A NEW LIQUID-LIQUID PARTITIONING SYSTEM FOR
BIOSEPARATIONS AT LOW TEMPERATURES

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Fritz J. and Dolores H. Russ
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Liqin Zhang

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Table of Contents

Chapter 1  Introduction..............................................1

Chapter 2  Literature Review...........................................3

2.1  Concept of Liquid-Liquid Partitioning............................3

2.2  The Choice of a Solvent System....................................3

2.2.1  The Thermodynamics of Phase Separation ..................3

2.2.2  Requirements for a Suitable Solvent System...............4

2.3  Partitioning of Proteins and Peptides............................5

2.3.1  Origination..................................................5

2.3.2  Partitioning in Aqueous Two-Phase Systems...............5

2.3.3  Two-Phase Aqueous Surfactant Systems.......................6

2.3.4  Liquid-Liquid Extraction with Reversed Micelles.........7

2.4  Extraction of Antibiotics........................................8

2.4.1  Solvent Extraction...........................................8

2.4.2  Extraction of Antibiotics with Reversed Micelles..........12

2.5  Acetonitrile-Water Liquid-Liquid Equilibrium................13

2.6  Advantages of the Two-Phase Acetonitrile-Water System......14
5.4 Partitioning of Human Hemoglobin in the ACN-Water Two-Phase System

5.5 The Effect of Temperature

5.6 The Effect of Salt Concentration

5.7 Separation of a Trypsin-Antimycin Mixture

Chapter 6 Conclusions and Recommendations

6.1 Conclusions

6.2 Recommendations

Bibliography

Appendix A - Wavelengths Analyzed for the Biomaterials

Appendix B - Calibration Curves of the Biomaterials (Absorbance vs. Concentration)

Appendix C - Contents of Buffer Solutions
<table>
<thead>
<tr>
<th>Table 1.</th>
<th>Biomaterials Used in Partitioning Experiments</th>
<th>26</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 2.</td>
<td>The Partition Coefficient of Biomaterials in Unbuffered ACN-Water System at -10.0 °C</td>
<td>30</td>
</tr>
<tr>
<td>Table 3.</td>
<td>The Effect of pH on the Partitioning of Antibiotics at -10.0 °C</td>
<td>38</td>
</tr>
<tr>
<td>Table 4.</td>
<td>The Effect of pH on the Separation of Trypsin-Antimycin Mixtures at -10.0 °C</td>
<td>59</td>
</tr>
</tbody>
</table>
List of Figures

Figure 1. Phase diagram of the ACN-water two-phase system (Pence and Gu, 1996) ................................................................. 13

Figure 2. Ionization states of amino acids at different pH values (Stryers, 1988) ........................................................................... 21

Figure 3. Shapes of equilibrium curves and their implications (Blumberg, 1988) ........................................................................... 24

Figure 4. Equilibrium isotherm of erythromycin obtained at -10.0 °C .......................... 32

Figure 5. Equilibrium isotherm of phenylalanine obtained at -10.0 °C ........ 33

Figure 6. The effect of pH on the partitioning of four proteins (ribonuclease A, transferrin, thyroglobulin and trypsin) at -10.0 °C ........................................................................................................... 35

Figure 7. The effect of pH on the partitioning of two amino acids (tryptophan, phenylalanine) at -10.0 °C ........................................ 36

Figure 8. The effect of pH on the partitioning of two peptides (val-ala-ala-phe, phe-gly-gly-phe) at -10.0 °C ........................................ 37

Figure 9. The effect of pH on the partitioning of fusidic acid at -12.0 °C. (Analyzed at 230 nm.) ......................................................... 39

Figure 10. The effect of pH on the partitioning of gramicidin D at -12.0 °C. (Analyzed at 404 nm.) ......................................................... 40
Figure 11. The effect of pH on the partitioning of human hemoglobin at -7.3 °C. (Analyzed at 404 nm.) .................................................. 41

Figure 12. The effect of pH on the partitioning of human hemoglobin at -7.3 °C. (Analyzed at 273 nm.) .................................................. 42

Figure 13. The bottom phase of human hemoglobin separated by unbuffered ACN-water system at -7.3 °C ........................................ 44

Figure 14. The lyophilized bottom phase of human hemoglobin separated by unbuffered ACN-water system at -7.3 °C .......... 45

Figure 15. The spectrum of buffered human hemoglobin solution at pH=2.40 (without ACN) at -7.3 °C ............................................. 46

Figure 16. The top phase of human hemoglobin separated by ACN-water system at pH=3.78 (-7.3 °C) ................................. 47

Figure 17. The lyophilized top phase of human hemoglobin separated by ACN-water system at pH=3.78 (-7.3 °C) ............. 48

Figure 18. The bottom phase of human hemoglobin separated by ACN-water system at pH=3.78 (-7.3 °C) .................. 49

Figure 19. The lyophilized bottom phase of human hemoglobin separated by ACN-water system at pH=3.78 (-7.3 °C) ............ 50

Figure 20. The effect of temperature on the partition coefficient of three amino acids (phenylalanine, tryptophan and tyrosine) in unbuffered ACN-water system ........................................ 51
Figure 21. The effect of temperature on the partitioning of two peptides (phe-gly-gly-phe, val-ala-ala-phe) in unbuffered ACN-water system .......................... 52

Figure 22. The effect of temperature on the partitioning of two antibiotics (spiramycin and tetracycline) in unbuffered ACN-water system .................................................. 53

Figure 23. The effect of temperature on the partitioning of two antibiotics (demeclocycline and vancomycin) in unbuffered ACN-water system .................................................. 54

Figure 24. The effect of temperature on the partitioning of three antibiotics (chloramphenicol, cycloheximide, erythromycin) in unbuffered ACN-water system .................................. 55

Figure 25. The temperature effect on fusidic acid, antimycin A and gramicidin D (antibiotics) in unbuffered ACN-water system .................................................. 56

Figure 26. The effect of salt concentration on the partitioning of erythromycin (antibiotic) in unbuffered ACN-water system at -10.0 °C ........................................... 57

Figure 27. The effect of salt concentration on the partitioning of phenylalanine (amino Acid) and phe-gly-gly-phe (peptide) in unbuffered ACN-water system at -10.0 °C .............. 58
Figure 28. The top phase of trypsin-antimycin mixture separated at pH=3.82 (-10.0 °C).................................60

Figure 29. The bottom phase of trypsin-antimycin mixture separated at pH=3.82 (-10.0 °C).................................61

Figure 30. Calibration curve of spiramycin...........................................69

Figure 31. Calibration curve of gramicidin D.................................69

Figure 32. Calibration curve of cycloheximide...............................70

Figure 33. Calibration curve of fusidic acid.................................70

Figure 34. Calibration curve of antimycin A.................................71

Figure 35. Calibration curve of tetracycline.................................71

Figure 36. Calibration curve of erythromycin...............................72

Figure 37. Calibration curve of chloramphenicol...........................72

Figure 38. Calibration curve of vancomycin.................................73

Figure 39. Calibration curve of demeclocycline............................73

Figure 40. Calibration curve of ribonuclease A.............................74

Figure 41. Calibration curve of transferrin .................................74

Figure 42. Calibration curve of thyroglobulin...............................75

Figure 43. Calibration curve of tryptophan.................................75

Figure 44. Calibration curve of phenylalanine.............................76

Figure 45. Calibration curve of tyrosine......................................76
Figure 46. Calibration curve of trypsin.............................................. 77
Figure 47. Calibration curve of phe-gly-gly-phe.................................. 77
Figure 48. Calibration curve of val-ala-ala-phe.................................... 78
Chapter 1 Introduction

Recently, there have been considerable interests in the efficient recovery, purification and separation of biological substances as the scale of production increases. Liquid-liquid partitioning of bioproducts allows continuous steady-state operation which is beneficial both for large scale production and quality control (Asenjo, 1994), an essential feature of modern process biotechnology. The liquid-liquid partitioning method is easier to scale up than the chromatographic method, another method of purification and separation for bioproducts that requires high operational costs.

