METHODS OF BIOCHEMICAL ANALYSIS

Edited by DAVID GLICK

VOLUME 29

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PREFACE

Annual review volumes dealing with many different fields of science have proved their value repeatedly and are now widely used and well established. These reviews have been concerned, not only with the results in the developing fields, but also with the techniques and methods employed, and they have served to keep the ever-expanding scene within the view of the investigator, applier, the teacher, and the student.

It is particularly important that review services of this nature should have included the area of methods and techniques, because it is becoming increasingly difficult to keep abreast of the manifold experimental innovations and improvements which constitute the limiting factor in many cases for the growth of the experimental sciences. Concepts and vision of creative scientists far outrun that which can actually be attained in present practice. Therefore, an emphasis on methodology and instrumentation is a fundamental need in order for material achievement to keep in sight of the advance of useful ideas.

The volumes in this series are designed to try to meet the need in the field of biochemical analysis. The topics to be included are chemical, physical, microbiological, and if necessary, animal assays, as well as basic techniques and instrumentation for the determination of enzymes, vitamins, hormones, lipids, carbohydrates, proteins and their products, minerals, antimetabolites, etc.

Certain chapters will deal with well-established methods or techniques which have undergone sufficient improvement to merit recapitulation, reappraisal, and new recommendations. Other chapters will be concerned with essentially new approaches which bear promise of great usefulness. Relatively few subjects can be included in any single volume, but as they accumulate, these volumes should comprise a self-modernizing encyclopedia of methods of biochemical analysis. By judicious selection of topics it is planned that most subjects of current importance will receive treatment in these volumes.

The general plan followed in the organization of the individual chapters is a discussion of the background and previous work, a critical evalua-

tion of the various approaches, and a presentation of the procedural details of the method or methods recommended by the author. The presentation of the experimental details is to be given in a manner that will furnish the laboratory worker with the complete information required to carry out the analysis.

Within this comprehensive scheme the reader may note that the treatments vary widely with respect to taste, and point of view. It is the Editor's policy to encourage individual expression in these presentations because it is stifling to originality and justifiably annoying to many authors to submerge themselves in a standard mold. Scientific writing need not be as dull and uniform as it too often is. In certain technical details, a consistent pattern is followed for the sake of convenience, as in the form used for reference citations and indexing.

The success of the treatment of any topic will depend primarily on the experience, critical ability, and capacity to communicate of the author. Those invited to prepare the respective chapters are scientists who either have originated the methods they discuss or have had intimate personal experience with them.

It is the wish of the Advisory Board and the Editor to make this series of volumes as useful as possible and to this end suggestions will be always welcome.

DAVID GLICK

CONTENTS

Interaction Between Biomolecules Studied by Phase Partition

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I. INTRODUCTION

The study of interactions between different biomolecules is of increasing importance for our understanding of the function of these molecules. All molecules of the cell interact more or less strongly with other molecules. Some interactions are strong and specific, giving rise to stable complexes such as oligomeric enzymes. Other interactions are weaker, and the complexes formed are so unstable that they dissociate when the cells are broken and the content diluted.

In this chapter I describe how partition between two immiscible aqueous phases, phase partition, can be used to detect and quantify interactions of, for example, the following types: protein–small ligand, nucleic acid–small ligand, protein–protein, protein–nucleic acid, and protein–lipid.

The principle of this method is as follows. Two substances are partitioned separately and together in a two-phase system. If there is no interaction, the two substances partition independent of each other; the presence of one substance does not influence the other. If there is interaction, the presence of one substance perturbs the partition of the other. The changes in partition can be used for calculation of dissociation constants.

II. PHASE SYSTEMS AND FACTORS DETERMINING PARTITION

The properties of polymer two-phase systems have been described in detail previously (Albertsson, 1971). See also Albertsson et al. (1981). The systems are obtained by dissolving two water-soluble polymers, above certain concentrations, in water. Salts and buffers can be added to give a desired ionic strength and pH. Detergents can be added in order to keep membrane proteins in solution (Albertsson, 1973). The most-used phase system contains dextran and polyethylene glycol (PEG). Partition in this phase system depends on several factors, as described in the following sections.

