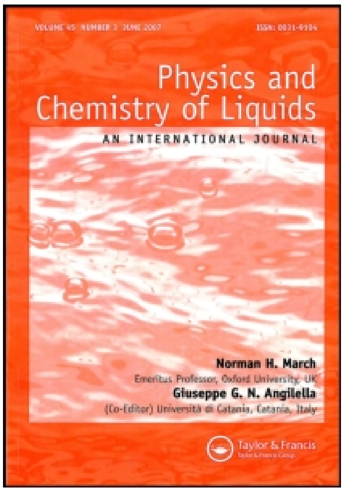

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PHASE SEPARATION OF POLYETHYLENE GLYCOL/SALT AQUEOUS TWO-PHASE SYSTEMS

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Salt in polyethylene glycol (PEG)/salt aqueous two-phase systems was excluded by PEG and concentrated in the solvent volume available for dissolution of salt (PEG-free solvent). The concentration of salt in the PEG-free solvent of the PEG-rich phase was the same as that at the critical point regardless of the compositions of the PEG/salt two-phase systems. This explained that the phase separation of PEG/salt two-phase systems occurs when the concentration of salt in the PEG-free solvent reaches its solubility limit. The concentration of salt required in the PEG-free solvent for the phase separation was lower with higher molecular weight of PEG. The solubility of salt in the PEG-free solvent decreased with increases in the molal surface tension increment of salt. The solubility limit of salt in the PEG-free solvent was 0.93 M for ammonium sulfate, 0.77 M for potassium phosphate, 0.75 M for sodium tartrate, 0.67 M for sodium phosphate, and 0.53 M for potassium citrate.

Keywords: Aqueous two-phase system; phase separation; excluded volume; PEG; solubility of salt

1. INTRODUCTION

Liquid-liquid extraction of biological products using aqueous two-phase systems provides significant advantages over conventional separation and overcomes the limitations of conventional methods

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currently used in bioprocesses with its simplicity and rapidity involving only mild conditions [1–3]. Polyethylene glycol (PEG)/dextran aqueous two-phase systems have received the most attention. Recently, more interest, however, has been shown in PEG/salt systems due to their lower viscosity and cost [4–7].

PEG/salt aqueous two-phase systems can be prepared by mixing PEG and a salt in water exceeding certain threshold concentrations [8, 9]. The resultant top phase is rich in PEG and the bottom phase is rich in salt. The mechanism of two-phase formation (i.e., phase separation), however, is not studied well yet.

The exclusion theory of proteins by PEG has been well established by studies of numerous laboratories [10–12]. Our earlier studies also demonstrated that PEG excludes salts as well as proteins [13]. In this study the phase separation of PEG/salt aqueous two-phase systems will be explained by applying the exclusion theory.

2. MATERIALS AND METHODS

Stock Solutions for Two-Phase Systems

Polyethylene glycol (PEG) solutions with various molecular weights, 300 (PEG300), 600 (PEG600), 1,450 (PEG1450), 3,350 (PEG3350), and 8,000 (PEG8000) (Sigma, Chem. Co., St. Louis, MO) were prepared in deionized water. The concentrations of the stock solutions for PEG1450, PEG3350, and PEG8000 were 50% (w/v). PEG300 (3.3 M) and PEG600 (1.7 M) are liquid at room temperature, so they were used directly without preparing stock solutions (100%, w/v). All PEG stock solutions were kept at room temperature.

Salt solutions of ammonium sulfate (4.0 M, $(NH_4)_2SO_4$), magnesium sulfate (2.3 M, $MgSO_4$), sodium sulfate (2.3 M, Na_2SO_4), sodium citrate (1.5 M, $Na_2C_6H_5O_7$), and sodium tartrate (2.0 M, $Na_2C_4H_4O_6$) (Mallinckrodt Inc., Paris, KY) were prepared in deionized water. The salt stock solutions were buffered with 10 mM potassium phosphate buffer and adjusted to pH 7.2. Potassium phosphate and sodium phosphate stock solutions were prepared by adding equal moles (1.75 M) of monobasic and dibasic in deionized water.

Phase Diagram and Critical Point

Phase diagrams of PEG/salt two-phase systems were constructed by titration as described in the procedure of Albertsson [8]. The tie lines were determined by the volume ratio of the top to the bottom phases. The critical point was determined by connecting the middle points of the tile lines.

Molar Excluded Volume of PEG and PEG-Free Solvent

The excluded volume of PEG, V_e, can be expressed as [10]:

$$V_e = 1 - \exp\left[-\alpha_s m_p\right] \qquad (1)$$

where m_p is the molar concentration of PEG and α_s is the molar excluded volume of PEG for salt.

