Preface

A mixture of two polymers, or one polymer and a salt, in an aqueous medium separates into two phases: this phenomenon is useful in biotechnology for product separations. Separation of biological molecules and particles in these *aqueous two-phase systems* (ATPS) was initiated over 40 years ago by P.-Å. Albertsson, and later proved to be of immense utility in biochemical and cell biological research. A boost in the application of ATPS was seen when problems of separations in biotechnology processes were encountered. Its simplicity, biocompatibility, and amenability to easy scaleup operations make the use of ATPS very attractive for large-scale bioseparations. Despite the advantages ATPS enjoys over other separation techniques, the application of two-phase systems has for a long time been confined to selected laboratories. Recent years have, however, shown a trend in which increasing numbers of researchers employ two-phase partitioning techniques in both basic and applied research.

Aqueous Two-Phase Systems: Methods and Protocols is a collection of cutting-edge methods intended to provide practical guidelines for those who are new to the area of separations in two-phase systems. Besides the established methods, many newly developed techniques with potential applications in biotechnology are also described. As an introduction, the first chapter provides a brief general overview of ATPS and its applications. The remainder of the volume is broadly divided into five sections. The first two sections are basic, describing methods for ATPS preparation and characterization, and the various partitioning techniques that may be employed. Multistage partitioning increases the resolving power of ATPS, allowing separation of materials differing only very slightly in physicochemical properties.

Partitioning applied to soluble molecules and particulates has been dealt with in the third section, where examples of different categories of materials are presented. Once the reader is acquainted with the methodology and the "tricks" to be used to obtain the desired partitioning, the separation technique may then be applied to any material of interest. Separation of particulates—including whole cells, membranes, and organelles—has been a major achievement of ATPS, one that greatly facilitates studies on cells and their properties. Purification of viruses is another successful example. With regard to soluble

molecules, partitioning has been most commonly applied to the separation of macromolecules, since their distribution between the two phases is influenced to a greater extent by a system variation than is the distribution of small molecules. This has enabled the application of ATPS even as an analytical tool to determine, e.g., the concentration and isoelectric point of proteins, molecular interactions, conformational changes of biomolecules, and so on. Lately, applications of ATPS in the separation of small molecules have also emerged. Molecules with defined properties are proving useful for understanding the interactions involved during partitioning, which would be helpful in the selection of appropriate phase systems for specific separation problems.

The main application of ATPS in biotechnology has been the isolation and purification of proteins; hence a significant part of *Aqueous Two-Phase Systems: Methods and Protocols*, compiled as Part IV, is devoted to this subject, including a glimpse of the large-scale handling of the two-phase separations. The real success of this technique has been in the extraction of proteins directly from crude feedstocks, where it has provided clarification, concentration, and even some purification in a single step. The extraction of proteins by spontaneous partitioning alone necessitates optimization of various parameters. The need to improve the selectivity of extractions has also led to exploitation of charge–charge, hydrophobic, and affinity interactions, in which specific binding groups are located in the phase used as the extractant. Integration of ATPS with other separation techniques provides scope for facilitating such selective extractions. Limiting the material costs for large-scale purposes still remains a challenge. The recycling of phase components is thus essential, which is easily done for some phase chemicals, but not for others. New phase materials with easy recyclability are being studied.

There has been interest in using aqueous two-phase systems in another area of biotechnology, i.e., *in situ* product recovery during biocatalytic processes. This concept has been presented in the last section of the volume. Analogous to aqueous systems are the newly developed polymer–polymer systems in organic solvents, which are useful with synthetic reactions.

My hope is that *Aqueous Two-Phase Systems: Methods and Protocols* will not only prove helpful in your research, but will also lead to discovery of the surprises and pleasures of aqueous two-phase systems separations. I wish to thank all the contributors to this volume for sharing their knowledge and practical experience with the reader. Special thanks are due to Associate Professor Göte Johansson, Emeritus Professor P.-Å. Albertsson, and Professor Bo Mattiasson for their useful suggestions.

