\pi^*$ , and hydrogen bond acceptor basicity,  $\Delta\beta$ , of the phases. The authors [30,31] also reported on the challenges regarding accurate determination of the solvent hydrogen bond donor acidity,  $\alpha$ , in such ATPSs due to effects of high salt concentrations on the solvatochromic probe used. We assumed that the challenges encountered by Huddlestone et al. [30,31] might be related to the low molecular weight of PEG used in the studies requiring using high total salt concentration (9–10 wt.%  $K_3PO_4$ ) for ATPS formation. Hence in this work we used PEG-8000 and PEG-10000 enabling us to decrease the salt concentration necessary for phase separation down to 6.3 wt.%  $Na_2SO_4$ .

It has been shown previously [32–35] that the osmolytes additives change the solvent properties of the phases under fixed salt composition of the system, while not being engaged in direct interactions with compounds (including proteins) being partitioned. Therefore these additives enable one to vary solvent properties of the polymer-salt ATPS without changing overall polymer and salt composition of the particular system.

The purpose of the present work was to explore if partitioning of different solutes in PEG- $Na_2SO_4$  ATPS is governed by the factors similar to those established in the ATPSs formed by two polymers. To this end, partitioning of several different organic compounds and proteins was examined in several PEG- $Na_2SO_4$  ATPSs in the presence of different nonionic additives.

## 2. Experimental

### 2.1. Materials

Polyethylene glycol-8000 (Lot 091M01372 V) with an average molecular weight ( $M_w$ ) of 8000 and polyethylene glycol-10000 (Lot 043K2522) with an average molecular weight ( $M_w$ ) of

10000 were purchased from Sigma-Aldrich (St. Louis, MO, USA). The solvatochromic probes 4-nitrophenol (reagent grade, >98%) was purchased from Aldrich (Milwaukee, WI, USA) and 4-nitroanisole (>97%, GC) was received from Acros Organics. Reichardt's carboxylated betaine dye, 2,6-diphenyl-4-[2,6-diphenyl-4-(4-carboxyphenyl)-1-pyridino]phenolate, sodium salt was kindly provided by Professor C. Reichardt (Philipps University, Marburg, Germany).

Sorbitol, trimethylamine N-oxide (TMAO), and trehalose were purchased from Sigma-Aldrich, and sucrose was received from USB (Cleveland, OH, USA). 4-Aminophenol, benzyl alcohol, caffeine, coumarin, methylantranilate, 4-nitrophenyl- $\alpha$ -D-glucopyranoside, phenol, 2-phenylethanol, vanillin, and o-phthalaldehyde (OPA) reagent (complete) were purchased from Sigma-Aldrich. All compounds were of 98–99% purity and used as received without further purification. All salts and other chemicals used were of analytical-reagent grade.

#### 2.1.1. Dinitrophenylated amino acids

Dinitrophenylated (DNP) amino acids—DNP-glycine, DNP-alanine, DNP-norvaline, DNP-norleucine, and DNP- $\alpha$ -amino- $n$ -octanoic acid, were purchased from Sigma-Aldrich. The sodium salts of the DNP-amino acids were prepared by titration.

#### 2.1.2. Proteins

$\alpha$ -Chymotrypsin from bovine pancreas,  $\alpha$ -chymotrypsinogen A from bovine pancreas, concanavalin A from *Canavalia ensiformis* (jack beans), lysozyme from chicken egg white, and papain from papaya latex were purchased from Sigma-Aldrich. Porcine pancreatic lipase was purchased from USB Corp. (Solon, OH, USA). All protein samples were characterized by SDS-PAGE electrophoresis in a microfluidic chip using Experion automated electrophoresis station (Bio-Rad, USA) under non-reduced conditions. All proteins were observed as single bands in the electrophoregrams.

## 2.2. Methods

### 2.2.1. Aqueous two-phase systems

Stock solutions of PEG 8000 (50 wt.%), PEG-10000 (50 wt.%) and  $Na_2SO_4$  (20.3 wt.%) were prepared in water. Sodium phosphate buffer (NaPB; 0.5 M, pH 6.8) was prepared by mixing 3.45 g of  $NaH_2PO_4 \cdot H_2O$  and 3.55 g  $Na_2HPO_4$  in 100 mL aqueous solution. Stock solutions of osmolytes: sorbitol (2 M), sucrose (1.8 M), trehalose (1.4 M), and TMAO (1.8 M) were prepared in water. A mixture of PEG-8000 or PEG-10000 and buffer was prepared by dispensing appropriate amounts of the aqueous stock PEG-8000,  $Na_2SO_4$  and NaPB solutions into a 1.2 mL microtube using a Hamilton (Reno, NV, USA) ML-4000 four-probe liquid-handling workstation. Appropriate amounts of water and/or stock osmolyte solutions were added to give the required ionic, polymer, and osmolyte composition of the final system with total weight of 0.5 g (after addition of the solute sample, see below). All aqueous PEG-8000- $Na_2SO_4$  two-phase systems had a fixed composition of 11.10 wt.% PEG-8000, 6.33 wt.%  $Na_2SO_4$  and 0.01 M NaPB, pH 6.8, with different 0.5 M osmolyte additive. The aqueous PEG-10000- $Na_2SO_4$  two-phase system had the same composition of 11.10 wt.% PEG-10000, 6.33 wt.%  $Na_2SO_4$  and 0.01 M NaPB, pH 6.8.

### 2.2.2. Partitioning experiments

The aqueous two-phase partitioning experiments were performed using an Automated Signature Workstation, ASW (Analiza, Cleveland, OH, USA). The ASW system is based on the ML-4000 liquid-handling workstation (Hamilton Company) integrated with a FL600 fluorescence microplate reader (Bio-Tek Instruments, Winooski, VT, USA) and a UV-VIS microplate spectrophotometer (SpectraMax Plus384, Molecular Devices, Sunnyvale, CA, USA).

Solutions of all organic compounds were prepared in water at concentrations of 2–5 mg/mL depending on the compound solubility. Solutions of all proteins were prepared in water at concentrations of 1–5 mg/mL. Varied amounts (0, 15, 30, 45, 60, and 75  $\mu\text{L}$ ) of a given compound solution and the corresponding amounts (75, 60, 45, 30, 15, and 0  $\mu\text{L}$ ) of water were added to a set of the same polymer/buffer mixtures with and without osmolyte additives. The systems were then vortexed in a Multi-pulse Vortexer and centrifuged for 60 min at  $3500 \times g$  at  $23^\circ\text{C}$  in a refrigerated centrifuge (Hettich Universal 320R, Germany) to accelerate phase settling. The upper phase in each system was partially removed, the interface discarded, and aliquots from the upper and lower phases were withdrawn in duplicate for analysis.

For the analysis of organic compounds partitioning, aliquots of 50–120  $\mu\text{L}$  from both phases were diluted up to 600  $\mu\text{L}$  in 1.2 mL microtubes. Water was used as diluent for all except phenol, and vanillin. 20 mM universal buffer with pH 12.4 was used as diluent (Universal buffer is composed of 0.01 M each of phosphoric, boric, and acetic acids adjusted to pH 12.4 with NaOH). Following vortexing and a short centrifugation (12 min), aliquots of 250–300  $\mu\text{L}$  were transferred into microplate wells, and the UV–VIS plate reader was used to measure optical absorbance at wavelengths previously determined to correspond to maximum absorption. The maximum absorption wavelength for each compound was determined in separate experiments by analysis of the absorption spectrum over the 240–500 nm range. In the case of the four aforementioned compounds the maximum absorption was found to be more concentration sensitive in the presence of the universal buffer at pH 12.4. In all measurements the same dilution factor was used for the upper and lower phases and correspondingly diluted pure phases were used as blank solutions.

For the analysis of the partitioning of proteins aliquots of 30  $\mu\text{L}$  from both phases were transferred and diluted with water up to 70  $\mu\text{L}$  into microplate wells. Then, the microplate was sealed, shortly centrifuged (2 min at 1500 rpm) and following the moderate shaking for 45 min in an incubator at  $37^\circ\text{C}$ , 250  $\mu\text{L}$  of o-phthalaldehyde reagent was added. After moderate shaking for 4 min at room temperature, fluorescence was determined using a fluorescence plate reader with a 360 nm excitation filter and a 460 nm emission filter, with a sensitivity setting of 100–125.

