COUNTER-CURRENT DISTRIBUTION OF INTERACTING MOLECULES

Simulation of distribution behaviour
and
application to protein-protein interactions

by

Lars Backman

AKADEMISK AVHANDLING

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Abstract

Associations of biological macromolecules with other macromolecules, with larger assemblies of macromolecules and with themselves are widely encountered phenomena. In principle, these interactions can be studied with any method able to differentiate between free molecules and complexes formed. The most extensively used techniques are sedimentation equilibrium and velocity, elastic light scattering and molecular sieve chromatography. This thesis describes an alternative technique; counter-current distribution in aqueous two-phase systems.

The counter-current distribution behaviour of a solute depends on its size and surface properties including charge and hydrophobicity. Since the surface properties of a complex formed most probably differ from those of the solutes participating in the association, complex formation should lead to changes in the average distribution behaviour of each solute. Consequently, the presence of one solute should affect the counter-current distribution of another solute if they interact with each other.

In order to establish the boundary conditions and the potential as well as limitations of the counter-current distribution technique, the distribution behaviour of homogeneous and heterogeneous association equilibria have been simulated.

The model developed for describing the distribution behaviour of heterogeneous associations has been tested using the well characterized interaction between bovine serum albumin and L-tryptophan. It was demonstrated that the theoretical model could predict the experimental distribution behaviour of these two molecules.

However, the primary aim of the counter-current distribution experiments has been to gain insight into protein-protein interactions. The metabolically linked enzymes, malate dehydrogenase and aspartate aminotransferase, have been studied in order to determine if there is also a physical link between these two enzymes. The results showed that the cytosolic enzymes as well as the mitochondrial forms associate while the cytoplasmic enzymes did not display any association with the mitochondrial forms. Thus, an organelle specific interaction between malate dehydrogenase and aspartate aminotransferase was demonstrated.

Hemoglobin and carbonic anhydrase are functionally linked through the Bohr effect. Thus, the binding of oxygen by hemoglobin in the lung capillaries is associated with the binding of protons which are formed by the catalytic action of carbonic anhydrase. From the counter-current distribution experiments it was possible to conclude that human carbonic anhydrase II, the high activity form, associates with human hemoglobin whereas carbonic anhydrase I, the low activity form, did not show any affinity for hemoglobin.

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...
you used to tell me about
Wonderful books you had just read,
And then I would imagine that I
Had read them, too
You read nothing but the best,
While I studied chemistry.

Kurt Vonnegut

The dissertation is based on the following papers, which are refered to by Roman numerals in the text.

I. Backman, L. and Shanbhag, V. (1979) Protein-protein interactions studies by counter-current distribution. I. Theoretical computations. *J. Chromat.* 171:1-13.

II: Backman, L. (1981) Protein-protein interactions studied by counter-current distribution. III. Simulation of self-associating systems. *manuscript.*

III. Backman, L. (1980) Protein-protein interactions studied by counter-current distribution. II. Test of the theoretical model using bovine serum albumin and L-tryptophan. *J. Chromat.* 196:207-216.

IV. Backman, L. and Johansson, G. (1976) Enzyme-enzyme complexes between aspartate aminotransferase and malate dehydrogenase from pig heart muscle. *FEBS Lett.* 65:39-43.

V. Backman, L. (1981) Binding of human carbonic anhydrase to human hemoglobin. *accepted for publication in Eur. J. Biochem.*

CONTENTS

PREFACE

I am very grateful to my teachers and collaborators Göte Johansson and Vithaldas Shanbhag for their never-ceasing enthusiasm and continous scientific support that have inspired me during the years I have been working with this thesis.

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Finally, I wish to thank all my colleagues at the department of Biochemistry for stimulating and inspiring me, not only in science, during these years.

PROLOGUE

A randomly ordered sequence of characters like

r t e t l e

makes no sense to us. We know that each symbol represents a constituent of the alphabet, but we can not discern any meaning. However, if the characters are ordered differently the word

l e t t e r

appears. This sequence has a meaning to us, it is a word with a well defined meaning. According to the dictionary the word »*l e t t e r*» means a character or sign representing a sound of which words in writing are formed. »*l e t t e r*» can also be a written message sent by one person to another. Nevertheless, at the word level it is not possible to distinguish between these two interpretations. If a number of correctly ordered sequences of characters are put together a sentence is created.

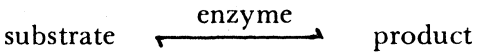
B i s a l e t t e r

At the sentence level it is then possible to discriminate between »*l e t t e r*» and »*l e t t e r*», and thus the correct interpretation is revealed due to the interplay between different words. Similarly, a number of sentences form a piece of text, which in turn, might interplay with other pieces of texts and thereby form a more complex assembly of symbols. Thus, the co-operation at one level accomplishes a more complex significance at a higher level.

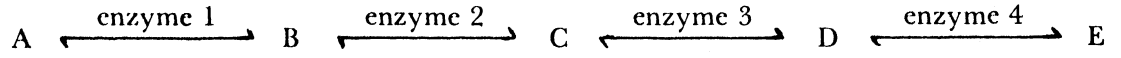
On the other hand, if for example the character »*e*» is removed from the set of symbols, the meaning at the word level in the example above will diminish. Accordingly, the interpretation at higher levels will also be affected.

This example shows that a meaning at one level does not originate from the constituents at lower levels alone but rather is accomplished by intermingled interactions and interplay between the constituents. However, some basic knowledge about the constituents at one level is necessary in order to understand the message at the next higher level.

In a living organism the basic characters can be represented by biological macromolecules such as nucleic acids, proteins, carbohydrates as well as by lipids. These basic entities of the biological alphabet can interplay or interact with one another and thereby form »words». For example, an enzyme catalyzes the conversion of a substrate into a product.

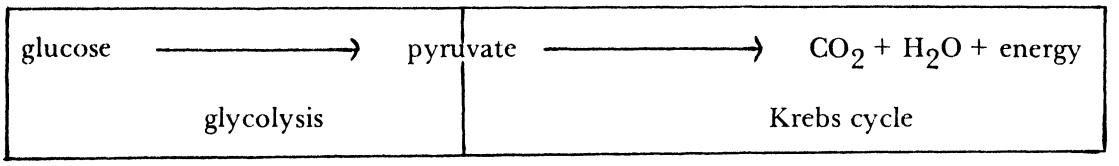


However, the particular product might be a substrate for another enzyme which in turn converts it into a second product and so on:



By such a functional interaction between a number of enzymes a biological »word», a metabolic sequence, is created. Similarly, other »words» like membrane and ribosome are created by a complex interplay between proteins and lipids, and between proteins and ribonucleic acids, respectively.

Biological »sentences» are created by physical or functional interactions between »words». For example, glycolysis, which metabolises glucose into pyruvate, and the Krebs cycle, which converts pyruvate to carbon dioxide, water and energy, form a biological »sentence». In this example the sentence might be interpreted as: The main energy producing machinery of oxygene consuming organisms.



Biological »pieces of texts» or supra-molecular assemblies such as the cell nucleus, mitochondria, chloroplasts, etc., are formed by complex interactions between different »sentences». The next level would be the cell itself and assemblies of cells to differentiated tissues, and finally the organism.

However, just as single letters fail to communicate any significant meaning, a complete description of the basic units of the biological alphabet does not clarify the complex machinery of living matter.

An enzyme is completely described kinetically by its particular characteristics such as K_M, V_{max}, rate constants and inhibitor effects. Hence, it may be expected that by combining the kinetic data of each enzyme in a metabolic sequence together with intracellular metabolite concentrations the over-all rate of the pathway would be achieved. However, this does not always yield a reasonable value as pointed out by Halper and Srere (1977). They calculated, from available kinetic data and intracellular substrate concentrations, that the rate of the citrate synthase reaction, which is the first step in the Krebs cycle, could only account for about 10% of the observed over-all rate of the Krebs cycle. This divergence can be explained on the basis of the observation of Green and co-workers (1965) among others, who demonstrated that the kinetic parameters can be affected by interplay with other cellular assemblies. They found that it was possible to obtain both from red blood cells and yeast cells membrane fractions that catalyze the complete glycolytic sequence of reactions without supplementary addition of enzymes with specific activities greater than those of whole homogenates. Likewise, it has been demonstrated in numerous cases that the binding of an enzyme to particular tissue fractions leads to significant alterations in the kinetic parameters (Masters, 1981).

Associations of biological macromolecules with other macromolecules, with larger assemblies of macromolecules and with other biomolecules have been the topic of a great deal of scientific consideration in the past. Examples of interacting systems that have been studied extensively are protein-nucleic acid (von Hippel and McGhee, 1972; Kurland, 1972; Elgin and Weintraub, 1974; Fitzimons and Wolstenholme, 1975; Kornberg, 1977; Brimacombe and Stöffler, 1978; Champoux, 1978), protein-membrane and protein-subcellular structure (Green et al, 1965; Gennis and Jonas, 1977; DePierre and Ernster, 1977; Masters, 1978; Op den Kamp, 1979; Wickner, 1979; Gratzer, 1981; Masters, 1981; Weatherbee, 1981) and protein-protein (see references marked with *). In many of these cases the physiological significance has also been established.