The solvent volume available for dissolution of salt (PEG-free solvent), V_a, is:

$$V_a = \exp\left[-\alpha_s m_p\right] \qquad (2)$$

By assuming that the solubility of a salt, m_s, is proportional to the available volume,

$$m_s = m_{sf} \exp\left[\alpha_s m_p\right] \qquad (3)$$

where m_{sf} is the solubility of a salt in the PEG-free solvent.

The logarithm of the solubility of salt in the PEG-rich phase ($\ln m_s$) was plotted against the concentration of the PEG-rich phase (m_p). The molar excluded volume of PEG for salt (α_s) was determined from the slope and the solubility of a salt in the PEG-free solvent (m_{sf}) was determined from the intercept.

Molar Hydrated Volume of PEG

The molar hydrated volume of PEG, V_h, was determined from [14]:

$$V_h = M(v_2 + \delta_1 v_1) \qquad (4)$$

where M is the molecular weight of PEG, v_1 is the specific volume of water and v_2 is the specific volume of PEG, δ_1 is hydration of PEG (g H_2O/g PEG).

The hydration of PEG was determined with a Differential Scanning Calorimeter by measuring non-freezable bound water. Various concentrations of PEG solutions (20 to 80%, w/v) were weighed in aluminium pans (8 to 15 mg). The aluminium pans were sealed tightly and weighed. Sample pans were cooled to −40°C and heated to 20°C at a rate of 10°C/minute at instrument sensitivity of 20 mcal/sec with a Differential Scanning Calorimeter equipped with an Intercooler (Perkin-Elmer DSC-2, Perkin-Elmer, Norwalk, CT). An empty pan was used to balance the heat capacity of the sample. The calibration of the instrument was carried out using indium and deionized water as standards.

Free water was calculated by measuring the enthalpies of melting (peak areas of free water). The content of free water was plotted against the content of total water, and non-freezable bound water was determined from the intercept of the plot.

3. RESULTS AND DISCUSSION

Molecular Weight of PEG

Phase diagrams of PEG/potassium phosphate aqueous two-phase systems were constructed. For higher molecular weights of PEG, lower concentrations of PEG and potassium phosphate were required to form two-phase systems. The critical point occurred at lower concentrations of both PEG and potassium phosphate and the distance of the critical point to the origin was shorter (Tab. I). Phase separation could be expressed in terms of the solubility of salt in PEG

TABLE I Critical points of PEG/potassium phosphate aqueous two-phase systems

Molecular Weight of PEG	*Concentration at Critical Point (M)* *PEG*	*Potassium Phosphate*	*Distance to Origin (M)*
300	0.55	1.42	1.52
600	0.23	1.08	1.10
1,450	0.07	0.85	0.86
3,350	0.03	0.67	0.67
8,000	0.01	0.54	0.54

solutions. All mixtures with salt concentrations higher than the solubility of salt in PEG solutions give rise to phase separation (above the binodial line), while mixtures which have salt concentrations lower than the solubility of salt (below the binodial line) do not.

Binodial lines on the phase diagrams constructed, therefore, were redrawn in terms of the solubility of potassium phosphate in PEG solutions. The logarithm of the solubility of potassium phosphate decreased linearly with increases in the concentration of PEG [13]. This suggests that the concentration of salt in the PEG-rich phase of PEG/salt two-phase systems (m_s) can be expressed in Eq. (3).

Equation (3) is an equation for the solubility of salt in PEG solutions described by current theories on the excluded volume effects of PEG [10–13]. Therefore, the decreases in the solubility of salt in PEG solutions could be explained with the exclusion of salt by PEG.

The slope of the solubility lines which gives the molar excluded volume of PEG for salt (α_s), increased with increases in the molecular weight of PEG. The molar excluded volume of PEG for potassium phosphate determined is given in Table II. The molar excluded volume of PEG for potassium phosphate determined was compared with the molar hydrated volume of PEG. The molar hydrated volume of PEG was determined from the hydration and specific volume of PEG. The molar excluded volume of PEG for potassium phosphate determined was about 2 times larger than the molar hydrated volume of PEG for all molecular weights of PEG tested. Therefore, potassium phosphate is sterically excluded by PEG.