The partition coefficient,  $K$ , defined as the ratio of the sample concentration in the upper phase to the sample concentration in the lower phase was determined as the slope of the compound concentration in the upper phase plotted as a function of the concentration in the lower phase averaged over the results obtained from two to four partition experiments carried out at the specified polymer, buffer, and osmolyte composition of the system, taking into consideration the corresponding dilution factors used in the experiment. The UV absorption measured in a given phase was used as a measure of a given organic compound concentration, and fluorescence intensity was used as a measure of the protein concentration. Deviation from the average  $K$ -value was consistently below 3% and in most cases lower than 2%.

### 2.2.3. Solvatochromic studies

All aqueous two-phase systems were prepared as described above with the total weight of 4g. Systems were centrifuged as described above and the phases were separated. The solvatochromic probes 4-nitroanisole, 4-nitrophenol and Reichardt's carboxylated betaine dye were used to measure the dipolarity/polarizability  $\pi^*$ , HBA basicity  $\beta$ , and HBD acidity  $\alpha$  of the media in the separated phases of ATPS. Aqueous solutions (ca. 10 mM) of each solvatochromic dye were prepared, and aliquots of 5–15  $\mu\text{L}$  of each dye were added separately to a total volume of 500  $\mu\text{L}$  of a given phase of each ATPS. A strong base was added to the samples (~5  $\mu\text{L}$  of 1 M NaOH to 500  $\mu\text{L}$  of a given phase) containing

Reichardt's carboxylated betaine dye to ensure a basic pH. A strong acid (~10  $\mu\text{L}$  of 1 M HCl to 500  $\mu\text{L}$  of the solution) was added to the phase containing 4-nitrophenol in order to eliminate charge-transfer bands of the phenolate anion that were observed in some solutions. The samples were mixed thoroughly in a vortex mixer and the absorption spectra of each solution were acquired. To check the reproducibility, possible aggregation and specific interactions effects, the position of the band maximum in each sample was measured in six separate aliquots. A UV–VIS microplate reader spectrophotometer SpectraMax Plus384 (Molecular Devices, Sunnyvale, CA, USA) with a bandwidth of 2.0 nm, data interval of 1 nm, and high resolution scan (~0.5 nm/s) was used for acquisition of the UV–Vis molecular absorbance data. The absorption spectra of the probes were determined over the spectral range from 240 to 600 nm in each solution. Pure osmolyte solutions or phase of ATPS containing no dye (blank) were scanned first to establish a baseline. The wavelength of maximum absorbance in each solution was determined as described by Huddleston et al. [31] using PeakFit software package (Systat Software Inc., San Jose, CA, USA) and averaged. Standard deviation for the measured maximum absorption wavelength was  $\leq 0.4$  nm for all dyes in all solutions examined.

The behavior of the probes (4-nitrophenol, and Reichardt's carboxylated betaine dye) in several solvents (water, n-hexane, methanol) was tested in the presence and absence of HCl (for 4-nitrophenol) and NaOH (for the betaine dye) at different concentrations of the probes, acid or base, and the maximum shifts of the probes were compared to reference values found in the literature and were within the experimental errors in all cases (data not shown).

The results of the solvatochromic studies were used to calculate  $\pi^*$ ,  $\alpha$ , and  $\beta$  as described by Marcus [36].

**2.2.3.1. Determination of the solvent dipolarity/polarizability  $\pi^*$ .** The  $\pi^*$  values were determined from the wave number ( $\nu_1$ ) of the longest wavelength absorption band of the 4-nitroanisole dye using the relationship:

$$\pi^* = 0.427(34.12 - \nu_1). \quad (2)$$

**2.2.3.2. Determination of the solvent hydrogen-bond acceptor basicity  $\beta$ .** The  $\beta$  values were determined from the wave number ( $\nu_2$ ) of the longest wavelength absorption band of the 4-nitrophenol dye using the relationship:

$$\beta = 0.346(35.045 - \nu_2) - 0.57\pi^*. \quad (3)$$

**2.2.3.3. Determination of the solvent hydrogen-bond donor acidity  $\alpha$ .** The  $\alpha$  values were determined from the longest wavelength absorption band of Reichardt's betaine dye using the relationship:

$$\alpha = 0.0649E_T(30) - 2.03 - 0.72\pi^* \quad (4)$$

The  $E_T(30)$  values are based on the solvatochromic pyridinium N-phenolate betaine dye (Reichardt's dye) as probe, and are obtained directly from the wavelength ( $\lambda$ , nm) of the absorption band of the carboxylated form, as,

$$E_T(30) = \left( \frac{1}{0.932} \right) \times \left[ \left( \frac{28591}{\lambda} \right) - 3.335 \right] \quad (5)$$

## 3. Results

Differences between the solvent properties of the coexisting phases (solvent dipolarity/polarizability,  $\Delta\pi^*$ , hydrogen bond donor acidity,  $\Delta\alpha$ , and hydrogen bond acceptor basicity,  $\Delta\beta$ ) were determined using solvatochromic dyes as described above. The values of these differences calculated as the difference between the measured value of a given solvent feature

**Table 1**  
Differences between the solvent properties of the phases and partition coefficients for simple organic compounds and free amino acids in PEG (8000 or 10000)-Na<sub>2</sub>SO<sub>4</sub>-0.01 M NaPB, pH 6.8 and PEG-8000-Na<sub>2</sub>SO<sub>4</sub>-0.5 M osmolyte-0.01 M NaPB, pH 6.8 ATPS (NaPB—sodium phosphate buffer).

Solvent properties <sup>d</sup>	0.01M NaPB <sup>a</sup>	0.01M NaPB <sup>b</sup>	0.5M Sorbitol <sup>c</sup>	0.5M Sucrose <sup>c</sup>	0.5M Trehalose <sup>c</sup>	0.5M TMAO <sup>c</sup>
$\Delta G(\text{CH}_2)$ (cal/mol)	-122 ± 1.8	-137 ± 5.8	-144 ± 2.4	-178 ± 3.5	-181 ± 3.5	-146 ± 2.4
$E$	0.048 ± 0.002	0.100 ± 0.004	0.102 ± 0.003	0.123 ± 0.006	0.125 ± 0.006	0.108 ± 0.002
$C$	0.067 ± 0.007	0.44 ± 0.017	0.67 ± 0.011	0.67 ± 0.02	0.71 ± 0.021	0.625 ± 0.007
$\Delta\pi^*$	-0.029 ± 0.003	-0.020 ± 0.003	-0.046 ± 0.004	-0.077 ± 0.005	-0.067 ± 0.004	-0.010 ± 0.003
$\Delta\alpha$	-0.128 ± 0.004	-0.075 ± 0.004	-0.248 ± 0.005	-0.228 ± 0.007	-0.247 ± 0.004	-0.208 ± 0.004
$\Delta\beta$	0.015 ± 0.004	0.013 ± 0.004	0.021 ± 0.008	0.028 ± 0.008	0.022 ± 0.007	0.021 ± 0.009
Compound	Partition coefficients					
4-Aminophenol	3.04 ± 0.049	3.165 ± 0.007	4.45 ± 0.013	4.55 ± 0.014	4.66 ± 0.014	4.11 ± 0.012
Benzyl alcohol	3.500 ± 0.007	3.55 ± 0.031	5.36 ± 0.017	6.11 ± 0.035	5.69 ± 0.068	3.86 ± 0.019
Caffeine	1.85 ± 0.039	1.984 ± 0.005	2.48 ± 0.010	2.49 ± 0.013	2.44 ± 0.012	2.45 ± 0.012
Coumarin	4.86 ± 0.014	4.55 ± 0.023	8.23 ± 0.024	8.46 ± 0.065	7.97 ± 0.039	6.27 ± 0.024
Glucopyranoside <sup>e</sup>	2.18 ± 0.023	2.405 ± 0.008	3.48 ± 0.011	3.75 ± 0.019	3.59 ± 0.016	2.87 ± 0.010
Methyl anthranilate	7.28 ± 0.035	7.29 ± 0.040	13.48 ± 0.091	14.21 ± 0.057	14.9 ± 0.22	9.06 ± 0.051
Phenol	4.60 ± 0.084	4.80 ± 0.020	8.84 ± 0.039	10.43 ± 0.078	9.86 ± 0.046	5.70 ± 0.020
2-Phenylethanol	4.16 ± 0.020	3.80 ± 0.029	6.67 ± 0.026	7.70 ± 0.032	6.87 ± 0.047	4.71 ± 0.020
Vanillin	6.78 ± 0.027	5.79 ± 0.018	11.11 ± 0.047	11.38 ± 0.046	11.00 ± 0.072	6.91 ± 0.021
DNP-Ala Na	3.70 ± 0.070	3.86 ± 0.025	6.21 ± 0.080	6.3 ± 0.29	6.88 ± 0.033	5.9 ± 0.18
DNP-NVal Na	4.73 ± 0.057	5.15 ± 0.022	8.72 ± 0.024	9.8 ± 0.25	10.7 ± 0.11	8.0 ± 0.18
DNP-NLeu Na	6.0 ± 0.11	6.4 ± 0.023	11.1 ± 0.13	13.2 ± 0.39	15.0 ± 0.12	10.7 ± 0.19
DNP-AO Na <sup>f</sup>	10.3 ± 0.19	12.34 ± 0.040	21.2 ± 0.98	28.7 ± 0.89	32.1 ± 0.28	20.2 ± 0.45