Acetonitrile (ACN) and water mixtures split into two liquid phases at a temperature below -1.32 °C (Pence and Gu, 1996). The top phase is acetonitrile-rich and the bottom phase is water-rich. Previous work (Gu et al., 1994) shows that at -17 °C the hGH120R protein, a human growth hormone antagonist, tends to partition into the water-rich bottom phase by greater than 99%. The ACN-rich phase could then be removed, thus reducing the amount of ACN remaining to be removed in the freezing drying up. This also brings up a potential liquid-liquid partition system for bioseparations. Since different substances may have different partition behavior, the potential partition discrimination of this system may lead to purification and separation of these materials.

When compared to the more traditional two-phase organic solvent-water systems (Johansson, 1985), two-phase aqueous polymer systems (Albertsson, 1971), two-phase aqueous surfactant systems (Liu et al., 1995), and two-phase extraction with reversed
micelles (Pires et al., 1996), the acetonitrile-water two-phase system at low temperatures offers a number of unique, desirable features.

The low temperature environment of this system offers better stability for the biomaterials. In some previous developed organic solvent-water systems, the high concentrations of organic solvent that extract the biomaterials, along with unfavorable temperatures (e.g., proteins favor temperatures at or under 4 °C), are often the major contributors to denaturation during the process. Since phase separation only occurs below -1.32 °C for the acetonitrile-water system, and 35% of the ACN-rich top phase is water at applicable temperatures (Pence and Gu, 1996), the chances for denaturation are reduced. Moreover, since acetonitrile is one of the most widely used mobile phases in reversed-phase liquid chromatography, the product of the partition step can be directly applied to high performance liquid chromatography (HPLC), the final purification step in a bioseparation process to yield high purity products.

In this work, the partition coefficient, which is defined as the ratio of the biomaterial concentration in the top phase to that in the bottom phase, is tested for 20 substances under various pH values and temperatures. They include common proteins with varying molecular weights, amino acids, peptides, and several antibiotics. Equilibrium isotherms were plotted for an amino acid and an antibiotic to reveal the mode of transfer of these molecules in this system. Experimental data were compared and analyzed to probe the mechanism behind the partitioning of different molecules.
2.1 Concept of Liquid-Liquid Partitioning

Liquid-liquid partitioning is the process of transferring a solute from one liquid phase to another immiscible or partially miscible liquid in contact with the first. Solvent extraction is an old concept, fully integrated into organic chemistry since the early days. The basis for this type of solvent extraction is preferential solubility, and its aim is recovery and/or purification in a system comprised of two immiscible liquids (Blumberg, 1988). The aspect that distinguishes the concept of liquid-liquid partitioning from the solvent extraction concept is that liquid-liquid partitioning relates to separation, and the two liquids are therefore intimate parts of the separation system.

2.2 The Choice of a Solvent System

2.2.1 The Thermodynamics of Phase Separation

A state of equilibrium is characterized as having a minimum Gibbs Free Energy at a given temperature, pressure, and composition (Walas, 1985).

The mathematical condition for phase separation is that

$$\frac{\partial G}{\partial x} = 0, \quad \frac{\partial^2 G}{\partial x^2} < 0.$$  \hspace{1cm} (1)

(G = The Gibbs Free Energy; x = molar fraction of a component of the mixture)

If there are values of compositions that satisfy these conditions, immiscibility will occur. The range of immiscibility can decrease or increase gradually by reason of temperature change or other factors.
2.2.2 Requirements for a Suitable Solvent System

A solvent system should provide suitable distribution ratios or partition coefficients for the partition separation. According to Morris and Morris (1964), a solvent system suitable for partitioning must fulfill certain conditions, including the most important listed here:

1. The components of the system must not react irreversibly with solutes.
2. The extraction phase must permit the ready recovery of the solutes from it.
3. The partition isotherms of the solutes in the selected system should be linear over a considerable concentration range.
4. The interfacial tension should be appropriate for contact in the solvent recovery system. High values for interfacial tension result in an increased energy requirement to maintain sufficient contact time. Low interfacial tensions result in the formation of stable emulsions that makes separation more difficult.

If a solvent system is to be applied to a large-scale production, where counter-current distribution is a powerful and well-studied separation method, it must always form two discrete liquid phases. This property must also be retained in the presence of appreciable concentrations of the solutes. The two phases should therefore differ in density, and neither should have a high viscosity; moreover, the interfacial tension should not promote the formation of stable emulsions.
2.3 Partitioning of Proteins and Peptides

2.3.1 Origination

The partition of proteins had its origin in the early 1950s when Craig searched for a two-phase system useful for protein separation in his newly invented counter-current distribution apparatus (Johansson, 1985). The systems tested were of the organic solvent-water type. The organic phases selected were butanol and ethanol so that they would form a butanol-water or an ethanol-aqueous salt solution. To increase the solubility of peptides and proteins in the organic phase, trichloroacetic acid or p-toluenesulfonic acid was added. In this kind of system, the organic solvent is totally immiscible with water, and the high concentration of the solvents may cause denaturation and precipitation effects, as well as the extreme partitioning of the proteins and peptides into one phase.

2.3.2 Partitioning in Aqueous Two-Phase Systems

Despite an intensive hunt for suitable two-phase systems for protein partitioning, a truly useful system remained elusive until a breakthrough occurred in 1958. Albertsson (1958) drew attention to aqueous two-phase systems in which both phases contain very high concentrations of water. The majority of research was performed in polyethylene glycol (PEG) - Dextran system (Albertsson, 1971). A mixture of Dextran and PEG dissolved in water is turbid above certain concentrations of the polymers. Two liquid layers in equilibrium are formed on standing, with the upper phase enriched in PEG while
the lower enriched in Dextran. Dextran and PEG are each fully water soluble, yet the two polymers are incompatible and separate into two aqueous phases.

Several general rules for the partitioning behavior of proteins in this system were stated:

1. The higher the concentration of phase-forming polymers, the more extreme is the partitioning of proteins into one of the phases. Most proteins tend to prefer the lower phase.

2. Protein degradation may alter the partitioning of a protein.

3. The partition coefficients change dramatically on increasing the salt concentration.

4. Changes in pH of the solution can result in significant change in the partition behavior.

5. The partition coefficient is independent of protein concentration.

In the years following, the aqueous systems were used primarily for separation purposes, both single-step extraction in protein purification and extraction in countercurrent distribution. This system does not involve any organic solvent and thus eliminates the problem existing in the conventional organic solvent-water systems.

2.3.3 Two-Phase Aqueous Surfactant Systems

Since the 1980's, there has been considerable interest in the utilization of two-phase aqueous surfactant systems to partition proteins and other biomaterials (Liu et al., 1995). Many surfactant systems can separate into two water-based, yet immiscible, liquid phases. Water-soluble proteins can partition unevenly between these phases while
maintaining their native confirmations and biological activities. One of the systems used was the zwitterionic surfactant dioctanoyl phosphatidyl-choline (C8-lecithin)-water two-phase system. A theoretical formulation was presented to describe and predict the partitioning of water-soluble proteins in two-phase aqueous surfactant systems containing cylindrical, noncharged micelles. The theory was based on the assumption that excluded-volume interactions between the globular hydrophilic proteins and the noncharged cylindrical micelles played the dominant role in determining the experimentally observed partitioning behavior.

The advantage of this system is that the self-assembling nature of the surfactant micelles present in the solution enables one to control and optimize the partition behavior by tuning micellar characteristics, including micellar shape and size. Moreover, the partitioning selectivity can be enhanced by utilizing mixed micelles containing surfactant-type ligands which can target a desired biomaterial.

2.3.4 Liquid-Liquid Extraction with Reversed Micelles

More recently, liquid-liquid extraction of proteins with reversed micelles was developed. Proteins were shown to be solubilized in organic solvents with surfactants, maintaining their functional properties, and to be transferred between an aqueous solution and a reversed micellar organic phase (Pires et al., 1996). The phase transfer depends on the specific characteristics of the proteins: the isoelectric point, size and shape, hydrophobicity, and charge distribution on the protein surface. The transfer also depends
on the pH, ionic strength, type of electrolyte, and surfactant concentration of the two-phase system. The driving forces involved in the transfer of proteins into reversed micelles are electrostatic interactions. This technology has been applied to different proteins and enzymatic systems including recombinant proteins at the laboratory scale.

