Korean J. of Chem. Eng., 9(4), 219-224 (1992)

EXPERIMENTAL STUDIES ON PHASE EQUILIBRIA OF PEG-WATER-DEXTRAN/AMMONIUM SULFATE SYSTEMS AND PARTITIONING OF ALBUMIN INTO TWO WATER-CONTINUOUS PHASES

Bong Kyu Kim, Yong-Byung Ban*** and Jong-Duk Kim***

Department of Chemical Engineering and Bioprocess ERC,
Korea Advanced Institute of Science and Technology, 373-1 Kusong-dong, Yusong-ku, Taejon 305-701
(Received 22 July 1992 • accepted 18 November 1992)

Abstract—The clouding points and equilibrium concentrations of PEG/dextran/water and PEG /ammonium sulfate/water systems were experimentally determined for different molecular weights of PEG by titration method and direct determination of concentrations. In phase diagrams of PEG-water with ammonium sulfate or dextran, the addition of salt or dextran induced the phase separation of PEG-rich phases near the PEG-lean phases having the different partitioning of PEG. The concentrations of PEG in PEG-rich phases increase as the amounts of PEG or salt and dextran increase, while the concentrations of PEG in aqueous media decrease in any cases. The higher the molecular weight of PEG has, the wider the two-phase regions are. In dextran DT10 systems, the partition coefficients of egg albumin into PEG-lean phases increase with concentrations of dextran and the molecular weight of PEG. In ammonium sulfate systems, the partitioning coefficients showed a maximum, having lower partitioning at the very high and low concentration of salts. It is also observed that as the amounts of albumin increase, the partitioning of albumin into PEG-lean phase increases.

INTRODUCTION

The partitioning of soluble substances such as proteins and nucleic acids in aqueous two-phase mixtures has been subjected to intensive research in efficient and large-scale separation processes of biochemical mixtures [1-4]. The complex particles obtained by disintegration of cells or cell organelles, in general, consist of very complicated mixtures differing in size, form and chemical compositions, and therefore it is commonly adopted to harvest the pure chemicals, combining different separation methods of centrifugation, chromatography, and solution methods with large-scale process [1]. In liquid-liquid two-phase separation, two water-soluble but incompatible polymers, such as dextran and polyethylene glycol (PEG) [1-3], are added to water, forming two aqueous phases. Thus far, many polymer-polymer-water or polymer-small molecules (low molecular weight components, in most cases, salts)-water systems, including multiphase systems with more than three phases have been investigated [1].

In two-phase separations, the distribution of substances plays the key role in successful applications together with the interfacial tension [5], the densities and viscosities of bulk phases [1]. Among others, the most important factor in the separation processes is the selective distribution between phases. This is different along with the concentrations of polymers, the molecular weight, and the chemical species, usually known in terms of partition coefficients $K(=C_b/C_t)$ [1-4], the ratio of the concentrations of protein in bottom phase (C_b) to those in the top phase (C_t) (in moles per liter). Experimentally, the phase diagrams, i.e., number of phases, and their compositions have been investigated by many investigators [1, 3, 6-10]. In theories, there have been some efforts devoted to the direct explanation of K by ionic interaction [11-16, 18, 19], but rarely discussed from the solution thermodynamics of the phase equilibria [17, 23-25].

In this paper, we report the phase diagrams of PEG-dextran-water systems and PEG-ammonium sulfate-water systems, and partitioning of dextran and PEG.

