PEG + Potassium Phosphate + Urea Aqueous Two-Phase Systems: Phase Equilibrium and Protein Partitioning

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Liquid−liquid equilibria of poly(ethylene glycol) (PEG) + potassium phosphate + urea + water aqueous two-phase systems were studied for different PEG molecular weights and pHs. Experimental techniques and analytical methods are described. Equilibrium data were obtained for PEG (1450, 3350, 10000) + potassium phosphate (pH 7 and 9) + urea (6 mass%) + water aqueous two-phase systems at 25 °C. Four tie lines were measured for each system. The partitioning behaviors of lysozyme, catalase, and β-galactosidase were studied in these systems at 25 °C, at pH 7 and 9, and at the urea concentrations 3 and 6 mass %.

Introduction

A biocompatible liquid−liquid system can arise when two polymers or a polymer and a salt are dissolved in water, with one of the components predominating in each phase.[1] An aqueous two-phase system is expected to be a promising means of separating biological materials because of the mild conditions provided by the phases and their different physical and chemical properties.[2]

Polymer−polymer aqueous two-phase systems were widely studied a number of years ago. However, electrolyte−polymer aqueous two-phase systems have some advantages such as low cost, low viscosity, and a short time for phase splitting.[3] Other advantages of such systems are that important properties, such as ionic strength and pH, can be adjusted conveniently.[4]

Experimental liquid−liquid equilibria (LLE) of systems containing two kinds of polymers have been frequently reported in the literature.[5−7] However, LLE of polymer + electrolytes + water systems are relatively scarce, at least for systems containing chaotropic compounds such as urea.[8,9]

As aqueous two-phase systems become increasingly popular in a number of large-scale and analytical applications, information on biomolecule partitioning in such systems becomes more important. The major factors that influence biomolecule partitioning include the concentration, molecular weight, and type of polymers used, pH, ionic strength, temperature, interfacial tension, and relative hydrophobicity.[10−14]

Aqueous two-phase systems containing urea have been used for the initial recovery steps of biomolecules from cell broth produced by recombinant *Escherichia coli*. For the solubilization of the inclusion bodies formed by the *E. coli* host, urea has to be added to the cell broth. High urea concentrations (30−35 mass %, ≅5−6 molar) are recommended. The further addition of poly(ethylene glycol) and salts such as sodium sulfate or potassium phosphate to the original system produces an aqueous two-phase system containing a high urea content.[9]

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Despite the projected importance of aqueous two-phase extractive techniques for future separation technology, no fully comprehensive theoretical framework exists for predicting protein partition coefficients. One obstacle to these investigations is the lack of an extensive database against which theories can be tested.[15]

Much empirical research has been devoted to the effects of selected independent factors and practical applications. Previous works have studied individual effects, which are important to protein partitioning.[3,12,16−20] Further progress in understanding protein partitioning in aqueous two-phase systems will require fundamental thermodynamic data.

In this paper, we present phase diagrams at 25 °C, at pH 7 and 9, and at the urea concentration 6 mass % for the PEG + potassium phosphate + urea + water system, using PEG of molecular weights 1450, 3350, and 8000. The partition coefficients of lysozyme, catalase, and β-galactosidase were determined in these systems using two urea concentrations, 3 and 6 mass %.

Experimental Section

Materials. The poly(ethylene glycol) (PEG), molecular weights 1450, 3350, and 8000, was purchased from Sigma. The polymers were analyzed by gel permeation chromatography (GPC) in a Waters chromatograph using the following conditions: water as the mobile phase at a rate of 0.8 μL/min, injection temperature of 40 °C, refraction index detector, sample injection of 100 μL. This methodology was used in order to obtain the polymers' molecular distribution and polydispersity index (M_w/M_n). The results are shown in Table 1. The water content of each polymer was determined by Karl Fisher titration using a Metrohm equipment. The amount of water ranges from (0.36 to 0.75) mass %, and it was considered in calculating the water concentration in the solutions. The potassium phosphate mono- and dibasic (minimum purity of 99.8 mass %) and urea (minimum purity of 99.5 mass %) were purchased from Merck, analytical reagent grade. All the reagents were used without further purification. Distilled water was used in all experiments.