The advantage of this system is the selective solubilization of proteins in reversed micelles. However, the disadvantage evolves when a back extraction step is needed for the recovery and concentration of the sample in a new aqueous phase. In some cases, the yield of back-extraction is very low, and in other cases, the situation worsens because the transfer of proteins into the organic phase seems to be irreversible.

2.4 Extraction of Antibiotics

2.4.1 Solvent Extraction

Organic solvents are used to recover antibiotics from their fermentation broth. One of the earliest and most well-studied processes is the recovery and purification of Penicillin. For example, Penicillin G and Penicillin V are soluble in many organic solvents and can be extracted with high efficiency into amyl acetate or butyl acetate at pH 2.5-3.0 (Vandamme, 1984). Most of the antibiotics tested in the acetonitrile-water system in this work have been reported to be isolated from fermentation broths by solvent extraction.
(1) Spiramycin and Erythromycin

Spiramycin is a 16-membered macrolide, while Erythromycin is a 14-membered one. The term macrolide was originally applied to a group of lipophilic basic antibiotics with a macrocyclic lactone ring in their chemical structure. Macrolides are produced extracellularly in the fermentation broth (Vandamme, 1984). The basic macrolide antibiotics can be extracted with a water-immiscible organic solvent such as methylisobutyl ketone or ethyl acetate and transferred to acidic water adjusted with hydrochloride, phosphoric acid, acetic acid, or citric acid.

(2) Gramicidin D

Gramicidin D is a linear peptide antibiotic produced from *Bacillus brevis* (Moo-Young, 1985). Adjustment of the filtrate of a culture of *B. brevis* to pH 4.5-4.8 by the addition of hydrochloric acid precipitates a mixture of antibiotics. The dried solid material is extracted with alcohol. The addition of saline to the alcoholic extract precipitates a mixture of antibiotics. Further extraction with acetone-ether mixture secures a soluble neutral fraction, the linear gramicidins. Countercurrent distribution of the neutral fraction in the solvent system benzene : chloroform : methanol : water = 15 : 15 : 23 : 7 (v/v) separates Gramicidin D from Gramicidin A, B, and C. (partition coefficient K of Gramicidin A: \( K_A = 0.56 \); partition coefficient of Gramicidin B: \( K_B = 0.30 \); partition coefficient of Gramicidin C: \( K_C = 1.56 \); partition coefficient of Gramicidin D: \( K_D = 16.65 \) ) (Weinstein, 1978).
(3) Cycloheximide

Cycloheximide is an effective protein synthesis inhibitor used as an agricultural fungicide. It was discovered in streptomycin-yielding cultures of *Streptomyces griseus*. Cycloheximide is soluble in polar solvents and streptomycin is not. In addition, cycloheximide is acid stable whereas Streptomycin is unstable in acid (Vandamme, 1984). These properties have aided in the development of recovery and purification procedures for cycloheximide. The recognized acid stability of cycloheximide resulted in sulfuric acid treatment of the fermentation broth as an initial step. Cycloheximide was recovered by eluting with 80% (v/v) acetone, distilling the acetone, and then extracting the remaining aqueous solution with chloroform.

(4) Fusidic Acid

Fusidic acid is a steroid antibiotic produced from *Fusidium coccineum*. Chemically, it belongs to a group of tetracyclic triterpenoic acids based on the structure of the hypothetic hydrocarbon fusidane. Solvent extraction techniques were found to be most suitable for the recovery and purification of fusidic acid. The filtered broth is adjusted to pH 6.8 with sulfuric acid and extracted with about 10% by volume of methyl isobutyl ketone using a countercurrent centrifugal extractor (Vandamme, 1984).
(5) Antimycin A

Antimycin A is a colorless crystalline lipophilic antibiotic practically insoluble in water (Vandamme, 1984). It is produced intracellularly from *Streptomyces kitasawaensis* and *S. griseus* (Moo-Young, 1985). After broth filtration, it is extracted by CH$_3$Cl and CH$_2$Cl$_2$.

(6) Tetracycline

Tetracycline is a polyketide produced by *Streptomyces aureofaciens*. Tetracycline is extracted from acid or alkaline medium by 1-Butanol. The antibiotic is extracted by solvent with salt, based on salting out (NaCl) from the aqueous to the organic phase (Weinstein, 1978).

(7) Chloramphenicol

Chloramphenicol is an antibacterial agent produced by *Streptomyces venezuelae*. The recovery of this antibiotic is limited to those used in lab-scale research experiments. It is readily extracted from clarified culture fluid at a slightly alkaline pH with a solvent such as ethyl acetate (Vandamme, 1984).

(8) Vancomycin

Vancomycin is a complex glycopeptide molecule containing glucose and amino vancosamine and the amino acids N-methyleucine, aspartic acid, phenylglycine, and
chloro-β-hydroxytyrosine. It is produced by *Streptomyces orientalis* (Moo-Young, 1985), but there is no report in existing literature showing this antibiotic being recovered by solvent extraction.

### 2.4.2 Extraction of Antibiotics with Reversed Micelles

Since conventional organic extraction is ineffective for some molecules because of their hydrophilic nature, reversed micelle systems are used for antibiotic separation and purification. As reviewed in Section 2.3.4, a reversed micelle system consists of an aqueous phase of bioproducts and an organic solution containing reversed micelles. The "water pool" of the reversed micelle inner core can serve as a carrier for bioproducts. In Hu and Gulari's work (1996), the reverse micelle system of sodium di-2-ethylhexyl phosphate (NaDEHP, an anionic surfactant) was used to extract two aminoglycoside antibiotics, neomycin and gentamycin. When the aqueous phase is contacted with the organic reversed micelle phase, the antibiotic molecules can be transferred from the aqueous phase to the polar core of reverse micelles through attractive electrostatic interaction during forward transfer. In backward transfer, the antibiotics loaded in the micelle phase are released back to an aqueous phase through breaking up the reverse micelles by using divalent cation solutions, such as Ca$^{2+}$ solutions.
2.5 Acetonitrile-Water Liquid-Liquid Equilibrium

Liquid-liquid equilibrium data for the acetonitrile-water system are available (Pence and Gu, 1996). The critical solution temperature (i.e., the highest temperature at which a liquid-liquid phase separation can occur) was found to be -1.32 °C. When temperature ranges from the critical point -1.32°C to -18.60 °C, the mole percentage of ACN in the water-rich bottom phase ranges from 36.5% to 13.4%, while that of the ACN-rich top phase ranges from 41.4% to 73.8%. It can then be concluded that both phases contain a considerable amount of water. The phase diagram of this system is shown in Figure 1.

Figure 1. Phase diagram of the ACN-water two-phase system (Pence and Gu, 1996).
2.6 Advantages of the Two-Phase Acetonitrile-Water System

When compared with all the two-phase systems studied for bioseparations, the two-phase acetonitrile-water system offers a number of unique, desirable features:

1. The system is used at low temperatures since the phase separation only occurs at under -1.32 °C. This low temperature system reduces the chances of denaturation.

2. Both phases contain a considerable amount of water, and this also reduces the chances of denaturation in organic solvents.

3. Only a binary ACN-water system is needed rather than more complex ternary systems.

4. Once the biomaterials are extracted, it is easy to separate them from the solvent system. This does not involve such steps as back-extraction that leads to low yield.

5. Since ACN is a widely used mobile phase in HPLC, the product of the partition step can be applied directly to HPLC, the final step for bioseparations.

6. The ACN-rich top phase can be removed right after HPLC at a sub-zero temperature, in place of using traditional methods to remove the organic solvent, such as evaporation which may cause denaturation.