<sup>a</sup> Data for osmolyte-free PEG-8000-Na<sub>2</sub>SO<sub>4</sub>-0.01 M NaPB, pH 6.8 ATPS.

<sup>b</sup> Data for osmolyte-free PEG-10000-Na<sub>2</sub>SO<sub>4</sub>-0.01 M NaPB, pH 6.8 ATPS.

<sup>c</sup> Data for PEG-8000-Na<sub>2</sub>SO<sub>4</sub>-0.5 M osmolyte-0.01 M NaPB, pH 6.8 ATPS.

<sup>d</sup> Parameters  $E$  and  $\Delta G(\text{CH}_2)$  values characterize the difference between the relative hydrophobicities of the coexisting phases of a given ATPS, parameter  $C$  value characterizes the difference between the electrostatic properties of the phases (for explanation see text),  $\Delta\pi^*$  characterizes the difference between the solvent dipolarity/polarizability of the phases,  $\Delta\alpha$  characterizes the difference between the solvent hydrogen bond donor acidity of the phases,  $\Delta\beta$  characterizes the difference between the solvent hydrogen bond acceptor basicity of the phases.

<sup>e</sup> p-Nitrophenyl- $\alpha$ -D-glucopyranoside.

<sup>f</sup> DNP-amino-n-octanoic acid sodium salt.

in the top phase and that of the same feature in the bottom phase are listed in Table 1. The difference between the solvent properties in the PEG-8000-Na<sub>2</sub>SO<sub>4</sub> ATPS clearly increases significantly in the presence of osmolyte additives. Osmolyte effects on the difference between the solvent hydrogen bond donor acidity,  $\Delta\alpha$ , between the two phases increases as follows: sorbitol  $\approx$  trehalose > sucrose > TMAO. Osmolyte effects on the difference between the solvent hydrogen bond acceptor basicity,  $\Delta\beta$ , between the two phases are very similar. The difference between the solvent dipolarity/polarizability,  $\Delta\pi^*$ , changes in the sequence: sucrose > trehalose > sorbitol > osmolyte free ATPS > TMAO.

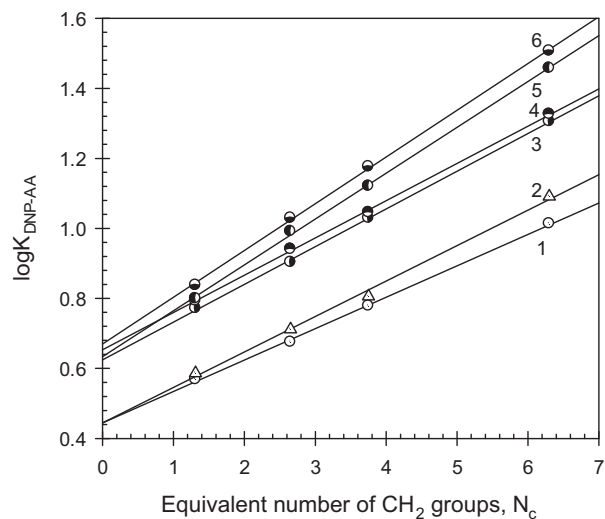
The difference between the hydrophobic and electrostatic properties of the coexisting phases was determined in each ATPS by partitioning of a homologous series of sodium salts of dinitrophenylated (DNP-) amino acids with the aliphatic alkyl side-chains of the increasing length (alanine, norvaline, norleucine, and  $\alpha$ -amino-n-octanoic acid). Partition coefficients of these compounds are listed in Table 1, and are presented graphically in Fig. 1, where the logarithms of their partition coefficients are plotted against the length of the side-chain expressed in equivalent number of methylene groups,  $N_c$ . It can be seen in Fig. 1 that the data in each ATPS may be described as [22–28]:

$$\log K_{\text{DNP-AA}}^i = C^i + E^i N_c \quad (6)$$

where  $K_{\text{DNP-AA}}$  is the partition coefficient of a DNP-amino acids Na-salt;  $N_c$  is the equivalent number of CH<sub>2</sub> groups in the side-chain [19–28],  $E$  and  $C$  are constants for a given  $i$ th ATPS characterizing the difference between the relative hydrophobicity and electrostatic properties of the phases correspondingly.

The values of the  $E^{(i)}$  and  $C^{(i)}$  coefficients determined for the ATPSs examined are listed in Table 1. As the standard free energy of transfer of a solute from the bottom phase to the top phase is described as:

$$\Delta G^0 = -RT \ln K \quad (7)$$



**Fig. 1.** Logarithm of the partition coefficient value,  $\ln K_{\text{DNP-AA}}$ , for sodium salts of DNP-amino acids with aliphatic side-chains in aqueous PEG-Na<sub>2</sub>SO<sub>4</sub> two-phase systems (ATPS) as a function of equivalent length of the side-chain,  $N_c$ , expressed in terms of equivalent number of CH<sub>2</sub> units: 1—PEG-8000-Na<sub>2</sub>SO<sub>4</sub>-0.01 M sodium phosphate buffer (NaPB), pH 6.8 ATPS; 2—PEG-10000-Na<sub>2</sub>SO<sub>4</sub>-0.01 M sodium phosphate buffer (NaPB), pH 6.8 ATPS; 3—PEG-8000-Na<sub>2</sub>SO<sub>4</sub>-0.5 M TMAO-0.01 M sodium phosphate buffer (NaPB), pH 6.8 ATPS; 4—PEG-8000-Na<sub>2</sub>SO<sub>4</sub>-0.5 M sorbitol-0.01 M sodium phosphate buffer (NaPB), pH 6.8 ATPS; 5—PEG-8000-Na<sub>2</sub>SO<sub>4</sub>-0.5 M sucrose-0.01 M sodium phosphate buffer (NaPB), pH 6.8 ATPS; 6—PEG-8000-Na<sub>2</sub>SO<sub>4</sub>-0.5 M trehalose-0.01 M sodium phosphate buffer (NaPB), pH 6.8 ATPS.

where  $R$  is the universal gas constant and  $T$  is the absolute temperature in Kelvin, it follows that

$$\Delta G^0(\text{CH}_2) = -RTE^* \quad (8)$$

where  $E^*$  is parameter  $E$  expressed in natural logarithm units;  $\Delta G^0(\text{CH}_2)$  is the standard free energy of transfer of a methylene



**Table 2**

Partition coefficients for proteins in PEG (8000 or 10000)-Na<sub>2</sub>SO<sub>4</sub>-0.01 M NaPB, pH 6.8 and PEG-8000-Na<sub>2</sub>SO<sub>4</sub>-0.5 M osmolyte-0.01 M NaPB, pH 6.8 ATPS (NaPB—sodium phosphate buffer).