Lysozyme (L7773, lot 16H6830) from chicken egg white, catalase (C9322, lot 88H72501) from bovine liver, and

10.1021/je000131f CCC: $20.00 © 2001 American Chemical Society
Published on Web 02/17/2001

Table 1. Polymer Characterization by GPC

PEG	avg molecular wt	polydispersity index (M_w/M_n)
1450	1468	1.03
3350	2938	1.04
8000	8768	1.09

β-galactosidase (G1875, lot 54H7025) from bovine liver, grade III crude, were purchased from Sigma. The isoeletric points (pI) of lysozyme, catalase, and β-galactosidase are 10.5, 5.6, and 4.6, respectively.

Phase Diagrams. Aqueous two-phase systems were prepared by weighing appropriate quantities of PEG, potassium phosphate, urea, and water on an analytical balance (A200 S Sartorius, Germany), accurate to ±0.0001 g. Liquid−liquid equilibrium cells were used to carry out the phase equilibrium determinations.[21] Typically 50 g of a system was prepared. The mixture was magnetically stirred for 50 min, after which the cell was tightly capped and then allowed to attain equilibrium for 5 h at 25 °C and at the desired pH value. The temperature in the cell was controlled within ±0.1 °C by a thermostated bath (Viscotherm VT2, Physica, Germany). The Henderson−Hasselbach equation[22] was used to determinate the ratio of mono- (M_1) and dibasic (M_2) salts necessary to bring the pH to 7 ($M_1/M_2 = 1.82$). For pH 9 only dibasic potassium phosphate was used. After equilibrium, the two phases became clear and transparent, and the interface became well defined. Samples from the top and bottom phases were collected, with the aid of syringes.

Protein Partitioning. Centrifuge tubes, volume 15 cm^3, were used to carry out the protein partitioning experiments. The tie lines were prepared from PEG, salt, and urea stock solutions. Proteins were dissolved in the PEG stock solution. Mixtures of known masses of the stock solutions were made up to a final mass of 12 g. All systems contained accurately known masses of the selected protein (approximately 50 mg). This mixture was gently stirred for 10 min at ambient temperature. Complete phase separation was achieved by centrifugation at 2900 g for 40 min at 25 °C (BR4i model, Jouan, France). After centrifugation the tubes were placed into a thermostatic bath at (25 ± 0.1) °C for 5 h to equilibrate. Aliquots of 5 mL were withdrawn using syringes. The top phase was sampled first, with care being taken to leave a layer of material at least 0.5 cm thick above the interface. The lower phase was sampled using a syringe with a long needle.[13]

Analysis of Phase Concentrations and pH Measurements. The concentration of salt was determined using simultaneously two methods: phosphate was determined by potentiometric titration, as described in detail by Greve,[23] and potassium was determined by means of an ion-selective electrode, as described by Hustedt et al.[24] The standard deviation of the salt mass percent by these methods was ±0.06%. The concentration of urea was determined following the micro-Kjeldahl procedure outlined by AOAC,[25] method 991.20, total nitrogen. The standard deviation of the urea weight percent by this method was ±0.07%. The concentration of water was determined by freeze-drying (EZ DRY model, FTS Systems, New York) at −54 °C and 13.32 Pa for 48 h. The standard deviation of the water mass percent by this method was ±0.03%. The PEG concentration was obtained by difference. The standard deviation of the PEG mass percent was ±0.02%. All concentrations were analyzed in triplicate. Measurements of the pH values of both phases were carried out using a Chemcadet 5986-50 pH meter (Cole Parmer Instrument Co.). Buffer solutions were used in the instrument calibration. The uncertainty of pH measurements is estimated to be 0.1 pH units.

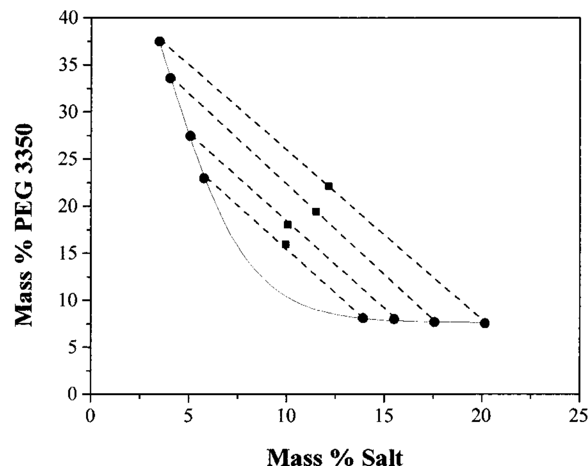

Figure 1. Phase diagram for PEG 3350 + potassium phosphate + water at 25 °C, 6 mass % urea, and pH = 7.