Thus, it can be concluded that nucleic acids, proteins, carbohydrates and lipids form the basis of life, but that life is created by interactions and co-operation between these basic units of the biological alphabet. Indeed, the motto of d'Artagnan is highly valid for living organisms;

Tous pour un, un pour tous

1. SOME ASPECTS OF PROTEIN–PROTEIN INTERACTIONS

Protein-protein interactions can be divided into two groups, heterogeneous and homogeneous interactions. The first type of association occurs between two or more dissimilar proteins,

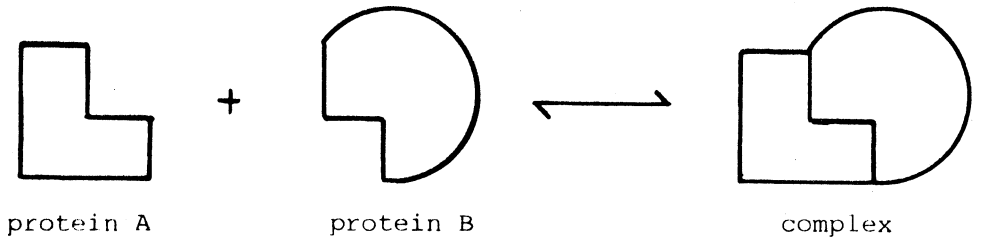


Figure 1. Schematic illustration of heterogeneous protein-protein interaction.

In the second type, also referred to as self-association or polymerization, proteins associate with themselves.

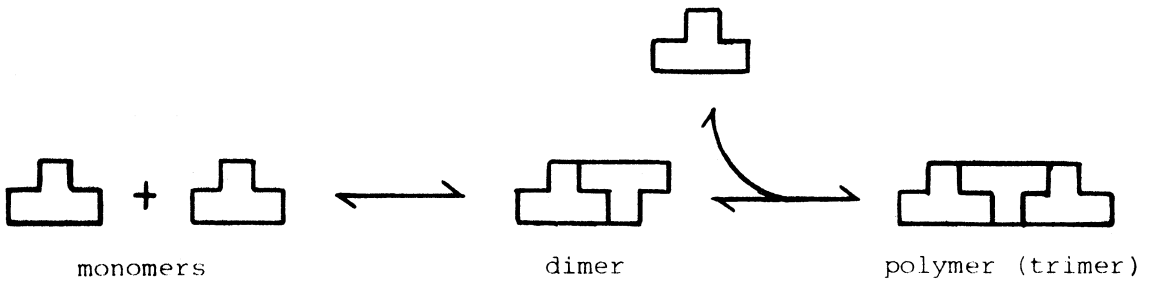


Figure 2. Schematic illustration of self-association.

A complex can be composed of a discrete number of a single protein molecule such as the shell of many special viruses (Casjens and King, 1975). More complex assemblies like bacteriophages (Casjens and King, 1975; Kellenberg, 1980), multienzyme complexes (Ginsburg and Stadtman, 1970; Reed, 1974; Lynen, 1980) and cytoskeletons (Gratzer, 1981) contain different numbers of various protein molecules. Another type is the linear complexes which are not always simple chain polymers, but rather helical or tubular structures, in which each protein molecule binds with many others. The coat protein (A-protein) of tobacco mosaic virus associates in acidic media to form structures with a helical arrangement very similar to that in tobacco mosaic virus (Casjens and King, 1975; Lauffer, 1978). Bacterial flagella is mainly composed of one single protein, flagellin, which is arranged in a helical cylinder (Gonzalez-Beltran and Burge, 1974; Calladine, 1975).

Muscle filaments are a good example of structures based on protein-protein interactions of different degrees of complexity. Actin, the main constituent of thin filaments in muscle cells, forms a helical polymer composed of two strands of globular actin molecules (Cold Spring Harbor Symposia, 1972; Oosawa and Asakuro, 1975). Tropomyosin and troponin, the other two constituents of thin filaments, have specific binding sites on the actin double helix. Thin filaments form together with thick filaments, which are bundles of associated myosin mole-

cules, the basic functional unit of the contractile system of muscle tissue (Murray and Weber, 1974).

All these assemblies based on protein-protein interactions share a common feature; they are relatively stable. Therefore, it has often been possible to isolate and purify the complete complex or at least a large part of it. In many cases it has also been possible to reconstitute the complexes in vitro from their constituents. As a consequence, these macromolecular assemblies have been studied with many different analytical approaches and are thus both structurally and functionally well substantiated. Weaker, reversible protein-protein interactions have been given increasing consideration lately, especially in view of their possible involvement in the organization and compartmentation within the cell and its organelles. Much of the interest has been directed towards the cytosol, containing as it does several major metabolic pathways and a number of so-called soluble enzymes. As cited earlier, the findings of Green and co-workers (1965), led them to assume that the entire glycolytic sequence was membrane bound and also to postulate that all major metabolic sequences might be associated with membranes in vivo. Support for the concept of a glycolytic complex has come from a variety of sources. Mowbray and Moses (1976) have been able to identify a multienzyme complex with glycolytic activity from Escherichia Coli. The localization of nine glycolytic enzymes within a microbody-like organelle, called a glycosome, has been established in trypanosomes by Opperdoes and Borst (1977). The emerging pattern in mammalian tissues is somewhat different. In this case, it seems that the glycolytic enzymes as well as other soluble enzymes are associated with structural components containing actin-like proteins (Masters, 1978; 1981).

In red blood cells, the band 3 protein (nomenclature according to Fairbanks et al, 1971) shows a very complicated association pattern. Band 3 is a transmembrane protein, very tightly bound to the membrane and thought to be responsible for anion transport (Rothstein et al, 1980). Yu and Steck have shown that band 3 exists as a non-covalent dimer in the membrane (Yu and Steck, 1975). The cytoskeleton is anchored to the membrane via band 2.1 protein which associates with both spectrin and band 3 protein (Sheetz and Casaly, 1980; Gratzer, 1981).

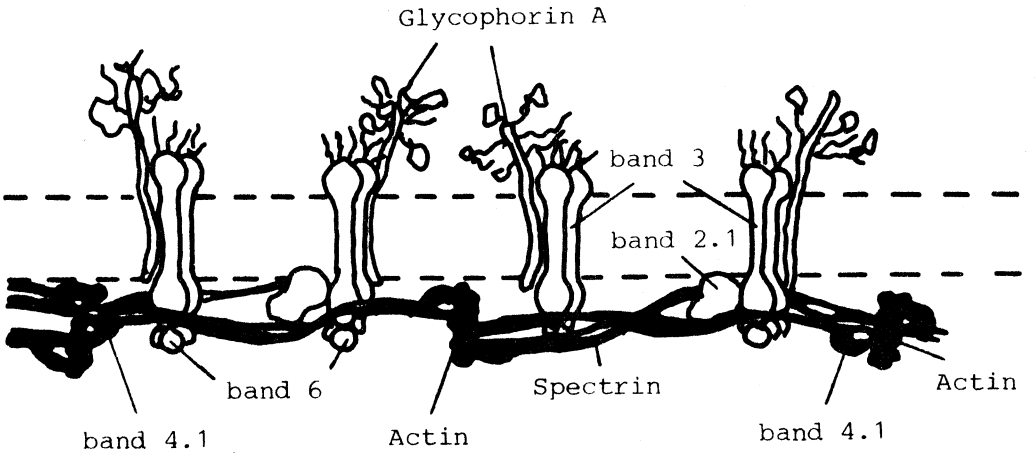


Figure 3. Schematic illustration of protein-protein interactions involving band 3 protein. Band 6 represents glyceraldehyde 3-phosphate dehydrogenase. Adapted from Gratzer (1981).

There is also evidence that the major sialoglycopeptide of the red blood cell membrane, glucophorin A (Cherry and Nigg, 1980) as well as glyceraldehyde 3-phosphate dehydrogenase (Kant and Steck, 1973) and aldolase (Strapazon and Steck, 1977) are associated with band 3 protein. Furthermore, the ^{31}P nuclear magnetic resonance studies of Fossel and Solomon (1976) have suggested that a complex consisting of glyceraldehyde 3-phosphate dehydrogenase, phosphoglycerate kinase, monophosphoglycerate mutase and band 3 protein exists in human red blood cells. This result has, however, been questioned and the observed shifts in the ^{31}P nuclear magnetic resonance spectra have been attributed to artefacts due to small pH changes in the media (Momsen et al, 1979). Yet another interaction involving band 3 protein is its suggested participation in the association of hemoglobin with the red blood cell membrane (Shaklai et al, 1977). Spectrin has also been proposed as a possible point of attachment of hemoglobin (Chaimanee and Yuthavong, 1977). Hemoglobin, in turn, interacts with both bovine and human carbonic anhydrases (Silverman et al, 1979; paper V).

Interactions between a number of enzymes catalyzing consecutive metabolic reactions have also been reported. For example, Fahien and co-workers (1979) have shown that glutamate dehydrogenase might form complexes with malate dehydrogenase and aminotransferases. Malate dehydrogenase, in turn, seems to associate both with citrate synthase (Halper and Srere, 1977) and with aspartate aminotransferase (Bryce et al, 1976; paper IV). Evidence, both kinetic and physical has also been obtained in favour of an interaction between the soluble enzymes aldolase and glyceraldehyde 3-phosphate dehydrogenase (Ovaldi et al, 1978).

The theoretical implications and possible advantages of interactions between metabolically linked enzymes have been discussed by a number of authors (Frieden, 1971; Friedrich, 1974; Keleti et al, 1977; Welch, 1977; Gaertner, 1978; Masters, 1978; 1981). It has been suggested that juxtapositioning of enzymes might lead to channelling and reduced transit times of intermediate metabolites and to increased possibilities for control and regulation. However, many significant questions concerning the physiological significance of these interactions still remain to be answered.

