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Partitioning behavior of amino acids in aqueous two-phase systems with recyclable volatile salts

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Abstract

As part of an ongoing research effort on aqueous two-phase systems (ATPSs) with volatile salts, this work describes the partitioning behavior of a series of amino acids, namely L-serine, glycine, L-alanine, L-valine, L-methionine, L-isoleucine, and L-phenylalanine, in these systems. The results show that amino acids partition in a similar way in polymer–volatile salt ATPSs and in traditional polymer–salt ATPSs. Increasing amino acid hydrophobicities lead to increasing partition coefficients. Moreover, the common linear relationship between the logarithm of the partition coefficient and the tie line length is observed here as well. Furthermore, the relation between relative partition coefficients and relative hydrophobicities of amino acids in the extraction systems investigated in this work is comparable to that in other extraction systems.

Keywords: Partitioning; Aqueous two-phase systems; Amino acids; Volatile salts

1. Introduction

Aqueous two-phase systems (ATPS) were first discovered by Beijerinck [1] in the city of Delft in the Netherlands at the end of the 19th century. Half a century later, Albertsson rediscovered the systems and started to work on ATPSs with the aim of using them for extraction purposes [2]. In the second half of the 20th century, there has been increasing attention focussed on these systems, especially for use in extraction processes for isolation and concentration of fermentation products. The main driving force for this increasing attention is the fact that ATPSs contain 60 to 80% water, which provides a very mild environment for large biomolecules such as proteins.

An important disadvantage of most of the ATPSs considered so far is the large consumption of auxiliary materials. In previous work [3,4], we presented a new type of ATPSs, namely based on polymers and volatile salts as phase forming components. These volatile salts are induced by dissolving combinations of NH_3 and CO_2 in water. These volatile components can be recycled via transfer to the gas phase. In a previous work [4], we focussed on establishing the system boundaries, especially regarding the ratio of dissolved NH_3 and CO_2. This ratio, named $R_{N/C}$ is defined as

$$R_{N/C} = \frac{m_{NH_3(aq)} + m_{NH_4^+} + m_{NH_2CO_2^-}}{m_{CO_2(aq)} + m_{HCO_3^-} + m_{CO_3^{2-}} + m_{NH_2CO_2^-}} \quad (1)$$

where m_i are molalities of the dissolved species i that originate from dissolved NH_3 and CO_2. Values for

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$R_{N/C}$ are particularly useful as a measure for the boundaries of these systems for convenient extraction processes. Systems with values for $R_{N/C}$ below 2.0 show no or limited potential because of the small operating area (or even absence of operating area). Systems with larger $R_{N/C}$'s are suitable for use in extraction processes. The molecular weight of polyethylene glycol (PEG) also influences the operating area. In conventional systems with relatively high molecular weight polymers, less polymer and salt are required than in systems with lower molecular weight polymers. The choice of a certain molecular weight of PEG is also influenced by other system properties such as pH, density difference, interfacial tension, and viscosity. Properties of the product and other solutes and particles that are present in the (pre-treated) fermentation broth or other relevant product-containing mixture also play an important role in the selection of a suitable extraction system. Therefore, experiments determining the partitioning behavior of solutes in extraction systems are usually necessary for obtaining optimal extraction processes.

There has been quite a number of partitioning studies of various components such as proteins, peptides, amino acids, other small organic molecules, and ions in polymer–salt ATPS. In this work, we will focus on amino acids. An important reason for this choice is the relative simplicity of these molecules in comparison to, for instance, proteins. In order to understand complex protein behavior in ATPS, it may be useful to examine the behavior of amino acids and peptides in such systems [5]. Amino acids only differ from each other by one characteristic group to which clear physical and chemical properties can be assigned. Another reason for preferring amino acids to proteins is that other researchers described partitioning of amino acids in other PEG–salt systems before. This provides us with the opportunity to compare the results obtained in this work with volatile salts to the results of other researchers with traditional salts such as sulfates and phosphates, and possibly find some dissimilar characteristics of the volatile salt systems.

