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"On the Collander equation": Protein partitioning in polymer/polymer aqueous two-phase systems

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ABSTRACT

Distribution coefficients of randomly selected proteins were measured in aqueous two-phase systems (ATPSs) formed by different combinations of Dextran-75 (Dex), Ficoll-70, polyethylene glycol-8000 (PEG), hydroxypropyl starch-100 (PES), and Ucon50HB5100 (Ucon, a random copolymer of ethylene glycol and propylene glycol) at particular polymer concentrations, all containing 0.15 M NaCl in 0.01 M phosphate buffer, pH 7.4. Most of the proteins in the PEG-Ucon system precipitated at the interface. In the other ATPSs, namely, PES-PEG, PES-Ucon, Ficoll-PEG, Ficoll-Ucon, and in Dex-PEG and Dex-Ucon described earlier the distribution coefficients for the proteins were correlated according to the solvent regression equation: $\ln K_i = a_{io} \ln K_o + b_{io}$, where K_i and K_o are the distribution coefficients for any protein in the *i*th and *o*th two-phase systems. Coefficients a_{io} and b_{io} are constants, the values of which depend upon the particular compositions of the two-phase systems under comparison.

1. Introduction

Aqueous two-phase systems arise in aqueous mixtures of different water-soluble polymers or a single polymer and a specific salt. When two specific polymers, e.g., Dex and PEG, are mixed in water above certain concentrations, the mixture separates into two immiscible aqueous phases. There is a clear interfacial boundary separating two distinct aqueous-based phases, each preferentially rich with one of the polymers. The aqueous solvent in both phases was demonstrated to provide media suitable for biological products [1–4]. These systems are unique because each of the phases contains over 80% water on a molal basis and yet the phases are immiscible and differ in their solvent properties [4,5], therefore, these systems can be used for differential distribution of solutes and particles.

Extraction in ATPSs has been clearly demonstrated as an efficient method for large scale recovery and purification of biological products [1–3,6,7]. Low cost, high capacity and easy scale-up are clear advantages of this technology. Partitioning in ATPSs may also be used for characterization of protein surface properties [4,8], changes in protein structure [9], conformation [10], ligand binding [1–3], etc. For successful utilization of partitioning in ATPSs it is important to understand the mechanisms of solute distribution in the systems as well as system properties at the molecular level.

The underlying concept for one current explanation for partitioning in ATPSs is that polymers and salts engaged in the formation of an ATPS are essentially neutral to the solute being partitioned and are important only in regard to their effects on the solvent features of the aqueous media in the coexisting phases. It should be mentioned that this concept is not applicable to ATPSs containing charged polymers or polymers with ligands for so-called affinity partitioning. This concept is based on experimental evidence indicating that (a) the solvent features of the aqueous media in the coexisting phases are different [4,5], and (b) there are clear similarities between partitioning of solutes in ATPS and in water-organic solvent systems [4,5,12–15].

Until now, the solvent properties of ATPSs examined were restricted to a limited number of systems formed by only two pairs of polymers, Dex-PEG and Dex-Ficoll, [4,11] and those formed by PEG and inorganic salts [5,12–16]. The free energy of transfer of a methylene group between the coexisting phases has been shown [4,5,11–22] to be important for characterization of ATPS of different polymer and salt composition.

One important consequence of the similarity between partitioning of solutes in ATPS and in water-organic solvent systems is the possible application of the Collander equation [23]. This equation

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describes a linear correlation between distribution coefficients of solutes in different biphasic systems. It was established by this group earlier [21] that the distribution coefficients for different randomly selected proteins in the Dex-PEG and Dex-Ucon ATPSs are correlated according to the so-called Collander equation or solvent regression equation. We explore in the present work the applicability of the solvent regression equation to ATPSs formed by different paired combinations of Dextran (Dex-75), Ficoll-70, polyethylene glycol (PEG-8000), hydroxypropyl starch (PES-100), and Ucon50HB5100 (a random copolymer of ethylene glycol and propylene glycol) at particular polymer concentrations, all containing 0.15 M NaCl in 0.01 M phosphate buffer, pH 7.4.