*To whom all correspondence should be addressed.
**Samsung General Chemicals Co. Ltd., Seoul Korea
***National Institute of Standards and Technology(NIST), Gaithersburg, MD 20899, USA

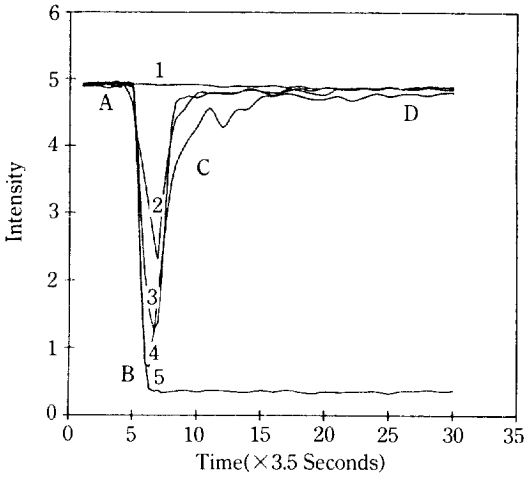

Fig. 1. Transmission intensities of 25% dextran solution with addition of the 20% PEG solution by 0.4 cc interval. The numbers are the sequential addition numbers. A represents the initial quiescent state, B the first appearence of cloudness, C the recovering state and D the final adjustment.

In addition, the partitioning of egg albumin was investigated in the aqueous two-phase separation.

EXPERIMENTS

1. Chemicals

Dextran (T10, M.W. = 105,000) purchased from Pharmacia and PEG (M.W., 3,400, 8,000) from Aldrich Co. Ltd., were used without further purifications. Egg albumin (Sigma Chemical Co.), ammonium sulfate (Kanto Chemical Co., 99%), succinic acid (Sigma Chemical Co.) and NaOH in buffer were used as purchases. Water was doubly distilled in our laboratory.

2. Determinations of Cloud Points

The cloud points were determined by the transmitted intensities of 632.8 nm He-Ne Laser, titrating the polymer stock solutions with polymeric or salt solutions. In Fig. 1, for an example, the intensities of transmitting light were plotted as the 20% PEG solution were added dropwise to the 25% dextran solution by 0.4 cc. The addition of drops continued until the turbidity of the solution remained unchanged after the solution turned turbid. The phase boundary was determined at the points where the solution became turbid. This method resulted in somewhat narrow separation regions, and therfore often adopted in primary studies.

3. Determinations of Equilibrium Concentrations

For PEG-dextran-water systems, the equilibrium concentration of dextran was directly measured by polarimeter (Rudolph Research) and that of PEG by refractometry (Bausch & Lomb). In PEG-ammonium sulfate-water system, the equilibrium concentration of salt was determined by Kjeldahl method. When the mixtures incubated at room temperature were separated into two phases, the upper phase rich in PEG were separated, and to determine the water content, dried until no weight changes were observed. For the salt concentrations in the lower phase, the 0.5 N NaOH was added in excess into the bottom phase and heated up to remove ammonia by

$$(NH_4)_2SO_4 + 2NaOH = Na_2SO_4 + 2H_2O + 2NH_3 \quad (4)$$

The excess NaOH in this reaction were titrated with 0.1 N HCl in the presence of phenol red. Then, the equilibrium concentrations of both phases or tie lines were evaluated.

4. Determination of Protein Partitioning

Since the isoelectric point of egg albumin is known to be 4.7 [26], the partition of egg albumin were determined at pH 4.7 maintained by a succinic acid and NaOH buffer. The bulk samples were prepared at a certain concentrations of polymer and protein, and incubated about 40 hours at 4℃, separating into two phases. The solutions of polymers and protein were eluted in a 1 cm ID gel permeation column, packed with sephadex G-75 gel. The gel was pretreated by swelling at 90℃ for 3 hrs and degassed for 9 hrs. The packing height was 30 cm. The elution speed was 0.6 cc/min and the aliquots were automatically selected through 4 way control valve. The partition coefficients were determined by the ratio of the elution intensities in recorder.