Rajni Hatti-Kaul

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The Phase Diagram

Anita Kaul

1. Introduction

The *phase diagram* delineates the potential working area for a particular two-phase system and is a "fingerprint" unique to that system under set conditions of, for example, pH, temperature and salt concentration. Information that can be generated from such a diagram (*see* **Fig. 1**) includes: the concentration of phase-forming components necessary to form a system with two phases that are in equilibrium, the subsequent concentration of phase components in the top and bottom phases, and the ratio of phase volumes. Present on the diagram is a *binodal* curve, which divides a region of component concentrations that will form two immiscible aqueous phases (i.e., above the curve) from those that will form one phase (i.e., at and below the curve). Coordinates for all "potential" systems will lie on a *tie-line*; the tie-line connects two *nodes* on the binodal, which represent the final concentration of phase components in the top and bottom phases. Moving along the tie-line coordinates denote systems with differing *total* compositions and volume ratios, but with the same *final* concentration of phase components in the top and bottom phases. Also present on the binodal is a *critical point*; just above this point the composition and volume of both phases are almost equal (as is partitioned material). A theoretical account of the phase diagram can be found in **refs. *1–3***.

1.1. The Binodal

For scientists new to the field of aqueous two-phase technology, it is advisable to start with construction of a binodal so that a systematic choice of systems can be used for preliminary partitioning experiments, e.g., systems are often chosen by extrapolating through the critical point. If, however, published phase diagrams are used, a few systems should be made up and the resultant

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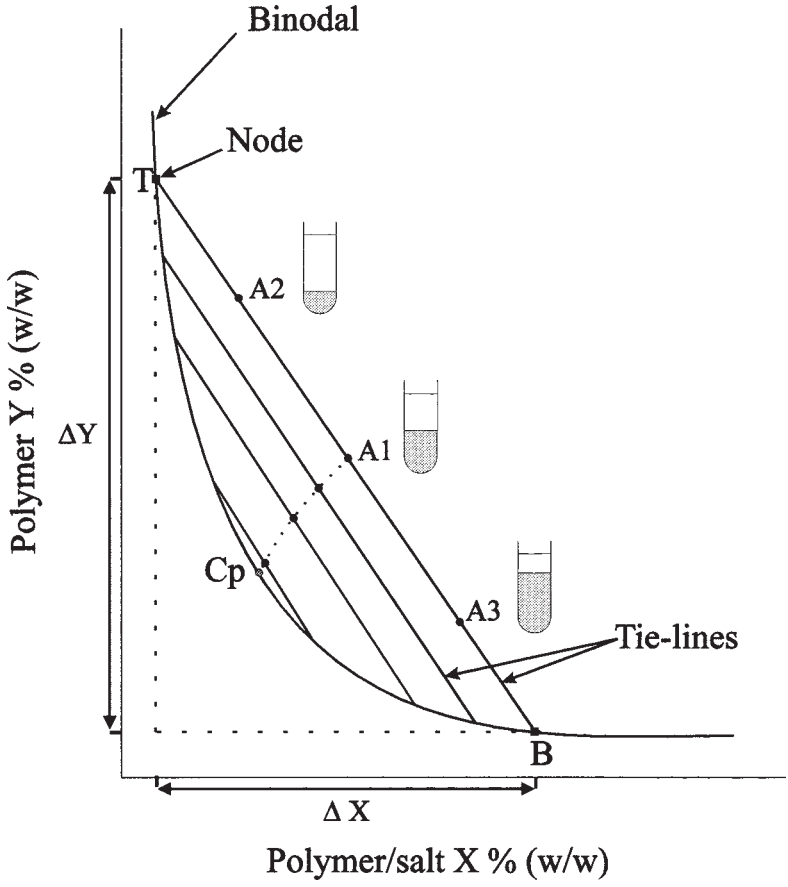