Several experimental studies to determine the partitioning behavior of amino acids in liquid–liquid systems are known. Examples of the systems that were used in those studies are water–octanol [6–8], water–butanol [9–11], water–butanol–ethanol [12], and a range of other water–solvent systems, i.e. [13]. Recently, some partitioning data of amino acids in aqueous reverse micellar systems has been published [14,15] as well as data for aqueous polymer–polymer systems [16–18]. For systems with thermoseparating polymers (copolymers of ethylene oxide and propylene oxide), some amino acid partitioning data is available as well [19,20].

Also in PEG–salt aqueous two-phase systems, several experimental investigations on partitioning of amino acids (and also other relatively small solutes such as peptides) have been reported. Diamond et al. [21] studied the partitioning of 20 amino acids and also several small peptides in systems composed of PEG 3400 and potassium phosphate. Partitioning of tysine, glutamic acid and phenylalanine in pure solutions and fermentation broth was studied by Chu et al. [22] in systems with $MgSO_4$, $(NH_4)_2SO_4$, Na_2SO_4, and K_2HPO_4 as phase forming salts. Eiteman and Gainer [5,23] determined the partitioning behavior of 11 amino acids and small peptides in systems composed of PEG 8000 and magnesium sulfate. Cohen et al. [24] used systems containing PEG 8000 and potassium phosphate to investigate partitioning of 7 amino acids. Grossmann et al. [25,26] determined the partitioning behavior of glycine, phenylalanine, glutamic acid, some small peptides, and lysozyme in systems of potassium phosphate and PEG 6000 or PEG 35000. The experimental conditions of the investigations mentioned in this paragraph are not identical: Temperatures ranged from 20°C to 35°C, pH's from 2 to 12, and overall amino acid concentrations from about 0.005 to 15 mg/g. Therefore, one has to be careful when comparing the results of different studies with one another. In this work, the partitioning behavior of seven amino acids has been studied at 25°C in aqueous two-phase systems with volatile salts at various NH_3/CO_2 ratios and with various molecular weights of PEG.

2. Materials and methods

2.1. Chemicals

Ammonium carbamate ($NH_4NH_2CO_2$, >99.5% purity) was obtained from Merck (Darmstadt, Ger-

many), ammonium bicarbonate (99.4% purity) and ammonia solution (25.0% w/w) from Baker (Deventer, The Netherlands). PEG 2000 and PEG 4000, both analytical grade, were purchased from Merck (Hohenbrunn bei München, Germany). Water was distilled and deionized with a Milli-Q Water System (Bedford, MA).

Glycine and L-alanine were purchased from Sigma (St Louis, MO), L-valine from Fluka (Buchs, Switzerland), and L-serine, L-methionine, L-isoleucine, and L-phenylalanine from Merck (Darmstadt, Germany).

2.2. Preparation of phase systems and samples

The experiments were performed in 100 ml glass bottles with a screw top and two side ports at different heights with septa allowing for sampling of both liquid phases. Seven amino acids (serine, glycine, alanine, valine, methionine, isoleucine, and phenylalanine) were weighed out first, followed by the polymer, ammonium bicarbonate and/or ammonium carbamate, and water. Ammonia solution was then added to the system with a $R_{N/C}$ ratio of 2.51. The total weight of these components was about 80 g in each bottle. The concentration of each amino acid in each bottle was about 1 m*M* resulting in solutions that can be characterized as dilute regarding the total amino acid concentration that is smaller than 0.1% w/w. A Mettler Toledo AB 204 balance (Mettler-Toledo, Greifensee, Switzerland) was used for determining the masses. The bottles were then placed in a thermostated water bath and magnetically stirred for at least 24 h to ensure complete equilibration. A JULABO U3 Thermostate (Julabo Labortechnik, Seelbach, Germany) maintained the temperature of the bath at 25°C. After equilibration of the systems, samples of approximately 10 ml of both phases were taken through the septums with syringes in a gentle way to avoid remixing of the phases.