RESULTS AND DISCUSSION

1. Cloud Points and Equilibrium Concentrations

As the titrations approach the concentrations of phase separation, the local fluctuations are much prolonged in longer time interval and the turbidities increase up to the concentrations of phase separation. Finally, the turbid mixtures could not recover their original brightness, indicating the phase separation in microscale. In the course of titration, the highest concentration before the phase separation is assigned to be the cloud points in our measurements. In Fig. 2, the ternary diagrams of PEG-ammonium sulfate-water systems were given for PEG molecular weights of 3,400. The circles represent the phase boundaries determined by the cloud point measurements. The

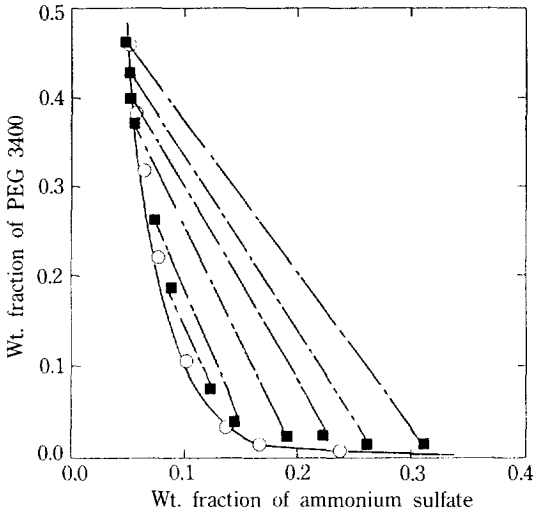

Fig. 2. Phase diagram of PEG 3400-ammonium sulfate-water systems at 25°C. The open circles (○) for the phase separation boundaries determined by cloud point measurements, and the filled squares (■) by equilibrium measurements.

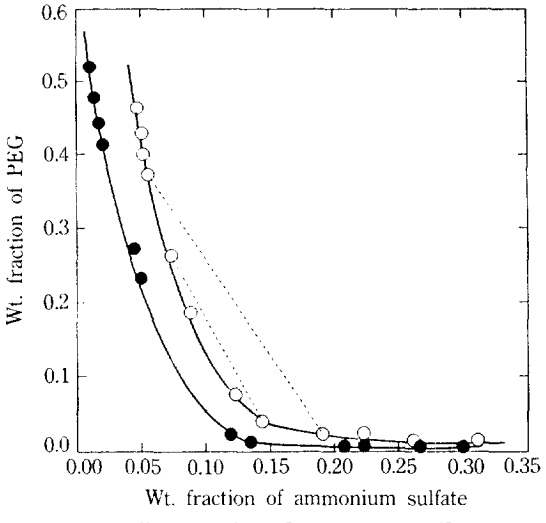

Fig. 3. Phase diagram of PEG-ammonium sulfate-water systems at 25°C. The open circles (○) represents for PEG 3400 and the filled circles (●) for PEG 8000.

squares and dotted lines stand for the equilibrium concentration measurements. In figures, the titration provides almost identical phase boundaries with equilibrium measurements although it gives no tie line data. The identical results were observed for the PEG-dextran-water system [26]. Therefore, it is concluded

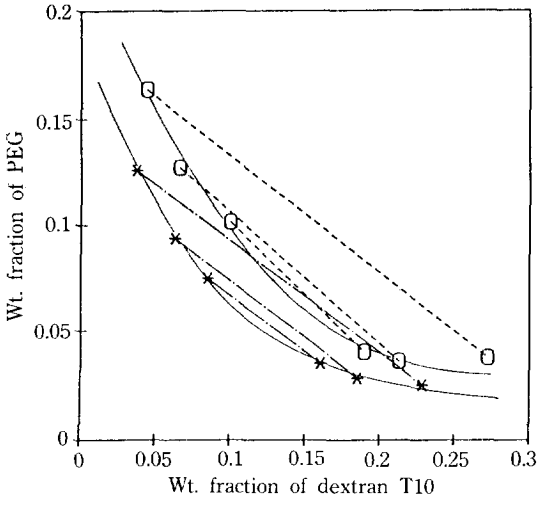

Fig. 4. Phase diagram of PEG-dextran T10-water systems at 25°C. The open circles (○) represent for PEG 3400 and the stars (*) for PEG 8000.

that the equilibrium measurements in both dextran and salts are reliable.

Further, the mixtures with dextran are separated into the PEG-rich top phase and dextran-rich bottom phase within 2-60 minutes, while, for the mixtures of salts it takes 2-15 minutes, and easily separated even at high water contents of 75 wt%.

