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A MODEL FOR THE BEHAVIOR OF VESICLES IN DENSITY GRADIENTS: IMPLICATIONS FOR FRACTIONATION

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SUMMARY

The need for high resolution in the fractionation of membranous vesicles is addressed in terms of a simple theoretical model. We argue that the equilibrium buoyant density of a semipermeable vesicle reflects an osmotic balance between the shrinking forces exerted by non-penetrating gradient solutes and expanding forces engendered by the excess of Donnan ions associated with charges fixed within the vesicle. The mathematical formulation derived from these assumptions leads directly to explicit predictions of density gradient design optimal for separating vesicle species according to their fixed charge content. Briefly, resolution is favored in gradients whose osmotic and ionic activities are minimal, under conditions which also minimize the fixed charge on the membrane. Available experimental data affirm this hypothesis.

INTRODUCTION

Equilibrium density gradient centrifugation is a principal technique in the fractionation of cellular organelles. The approach assumes that the subcellular elements achieve buoyant equilibrium at characteristic densities. However, membranous organelles and vesicles do not possess invariant densities; being bounded by closed and semipermeable membranes, their internal volume varies in response to the milieu. Their buoyancy, unlike that of solid particles and open sheets, is strongly influenced by the changing osmotic activity of the density gradients they traverse. This phenomenon has been analyzed by assuming that the density of a vesicular particle is related to a simple osmotic equilibrium across its membrane[1-3]. This treatment, however, does not explain the characteristic influence of pH, ionic strength, and divalent cations on the buoyancy of vesicles[4,5].

To account more fully for the effects of gradient composition on the density of vesicles, we offer a simple model. As previously proposed[5], we stress that the charges sequestered within the vesicle lumen are a major determinant of its equilibrium density. The formulation is not only compatible with features of vesicle behavior, but it also affords several predictions which should extend the usefulness of equilibrium

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density gradient centrifugation in the resolution of mixtures of closely related vesicles. A partial description of this model has appeared elsewhere[6].

THE MODEL

We regard a vesicle as a solvent compartment bounded by a flexible, selectively permeable membrane, akin to a dialysis bag (Fig. 1). Fixed within the vesicle are nondiffusible charges which can arise from ionic groups of either the inner surface of the membrane or enclosed protein. The surrounding gradient medium is a solution of (1) permeant salts, in Donnan equilibrium with the charges in the vesicle interior, and (2) uncharged, non-penetrating solutes*. The osmotic activity of these solutes tends to shrink the vesicle, while the excess of diffusible ions constrained within the vesicle by the Donnan effect[7] creates an expansive osmotic force. Solvent and ions move across the membrane until a Donnan–osmotic equilibrium is attained. In this way, the aqueous volume, and hence the density, of a vesicle is governed by the ambient medium. A sedimenting particle will come to rest at that point in a density gradient where both Donnan–osmotic equilibrium and buoyant equilibrium obtain. A formulation describing these events follows.

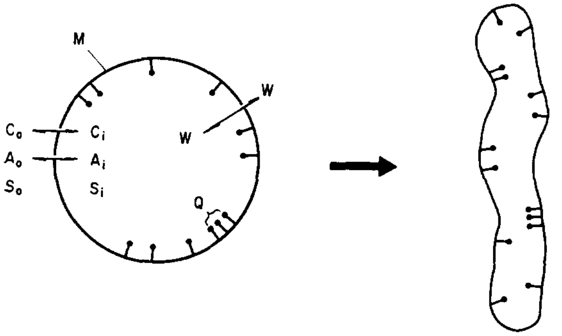

Fig. 1. The hypothetical vesicle undergoing osmotic shrinkage. Solvent (W), univalent cations (C) and univalent anions (A) pass freely through the membrane (M), while impermeant, uncharged solutes (S) and fixed charges (Q) cannot. Subscript 'o' denotes the outside medium, while subscript 'i' symbolizes the vesicle interior.

The attainment of Donnan–osmotic equilibrium

At osmotic equilibrium, the solute activities on the two sides of the vesicle membrane are equal; *i.e.*

$$S_o + C_o + A_o = S_i + C_i + A_i \tag{1}$$

Here S_0 and S_i are the concentrations of impermeant, uncharged solute in the bulk and vesicle compartments, respectively. C_0 and A_0 denote the permeant, univalent cation and anion concentrations in the bulk medium, while C_i and A_i are the corresponding concentrations within the vesicle. (All concentrations are molal and represent the solute activities at Donnan and osmotic equilibrium.)