Protein	Partition coefficients					
	0.01 M NaPB <sup>a</sup>	0.01 M NaPB <sup>b</sup>	0.5 M Sorbitol <sup>c</sup>	0.5 M Sucrose <sup>c</sup>	0.5 M Trehalose <sup>c</sup>	0.5 M TMAO <sup>c</sup>
α-Chymotrypsinogen	0.429 ± 0.003	0.379 ± 0.001	0.204 ± 0.001	0.157 ± 0.001	0.119 ± 0.001	0.456 ± 0.002
Chymotrypsin	0.117 ± 0.001	0.0924 ± 0.0003	0.0576 ± 0.0002	0.047 ± 0.001	0.0271 ± 0.0007	0.112 ± 0.001
Concanavalin A	0.192 ± 0.001	0.195 ± 0.002	0.159 ± 0.0007	0.144 ± 0.001	0.116 ± 0.0006	0.182 ± 0.001
Lipase	0.618 ± 0.001	0.640 ± 0.002	0.569 ± 0.002	0.537 ± 0.002	0.516 ± 0.003	0.595 ± 0.002
Lysozyme	0.406 ± 0.003	0.411 ± 0.003	0.145 ± 0.002	0.055 ± 0.0003	0.045 ± 0.0004	0.297 ± 0.002
Papain	2.22 ± 0.011	1.913 ± 0.008	2.21 ± 0.011	2.220 ± 0.006	1.374 ± 0.008	2.29 ± 0.010

<sup>a</sup> Data for osmolyte-free PEG-8000-Na<sub>2</sub>SO<sub>4</sub>-0.01 M NaPB, pH 6.8 ATPS.

<sup>b</sup> Data for osmolyte-free PEG-10000-Na<sub>2</sub>SO<sub>4</sub>-0.01 M NaPB, pH 6.8 ATPS.

<sup>c</sup> Data for PEG-8000-Na<sub>2</sub>SO<sub>4</sub>-0.5 M osmolyte-0.01 M NaPB, pH 6.8 ATPS.

group from one phase to another. The  $\Delta G^0(\text{CH}_2)$  values calculated from the experimental data with Eqs. 7 and 8 are listed in Table 1. The data in Table 1 show that the difference between the relative hydrophobicity of the phases increases in the sequence: osmolyte-free ATPS < sorbitol ≤ TMAO < sucrose ≤ trehalose. The essentially similar sequence is observed for the difference between the electrostatic properties of the phases: osmolyte-free ATPS < TMAO < sorbitol ≈ sucrose ≤ trehalose.

In order to check if the molecular weight of PEG is important for the solute partitioning in terms of solute–solvent interactions (see below) we attempted to use ATPSs based on PEG-600-Na<sub>2</sub>SO<sub>4</sub>, PEG-4000-Na<sub>2</sub>SO<sub>4</sub>, and PEG-10000-Na<sub>2</sub>SO<sub>4</sub>. We found out that the solvatochromic measurements could not be reliably performed in the salt-rich phase of ATPS when Na<sub>2</sub>SO<sub>4</sub> concentration exceeds 11–12 wt.%. Beyond 12 wt.%, Na<sub>2</sub>SO<sub>4</sub> it is not possible to determine the a peak maximum for the solvatochromic absorption peak using the method employed. According to Huddleston et al. [30,31] who observed similar phenomena in the presence of potassium phosphate, the aforementioned challenges may be explained by aggregation of the dye due to high salt concentration. Therefore, we omitted measurements in PEG-600-Na<sub>2</sub>SO<sub>4</sub> and PEG-4000-Na<sub>2</sub>SO<sub>4</sub> ATPSs where the salt concentrations necessary for the formation of two-phase systems were too high, and used only PEG-10000-Na<sub>2</sub>SO<sub>4</sub> ATPS with the overall composition identical to that used in PEG-8000-Na<sub>2</sub>SO<sub>4</sub> ATPS. Differences between the solvent properties of the two phases in this ATPS are presented in Table 1 together with partition coefficients for nine organic compounds examined here.

Partitioning of six proteins was examined in all aforementioned ATPSs and the corresponding partition coefficients are listed in Table 2.

#### 4. Discussion

Analysis of partition coefficients determined in this study for various organic compounds and proteins listed in Tables 1 and 2 shows that the so-called Collander solvent regression equation (see, e.g., in [22,32–35]) holds for all compounds (including proteins) in the PEG-8000-Na<sub>2</sub>SO<sub>4</sub> and PEG-10000-Na<sub>2</sub>SO<sub>4</sub> ATPSs as shown in Fig. 2a. The linear relationship plotted in Fig. 2a may be described as:

$$\log K_i^{\text{PEG-10000-Na}_2\text{SO}_4} = -0.02_{\pm 0.01} + 1.02_{\pm 0.02} \times \log K_i^{\text{PEG-8000-Na}_2\text{SO}_4} \quad (9)$$

$$N = 15; r^2 = 0.9950; \text{SD} = 0.043; F = 2587$$

where  $K_i^{\text{PEG-8000-Na}_2\text{SO}_4}$  and  $K_i^{\text{PEG-10000-Na}_2\text{SO}_4}$  are partition coefficients for the *i*th compound in PEG-8000-Na<sub>2</sub>SO<sub>4</sub> and PEG-10000-Na<sub>2</sub>SO<sub>4</sub> ATPS correspondingly; *N*—number of compounds;

$r^2$ —correlation coefficient; SD—standard deviation; *F*—ratio of variance.

Similar relationship is observed between partition coefficients for all compounds examined in the PEG-8000-Na<sub>2</sub>SO<sub>4</sub> ATPS and same ATPS containing 0.5 M TMAO. The relationship is plotted in Fig. 2b and it may be described as:

$$\log K_i^{\text{PEG-8000-Na}_2\text{SO}_4\text{-TMAO}} = 0.03_{\pm 0.02} + 1.08_{\pm 0.03} \times \log K_0^{\text{PEG-8000-Na}_2\text{SO}_4} \quad (10)$$

$$N = 15; r^2 = 0.9918; \text{SD} = 0.059; F = 1582$$

where  $K_i^{\text{PEG-8000-Na}_2\text{SO}_4\text{-TMAO}}$  is partition coefficient for the *i*th compound in PEG-8000-Na<sub>2</sub>SO<sub>4</sub> ATPS containing 0.5 M TMAO; all the other parameters are as defined above.

Partition coefficients for all compounds in ATPS containing 0.5 M trehalose and 0.5 M sucrose fit the Collander relationship very well (see Fig. 2c) and may be described as:  $(11) \log K_i^{\text{PEG-8000-Na}_2\text{SO}_4\text{-trehalose}} = -0.07_{\pm 0.02} + 1.06_{\pm 0.02} \times \log K_i^{\text{PEG-8000-Na}_2\text{SO}_4\text{-sucrose}}$