Fig. 1. Illustration of the phase diagram. Bottom phase polymer/salt X (% w/w) is plotted on the abscissa and top phase polymer Y (% w/w) is plotted on the ordinate. A1, A2, and A3 represent the total compositions (●) of three systems lying on the same tie-line with different volume ratios. The final composition of the top and bottom phase is represented by nodes T and B (■), respectively. The ratio of the segments AB (top phase) and AT (bottom phase) can be approximated graphically by the volume ratio of the two phases. The critical point, Cp (○) is determined by extrapolation (------) through the midpoints of a number of tie-lines. The difference in concentration of component X and Y between the two phases is represented by ΔY and ΔX.

volume ratios and/or phase compositions should be compared to those predicted; thus, ensuring that similar experimental conditions have been achieved. Chemicals commonly used for two-phase formation are described in the following chapter. Novel systems that comprise thermoseparating polymers and affinity ligands are also reported in Chapters 26 and 29–31.

Three methods for the preparation of a binodal are described. When phase components are mixed, only when immiscibility occurs does the resultant mixture become "turbid;" this permits a "visual" measurement of the binodal. Turbidometric titration is a relatively quick and commonly used method for determination of the binodal. A series of systems of known total composition and weight are prepared. Upon dilution with the appropriate solvent, the mixture will eventually turn "clear" when one phase is formed. The resultant composition, at the point of transition, is calculated and lies on the binodal. The cloud-point method follows a similar principle. A concentrated stock of component 1 (e.g., dextran or phosphate) is added drop-wise to a known amount of a concentrated stock of component 2 (e.g., PEG). At a critical point—the cloud point—the mixture will become turbid and is indicative of two-phase formation. The composition, just prior to two-phase formation, is calculated and provides a point on the binodal. The mixture is then diluted to below the cloud point and the procedure is repeated. An alternative method is by determination of the nodes for a series of systems, thus, providing points on the binodal. The former methods are relatively inaccurate when using polymers that are polydisperse. Such polymers produce a gradual decrease/increase in turbidity rather than a sharp change, making the point of transition awkward to calculate.

1.2. Tie-Line Length

The tie-line length (TLL) has the same units as the component concentrations (i.e., %w/w) and is often used to express the effect of system composition on partitioned material. The ratio of segments AB and AT (*see* **Fig. 1**) can be estimated graphically by using the weight ratio $V_t p_t / V_b p_b =$ AB/AT where V and ρ are the volume and density of the top (*t*) and bottom (*b*) phase. A more precise method is by analysis of the top and bottom phase composition where the $\mathrm{TLL} = (\Delta X^2 + \Delta Y^2)^{1/2}$, *X* denotes the concentration of component 1 and *Y*, the concentration of component 2 (*see* **Fig. 1**). However, if the total composition and weight ratio are known, then analysis of one phase is sufficient by using, $V_t p_t / V_b p_b = X_b - X_0 / X_0 - X_t$ where X denotes the concentration of component 1 in the top phase (*t*), bottom phase (*b*) and the total system (0) (the same holds true for component 2) ***(1)***. Tie-lines are commonly parallel and hence the slope of the tie-line (STL) can also be calculated, $\mathrm{STL} = \Delta Y_1 / \Delta X_2$ thus, facilitating the construction of further tie-lines ***(3)***. Phase density can be determined using a pycnometer or by weighing a known volume of phase in a volumetric flask using an analytical balance. Analysis of phase composition is routinely carried out by using a combination of either optical rotation (for phase components containing an asymmetric center that can rotate a plane of polarized light), refractive index, dry weight, or conductivity measurements ***(4)***.

1.3. Critical Composition

As tie-lines decrease in length, they ultimately approach a critical point on the binodal where the TLL = 0. At this point the composition and volume of the two phases theoretically become equal. The critical composition can be determined by trial and error where a one-phase system, just below the binodal, forms a two-phase system with approximately equal volumes of the two phases on addition of one component. A less tedious method is by extrapolation through the mid-point of a number of tie-lines near the binodal (*see* **Fig. 1**).