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*In gradients formed of solutes which penetrate the membrane barrier, the vesicle would achieve buoyant equilibrium at a density equal to that of its membrane; we shall not consider this situation further.

We now seek an expression describing the distribution of small ions across the membrane. Because of the charges fixed within the vesicle, ions accumulate in its lumen according to the Donnan equilibrium[7]. If the effective concentration of fixed anions within the vesicle is Z (equiv/kg water), the concentration of diffusible cations in the vesicle is

$$C_i = \frac{1}{2}\left(\sqrt{Z^2 + 4C_o^2} + Z\right) \tag{2}$$

Similarly, the diffusible anion concentration in the vesicle is

$$A_i = \frac{1}{2}\left(\sqrt{Z^2 + 4A_o^2} - Z\right) \tag{3}$$

The difference between C_i and A_i is Z, satisfying electroneutrality. The total concentration of diffusible ions within the vesicle is

$$C_i + A_i = \sqrt{Z^2 + 4E^2} \tag{4}$$

where $E \cong C_0 \cong A_0$*

We shall restrict ourselves, for now, to 'empty' vesicles, where S_i is negligible. Eqn. 1 may then be written

$$S_o + 2E = (C_i + A_i) = \sqrt{Z^2 + 4E^2} \tag{5}$$

Squaring both sides and rearranging gives

$$Z^2 = S_o^2 + 4S_oE \tag{6}$$

and, therefore

$$Z = \sqrt{S_o^2 + 4S_oE} \tag{7}$$

If we assume that molality is about molarity in the vesicle water compartment, we may express the fixed charge concentration as $Z = Q/V_W$ (equiv per l of vesicle solvent). Then the aqueous volume of the vesicle is**

$$V_W = \frac{Q}{Z} = \frac{Q}{\sqrt{S_o^2 + 4S_oE}} \tag{8}$$

If we take V_W to be the only vesicle attribute which varies in response to the solute gradient, we can now relate the density of a vesicle to its environment.

The instantaneous density of vesicles

The buoyant density of a vesicle, D, can be resolved into the mass (μ, kg) and volume (V, l) contributions of its hydrated membrane (M) and solvent (W) compartments. Thus,

$$D = \frac{\mu_M + \mu_W}{V_M + V_W} \tag{9}$$

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* We have assumed (1) that the effective fixed charges are entirely anions; and (2) that the medium volume is much larger than the intravesicular space, so that the solute concentrations in the medium are not altered by equilibration with the vesicles. This assumption should be reevaluated in the case of concentrated suspensions of vesicles with large aqueous spaces.

** This proposition is not unique to our study. General descriptions of the relationship between erythrocyte volume and external solutes have been derived, of which Eqn. 8 is but a special case (*cf.* ref. 8).

If the densities of these compartments are D_M and D_W, respectively

$$D = \frac{\mu_M + D_W \cdot V_W}{\mu_M/D_M + V_W} \tag{10}$$

Since $V_M = Q/Z$, we may rewrite Eqn. 10 as

$$D = \frac{\mu_M + D_W \cdot \frac{Q}{Z}}{\frac{\mu_M}{D_M} + \frac{Q}{Z}} = \frac{Z + D_W \cdot \frac{Q}{\mu_M}}{\frac{Z}{D_M} + \frac{Q}{\mu_M}} \tag{11}$$

Since the vesicle we envision bears its Donnan fixed charges exclusively on the inner aspect of the membrane, Eqn. 11 can be simplified by introducing the parameter $\hat{Q} = Q/\mu_M$ (equiv/kg membrane), the charge per membrane mass. Then

$$D = \frac{Z + D_W \hat{Q}}{\frac{Z}{D_M} + \hat{Q}} \tag{12}$$

Eqn. 12 relates the instantaneous vesicle density to the two characteristic attributes of its membrane ($\hat{Q}$ and D_M) and to the two relevant features of the bulk medium: the permeant solvent density, D_W, and the Donnan–osmotic parameter, $Z = \sqrt{S_0{}^2 + 4\,S_0E}$.

The attainment of buoyant equilibrium

A vesicle which comes to osmotic equilibrium at some point in a gradient will, in general, differ in density from the surrounding medium. It therefore moves under the influence of the centrifugal field until it achieves osmotic equilibrium at a density matching its own. In order to define buoyant equilibrium in terms of our model, we must introduce an additional expression, relating the osmotic activity and density of a given gradient zone, namely:

$$S_0 = K(D_G - D_W) \tag{13}$$

Here D_G is the density of the gradient solution whose osmotic activity is S_0; K is an empirical coefficient relating the two variables*.