$$N = 15; r^2 = 0.9961; \text{SD} = 0.060; F = 3325$$

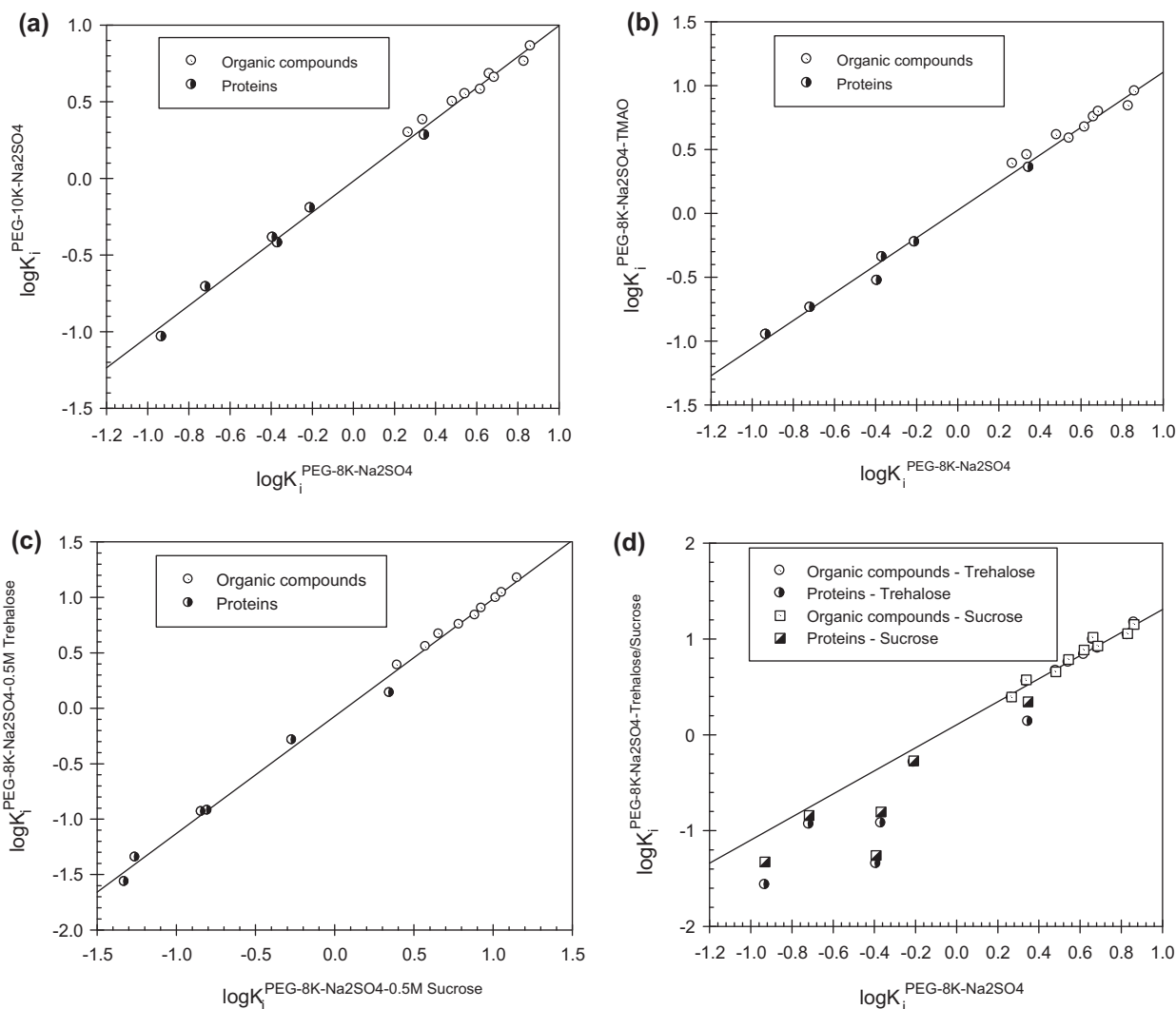
where  $K_i^{\text{PEG-8000-Na}_2\text{SO}_4\text{-trehalose}}$  and  $K_i^{\text{PEG-8000-Na}_2\text{SO}_4\text{-sucrose}}$  are partition coefficients for the *i*th compound in PEG-8000-Na<sub>2</sub>SO<sub>4</sub> ATPS containing 0.5 M trehalose and 0.5 M sucrose correspondingly; all the other parameters are as defined above. Similar relationship was established [33] previously in dextran-PEG ATPS with the same osmolyte additives.

Analysis of the partition coefficients for all compounds in other PEG-8000-Na<sub>2</sub>SO<sub>4</sub> ATPSs containing 0.5 M osmolyte and in osmolyte-free PEG-8000-Na<sub>2</sub>SO<sub>4</sub> ATPS shows that the Collander relationship holds for small organic compounds but not for proteins. Typical relationship is shown in Fig. 2d for trehalose. The data for partition coefficient for the same compounds and proteins in the presence of 0.5 M sucrose are presented for comparison. The relationship obtained for trehalose may be described as:

$$\log K_i^{\text{PEG-8000-Na}_2\text{SO}_4\text{-trehalose}} = -0.16_{\pm 0.06} + 1.6_{\pm 0.11} \times \log K_i^{\text{PEG-8000-Na}_2\text{SO}_4} \quad (12)$$

$$N = 15; r^2 = 0.9447; \text{SD} = 0.23; F = 222$$

where all the parameters are as defined above. The reduced  $r^2$  and *F* values as well as the increased SD value indicate that the relationship in question describes a trend rather than reliable correlation, and it is readily seen from Fig. 2d that proteins fit the relationship rather poorly. It should be noted that the data for partition coefficients of organic compounds in the presence of 0.5 M sucrose



**Fig. 2.** (a) Logarithms of partition coefficients for organic compounds and proteins in PEG-8000- $\text{Na}_2\text{SO}_4$ -0.01 M sodium phosphate buffer (NaPB), pH 6.8 ATPS versus those for the same compounds and proteins in PEG-10000- $\text{Na}_2\text{SO}_4$ -0.01 M sodium phosphate buffer (NaPB), pH 6.8 ATPS; (b) Logarithms of partition coefficients for organic compounds and proteins in PEG-8000- $\text{Na}_2\text{SO}_4$ -0.5 M TMAO-0.01 M sodium phosphate buffer (NaPB), pH 6.8 ATPS versus those for the same compounds and proteins in PEG-8000- $\text{Na}_2\text{SO}_4$ -0.01 M sodium phosphate buffer (NaPB), pH 6.8 ATPS; (c) Logarithms of partition coefficients for organic compounds and proteins in PEG-8000- $\text{Na}_2\text{SO}_4$ -0.5 M trehalose-0.01 M sodium phosphate buffer (NaPB), pH 6.8 ATPS versus those for the same compounds and proteins in PEG-8000- $\text{Na}_2\text{SO}_4$ -0.5 M sucrose-0.01 M sodium phosphate buffer (NaPB), pH 6.8 ATPS; (d) Logarithms of partition coefficients for organic compounds and proteins in PEG-8000- $\text{Na}_2\text{SO}_4$ -0.5 M trehalose (or sucrose)-0.01 M sodium phosphate buffer (NaPB), pH 6.8 ATPS versus those for the same compounds and proteins in PEG-8000- $\text{Na}_2\text{SO}_4$ -0.01 M sodium phosphate buffer (NaPB), pH 6.8 ATPS (line described the linear relationship observed for organic compounds).

fit the above relationship perfectly, while those for proteins clearly deviate from it.

It has been shown [33,35] previously that logarithms of partition coefficients of proteins in dextran-PEG-0.5 M osmolyte ATPS, all containing 0.01 M sodium/potassium phosphate buffer, pH 7.4 are linearly interrelated in a three dimensional space. Analysis of the partition coefficients listed in Tables 1 and 2 show that similar relationships exists for all compounds examined in PEG- $\text{Na}_2\text{SO}_4$ -0.5 M osmolyte ATPS as well. The relationship illustrated graphically in Fig. 3 is observed between logarithms of partition coefficients of compounds (including proteins) in PEG- $\text{Na}_2\text{SO}_4$ , PEG- $\text{Na}_2\text{SO}_4$ -0.5 M sucrose, and PEG- $\text{Na}_2\text{SO}_4$ -0.5 M sorbitol ATPSs. This relationship may be described as:

$$\begin{aligned} \log K_i^{\text{PEG-8000-Na}_2\text{SO}_4\text{-sorbitol}} \\ = 0.02_{\pm 0.01} + 0.55_{\pm 0.07} \times \log K_i^{\text{PEG-8000-Na}_2\text{SO}_4} \\ + 0.55_{\pm 0.04} \times \log K_i^{\text{PEG-8000-Na}_2\text{SO}_4\text{-sucrose}} \end{aligned} \quad (13)$$

$$N = 15; r^2 = 0.9985; \text{SD} = 0.033; F = 4087$$

where all the parameters are as defined above.