In general, this new method of bioseparation at low-temperature using the ACN-water system should be particularly useful in large-scale operations. As such, this system appears to be very promising from an application point of view.
Chapter 3 Theory and Model

3.1 The Distribution of Particles Due to Brownian Motion and Interfacial Forces

The mechanism governing partition is largely unknown. Qualitatively, it can be described as follows. When a particle is suspended in a phase it interacts with the surrounding molecules in a complicated manner. Various bonds, such as hydrogen, ionic, and hydrophobic, are probably involved, together with other weak forces. Their relative contributions are difficult to estimate, however, their net effect is likely to be different in the two phases. If the energy needed to move a particle from one phase to the other is $\Delta E$, the following relation, according to the theory of Brownian motion, should hold true (Albertsson, 1971):

$$K = \frac{C_{\text{top}}}{C_{\text{bottom}}} = \exp(-\Delta E / kT)$$

(2)

where,

$C_{\text{top}}$ = concentration of particles in the top phase

$C_{\text{bottom}}$ = concentration of particles in the bottom phase

$k =$ Boltzmann constant = $1.380 \times 10^{-23}$ J/(K·molecule)

$T =$ temperature (K)

$K =$ partition coefficient

$\Delta E$ is calculated for a spherical particle of radius $R$, when it is assumed to have a perfectly uniform interface separating it from the surrounding medium, and gravitational forces are neglected (Albertsson, 1971). The following formula is derived:

$$K = \exp(A\lambda / kT)$$

(3)
where,

\[
\lambda = -(\gamma_{p1} - \gamma_{p2})
\]  

(4)

A = surface area of the particle

\(\gamma_{p1}\) = the particle-liquid interfacial tension between the particle and the top phase

\(\gamma_{p2}\) = the particle-liquid interfacial tension between the particle and the bottom phase

\(k\) = Boltzmann constant = \(1.380 \times 10^{-23}\) J/(K·molecule)

\(T\) = temperature (K)

The surface area A should be replaced by the molecular weight for smaller and less spherical molecules (Albertsson, 1971).

As may be seen from Equation (3), if \(\gamma_{p1} < \gamma_{p2}\), K increases when surface area increases; and if \(\gamma_{p1} > \gamma_{p2}\), K decreases when surface area increases. Therefore, a more unilateral distribution can be obtained and two kinds of particles will be more completely separated the larger their particle sizes, if for one species \(\gamma_{p1} > \gamma_{p2}\) (particle favors bottom phase) and for the other \(\gamma_{p1} < \gamma_{p2}\) (particle favors top phase). From a theoretical point of view two-phase systems seem to be very suitable for the fractionation of large particle-weight substances.

3.2 The Donnan Effect: The Partition Potential

When a charged biomolecule distributes unequally between two phases, it causes an electrical potential between the phases. This can be countered by the addition of a salt which partitions differently into the two phases, stabilizing the electrical potential. In
other words, salt can be used to “push” a biomolecule from one phase to another by exploitation of the Donnan effect (Albertsson, 1971). Previous work shows that unlike many two-phase systems used for protein partitioning, salt concentration does not seem to be an important factor in the ACN-water system. This, coupled with the extreme partition coefficient observed (i.e., virtually complete partitioning to one phase), suggests that the Donnan Effect is not a major factor to protein partitioning in this system. It appears to be governed mainly by protein surface-solution interactions (Pence, 1996).

### 3.3 Models of Partitioning

Many complicated thermodynamic models have been developed over the last two decades to account for the influence of system factors. These models start with the fact that at equilibrium the chemical potential of a given species will be equal in the two phases. By finding the appropriate form for the chemical potential of the particle in each phase, the partition coefficient for the particle can be found as a function of the system variables. Among them, Baskir’s Model accounts for all known factors that affect the partition coefficient. In this model, the chemical potential of the particle is assumed to be a function of the particle concentration, the particle surface energy and surface area, the charge on the particle, and the electrostatic potential difference between the two phases (Baskir, 1989). Gravitational effects are assumed to be negligible. Thus, the form of the chemical potential is

\[ \mu_i = \mu_i^0 + kT \ln a_i + A \gamma_i + zF \psi_i / N \]  

(5)
where,

\( \mu_i^\circ \) = the standard state chemical potential of the protein in phase \( i \)

\( a_i \) = protein activity in phase \( i \)

\( A \) = surface area of the particle

\( \gamma \) = the interfacial tension of the particle in phase \( i \)

\( z \) = the total charge on the particle

\( F \) = Faraday’s constant

\( \psi_i \) = the electrical potential in phase \( i \)

\( N \) = Avogadro’s number

For a two-phase system, setting \( f_i = f_i \) and \( a_i = f_i m_i \)

where,

\( f_i \) = the protein activity coefficient in phase \( i \)

\( m_i \) = the molar concentration of the protein in phase \( i \),

and assuming an infinitely dilute solution, Equation (4) becomes

\[-kT\ln K_i = \Delta \mu_i^\circ + A \Delta \gamma + z_i F \Delta \psi / N\] (6)

According to this model, for the same particle, changes in the partition coefficient \( K \) depends on the changes of the particle charge \( z_i \), the particle surface energy \( \Delta \gamma \), or the Donnan effect. Although this model has the limitations that it examines only effects of particle characteristics, does not analyze in detail the contribution of the phase environment, it can be used to analyze the system qualitatively.
3.4 Protein Denaturation in the System

Denaturation of a protein occurs when the tertiary structure is destroyed and random polypeptide chains are formed (Scopes, 1982). These chains tend to aggregate and thus cause precipitation out of solution. However, if there is little salt present and the solution pH is far from the protein's isoelectric point, the denatured protein may remain in the solution. Therefore, other methods, such as inspection of the UV spectrum, will be used along with the observation of precipitation to determine denaturation. Denaturation of a protein can be described by the following first order process:

\[
d\ln k_{\text{den}}/dT = E/(RT^2),
\]

(7)

in which \( k_{\text{den}} = \) the denaturation rate constant

- \( E = \) the activation energy
- \( R = \) the gas constant
- \( T = \) the absolute temperature

The overall activation energy is affected by the organic solvent concentration and solution pH, and the energy available to the protein is affected by temperature. An unfavorable pH value can change the charges on the side chain amino acids of the protein and, thus, cause electrostatic repulsion in the molecule or loss of electrostatic attraction that holds the protein together. The protein then breaks apart, losing its tertiary structure and becoming denatured. Organic solvents can cause denaturation because the organic molecules can slip inside the protein during natural flexing of the protein molecule and attach to the hydrophobic sites inside the protein molecule. The internal
hydrophobic interactions that sustain the protein structures are thus disrupted by those intruding organic molecules. Since the flexing of the protein molecule is a highly temperature dependent phenomenon (Wheelright, 1991), the risk of denaturation by organic solvents can be greatly reduced by operating at sub-zero temperatures.

Denatured proteins generally partition differently from proteins in their native state because the denatured protein has a significantly greater surface area than the native protein, and the exposed surface is much more hydrophobic (Baskir, 1989).

Although the sub-zero operating temperature should reduce the chances of denaturation, effort must still be made to ensure that it does not occur. Methods of monitoring denaturation include observance of a precipitate and changes in the protein’s absorbance spectrum (Kirschenbaum, 1972). The true test is, of course, to check the protein’s bioactivity.

3.5 Ionization States of Amino Acids

The ionization states of amino acids as a function of pH might be used to explain why changes of pH can change the partition coefficients of amino acids, peptides, and proteins. Amino acids in solution at neutral pH are predominantly dipolar ions. In the dipolar form of an amino acid, the amino group is protonated (-NH$_3^+$) and the carboxyl group is dissociated (-COO$^-$). The ionization state of an amino acid varies with pH (Fig. 2). In an acid solution (e.g., pH=1), the net charge of an amino acid is positive. In an alkaline solution (e.g., pH=11), the net charge of the molecule is negative. Since peptides
and proteins are made up of amino acids, this can also apply to the ionization states of the side chains on the exposed surfaces of peptides and proteins, which may contribute to their partition behavior.

![Figure 2](image.png)

**Figure 2.** Ionization states of amino acids at different pH values (Stryers, 1988).

### 3.6 Controlled pH Extraction for Weak Acids and Bases

Consider the behavior of weak acids and weak bases that ionize according to the following general equations (Robinson and Cha, 1985):

\[
HA \rightleftharpoons H^+ + A^- \quad \text{(weak acid)}
\]  

(8)
\[ \text{BH}^+ \rightleftharpoons B^0 + H^+ \] (weak base) \hspace{1cm} (9)

It is assumed that the ionized species (A⁻ or BH⁺) are not significantly soluble in an organic phase. The un-ionized species, HA or B₀, may be soluble in both the organic and aqueous phases.