2. SOME METHODOLOGICAL CONSIDERATIONS

Reversible molecular interactions can be studied by any method able to distinguish between individual molecules and complexes. For practical reasons, however, molecular associations are generally studied by techniques able to discriminate features of free individual moelcules from features induced by complex formation.

The most obvious consequence of complex formation is the strict additive relation between the molecular weights of the complex and its constituents. For example, consider a solute which self-associates according to

$$A + A \rightleftharpoons A_2$$

Obviously, the molecular weight of the complex must be twice that of the monomer.

In accordance with the law of mass action the fractional amount of the complex must increase with increasing concentration. Accordingly, the average molecular weight also increases with increasing concentration.

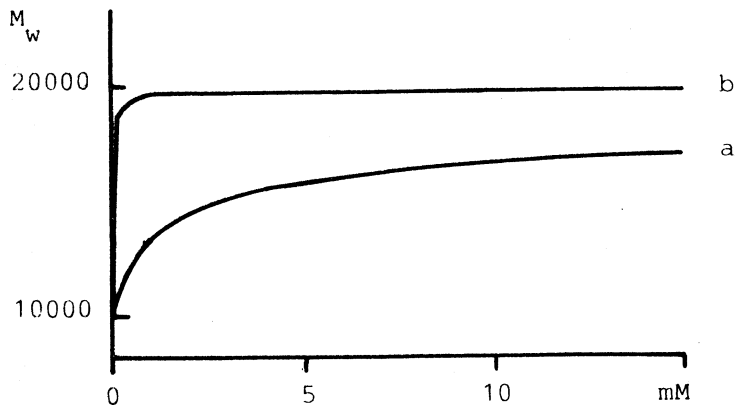


Figure 4. Calculated weight average molecular weights for a monomer-dimer equilibrium. Equilibrium constants were 1000 M^{-1} (curve a) and $1 \cdot 10^6$ M^{-1} (curve b).

Methods, such as sedimentation equilibrium and velocity, membrane osmometry and elastic light scattering, which yield molecular weights, can thus be used for monitoring molecular associations.

Due to the larger molecular size of a complex, an association will be manifested by a concentration dependent elution profile in molecular sieve chromatography.

Another inevitable effect of an association is that the molecules within the complex must be in physical contact with each other and consequently, that some surface area of each molecule is removed from contact with surrounding medium. Therefore, the surface properties of the complex would most probably differ from those of the interacting molecules. Thus, the net

charge of a complex and hence also its electrophoretic mobility might be different. This in turn will cause the apparent mobility to display a concentration dependency. Similarly, the solubility and hydrophobicity of a complex might differ from that of the free molecules and thereby lead to altered average characteristics. A complex formation might also cause conformational changes in the associated molecules which can lead, for example, to new spectral properties or to new regulatory mechanisms in the case where an enzyme is involved in the association.

However, all methods available for the study of molecular interactions have their own distinctive features. Some are more useful for a thermodynamic analysis of an association whereas others are more suited for descriptive studies.

3. EQUILIBRIUM METHODS

A homogeneous or heterogeneous molecular interaction in solution leads to the formation of molecular species with molecular weights higher than those of the reacting molecules. Consequently, weight average and number average molecular weights, defined by

$$M_w = \frac{\Sigma\, c_i \cdot M_i}{\Sigma\, c_i}$$

and

$$M_n = \frac{\Sigma\, c_i}{\Sigma\, (c_i/M_i)}$$

as well as other types of molecular weight averages will vary with the degree of association. Therefore, a concentration-dependent change in average molecular weight is indicative of an association and the average values can thus be used for thermodynamical analysis of interactions.

Average molecular weights or their apparent values in non-ideal solutions can be obtained by three basic methods; sedimentation equilibrium, elastic light scattering and membrane osmometry.

Sedimentation equilibrium

The theory for sedimentation equilibrium of homogeneous associations has been treated very extensively (Teller, 1973; Kim et al, 1977; Adams et al, 1978a). Theoretical treatment of heterogeneous associations, on the other hand, has been less comprehensive (Nichol and Ogston, 1965; Chun and Kim, 1970; Pekar et al, 1971a; 1971b; Teller, 1973).

For a thermodynamic analysis of molecular interactions based on sedimentation equilibrium experiments it is assumed that the partial specific volumes and the refractive index increments of all molecular species are equal (Adams et al, 1978a). Furthermore, it is also usually assumed that the associating system is incompressible and, especially in the case of heterogeneous associations, that the system is ideal in that the activity coefficient of each species is unity (Nichol and Ogston, 1965).

One general approach in the treatment of experimental data is to use molecular weight averages calculated at single points in the concentration distribution in the cell. The apparent weight average molecular weight, M_w^{app}, at radical distance, r, may be obtained from

$$M_w^{app} = \frac{d(\ln\, \bar{c}(r))}{d(r^2)} \cdot \frac{1}{\Phi_A}$$

where $\bar{c}(r)$ is the total concentration of all species at r and

$$\Phi_A = \frac{\omega^2 (\delta\rho/\delta c_A)}{2 \cdot R \cdot T}$$

where ω is the angular velocity, $(\delta\rho/\delta c_A)$ is the density increment replacing the conventional $(1-\bar{\nu}_A\rho)$ term, R is the gas constant and T is the absolute temperature (Howlett and Nichol, 1972). Other apparent molecular weight averages such as the apparent number average molecular weight and the Z average molecular weight can also be evaluated from the experimental data (Teller, 1973; Kim et al, 1977). From these averages at each point it is then possible to determine the molecular weight and stoichiometry as well as equilibrium constants of the association (Teller, 1973; Kim et al, 1977; Adams et al, 1978a). However, this approach is usually applicable to self-associations only. It is also possible to analyse the concentration distribution directly by expressing the distribution as a sum of exponentials in the constituents (Chun and Kim, 1970; Teller, 1973; Aure and Rohde, 1977; Kim et al, 1977). In this case it is generally required that the molecular weights of the species are known but, on the other hand, rather complex reaction stoichiometries can be solved (Chun and Kim, 1970; Teller, 1973). Various graphical techniques have also been developed for the interpretation of sedimentation equilibrium data (Kim et al, 1977; Stafford, 1980).

Sedimentation equilibrium has mainly been utilized for the investigation of self-associating proteins. Lysozyme, β-lactoglobulins and α-chymotrypsin among many others are examples of proteins that have been studied extensively by sedimentation equilibrium. Other examples of the application of this method for the study of self-associating proteins can be found in the reviews of Adams et al (1978a) and Kim et al (1977). Reports on sedimentation equilibrium studies of heterogeneous protein-protein interactions are, on the other hand, very few (Howlett and Nichol, 1972).

Membrane osmometry

When membrane osmometry is used to study associations one first evaluates the apparent number average molecular weight, M_n^{app}, which can be obtained from the relation

$$\frac{1000 \cdot \pi}{R \cdot T} = \frac{\bar{c}}{M_n^{app}}$$

where π is the osmotic pressure in atmospheres, R is the gas constant, T is the absolute temperature and $\bar{c}$ is the total concentration. From the number average molecular weight it is possible to calculate the apparent weight average molecular weight and the apparent weight fraction of monomer (Adams et al, 1978b).

In contrast to sedimentation equilibrium and light scattering it is only necessary to assume, in non-ideal cases, that the natural logarithm of the activity coefficient of species *i* can be represented by a Taylor series expansion

$$\ln\gamma_i = M_i \cdot \sum_k B_{ik} \cdot c_k$$

where B is the second virial coefficient (Adams et al, 1977; 1978b). The theoretical treatment of how to obtain the stoichiometry and equilibrium constants as well as molecular weight have been described extensively by Adams and co-workers (1977; 1978b). In general, experimental data obtained from membrane osmometry can be treated similarly to those obtained from sedimentation equilibrium.

Although membrane osmometry is a simple technique to master, it has not been frequently used for studying protein associations. The technique has, however, been applied primarily to investigate self-associating proteins such as tobacco mosaic virus protein (Banerjee and Lauffer, 1966) and various histones (Diggle and Peacock, 1971) but also to heterogeneous associations (Crouch and Kupke, 1980).

Elastic light scattering

The third general technique for obtaining molecular weight averages is elastic light scattering experiments. This method yields the apparent weight average molecular weight, M_w^{app}, as primary data through the following relation (Tanford, 1965; van Holde, 1971)

$$\frac{K \cdot \bar{c}}{\triangle R_\theta} = \frac{1}{M_w^{app}}$$

where $\triangle R_\theta$ is the excess reduced scattering, that is

$$\triangle R_\theta = R_\theta^{solution} - R_\theta^{solvent}$$

The Rayleigh ratio, R_θ, is defined by

$$R_\theta = \frac{i_\theta}{I_o} \cdot \frac{r^2}{1 + \cos^2\theta}$$

where r is the distance from the centre of the light scattering cell to the detector, I_o and i_θ are the respective intensities of the incident light and that scattered at an angle θ with respect to the incident.