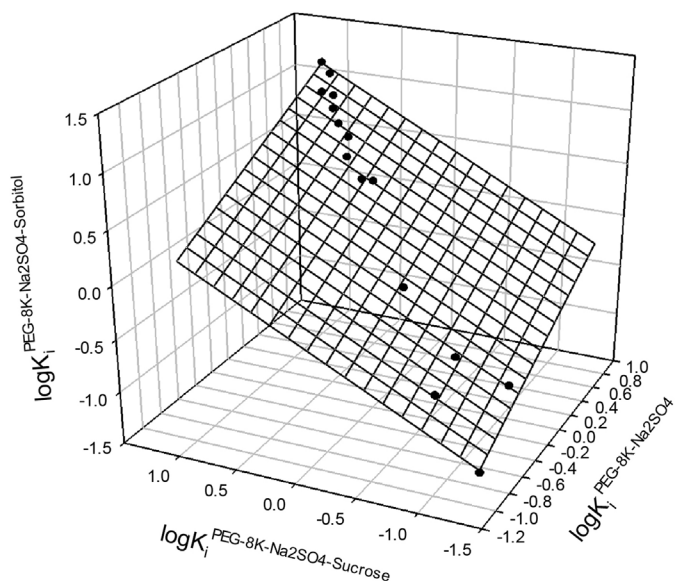
Similar relationship for the PEG- $\text{Na}_2\text{SO}_4$ -TMAO, PEG- $\text{Na}_2\text{SO}_4$ -0.5 M sorbitol, and PEG- $\text{Na}_2\text{SO}_4$ -0.5 M trehalose ATPSs is illustrated graphically in Fig. 4 and may be described as:

$$\begin{aligned} \log K_i^{\text{PEG-8000-Na}_2\text{SO}_4\text{-TMAO}} \\ = -0.01_{\pm 0.03} + 1.3_{\pm 0.2} \times \log K_i^{\text{PEG-8000-Na}_2\text{SO}_4\text{-sorbitol}} \\ - 0.4_{\pm 0.19} \times \log K_i^{\text{PEG-8000-Na}_2\text{SO}_4\text{-trehalose}} \end{aligned} \quad (14)$$

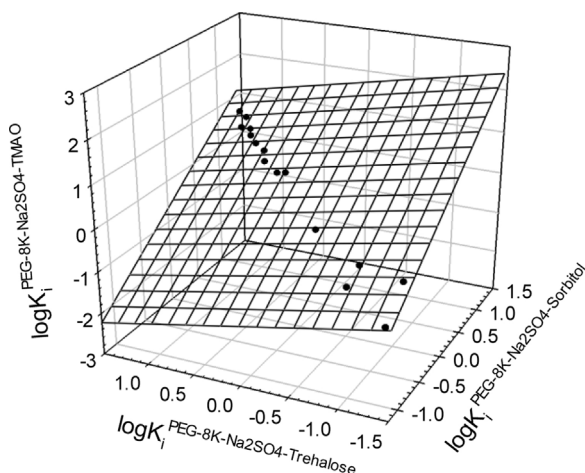
$$N = 15; r^2 = 0.9906; \text{SD} = 0.066; F = 635$$

where all the parameters are as defined above.

It was suggested previously [37,38] that the relationships of the type represented by Eqs. 13 and 14 imply that the compounds respond to their environment in aqueous solutions in the compound structure- and environment-specific manner, and also that



**Fig. 3.** Logarithms of partition coefficients for organic compounds and proteins in PEG-8000-Na<sub>2</sub>SO<sub>4</sub>-0.5 M sorbitol-0.01 M sodium phosphate buffer (NaPB), pH 6.8 ATPS versus those for the same compounds and proteins in PEG-8000-Na<sub>2</sub>SO<sub>4</sub>-0.01 M sodium phosphate buffer (NaPB), pH 6.8 ATPS and in PEG-8000-Na<sub>2</sub>SO<sub>4</sub>-0.5 M sucrose-0.01 M sodium phosphate buffer (NaPB), pH 6.8 ATPS.



**Fig. 4.** Logarithms of partition coefficients for organic compounds and proteins in PEG-8000-Na<sub>2</sub>SO<sub>4</sub>-0.5 M TMAO-0.01 M sodium phosphate buffer (NaPB), pH 6.8 ATPS versus those for the same compounds and proteins in PEG-8000-Na<sub>2</sub>SO<sub>4</sub>-0.5 M sorbitol-0.01 M sodium phosphate buffer (NaPB), pH 6.8 ATPS and in PEG-8000-Na<sub>2</sub>SO<sub>4</sub>-0.5 M trehalose-0.01 M sodium phosphate buffer (NaPB), pH 6.8 ATPS.

the responses are governed by changes in the compound–water interactions possibly originating from the compound dipole–ion interactions.

#### 4.1. Organic compound–water interactions in PEG-sodium sulfate ATPS

The partition coefficients for organic compounds listed in Table 1 were examined with Eq. (1). The solute-specific coefficients were determined by multiple linear regression analysis using the procedure described by Ab Rani et al. [39]. According to this procedure [39], the *p*-value was used for a given compound as a test for significance for each solute-specific coefficient in Eq. (1). In view of the small number of six ATPSs employed (and additional condition of partition coefficient *K*-value = 1 for the compound in the

theoretical critical point in an ATPS, when both phases have identical composition; i.e., zero difference between each of the solvent properties of the phases [22]), we have chosen to use the maximum statistical significance value of  $p \leq 0.1$ . If all four coefficients ( $S_s$ ,  $A_s$ ,  $B_s$ , and  $C_s$ ) proved statistically significant ( $p \leq 0.1$ ), the correlation was accepted. If one or more values reveal a *p*-value  $> 0.1$ , then equations contained different combinations of coefficients were examined. The equation with a set of coefficients providing *p*-values for all parameters below or equal to 0.1 was accepted.

The solute-specific coefficients determined for each compound are presented in Table 3 together with the corresponding *p*-values (except the cases when  $p < 0.001$ ).

It has been reported [40] recently that there seems to be a cooperativity between the different types of solute–water interactions due to which the solute-specific coefficients are linearly interrelated. The interrelationship between the solute-specific coefficients presented in Table 3 is illustrated graphically in Fig. 5, and it may be described as:

$$B_s^i = -0.5 \pm 0.3 + 0.8 \pm 0.16 \times C_s^i - 0.11 \pm 0.04 \times S_s^i \quad (15)$$

$$N = 9; r^2 = 0.8785; SD = 0.20; F = 21.7$$

where  $B_s^i$ ,  $C_s^i$ , and  $S_s^i$  are solute-specific coefficients for the *i*th compound; all the other parameters are as defined above.

Analysis of solute-specific coefficients presented in Table 3 for compounds with solute-specific coefficients determined [40] in different multiple polymer–polymer ATPS containing 0.15 M Na<sub>2</sub>SO<sub>4</sub> in 0.01 M NaPB, pH 7.4 shows the linear relationship for solute-specific coefficients  $S_s$  representing contributions of dipole–dipole and induced dipole–dipole solute–water interactions in the partition coefficients of compounds in the corresponding ATPS. The relationship illustrated graphically in Fig. 6 may be described as:

$$\begin{aligned} S_s^{i \text{ PEG-Na}_2\text{SO}_4} \\ = -0.3 \pm 0.16 + 0.74 \pm 0.05 \times S_s^{i \text{ polymer-polymer-0.15 M Na}_2\text{SO}_4-0.01 \text{ M NaPB}} \end{aligned} \quad (16)$$

$$N = 10; r^2 = 0.9623; SD = 0.20; F = 204$$

where  $S_s^{i \text{ PEG-Na}_2\text{SO}_4}$  and  $S_s^{i \text{ polymer-polymer-0.15 M Na}_2\text{SO}_4-0.01 \text{ M NaPB}}$  are the solute specific coefficients  $S_s$  for *i*th organic compound determined in PEG-Na<sub>2</sub>SO<sub>4</sub>-0.01 M NaPB, pH 6.8 ATPS (Table 3) and in polymer–polymer ATPS containing 0.15 M Na<sub>2</sub>SO<sub>4</sub> in 0.01 M NaPB, pH 7.4 [40]. It should be mentioned that only compounds with determined  $S_s$  values in both types of ATPS were considered.