In the case of a partially ionized weak base distributed between an aqueous phase and a non-ionizing organic phase solvent, assume that the ionized species, BH⁺, is not appreciably soluble in the organic phase. In this case, the observed or actual partition coefficient will be as follows:

\[
K = [B^0]_o / ([B^0]_w + [BH^+]_w)
\]

(10)

where,

\[ K = \text{actual partition coefficient} \]

\[ [\ ]_o = \text{concentration of a certain species in the organic phase} \]

\[ [\ ]_w = \text{concentration of a certain species in the aqueous phase} \]

The ionization constant of a base is defined by the following:

\[
k = [B^0]_w [H^+]_w / [BH^+]_w
\]

(11)

Solving for [BH⁺]_w and substituting this result into Equation (10) yields the equation:

\[
K = [B^0]_o / ( ([B^0]_w (1+[H^+]_w/k))
\]

(12)

If we now assume that the ratio of the concentrations of un-ionized species in the two phases is unaffected by the presence of BH⁺, the definition of \( K' = [B^0]_o / [B^0]_w \) can be used to write the following equation:

\[
K = K^2 / (K' + [H^+])
\]

(13)
in which $K$' is the intrinsic partition coefficient and applies at extreme pH values when all of the species in question are present in the un-ionized form.

Since $pH = -\log_{10}[H^+]$ and $pk = -\log_{10}k$, Equation (13) can be written in logarithmic form as the following:

$pk - pH = \log_{10}(K'/K - 1)$

As the pH of aqueous phase is lowered, $K$ will decrease, and vice versa. ($K$ can never be greater than $K'$, which is a set value for a certain species in a certain system.) Similarly, for acids, $pH - pk = \log_{10}(K'/K-1)$. As the pH of the aqueous phase is raised, $K$ decreases.

In this work, the ACN-water system has water in both phases instead of the case described above that applies to two immiscible phases. Therefore, these equations cannot be used to calculate the partition coefficient quantitatively. However, the theory can still be applied qualitatively to explain the partition behavior of weak bases and weak acids under the change of pH. According to this theory, weak acids prefer the organic-rich top phase at lower pH values, while weak bases tend to favor the top phase at higher pH values.

3.7 The Shapes of Equilibrium Curves and Their Implications

According to Blumberg (1988), the shapes of the equilibrium curves that can be anticipated are of three types, as shown in Figure 3. The first is stoichiometrically controlled, as in Figure 3(a), and applies when molecular ratios are limiting as in compound formation or in ion-pair formation. The second is controlled by concentration
as with solvating reagents, shown in Figure 3(b), where the extracting solvent is in competition with the initial solvent. The third case, given in Figure 3(c), is simply a function of relative solubility of the solute in the competing solvent without strong interactions.

Figure 3. Shapes of equilibrium curves and their implications (Blumberg, 1988).
Chapter 4 Experimental Procedure and Apparatus

4.1 Materials

The water used in this work was purified from a standard in-house deionized cartridge system. The ACN used was purchased from Fisher Scientific (Pittsburgh, PA). It was HPLC grade and had a minimum stated purity from the manufacturer of 99.9%.

Buffers:

All buffers, acids and bases used were obtained from Fisher Scientific. They are as follows: 0.1 Molar Hydrochloric Acid (Certified Grade), 0.1 Molar Sodium Hydroxide (Certified Grade), Buffer Solutions (pH=2.00, 4.00, 7.00, 10.00, Certified Grade).

Proteins:

Trypsin, Hemoglobin (human), Thyroglobulin, Transferrin, Ribonuclease A. These proteins were all obtained from Sigma Chemical (St. Louis, MO).

Amino Acids:

L-Phenylalanine, L-Tryptophan, L-Tyrosine. All amino acids were obtained from Sigma Chemical (St. Louis, MO).

Peptides:

Val-Ala-Ala-Phe, Phe-Gly-Gly-Phe. All peptides were obtained from Sigma Chemical (St. Louis, MO).

Antibiotics:

Erythromycin, Chloramphenicol, Vancomycin, Spiramycin, Gramicidin D, Cycloheximide, Fusidic Acid, Antimycin, Tetracycline, Demeclocycline. All antibiotics
were obtained from Sigma Chemical (St. Louis, MO).

The biomaterials used in the partitioning experiments are listed below in Table 1.

<table>
<thead>
<tr>
<th>Proteins</th>
<th>Molecular Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ribonuclease A</td>
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</tr>
<tr>
<td>Trypsin</td>
<td>15,100</td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>64,000</td>
</tr>
<tr>
<td>Transferrin</td>
<td>77,000</td>
</tr>
<tr>
<td>Thyroglobulin</td>
<td>669,000</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Amino Acids</th>
<th>pI</th>
<th>pK$_1$</th>
<th>pK$_2$</th>
<th>Molecular Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenylalanine</td>
<td>5.48</td>
<td>1.83</td>
<td>9.13</td>
<td>165</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>5.66</td>
<td>2.20</td>
<td>9.11</td>
<td>181</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>5.89</td>
<td>2.38</td>
<td>9.39</td>
<td>204</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Peptides</th>
<th>Molecular Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Val-Ala-Ala-Phe</td>
<td>406</td>
</tr>
<tr>
<td>Phe-Gly-Gly-Phe</td>
<td>426</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Molecular Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloramphenicol</td>
<td>323.1</td>
</tr>
<tr>
<td>Cycloheximide</td>
<td>281.4</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>733.9</td>
</tr>
<tr>
<td>Fusidic Acid</td>
<td>516.7</td>
</tr>
<tr>
<td>Antimycin A</td>
<td>548.6</td>
</tr>
<tr>
<td>Spiramycin</td>
<td>860.0</td>
</tr>
<tr>
<td>Gramicidin D</td>
<td>1141.5*</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>444.4</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>1485.7</td>
</tr>
<tr>
<td>Demeclocycline</td>
<td>501.3</td>
</tr>
</tbody>
</table>

Table 1. Biomaterials Used in Partitioning Experiments

(* There is no report in existing literature for the molecular weight of gramicidin D. The listed value is for gramicidin S, a similar linear peptide.)
Apparatus

A Neslab (Portsmouth, NH) RTE-100LP low temperature bath (± 0.1 °C precision) filled with 60% (wt/wt) ethylene glycol and 40% water was used for temperature control. The freezing point of the ethylene glycol-water mixture of this composition is -48°C (Merck Index, 1989). Temperatures were measured with an ASTM-62C (0.1 °C subdivisions) thermometer from Fisher Scientific. All pH measurements were performed with a Model 476530 general purpose pH electrode from Fisher Scientific.

Sample Preparation

Solutions with appropriate concentrations for UV detection were prepared. Concentrations varied from 0.1 - 4.0 mg/ml for different substances which have different UV absorbance. Water solutions were prepared for hydrophilic substances and they were then buffered to the pH desired with an appropriate buffer. 0.1 M NaOH and 0.1 M HCl were used to carry out minor adjustments of the pH to the desired point if the pH tested was different from the buffer pH. pH measurements were performed prior to the addition of ACN, due to the difficulty of pH measurements in the presence of an organic solvent. It was assumed that the pH did not vary significantly due to the presence of the buffer. The solution was then mixed with ACN at a water : ACN volumetric ratio of 1 : 1.5. For hydrophobic substances, solutions were prepared in ACN and then mixed with buffered water with the ratio of 1 : 1.5 (water : ACN). Mixing was performed by inverting the test
tubes several times. Samples were put into the temperature bath and were allowed to equilibrate overnight to form a two-phase system.