$\bar{c}$ is the total concentration and K is an optical constant

$$K = \frac{2\pi^2 \cdot n_o^2 (\delta n/\delta \bar{c})^2}{N \cdot \lambda^4}$$

The value of K depends on temperature, the nature of the solvent-solute combination and on λ, the wave length of the light used, as these affect the values of the refractive indices of the solvent (n_o) and of the solute (n) as well as the refractive index increment ($\delta n/\delta \bar{c}$). N is Avogadro's number. However, for large macromolecules it is necessary to extrapolate the values of $K\bar{c}/\triangle R_\theta$ to $\theta = 0^o$.

The apparent weight average molecular weight can be calculated from light scattering data under the assumption that the refractive index increments of all the species in an association equilibrium are equal and that the activity coefficients can be expressed as above. It is there-

fore possible to analyse light scattering data in a manner similar to sedimentation equilibrium data.

It is also possible to use light scattering for determining the kinetics of an interaction. In addition, with large molecules, where there is an angular dependence of the intensity of scattered light, it is possible to obtain the radius of gyration (Tanford, 1965).

Elastic light scattering has, like the other equilibrium methods, mostly been used for studying protein self-associations (Bruzzesi et al, 1965; Kumosinski and Timasheff, 1966; Resiler and Eisenberg, 1971; Pletcher et al, 1980).

4. TRANSPORT METHODS

The concentration distribution generated by a transport process, like sedimentation velocity, electrophoresis or molecular sieve chromatography, is dominated by diffusion which leads to dispersion of the initial solute zone. Diffusion gives rise to Gaussian shaped concentration distributions or concentration gradients, depending on the process, provided that no association takes place. If, however, there is interaction the shape of the distribution or gradient will be characteristically affected (Cann, 1970; Gilbert and Gilbert, 1973). Thus, the appearance of non-Gaussian shaped concentration distributions or concentration gradients are indicative of associations.

In molecular sieve chromatography, for example, the Gaussian shape is disturbed by the tendency of larger molecules to move faster within the column than smaller ones. At the trailing edge, both diffusion and association cause a continuous decrease in concentration and this promotes dissociation and decreases the transport velocity. This leads to a continuous dispersion of the trailing boundary. Those molecules that move ahead into a region of lower concentration are also slowed down due to dissociation. However, at the leading edge this causes a continuous sharpening of the boundary (Ackers, 1975).

The theory describing the transport-behaviour of rapidly re-equilibrating self-associating systems was first presented by Gilbert (1955). Neglecting the effect of diffusion on the shape of sedimentation velocity patterns, Gilbert showed how to interprete such patterns properly in order to obtain both stoichiometries and equilibrium constants of self-associations. Later, the Gilbert theory was verified by Townend and co-workers in their studies on the self-association of β-lactoglobulin (Townend et al, 1960).

As diffusion, in general, does not introduce totally new features into a boundary, the essential features predicted by the diffusion-free treatment of homogeneous (Gilbert, 1955) and heterogeneous (Gilbert and Jenkins, 1959) associations are useful in illustrating the typical features of reaction boundaries. However, for a more detailed description of the transport behaviour of an interacting system this procedure is not suitable.

In sedimentation velocity, free diffusion is influenced by a centrifugal force which causes a directed flow of solute molecules. This transport process is described by the continuity equation, which for an ideal transport behaviour of a single non-interacting solute is given by

$$\frac{\delta c}{\delta t} + \frac{1}{r} \cdot \frac{\delta (rJ)}{\delta r} = 0$$

where c is the solute concentration, r is the radial distance and J is the flux, defined by

$$J = s \cdot \omega^2 \cdot r \cdot c - D \frac{\delta c}{\delta r}$$

in which s is the sedimentation coefficient, ω is the angular velocity and D is the diffusion coefficient (Cann, 1970; Claverie et al, 1975).

However, in non-ideal cases and in associating systems where the sedimentation and the diffusion coefficients are functions of the solute concentrations the continuity equation may be written as

$$\frac{\delta c_i}{\delta t} + \frac{1}{r}\frac{\delta (r J_i)}{\delta r} = f_i$$

where f_i describes the interrelation between the ith species and all the others (Cohen and Claverie, 1975; Claverie, 1976).

In general, the conservation of mass for a transport process is (Claverie, 1976)

$$\frac{\delta c}{\delta t} + \frac{\delta J}{\delta x} = f$$

In electrophoresis the flux J is expressed by (Cann, 1970)

$$J = \mu \cdot E \cdot c - D \frac{\delta c}{\delta x}$$

where μ is the electrophoretic mobility and E is the electric field strength, whereas in molecular sieve chromatography (Ackers, 1975)

$$J = \frac{F}{\xi \cdot A} \cdot c - L \frac{\delta c}{\delta x}$$

in which F is the bulk flow rate, ξ is the accessible volume fraction, A is the cross-section area of the column and L is the axial dispersion.

It is immediately evident that the flux equations are very similar to each other; consisting of a driving force term and a diffusion term. On account of these analogies, the theoretical treatment concerning sedimentation velocity is also applicable to electrophoresis and molecular sieve chromatography and vice versa.

The continuity equation provides the starting point for simulations of associating systems undergoing transport processes. A number of different approaches for simulation have been described in the literature, see for example the reviews on molecular sieve chromatography by Ackers (1975), on electrophoresis by Cann (1970) and on sedimentation velocity by Cox (1978) and by Gilbert and Gilbert (1978).

The most straightforward approach to evaluate experimental transport data is to fit simulated patterns of different associating systems to the experimental patterns. The best fitting model might yield the stoichiometry and the equilibrium constant of the interaction. However, other approaches are of course also possible. For example, in molecular sieve chromatography use can be made of the dependence of the weight average partition coefficient upon the solute concentration. The dependence is determined by the equilibrium constants of the interaction and the partition coefficients of the individual associating molecules (Ackers, 1975). A similar procedure applicable to sedimentation velocity makes use of the weight average sedimentation coefficient (Weirich et al, 1975).

Furthermore, as first described by Gilbert (1955) and later extended by Cox (1969), the shape of the concentration gradient of a self-associating system may give information about the stoichiometry of the association. In the case of monomer-dimer equilibrium, the gradient should be unimodal, whereas if higher polymers are present the pattern should, under certain circumstances, display a bimodality or show a tendency to develop a minimum (Cox, 1969).

Continuous transport techniques have primarily, like equilibrium techniques, been applied experimentally to self-associating systems. Many examples are cited in the reviews of Ackers (1975), Cann (1970), Cox (1978) and Gilbert and Gilbert (1978). However, transport experiments have also been used to investigate heterogeneous protein-protein interactions. For example, the binding of aldolase to muscle proteins (Walsh et al, 1980) and the complex formation of α_{S1}- and β-casein (Payens and Nijhuis, 1974), the nitrogenase complex (Davis and Chen, 1979) and the interaction between myoglobin and ovalbumin (Gilbert and Kellett, 1971) and between Bence-Jones proteins (Stevens and Schiffer, 1981) have been investigated by electrophoresis, sedimentation velocity and molecular sieve chromatography, respectively.

5. MISCELLANEOUS METHODS

Many other methods, such as various spectroscopic techniques, calorimetry and isoelectric focusing, have been described and used for analysis of associating protein systems (Hirs and Timasheff, 1979). Some of these methods are intended for qualitative use only whereas others are also useful for thermodynamic interpretation of experimental results. In this part I would like to mention two interesting approaches, namely chemical cross-linking and co-precipitation in the presence of polyethylene glycol.

Chemical cross-linking

An obligatory consequence of a homogeneous or heterogeneous interaction is that the average distance between the associating macromolecules must be shorter than between non-associating molecules. Therefore bifunctional cross-linking reagents of defined length should serve as a »measure» of the average distance between macromolecules in solution. This nearest neighbour approach has been applied by Fahien and co-workers in combination with molecular sieve chromatography, for investigation of the possible complex formation between glutamate dehydrogenase and malate dehydrogenase (Fahien et al, 1979). They found that when these two enzymes were incubated with the bifunctional cross-linker, 3,3′-dithiobispropionimidate, and chromatographed on Sephadex G-200, about half of the malate dehydrogenase activity was eluted with glutamate dehydrogenase in the void volume. They could exclude the possibility that malate dehydrogenase was in the void volume as a result of cross-linking of two or more malate dehydrogenase molecules, since none, of this enzyme could be detected in the void volume when it alone was incubated with the cross-linker. Hence, their results strongly indicate the formation of a complex.

A similar approach was used by Knull (1980), who found that glutaraldehyde treatment of synaptosomes cross-linked glyceraldehyde 3-phosphate dehydrogenase, aldolase, pyruvate kinase and lactate dehydrogenase to the synaptosomal membrane. Though, the result does not specifically indicate the existence of a complex of glycolytic enzymes it definitely suggests that compartmentation occurs in isolated nerve endings.

Co-precipitation

It has long been known that polyethylene glycol can be used to precipitate proteins (Juckes, 1971). With this in mind together with the hypothesis that non-ionic polymers provide an environment comparable to the cellular environment, Halper and Srere (1977) have studied the polyethylene glycol induced co-precipitation of pig heart citrate synthase and mitochondrial malate dehydrogenase. They found that a large amount of both enzymes precipitated when mixed in the presence of polyethylene glycol, whereas very little of either enzyme was precipitated in the absence of the other enzyme. Furthermore, no protein was precipitated when cytosolic malate dehydrogenase was mixed with citrate synthase in the presence of the synthetic polymer. They concluded therefore that mitochondrial malate dehydrogenase and citrate synthase associate specifically with each other.