Comparison of the solute-specific coefficient  $C_s$  values determined for the same compounds in the PEG-Na<sub>2</sub>SO<sub>4</sub> ATPS and in polymer–polymer ATPS containing 0.15 M Na<sub>2</sub>SO<sub>4</sub> in 0.01 M NaPB, pH 7.4 [40] shows that those determined in the former (see Table 3) exceed the  $C_s$  values determined in the latter from 1.2-fold to 5.6-fold. It seems possible to explain these observations assuming that the differences between the electrostatic properties of the phases in the PEG-Na<sub>2</sub>SO<sub>4</sub> ATPSs used in this study varied from 0.067 to 0.71, while in polymer–polymer ATPSs containing 0.15 M Na<sub>2</sub>SO<sub>4</sub> in 0.01 M NaPB, pH 7.4 [40] it varied from -0.011 to 0.456. The sodium sulfate concentrations in the top and bottom phases of the PEG-8000-Na<sub>2</sub>SO<sub>4</sub> ATPS used was reported [20] to be ~0.69 M and 0.24 M correspondingly (with the difference between the phases of ca.0.45 M Na<sub>2</sub>SO<sub>4</sub>), while in polymer–polymer ATPS used in [40] the difference between the Na<sub>2</sub>SO<sub>4</sub> concentrations in the phases is less than 0.15 M Na<sub>2</sub>SO<sub>4</sub>. Therefore it seems reasonable that the contribution of electrostatic ion–dipole solute–solvent interactions in the partition coefficients of solutes in PEG-Na<sub>2</sub>SO<sub>4</sub> ATPS exceed those in the polymer–polymer ATPS containing 0.15 M Na<sub>2</sub>SO<sub>4</sub>.

**Table 3**  
Solute-specific coefficients<sup>a</sup> (see Eq. 1) for organic compounds in PEG-Na<sub>2</sub>SO<sub>4</sub>-0.01 M NaPB (calculated by multiple linear regression analysis from data in Table 1. NaPB—sodium phosphate buffer, pH 6.8.

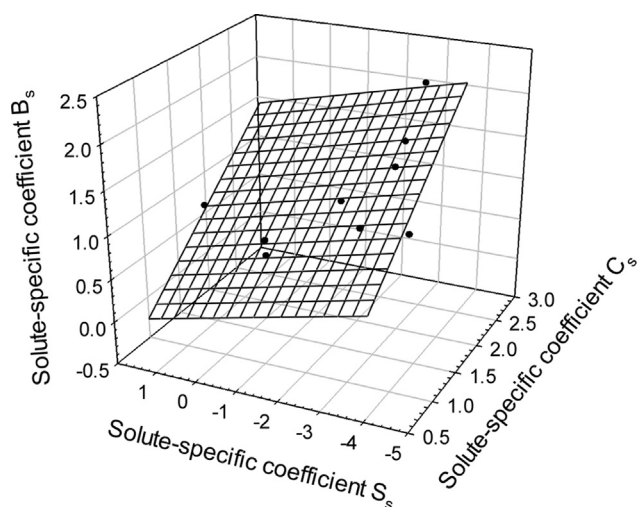
Compound	$S_s$	$A_s$	$B_s$	$C_s$	$N$ ; SD; $F^c$
4-Aminophenol; $p$ -values <sup>b</sup>	$-0.7 \pm 0.21$ ; 0.04	–	$0.8 \pm 0.13$ ; 0.004	$1.24 \pm 0.05$	7; 0.01; 7030
Benzyl alcohol; $p$ -values <sup>b</sup>	$-2.9 \pm 0.55$ ; 0.006	–	$1.1 \pm 0.33$ ; 0.03	$1.3 \pm 0.1$ ; 0.0002	7; 0.03; 1304
Caffeine; $p$ -values <sup>b</sup>	–	–	$0.3 \pm 0.1$ ; 0.08	$0.72 \pm 0.05$	7; 0.01; 2638
Coumarin	–	–	–	$1.40 \pm 0.03$	7; 0.05; 1673
Glucopyranoside <sup>d</sup> ; $p$ -values <sup>b</sup>	$-1.4 \pm 0.4$ ; 0.02	–	–	$0.72 \pm 0.03$	7; 0.02; 1637
Methyl anthranilate; $p$ -values <sup>b</sup>	$-3 \pm 0.82$ ; 0.02	–	$1.7 \pm 0.49$ ; 0.02	$2.1 \pm 0.16$ ; 0.0002	7; 0.04; 1366
Phenol; $p$ -values <sup>b</sup>	$-3.8 \pm 0.48$ ; 0.001	–	$1 \pm 0.99$ ; 0.025	$1.51 \pm 0.09$	7; 0.02; 2839
2-Phenylethanol; $p$ -values <sup>b</sup>	$-2.4 \pm 0.87$ ; 0.04	–	–	$1.09 \pm 0.07$	7; 0.05; 818
Vanillin; $p$ -values <sup>b</sup>	$-3 \pm 1.4$ ; 0.1	–	$1.5 \pm 0.85$ ; 0.1	$1.9 \pm 0.27$ ; 0.002	7; 0.07; 382
DNP-Ala Na; $p$ -values <sup>b</sup>	$-0.5 \pm 0.14$ ; 0.03	–	$0.52 \pm 0.08$ ; 0.004	$1.39 \pm 0.03$	7; 0.007; 23785
DNP-NVal Na; $p$ -values <sup>b</sup>	$1.5 \pm 0.31$ ; 0.009	–	$0.8 \pm 0.18$ ; 0.01	$1.67 \pm 0.06$	7; 0.01; 7372
DNP-NLeu Na; $p$ -values <sup>b</sup>	$-1.7 \pm 0.56$ ; 0.04	–	$1 \pm 0.3$ ; 0.04	$1.9 \pm 0.1$	7; 0.03; 2876
DNP-AO Na; $p$ -values <sup>b</sup>	$-2.8 \pm 0.73$ ; 0.02	–	$2.1 \pm 0.44$ ; 0.009	$2.7 \pm 0.14$	7; 0.03; 2774

<sup>a</sup> Solute specific coefficients represent the following solute–water interactions:  $S_s$ —dipole–dipole interactions;  $A_s$ —hydrogen bonding with solute as a donor;  $B_s$ —hydrogen bonding with solute as an acceptor;  $C_s$ —induced dipole–ion interactions.

<sup>b</sup> Statistical significance  $p$ -value (not shown for  $p < 0.0001$ ).

<sup>c</sup>  $N$ —number of experimental data (partition coefficients under different conditions, including  $K = 1$  when properties of the phases are identical (see in text); SD—standard deviation;  $F$ —ratio of variance.

<sup>d</sup>  $p$ -Nitrophenyl- $\alpha$ -D-Glucopyranoside.

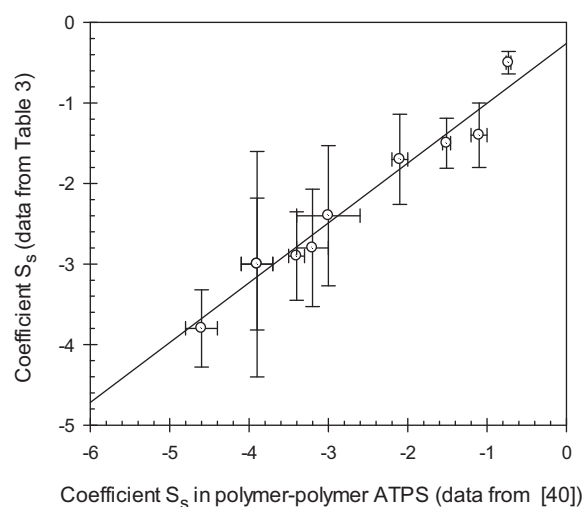


**Fig. 5.** Interrelationship between solute-specific coefficients  $B_s$  for organic compounds and solute-specific coefficients  $C_s$  and  $S_s$  for the same compounds.

It should be mentioned that Willauer et al. in the study [41] of partitioning of a set of 29 organic compounds in PEG-2000-K<sub>3</sub>PO<sub>4</sub> ATPS described partition behavior of compounds by the so-called linear free energy relationship (LFER) model by Abraham [42–45]. It was concluded [41] that partitioning of organic compounds in PEG-salt ATPS is governed by the solute size, basicity, and aromaticity or halogenicity. The results obtained here and discussed below show that the molecular size of the solute is not the factor governing the solute partition behavior in PEG-Na<sub>2</sub>SO<sub>4</sub> ATPS.

#### 4.2. Protein–water interactions in PEG-sodium sulfate ATPS

It should be noted that essentially all the proteins examined (except for papain) distribute into lower salt-rich phase in clear contradiction with the aforementioned conclusion by Willauer et al. [41] that the increasing molecular size of a solute drives the solute partitioning into PEG-rich phase. Partition coefficients of papain ( $K = 2.22$ ; molecular weight of ca. 23,400 Da) in PEG-8000-Na<sub>2</sub>SO<sub>4</sub> ATPS is very close to that of  $p$ -nitrophenyl- $\alpha$ -D-glucopyranoside ( $K = 2.18$ ; molecular weight  $\sim 301$  Da) may serve as additional illustration of the contradiction.