2. Experimental

2.1. Materials

2.1.1. Polymers

All polymers were used without further purification. Dextran 75 (lot 115195), weight-average molecular weight (M_w) ≅ 75,000 was purchased from USB (Cleveland, OH, USA). Polyethylene glycol 8000 (lot 69H00341), M_w = 8000 was purchased from Sigma–Aldrich (St. Louis, MO, USA). Ucon 50-HB-5100 (lot SJ1955S3D2), M_w = 3930 was purchased from Dow-Chemical (Midland, MI, USA). Ficoll 70 (lot 302970), M_w ≅ 70,000 was purchased from GE Healthcare Biosciences AB (Sweden). Reppal PES-100 (lot D702-09/01), M_w ≅ 1,00,000 was purchased from REPPE AB (Växjö, Sweden).

2.1.2. Proteins

Chicken egg lysozyme (#L-6876), bovine α-chymotrypsinogen A (#C-4879), bovine hemoglobin (#H-2500), horse heart cytochrome *c* (#C-7752), bovine ribonuclease B (#R-7884), bovine ribonuclease A (#R-5000), bovine trypsinogen (#T-1143), human hemoglobin (#H-7379), horse myoglobin (#M-0630), and bovine β-lactoglobulin (#L-3908) were purchased from Sigma. Porcine lipase (#18480), and human transferrin (#22508) were purchased from USB.

2.1.3. Others

o-Phthaldialdehyde (OPA) reagent solution (complete) was purchased from Sigma. All salts and other chemicals used were of analytical-reagent grade.

2.2. Methods

2.2.1. Phase diagrams

The phase diagrams were reported previously [22].

2.2.2. Partitioning

2.2.2.1. Phase systems. A mixture of polymers was prepared by dispensing appropriate amounts of the aqueous stock polymer solutions into a 1.2 mL microtube using a Hamilton Company (Reno, NV, USA) ML-4000 four-probe liquid-handling workstation. Appropriate amounts of stock buffer solutions were added to give the required ionic and polymer composition of the final system with total volume of 0.5 mL. All two-phase systems had the polymer compositions indicated in Table 1 and salt composition of 0.15 M NaCl in 0.01 M sodium phosphate buffer (NaPB), pH 7.4.

2.2.2.2. Partitioning experiments. An automated instrument for performing aqueous two-phase partitioning, Automated Signature Workstation, ASW (Analiza, Inc., Cleveland, OH, USA) was used for the partitioning experiments. 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 Plus^{384}; Molecular Devices, Sunnyvale, CA, USA). Solutions of all proteins were prepared in water at concentrations of 1–5 mg/mL. Varied amounts (e.g., 0, 15, 30, 45, 60, and 75 μL) of a given protein solution and the corresponding amounts (e.g., 100, 85, 70, 55, 40 and 25 μL) of water were added to a set of the same polymer/buffer mixtures. The systems were vortexed in a Multi-pulse vortexer and centrifuged for 30 min at 3000 × *g* at 23 °C (refrigerated centrifuge Jouan, BR4i) to accelerate phase settling. The top phase in each system was removed, the interface discarded, and aliquots of 20–70 μL from the top and bottom phases were withdrawn in duplicate for analysis. These aliquots were combined with 250 μL of *o*-phthaldialdehyde reagent solution (complete) in microplate wells. After moderate shaking for 2 min at room temperature, fluorescence was determined with a fluorescence plate reader with a 360 nm excitation filter and a 460 nm emission filter, and with a 100–125 sensitivity setting.

The distribution coefficient, *K*, is defined as the ratio of the sample concentration (mg/mL) in the top phase to the sample concentration (mg/mL) in the bottom phase. The *K* value for each solute was determined as the slope of the concentration in the top phase plotted as a function of the concentration in the bottom phase averaged over the results obtained from two to four partition experiments carried out at the specified ionic composition of the system (0.15 M NaCl in 0.01 M NaPB, pH 7.4). The deviation from the average *K* value was always less than 5%, and in most cases lower than 2%.