The concentrations of proteins (top and bottom) were determined by spectrophotometry. A sample of each phase was mixed with distilled water, and its absorbance was read at 280 nm using a spectrophotometer (Hach DR-4000U). The standard deviation for the protein concentration was ±0.01 mg/g. The protein concentrations were also analyzed in triplicate.

Results and Discussion

Liquid−Liquid Equilibrium. Phase diagrams for aqueous two-phase systems containing PEG (1450, 3350, and 8000) + potassium phosphate + urea at 25 °C and at pH 7 and 9 were experimentally determined. A typical phase diagram is shown in Figure 1. The tie lines were determined by linear regression of each corresponding set of total, bottom phase, and top phase concentrations. As can be seen in Figure 1, good linear fittings were obtained for the experimental data.

The tie line concentrations were confirmed, within an experimental error of 4%, by performing mass balance based on the equilibrium compositions. These values are in good agreement with results from the literature.[5,21,26,27]

The experimental liquid−liquid equilibrium results for these aqueous two-phase systems, including the pH values for both phases, are given in Tables 2 and 3. All concentrations are expressed as mass percentages. For each polymer−salt combination four tie lines were determined.

In all systems studied here (PEG + salt + urea) the concentration of PEG and salt required to exhibit phase splitting is higher than that in PEG + salt systems without urea, as compared to data reported by Silva et al.[21]

Analysis of the binodal curves from the systems studied with urea revealed the effect of PEG molecular weight on phase separation. Figure 2 shows the binodal curves for PEG (1450, 3350, and 8000) + potassium phosphate + urea + water. The decrease in PEG molecular weight caused a decrease in the size of the two-phase region, a behavior already reported in the literature for similar systems.[28−30] The difference in position between the binodal curves begins to decrease at higher molecular weights, with the binodal curves for PEGs 3350 and 8000 being very close. In fact, the addition of urea displaced the binodal curve toward higher concentrations (Figure 3), so that these curves for different PEG molecular weights became closer.

As can be seen in Tables 2 and 3, the pH values for the top phase were lower than those measured for the bottom

Table 2. Phase Compositions for the PEG + Potassium Phosphate + Water System at 25 °C, pH = 7, and 6 mass % Urea

system	total composition			top phase				bottom phase			
	salt (mass %)	PEG (mass %)	urea (mass %)	pH	salt (mass %)	PEG (mass %)	urea (mass %)	pH	salt (mass %)	PEG (mass %)	urea (mass %)
PEG 1450	11.47	20.09	5.93	6.7	5.79	31.85	4.65	6.7	16.60	10.60	6.07
	12.43	22.26	5.99	6.6	3.54	39.11	4.39	6.9	18.45	10.48	7.43
	13.61	23.39	6.02	6.5	2.70	42.98	3.82	6.9	20.75	10.12	7.76
	14.61	24.59	6.02	6.5	2.47	45.78	3.78	6.7	23.71	9.95	9.15
PEG 3350	9.97	15.87	6.04	6.9	5.77	22.95	8.19	7.0	13.94	8.09	5.33
	10.08	17.98	6.04	6.8	5.07	27.41	8.40	6.9	15.52	7.98	5.87
	11.52	19.36	6.01	6.8	4.04	33.56	4.82	6.9	17.58	7.64	6.18
	12.16	22.09	6.07	6.7	3.49	37.47	4.58	6.9	20.16	7.53	9.57
PEG 8000	10.01	15.88	6.07	6.8	5.07	28.73	8.38	6.9	13.48	7.20	5.54
	10.36	17.91	6.03	6.8	4.32	33.04	8.57	6.9	14.83	7.08	5.81
	11.56	19.80	5.93	6.7	3.73	37.79	4.85	6.9	17.03	6.95	6.36
	12.53	21.99	6.14	6.7	3.25	41.47	3.28	6.9	19.84	6.07	12.01