1. Molecular Weight of the Partitioned Substance

Small molecules usually partition with a partition coefficient close to 1. The larger the molecular weight, the more one-sided the partition. Proteins usually have a K value in the range 0.1–10, whereas DNA can have K values in the range 0.001–100.

2. Charge of the Partitioned Molecules

There is an electrical potential difference between the phases, (Albertsson, 1971). The sign and magnitude of this potential depends on the salts present. This potential influences the partition of charged molecules such as proteins and nucleic acids. The salt composition of the phase system therefore has a great influence on the partition of macromolecules. If charged groups are bound covalently to polyethylene glycol, the partition of proteins is highly charge-dependent and therefore also pH-dependent (Johansson et al., 1973).

3. Ionic Composition of the Phase System

The ionic composition has a strong influence on the partition of charged macromolecules. It is mainly the types of ions present and the ratio between the concentration of different ions that determine the partition.

4. Molecular Weight of Polymers

In most cases the partition depends strongly on the molecular weight of the polymers used. There is a general rule that if the molecular weight of one polymer is decreased, the partitioned substance favors the phase of this polymer more. Thus proteins, for example, favor the PEG-rich phase more if a lower-molecular-weight PEG is used, and they favor the dextran-rich phase more if its molecular weight is decreased. Small molecules like sucrose, amino acids, and small protein molecules, however, do not depend much on the molecular weight of the phase polymers.

5. Hydrophobic and Biospecific Affinity Effects

When hydrophobic groups or biospecific ligands are covalently bound to the polymers, these can strongly influence the partition (Shanbhag and Johansson, 1974; Flanagan and Barondes, 1975). See Albertsson (1978) for further references.

6. Possibilities to Adjust the Partition Coefficient

The following methods can be used to adjust the partition coefficient to a desired value.

A. CHANGING THE IONIC COMPOSITION

For negative proteins a higher K value is obtained by using ions in the following series $Li^+ > Na^+ > K^+$ or $HPO_3^{2-} \geq SO_4^{2-} > Cl$. For pos-

itively charged macromolecules the opposite is true. This salt effect can be used to increase the difference in partition between two proteins according to their charge.

B. USING CHARGED POLYMERS

If charged groups are attached covalently to the polymers, these can influence the partition of charged molecules. For example, trimethylamino-polyethylene glycol has been used to steer the partition of proteins (Johansson et al., 1973; Backman and Johansson, 1976).

C. CHANGING MOLECULAR WEIGHT OF THE PHASE POLYMERS

For a given ionic composition and pH, the partition coefficient can be decreased by using a lower-molecular-weight fraction of dextran or a higher-molecular-weight fraction of PEG. Since this effect of the molecular weight of the polymers is stronger on large protein molecules than on small protein molecules, it can be used to increase the difference in partition between two proteins having different molecular weights.

D. LIGANDS COVALENTLY BOUND TO THE POLYMERS

See Johansson (1976).

E. MODIFYING THE PARTITIONED MOLECULE

When a partitioned molecule is bound to a polymer, for example, a protein to PEG, its partition is shifted toward the PEG-rich upper phase. This binding can be either by a noncovalent interaction, such as when palmitoyl-PEG is bound to protein (Shanbhag and Johansson, 1979; Axelsson and Shanbhag), or it can be covalent, as, for example, when monomethoxy polyethylene glycol is coupled to a protein with triazin as coupling agent (Abuchowski et al., 1976; Mattiasson and Ling, 1980).

7. Other Phase Systems

Dextran and Ficoll also form a two-phase system with water. The settling time is rather long, however (it can be shortened by centrifugation). When PEG is added, the settling time is reduced considerably, and a system of 6% dextran, 5% Ficoll, and 2% PEG 6000 does, in fact, settle in a shorter time than the dextran-PEG system. The dextran-Ficoll and dextran-Ficoll-PEG systems seem to dissolve proteins better than the dextran-PEG systems and may therefore be advantageous for membrane particles that show a tendency for aggregation.

One can also utilize phase systems composed of one polymer only, for example, PEG and a high concentration of certain salts like ammonium or magnesium sulfate and potassium phosphate (Albertsson, 1971).