2.2.3. Electrophoresis

All protein preparations were characterized by SDS-PAGE electrophoresis in a microfluidic chip using Bioanalyzer 2100, Protein 200 Plus Assay (Agilent Technologies, USA) under non-reduced conditions. All the proteins except two were observed as single bands in the electrophoregrams, while both gamma-globulins under study

Table 1
Polymer compositions[a] of the phases in the aqueous two-phase systems used for partitioning, difference between the relative hydrophobic character of the coexisting phases represented by coefficient E[b], and difference between the polar/electrostatic character of the coexisting phases represented by coefficient C[b]

Polymer 1	Polymer 2	Total composition		Top phase		Bottom phase		E[b]	C[b]
		Polymer 1	Polymer 2	Polymer 1	Polymer 2	Polymer 1	Polymer 2		
Dextran	PEG	12.4	6.1	0.31	13.0	22.4	0.53	0.062 ± 0.002	−0.089 ± 0.007
Dextran	Ucon	12.4	10.1	0.16	18.3	26.5	0.59	0.196 ± 0.005	0.004 ± 0.017
PEG	Ucon	15.0	30.0	0.36	50.3	35.5	1.58	0.284 ± 0.019	1.299 ± 0.067
Ficoll	PEG	15.1	7.90	9.55	11.7	24.0	1.83	0.022 ± 0.002	−0.211 ± 0.007
Ficoll	Ucon	13.0	9.93	2.90	16.4	24.5	2.54	0.127 ± 0.008	−0.072 ± 0.029
PES	PEG	15.2	6.96	3.67	12.3	29.6	0.37	−0.034 ± 0.0003	−0.300 ± 0.0007
PES	Ucon	12.9	7.68	2.76	13.5	24.0	1.32	0.082 ± 0.013	−0.266 ± 0.023

[a] Polymer concentrations are given in % (m/m).
[b] Coefficients *C* and *E* calculated from experimental data on partitioning of sodium salts of dinitrophenyl-amino acids with aliphatic side chain described by Eq. (2) as reported in [21].

Table 2
Distribution coefficients, *K*, for proteins examined in the ATPS indicated

Protein	M_w (kD)	pI[a]	Dex-PEG	Dex-Ucon	PES-PEG	PES-Ucon	Ficoll-PEG	Ficoll-Ucon	PEG-Ucon
RNAse A	13.7	9.6	0.489	0.247	0.604	0.506	0.466	0.25	~0.014*
RNAse B	~15.0	~9.45	0.455	0.265	0.703	0.63	0.440	0.237	–
Chymotrypsinogen	~25.7	9.0	2.71	1.78	2.98	1.80	1.04	0.638	0.0098
Trypsinogen	23.7	9.3	0.89	0.702	0.967	0.779	0.580	0.345	0.015
Lysozyme	14.3	11.4	2.36	2.95	1.29	1.28	0.91	1.00	0.036
Hemoglobin bovine	64.5	6.8	0.074	0.053	0.148	0.208	0.094	0.052	*
Hemoglobin human	64.5	6.8	0.131	0.117	0.22	0.282	0.153	0.074	*
Lactoglobulin	18.4	5.2	0.071	0.033	0.213	0.176	0.112	0.044	*
Transferrin	77.0	5.7	0.0084	0.0015	0.052	0.042	0.019	0.0035	*
Myoglobin	17.6	7.3	0.161	0.080	0.340	0.310	0.258	0.154	0.065
Lipase	48	5.2	0.716	0.658	0.75	0.78	0.733	0.634	0.358
γ-Globulin human[b]	~160	~6.8	0.043	0.014	–	–	–	–	–
γ-Globulin bovine[b]	~180	~6.5	0.024	0.007	–	–	–	–	–
Cytochrome c	12.4	10.0	0.29	0.12	–	–	–	–	–

[a] p*I* – isoelectric point.
[b] Heterogeneous preparations.
* Observed protein precipitation.

displayed a series of overlapping bands and were judged to be heterogeneous.