2.3. Analyses of samples

The density was determined using a Anton Paar DMA 48 density meter (Anton Paar, Graz, Austria) with an accuracy of better than 0.1 kg/m^3. The density meter was calibrated against air and water at 25°C before every set of measurements.

The pH was measured with a Metrohm 691 pH meter (Metrohm, Herisau, Switzerland). The pH meter was calibrated before each set of measurements and has an accuracy that was determined to be ± 0.05 pH-scale units.

The concentrations of PEG and salts were determined by measurement of Total Organic Carbon (TOC) and Inorganic Carbon (IC) of the samples using a Shimadzu TOC-SOSGA Total Organic Carbon Analyzer (Shimadzu, Tokyo, Japan) with a stated precision of $\pm 2\%$. Firstly, the samples were diluted ten times immediately after collecting them from the phase systems to avoid formation of a second liquid phase due to, for instance, temperature change. These diluted samples were then diluted by weight to approximately 0.05 g PEG/kg and then injected in the TOC Analyzer. A part of the sample is automatically injected in the combustion tube and through catalytic oxidation at 680°C, this sample is completely oxidized to CO_2 and H_2O. The dried CO_2 containing gas is then passed through a CO_2 specific no-dispersive infrared detector (NDIR) to quantify the total amount of CO_2, and consequently the Total Carbon (TC) amount. The amount of Inorganic Carbon (IC) is measured at room temperature in the same apparatus by passing another part of the sample through an acidic solution so that all IC reacts to CO_2 which is quantified by the same detector. The TOC is calculated by subtracting the IC value from the TC value. The amounts of amino acids were subtracted from the TOC values in order to obtain PEG concentrations. Salt concentrations were calculated from IC values assuming that the compositions of the dense and less dense phase were similar regarding equality of all salt component concentration ratios between the dense and less dense phase. In this way, there is no difference in salt composition between the phases, and only a difference in salt concentration. This assumption seems very relevant, for instance because the pH difference between both phases was almost equal to zero in all cases.

The amino acid concentrations were determined by an HPLC-method with pre-column derivatization with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) using the Waters AccQ-Fluor Reagent

Kit. The standards were prepared from the Amino Acid standard H (Pierce nr. 20 088) to which asparagine, glutamine, tryptophan, and ornithine were added. The samples were diluted ten times immediately after collecting them from the phase systems to avoid formation of a second liquid phase. The ammonia was removed from the diluted samples by adding 250 μl borate buffer (1 *M*, pH = 12.5) to 700 μl sample and 70 μl internal standard (2.5 m*M* α-amino butyric acid) and then flush this mixture with nitrogen gas for 20 min at 75°C. Next, 10 μl of the resulting mixture was mixed with 70 μl of borate buffer (1 *M*, pH = 12.5) and 20 μl AQC reagent, and heated for 10 min at 55°C. These mixtures were analyzed using two 510 HPLC pumps, a M680 Gradient Controller, a M717 autosampler, a TCM column heater, a Nova-pak C18 column, and a M470 fluorescence detector ($\lambda_{excitation}$ = 245 nm, $\lambda_{emission}$ = 395 nm), all from Waters. Eluent A was 60 m*M* ammonium acetate (Aldrich, pH = 5.0) and eluent B a 50% v/v mixture of 60 m*M* ammonium acetate and acetonitrile (Rathburn). The elution conditions were as follows: in 27 min from 97% eluent A to 89% A, then in 22 min to 54% A. The total eluent flow was 1 ml/mm and the column temperature was 28°C.

