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Temperature-dependent phase inversion and its effect on partitioning in the poly(ethylene glycol)–ammonium sulfate aqueous two-phase system

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Abstract

As temperature is increased, the poly(ethylene glycol) (PEG)–ammonium sulfate aqueous two-phase system exhibits a phase inversion. Specifically, the PEG-enriched phase —which at low temperature is the less dense upper phase— becomes the lower phase at elevated temperatures. The effect depends on the concentrations of PEG and ammonium sulfate in the system, with lower concentrations of either phase-forming constituent causing the inversion to occur at a lower temperature. The partitioning of several solutes is examined in this phase system at temperatures of 20–70°C. The results show that the logarithm of the partition coefficient of each solute is proportional to the PEG concentration difference between the phases (*i.e.*, upper phase concentration minus lower phase concentration), whether this difference has a positive or negative value.

1. Introduction

Albertsson [1] showed that two liquid "aqueous" phases form when threshold concentrations of two water-soluble, but mutually incompatible, components in water are exceeded. One type of aqueous two-phase system is formed when a polymer such as poly(ethylene glycol) (PEG) and a salt are dissolved together in water. The boundary between the two-phase region and the one-phase region on axes of component concentrations is referred to as the binodal, and its location for a particular pair of components depends on the system's temperature. Albertsson [2] noted that for polymer–polymer systems, the polymer which concentrates in the lower, denser phase usually does so at all component compositions. Furthermore, for PEG–salt systems, the PEG has been observed to enrich the upper, less dense phase, while the salt has been observed to enrich the lower phase. Theories of two-phase formation and partitioning in these systems have recently been reviewed [3].

A fundamental result of numerous theoretical and experimental studies concerning partitioning is that the logarithm of the partition coefficient (K) is proportional to the concentration difference of PEG between the phases by a proportionality constant, A. Mathematically,

$$\ln K = A(w_2' - w_2'') = A\,\Delta w_2 \qquad (1)$$

where w_2 indicates the concentration of component 2 (by convention, PEG) in the upper ($'$) and lower ($''$) phase. This equation describes a property of a majority of phase systems which is that the tie lines are parallel. The relationship of tie line length to partition coefficients arises from

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theoretical treatments [4–6] relying on the osmotic pressure virial expansion of Edmond and Ogston [7]. This result also arises from similar derivations [8,9] using Flory–Huggins [10,11] polymer solution theory, and approaches [12–14] using the Hill constant pressure theory [15].

In PEG–salt phase systems the upper phase has been observed to be the PEG-enriched phase, in which case the PEG concentration difference, Δw_2, is positive. The conclusion drawn from these studies is straightforward: in order to increase the partition coefficient of a solute which partitions preferentially into the upper phase —and hence having a partition coefficient greater than unity— one must increase the tie line length by increasing the concentration of one or both of the phase forming components. Similarly, in order to increase the partition coefficient of a solute partitioning preferentially into the lower phase (*i.e.*, move K closer to unity), one must decrease the tie line length.

An additional method which may be used to influence the binodal and thereby affect the tie line length is to alter the temperature of the phase system. The few early studies concerning the effect of temperature on phase systems and on the partition coefficient of solutes distributed in these phase systems [16–18] did not clarify this effect. Forciniti and Hall [14] noted that the concentration difference of PEG between the phases was independent of temperature while the concentration difference of dextran was sensitive to temperature in PEG–dextran systems. In general, the tie line length decreased with increasing temperature in these systems. Recent theoretical considerations [19] suggest the change in partitioning of model proteins with temperature is a result of the combination of repulsive and attractive forces for a particular protein. In the PEG–dextran phase systems almost exclusively studied, PEG remains the predominant upper phase component for the entire temperature range.

Cohen [20] noted that at 60°C the lower phase of a PEG–ammonium sulfate system of particular concentration becomes PEG-enriched, but only very limited studies were performed. In this current study, additional investigations of this unique phase system are conducted to determine the conditions under which the observed phase inversion occurs. In addition to studying the properties of this phase system, the partitioning of several compounds is determined as a function of temperature.