**Fig. 6.** Relationship between solute specific coefficients  $S_s$  for organic compounds determined in PEG-Na<sub>2</sub>SO<sub>4</sub>-0.01 M sodium phosphate buffer, pH 6.8 ATPS and solute specific coefficients  $S_s$  for the same compounds determined in polymer–polymer ATPS containing 0.15 M Na<sub>2</sub>SO<sub>4</sub> in 0.01 M sodium phosphate buffer, pH 7.4 ATPS (data taken from [40]).

The solute-specific coefficients for proteins were determined similarly to those for organic compounds from the solvent properties of ATPSs (Table 1) and from proteins partition coefficients (Table 2), and are listed in Table 4. It should be noted that in contrast to small organic compounds, the solute-specific coefficients  $A_s$  are quite significant for three out of six proteins studied. Contributions of dipole–dipole interactions (solute-specific coefficient  $S_s$ ) into the partition coefficients are large for three proteins ( $\alpha$ -chymotrypsinogen,  $\alpha$ -chymotrypsin, and lysozyme), while the contributions of electrostatic interactions in the PEG-Na<sub>2</sub>SO<sub>4</sub> ATPS containing 0.01 M NaPB, pH 6.8 are less significant than those determined for the same proteins in dextran-PEG ATPS containing 0.01 M K/NaPB, pH 7.4 [35]. As an example, for  $\alpha$ -chymotrypsinogen, the coefficient  $C_s = 7.6 \pm 0.02$  in dextran-PEG ATPS [35] and  $-1.2 \pm 0.21$  in PEG-Na<sub>2</sub>SO<sub>4</sub> ATPS (Table 4), for concanavalin A,  $C_s = 5.1 \pm 0.2$  in dextran-PEG ATPS [35] and  $-1.9 \pm 0.2$  in PEG-Na<sub>2</sub>SO<sub>4</sub> ATPS (Table 4). The decreasing electrostatic interactions of proteins with the solvent appear to agree with the suggested by Ninham et al. [46,47] changes in the nature of



**Table 4**

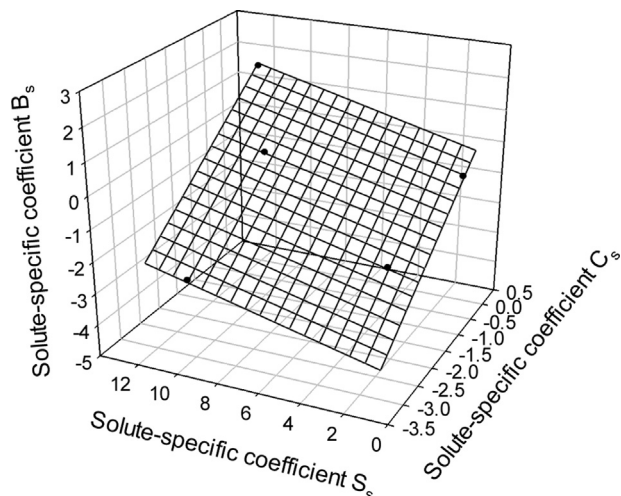
Solute-specific coefficients<sup>a</sup> (see Eq. 1) for proteins in PEG–Na<sub>2</sub>SO<sub>4</sub>–0.01 M NaPB (calculated by multiple linear regression analysis from data in Table 1. NaPB—sodium phosphate buffer, pH 6.8.

Protein	S <sub>s</sub>	A <sub>s</sub>	B <sub>s</sub>	C <sub>s</sub>	N; SD; F <sup>c</sup>
α-Chymotrypsinogen; <i>p</i> -values <sup>b</sup>	9.6 ± 0.96; 0.04	24 ± 7.1; 0.03	–	–1.2 ± 0.21; 0.006	7; 0.04; 518
Chymotrypsin; <i>p</i> -values <sup>b</sup>	9.9 ± 1.1; 0.003	33.2 ± 7.7; 0.02	–2.3 ± 0.5; 0.02	–3.2 ± 0.3; 0.001	7; 0.04; 1221
Concanavalin A; <i>p</i> -values <sup>b</sup>	2.4 ± 1.1; 0.1	–	–2.2 ± 0.6; 0.03	–1.9 ± 0.2; 0.0007	7; 0.05; 469.8
Lipase; <i>p</i> -values <sup>b</sup>	0.8 ± 0.28; 0.04	–	–0.4 ± 0.17; 0.07	–0.48 ± 0.05; 0.0008	7; 0.01; 644.5
Lysozyme; <i>p</i> -values <sup>b</sup>	12 ± 2.4; 0.005	–	1.7 ± 0.58; 0.04	–	7; 0.11; 174.8
Papain; <i>p</i> -values <sup>b</sup>	4 ± 2; 0.1	23 ± 4.6	–	–	7; 0.09; 36.2

<sup>a</sup> Solute specific coefficients represent the following solute–water interactions: S<sub>s</sub>—dipole–dipole interactions; A<sub>s</sub>—hydrogen bonding with solute as a donor; B<sub>s</sub>—hydrogen bonding with solute as an acceptor; C<sub>s</sub>—induced dipole–ion interactions.

<sup>b</sup> Statistical significance *p*-value (not shown for *p* < 0.0001).

<sup>c</sup> N—number of experimental data (partition coefficients under different conditions, including *K* = 1 when properties of the phases are identical (see in text); SD—standard deviation; F—ratio of variance.



**Fig. 7.** Interrelationship between solute-specific coefficients B<sub>s</sub> for proteins and solute-specific coefficients C<sub>s</sub> and S<sub>s</sub> for the same proteins.

protein–ion interactions in the presence of high salt concentration exceeding 0.2 M.

Analysis of the data in Table 4 confirms that the linear relationship similar to the one found for organic compounds exists for proteins as well. This relationship illustrated graphically in Fig. 7 may be described as:

$$B_s^i = -0.1_{\pm 0.2} + 1.20_{\pm 0.08} \times C_s^i - 0.16_{\pm 0.02} \times S_s^i \quad (17)$$

$$N = 5; r^2 = 0.9931; SD = 0.20; F = 144.5$$

where B<sub>s</sub><sup>i</sup>, C<sub>s</sub><sup>i</sup>, and S<sub>s</sub><sup>i</sup> are solute-specific coefficients for the *i*th protein; all the other parameters are as defined above. (Papain was not considered for the above relationship because its solute-specific coefficients B<sub>s</sub> and C<sub>s</sub> could not be determined.)

The limited number of proteins examined here prevents any general conclusion. It should be emphasized, however, that the partition behavior of both small organic compounds and proteins in PEG–Na<sub>2</sub>SO<sub>4</sub> ATPS can be described in terms of solute–solvent interactions.

It seems also important that the results obtained in our study indicate that the solute-specific coefficients representing contributions of different types of solute–solvent interactions under the conditions explored differ from those determined at relatively low salt concentrations [35], supporting the previously suggested hypothesis that the solute–solvent interactions depend upon the solvent environment as well as upon the solute structure. Further studies are clearly necessary for gaining better insight into mechanisms of these interactions, and these studies are currently in progress in our laboratories.

## 5. Conclusions

It is experimentally established that partition behavior of small organic compounds and proteins in aqueous PEG–Na<sub>2</sub>SO<sub>4</sub> two-phase system is governed by solute–water interactions. The experimental data obtained show that the compound partition behavior in PEG–salt ATPS does not depend on the molecular volume of the compound. The data obtained agree with the assumption that polar organic compounds and proteins respond to their environment in aqueous media by changing contributions of different types of solute–water interactions in the solute- and ionic composition-specific manner. Both polar organic compounds and proteins demonstrate cooperativity of different types of solute–water interactions found to be linearly interrelated. Solvent properties of aqueous media in the coexisting phases of polymer-salt ATPS may be quantified using the solvatochromic comparison method provided the salt concentrations in both phases are below the level prohibiting solvatochromic measurements.

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