Buoyant equilibrium (where $D_{eq} = D_G$) may now be defined by combining Eqns. 7, 12 and 13 as an equation cubic in D_{eq}:

$$D_{eq}{}^3K^2 + D_{eq}{}^2(4EK - 2D_MK^2 - D_WK^2) + D_{eq}(D_M{}^2K^2 + 2K^2D_WD_M - 8EKD_M - D_M{}^2\hat{Q}^2) + D_M{}^2(\hat{Q}^2D_W - K^2D_W + 4EK) = 0 \tag{14}$$

The variables in this equation have been previously introduced in describing the gradient and the vesicle. Although cubic, Eqn. 14 seems, in general, to have only one real root for simple gradient systems such as sucrose; *i.e.* a single equilibrium density obtains for each vesicle species. However, because of the non-ideal osmotic properties of solutions of high polymers*, one can predict more than one isopycnic density for a

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* Osmotic activity does not vary linearly with concentration (hence density) in gradients of high polymers, such as dextran and Ficoll. The large second virial coefficient describing the solution properties of these polymers dominates over the ideal colligative term at the concentrations employed in density gradients[9,10].

given vesicle type in such gradients, perhaps accounting for the anomalous behavior of microsomes in Ficoll gradients[4].

LIMITATIONS

Undoubtedly, the model does not fully encompass the complexity of biological membranes and their deviation from physicochemical ideality. For example: (1) Charges anchored to the membrane may produce anomalous distributions and activities of their Donnan counterions at the osmotic boundary. (2) Further, the effective charge, Q, probably differs from the net fixed charge usually assumed in Donnan equilibria. (3) Forces other than Donnan–osmotic have been neglected. Electrostatic repulsions among fixed charges of the same sign would contribute to the expansive potential and, in general, mimic Donnan effects. In addition, mechanical (elastic) forces may dominate as vesicles expand or collapse fully. Small vesicles could become maximally inflated before reaching the buoyant equilibrium point described in Eqn. 14. For example, a vesicle of internal radius = 500 Å, wall thickness = 75 Å, and wall density = 1.15 cannot achieve a density below 1.058; a similar vesicle of radius = 1000 Å can equilibrate below D = 1.032. Thus, high resolution fractionation of homologous vesicles according to size may be possible using isopycnic centrifugation. (4) We have neglected the charges fixed to the outer surface of the membrane; these could alter equilibrium density by affecting the Donnan–osmotic equilibrium[11], the electrostatic potential, and the hydration of the membrane. (5) The above formulation assumed that no soluble solutes were held within the vesicle. While vesicles derived from plasma membrane[12] and endoplasmic reticulum[13] have been so prepared, native organelles contain characteristic macromolecules. In that case S_i will be finite, but in the case of charged proteins, will still be much less significant than the Donnan contribution of the macromolecule. Eqn. 11 can be re-stated to accommodate this more general case: let μ_M and D_M be redefined as the total mass and density of non-diffusible hydrated solids, and Q as the total effective intravesicular charge. Eqn. 11 then suggests that vesicles with identical membrane properties will fractionate according to their content of soluble protein[*].

PREDICTIONS

Several of the implications of the model are supported by experimental data. (1) An important deduction from Eqn. 12 is that the size of a membranous

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[*] In the general case, $S_i \neq 0$. Eqn. 7 then becomes

$$Z = \sqrt{(S_o - S_i)^2 + 4E(S_o - S_i)} \tag{7a}$$

and Eqn. 8 becomes

$$V_W = \frac{N_i(S_o + 2E) \pm \sqrt{Q^2(S_o^2 + 4ES_o) + 4E^2N_i^2}}{S_o^2 + 4S_oE} \tag{8a}$$

where N_i is the vesicle osmotic load, such that $S_i = N_i/V_W$ (M) and intravesicular molality is molarity. Only the positive radical seems to be physically meaningful.

The general expression for instantaneous vesicle density is then obtained by substituting from Eqn. 8a into Eqn. 10, with D_M, μ_M and Q redefined for the protein-filled vesicle. The expression for equilibrium density (Eqn. 14) can likewise be generalized. The heuristic advantages of the text presentation appear to justify our simplifying assumptions.

vesicle does not influence its equilibrium buoyant density, except for very small vesicles at their elastic limits, as discussed above. Thus, a dispersion of vesicles of common origin should equilibrate at a single density, independent of membrane mass. We have observed (Fig. 2) that whole erythrocyte ghosts and their vesicles of various sizes fulfill this expectation.