2. Materials and methods

Three different PEG–ammonium sulfate aqueous two-phase systems were prepared: 7% PEG–9% salt, 6% PEG–10% salt and 7% PEG–11% salt. Reported values are in mass fractions of the compounds in the total system, that is, mass of component per mass of phase system. The particular polymer used in these solutions was PEG with a molecular mass of 8000. A 20-ml volume of each phase system containing 5–10 mg of the desired solute was refrigerated at 4°C. Systems were withdrawn when needed and placed in a bath at the desired temperature (±0.1°C). After two days of mixing and one day of equilibration, the phases were separated carefully with Pasteur pipettes and analyzed. PEG concentrations in each phase were determined by the method of Skoog [21].

The following solutes were used for partitioning studies: tryptamine, phenylalanine, tryptophol, leucine–phenylalanine (Sigma, St. Louis, MO, USA) and pentanol (Aldrich, Milwaukee, WI, USA).

A Hewlett-Packard HP-5890 gas chromatograph housing an 8 ft. × 2 mm I.D. Supelco Carbopack FT-A column (1 ft. = 30.48 cm) was used to determine the partition coefficients of pentanol. Other solutes were analyzed by liquid chromatography. The HPLC system comprised a Gilson Model 306 pumps, 231 autosampler, and an Applied Biosystems 759A UV–Vis detector. The column was a Waters Radial-Pak C_8, with eluent and detector settings appropriate to separate the pure solute of interest from impurities arising from the PEG and solute sample.

3. Results and discussion

Table 1 shows the properties of the three PEG–ammonium sulfate phase systems studied over the temperature range of 20–70°C.

The 7% PEG–9% salt solution (7/9) did not become biphasic until 30°C was exceeded. At 40°C the upper phase was PEG-enriched. Between 40 and 50°C this phase system experienced a phase inversion. The upper, PEG-enriched phase, which made up about thirty percent of the total system volume, migrated down the test tube beneath the previously lower phase. In the 50 and 60°C systems, the lower phase was PEG-enriched. The concentration of PEG in the PEG-enriched phase increased with increasing temperature, from 0.191 to 0.306.

A second PEG–ammonium sulfate phase system with slightly more salt and less PEG was also prepared and studied as a function of temperature. In this 6% PEG–10% salt system (6/10), the two-phase region was entered at temperatures less than 30°C. Like the previous system, a phase inversion occurred between 40 and 50°C. Once again the concentration of PEG in the PEG-enriched phase increased with increasing temperature, in this case from 0.165 to 0.354. For each of the three temperatures at which two-phase systems occurred for the 7/9 and 6/10 systems, the concentration of PEG in the PEG-enriched phase was greater in the 6/10 system.

The third PEG–ammonium sulfate phase system prepared contained 7% PEG and 11% salt (7/11). This system was biphasic below 20°C, and PEG remained the predominant upper phase component until 70°C. Of the three systems studied, the 7/11 phase system contained the highest concentration of PEG in the PEG-enriched phase at any given temperature. Although the highest temperature was required for the 7/9 phase system to become biphasic, this phase system exhibited a phase inversion at the lowest temperature. In all three phase systems the absolute value of the PEG concentration difference between the phases increased markedly with increasing temperature.

These results suggests two methods which may be used to cause a phase system to invert. One method would be to alter the temperature of a phase system. For example, by increasing the temperature from 40 to 50°C, the upper phase in the 6/10 system may be changed from PEG-enriched to salt-enriched. A second method to invert a phase system would be to change the concentration of one of the phase forming components. For example, a 7/9 system at 50°C (in which the lower phase is PEG-enriched) may be inverted by increasing the salt concentration to 11%. Interestingly, this same increase in salt concentration at 30°C would cause a single phase solution to become biphasic.