2. Effect of Molecular Weights of PEG

The equilibrium concentrations of the ternary mixtures of PEG-dextran-water and PEG-ammonium sulfate-water systems were determined from the incubated mixtures. Figs. 3 and 4 (also Tables 1 and 2) show the phase separation regions of the ternary systems of different molecular weights of PEG, 3,400 and 8,000. Near the water-rich phase, two-phase separation regions broaden as the molecular weight increases for both salt and dextran, consistent with Albertson's [1]. As the molecular weight of PEG increases, the solubilities of PEG in dextran or ammonium sulfate solution (bottom phase), and those of dextran or ammonium sulfate in PEG solutions (top phase) decrease. Therefore, the molecular weight of PEG affects the separation of the water-rich and also the concentration of water-rich phases. Further, it is very important to observe the decrease of the amounts of water in bottom phases.

3. Effect of Salts and Dextran

In Fig. 4, the effect of ammonium sulfate in PEG-ammonium sulfate-water system was shown. As the amount of salt in the bottom phase increases, water was extracted from the PEG-rich phase. In results,

Table 1. Equilibrium concentrations of PEG-ammonium sulfate-water systems at 25°C

PEG 3400 (weight fraction)

top phase			bottom phase		
Salt	PEG	Water	Salt	PEG	Water
0.0475	0.4623	0.4902	0.3114	0.0156	0.6730
0.0510	0.4280	0.5210	0.2616	0.0150	0.7234
0.0518	0.3993	0.5489	0.2230	0.0248	0.7522
0.0558	0.3715	0.5727	0.1909	0.0237	0.7854
0.0735	0.2627	0.6638	0.1440	0.0399	0.8161
0.0884	0.1864	0.7252	0.1229	0.0754	0.8017

PEG 8000 (weight fraction)

top phase			bottom phase		
Salt	PEG	Water	Salt	PEG	Water
0.0110	0.5200	0.4690	0.3000	0.0068	0.6932
0.0143	0.4770	0.5087	0.2666	0.0070	0.7264
0.0175	0.4420	0.5405	0.2235	0.0079	0.7686
0.0205	0.4127	0.5668	0.2079	0.0078	0.7843
0.0446	0.2721	0.6833	0.1350	0.0133	0.8517
0.0500	0.2320	0.7180	0.1195	0.0227	0.8578

Table 2. Equilibrium concentrations of PEG-dextran T10-water systems at 25°C

PEG 3400 (weight fraction)

top phase			bottom phase		
dextran	PEG	Water	dextran	PEG	Water
0.0433	0.1672	0.7895	0.2756	0.0309	0.6935
0.0687	0.01292	0.8021	0.2123	0.0385	0.7492
0.0984	0.01037	0.7979	0.1921	0.0423	0.7656

PEG 8000 (weight fraction)

top phase			bottom phase		
dextran	PEG	Water	dextran	PEG	Water
0.0384	0.1273	0.8343	0.2302	0.0257	0.7441
0.0641	0.0947	0.8412	0.1862	0.0286	0.7852
0.0861	0.0747	0.8392	0.163	0.0332	0.8038

the PEG-rich phase would be more concentrated in PEG and the mutual solubility of PEG and water may be reduced by the existence of salt, and therefore, the concentrations of PEG in the water-rich phase were significantly reduced. In dextran, the tendency of phase separation by PEG was observed identical as the salt did. However, a strong discrepancy in the degree of phase separation was observed. In Fig. 5, the phase separation of PEG-8,000-dextran T10-water and PEG-8,000-ammonium sulfate-water systems were given with a tie line crossing each other. If a sample was taken at the point of crossing the tie lines, the