Another example of the application of the technique of co-precipitation in polyethylene

glycol media is the study by Fahien and Kmiotek (1979). They investigated the effect of some aminotransferases and malate dehydrogenase on the precipitation of glutamate dehydrogenase and observed that aspartate and ornithine aminotransferase as well as mitochondrial malate dehydrogenase enhanced the precipitation of glutamate dehydrogenase and vice versa, thus indicating an association between these enzymes in the presence of polyethylene glycol.

6. COUNTER–CURRENT DISTRIBUTION

Partition between two immiscible phases, where one of the constituents is usually an organic solvent, is a classical method for separation of different components in a mixture. The use of partition is also widely applied for the study of complexation or chelation of metal ions (Irving and Williams, 1961; Dyrssen et al, 1967).

Since phase systems containing organic solvents have deleterious effects on biological material, the wide use of the partition technique for the study of biological macromolecules was hindered until about 25 years ago. It was then that Albertsson utilized the general phenomenon of immiscibility of two different polymers above a certain concentration to obtain aqueous phase systems (Albertsson, 1958).

He demonstrated the applicability of a large number of aqueous two-phase systems for separation of cell particles. Since then, the versatility of aqueous polymer two-phase system, especially systems containing dextran and polyethylene glycol, for separation and studies of biological materials, such as proteins, nucleic acids, viruses, mitochondria, choloroplasts, cell membranes and whole cells, has been well established (Albertsson, 1971; 1978). Neither dextran nor polyethylene glycol, in the concentration range used in these two-phase systems, have been observed to have any deleterious effects on biological material. It seems rather that the polymers stabilize structure and biological activity.

Partition in dextran-polyethylene glycol-water two-phase systems

When aqueous solutions of dextran and polyethylene glycol are mixed in certain proportions the mixture separates into two phases. The upper phase is enriched in polyethylene glycol whereas most of the dextran is found in the lower phase. Although enriched in one of the polymers, both phases are composed mostly of water which constitutes about 75-90% of each of the phases.

The partition behaviour of a macromolecule can be described by its partition coefficient, K, defined as

$$K = \frac{\text{concentration in upper phase}}{\text{concentration in lower phase}} \qquad (1)$$

The partition of macromolecules between the two phases depends upon their size and surface properties including charge and hydrophobicity as well as on the composition of the two-phase system (Albertsson, 1971). Thus, the partition coefficient can be split into two terms

$$K = K_0 \cdot K_{el} \qquad (2)$$

where K_0 is the contribution from size and surface properties other than charge. K_{el} is the contribution from the net charge of the macromolecule and is a function of the interfacial potential between the two phases. The interfacial potential between the phases arises from an unequal partition of ions of the electrolytes included in the two-phase system (Johansson, 1970).

With an excess of salt it has been shown that the partition of a protein is a function of the interfacial potential, Ψ, as

$$\ln K = \ln K_0 + \frac{F \cdot Z}{R \cdot T} \cdot \Psi \qquad (3)$$

It is evident that K_0 is the value of the partition coefficient when the interfacial potential is zero or when the protein net charge, Z, is zero. F, R and T are the Faraday constant, the gas constant and the absolute temperature, respectively (Albertsson, 1971; Johansson, 1974). Since the partition coefficient changes exponentially with Z even a small potential will strongly influence the partition coefficient if Z is large. This is also true for other charged macromolecules included in the system.

Higher interfacial potentials and, therefore, greater dependence of partition on protein net charge can be achieved by covalent binding of charged groups to polyethylene glycol. Since this polymer is enriched in the upper phase, the bound charged groups are also enriched in the upper phase. This extreme partition of charged groups causes a high interfacial potential (Johansson, 1974).

The partition of a macromolecule can be changed by simply adjusting the pH of the buffer included in the two-phase system. The partition can alternatively be adjusted by changing the salt. There are also other possibilities for influencing the partition of a solute, for example, biospecific ligands or hydrophobic groups can be bound covalently to one of the phase polymers. As the polymers are enriched in the corresponding phases, proteins that bind such ligands should acquire an increased affinity for the phase containing the modified polymer (Albertsson, 1978).

Determination of the partition coefficient

The characteristic partition coefficient of a protein, in a particular phase system, can be easily obtained by determining the protein concentration in each phase. A more accurate determination of the partition coefficient can, however, be achieved by using a multi-stage procedure such as counter-current distribution (Craig, 1960).

In counter-current distribution a solute is subjected to a series of successive partitions and bulk transfers along with one of the phases. This leads to a continuous spreading of material into an increasing number of chambers or tubes. The distribution of a homogeneous solute subjected to counter-current distribution is not only as in single-step partitions dependent on the partition coefficient of the solute but also on the volume of the two phases. From the final distribution it is thus possible to obtain the partition coefficient as assuming that the solute initially was only introduced into chamber zero, the concentration, $[S]^{in}$, in the ith chamber after n transfers is given by

$$[S]^{in}_{cal} = [S]^{00} \binom{n}{i} \frac{G^i}{(1 + G)^n} \qquad (4)$$

where $[S]^{00}$ is the initial concentration in the *zero*th chamber and G is the partition ratio, defined as

$$G = \frac{S_u}{S_l} \tag{5}$$

where S_u and S_l are the fractions of solute in the upper and lower phase, respectively.

The partition ratio and the partition coefficient are related by

$$G = K \frac{V_u}{V_l} \tag{6}$$

in which V_u and V_l are the volumes of the upper and lower phase, respectively.

The most convenient way to determine the partition ratio and thus also the partition coefficient from an experimental counter-current distribution is to minimize

$$U = \sum_{i=0}^{n} ([S]_{exp}^{in} - [S]_{cal}^{in})^2 \cdot \omega_{c,i} \quad U = \min \tag{7}$$

by non-linear regression. $[S]_{exp}^{in}$ and $[S]_{cal}^{in}$ are the experimental and calculated concentrations in the ith chamber, respectively. The weight factor, $\omega_{c,i}$, is defined by

$$\omega_{c,i} = \frac{[S]_{exp}^{in}}{[S]_{exp}^{max}} + 1 \tag{8}$$

where $[S]_{exp}^{max}$ is the highest experimentally observed concentration of S. It is included to compensate for the non-ideal behaviour of experimental counter-current distributions in areas far from peak maxima.

Equation 4 is not very practical for computations as it includes factorial expressions. However, if the number of transfers is large, equation 4 can be approximated by Laplace's formula (Shchigolev, 1965) and the concentration distribution is thus given by

$$[S]_{cal}^{in} = [S]_{cal}^{00} \frac{G}{\sqrt{2\pi nG}} \cdot e^{\frac{-(i + iG - nG)^2}{2nG}} \tag{9}$$

This function is much easier to handle in a computer. Furthermore, it has a derivative and can therefore be incorporated into standard non-linear regression computer programs. In fact, if $n > 5$ the approximated concentration distribution satisfactorily represents the exact distribution (equation 4). The parameters describing the best fitting theoretical distribution, G and $[S]_{cal}^{00}$, can therefore very easily be determined by minimizing equation 7.

Study of molecular associations using two-phase systems

One consequence of complex formation, as mentioned earlier, would be that the surface properties of the complex and those of the free constituents will differ in some respect. Accordingly, the partition behaviour of the complex most probably is different from those of the

associating solutes. It should thence be possible to study molecular associations by the partition technique. In cases where the interaction leads to large changes in average partition behaviour due to large differences in partition of the complex and its constituents and tight association the interaction can easily be monitored by single-step partitions (Albertsson, 1978). If, however, the variation in average partition behaviour is not large counter-current distribution is more suitable.

For the description of the counter-current distribution behaviour of an associating system it is necessary to assume that:

a. the association is freely reversible;
b. re-equlibration upon dilution is more rapid than phase separation;
c. it should be possible to determine the total concentration of each associating solute;
d. the partition coefficients must not display any concentration dependence;
e. the two phases are immiscible and no volume change occurs upon mixing and equilibration;
f. equilibrium within and between the phases is established before a transfer;
g. all solutions are thermodynamically ideal.

The applicability of the partition or the counter-current distribution technique for studying molecular associations is by no means limited to reversible interactions. However, the forthcoming theoretical models were developed for reversible interactions.

After each transfer in counter-current distribution, the two phases are thoroughly mixed and therefore neither the phases nor the solutes are in equilibra. Both phase separation and re-equilibration of the associating system start immediately after the mixing procedure. In order to avoid serious complications in terms of kinetic effects on the partition behaviour, it is necessary that the rate of re-equlibration must exceed that of phase separation.

In general, the time between successive transfers is in the range of 5-10 minutes but the time can be increased if necessary without complications except that the total time required to complete the experiment will be longer. There is, however, no formal hindrance to the inclusion of kinetic effects in theoretical models describing the distribution behaviour of interacting systems (Oberhausen et al, 1965) but the analysis of experimental counter-current distribution data becomes formidable.

In order to evaluate experimental counter-current distributions qualitatively it is necessary to determine the concentration in each chamber of each species participating in the association. When dealing with weak reversible interactions, it is usually impossible to measure the concentration of complexes formed. Instead it seemed fruitful to assume that it is possible to dissociate any complex formed prior to measurement. The contents in each chamber are thus expressed in terms of total concentration of each interacting solute. This assumption is simple to verify since there should be a linear relationship between the amount of solute and the measurable quantity.

Evidently, the partition coefficient of each species involved in the association must be independent of its own concentration as well as the presence of other species. Although the number of proteins studied is limited, the experiments indicate that the partition coefficient is constant at different protein concentrations and not dependent on the presence of other non-interacting proteins.