3. Results and conclusions

Firstly, it has to be noted that the influence of the dissolved amino acids on the phase behavior of the extraction systems themselves can be neglected. A number of measurements of the concentrations of polymers and salts have shown that the phase compositions are hardly influenced by the small amounts of added amino acids so that the concentrations of polymer and salt in both phases do not differ between systems with and without amino acids. This is shown in Figs. 1 and 2. Fig. 1 shows the phase diagram of ATPSs composed of PEG 4000 and volatile salts with $R_{N/C}$ = 2.0, both with and without the seven amino acids added. The phase compositions, binodals, and tie lines are almost identical, especially within experimental error. Fig. 2 shows phase densities of top and bottom phases of the same systems also indicating that the addition of amino acids hardly influences the phase properties. Similar correlations were observed for pH values.

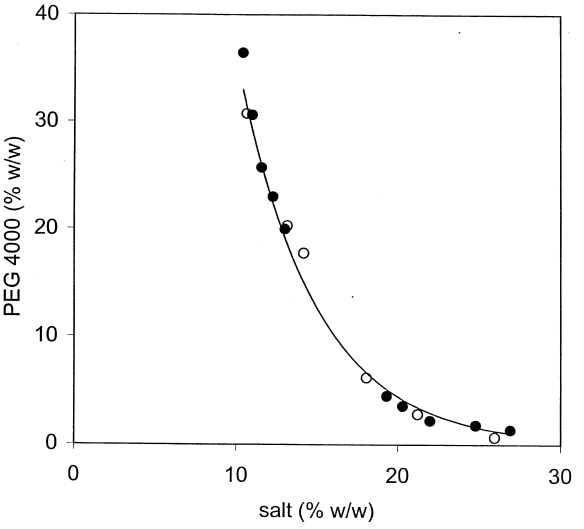

Fig. 1. Phase compositions of aqueous two-phase systems with and without amino acids (PEG 4000; $R_{N/C}$ = 2.0). ● without amino acids; ○ with amino acids. Line is shown as guidance to eyes. Values for systems without amino acids from [3].

Table 1 gives the results of the partitioning experiments. The values for $R_{N/C}$ of 2.0 are exact

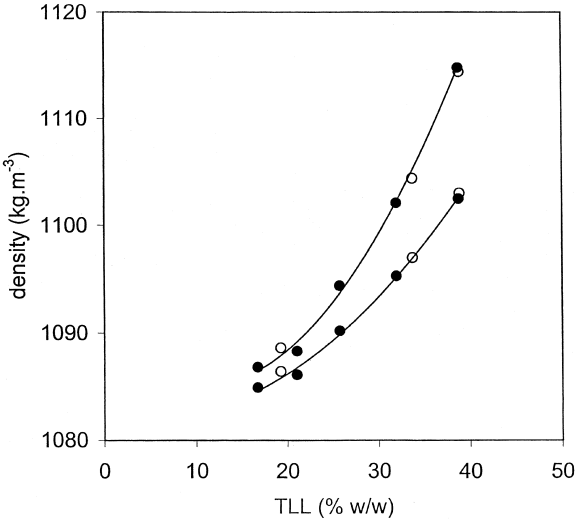

Fig. 2. Phase densities of aqueous two-phase systems with and without amino acids (PEG 4000; $R_{N/C}$ = 2.0). ● without amino acids; ○ with amino acids. Lines are shown as guidance to eyes. Values for systems without amino acids from [3].

Table 1
Partition coefficients of amino acids in aqueous two-phase systems with PEG molecular weights of 2000 and 4000, and with $R_{N/C}$ values of 2.0 and 2.51