**Partition - Coefficient Measurement**

After phase separation, top and bottom phase samples were taken with disposable glass pipettes and their compositions were determined with a Beckman DU 640 Spectrophotometer (from Beckman Instrument, Inc., Fullerton, CA). The partition coefficient \(K\) is defined as the ratio of the biomaterial's concentration in the top phase \(C_t\) to that in the bottom phase \(C_b\), i.e., \(K = \frac{C_t}{C_b}\). For the measurement of \(K\), the concentration of a certain biomaterial in each coexisting phase was determined by measuring the UV absorbance at its high response wavelength (see Appendix A). Spectrophotometric measurements were also performed on all buffers and solvents to make sure that they did not absorb at the measured wavelengths. Calibration curves were made for all the biomaterials involved to make sure that absorbance vs. concentration was linear in the tested range (see Appendix B). If there was a significant change of spectrum from the native one (i.e., fresh Sigma sample dissolved in pure water) for a certain sample after partitioning, it was then lyophilized to eliminate the organic solvent, redissolved, and scanned again on the spectrophotometer. The spectrum of the freeze-dried sample was compared with that of the native sample. If there was still significant difference, denaturation would be determined.
Chapter 5 Results and Discussion

5.1 Partition Coefficient of Various Types of Biomaterials in Unbuffered Systems

Table 1 shows the experimentally determined partition coefficient values in the acetonitrile-water system without adding a buffer at -10 °C. Most compounds, including all the proteins, peptides, amino acids and some of the antibiotics, have $K < 1$, indicating that they prefer the water-rich bottom phase. The ones that have $K > 1$, all of them antibiotics, prefer the acetonitrile-rich top phase.

All of the proteins tested have partition coefficients less than 0.01. This means that proteins tend to stay exclusively (more than 99%) in the bottom phase with comparable volumes of the top and bottom phases of the tested system. Smaller molecules such as amino acids and short-chain peptides may partition into both phases.

The order for proteins favoring the bottom water-rich phase is thyroglobulin > transferrin > trypsin > ribonuclease A. This observed trend is consistent with the notion that interfacial energy between the biomolecule and the two-phase system plays the dominant role in determining the observed partitioning behavior, since thyroglobulin has the largest size (Molecular Weight: 669 K), followed by transferrin (77 K), trypsin (15.1 K), and ribonuclease A (13.5 K).
<table>
<thead>
<tr>
<th>Material</th>
<th>K</th>
<th>SD*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ribonuclease A</td>
<td>0.067</td>
<td>0.005</td>
</tr>
<tr>
<td>Trypsin</td>
<td>0.046</td>
<td>0.008</td>
</tr>
<tr>
<td>Transferrin</td>
<td>0.015</td>
<td>0.002</td>
</tr>
<tr>
<td>Thyroglobulin</td>
<td>0.008</td>
<td>0.002</td>
</tr>
<tr>
<td>Human Hemoglobin</td>
<td>0.023 (denatured)</td>
<td>0.009</td>
</tr>
<tr>
<td>Val-Ala-Ala-Phe</td>
<td>0.063</td>
<td>0.012</td>
</tr>
<tr>
<td>Phe-Gly-Gly-Phe</td>
<td>0.26</td>
<td>0.015</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.097</td>
<td>0.009</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>0.131</td>
<td>0.011</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>0.183</td>
<td>0.019</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>0.039</td>
<td>0.004</td>
</tr>
<tr>
<td>Spiramycin</td>
<td>0.249</td>
<td>0.020</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>0.248</td>
<td>0.038</td>
</tr>
<tr>
<td>Demeclocycline</td>
<td>0.477</td>
<td>0.022</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>2.217</td>
<td>0.108</td>
</tr>
<tr>
<td>Cycloheximide</td>
<td>5.52</td>
<td>0.622</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>6.248</td>
<td>0.905</td>
</tr>
<tr>
<td>Fusidic Acid</td>
<td>7.146</td>
<td>1.019</td>
</tr>
<tr>
<td>Gramicidin D</td>
<td>25.098</td>
<td>2.011</td>
</tr>
<tr>
<td>Antimycin</td>
<td>60.418</td>
<td>4.009</td>
</tr>
</tbody>
</table>

Table 2. The Partition Coefficient of Biomaterials in Unbuffered ACN-Water System at -10.0 °C (SD*: Standard Deviation of 3 Experimental Data)
As observed in the experiments, phe-gly-gly-phe is more reluctant to dissolve in pure ACN than val-ala-ala-phe and has a higher partition coefficient than the latter. Antimycin, an antibiotic that is practically insoluble in water, has the highest partition coefficient among the tested species. With comparable volumes of the two coexisting phases, it partitions into the ACN-rich top phase by greater than 90%. However, vancomycin, a hydrophilic antibiotic, has a fairly low partition coefficient of 0.039 in this system.

It is clear that for large molecules, interfacial energy plays the dominant role in determining the partition behavior. For smaller molecules such as amino acids, short-chain peptides, and antibiotics, the mode of transfer seems to be controlled by the relative solubility of the solute in ACN -- in other words, by hydrophobicity of the solute.

5.2 The Shapes of Equilibrium Curves and Their Implications

The equilibrium isotherm of erythromycin is presented in Figure 4. Concentration is expressed as UV absorbance at 288 nm. In the tested range (concentration of erythromycin up to 16.0 mg/ml), absorbance vs. concentration is linear (see Appendix B). According to Blumberg (1988), the shape of the equilibrium curve gives a practical presentation of the distribution behavior of the solute as a function of its concentration. As stated in Section 3.7, different shapes of isotherms indicate different mechanisms of partitioning. The linear relationship shown in Figure 4 indicates that the mode of transfer is simply a function of relative solubility of the solute in the competing solvent ACN,
without strong interactions for erythromycin. A similar equilibrium isotherm for phenylalanine was obtained, as shown in Figure 5.

Figure 4. Equilibrium isotherm of erythromycin obtained at -10.0 °C.
Figure 5. Equilibrium isotherm of phenylalanine obtained at -10.0 °C.
5.3 The Effect of pH

Figures 6 to 8 and Table 2 show the experimental partition coefficient $K$ values at different pH values. For proteins, peptides, and amino acids, the partition coefficient increases with decreasing pH values. This might be due to the selective solvation properties (Coetzee and Ritchie, 1969) of the ACN-water mixture. Selective solvation means that the solution composition surrounding an ion varies significantly from the bulk solution. For example, when silver nitrate is dissolved in a 50% (mole/mole) solution of water and ACN, the positively charged silver ion is surrounded by seven ACN and two water molecules, while the negatively charged nitrate ion is surrounded by two ACN molecules and four water molecules. At lower pH values, the compounds tested became positively charged. This may explain the tendency of transferring into the ACN-rich top phase.
Figure 6. The effect of pH on the partitioning of four proteins (ribonuclease A, transferrin, thyroglobulin and trypsin) at -10.0 °C.
Figure 7. The effect of pH on the partitioning of two amino acids (tryptophan, phenylalanine) at -10.0°C.
Figure 8. The effect of pH on the partitioning of two peptides (val-ala-ala-phe, phe-gly-gly-phe) at -10.0 °C.
<table>
<thead>
<tr>
<th>Chemicals</th>
<th>pH</th>
<th>K</th>
<th>SD*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spiramycin</td>
<td>4</td>
<td>0.193</td>
<td>0.014</td>
</tr>
<tr>
<td></td>
<td>no buffer</td>
<td>0.249</td>
<td>0.020</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>1.854</td>
<td>0.108</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>4</td>
<td>0.012</td>
<td>0.002</td>
</tr>
<tr>
<td></td>
<td>no buffer</td>
<td>0.039</td>
<td>0.004</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0.024</td>
<td>0.004</td>
</tr>
<tr>
<td>Cycloheximide</td>
<td>4</td>
<td>5.511</td>
<td>0.603</td>
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<td></td>
<td>no buffer</td>
<td>5.52</td>
<td>0.622</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>2.668</td>
<td>0.214</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>4</td>
<td>0.279</td>
<td>0.019</td>
</tr>
<tr>
<td></td>
<td>no buffer</td>
<td>2.217</td>
<td>0.108</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>2.888</td>
<td>0.134</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>4</td>
<td>0.316</td>
<td>0.034</td>
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<td></td>
<td>no buffer</td>
<td>0.214</td>
<td>0.038</td>
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<td></td>
<td>10</td>
<td>0.127</td>
<td>0.019</td>
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<td>Chloramphenicol</td>
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<td>5.886</td>
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<td>no buffer</td>
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<td></td>
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<td>Antimycin</td>
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<td>no buffer</td>
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<td>10</td>
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<td>no buffer</td>
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<tr>
<td></td>
<td>10</td>
<td>0.72</td>
<td>0.043</td>
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</table>

Table 3. The Effect of pH on the Partitioning of Antibiotics at -10.0 °C

(* SD: Standard Deviation of Three Experimental Data)
The partition behavior of antibiotics seems to follow the rule of extractive separation of weak acids and bases, as discussed in Chapter 3. Weak bases favor the ACN-rich top phase at high pH values, while weak acids favor the top phase at low pH values.