1.4. Operation Point

Once a phase diagram is constructed, the point of operation is dependent on acquiring 1) the desired conditions for partition of the target molecule(s) ***(5)***, cells or cell particles ***(6)*** and 2) an extreme phase volume ratio (if a concentration step is required). However, if systems are to be scaled-up and/or phases are to be recycled, then the working point should also take into account the position relative to the binodal. In such cases, small variations in phase composition are likely to occur and the precision that is achieved at bench-scale may not as easily be achieved at large scale. A system should therefore be "robust," and the consequence of any changes on the physical and chemical characteristics of the system should be considered. Check points should include: 1. dramatic changes in composition may occur near the critical point, and 2. a one phase system may form near the binodal. This may be owing to an unaccounted dilution effect or a variation in concentration of component 1 or component 2. Therefore, systems will differ in stability and the result of an individual change, or a combination of changes, will give a corresponding deviation in the tie-line length and, thus, in the composition and volume of the phases. The behavior of the partitioned material may, as a result, differ from that which would have been obtained in the "selected" system and the separation/purification is rendered ineffective.

2. Materials

1. Chemicals: A variety of phase chemicals and their stock preparation and storage can be found in Chapter 3. Stock solutions are prepared by weight and, therefore, the hydration of any salts used should be accounted for. For polymeric systems, each polymer may be made up in buffer, e.g., 10–100 m*M* phosphate buffer, or double-distilled water. Alternatively, make a concentrated stock of buffer at the required pH and add to the system to obtain the desired final concentration.
   Typical concentrations of stock solutions (*see* **Note 1**) are as follows.
   a. 20–30% (w/w) for various glucose polymers, e.g., dextran (average molecular weights ranging from 3400–460,000) (Amersham Pharmacia Biotech AB, Uppsala, Sweden), Reppal PES 100 (M_r 100,000) and Reppal PES 200 (M_r 200,000) and amylopectins (both from Carbamyl AB, Kristianstad, Sweden).

b. 30–50% (w/w) for PEGs (*see* **Note 2**). A range of molecular weights can be used e.g., 300–20,000 (low molecular weights are in liquid form and may therefore be used as 100%) (Serva, Heidelberg/New York).
c. 20% (w/w) for $MgSO_4$, 30–40% (w/w) for phosphate (mixture of the monobasic and dibasic salt at the required pH, *see* **Note 3**) and 25% (w/w) for citrate (mixture of trisodium citrate and citric acid at the required pH). All salts should be of analytical grade.

2. Apparatus: For phase analysis, a refractometer (Carl Zeiss, Germany), polarimeter (for optically active components) (Optical Activity Ltd, UK) and a conductivity meter (for systems with salt) (Metrohm, Switzerland) are required (*see* **Subheading 3.2.**).

3. Methods

3.1. Determination of the Binodal

By convention, the component predominantly in the bottom phase is plotted as the abscissa and the component predominantly in the top phase is plotted as the ordinate. The three methods are illustrated graphically in **Fig. 2** (*see* **Note 4**).

3.1.1. Turbidometric Titration

1. In test tubes, using the appropriate stock solutions, prepare systems with different compositions of known weight. Account for the additional volume owing to titration, e.g., if 5 g systems are prepared, 10 mL test tubes should be used (*see* **Note 5**). As an example **Table 1** shows systems that can be used for various PEG-phosphate and PEG-dextran systems, and the necessary calculations. This table may be reproduced in a spreadsheet program to allow ease of calculation.
2. Note the weight of the test tube and titrate, drop-wise, with the appropriate diluent (*see* **Note 7**) until the system just turns clear, i.e., one phase is formed. This can be carried out while the system is continually being mixed or by adding a drop, mixing, adding a second drop, and so on. To ensure that a one-phase system has formed, systems should be centrifuged (e.g., 1000–2000*g*, 5 min) (*see* **Note 8**).
3. Note the final weight of the test tube and calculate the weight of diluent added just prior to one-phase formation.
4. Because the number of systems titrated is proportional to the number of points on the binodal, greater accuracy is achieved with a greater number of systems (*see* **Note 9**).

