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# GEOMETRICAL CONSIDERATIONS IN THE SEPARATION OF BIOLOGICAL PARTICLES BY AFFINITY PARTITIONING

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Highly specific and selective purification techniques are urgently needed in such diverse biological fields as immunology, the neurosciences, and cell biology. Often, only a very highly specific subpopulation of cell particles is required for a given clinical or basic research application. When purifying a specific particle population from an extremely heterogeneous mixture, only methods that are based upon the binding of specific ligands to membrane surface components are likely to yield the required selectivity. Affinity partitioning, a new separation technique, is based upon the distribution of biological particles between aqueous polymer phase systems (cf. D. Brooks) and has advantages over affinity chromatography in applications to the purification of cells and cell particles. In affinity partitioning separations, a specific ligand-polymer that binds to biospecific sites is added to the aqueous polymer phase systems. The binding of the ligand-polymer to a subpopulation of biological membranes changes the distribution of that subpopulation, thus effecting a selective change in distribution of the particles. Application of affinity partitioning to several interesting biological problems has been hindered by a lack of complete experimental and theoretical foundations for the technique. New ground-based and zero g experiments, designed to increase our experimental and theoretical understanding of affinity partitioning, are likely to yield new insights and will allow the application of affinity partitioning to a number of important biological problems. Geometrical considerations that may influence the distribution of biological membranes will be described.

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## INTRODUCTION

Highly specific and selective purification techniques are urgently needed in such diverse biological fields as immunology, cell biology, and the neurosciences. Often, a highly specific subpopulation of cell or cell particles is required for clinical or basic research applications. When purifying a specific particle population from an extremely heterogeneous mixture, only methods that are based upon the binding of specific ligands to membrane surface components are likely to yield the required selectivity. Affinity partitioning (1-3) is a promising new affinity separation technique based upon the phase partition method for the distribution of biological particles between aqueous polymer phase systems (4). In affinity partitioning a specific ligand is covalently attached to one of the water soluble polymers that make up the phases. Binding of a ligand-polymer to biospecific binding sites selectively changes the distribution of particles that are rich in the binding sites. Affinity partitioning has advantages over affinity chromatography in applications to the purification of cells and cell particles. Often biological particles are bound irreversibly to the ligand matrices used in affinity chromatography. In affinity partitioning separations, the purified fractions are recovered from the phases by addition of sufficient water to break the phases, and concentrated by centrifugation. Unfortunately, application of affinity partitioning to several interesting biological problems has been hindered by a lack of adequate experimental and theoretical foundations for the technique.

## THEORETICAL DESCRIPTION OF THE AFFINITY PARTITIONING EFFECT

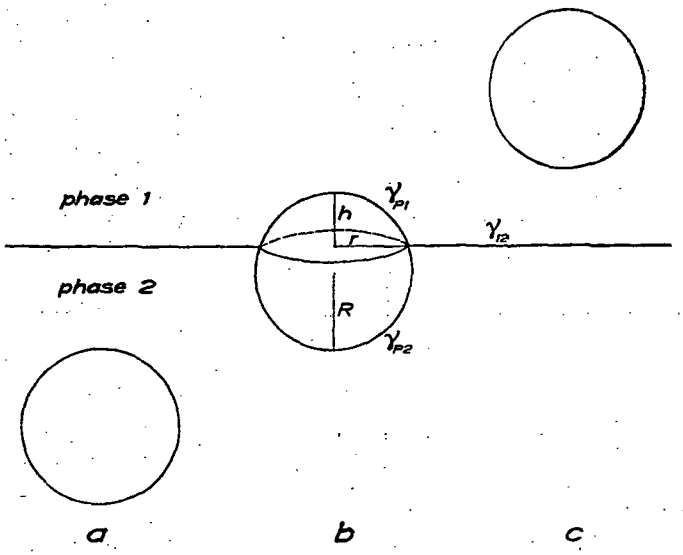
The interfacial surface tension between the phases in typical poly(ethylene oxide)-dextran phase systems is from 0.001 to 0.1 dynes/cm. For cells and subcellular particles, the energy released by reduction of the interfacial surface area upon adsorption into the interface (surface tension X area of particle cross section) is many-fold 3/2 kT (see figure). The energy transferred to cell particles by collisions with solvent molecules is on the order of 3/2 kT. Therefore, cells and subcellular particles that distribute into the interfacial layer are not displaced by molecular collisions. In contrast, soluble proteins of molecular weight one million or less are not significantly bound in the interface because of their low cross sectional area. This considerably simplifies our task of developing a theory based upon thermodynamic considerations to describe the distribution of proteins containing biospecific binding sites. For a protein consisting of n binding sites, the distribution of the complex formed between the protein and n ligand-polymers is quantitatively described by the following expression (1):

$$K_{PL_n} = \left[\frac{k_{aT}}{k_{aB}}\right]^n \cdot K_L{}^n \cdot K_o \quad ,$$

where $K_{PL}$ is the partition coefficient of the complex, $k_{aT}$ is the association constant of the complex in the top phase, $k_{aB}$ the association constant in the bottom phase and $K_L$ and $K_O$ are the partition coefficients of the free ligand-polymer and protein, respectively. By substituting typical values into the above expression ($K_L$=3, $K_O$=1, n=4 and $\frac{k_{aT}}{k_{aB}} \simeq 1$), we find that $K_{PL}$=81 and the affinity partitioning effect is significant. This theory has been validated in a number of cases and appears to describe quantitatively the distribution of proteins containing biospecific binding sites.