Extended pH studies were performed on two antibiotics having high partition coefficients in unbuffered systems: fusidic acid and gramicidin D. Experiments were conducted under -12.0 °C in place of -10.0 °C for these two biomaterials. This change of temperature should not affect the trend of the change of the partition coefficient with pH. As shown in Figures 9 and 10, when pH decreases, partition coefficient increases.

![Graph showing the effect of pH on the partitioning of fusidic acid at -12.0 °C. Analyzed at 230 nm.](image)

Figure 9. The effect of pH on the partitioning of fusidic acid at -12.0 °C. (Analyzed at 230 nm.)
Figure 10. The effect of pH on the partitioning of gramicidin D at -12.0 °C. (Analyzed at 281 nm.)
5.4 Partitioning of Human Hemoglobin in the ACN-Water Two-Phase System

Figures 11 and 12 illustrate the different partition coefficients observed for human hemoglobin at two different wavelengths, 273 nm and 404 nm.

Figure 11. The effect of pH on the partitioning of human hemoglobin at -7.3 °C. (Analyzed at 404 nm.)
Hemoglobin has a nonpolypeptide unit, and a heme group, which contains an iron complex that binds oxygen and gives hemoglobin its distinctive color. According to Wuenschell et al. (1992), the maximum absorbance of Iron (II) complexes of ligands in hemoglobin is at the wavelength of 415-418 nm. As seen in Figures 11 and 12, it is very likely that the hemoglobin has split into two parts: the heme group, which is a small prosthetic group ($\lambda_{\text{max}} = 404 \text{ nm}$), and the remaining apoprotein part, which is similar to a typical protein ($\lambda_{\text{max}} = 273 \text{ nm}$). These two parts partition differently into the coexisting
two phases. The smaller heme group (404 nm) partitions more readily into the top phase, while the much larger apoprotein part (273 nm) tends to remain in the bottom phase.

It should be noted that the apoprotein part partitions differently from the other normal proteins since its partition coefficient is much larger, which might be due to denaturation effect. Since their tertiary structures have been destroyed and the more hydrophobic inner side chains may be exposed to the outer environment, denatured proteins usually partition differently from the normal proteins.

In this work, efforts were made to determine whether it is the presence of ACN, the unfavorable pH values, or the combination of these factors that causes denaturation of human hemoglobin. In Figure 13, the spectrum of human hemoglobin dissolved in the unbuffered system was compared with the native protein sample. Then, the sample in the unbuffered system was freeze-dried to remove the organic solvent and was dissolved again in water. The spectrum of the redissolved sample was compared with the native spectrum in Figure 14. From these figures, it can be concluded that the presence of ACN is not the major contributor to hemoglobin denaturation.
Figure 13. The bottom phase of human hemoglobin separated by unbuffered ACN-water system at -7.3 °C.
Figure 14. The lyophilized bottom phase of human hemoglobin separated by unbuffered ACN-water system at -7.3 °C.

In Figure 15, the spectrum of human hemoglobin in a buffered water solution (without ACN) is compared to the native spectrum. It is clear that there is significant change in the spectrum, especially at the 404 nm peak. It seems that unfavorable pH is the major contributor to the denaturation of hemoglobin. When this buffered solution (without ACN) was freeze-dried, it would not redissolve in water, and this is an indication of denaturation.
Figure 15. The spectrum of buffered human hemoglobin solution at pH=2.40 (without ACN) at -7.3 °C.

Human hemoglobin solution was buffered to pH = 3.78 and was mixed with ACN. After equilibrating overnight, the spectrum of the top phase was compared with the native one (Figure 16). Native sample refers to fresh sample from sigma dissolved in pure water at 25 °C. The top phase was also lyophilized. The lyophilized sample was reluctant to redissolve in water. The sample was then centrifuged and the solution was taken and scanned on the UV spectrophotometer (Figure 17). Similarly, the spectra of the bottom phase were shown in Figures 18 and 19, each comparing with that of the native sample.
Native sample refers to the protein obtained from Sigma directly dissolved in pure water at 25 °C. Similar results were obtained at other pH values.

Figure 16. The top phase of human hemoglobin separated by ACN-water system at pH=3.78 (-7.3 °C).
Figure 17. The lyophilized top phase of human hemoglobin separated by ACN-water system at pH=3.78 (-7.3 °C).
Figure 18. The bottom phase of human hemoglobin separated by ACN-water system at pH=3.78 (-7.3 °C).
Figure 19. The lyophilized bottom phase of human hemoglobin separated by ACN-water system at pH=3.78 (-7.3 °C).

It can be concluded that an unfavorable pH and a high concentration of salt are major contributors to the hemoglobin denaturation, but not the organic solvent ACN. The hemoglobin molecule splits into two parts upon denaturation: the small heme group and the large apoprotein. The tertiary structure of the apoprotein is destroyed. The heme group favors the top phase, while the much larger apoprotein favors the bottom. Actually, this is where the different partition behavior of large and small molecules was noticed and studied.
5.5 The Effect of Temperature

Figures 20 to 25 show the experimental data of partition coefficients of amino acids, peptides, and antibiotics as a function of temperature between -2.5 °C and -15.0 °C. The lowest temperature used was -15.0 °C since the whole two-phase system tends to freeze at a temperature below -20 °C.

![Graph showing the effect of temperature on the partition coefficient of three amino acids](image)

Figure 20. The effect of temperature on the partition coefficient of three amino acids (phenylalanine, tryptophan and tyrosine) in unbuffered ACN-water system.
At the critical point $T_c = -1.32 \, ^\circ C$, the ACN/water compositions of the coexisting two phases are identical, thus the partition coefficient of a biomaterial is equal to unity. As temperature decreases further below the critical point, partition coefficient $K$ decreases for the species that have $K < 1$, while $K$ increases for those having $K > 1$. As $(T_c - T)$ increases, the difference of the ACN compositions of the two coexisting phases increases, and $K$ deviates further from unity.

Figure 21. The effect of temperature on the partitioning of two peptides (phe-gly-gly-phe, val-ala-ala-phe) in unbuffered ACN-water system.
Figure 22. The effect of temperature on the partitioning of two antibiotics (spiramycin and tetracycline) in unbuffered ACN-water system.
Figure 23. The effect of temperature on the partitioning of two antibiotics (demeclocycline and vancomycin) in unbuffered ACN-water system.
Figure 24. The effect of temperature on the partitioning of three antibiotics (chloramphenicol, cycloheximide, erythromycin) in unbuffered ACN-water system.
Figure 25. The temperature effect on fusidic acid, antimycin A and gramicidin D (antibiotics) in unbuffered ACN-water system.
5.6 The Effect of Salt Concentration

The effect of salt (potassium chloride) on the partitioning of erythromycin is shown in Figure 26. The partition coefficients of the antibiotics were tested in the ACN-water system without salt, with 1M KCl, and with 3M KCl (saturated at the tested temperature of -10.0 °C). With increasing salt concentration, the partition coefficient of erythromycin increases. This is consistent with the notion that salt is more soluble in water and thus “pushes” the antibiotic from the water-rich bottom phase to the ACN-rich top phase.

![Graph showing the effect of salt concentration on the partitioning of erythromycin](image)

Figure 26. The effect of salt concentration on the partitioning of erythromycin (antibiotic) in unbuffered ACN-water system at -10.0 °C.
However, as shown in Figure 27, the partition coefficients of phenylalanine and phe-gly-gly-phe decrease when the salt concentration increases. It is possible that the amino acid and the peptide can bind with the salt and thus tend to remain in the bottom phase. When the salt solution in the bottom phase became saturated (3M KCl at -10.0 °C), the salt itself started to partition into the top phase, and so did the biomolecules that bound with the salt.