When aqueous solutions of dextran and polyethylene glycol are mixed in certain propor-

tions the mixture will separate into two phases even if the process of mixing is repeated. Therefore, it can be presumed that the two phases are immiscible. This presumption is supported by the fact that the partition of proteins is not affected by repeated mixing and separation of the phases.

Although equilibrium between the phases can always be achieved, attainment of equilibrium within the phases is dependent on the kinetics of the association. Therefore it is difficult to ascertain whether chemical equilibrium is attained without a knowledge of the rate constants.

The presence of high concentrations of polymers in the two-phase systems undoubtedly alters the solvation properties of water. It can thus be directly concluded that neither of the phases are thermodynamically ideal. Due to the excluded volume effect (Tanford, 1965; Topchieva, 1980) the effective concentration of added protein or other biological macromolecules can be expected to be higher in solutions containing inert polymers than in ordinary buffer solutions. Consequently, according to the law of mass action the equilibrium in macromolecular associations would be shifted towards complex formation in the presence of polymers. Nichol and co-workers have shown that this is actually the case in the presence of polyethylene glycol (Nichol et al, 1981). However, at the moment there is insufficient cause to include non-ideality terms in the theoretical description of distribution behaviour, although it would be possible. One reason for this is that, right now, we are not primarily interested in determining the true equilibrium constants but rather in estimating the strength of association.

The models

If a heterogeneous association of the type

$$A + B \rightleftharpoons C \tag{10}$$

may occur in a two-phase system that fulfils the requirements listed above then the equilibrium within and between the phases in each chamber can be described completely by

$$\begin{array}{ccc} A_u + B_u & \xrightleftharpoons{K^u} & C_u \quad \text{upper phase} \\ \Big\updownarrow K_A & & \Big\updownarrow K_B \qquad \Big\updownarrow K_C \\ A_l + B_l & \xrightleftharpoons{K^l} & C_l \quad \text{lower phase} \end{array} \tag{11}$$

where the subscripts u and l refer to the upper and lower phase, respectively. K^u and K^l are the association constants in respective phase, defined on a molar scale and the partition coefficients, K_A, K_B and K_C, are defined as before.

It can be shown (paper I) that the association constant in the upper phase is dependent on that in the lower phase as

$$K^u = K^l \frac{K_C}{K_A K_B} \tag{12}$$

The total concentration of A in the ith chamber after n transfers is given by

$$[A]_{tot}^{in} = p\,([A]_u^{in} + [C]_u^{in}) + q\,([A]_l^{in} + [C]_l^{in}) \tag{13}$$

where p and q are the fractional volumes of upper and lower phase, respectively. The analogous expression for the total concentration of B is obtained by substituting A for B in the above equation.

By appropriate substitutions, equation 13 can be rewritten as

$$[A]^{in}_{tot} = [A]^{in}_{l} (a + \gamma K^{l} [B]^{in}_{l})$$
$$[B]^{in}_{tot} = [B]^{in}_{l} (\beta + \gamma K^{l} [A]^{in}_{l}) \qquad (14)$$

in which $a = p \cdot K_A + q$, $\beta = p \cdot K_B + q$ and $\gamma = p \cdot K_C + q$.

Self-associations, represented by equilibria of the type

$$j\ P \longleftrightarrow P_j \qquad j = 2, 3.... \qquad (15)$$

$$j\ P \longleftrightarrow r\ P_2 + s\ P_3 \qquad (16)$$

and related equilibria, can be treated in an analogous manner (paper II). Consequently, the total solute concentration, expressed as monomeric concentration in the ith chamber after n transfers is obtained by summation of all species in solution at equilibrium

$$[P_1]^{in}_{tot} = \sum_{j=1} (p \cdot j \cdot [P_j]^{in}_{u} + q \cdot j \cdot [P_j]^{in}_{l}) \qquad (17)$$

which can be rewritten in the same form as equation 14.

$$[P_1]^{in}_{tot} = \sum_{j=1} j ([P_1]^{in}_{l})^{j} \cdot a_j \qquad (18)$$

where

$$a_j = (p \cdot K_{P_j} + q) \cdot K^{l}_{1j} \qquad (19)$$

or

$$a_j = (p \cdot K_{P_j} + q) \cdot \prod_{\substack{g=0 \\ h=g+1}}^{j-1} K^{l}_{gh} \qquad (20)$$

depending on the type of homogeneous equilibrium. In equation 20, $K_{01} = 1$ by definition.

Hence, given the initial total concentrations and assigning values to the association constant(s) in the lower phase (or upper phase) and to the partition coefficients, equations 14 and 18 can be used to calculate the equilibrium concentrations of associating solutes in each lower phase. From these values, the other equilibrium concentrations in each chamber can be calculated. By moving each upper phase to the next chamber in sequence and the upper phase of the last chamber to chamber *zero*, a transfer is simulated. The new total solute concentration(s) in each chamber can then be calculated. Thereafter, using these new values of total concentration, equation 14 or 18 is solved for each solute-containing chamber. This yields the new equilibrium concentrations in these chambers. Thus, by repeating this iterative process the final simulated counter-current distribution pattern is obtained for n transfers (paper I

and II). Figure 5 shows a series of simulated distribution patterns of a monomer-dimer equilibrium.

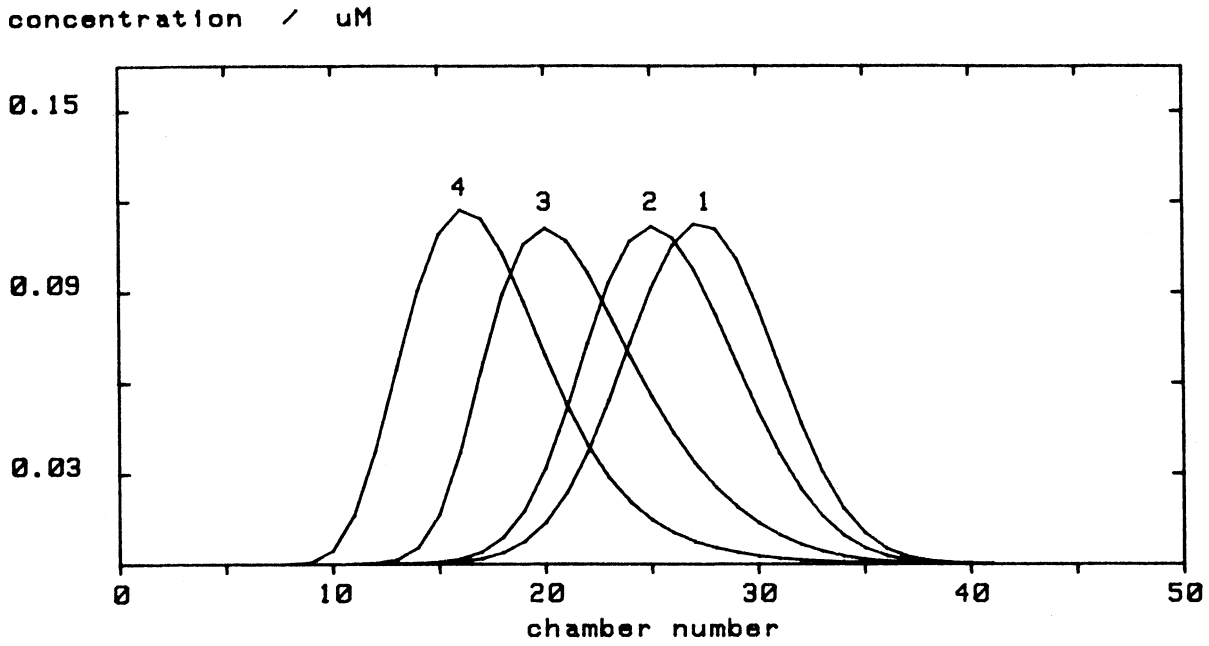


Figure 5. Simulated counter-current distribution patterns of a monomer-dimer equilibrium. Partition coefficients were K_{P_1} = 1.2 and K_{P_2} = 0.4, the initial solute concentration was 1 mM and the association constant in the lower phase, K_{1_2}, was (curve 1) 0, (curve 2) 1000, (curve 3) $1 \cdot 10^4$ and (curve 4) $1 \cdot 10^5$ M^{-1}, respectively.

Simulation of counter-current distribution patterns

Since a large number of simulated counter-current distribution patterns have been presented in papers I and II as well as by Bethune and Kegeles (1961a, 1961b) for different types of equilibria and different initial conditions, I will herein merely point out some typical features of the distribution behaviour of interacting systems.

The first step in a simulation experiment is to set up the frame of reference; the non-interacting distribution patterns. This can be done by inserting the appropriate values of the partition coefficients in either the binomial experession (equation 4) or in its Laplace's approximation (equation 9). The theoretical models can equally well be used by setting all association constants equal to zero. The reference patterns calculated by the two methods should be indistinguishable from each other and can thus also serve as a check for the computer programs. A series of counter-current distribution patterns simulating association can then be generated by assigning values to the initial concentration(s), to the partition coefficients of complexes formed and to the association constants. This is suitably done by varying only one of the parameters at a time. Typical simulation experiments are shown in figures 5 and 6.

The most apparent feature of the distribution behaviour of a heterogeneous associating system is the mutual influence of the solutes on their respective distributions as illustrated in figure 6.