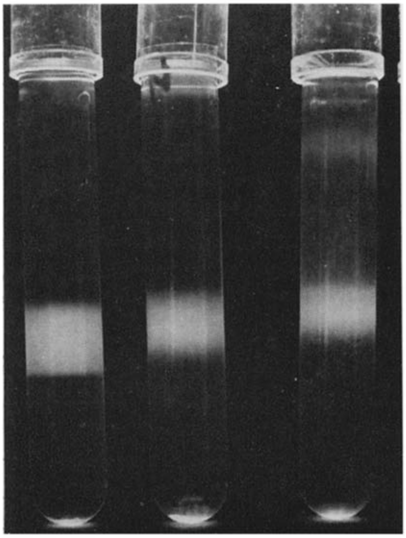

Fig. 2. The influence of vesicle size on its equilibrium density. Whole human erythrocyte ghosts (left) and small (about 0.1–1 μ diameter) vesicles derived therefrom by homogenization with a No. 27-gauge needle (middle) and by nitrogen decompression (right) were centrifuged for $10^8 \times g \cdot$min through a 1.01–1.07 density gradient of Dextran-110 (Pharmacia Fine Chemicals) in 15 mM NH_4HCO_3 (pH 8).

(2) Eqn. 12 suggests that if D_M is independently determined (*e.g.* on glycerol gradients), one can compute $\hat{Q}$ by measuring D and Z at buoyant equilibrium. In view of the limitations discussed above, such values cannot be considered accurate. Nevertheless, total charge densities on the order of 0.1 mequiv/g membrane have been estimated in this fashion for human erythrocyte membranes, in good agreement with values in studies on cation binding sites; *viz.* around 0.16 mequiv/g membrane[14].

(3) Eqn. 7, $Z = \sqrt{S_0^2 + 4\,S_0E}$, has a hidden meaning: it says that the fixed charge concentration in a vesicle is a function of the medium and not the vesicle. Thus, all vesicles in osmotic equilibrium with a given medium have the same fixed charge concentration, Z, irrespective of the size of their membrane or their charge. We have tested this prediction in a simple, macroscopic system. Dialysis bags were loaded with measured amounts of impermeant polyelectrolyte (*e.g.* poly-L-ornithine) and equilibrated against media containing various concentrations of permeant, univalent electrolyte (KBr) and impermeant, uncharged solute (Dextran-110). These model vesicles followed Eqn. 7 over a 10-fold range of Z, S_0, and E values.

(4) The effect of electrolyte activity on D is illustrated in Fig. 3A. The similarity of these theoretical curves to experimental findings[4] is evident. Fig. 3B depicts the influence of the osmotic activity of the medium on vesicle density; these results conform well to published observations[2,3].

The model also accounts for the influence of pH and divalent cations on D, if we assume that these act by titrating fixed charges within the vesicle. Fig. 3C illustrates how reducing the membrane charge increases the vesicle density, as observed experimentally[4,5]. This close correspondence between theory and practice points to the importance of the Donnan–osmotic equilibrium in the density behavior of vesicles and indicates the need to evaluate these parameters in experimental design.

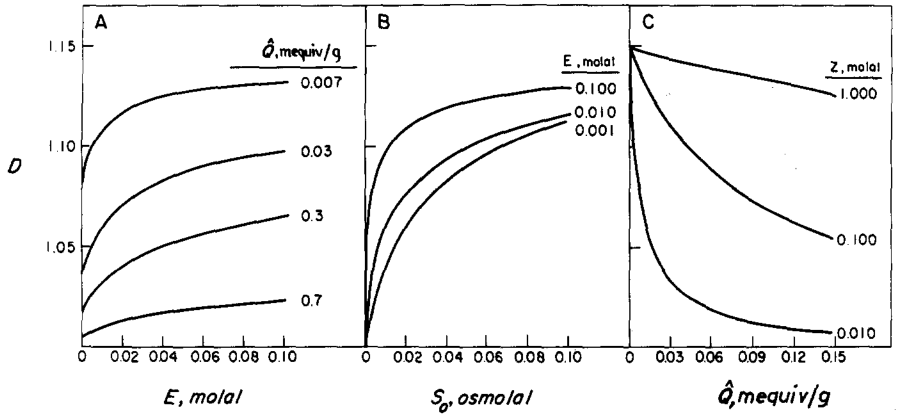

Fig. 3. The influence of experimental variables on the density of vesicles. Hypothetical curves were generated from Eqn. 12, assuming $D_W = 1.00$ and $D_M \times 1.15$. A. The effect of ionic strength; $S_0 = 0.01$ throughout. B. The effect of osmotic activity; $\hat{Q} = 0.03$ throughout. C. The effect of membrane charge density.