Figure 27. The effect of salt concentration on the partitioning of phenylalanine (amino acid) and phe-gly-gly-phe (peptide) in unbuffered ACN-water system at -10.0 °C.
5.7 Separation of a Trypsin-Antimycin Mixture

Because some substances tend to remain in the water-rich phase while others favor the ACN-rich top phase, it is possible to separate these species that have different partition behavior by allowing the phase separation to occur. In this fashion, trypsin (a hydrophilic protein) was separated from antimycin A (a hydrophobic antibiotic), as seen in Figures 28 and 29. In these two figures “original” refers to the trypsin-antimycin solution before separation.

In Table 4, the fraction of the antimycin extracted to the top phase and the fraction of the trypsin remained in the bottom phase, both under different pH environments, were listed. It is clear that the addition of acids improved the separation. Separation experiments were not conducted under alkaline pH values because the spectrum of antimycin was observed to be changed in the presence of bases.

<table>
<thead>
<tr>
<th>pH</th>
<th>The Fraction of Antimycin Extracted to the Top Phase</th>
<th>The Fraction of Trypsin Remained in the Bottom Phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.01</td>
<td>95.8%</td>
<td>98.8%</td>
</tr>
<tr>
<td>3.82</td>
<td>93.1%</td>
<td>97.7%</td>
</tr>
<tr>
<td>unbuffered</td>
<td>84.6%</td>
<td>87.7%</td>
</tr>
</tbody>
</table>

Table 4. The Effect of pH on the Separation of Trypsin-Antimycin Mixtures at -10.0 °C
Figure 28. The top phase of the trypsin-antimycin mixture separated at pH=3.82 (-10.0 °C).
Figure 29. The bottom phase of trypsin-antimycin mixture separated at pH=3.82 (-10.0 °C).
Chapter 6  Conclusions and Recommendations

6.1 Conclusions

The partition coefficient of some biomaterials have been obtained, including some common proteins, peptides, amino acids, and antibiotics. The effect of pH and the mechanism behind the partitioning behavior of different molecules were probed. pH affects the partition behavior of proteins, peptides, and amino acids as a protonization force. Antibiotics behave as common weak bases or weak acids with the change of pH in the ACN-water two-phase system.

For large molecules such as proteins, interfacial energy seems to be the dominant factor for determining the partition behavior. For small molecules, partition coefficients are independent of solute concentrations. The linear equilibrium curves suggest that the mode of transfer of these molecules in the ACN-water two-phase system is a function of the relative solubility of the solute in the competing solvent ACN. Accordingly, as temperature decreases away from the critical point and the difference between the phase compositions increases, the partition coefficient of a biomaterial deviates further from unity.

Partition coefficients of some biomaterials indicated that the ACN-water system can be used for bioseparations since it has partition discrimination for different kinds of biomaterials.

At low temperatures, the two-phase acetonitrile-water system exhibits several unique features compared with the more traditional liquid-liquid two-phase partitioning
methods. It is clear that the ACN-water two-phase system is very promising for use in large-scale operations.

6.2 Recommendations

The effect of salt concentration on the partitioning of antibiotics, amino acids, and peptides should be further investigated. Establishing a thermodynamic model of partitioning in the ACN-water liquid-liquid two-phase system that includes the factors affecting phase transfer, such as the specific characteristics of the biomaterials (isoelectric point, size, shape, hydrophobicity, charge distribution, etc.) and the pH, ionic strength of the two-phase system, is also of interest.

The success of separating a protein and an antibiotic creates interest in the possibility of purifying other pairs of biomaterials that have different partition behavior in this system and in other liquid-liquid systems at low temperatures. Since most of the biomaterials tested have their characteristic absorbance wavelength at around 280 nm, UV spectra scanned by a spectrophotometer are not able to determine the concentrations of the biomaterials in the coexisting phases. Therefore, high performance liquid chromatography (HPLC) should be used to determine the concentrations of these substances in separation experiments, especially when more than two biomaterials are involved.

Several other aqueous-organic solutions that separate at low temperatures have been identified. The phase envelopes of the isopropyl alcohol-water system and the
acetone-water system are currently under investigation in the Biochemical Engineering Lab at Ohio University. They have a reported critical solution temperature of -23 °C and -11 °C, respectively (Francis, 1961).
Bibliography


Appendix A  Wavelength Analyzed for the Biomaterials

The concentration of a certain biomaterial in each coexisting phase was determined by measuring the UV absorbance at its high response wavelength on a Beckman DU 640 Spectrophotometer. These wavelengths are listed below.

<table>
<thead>
<tr>
<th>Material</th>
<th>Wavelength (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ribonuclease A</td>
<td>276</td>
</tr>
<tr>
<td>Trypsin</td>
<td>276</td>
</tr>
<tr>
<td>Human Hemoglobin</td>
<td>273, 404</td>
</tr>
<tr>
<td>Transferrin</td>
<td>279</td>
</tr>
<tr>
<td>Thyroglobulin</td>
<td>279</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>257</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>280</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>275</td>
</tr>
<tr>
<td>Val-Ala-Ala-Phe</td>
<td>257</td>
</tr>
<tr>
<td>Phe-Gly-Gly-Phe</td>
<td>257</td>
</tr>
<tr>
<td>Spiramycin</td>
<td>230</td>
</tr>
<tr>
<td>Gramicidin D</td>
<td>281</td>
</tr>
<tr>
<td>Cycloheximide</td>
<td>288</td>
</tr>
<tr>
<td>Fusidic Acid</td>
<td>260</td>
</tr>
<tr>
<td>Antimycin A</td>
<td>321</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>272</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>288</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>274</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>279</td>
</tr>
<tr>
<td>Demeclocycline</td>
<td>371</td>
</tr>
</tbody>
</table>
Appendix B  Calibration Curves of the Biomaterials

(Absorbance vs. Concentration)
Figure 30. Calibration curve of spiramycin.

Figure 31. Calibration curve of gramicidin D.
Figure 32. Calibration curve of cycloheximide.

Figure 33. Calibration curve of fusidic acid.
Figure 34. Calibration curve of antimycin A.

Figure 35. Calibration curve of tetracycline.
Figure 36. Calibration curve of erythromycin.

Figure 37. Calibration curve of chloramphenicol.
Vancomycin

\[ y = 4.331x \]
\[ R^2 = 0.9985 \]

Figure 38. Calibration curve of vancomycin.

Demeclocycline

\[ y = 23.15x \]
\[ R^2 = 0.9989 \]

Figure 39. Calibration curve of demeclocycline.
Figure 40. Calibration curve of ribonuclease A.

Figure 41. Calibration curve of transferrin.
Figure 42. Calibration curve of thyroglobulin.

Figure 43. Calibration curve of tryptophan.
Figure 44. Calibration curve of phenylalanine.

Figure 45. Calibration curve of tyrosine.
Figure 46. Calibration curve of trypsin.

Figure 47. Calibration curve of phe-gly-gly-phe.
Figure 48. Calibration curve of val-ala-ala-phe.
### Appendix C  Contents of Buffer Solutions

1. Buffer Solution pH 2.00 ± 0.02  
   (0.05 M)  Certified Grade  
   **Contents:**  Potassium Chloride  
                  Hydrolic Acid  
                  Formaldehyde  
                  Water  

2. Buffer Solution pH 4.00 ± 0.02  
   Certified Grade  
   **Contents:**  Acetic Acid  
                  Sodium Acetate  
                  Formaldehyde  
                  Methanol  
                  Water  

3. Buffer Solution pH 7.00 ± 0.01  
   (0.05 M)  Certified Grade  
   **Contents:**  Sodium Phosphate, Dibasic  
                  Potassium Phosphate, Monobasic  
                  Water  

4. Buffer Solution pH 10.00 ± 0.01  
   (0.05 M)  Certified Grade  
   **Contents:**  Potassium Borate, Tetra  
                  Potassium Carbonate  
                  Potassium Hydroxide  
                  Water  

All buffers were obtained from Fisher Scientific (Pittsburgh, PA)