Since phase inversion involves the lower phase becoming PEG-enriched, the sign of Δw_2 in Eq. 1 changes from positive to negative. The question immediately arises as to whether, as Eq. 1 suggests, the logarithm of a solute's partition coefficient in these phase systems also alters sign.

Pentanol, a relatively hydrophobic uncharged model compound, was initially selected for partitioning studies. Fig. 1 shows the results of partitioning pentanol in the phase systems listed in Table 1 as a function of temperature. In general, the pentanol partitioned into the same phase in which PEG resided. At low temperatures, in phase systems which have their upper phase PEG-enriched, pentanol had a partition

Table 1
PEG concentration (mass fraction) in upper (') and lower (") phase of three PEG–ammonium sulfate aqueous two-phase systems as a function of temperature

T (°C)	7% PEG–9% salt		6% PEG–10% salt		7% PEG–11% salt	
	w_2'	w_2''	w_2'	w_2''	w_2'	w_2''
20	One Phase		One Phase		0.182	0.015
30	One Phase		0.165	0.028	0.228	0.015
40	0.191	0.015	0.194	0.013	0.276	0.013
50	0.0084	0.251	0.010	0.280	0.327	0.0045
60	0.0073	0.306	0.0027	0.354	0.369	0.0034
70	–	–	–	–	0.0036	0.428

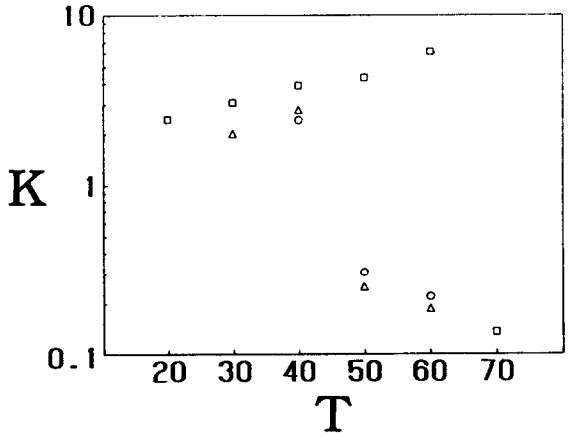

Fig. 1. Observed partition coefficients (K) of pentanol in three PEG–ammonium sulfate aqueous two-phase systems *versus* equilibration temperature. Phase systems: 7% PEG–9% salt (○), 6% PEG–10% salt (△) and 7% PEG–11% salt (□).

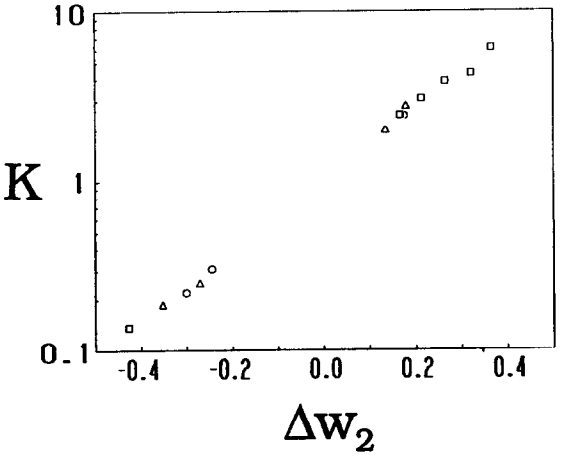

Fig. 2. Observed partition coefficients (K) of pentanol in three PEG–ammonium sulfate aqueous two-phase systems *versus* PEG concentration difference between the phases (Δw_2). Phase systems: 7% PEG–9% salt (○), 6% PEG–10% salt (△) and 7% PEG–11% salt (□).

coefficient greater than one. The partition coefficient in a particular system was observed to increase with increasing temperature until the phases inverted. After the inversion temperature, the partition coefficient became less than 1, and a continued increase in temperature resulted in a decrease in the partition coefficient.