Although affinity partitioning has proved useful in the purification of enzymes difficult to purify by affinity chromatography (5,6), its most promising applications are in the purification of intact cells and subcellular particles. Our efforts have been directed at the purification of membrane fragments enriched in cholinergic neurotransmitter receptors. Subcellular particles containing nicotinic cholinergic receptors from the electroplax of Torpedo californica are 0.3μ to 0.4μ in diameter, each containing 1,000 sites that bind cholinergic ligands or 30,000 per square μ. If the above expression correctly approximates the affinity partitioning of subcellular particles, then even particles containing as few as ten binding sites should be easily separated from other particles by affinity partitioning.

Unfortunately, the above theory does not quantitatively describe the distribution of nicotinic cholinergic receptor-containing membranes. By blocking various fractions of the binding sites with α-bungarotoxin, we have shown that at least 5,000 binding sites per square micron or 150 binding sites per particle are required for a significant affinity partitioning effect. Albertsson (4, pp 58-65)




A spherical particle at three different positions in a liquid two-phase system. $\gamma_{12}$ is the interfacial tension between the two phases; $\gamma_{P1}$ between the particle and phase 1; and $\gamma_{P2}$ between the particle and phase 2.

From Albertsson (4).

concluded, based upon geometrical and thermodynamic grounds, that the distribution of particles between the interface and one of the phases is given by

$$\frac{C_{min}}{C_1} = \exp\left[\frac{\pi R^2(\gamma_{P2}-\gamma_{P1}-\gamma_{12})^2}{\gamma_{12}\,kT}\right],$$

where $C_{min}$ is the number of particles per $cm^3$ in a thin layer of the interface and $C_1$ refers to the concentration of particles per ml in one of the phases. This formulation assumes that the surface tension energy released by a particle in the interface is on the order of 3/2·kT, the translational energy of a molecule. Thus, in the limiting case, where the absolute value of $\gamma_{P2}-\gamma_{P1}$ is much less than $\gamma_{12}$, the radius of a particle of 300 Å would release an interfacial energy on the order of 3/2·kT. The radius 300 Å is calculated assuming a surface tension of 0.001 dyne/cm and a temperature (T) of 277 degrees Kelvin. Therefore, both $\gamma_{12}$ and particle radius must be taken into account when evaluating the distribution of particles of radii larger than approximately 300 Å.

In affinity partitioning the value $\gamma_{12}$ remains constant, but the value of $\gamma_{P2}-\gamma_{P1}-\gamma_{12}$, or $\Delta\gamma$, is changed upon the addition of the affinity partitioning reagents. The addition of the ligand-polymer produces a change which is equal to the density (d) of the binding sites times the energy ($\varepsilon$) contribution of each ligand-polymer bound to a specific site, i.e., $\Delta\gamma_f=\Delta\gamma_i+d\varepsilon$, where $\Delta\gamma_i$ is the initial value in absence of affinity partitioning reagents and $\Delta\gamma_f$ is the value in phase systems containing ligand-polymer. It is apparent that a substantial affinity partitioning effect is favored by the maintenance of $\Delta\gamma_i$ as low in absolute magnitude as possible while striving to maintain $\varepsilon$ as large as possible. Since conditions have been achieved where particles as large as red blood cells partition between the phases and the interface, it was assumed that the value of $\pi R^2\cdot\Delta\gamma_i$ was on the order of kT. The value of $\varepsilon$ is estimated to be on the order of kT per binding site. Therefore, it was concluded that only a low density of binding sites should be required to achieve a significant affinity partitioning effect. That 10,000 per $\mu^2$ is required indicates that one or more of the assumptions is not correct, and that nonideal considerations make a significant impact on the final result.

Possible explanations for the above discrepancy may be: 1) thermodynamic equilibrium is not achieved during the distribution of the particles among the phases and interface; 2) entropic effects on the binding of high densities of ligand-polymers to cell surface membranes are not considered; and 3) linear tension energies are not evaluated (7). Two experimental observations may help to resolve these points. First, microscopic observations indicate that the distribution of red blood cells in phase droplets is predominately into the droplet interface even when red blood cells ultimately are distributed into the top phase (8,9). This indicates that the final distribution of particles between the bulk phases and the microscopic distribution during phase separation are at variance. This may result in incomplete attainment of equilibrium during bulk phase separation. A second observation is that affinity partitioning separations are best achieved with systems near the critical point of the phase diagram, where the phases become almost equal in composition and isopycnic.

## EXPERIMENTS AT REDUCED AND ZERO X g

When phase systems near the critical point are compared with phase systems far from the critical point, two important parameters co-vary: the interfacial surface tension and the density difference between the phases. The density difference may be corrected by appropriate reduction of g forces. A further complication is the effect of phase droplet curvature on the microscopic distribution of biological particles. During phase separation driven by surface tension and gravitational forces the geometry of phase droplets is complex. Shortly after phase separation has begun at 1 x g, there is a large range in the distribution of droplet curvature at different depths in the tube. At the bottom of the tube, droplets of top phase are found surrounded by bulk bottom phase, while at the top of the tube the situation is reversed. The geometry is considerably simpler during phase separation at 0 x g, where phase separation is driven only by the reduction of interfacial surface area. The direction of the curvature at 0 x g is dependent on the overall ratio of the two phase volumes. As phase droplets collide, phase coalescence occurs. The average radius of phase droplets increases, and the biological particles interact with a relatively uniform population of phase droplets. Under these conditions we may expect the microscopic distribution of particles between the droplets to approach equilibrium as coalescence continues because, as the droplet radii increase, we approach a planar interface. Should the microscopic distribution depend on the curvature of the phase droplets, the distribution found after 0 x g incubation followed by 1 x g phase separation would be quite different from that observed when both processes occur at 1 x g. Further analysis of these interesting interfacial phenomena should lead to new theoretical insights which may be applied to 1 x g separation and help in the design of suitable phase systems for affinity partitioning of particles.

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