Top phase			Bottom phase			Partition coefficients						
PEG (% w/w)	Salt (% w/w)	Density (kg/m^3)	PEG (% w/w)	Salt (% w/w)	Density (kg/m^3)	K_{ser} (–)	K_{gly} (–)	K_{ala} (–)	K_{val} (–)	K_{met} (–)	K_{ile} (–)	K_{phe} (–)
PEG 2000; $R_{N/C}=2.0$												
28.0	14.5	1101.7	4.2	26.5	1112.2	0.39	0.43	0.52	0.70	0.89	0.89	1.65
30.5	13.7	1102.9	3.6	27.2	1114.6	0.38	0.42	0.47	0.69	0.90	0.89	1.73
40.3	11.7	1112.5	2.2	33.1	1134.1	0.23	0.26	0.34	[a]	0.76	0.75	1.71
45.5	11.3	1118.7	2.0	35.9	1146.8	0.17	0.20	0.28	0.48	0.76	0.74	1.88
PEG 4000; $R_{N/C}$ 2.0												
20.0	13.0	1084.9	4.4	19.3	1086.6	0.61	0.64	0.68	0.80	0.88	0.89	1.27
21.2	12.7	1085.6	3.8	20.1	1087.6	0.54	0.57	0.59	0.76	0.87	0.88	1.36
31.3	10.9	1096.2	1.7	24.9	1103.5	[a]	[a]	0.45	0.69	[a]	0.79	1.39
37.3	10.4	1103.5	1.4	27.3	1116.7	0.27	0.31	0.36	[a]	0.75	0.73	1.64
PEG 2000; $R_{N/C}=2.51\pm0.02$												
27.6	16.7	1093.8	5.4	28.8	1100.9	0.48	0.51	0.62	0.79	0.96	0.94	1.51
33.0	15.7	1097.3	2.9	32.7	1108.2	0.40	0.42	0.48	0.66	0.85	0.86	1.48
36.2	14.7	1099.7	2.6	33.8	1112.8	0.31	0.39	0.43	0.70	0.95	0.94	1.88
PEG 4000; $R_{N/C}=2.51\pm0.02$												
18.4	15.7	1078.0	6.1	21.1	1078.8	0.69	0.71	0.75	0.83	0.90	1.01	1.20
23.0	14.6	1080.3	3.9	23.2	1081.8	0.47	0.51	0.53	0.76	0.81	0.98	1.37
23.8	14.3	1080.6	3.8	23.4	1082.3	0.52	0.53	0.58	0.72	0.85	0.98	1.33
28.5	13.3	1085.0	1.6	27.5	1088.7	0.33	0.37	0.41	0.57	0.71	0.95	1.31

[a] Not measured. (Data for PEG and salt concentrations and densities are interpolated from the corresponding aqueous two-phase systems without amino acids [4].)

numbers; the values of 2.51 have a spread due to small experimental variations. Because of the observations described in the previous paragraph, most polymer and salt concentrations were not measured for these systems, and all were interpolated from the corresponding systems without amino acids [4]. Partition coefficients were calculated on the basis of molalities. Figs. 3–6 show the dependence of partition coefficients for all amino acids on the tie line length (TLL) for the four systems, differing in polymer molecular weight and NH_3/CO_2 ratio, that were investigated. Plotting the (natural) logarithm of the partition coefficients versus the TLL (or versus the concentration difference of polymer or salt between two coexisting phases) results in linear relationships that have been reported frequently in literature for ATPS, i.e. [2,5,21]. Deviations from this linearity are especially visible at low TLLs and are partly due to the specific properties of the phase systems, such as the very small density difference, and experimental errors that result from these properties. These systems with extremely small physical phase differences make taking and handling samples very difficult. Small fluctuations in temperature and pressure and also minor shaking of the bottles that is inevitable when taking the bottles out of the water bath or when inserting a syringe, can result in minor remixing of the phases or entrainment of droplets of one phase into another.

The effect of polymer molecular mass on solute partitioning in aqueous two-phase systems has been described before, i.e. [2,27]. In general, an increase in PEG molecular mass decreases solute partition coefficients, but the influence on small solutes such as amino acids is almost negligible [26]. When comparing Figs. 3 and 5 (PEG 2000) with Figs. 4 and 6 (PEG 4000) it appears that the PEG molecular weight has a small influence on the partition co-