As noted in the introduction, the logarithm of the partition coefficient theoretically may be considered proportional to the tie line length, and hence the PEG concentration difference between the phases. Fig. 2 shows the pentanol partitioning data as a function of the PEG concentration difference, as calculated from the values listed in Table 1. As this figure indicates, the logarithm of the partition coefficient was proportional to the PEG concentration difference between the phases, regardless of whether PEG was the principal upper or lower phase component.

Additional simple solutes were partitioned in these phase systems. Fig. 3 shows the partition coefficients of tryptophol as a function of temperature in the aqueous two-phase systems. Fig. 4 shows data for this solute as functions of the PEG concentration difference between the phases. The results for uncharged tryptophol were similar to those for pentanol, except that the values of the partition coefficients were about five times greater (or less after phase inversion) for tryptophol. Fig. 4 indicates that for this solute also, the logarithm of the partition coefficient was proportional to the PEG concentration difference between the phases. Only at partition coefficients far from unity did the

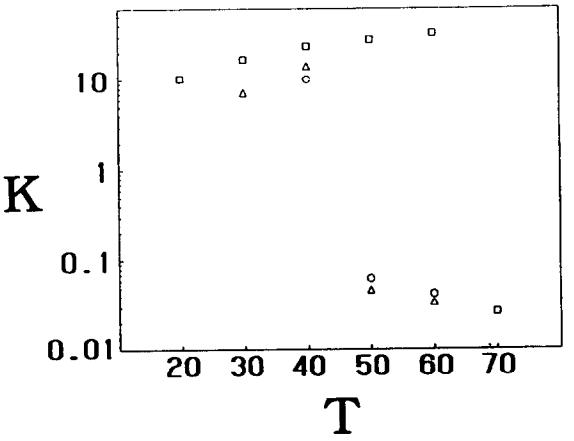

Fig. 3. Observed partition coefficients (K) of tryptophol in three PEG–ammonium sulfate aqueous two-phase systems *versus* equilibration temperature. Phase systems: 7% PEG–9% salt (○), 6% PEG–10% salt (△) and 7% PEG–11% salt (□).

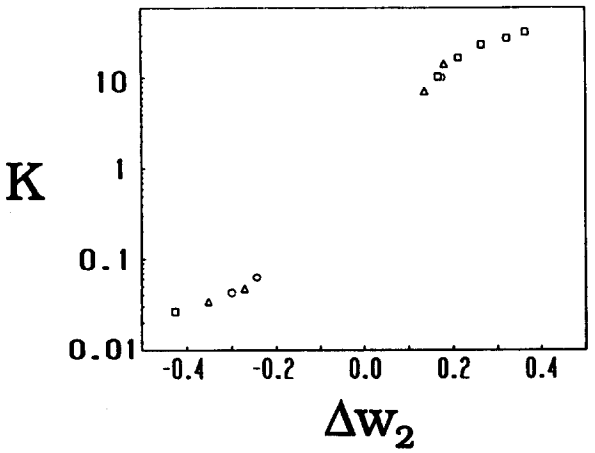

Fig. 4. Observed partition coefficients (K) of tryptophol in three PEG–ammonium sulfate aqueous two-phase systems *versus* PEG concentration difference between the phases (Δw_2). Phase systems: 7% PEG–9% salt (○), 6% PEG–10% salt (△) and 7% PEG–11% salt (□).

results begin to deviate from this theoretical proportionality, an observation explained by Cohen [20].

A less hydrophobic solute, phenylalanine, was also selected to determine the generality of the observed partitioning behavior. Fig. 5 shows the partition coefficients of this solute *versus* temperature. Since the phase systems studied were in the pH range of 5.3–5.8, phenylalanine had essentially no net charge in the aqueous two-phase systems studied. For this particular solute, the partition coefficients were less than 1 at low temperatures. At elevated temperatures, for systems in which the lower phase was PEG enriched, phenylalanine had partition coefficients greater than 1. Fig. 6 shows the partitioning data as a function of the PEG concentration difference between the phases. Once again, the logarithm of the partition coefficient was proportional to the PEG concentration difference, although for this solute the partition coefficient decreased with increasing concentration difference.