(5) The most important predictions relate to the experimental conditions which favor the separation of closely related vesicles. Fig. 3C illustrates that at $Z = 1$ molal (representing sucrose gradients) vesicle densities are close to that of their membranes, D_M, and are rather insensitive to variations in $\hat{Q}$. In contrast, the curve where $Z = 0.010$, *e.g.* dextran or Ficoll gradients, shows that D varies widely with $\hat{Q}$ in the low charge density range. Therefore, the experimental conditions optimal for separating vesicles as a function of their sequestered charges obtain when osmotic activity, ionic strength and fixed charge density are minimized.

Illustrations of these postulates are at hand. (1) The separation of plasma membrane vesicles from endoplasmic reticulum membranes was incomplete in sucrose[15] (high Z) but quite satisfactory in Ficoll (polysucrose, low Z) gradients, provided that $\hat{Q}$ was reduced by titration with H^+ or divalent cations [5,12]. (2) Similarly, the separation of mammary tumor virus from vesicular membrane fragments in mouse milk was effective on polysucrose gradients but not on sucrose or salt gradients[16]. (3) These fractionation principles are perhaps epitomized in the resolution of inside-out from right-side-out erythrocyte membrane vesicles[17]. These two species are not separated on sucrose and glycerol gradients, where they share the density of intact ghosts. In contrast, the inverted membranes equilibrate at significantly lower densities than their normal counterparts on high molecular weight dextran gradients (Fig. 4). The fractionation is thought to derive from the asymmetric distribution of charges across the red cell membrane. Internalization of the many sialic acid anions located on the outer surface should produce vesicles with higher $\hat{Q}$, hence lower density, than species*. In this regard, we have found that pre-treatment of ghosts with sialidase converts their inverted vesicles to high density forms. (The red cell vesicles also respond to ionic strength, pH, and divalent cations as predicted by the model.) (4) Fig. 3C suggests that fractionation of vesicles with sufficiently high $\hat{Q}$ values is favored by high Z, since the low Z curve flattens in that region. The partial purification of Ehrlich ascites plasma membrane vesicles from microsomal mixtures is thus more effective in sucrose[15] than polysucrose[4, 5] at high $\hat{Q}$. Indeed, this sucrose fractionation approach was applied with considerable success to rat liver surface membrane isolation[18].

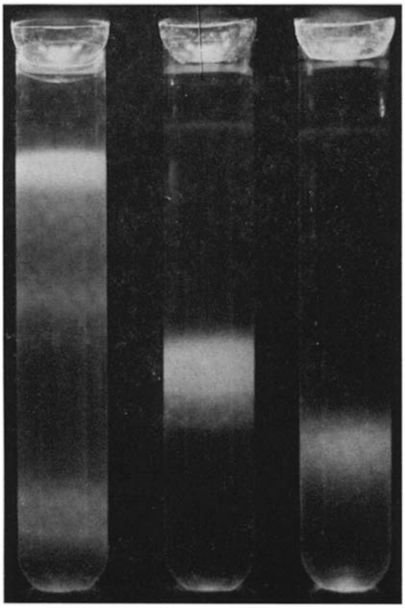

Fig. 4. Equilibrium distribution of inside-out and right-side-out vesicles in dextran, sucrose and glycerol gradients. Erythrocyte membrane vesicle mixtures, prepared as in STECK *et al.*[17], were centrifuged (10^8 $g \cdot$min) in continuous gradients of Dextran-110 (D_G = 1.01–1.07) (left), sucrose (D_G = 1.08–1.16) (middle) and glycerol (D_G = 1.08–1.16) (right), all in 0.5 mM phosphate buffer (pH 8) and 0.1 mM $MgSO_4$. The top two bands of the dextran gradient contained inside-out vesicles, while the bottom fraction was normal in orientation.

In conclusion, we regard our model as a simple, reasonable description of the buoyant behavior of vesicles and as a working hypothesis which predicts conditions for the improved fractionation of vesicle mixtures on density gradients.

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*These considerations support a Donnan–osmotic rather than an electrostatic model, since repulsion between fixed charges of like sign should be little affected by the membrane orientation alone.

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