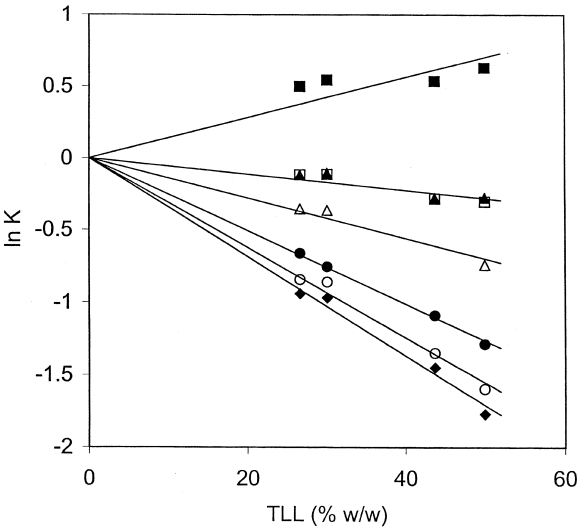

Fig. 3. Partition coefficients of amino acids as a function of the tie line length (PEG 2000; $R_{N/C}=2.0$). ◆ serine; ○ glycine; ● alanine; △ valine; ▲ methionine; □ isoleucine; ■ phenylalanine. Lines represent linear fits to data.

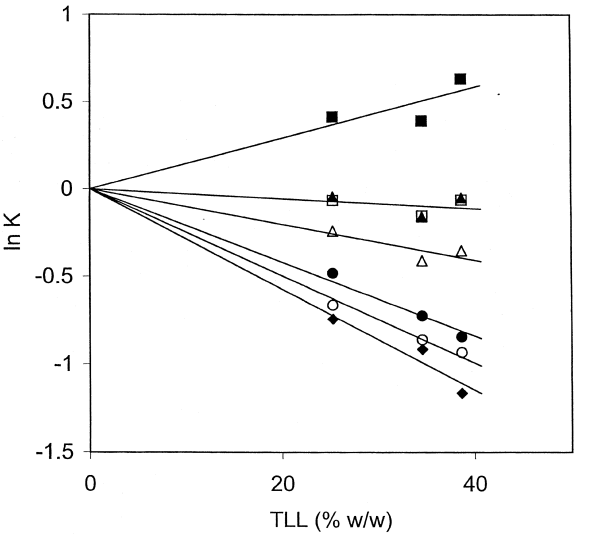

Fig. 5. Partition coefficients of amino acids as a function of the tie line length (PEG 2000; $R_{N/C}=2.51$). ◆ serine; ○ glycine; ● alanine; △ valine; ▲ methionine; ■ isoleucine; □ phenylalanine. Lines represent linear fits to data.

efficients. The partition coefficients in the PEG 4000 systems are somewhat smaller than those in the respective PEG 2000 systems at equal TLL, but this effect can also be due to other differences such as the concentration of salt.

Also the NH_3/CO_2 ratio does not affect the

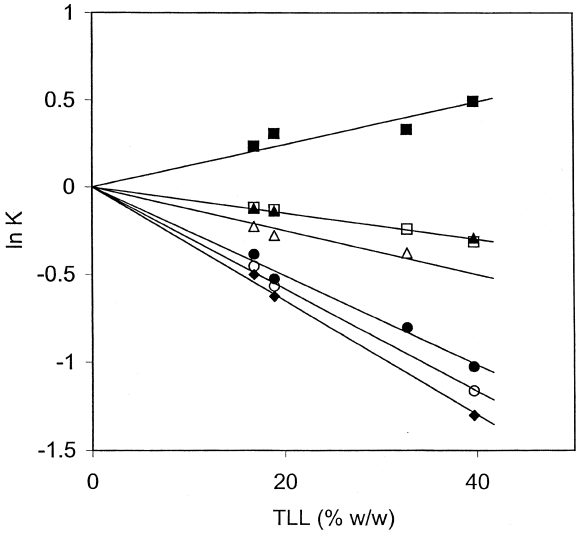

Fig. 4. Partition coefficients of amino acids as a function of the tie line length (PEG 4000; $R_{N/C}=2.0$). ◆ serine; ○ glycine; ● alanine; △ valine; ▲ methionine; ■ isoleucine; □ phenylalanine. Lines represent linear fits to data.