3.1.2. Cloud Point Method

1. Weigh 5 g of a stock solution of component X into a 25-mL conical flask.
2. Weigh the flask and add, drop-wise, a stock solution of component Y until the first sign of turbidity, i.e., the cloud point. Mix as previously noted.
3. Note the weight of component Y necessary for the mixture to cloud. This provides the first point on the binodal. Refer to **Table 2** for calculations.
4. Add a known weight of diluent (*see* **Note 7**) to below the cloud point and repeat as noted.

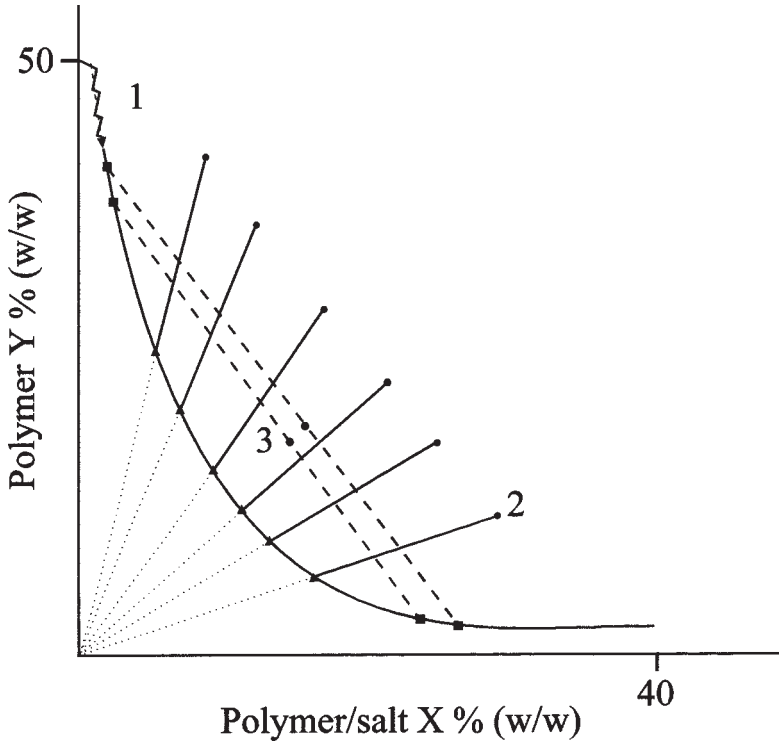

Fig. 2. Graphical representation of three methods used to determine the binodal. **1.** illustrates the cloud point method, where a concentrated stock of component 1, i.e., polymer/salt X is added to a concentrated stock of component 2, i.e., polymer Y. The solution is repeatedly taken above and below the cloud point; the binodal lies between these two points (shown by the "zig zag" line). **2.** illustrates turbidometric titration where a series of systems (●) are prepared and titrated until a one-phase system is formed—the binodal lies just above this point (▲). **3.** illustrates the determination of nodes (■) for systems, which is accomplished by preparing a series of systems lying on different tie-lines (------●------) and analyzing the concentration of components in the top and bottom phase.

3.1.3. Node Determination

1. Prepare a series of systems and analyze the phase composition of the top and bottom phase. (*See* **Subheading 3.2.**).
2. To aid in the selection of systems, start with, e.g., 5% (w/w) of component X and 5% (w/w) of component Y. If the resultant mixture forms one phase, then prepare additional systems in, e.g., 2% (w/w) increments until two phases form. Continue to prepare additional systems in this way for phase concentration analysis.

Table 1
System Composition for Various PEG-Phosphate (bold) and PEG-Dextran (unbold) Systems (*see* Note 6) and the Necessary Calculations for Binodal Determination Using Turbidometric Titration