The solubility of potassium phosphate in the PEG-free solvent was determined from the intercept of the solubility line. The solubility of potassium phosphate in the PEG-free solvent decreased with increases

TABLE II Molar excluded volumes of PEG for potassium phosphate and molar hydrated volumes of PEG

Molecular Weight of PEG	*Molar Excluded Volume of PEG (L/mole)*	*Molar Hydrated Volume of PEG (L/mole)*
300	1.5	0.6
600	2.6	1.3
1,450	7.3	3.1
3,350	14.5	6.4
8,000	29.0	13.8

in the molecular weight of PEG (Tab. III). The solubility of potassium phosphate in the PEG-free solvent determined from the intercept was about the same as the concentration of potassium phosphate in the PEG-free solvent at the critical point calculated with Eq. (3). The solubility of potassium phosphate in the PEG-free solvent decreased with increases in the molecular weight of PEG. Therefore, the concentration of potassium phosphate required in the PEG-free solvent for phase separation of PEG/potassium phosphate two-phase systems is lower with higher molecular weight of PEG. This explains that phase separation of PEG/salt two-phase systems occurs at lower concentrations of salt with higher molecular weights of PEG.

Type of Salt

Phase diagrams of PEG8000/salt two-phase systems for ammonium sulfate, sodium phosphate, sodium tartrate, and potassium citrate were constructed. The critical points of two-phase systems determined are given in Table IV. The critical concentration of PEG was the same for all PEG/salt two-phase systems tested. Therefore, the critical concentration of PEG is independent of the type of salt. The critical concentration of salt, however, was different with different types of salt. Therefore, the critical concentration of salt is dependent on the type of salt. The larger molal surface tension increment of the salt, the lower critical concentration of the salt (Tab. IV). In addition, a salt with the low surface tension increment (sodium chloride) did not form two-phase systems with PEG. Therefore, interfacial tension between PEG molecules and the PEG-free solvent might be one of the important parameters determining phase separation.

TABLE III Solubilities of potassium phosphate in the PEG-free solvent

Molecular Weight of PEG	*Solubility of Potassium Phosphate in PEG-Free Solvent (M)*	
	from Intercept	*at Critical Point*
300	3.3	3.2
600	1.8	2.0
1,450	1.4	1.6
3,350	1.0	1.1
8,000	0.7	0.8

TABLE IV Critical points of PEG8000/salt two-phase systems

Salt	*Concentration at Critical Point (M)*		*Molal Surface*[a] TENSION *Increment of Salt*
	PEG	*Salt*	*(dyne kg/cm mole)*
Sodium Chloride	–[b]	–[b]	1.64
Magnesium Sulfate	0.013	1.04	2.10
Ammonium Sulfate	0.013	0.67	2.16
Potassium Phosphate	0.013	0.54	–
Sodium Tartrate	0.013	0.53	2.35
Sodium Phosphate	0.013	0.46	2.66
Potassium Citrate	0.013	0.40	3.12

[a] Melander and Horvath [15].
[b] did not form two-phase systems.

When binodial lines of PEG/salt two-phase systems were expressed again in terms of the solubility of salt in PEG8000 solutions, the logarithm of the solubility of all salts tested again decreased linearly with increases in the concentration of PEG8000. Therefore, all salts tested were excluded by PEG. The molar excluded volume of PEG8000 for salt which was determined from the slope of the plot was about the same for all salts (Tab. V). This suggests that the excluded volume effects of PEG for salts tested is independent of the type of salt. This agrees with the critical concentration of PEG. The concentration of PEG at the critical point of PEG8000/salt two-phase systems was independent of the type of salt (Tab. IV). The solubilities of salt in the PEG-free solvent determined from the intercept of the solubility lines were again the same as the concentrations of salt in the PEG-free solvent at the critical points, which were calculated with Eq. (3) (Tab. V).

TABLE V Molar excluded volume of PEG8000 for salt

Salt	*Molar Excluded Volume of PEG8000*	*Solubility of Salt in PEG-Free Solvent (M)*	
	(L/mole)	*from intercept*	*at Critical Point*[a]
Ammonium Sulphate	25	0.93	0.93
Sodium Phosphate	25	0.67	0.67
Potassium Phosphate	29	0.79	0.77
Sodium Tartrate	28	0.76	0.75
Potassium Citrate	23	0.52	0.52

[a] Calculated with Eq. (3).

Phase Separation

As discussed in previous sections salt is excluded by PEG; consequently this increases the concentration of salt in the PEG-free solvent. When the concentration of salt in the PEG-free solvent reaches its solubility limit in the PEG-free solvent, phase separation occurs. If it is true, the concentration of salt in the PEG-free solvent of the PEG-rich phase should be the same regardless of the concentration of PEG in the PEG-rich phase.