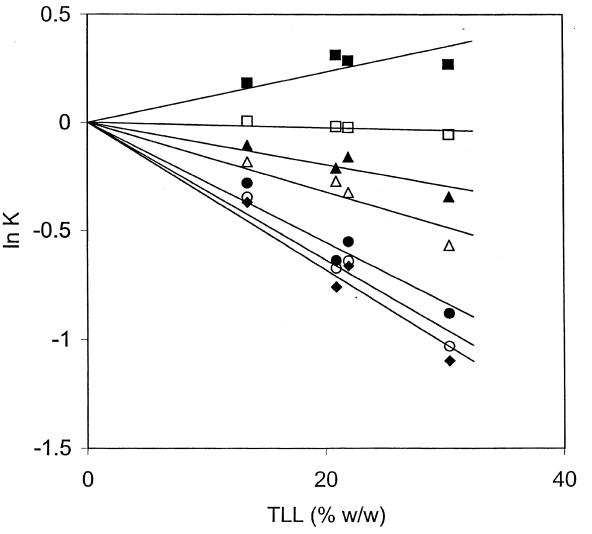

Fig. 6. Partition coefficients of amino acids as a function of the tie line length (PEG 4000; $R_{N/C}=2.51$). ◆ serine; ○ glycine; ● alanine; △ valine; ▲ methionine; ■ isoleucine; □ phenylalanine. Lines represent linear fits to data.

partition coefficients to a large extent. The partition coefficients in phase systems with $R_{N/C}=2.0$ seem to be a little smaller than those in phase systems with $R_{N/C}=2.51$. This observation can be explained by taking into account the difference in hydrophobic and electrostatic properties of the salt-rich phase due to the presence of different amounts of uncharged ammonia.

When regarding the partition coefficients in relation to the properties of the individual amino acids, some solute properties are of importance, such as size and polarity. An important property influencing partition coefficients of amino acids as well is hydrophobicity. Nozaki and Tanford [28] calculated a hydrophobicity scale for amino acids based on the free energy of transfer of amino acid side chains from an organic solvent to water. The larger the hydrophobicity of an amino acid, the larger the affinity for the more hydrophobic PEG-rich phase, and, consequently, the larger the partition coefficient. Experimental studies on partitioning of amino acids in polymer–salt aqueous two-phase systems have proven the validity of such a statement [5,21]. Amino acid partitioning studies in other phase systems such as water–butanol mixtures [11] show similar results.

The results in the present work also show the same dependence of amino acid partition coefficients on hydrophobicity. The partition coefficients in volatile salt aqueous two-phase systems are quite similar to those of for instance Eiteman and Gainer [5] in systems containing magnesium sulfate. Fig. 7 shows the relationship between partition coefficients and hydrophobicity (hydrophobicity scale of Nozaki and Tanford [28]) for phase systems located on four different tie lines in systems with PEG 4000 and $R_{N/C}=2.0$. As expected, the longer a tie line, the more extreme the partition coefficients. Also, the larger the hydrophobicity, the larger the partition coefficient.

In several publications (i.e. [5,28,29]) it has been shown that a hydrophobicity scale can be used to obtain at least qualitative information about the relative phase behavior of molecules of similar structure. Amino acids do indeed have alike structures and can be regarded as a homologous series. Van der Wielen and Rudolph [29] have shown that the relative phase behavior of amino acids can be