	A	B	C	D	E	F	G	H	I	J
1										
2		**Stock % (w/w)**								
3	**Component X**	40								
4										
5	**Component Y**	50								
6										
7	**Salt *eg* NaCl**	100								
8										
9	**Final weight**									
10	**of system (g)**	5								
11										
12										
13	**System composition**			**Amount of stock**					**Final composition**	
14										
15	**X**	**Y**	**Salt**	**X stock**	**Y stock**	**Salt**	**Buffer**	**Diluent**	**X *final***	**Y *final***
16	**% (w/w)**	**% (w/w)**	**% (w/w)**	**g**	**g**	**g**	**g**	**g**	**% (w/w)**	**% (w/w)**
17										
18	**7.0** / 1.0	**27.5** / 7.0								
19	**7**	**27.5**	**6.0**	**0.88**	**2.75**	**0.3**	**1.07**	d		
20				=(A19*B10)/B3	=(B19*B10)/B5	=(C19*B10)/B7	=B10-(D19+E19+F19)		=(A19*B10)/(B10+H19)	=(B19*B10)/(B10+H19)
21	**7.5** / 2.0	**25.0** / 6.0								
22	**8.0** / 3.0	**22.5** / 5.5								
23	**8.5** / 4.0	**20.0** / 5.0								
24	**11.0** / 4.5	**17.5** / 4.5								
25	**12.5** / 6.0	**15.0** / 4.0								
26	**13.5** / 7.0	**12.5** / 3.5								
27	**15.0** / 9.0	**10.0** / 3.0								
28	**17.5** / 10.0	**7.5** / 2.5								
29	**20.0** / 11.0	**5.0** / 2.0								
30	**22.5** / 13.0	**2.5** / 1.0								

X, concentration of polymer/salt X; Y, concentration of polymer Y; X, Y final, composition on the binodal; d, amount of diluent required just to pne-phase formation. Example shown has the necessary calculations below the values.

Table 2
Calculations for Binodal Determination Using the Cloud Point Method

	A	B	C	D	E	F	G
1							
2		**Stock % (w/w)**					
3	**Component X**	40					
4							
5	**Component Y**	50					
6							
7							
8	**Amount of stock**			**Final composition**			
9							
10	**X stock**	**Y stock**	**Total Y stock**	**X final**	**Y final**	**Dilutent**	**Total weight**
11	**g**	**g**	**g**	**% (w/w)**	**% (w/w)**	**g**	**g**
12							
13	5	y		=(B3*A13)/(A13+B13)	=(B5*B13)/(A13+B13)	d	
14		*y1*	=B13+B14	=(B3*A13)/(A13+F13+C14)	=(B5*B13)/(A13+F13+C14)	d*1*	=F13+F14
15		*y2*	=C14+B15	=(B3*A13)/(A13+G14+C15)	=(B5*B13)/(A13+F14+C15)	d*2*	=G14+F15
16		*y3*	=C15+B16	=(B3*A13)/(A13+G15+C16)	=(B5*B13)/(A13+F15+C16)	d*3*	=G15+F16
17		*y4*	=C16+B17	=(B3*A13)/(A13+G16+C17)	=(B5*B13)/(A13+F16+C17)	d*4*	=G16+F17

Example shown starts with 5 g of the stock solution of component X. A known amount of the stock solution of component Y is required to cloud the solution. X, Y final: concentration of component X and Y at the point of clouding, y: amount of stock Y required just prior to clouding and d: diluent necessary to reach below the cloud point.

3.2. Tie-Line Determination

Tie-line measurement for polymeric systems containing one optically active component is described in **Subheading 3.2.1.**, e.g., PEG-dextran, PEG-hydroxypropyl starch, PEG-Ficoll, and ethylene oxide-propylene oxide-Reppal PES 100 (*see* Chap. 26 for thermoseparating polymers; *see* **Subheading 3.2.2.** for polymer-salt systems).

3.2.1. Polymeric Systems Containing One Optically Active Polymer

1. Prepare a standard curve for the optically active component, in the range of 0–10% (w/v), i.e., within the linear range (*see* **Note 10**), with the same samples prepare a second standard curve for the refractive index measurement. Prepare a third standard curve for the refractive index of the second polymer also in the range of 0–10% (w/v). If the system is prepared in a buffer then the standard curves for the pure components should be made with the same buffer, because salts also contribute to the refractive index (*see* **Note 11**).
2. Prepare the phase system for analysis, making sure that the phase components are mixed thoroughly; allow phases to separate. To ensure complete separation, centrifuge at a low speed (e.g., 1000–2000*g*, 5 min). The size of the system should be sufficient to allow the removal of at least 5 g of top and bottom phase for phase concentration analysis and a further amount for density measurements.
3. Separate the top and bottom phases making sure not to cause phase mixing.
4. Make appropriate dilutions, e.g., dilute 5 g of phase, with the appropriate solvent, to 25 mL in a volumetric flask.
5. Measure the optical rotation for each phase and calculate the respective concentrations.
6. The concentration of the second component is determined by measuring the refractive index of the top and bottom phase and by subtracting the refractive index contribution made by the optically active component.