Table 3. Phase Compositions for the PEG + Potassium Phosphate + Water System at 25 °C, pH = 9, and 6 mass % Urea

system	total composition			top phase				bottom phase			
	salt (mass %)	PEG (mass %)	urea (mass %)	pH	salt (mass %)	PEG (mass %)	urea (mass %)	pH	salt (mass %)	PEG (mass %)	urea (mass %)
PEG 1450	10.62	19.95	5.96	8.5	4.94	31.71	4.68	8.9	15.75	10.46	6.16
	12.49	21.99	6.02	8.5	3.60	38.84	4.39	8.9	18.51	10.21	7.46
	13.67	23.46	6.05	8.5	2.76	43.05	3.85	8.7	20.81	10.19	7.79
	14.59	24.59	6.00	8.3	2.45	45.72	3.76	8.8	23.27	9.99	9.13
PEG 3350	9.87	15.63	6.00	8.9	4.61	26.70	7.97	9.0	12.63	10.39	4.92
	10.33	17.88	6.15	8.6	3.59	32.26	8.04	9.0	15.11	8.33	5.74
	11.31	19.58	6.08	8.4	3.31	36.04	4.97	9.0	18.06	6.91	6.22
	12.33	21.87	6.12	8.3	2.67	43.50	3.39	9.0	19.99	6.25	11.06
PEG 8000	10.01	15.95	6.24	8.7	3.63	30.69	7.99	9.0	14.14	8.63	4.76
	10.26	17.80	5.98	8.7	3.24	34.44	8.23	9.0	15.54	7.29	5.77
	11.39	19.75	5.94	8.5	2.74	38.73	4.99	9.0	17.64	7.16	6.15
	12.18	21.69	5.99	8.6	2.33	42.30	5.42	9.2	19.78	7.05	9.51

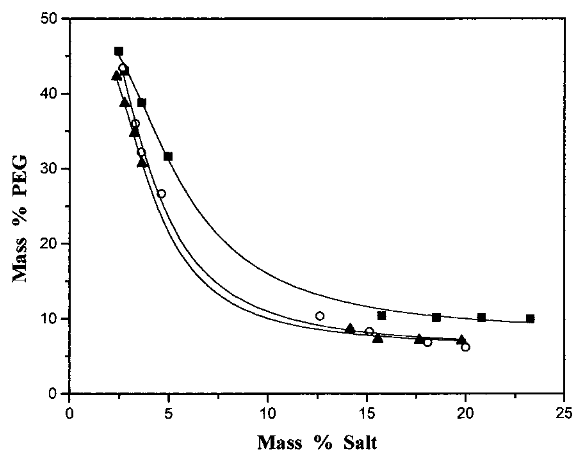

Figure 2. Binodal curves at 25 °C and pH 9 for the systems PEG + potassium phosphate + urea: (■) PEG 1450; (○) PEG 3350; (▲) PEG 8000.

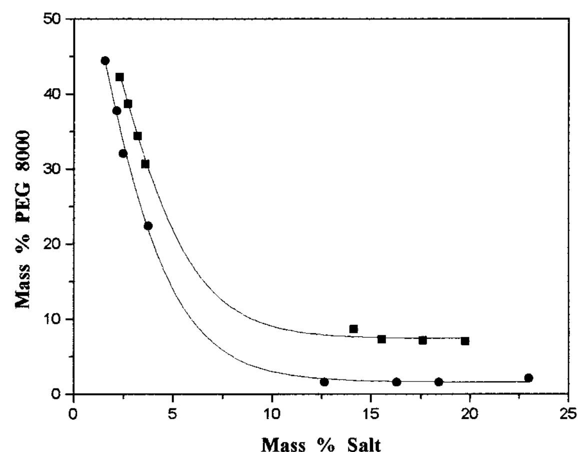

Figure 3. Binodal curves for the systems PEG 8000 + potassium phosphate + urea (■, this work) and PEG 8000 + potassium phosphate (●, Silva et al.)[21] at 25 °C and pH 9.

phase. In the case of the systems at pH 7, this observed difference has an average value of 0.2 pH units. For the systems at pH 9, the difference between the pH values of both phases was about 0.4 pH units.

Protein Partitioning. Lysozyme, catalase, and β-galactosidase were partitioned at 25 °C in the PEG + potassium phosphate + urea + water systems at different pH values. Two urea concentrations, 3 and 6 mass %, were used. Partitioning at three different tie lines was determined for each system. The partition coefficient (K) was calculated as $K = C_T/C_B$, where C_T and C_B are the protein concentrations in mg/g of the top and bottom phases, respectively. For the partition coefficients the mean deviation was ±0.02. Tables 4–6 show the partition coefficients of lysozyme, β-galactosidase, and catalase at two different pHs in the PEG + potassium phosphate + urea + water systems. It should be noted that the protein concentration in the total system was always very low, so that one can neglect its influence upon the equilibrium concentrations of the other components.