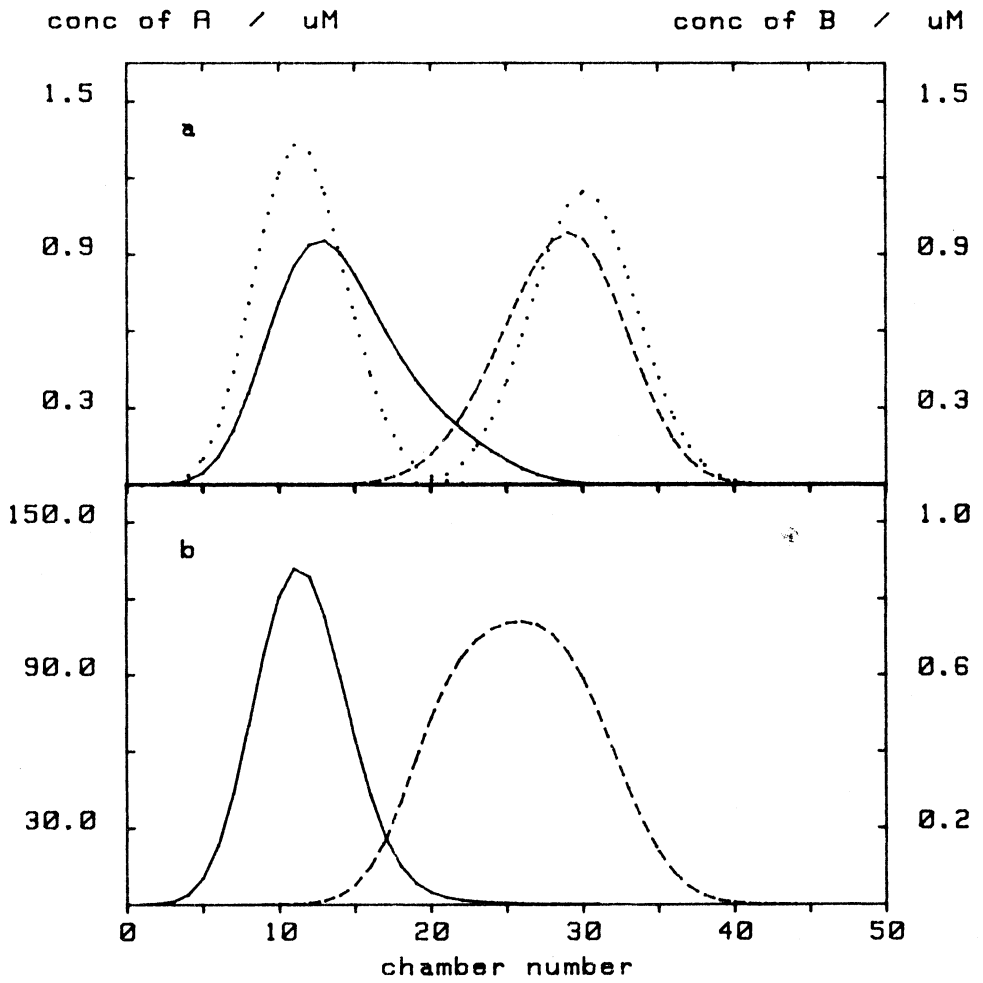


Figure 6. Simulated counter-current distribution patterns of a heterogeneous association of the type A + B $\longleftrightarrow$ C. The partition coefficients used were, K_A = 0.3, K_B = 1.5 and K_C = 0.9.
a) the initial concentration of both solutes were 0.01 mM and the association constant in the lower phase were 0 (dotted lines) and $1 \cdot 10^6$ M^{-1}, respectively. b) the initial concentration of A was 1 mM and that of B 0.01 mM and the association constant was $1 \cdot 10^6$ M^{-1}. Full drawn lines represent the distribution of A and dashed lines the distribution of B.

The mutual influence is an obligatory effect of the law of mass action; if the association constant or the initial concentrations are increased the amount of complex formed must increase and consequently, the changes in shape and position of the distribution patterns become more pronounced. However, if one of the interacting solutes is in excess, only a small fraction of that solute will be confined within the complex and, hence, its distribution will not be affected by the presence of the other solute (figure 6b).

The distribution behaviour of self-associating systems are similar, that is the higher the solute concentration or the larger the association constants the greater is the change in distribution as illustrated in figure 5.

Simulation of different types of self-associating systems has revealed that the shape of the counter-current distribution patterns are also dependent on the stoichiometry of the association (paper II). Those, sometimes subtle distinctions in shape between patterns for different types of associations can be enlarged by utilizing the concentration gradient curves, that is the first derivative of the distribution patterns.

The most remarkable feature in the distribution patterns of self-associating solutes is that one of the boundaries is hypersharpened while the other is dispersed. Which of the edges that is hypersharpened depends on the values of the partition coefficients of the associating species. This hypersharpening effect is similar to that seen in molecular sieve chromatography of a self-associating solute (Ackers, 1975).

According to the Gilbert theory (Gilbert, 1955), self-associating solutes forming polymeric species higher than dimers should yield bimodal concentration gradient curves when subjected to transport processes. However, the simulations presented in paper II as well as those of Cox (1971) show that this is not necessarily the case. On the other hand, an monomer-dimer equilibrium will always give rise to unimodal gradient curves. In other words, under certain circumstances, equilibria of different stoichiometries cause distinctive counter-current distribution patterns as well as concentration gradient curves.

Although simulations of heterogeneous associations only have been performed on 1:1 interactions it seems that different stoichiometries would probably be characterized by typical distribution patterns and gradient curves.

For a particular type of interaction, the shape and position of the counter-current distribution patterns and thus also the concentration gradient curves depend on the partition coefficients, the association constants, the initial solute concentrations as well as the number of transfers. Therefore, it must be stressed that the typical features of each type of equilibrium would be more or less preserved depending on the initial conditions.

Initially the solute or solutes can be introduced into the chambers in different ways; in small zone counter-current distribution the material is introduced into a small number of chambers. If the material, on the other hand, is initially added to a large number of chambers, a region of constant solute concentration will persist throughout the distribution experiment and a moving boundary is created. There are also other possible set-ups, for example, one solute of a heterogeneous association may be added to all chambers whereas the other is added to one single chamber as was done in the second model described in paper I. However, the typical features of any interaction mode are independent of the way the material is introduced initially. Although the earmarks of each type of equilibrium are better preserved in moving boundary counter-current distribution than in small zone counter-current distribution, there are no principal differences in features between the two different models. This implies, though, that weaker associations can be studied by the moving boundary technique rather than by small zone experiments.

Simulation experiments may provide some guidance regarding the choice of the variable experimental parameters, that is the initial solute concentrations and partition coefficient of the solutes, in designing real experiments in order to attain the optimal conditions for studying associations. Furthermore, the simulation might also give some ideas of the expected experimental results.

Analysis of experimental counter-current distributions

The analysis of experimental counter-current distributions can be divided into two parts; detection of and quantification of the interaction.

It is reasonable to conclude that a solute self-associates whenever its distribution behaviour exhibits a concentration dependency. A heterogeneous association is similarly manifested if the presence of one of the solutes exerts an influence on the counter-current distribution of the other solute. Hence, an interaction is easily detected by comparing non-interacting counter-current distribution patterns with patterns obtained in the presence of other solutes or at higher initial concentrations.

Figure 7 shows counter-current distribution experiments on bovine hemoglobin and carbonic anhydrase, distributed separately and together at increasing initial concentrations of the proteins. Apparently, the presence of hemoglobin affects the distribution behaviour of carbonic anhydrase, and the effect increases with increasing initial concentration. It was therefore concluded that bovine hemoglobin associates with bovine carbonic anhydrase (Silverman et al, 1979).

The quantification of an interaction is more complicated. First, the non-interacting distribution patterns have to be established, as before, in order to determine the individual partition coefficients of the associating solutes. This is most conveniently done by distributing the solutes separately or together at very low initial concentrations and then analysing the patterns as described above by non-linear regression analysis.

The experimental patterns obtained at different initial solute concentrations can then be analysed in order to determine the unknown parameters, that is the association constants, the partition coefficients of complexes formed and stoichiometry. It might be useful to first evaluate the concentration gradient curves, since it may be possible to exclude some types of equilibria on the basis of the shape of these. Following that, series of patterns must be generated for plausible models, using the known values of the initial solute concentration and the partition coefficient of the associating solutes and assigning values to the unknown parameters. Among the generated patterns it should be possible to find a set of best fitting patterns and thus also the values of the unknowns. In practice, the best fitting process is performed on a computer with a suitable optimizing procedure, such as SIMPLEX (Nelder and Mead, 1965).

Since the partition coefficients of the various species of an association equilibrium are not related to each other similar to their molecular weights, the complexity of the analysis increases enormously for more complicated types of equilibria. In addition, the best fitting process usually yields a better fit when the number of unknown parameters is larger. It may thus be hazardous to discriminate between different types of equilibria solely on the basis of best fit.

The theoretical model developed for small zone counter-current distribution of associating systems of the type $A + B \rightleftharpoons C$, has been tested to reveal if the model was realistic. For this purpose, bovine serum albumin and L-tryptophan were used (paper III) as the association of L-tryptophan with serum albumin is well characterized by different methods and is known to be a one-to-one complex on molar basis. The experimental distribution behaviour agreed with that predicted by the model for this type of interacting system. Furthermore, the best fit analysis yielded unequivocal values of both association constant and of partition coefficient of the complex. The higher association constant obtained can be reasonably attributed to the presence of synthetic polymers. Therefore, it was concluded that the model described the experimental results very well.