3. Results and discussion

3.1. Protein partitioning

Previously [22] we showed that distribution coefficients for randomly selected proteins in Dex-PEG and Dex-Ucon ATPSs are correlated according to the Collander equation [23] or solvent regression equation [22–27]:

$$\ln K_i = a_{io} \ln K_o + b_{io} \quad (1)$$

where K_i and K_o are distribution coefficients for any given solute in the *i*th and *o*th two-phase systems; a_{io} and b_{io} are constants, the values of which depend upon the particular composition of the *i*th and *o*th two-phase systems under comparison.

The distribution coefficients for all of the proteins studied in the ATPSs under consideration (listed in Table 1) are presented in Table 2. It should be noted that upon introduction into the PEG-UCON system most of the proteins either partially or completely precipitated and their distribution behavior could not be analyzed. ATPS formed by these two polymers at relatively high concentrations which served as precipitating agents is not suitable for partitioning of hydrophilic proteins and is not considered further in this work. In all of the other ATPSs described in Tables 1 and 2, the protein distribution coefficients are clearly correlated according to Eq. (1). Fig. 1 presents typical data, where logarithms of the distribution coefficients in different ATPSs are plotted versus those in the Dex-PEG system. It should be mentioned that the molecular weights of the proteins studied varied from ~14,000 (ribonuclease, lysozyme) to ~77,000 (transferrin), with isoelectric points (p*I*) from 5.2 (lipase, lactoglobulin) to 11.4 (lysozyme) (Table 2). Gammaglobulins studied previously in Dex-PEG and Dex-Ucon systems were not examined here, since the protein preparations were highly heterogeneous.

All of the data presented in Table 2 were processed according to Eq. (1). The coefficients a_{io} and b_{io} values with corresponding correlation coefficients, r^2, and number of proteins examined in a given pair of ATPSs, *N*, are presented in Table 3 with different ATPSs used as the reference systems. As expected, the linear correlations shift depending on the ATPS used as a reference. The observed correlations indicate that neither the charge nor the molecular weight of a protein is the sole factor governing its partition behavior.

3.2. Physico-chemical characterization of the phases

One current approach used to characterize the properties of coexisting phases in a given ATPS is derived from partitioning a homologous series of solutes in the system [4,5,11–22]. According to this approach, the distribution coefficients observed are examined with regard to the structural changes within the solutes' series. For any homologous series of solutes with varied aliphatic alkyl chain length the distribution coefficient in a particular *i*th ATPS may be described as:

$$\ln K_{ji} = C_i + E_i(N_C)_j \quad (2)$$

where $\ln K_{ji}$ is the natural logarithm of the distribution coefficient, *K*, of a *j*th member of the homologous series with the corresponding $(N_C)_j$ length of the aliphatic chain of a given solute represented by the equivalent number of CH_2 groups; E_i is an average $\ln K$ increment per CH_2 group; C_i represents the total contribution of the non-alkyl part of the structure of the solute in the series into $\ln K_{ji}$. We used a series of sodium salts of dinitrophenylated amino acids with aliphatic side chains of different length [21,22], in which coefficient *C* represents the contribution of the charged, non-alkyl part of a DNP-amino acid structure into $\ln K_i$.