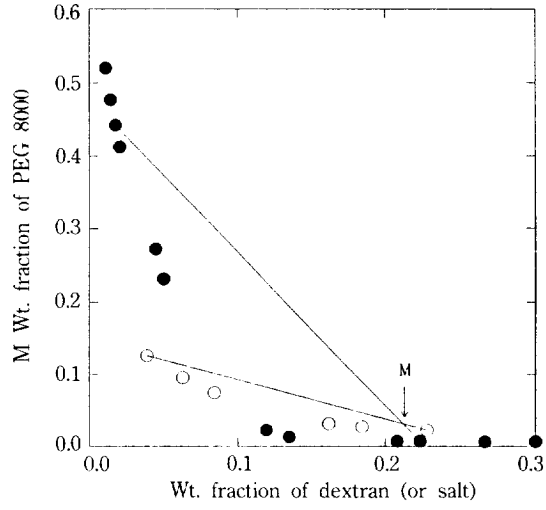

Fig. 5. Phase separations of dextran T10/ammonium sulfate-PEG 8000-water systems. A cross point of two tie lines M can be separated with different fashion along with the tie lines. The open circles (○) represents the dextran T10 system and the filled circles (●) for the ammonium sulfate system.

two systems could be separated as shown. In dextran systems, the relative partitioning of PEG into PEG-rich phase is relatively small, while for salt most of PEG resides in PEG-rich phase.

The length of tie line(L) defined by the distance between the concentrations in phase are important in separating the ingradients. For ternary systems, the length can be given by,

$$L=[\sum_{i,j=1}^{3}(X_i-X_j)^2]^{1/2}$$

where X_i is the mole fraction of i species. As the phase separation approaches the critical point, the length of tie line becomes zero because the X_i and X_j become identical. Following the scaling law along the critical isochore, the phase envelope can be described by the exponent of the shape of coexistence β. Near the critical points, the ratio of activity of i species at bottom phase to that of the critical point is almost one and then linearized to give a slope. In Fig. 6, it is interesting that all have apparently identical slopes.

3. Partitioning of Albumin

The amount of albumin in each phase was determined by gel permeation chromatography and the partition coefficients K was determined by the area ratio of eluent peaks between two phases. Table 3 summarized the partitioning of albumin into the dextran-rich

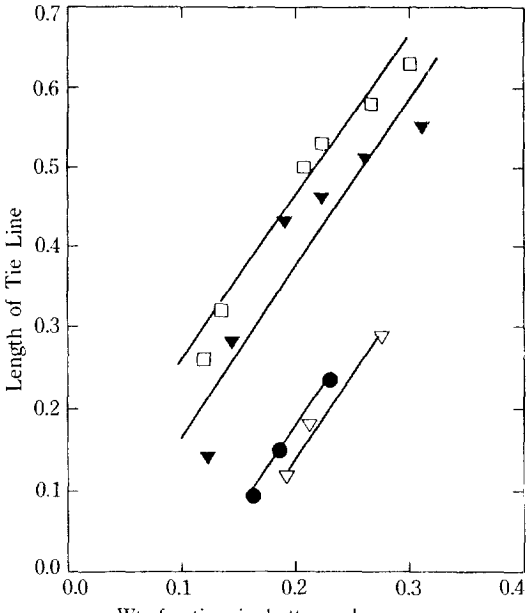

Fig. 6. The length of tie lines near the critical point. Dextran and ammonium sulfate systems have the similar slopes.

(▽) PEG 3400-dextran T10-water
(●) PEG 8000-dextran T10-water
(▼) PEG 3400-ammonium sulfate-water
(□) PEG 8000-ammonium sulfate-water

Table 3. Partition coefficients of egg albumin in PEG-dextran-water systems at 4°C

PEG/Dextran T10 (wt%)	Albumin mg/g	Conc. of EA in PEG -rich phase, mg/ml	Partition coefficient
(PEG 3400)			
7/9	2	1.12	6.75
7/10	2	0.635	8.33
	5	1.94	8.33
	8	2.56	9.0
7/11	2	0.57	10.0
7/12	2	0.36	11.1
8/11	2	0.256	17.5
	5	0.952	19.2
	8	1.69	20.0
(PEG 8000)			
10/11	2	1.6	4.17
10/12	2	1.5	5.81
10/13	2	1.22	7.69
10/14	2	1.12	7.69
10/15	2	0.92	9.09
9/12	2	1.53	5.0