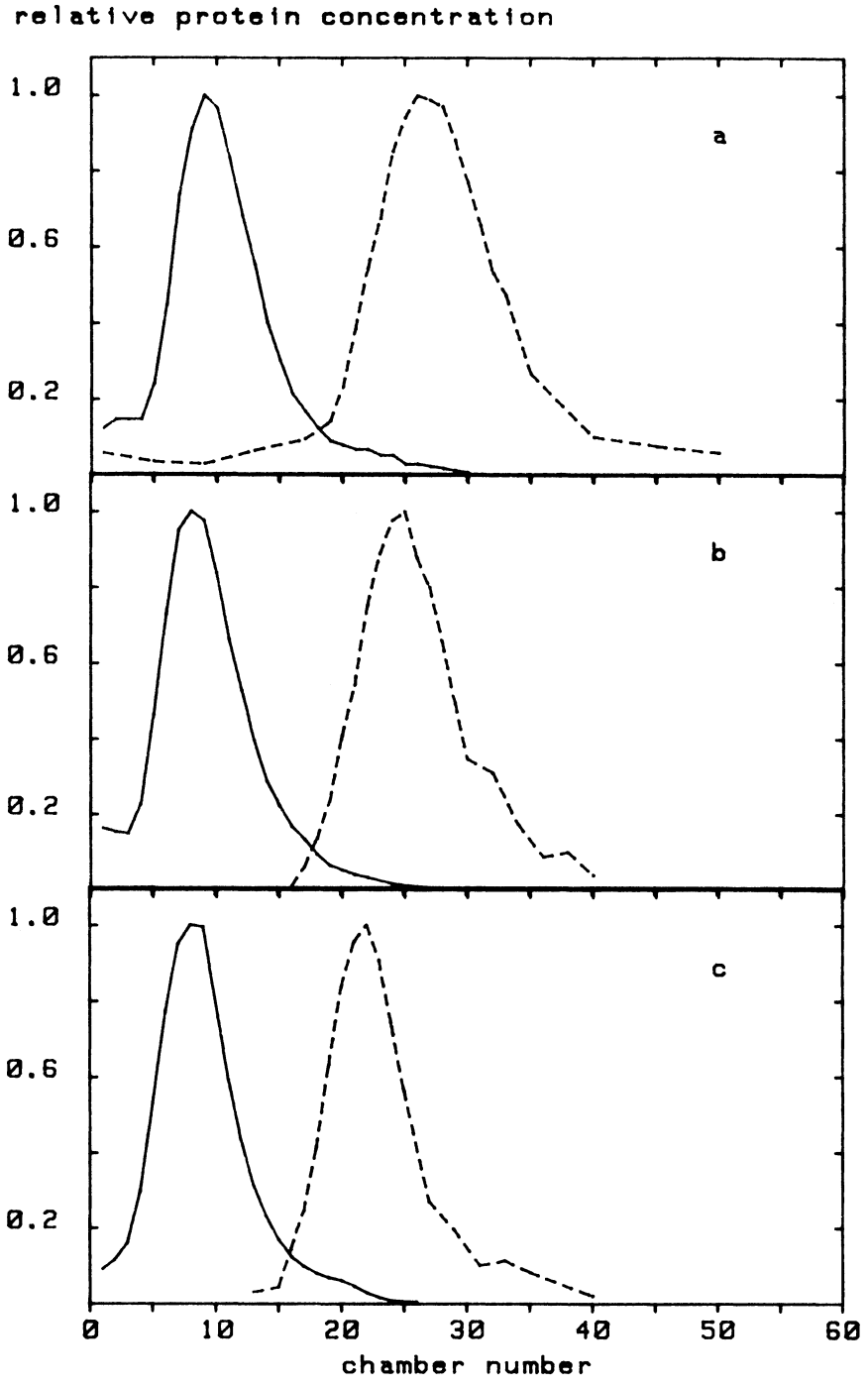


Figure 7 Counter-current distribution of bovine hemoglobin and carbonic anhydrase. Hemoglobin (full drawn lines) and carbonic anhydrase (dashed lines) were distributed separately (a) and together (b and c). The initial concentrations of hemoglobin and carbonic anhydrase were b) 0.1 mM and 0.0125 mM, and c) 0.2 mM and 0.025 mM, respectively. Adapted from Silverman and co-workers (1979).

Experimental results

One of the first applications of counter-current distribution in aqueous polymer two-phase systems for the study of protein-protein interactions was presented by Backman and Johansson in 1976 (paper IV) and involved the study of a possible association between malate dehydrogenase and aspartate aminotransferase, two metabolically linked enzymes.

The differences between the counter-current distribution behaviour of the enzymes when they were mixed and when they were distributed separately clearly indicated an association between them. Furthermore, the cytoplasmic forms of malate dehydrogenase and aspartate aminotransferase associated with each other as did the mitochondrial forms, whereas no association between a cytoplasmic and a mitochondrial form could be detected showing that the interaction was specific.

At the same time Bryce and co-workers presented evidence that labelled oxaloacetate produced by aspartate aminotransferase was converted into malate by malate dehydrogenase before it equilibrated with the pool of unlabelled oxaloacetate (Bryce et al, 1976). Although no physical evidence of an association between these two enzymes was found by molecular sieve chromatography, one possible explanation of the kinetic results was that oxaloacetate was confined within a complex of malate dehydrogenase and aspartate aminotransferase.

Another pair of metabolically linked proteins that have been studied by the counter-current distribution technique is hemoglobin and carbonic anhydrase (paper V; Silverman et al, 1979).

These two proteins are metabolically connected in that hemoglobin can act as a proton transfer agent in the repeated hydration-dehydration reaction catalyzed by carbonic anhydrase. Furthermore, Forster and Steen (1968) have shown that the response of the Bohr off-shift to an increased concentration of protons outside the red blood cell is dependent on the protons produced in the cell by the carbonic anhydrase-catalyzed hydration of CO_2. In the same experiment, the rate of the Bohr shift was dramatically decreased when carbonic anhydrase activity was inhibited, thus indicating a functional link between carbonic anhydrase and hemoglobin. This was later corroborated by the finding of Silverman and co-workers (1979) who found that the counter-current distribution of bovine carbonic anhydrase was influenced by the presence of hemoglobin (figure 7); indicating an interaction between these two proteins.

In paper V similar evidence are presented, indicating that human carbonic anhydrase associates with human CO-hemoglobin. In human red blood cells carbonic anhydrase appears in two forms, carbonic anhydrase I and II. There appears to be distinct differences between the affinity of these two forms for CO-hemoglobin; the high activity form, carbonic anhydrase II, apparently interacts with hemoglobin whereas the low activity form does not.

Non-linear regression analysis of the experimental patterns according to the only descriptive model so far available, that is for equilibrium of the type $A + B \rightleftharpoons C$, revealed that this model did not predict the distribution behaviour of this associating system very well. One reason for this could be that the stoichiometry of the association is not one-to-one on molar basis. The discrepancy between the experiments and the model might also depend on kinetic effects, that is the equilibrium within the phases is slower than phase separation.

EPILOGUE

From the results presented in this thesis it is apparent that counter-current distribution in aqueous two-phase systems provides an alternative to other methods for the study of biological macromolecular associations.

Both single-step partitions (Patton et al, 1978; Petersen, 1978, Fex et al, 1979; Middaugh and Lawson, 1980) and counter-current distribution (paper IV and V; Silverman et al, 1979) have been used successfully for the study of interactions between associating proteins. The method is especially well suited for the study of weak interactions since the polymers used in the two-phase systems strengthen the association of macromolecules (Laurent, 1971; Topchieva, 1980; Nicol et al, 1981; Shanbhag and Backman, 1981), probably due to excluded volume interactions. In addition, both phases of dextran-polyethylene-water two-phase systems contain mostly water and stabilize structure as well as biological activity.

Depending on the type of interaction and association strength presumed, it is possible to choose an experimental set-up such that the alteration in distribution behaviour due to association is as large as possible. It is thus possible to choose among different experimental modes, from small zone counter-current distribution to moving boundary counter-current distribution. Simulation experiments can be of great help for this purpose and may also be of some guidance in the choice of the variable parameters, that is the partition coefficients of the associating solutes and the initial solute concentrations.

The simulated patterns can also be used for a thermodynamic analysis of experimental counter-current distribution patterns. By generating patterns according to different theoretical models and the experimental mode, sets of best fitting theoretical patterns can be found to match the corresponding experimental patterns and, consequently, the association constant can also be determined.

Partition and counter-current distribution in aqueous two-phase systems are nondestructive and therefore it is possible to recover the material after the experiment is completed.

The major disadvantage of the counter-current distribution technique is that there is no correlation between stoichiometry and partition coefficients as there is between stoichiometry and molecular weights. This makes it very difficult to analyse, at present, complex heterogeneous associations in terms of thermodynamical parameters. However, simulations of the distribution behaviour of self-associating systems have shown that the stoichiometry might be obtained from the corresponding concentration gradient curves. It is possible that the gradient curves might also be of use for analysis of heterogeneous associations. A very attractive possibility is the future development of two-phase systems in which the partition behaviour is truly molecular weight-dependent. This would make the analysis of counter-current distribution experiments on interacting macromolecules much easier.

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