3.2.2. Polymer-Salt Systems

1. Prepare a standard curve for the conductivity of the salt within the linear range (in %w/v).
2. Prepare phase systems as previously noted and remove 5 g samples from the top and bottom phase and dilute with water (e.g., 1/5) and freeze-dry. Note the dry weight.
3. Remove a further sample from the top and bottom phase, dilute with water, and measure the conductivity of each phase. Calculate the concentration of salt and subtract the weight contribution from the dry weight of the sample.

4. Notes

1. For greater accuracy, the concentration of all stocks should be analyzed using the appropriate method, i.e., refractive index for PEGs, optical rotation for glucose polymers, and conductivity for salts.

2. PEGs are hygroscopic and in humid conditions will pick up water. When preparing standard curves, take into account any absorbed water by, for example, determining the concentration by freeze drying.
3. K_2HPO_4 and NaH_2PO_4 display greater solubilities than their respective monobasic and dibasic salts.
4. The shape and position of the binodal is dependent on the chemicals used.
   a. Generally, high-polymer molecular weights, the addition of salt, lowering the temperature, and the presence of cell debris *(7)* all serve to decrease the concentration of phase forming chemicals necessary to form two phases.
   b. Polymer-salt systems and an increased difference in the molecular weight of the two polymers in polymer–polymer systems, both contribute to the asymmetry of the binodal.
5. When measuring the volume of the two phases, graduated test tubes are used that are further calibrated for increased accuracy. Alternatively, graduated pipets with the top end cut off and the bottom end sealed can be used.
6. Concentrations in bold face can be used for PEG-phosphate systems for PEG molecular weights ranging from 1500–20,000 at 20°C and pH 7.0. Other concentrations can be used for PEG 6000-Dextran T500 (M_r 500 000) systems at 20°C. Generally, for PEG-salt systems, higher concentrations of components are required to form two phases, when compared to polymer–polymer systems. For this method, compositions should be chosen with this in mind. If systems are prepared and form one phase, then add a known weight of component X until two phases form, calculate the resultant composition, and begin to titrate.
7. When systems are prepared in a buffer or have added salts, e.g., NaCl, the same solvent should be used when titrating/diluting, thus ensuring that only concentrations of component X and Y are decreasing. If, for example, PEG 4000–phosphate with 6% (w/w) NaCl or PEG 6000–dextran in 10 m*M* phosphate is used, then accordingly 6% (w/w) NaCl or 10 m*M* phosphate is required, respectively.
8. Centrifugation is especially advisable when using polymers that are polydisperse.
9. Generally, only the midsection of the binodal is resolved using this method. Extremes of the binodal are difficult to measure because points are almost parallel to the axes and, therefore, the cloudpoint method or determination of the nodes for systems far from the critical point can be used for this purpose.
10. The polymer concentration can also be determined with the following equation, $[P]_v = \frac{\phi}{1\,[\alpha]}$ where $[P]_v$ is the sample polymer concentration (g/100 mL); ϕ, the optical rotation; l the sample tube length in decimeters and α the specific optical rotation (°mL/dm/g) at 25°C with the sodium D line. The sample concentration can then be converted to % (w/w) (i.e., $[P]_w$ by using the following equation, $[P]_w = [P]_v / \rho$ where ρ is the density of the phase (the same equation is used for $[P]_v$ obtained by refractive index measurement).
11. If systems are made up with buffer or others salts, conductivity measurements should be carried out to ensure that their partition is equal between the two phases (and therefore that the refractive index contribution is the same for the two phases).

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