The highest values for the lisozyme partition coefficients were obtained in the PEG 1450 + salt + urea 3 mass % system at pH 9, and the lowest ones were obtained in the PEG 8000 + salt + urea 6 mass % system at pH 7. Table 4 shows that the partition coefficient decreases with increasing PEG molecular weight. In general, the partition coefficient increases as PEG concentration increases, except in the systems PEG 1450 + urea 3 mass % and PEG 8000 + urea 6 mass %, both at pH 9.

The highest values for β-galactosidase partition coefficients were obtained in PEG 3350 + salt + urea 3 mass % and PEG 1450 + salt + urea 3 mass % systems at pH 9,

Table 4. Partition Coefficients of Lysozyme at 25 °C in PEG + Potassium Phosphate + Urea + Water Systems

system	mass % PEG/salt	mass % urea	partition coefficient	
			pH = 7	pH = 9
PEG 1450	24.69/15.28	3.11	1.20	4.15
	25.28/15.16	3.05	1.36	3.14
	26.47/14.92	2.99	1.54	2.92
PEG 3350	24.48/15.33	3.09	1.42	1.81
	25.13/15.14	3.16	1.30	1.77
	26.51/14.73	2.99	1.43	2.14
PEG 8000	24.58/15.29	3.07	0.38	1.04
	25.21/15.24	3.04	0.46	0.91
	26.38/14.90	2.99	0.52	1.67
PEG 1450	21.80/13.62	6.06	1.05	2.05
	22.70/13.64	6.12	1.26	2.21
	24.04/13.59	6.03	1.41	2.48
PEG 3350	21.75/13.57	6.04	0.95	1.10
	22.58/13.58	6.14	1.29	1.13
	23.86/13.65	6.05	1.31	1.21
PEG 8000	21.89/13.63	6.17	0.26	2.37
	22.48/13.67	6.09	0.28	1.35
	23.95/13.59	5.97	0.46	0.66

Table 5. Partition Coefficients of β-Galactosidase at 25 °C in PEG + Potassium Phosphate + Urea + Water Systems

system	mass % PEG/salt	mass % urea	partition coefficient	
			pH = 7	pH = 9
PEG 1450	24.61/15.28	3.15	0.26	3.93
	25.22/15.25	3.02	0.39	1.52
	26.44/14.88	2.97	0.55	1.42
PEG 3350	24.74/15.24	3.06	2.76	3.25
	25.19/15.13	3.08	2.42	2.47
	26.34/14.90	2.97	2.24	3.20
PEG 8000	24.10/15.20	3.12	0.48	1.43
	25.21/15.14	3.01	0.52	1.22
	26.38/14.87	2.92	0.56	2.47
PEG 1450	21.92/13.62	5.98	0.54	1.17
	22.91/13.49	6.06	0.64	1.53
	24.13/13.48	6.10	0.70	1.85
PEG 3350	21.85/13.55	6.12	1.47	2.41
	22.50/13.59	6.04	1.90	2.41
	23.95/13.51	5.95	1.98	2.22
PEG 8000	21.86/13.63	6.09	0.37	1.78
	22.58/13.62	6.08	0.48	0.80
	24.02/13.40	5.99	1.22	0.58

Table 6. Partition Coefficients of Catalase at 25 °C in PEG + Potassium Phosphate + Urea + Water Systems

system	mass % PEG/salt	mass % urea	partition coefficient	
			pH = 7	pH = 9
PEG 1450	24.65/15.30	3.12	0.36	4.79
	25.30/15.22	3.09	0.53	3.53
	26.48/14.88	2.95	0.64	3.26
PEG 3350	24.52/15.25	3.04	1.61	2.52
	24.92/15.06	3.11	1.45	2.02
	26.39/14.83	2.94	1.55	2.57
PEG 8000	24.74/14.36	3.07	0.94	3.31
	25.05/15.19	3.04	0.99	3.08
	26.39/14.87	2.92	0.96	3.96
PEG 1450	21.95/13.57	6.11	0.67	3.02
	22.59/13.71	6.13	0.85	3.68
	24.15/13.45	5.94	0.93	3.57
PEG 3350	21.56/13.80	6.08	1.75	1.74
	22.50/13.63	6.05	2.05	1.80
	23.98/13.57	5.92	2.14	1.71
PEG 8000	21.87/13.59	6.02	0.80	5.38
	22.55/13.72	6.02	0.96	1.69
	24.07/13.48	5.93	2.25	1.49

and the lowest ones were obtained in the PEG 1450 + salt + urea 3 mass % system at pH 7. Table 5 shows that the partition coefficient increases as PEG molecular weight changes from 1450 to 3350 and decreases as the PEG molecular weight changes from 3350 to 8000. In general,