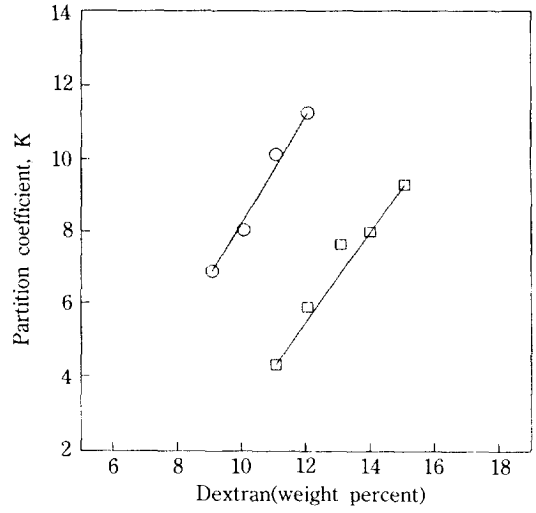

Fig. 7. Partition coefficients of egg albumin in dextran (M.W. 10,500)-PEG-water mixtures at 4°C and pH=4.7. The open circles (○) represent for PEG 8000 and the open squares (□) for PEG 3400.

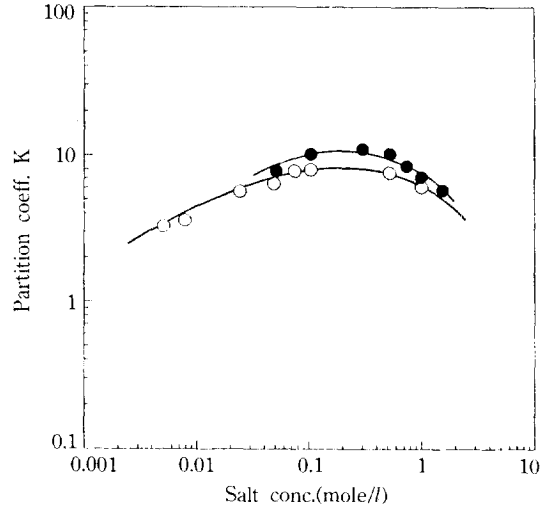

Fig. 8. The effect of salts on the partition coefficients of egg albumin. The filled circles (●) represent the partitioning of egg albumin in 7% PEG 8000-9% dextran T10 with ammonium sulfate and the open circles (○) for human serum albumin with KCl [1].

phase in the PEG-dextran-water systems. Fig. 7 shows the partition coefficients of egg albumin in PEG-8,000 (circles) and in PEG-3,400 (squares). The partitioning of albumin increases as the concentrations of dextran increase. Also the molecular weight of dextran has

the same effects as the molecular weight of PEG does [1]. As the molecular weight increases, the top phase becomes the more hydrophobic, and the hydrophilic proteins likely partitions into the bottom phase. In Fig. 8, the partition coefficients of egg albumin were given with the human serum albumin [2]. As the concentrations of salts increase, the partitioning coefficients increase at the low salt concentrations, but decrease at the high salt concentration. At the low concentration of salt, the salt may bind to the protein working as a buffer, but at the high concentration of salt, the most salt itself partitions into bottom phases and salting-out the protein and PEG.

In general, the distribution coefficients depends on the amounts of chemicals and their environments, such as the water content, ionic compositions, osmotic pressure, and denaturating effects. For the partition coefficients, the existence of extrema can be expected as a result of the electrochemical interaction since the solubilities of proteins are highly sensitive to the pH or electrolytes in the aqueous mixtures.