It was previously observed [22] that the difference in the relative hydrophobic character of the coexisting phases, represented

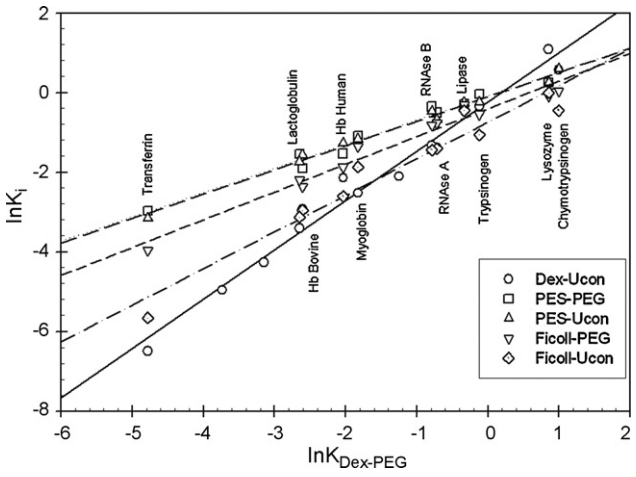

Fig. 1. Natural logarithms of distribution coefficients for proteins indicated in ATPSs Dex-Ucon, Ficoll-Ucon, Ficoll-PEG, PES-Ucon, and PES-PEG versus natural logarithms of distribution coefficients for the same proteins in Dex-PEG system (see Table 1 for polymer compositions of ATPSs and Table 2 for *K*-values for the proteins).

Table 3
Coefficients a_{io} and b_{io} in solvent regression equation (Eq. (1)) with different ATPSs used as a reference system (N – number of proteins; r^2 – regression coefficient; * – data from [21])

ATPS	Reference	a_{io}	b_{io}	N	r^2
Dex-PEG	Dex-PEG	1.000	0.000		
Dex-Ucon*		1.24 ± 0.05	−0.2 ± 0.1	14	0.9780
PES-PEG		0.61 ± 0.03	−0.09 ± 0.07	11	0.9739
PES-Ucon		0.61 ± 0.02	−0.11 ± 0.04	11	0.9890
Ficoll-PEG		0.70 ± 0.04	−0.41 ± 0.09	11	0.9677
Ficoll-Ucon		0.92 ± 0.07	−0.7 ± 0.2	11	0.9465
Dex-PEG*		0.79 ± 0.03	0.20 ± 0.1	14	0.9780
Dex-Ucon	Dex-Ucon	1.000	0.000		
PES-PEG		0.46 ± 0.04	−0.1 ± 0.1	11	0.9186
PES-Ucon		0.49 ± 0.02	−0.02 ± 0.07	11	0.9746
Ficoll-PEG		0.56 ± 0.04	−0.3 ± 0.1	11	0.9451
Ficoll-Ucon		0.75 ± 0.05	−0.6 ± 0.1	11	0.9595
Dex-PEG		1.60 ± 0.09	0.1 ± 0.1	11	0.9739
Dex-Ucon		2.0 ± 0.2	0.0 ± 0.3	11	0.9186
PES-PEG	PES-PEG	1.000	0.000		
PES-Ucon		0.97 ± 0.07	−0.1 ± 0.1	11	0.9608
Ficoll-PEG		1.18 ± 0.07	−0.26 ± 0.09	11	0.9738
Ficoll-Ucon		1.6 ± 0.1	−0.5 ± 0.2	11	0.9387
Dex-PEG		1.62 ± 0.06	0.16 ± 0.08	11	0.9890
Dex-Ucon		2.0 ± 0.1	0.0 ± 0.1	11	0.9746
PES-PEG		1.00 ± 0.06	0.03 ± 0.08	11	0.9608
PES-Ucon	PES-Ucon	1.000	0.000		
Ficoll-PEG		1.13 ± 0.07	−0.29 ± 0.09	11	0.9679
Ficoll-Ucon		1.5 ± 0.1	−0.6 ± 0.2	11	0.9510
Dex-PEG		1.39 ± 0.09	0.5 ± 0.1	11	0.9677
Dex-Ucon		1.7 ± 0.1	0.41 ± 0.2	11	0.9451
PES-PEG		0.83 ± 0.05	0.19 ± 0.08	11	0.9738
PES-Ucon		0.85 ± 0.05	0.22 ± 0.09	11	0.9679
Ficoll-PEG	Ficoll-PEG	1.000	0.000		
Ficoll-Ucon		1.321 ± 0.06	−0.2 ± 0.1	11	0.9808
Dex-PEG		1.03 ± 0.08	0.7 ± 0.2	11	0.9465
Dex-Ucon		1.28 ± 0.09	0.7 ± 0.2	11	0.9595
PES-PEG		0.59 ± 0.05	0.2 ± 0.1	11	0.9387
PES-Ucon		0.63 ± 0.05	0.3 ± 0.1	11	0.9510
Ficoll-PEG		0.74 ± 0.04	0.13 ± 0.09	11	0.9808
Ficoll-Ucon	Ficoll-Ucon	1.000	0.000		