When three polymers are used, a three-phase system can be obtained, and this has been used for partition of proteins (Hartman et al., 1974).

III. THEORY

The following symbols are used to denote concentrations, partition coefficients, and dissociation constants:

$[A^0]_t$ = total concentration of A in top phase
$[B^0]_t$ = total concentration of B in top phase
$[A]_t$ = concentration of free A in top phase
$[B]_t$ = concentration of free B in top phase
$[AB]_t$ = concentration of complex AB in top phase; by replacing the subscript t with b, corresponding symbols for the bottom phase are obtained
K_A, K_B, K_{AB} = partition coefficients for A, B, and AB, respectively
K_t, K_b = dissociation constants in the top and bottom phase, respectively

1. Interactions Between Two Molecules A and B

Two cases will be considered.

A. INTERACTION BETWEEN TWO MOLECULES A AND B HAVING THE PARTITION COEFFICIENTS K_A AND K_B

In this case we have the equilibria shown in Figure 1. It is assumed that A and B form a 1:1 complex. There are two dissociation equilibria, one for each phase, and three partition equilibria. If the total concentrations of A and B can be determined in each phase, the equilibrium constants

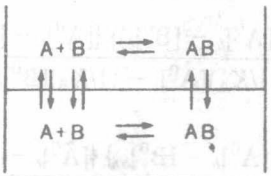

Figure 1. Equilibria for two interacting species A and B in a two-phase system.

can be calculated. In this manner interactions between molecules can be detected and studied quantitatively.

The following equations can be written:

$$K_A = \frac{[A]_t}{[A]_b} \tag{1}$$

$$K_B = \frac{[B]_t}{[B]_b} \tag{2}$$

$$K_{AB} = \frac{[AB]_t}{[AB]_b} \tag{3}$$

$$[A]_t + [AB]_t = [A^0]_t \tag{4}$$

$$[B]_t + [AB]_t = [B^0]_t \tag{5}$$

$$[A]_b + [AB]_b = [A^0]_b \tag{6}$$

$$[B]_b + [AB]_b = [B^0]_b \tag{7}$$

$$K_t = \frac{[A]_t[B]_t}{[AB]_t} \tag{8}$$

$$K_b = \frac{[A]_b[B]_b}{[AB]_b} \tag{9}$$

If we take a sample from the top phase and dilute it such that the complex AB dissociates, and if we can assay A and B separately, for example, by an enzymatic, immunological, or radioactive assay, then we can determine the total concentration of A and B in the upper phase. In the same way, the total concentration of A and B in the bottom phase can be determined. Thus $[A^0]_t$, $[B^0]_t$, $[A^0]_b$, and $[B^0]_b$ will be known. Both K_A and K_B can be determined by measuring the partition coefficients of the proteins separately. The remaining nine unknowns can be solved by means of the above equations. We obtain the following relationships for the dissociation constants and the partition coefficient of the complex:

$$K_t = \frac{\{[A^0]_t - [B^0]_t - K_B([A^0]_b - [B^0]_b)\}\{[A^0]_t - [B^0]_t - K_A([A^0]_b - [B^0]_b)\}}{(K_B - K_A)[(1/K_A)[A^0]_t - (1/K_B)[B^0]_t - [A^0]_b + [B^0]_b]} \tag{10}$$

$$K_b = \frac{\{[A^0]_t - [B^0]_t - K_B([A^0]_b - [B^0]_b)\}\{[A^0]_t - [B^0]_t - K_A([A^0]_b - [B^0]_b)\}}{(K_A - K_B)(K_A[A^0]_b - K_B[B^0]_b - [A^0]_t + [B^0]_t)} \tag{11}$$

$$K_{AB} = K_A K_B \frac{[B^0]_t/K_B - [A^0]_t/K_A + [A^0]_b - [B^0]_b}{K_A[A^0]_b - K_B[B^0]_b - [A^0]_t + [B^0]_t}$$

The following relation also holds:

$$\frac{K_t}{K_b} = \frac{K_A K_B}{K_{AB}} \tag{12}$$

Hence the dissociation constants and the partition coefficient of the complex can be determined by one partition experiment only.