Two additional solutes (the dipeptide leucine–phenylalanine and tryptamine) were examined in these three phase systems. Fig. 7 shows the logarithm of the partition coefficient of the dipeptide versus the tie line length. The proportionality predicted by Eq. 1 was again supported. Fig. 8 shows the data for tryptamine, a positively charged analogue of tryptophol. The logarithm of this solute's partition coefficient also was observed to be proportional to the tie line length. The actual value of the partition

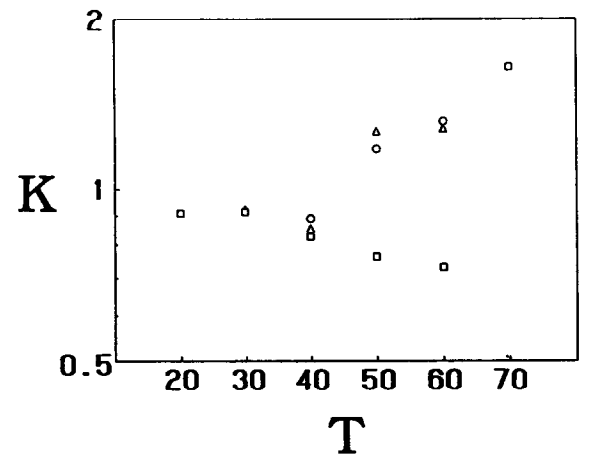

Fig. 5. Observed partition coefficients (K) of phenylalanine in three PEG–ammonium sulfate aqueous two-phase systems *versus* equilibration temperature. Phase systems: 7% PEG–9% salt (○), 6% PEG–10% salt (△) and 7% PEG–11% salt (□).

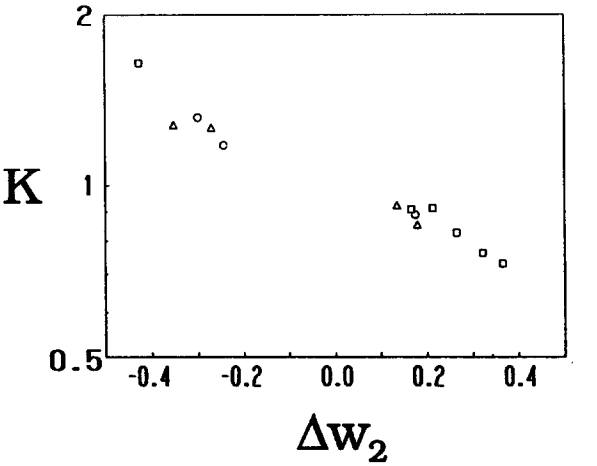

Fig. 6. Observed partition coefficients (K) of phenylalanine in three PEG–ammonium sulfate aqueous two-phase systems *versus* PEG concentration difference between the phases (Δw_2). Phase systems: 7% PEG–9% salt (○), 6% PEG–10% salt (△) and 7% PEG–11% salt (□).

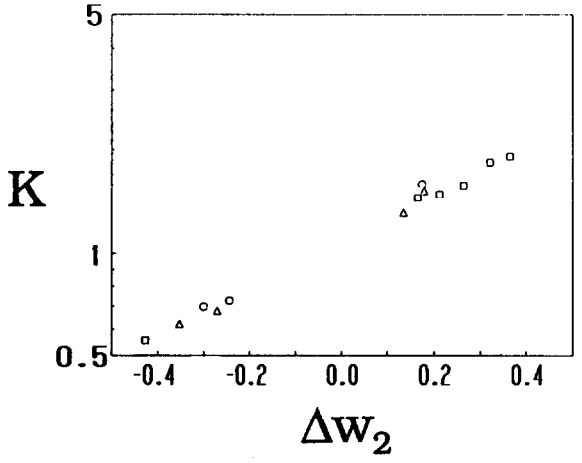

Fig. 7. Observed partition coefficients (K) of leucine–phenylalanine in three PEG–ammonium sulfate aqueous two-phase systems *versus* PEG concentration difference between the phases (Δw_2). Phase systems: 7% PEG–9% salt (○), 6% PEG–10% salt (△) and 7% PEG–11% salt (□).

coefficient in any given system was lower for tryptamine than for tryptophol.