by coefficient E, cannot serve as a single measure of the phase properties governing the partitioning of a solute in ATPSs. The relationships between coefficients a_{io} (Table 3) and coefficients E and C in the reference ATPS were examined in order to explore if coefficients E and C in Eq. (2) or a combination of the two may characterize the properties of the ATPS which influence the partitioning of proteins. The typical data observed when PES-PEG system is the reference system are presented in Fig. 2.

All of the coefficients a_{io} listed in Table 3 are correlated to the ratios of the coefficients E_i/E_o and C_i/C_o (coefficients E_i and C_i are listed in Table 1, and the subscript o denotes the particular reference system) according to the following equation:

$$\frac{1}{a_{io}} = \alpha_o + \beta_o^* \frac{E_i}{E_o} + \gamma_o^* \frac{C_i}{C_o} \quad (3)$$

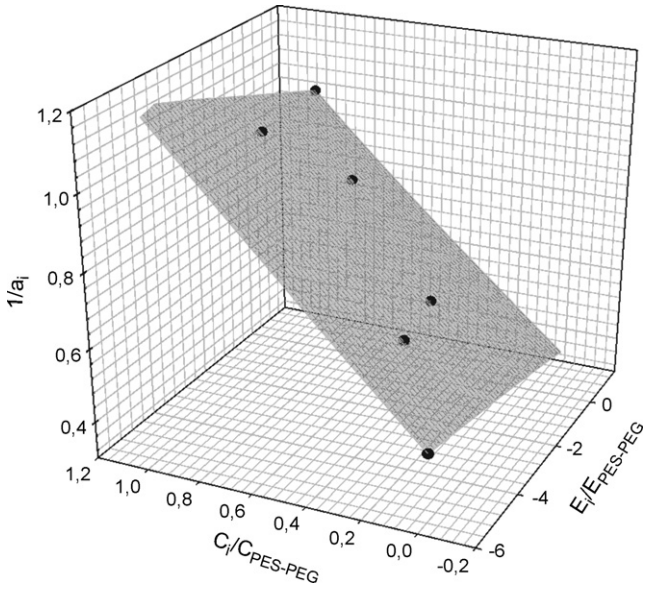

Fig. 2. 3-D plot of coefficient a_{io} in the solvent regression equation (Eq. (1)) as a function of the ratios of coefficients E_i/E_o and C_i/C_o in Eq. (2). System PES-PEG was used as the reference system (with coefficients C_o and E_o) and E_i and C_i are coefficients in Eq. (2) for the systems Dex-PEG, Dex-Ucon, PES-PEG, PES-Ucon, and Ficoll-Ucon (see text for explanation.).

where α_o, β_o, and γ_o are coefficients dependent upon the ATPS used as a reference. The values of these coefficients are presented in Table 4 together with the corresponding correlation coefficients.

It is important to highlight that the slope of the solvent regression equation (Eq. (1)) for various proteins is correlated with coefficients E_i and C_i which were determined from separate experiments with the homologous series of DNP-amino acids [21,22] and used here to characterize the differences between the solvent properties of the aqueous media in the coexisting phases of ATPSs. Coefficient a_{io} in Eq. (1) may be simplified:

$$a_{io} = \frac{E_o C_o}{\alpha_o E_o C_o + \beta_o E_i C_o + \gamma_o E_o C_i} \quad (4)$$

where all of the parameters are as defined above. This result strongly suggests that both E and C can be used as solvent descriptors in polymer/polymer ATPSs.