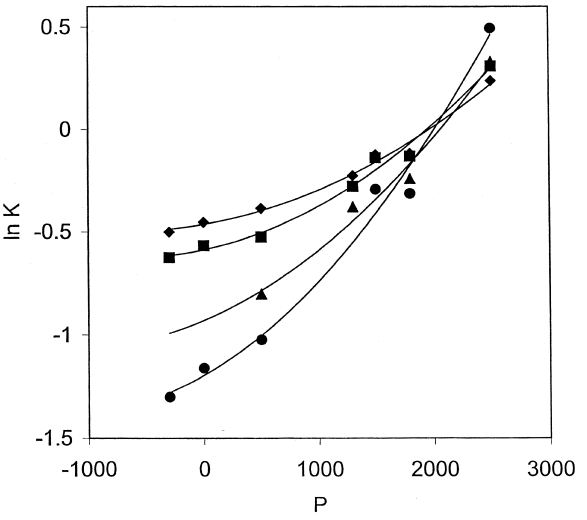

Fig. 7. Partition coefficients of amino acids as a function of their relative hydrophobicity (P) for four two-phase systems located on different tie-lines (PEG 4000; $R_{N/C}=2.0$). Tie line length increases as follows: ◆■▲●. Lines are shown as guidance to eyes.

correlated to their relative hydrophobicities, even when comparing quite different separation processes such as aqueous two-phase extraction, solvent extraction, HPLC, cation exchange, and centrifugal partition chromatography. Fig. 8 shows relative partition coefficients of various amino acids as a function of their relative hydrophobicity, according to the scale of Nozaki and Tanford [28], in a number of different extraction systems. The systems are ATPSs with PEG and volatile salts, ATPSs with PEG and $MgSO_4$, ATPSs with PEG and KH_2PO_4, and systems composed of L-butanol and water. The experimental data originate from this work, [5], [30], and [11], respectively. The partition coefficients K_i of the amino acids i have been scaled relative to the partition coefficients of glycine and phenylalanine in identical systems. This scaling method is analogous to that given in [29]:

$$\frac{\ln\left(\frac{K_i}{K_{Gly}}\right)}{\ln\left(\frac{K_{Phe}}{K_{Gly}}\right)} \qquad (2)$$

When regarding Fig. 8 it is evident that the amino acids show quite similar relative partitioning behavior in all regarded systems. Most deviations from

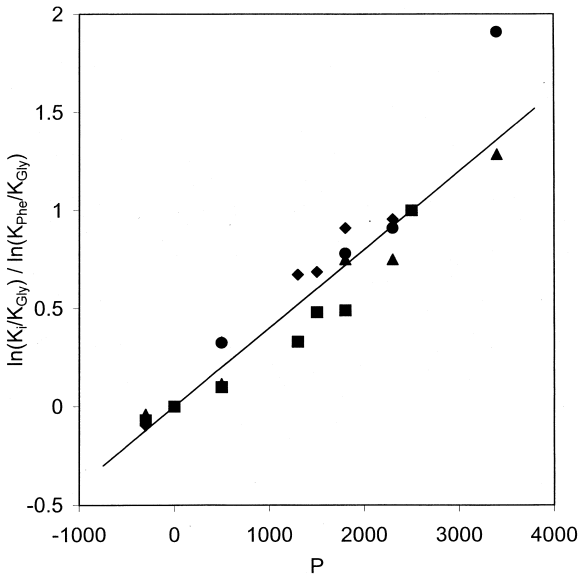

Fig. 8. Relative partition coefficients of amino acids as a function of their relative hydrophobicity for four different extraction systems. ■ aqueous two-phase systems with PEG 4000 and volatile salts; ● aqueous two-phase systems with PEG 8000 and $MgSO_4$ ◆ aqueous two-phase systems with PEG 600 and KH_2PO_4 ▲ systems with L-butanol and water. The line indicates behavior in conformance with the hydrophobicity scale.

the drawn line can be attributed to experimental variations, especially in the analysis of phenylalanine and tryptophane. The similarities imply that the hydrophobicity scale that has been applied, can describe the partitioning behavior of amino acids in a qualitative and semi-quantitative way, at least for the extraction systems that have been regarded here. It also shows that the use of aqueous two-phase systems with dissolved NH_3 and CO_2 as phase forming salts cause no adverse abnormalities to the partitioning behavior of amino acids.

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