Table VI shows the concentration of ammonium sulfate in the PEG-free solvent of the PEG-rich top phase of PEG8000/ammonium sulfate two-phase systems, which was calculated with Eq. (3). The concentration of ammonium sulfate in the PEG-free solvent of the PEG-rich phase was the same as that at the critical point regardless of the compositions of the PEG/ammonium sulfate two-phase systems. This was true for all other salts tested (Tab. VII). Therefore, phase separation of PEG/salt two-phase system occurs when the concentration of salt in the PEG-free solvent reaches its solubility limit by PEG exclusion. The concentrations of potassium phosphate in the PEG-free solvent of the PEG-rich phase were compared with those in the salt-

TABLE VI Concentrations of ammonium sulfate in the PEG-free solvent of the PEG-rich top phase of PEG8000/ammonium sulfate two-phase systems

*Concentration of PEG*8000	*Concentration of Ammonium Sulfate (M)*	
in PEG-Rich Phase (M)	*in PEG-Rich Phase*	*in PEG-Free Solvent*
0.041	0.33	0.94
0.035	0.38	0.93
0.031	0.42	0.92
0.028	0.45	0.92
0.025	0.49	0.92
0.023	0.52	0.93
0.021	0.54	0.92
0.019	0.57	0.92
0.018	0.59	0.93
0.017	0.61	0.93
0.016	0.63	0.93
0.015	0.64	0.93
0.014	0.66	0.93
0.013 [a]	0.67 [b]	0.93 [c]

[a] Concentration of PEG at the critical point.

[b] Concentration of ammonium sulfate at the critical point.

[c] Solubility of ammonium sulfate in the PEG-free solvent at the critical point.

TABLE VII Solubility of salt in the PEG-free solvent of the PEG-rich top phase of PEG8000/salt two-phase systems

Salt	*Solubility of Salt in PEG-Free Solvent (M)*	
	PEG-Rich Top Phase	*Critical Point*
Ammonium Sulfate	0.93	0.93
Potassium Phosphate	0.77	0.79
Sodium Tartrate	0.75	0.77
Sodium Phosphate	0.67	0.64
Potassium Citrate	0.53	0.54

rich phase of various compositions of PEG/potassium phosphate two-phase systems (Tab. VIII). The concentration of salt in the PEG-free solvent of the PEG-rich phase was always lower than that in the salt-rich phase.

The solubility of salt in the PEG-free solvent might be interdependent with the surface tension. The surface tension of salt solutions increases with increases in the concentration of salt [15]. Salt

TABLE VIII Concentrations of potassium phosphate in the PEG-free solvent of the PEG-rich phase and in the salt-rich phase of PEG8000/potassium phosphate two-phase systems

Concentration of PEG8000	*Concentration of Potassium Phosphate (M)*	
in Two-Phase System (M)	*PEG-Free Solvent of PEG-Rich Phase*	*Potassium Phosphate-Rich Phase*
PEG8000/Potassium Phosphate (0.75 M)		
0.006	0.76	0.81
0.013	0.80	0.98
0.019	0.77	1.18
0.025	0.75	1.44
0.031	0.75	1.73
PEG8000/Potassium Phosphate (1.00 M)		
0.006	0.78	1.13
0.013	0.80	1.31
0.019	0.74	1.55
0.025	0.79	1.79
0.031	0.77	2.08
PEG8000/Potassium Phosphate (1.25 M)		
0.006	0.75	1.42
0.012	0.76	1.70
0.019	0.79	1.89
0.025	0.76	2.13
0.031	0.76	2.39

is excluded by PEG and concentrated in the PEG-free solvent. Therefore, the surface tension of PEG-free solvent increases with exclusion of salt by PEG. When the surface tension of the PEG-free solvent reaches to a certain level, phase separation initiates. This agrees with the results shown in Tables IV and V. The solubility of salt in the PEG-free solvent decreases with increases in the molal surface tension increment of salt.

4. CONCLUSIONS

Salt in PEG/salt aqueous two-phase systems was excluded by PEG and concentrated in the PEG-free solvent. The concentration of salt in the PEG-free solvent of the PEG-rich phase was the same as that at the critical point regardless of the composition of the PEG/salt two-phase systems. Therefore, the phase separation of PEG/salt two-phase systems occurred when the concentration of salt in the PEG-free solvent reached its solubility limit.

The solubility limit of salt in the PEG-free solvent decreased with increases in the molecular weight of PEG. The concentration of salt required in the PEG-free solvent for the phase separation was lower with higher molecular weight of PEG. This explained that the phase separation of PEG/salt two-phase systems occurs at lower concentrations of salt with higher molecular weights of PEG.

The solubility of salt in the PEG-free solvent decreased with increases in the molal surface tension increment of salt. Therefore, the concentration of salt required for the phase separation of PEG/salt two-phase systems was different with different types of salt. The solubility limit of salt in the PEG-free solvent was 0.93 M for ammonium sulfate, 0.77 M for potassium phosphate, 0.75 M for sodium tartrate, 0.67 M for sodium phosphate, and 0.53 M for potassium citrate.

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