It was suggested earlier that coefficients a_{io} and b_{io} in the solvent regression equation may be represented as [27]:

$$a_{io} = \frac{E_i}{E_o} \quad \text{and} \quad b_{io} = E_i \left(\frac{C_i}{E_i} - \frac{C_o}{E_o} \right) \quad (5)$$

Using the solvent regression equation to compare the distribution coefficients of proteins and other solutes in ATPSs formed by pairs of polymers of the same chemical nature but different molecular weights ([4], pp. 268–276) confirmed this suggestion.

It follows from the data obtained for proteins in this study as well as from previously reported data [21] for homooligopeptides and monosaccharides that Eq. (5) does not hold for the comparison of ATPSs formed by pairs of polymers of different chemical nature.

Table 4
Coefficients α, β, and γ in Eq. (3) with different ATPSs used as a reference system (r^2 – correlation coefficient; N = 6 – number of ATPSs used in analysis)

Reference ATPS	α_o	β_o	γ_o	r^2	F^*	SD*
Dex-PEG	0.68 ± 0.08	0.05 ± 0.03	0.30 ± 0.03	0.9903	154	0.04
Dex-Ucon	0.91 ± 0.09	0.1 ± 0.1	−0.017 ± 0.001	0.9932	210	0.05
PES-PEG	0.36 ± 0.02	−0.028 ± 0.004	0.68 ± 0.03	0.9980	747	0.01
PES-Ucon	0.42 ± 0.05	0.04 ± 0.02	0.54 ± 0.05	0.9911	167	0.03
Ficoll-PEG	0.48 ± 0.04	0.013 ± 0.005	0.52 ± 0.03	0.9963	403	0.02
Ficoll-Ucon	0.67 ± 0.07	0.07 ± 0.05	0.24 ± 0.02	0.9933	222	0.04

* F – ratio of variance and SD – standard deviation.

This fact may be explained qualitatively as the result of different effects of phase-forming polymers on the solvent features of the aqueous media in the coexisting phases of ATPSs. Separation of the various types of protein-water molecular interactions into just two classes of interactions involving nonpolar and polar groups implied by Eq. (5) is obviously an oversimplification. Molecular interactions of water with DNP-amino acids used for the characterization of the solvent features of coexisting aqueous phases in a given ATPS are unlikely to completely represent the much more complex variety of molecular interactions involved in protein-water interactions. This likely explains the fact that no reliable correlation between the coefficient b_{io} values and coefficients C and E could be found. In order to explore the relative roles of different protein-water molecular interactions in protein partitioning in ATPSs an additional study is necessary and currently in progress.

It should also be mentioned that 3 out of 10 ATPSs, Dex-Ficoll, Dex-PES, and Ficoll-PES displayed distribution behavior of proteins different from that observed in this study. Protein partitioning in these three systems and the solvent features of the phases in these systems as compared to those of the ATPSs examined here are the subject of our current studies.

4. Conclusions

Distribution coefficients of randomly selected proteins were measured in ATPSs formed by different paired combinations of Dextran-75 (Dex), Ficoll-70, polyethylene glycol-8000 (PEG), hydroxypropyl starch-100 (PES), and Ucon50HB5100 at specific polymer concentrations, all containing 0.15 M NaCl in 0.01 M phosphate buffer, pH 7.4. Most of the proteins in the PEG-Ucon system precipitated at the interface. In PES-PEG, PES-Ucon, Ficoll-PEG, Ficoll-Ucon, Dex-PEG and Dex-Ucon ATPSs the distribution coefficients for the proteins were correlated according to the solvent regression equation: $\ln K_i = a_{io} \ln K_o + b_{io}$, where K_i and K_o are distribution coefficients for any protein in the ith and oth two-phase systems and coefficients a_{io} and b_{io} are constants, the values of which depend upon the particular composition of the systems under comparison. These results suggest that the protein-solvent interactions in these systems are similar.

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