SUMMARY

The phase diagrams and equilibrium concentrations of PEG/dextran/water and PEG/ammonium sulfate/water systems with different molecular weights of PEG were experimentally determined by measuring the concentration of PEG, dextran and/or ammonium sulfate. In phase diagrams of PEG-water with ammonium sulfate or dextran, the addition of salt or dextran induced the phase separation with different partition of PEG, but salt and dextran were accumulated in aqueous media in any cases. In PEG/water/dextran systems, the partition coefficients of egg albumin increase with the molecular weight of PEG, and the concentrations of dextran. For salt systems, the partitioning of albumin showed the optimal conditions along with the concentrations of salts.

REFERENCES

1. Albertsson, P. A.: "Partition of Cell Particles and Macromolecules", 3rd ed., Wiley Interscience, N. Y. (1986).
2. Baskir, J. N., Hatton, T. A. and Suter, U. W.: *Biotech. Bioeng.*, **34**, 541 (1989).
3. Walter, H., Brooks, D. E. and Fisher, D.: "Partitioning in Aqueous Two Phase Systems", Academic Press, London (1985).
4. Abbot, N. L. and Hatton, A.: *Chem. Eng. Prog.*, August, 31 (1988).
5. Albertsson, P. A. and Ryden, J.: *J. Colloid. Interface Sci.*, **37**, 219 (1971).
6. Lou, J.: *Acta. Chem. Scand.*, **B37**, 241 (1983).
7. Albertsson, P. A.: *Archiv. Biochem. and Biophys. Suppl.*, **1**, 264 (1962).
8. Hustedt, H., Kroner, K. H., Stach, W. and Kula, M. R.: *Biotech. and Bioeng.*, **20**, 1989 (1978).
9. Albertsson, P. A., Sasakawa, S. and Walter, H.: *Nature*, **228**, 1329 (1970).
10. Alam, S.: Ph. D. Thesis, Illinois Institute of Technology, Chicago, Illinois (1988).
11. Bronsted, J. N.: *Z. Phys. Chem. A (Bodenstein-Festband)*, **155**, 257 (1931).
12. Bronsted, J. N. and Warming, E.: *Z. Phys. Chem. A.*, **155**, 343 (1931).
13. Gorson, D.: *Biochim. Biophys. Acta*, **602**, 269 (1980).
14. Brooks, D. E., Sharp, K. E. and Fisher, D. E.: in "Partitioning in Aqueous Two-Phase Systems", Walter, H., Brooks, D. E. and Fisher, D., eds. Academic, New York (1985).
15. King, R., Blanch, H. W. and Prausnitz, J. M.: *AIChE J.*, **34**, 1585 (1988).
16. Kim, C. W.: Ph. D. Thesis, Massachusetts Institute of Technology, Cambridge, MA (1986).
17. Kang, C. H. and Sandler, S. I.: *Fluid Phase Equilibria*, **38**, 245 (1987).
18. Haynes, C. A., Blanch, H. W. and Prausnitz, J. M.: *Fluid Phase Equilibria*, **53**, 463 (1989).
19. Haynes, C. A., Beynon, R. V., King, R. S., Blanch, H. W. and Prausnitz, J. M.: *J. Phys. Chem.*, **93**, 5612 (1989).
20. Huggins, M. L.: *J. Phys. Chem.*, **46**, 151 (1942).
21. Flory, P. J.: *J. Chem. Phys.*, **12**, 425 (1944).
22. Flory, P. J.: "Principles of Polymer Chemistry", Cornell Univ. Press (1971).
23. Kim, Y.-C. and Kim, J.-D.: *Fluid Phase Equilibria*, **41**, 229 (1988).
24. Kim, J.-D., Kim, Y.-C. and Ban, Y.-B.: *Fluid Phase Equilibria*, **53**, 323 (1989).
25. Kim, B. K.: Master Thesis, KAIST, Seoul, (1989).
26. Ban, Y.-B. and Kim, J.-D.: *Polymer Prep.*, **30**, 94 (1989).
27. Ban, Y.-B. and Kim, J.-D.: in "Computer Simulation of Polymers", ed. by Roe, R. J., Prentice Hall (1991).
28. Shultz, A. R. and Flory, P. J.: *J. Am. Chem. Soc.*, **74**, 4760 (1952).