It is assumed that a 1:1 complex between A and B is formed. Also, the partition coefficients K_A and K_B must be different. If they are only slightly different, the method is not very accurate, and if they are identical, the calculation cannot be used.

The partition coefficient of the complex, K_{AB}, can also be determined if an excess of A over B is added to the system. If the excess is so large that all B is in the complex, its partition coefficient can be determined. The following expression for the dissociation constant in the bottom phase can then be written:

$$K_b = \frac{([A^0]_b - \varphi)\,([B^0]_b - \varphi)}{\varphi} \tag{13}$$

where

$$\varphi = \frac{[A^0]_t - K_A[A^0]_b}{K_{AB} - K_A} \tag{14}$$

The dissociation constant in the top phase is obtained from (13). In this case K_A and K_B may be similar, provided that they are different from K_{AB}.

B. THE PARTITION OF A IS ONE-SIDED, THAT IS, THE PARTITION COEFFICIENT OF A IS EITHER 0 or ∞

In this case we have the situation as shown in Figure 2.

The partition coefficient of B, K_B, is determined by partitioning B alone. In the equilibrium-partition experiment we determine the concentration of B in the upper phase $[B]_t$ and we can thereby calculate the concentration of free B in the lower phase $[B]_b$

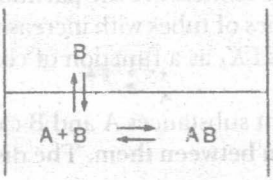

Figure 2. The same as Figure 1, but the partition coefficients of A and AB are so low that their concentration in the upper phase can be neglected.

since

$$[B]_b = \frac{[B]_t}{K_B} \tag{15}$$

If we know the total amounts of A and B added to the system and if we know the volumes of the phases, we can calculate both $[A]_b$ and $[AB]_b$ and therefore also the dissociation constant. We may also employ the Scatchard plot, that is, we plot bound/free versus bound in order to obtain both the dissociation constant and the number of binding sites.

2. Association of Two Identical Molecules

The association of two molecules of A to the dimer A_2 can also be detected and studied quantitatively (Middaugh and Lawson, 1980) by partition. In this case we can use (10) and (11) and set B equal to A and K_{AB} equal to K_{A_2}, the partition coefficient of the dimer. This can be determined by extrapolation of the partition coefficient at a high concentration when the dimers dominate.

The following expression can then be written for the dissociation constant of the A_2 complex in the lower phase:

$$K_b = 2\,\frac{([A^0]_t - [A^0]_b K_{A_2})^2}{(K_{A_2} - K_A)\,([A^0]_t - K_A[A^0]_b)} \tag{16}$$

and for the upper phase,

$$K_t = \frac{(K_A)^2}{K_{A_2}} K_b \tag{17}$$

Treatment of Data

According to (10) and (11), K_t and K_b can be calculated from one partition experiment only. However, more-reliable values of the dissociation constants are obtained if the partition is carried out at different concentrations of A and B. The dissociation constant so obtained should be independent of concentration for a 1:1 complex. However, one has to be sure that the concentrations of A and B are chosen such that part of A or B is free and the rest bound. Also one should check that K_A and K_B are independent of concentration in the concentration range used. A convenient approach is therefore to measure the partition coefficients of A and B alone and mixed in a series of tubes with increasing concentrations of A and B, and then plot K_t and K_b as a function of concentration, as seen in Figure 5.

Partition of two different substances A and B can therefore be used to detect if there is interaction between them. The dissociation constant can

be determined easily in the case of a 1:1 complex. In the special case when one of the substances has a very low or a very high K value, the number of binding sites can also be determined from the Scatchard plot.

However, one may also get an indication of the surface of contact between the two molecules A and B. This information can be obtained by comparing the partition coefficient of the complex, K_{AB}, with K_A and K_B.