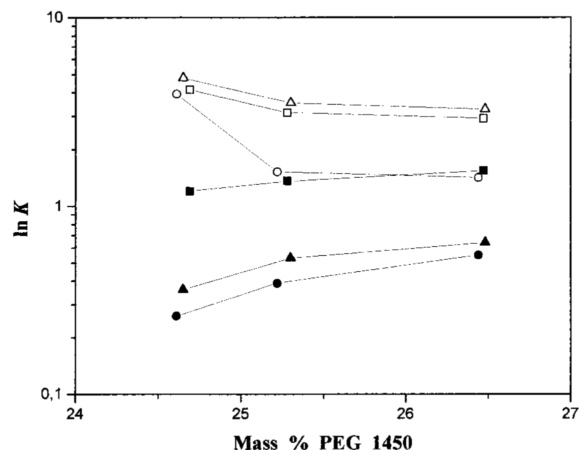

Figure 4. Protein partition coefficients in PEG 1450 + potassium phosphate + 3 mass % urea (■ and □, lysozyme; ● and ○, β-galactosidase; ▲ and △, catalase; pH = 7, solid symbols; pH = 9, open symbols).

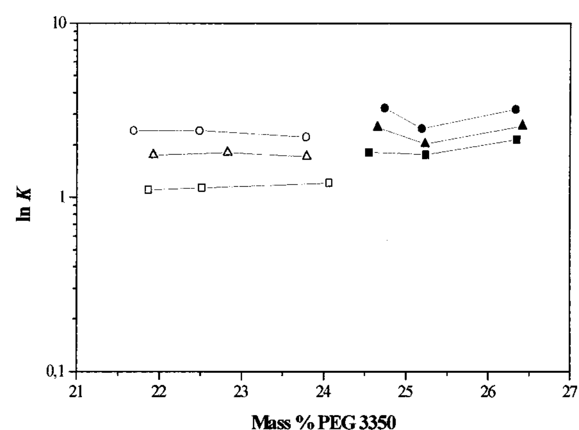

Figure 5. Protein partition coefficients in PEG 3350 + potassium phosphate + urea at pH 9 (■ and □, lysozyme; ● and ○, β-galactosidase; ▲ and △, catalase; 3 mass % urea, solid symbols; 6 mass % urea, open symbols).

the partition coefficient increases as PEG concentration increases, except in PEG 3350 + urea 3 mass % for both pHs and in PEG 1450 + urea 3 mass %, PEG 3350 + urea 6 mass %, and PEG 8000 + urea 6 mass % at pH 9.

The highest values for catalase partition coefficients were obtained in PEG 1450 + salt + urea 3 mass % and PEG 8000 + salt + urea 6 mass % systems, both at pH 9, and the lowest ones were obtained in the PEG 1450 + salt + urea 3 mass % system at pH 7. Concerning the influence of PEG molecular weight, the partition coefficients of catalase at pH 7 exhibit a behavior similar to that observed for β-galactosidase: the partition coefficients increase as the molecular weight changes from 1450 to 3350 and decrease as the molecular weight changes to 8000. At pH 9 the opposite behavior was observed in most cases, since the lowest values for the partition coefficient were obtained for the molecular weight 3350. In general, at pH 7 the partition coefficient increases as the PEG concentration increases, except in PEG 3350 + urea 3 mass % and PEG 8000 + urea 3 mass %. At pH 9 this trend was not observed and for the PEG 1450 + urea 3 mass % and PEG 8000 + urea 6 mass % systems the obtained behavior was exactly the opposite one.

In PEG + salt + urea systems at pH 9, the proteins partition preferentially to the top phase ($K > 1$). In general the partition coefficients at pH 9 were higher than those at pH 7 (Figure 4). In most cases the partition coefficients

decrease as the urea concentration changes from 3 to 6 mass %, as can be seen in Figure 5.

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Received for review May 1, 2000. Accepted November 20, 2000. This work was supported financially by research grants from Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP−Proc. 1997/10630-7), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq−Proc. 521011/95-7 + 466680/00-7), and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES/PICDT).

JE000131F