For all five solutes studied, the data were regressed to the linear equation $\ln K = \ln K_0 + A\Delta w_2$. Table 2 shows the results of these regressions. Each of the five best-fitting lines passed very near a "y-intercept" of $K_0 = 1$, supporting

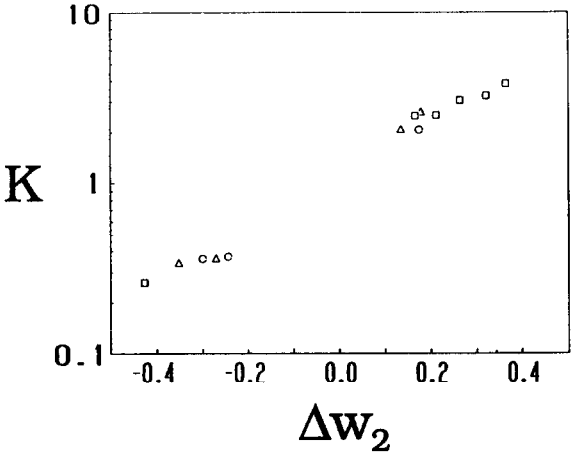

Fig. 8. Observed partition coefficients (K) of tryptamine in three PEG–ammonium sulfate aqueous two-phase systems *versus* PEG concentration difference between the phases (Δw_2). Phase systems: 7% PEG–9% salt (○), 6% PEG–10% salt (△) and 7% PEG–11% salt (□).

Table 2
Results of regressing the best-fitting line ($\ln K = \ln K_0 + A\Delta w_2$) through the observed data for each of the five solutes studied (R = correlation coefficient for $n = 13$)

Solute	A	K_0	R
Tryptophol	5.495	4.66	0.986
Pentanol	4.565	1.12	0.995
Tryptamine	3.074	1.30	0.988
Leu–Phe	1.598	1.09	0.992
Phenylalanine	−0.866	1.03	0.968

the principle that at a PEG concentration difference between the phase of zero (the plait point), any solute should distribute equally between the phases. The slope of the line, A, for each of the best-fitting lines were calculated to be different for the five solutes. Although only four uncharged solutes were examined, the calculated value of A was related to the hydrophobicity of the solutes: the greater the value of A, the greater the solute's hydrophobicity [22]. The exception of this observation was the one charged solute studied, tryptamine, which has a hydrophobicity essentially equivalent to tryptophol. In this case the calculated slope, A, was much less for tryptamine than for its uncharged analogue, tryptophol. The results support previous predictions [23] than the presence of a charge appears to shift the partition coefficients of solutes.

4. Conclusions

The PEG–ammonium sulfate aqueous two-phase system has a previously unobserved property of inverting the phases as the temperature is increased. The inversion takes place at different temperatures in phase systems of different PEG and/or salt concentration. In the three phase systems studied, the lower the component concentrations, the lower the temperature necessary for phase inversion. The logarithm of the partition coefficients of several solutes are nevertheless proportional to the PEG concentration difference between the phases. Several other PEG–salt phase systems have been studied briefly, and

presently no other phase system has been found which exhibits this phenomenon. It appears that altering temperature may serve as an additional and useful tool to shift the partitioning of compounds in aqueous two-phase systems. Clearly, additional studies are needed to determine the effect of this phase inversion on the complex partitioning of larger solutes such as proteins.

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