We use the following argument. First we assume that the free energy of transfer ΔG_A of a particle A between one phase and the other is the difference between the surface free energy G^s of the particle in the two phases:

$$RT \ln K_A = \Delta G^s{}_A = G^s{}_{A,bottom} - G^s{}_{A,top} \tag{18}$$

In the same way for particle B,

$$RT \ln K_B = \Delta G^s{}_B = G^s{}_{B,bottom} - G^s{}_{A,top} \tag{19}$$

Further we assume that the free energy of transfer of the complex is the difference between the surface free energy of the complex in the lower and the upper phase.

$$RT \ln K_{AB} = \Delta G^s{}_{AB} = G^s{}_{AB,bottom} - G^s{}_{AB,top} \tag{20}$$

If there is a small contact area between the two interacting particles, then the surface of the complex is the sum of the surfaces of the two particles. Therefore,

$$\Delta G^s{}_{AB} = \Delta G^s{}_A + \Delta G^s{}_B \tag{21}$$

and

$$RT \ln K_{AB} = RT \ln K_A + RT \ln K_B \tag{22}$$

or

$$K_{AB} = K_A K_B \tag{23}$$

If, on the other hand, the contact surface between the two interacting particles is large, (21) no longer holds. In this case, surfaces of each particle "disappear" from contact with the surrounding phase. We call the contact surface of particles A and B a_c and b_c, respectively, and the remaining surfaces that are exposed to the surrounding liquid a_e and b_e, respectively (Figure 3). The partition coefficient of particle A is determined by the surfaces a_c and a_e such that

$$RT \ln K_A = \Delta G^s{}_{a_c} + \Delta G^s{}_{a_e}$$

where $\Delta G^s{}_{a_c}$ and $\Delta G^s{}_{a_e}$ are the surface free energies of transfer of the

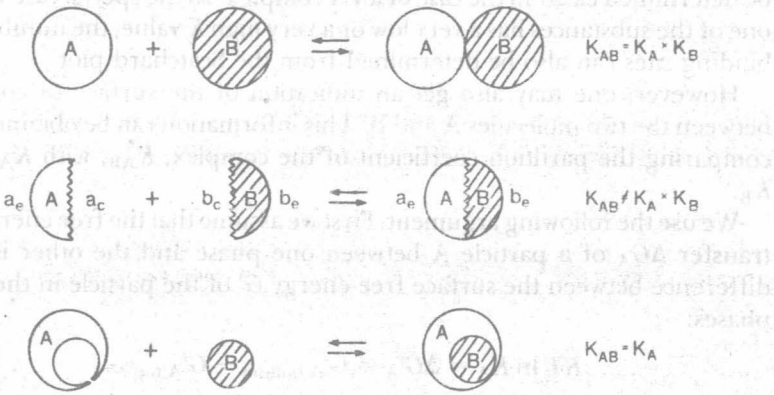

Figure 3. Three different models for complex formation between A and B. *(Upper)* Molecules A and B have a very small surface of contact. In this case the partition coefficient K_{AB} should be a product of the partition coefficients of A and B (see text). This model might represent some protein–protein interactions. *(Middle)* Molecules A and B have a very large surface of contact. In this case one would expect that the partition coefficient of the complex would differ from the product of the partition coefficients of A and B. An example of this model might be interactions between subunits of proteins, for example hemoglobin. An extreme example of this case is the complex between two single-stranded nucleic acids. *(Lower)* Molecule A encloses B in the complex. In this case the partition coefficient of the complex would be the same as that of A, since A and AB expose the same surfaces to the medium. An example of this case is when a detergent micelle encloses a hydrophobic protein or when a ligand is buried in a crevice of an enzyme.

surfaces a_c and a_e, respectively. In the same way the partition coefficient of particle B depends on its surfaces such that

$$RT \ln K_B = \Delta G^s{}_{b_c} + \Delta G_{b_e} \tag{24}$$

where $\Delta G^s{}_{b_c}$ and $\Delta G^s{}_{b_e}$ are the surface free energies of transfer of the surfaces b_c and b_e, respectively.

The partition coefficient of the complex depends on its surfaces as follows:

$$RT \ln K_{AB} = \Delta G^s{}_{a_e} + \Delta G^s{}_{b_e} \tag{25}$$

We then get the following relation between the partition coefficient of the complex AB and the partition coefficients of A and B:

$$\ln K_{AB} = \ln K_A + \ln K_B - \frac{\Delta G^s{}_{a_c} + \Delta G^s{}_{b_c}}{RT} \tag{26}$$

The last term is zero when