A Method for Measuring Nonelectrolyte Partition Coefficients between Liposomes and Water*

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Summary. This series of papers describes methods for measuring solute partition coefficients between membrane suspensions and water, and applies these methods to the system dimyristoyl lecithin:water. Knowledge of partition coefficients is relevant to analyzing permeability coefficients, probing membrane structure, and evaluating permeation models. The theoretical section of this first paper derives equations to correct measured partition coefficients for the effects of trapped water in the precipitate, tritium exchange with lipids or membranes, and nonsolvent water adjacent to membranes. The experimental section presents methods for measuring partition coefficients in liposomes of dimyristoyl lecithin, including techniques for minimizing effects of counter drift and quenching on double-label radioactive counting. These methods offer advantages in measuring membrane:water partition coefficients that are not high.

A partition coefficient (K) is the ratio, at equilibrium, of a solute's concentrations in two immiscible phases between which solute can migrate. In the present series of papers we describe methods for measuring nonelectrolyte partition coefficients between membrane suspensions and water, and we apply these methods to aqueous suspensions of dimyristoyl lecithin liposomes. The methods should also be applicable to determining partition coefficients between biological membranes and water.

These measurements are relevant to at least three general problems in membrane biology. First, the solute permeability coefficient (P), as commonly measured in biological membranes, is a black-box quantity which actually depends upon the solute's membrane:water K, the solute's diffusion coefficient within the membrane D, the membrane thickness x_o, and the resistances r' and r'' of the two membrane:water interfaces to the

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* These papers are dedicated to the memory of Aharon Katchalsky, admired teacher and friend.

solute's flow [*see* Eq. (A.22) in Appendix of Diamond and Katz, 1974]. Thus, any attempt to obtain a detailed understanding of permeability must first confront the problem of resolving the dependence of P on K, D, r', r'' and x_o. Second, K's are likely to vary over orders of magnitude for a given solute in different membranes and thus may provide sensitive probes of membrane structure. Finally, permeability coefficients of different non-electrolytes in a given biological membrane usually correlate closely with the nonelectrolytes' K's between water and bulk nonpolar solvents, such as ether, octanol or olive oil. However, there are systematic deviations from these correlations, such as that branched solutes are less permeant, and small polar solutes are often more permeant, than predicted on this basis (Collander & Bärlund, 1933; Collander, 1954; Wartiovaara & Collander, 1960; Diamond & Wright, 1969; Wright & Diamond, 1969; Hingson & Diamond, 1972). Since phospholipid bilayers are likely to provide better models of biological membranes than do bulk solvents, estimates of K's for bilayers may be helpful in interpreting these deviations, whose origin has been debated.

Outline of These Papers

Our presentation is organized so that readers interested primarily in the numerical values and interpretation of partition coefficients, and only incidentally in problems of measuring partition coefficients, may skip directly to the third and fourth papers.

This first paper of the series describes the underlying theory for measuring K's in membrane suspensions in general, and describes experimental methods for measuring K's of nonelectrolytes between dimyristoyl lecithin liposomes and water in particular. *We emphasize at the outset that the measurements provide an average value of a partition coefficient for the whole lipid phase* $\left(i.e., \left[\int_{0}^{x_0} K(x)\,dx \right] \middle/ x_o \text{ in the terminology of Diamond and Katz, 1974} \right)$, *and that the actual local partition coefficient* $K(x)$ *probably varies with position in the membrane* (*see* Diamond & Katz, 1974, for further discussion). Because the final estimate of K results from a sequence of many independent measurements, and because many solutes of interest have fairly low $K's$, an accumulation of small errors at each stage could yield a large error in the final estimate. We have therefore observed many experimental precautions and describe these precautions in detail so that our results can be reproduced, even though some of these precautions may prove eventually to be unnecessary.

The second paper (Katz & Diamond, 1974a – referred to as paper II) describes an estimation of the amount of nonsolvent water in liposomes and discusses the significance and origin of this phenomenon.

The third paper (Katz & Diamond, 1974b – referred to as paper III) presents the measurements of K's as a function of temperature, and extracts partial molar free energies, enthalpies and entropies of partition and of solution in lecithin.

The final paper (Diamond & Katz, 1974 – referred to as paper IV) discusses the interpretation of the measured K's in terms of solute structure, membrane structure and permeability measurements.

Symbols

b subscript referring to carbon-14
c concentration
C radioactive disintegrations per minute, per gram of supernatant or precipitate
D diffusion coefficient
f grams of nonsolvent water in liposomes, per gram of lipid
h radioactive counts per minute
K partition coefficient
l subscript referring to liposome phase or lipid phase
L grams of lipid per gram of precipitate
M weight of a phase, in grams
N weight of solute, in grams
o subscript referring to supernatant
p subscript referring to precipitate
P permeability coefficient
q subscript referring to trapped water in the precipitate
r resistance of membrane:water interface to permeation
R the gas constant
s subscript referring to solute
t subscript referring to tritium
T absolute temperature
w subscript referring to water phase
x distance along axis perpendicular to plane of membrane
x_o membrane thickness
α disintegrations per minute of tritium, per gram of lipid, arising from exchangeable hydrogens of the lipid
δ radioactive disintegrations per minute
ε radioactive counting efficiency (counts per disintegration)
ρ density
σ specific activity, expressed as disintegrations per minute per gram

Theory

Definition of Partition Coefficient

Consider a three-component system consisting of a solute (abbreviated s) plus two solvents: water (w) and a water-immiscible lipid solvent (l). K,

the solute's partition coefficient on a weight basis [1], is defined by the relation

$$K = \frac{c_{sl}}{c_{sw}} = \frac{N_{sl}/M_l}{N_{sw}/M_w} \tag{1}$$

where c_{sl} and c_{sw} are the solute's respective concentrations in phases l and w at equilibrium (expressed in grams of solute per grams of phase), M_l and M_w are the weights of each phase, and N_{sl} and N_{sw} are the weights of solute in each phase.

Ideally, K could be obtained from four independent determinations: the weight of each phase and the weight of solute in each phase, or any four independent measurements of combinations of these four quantities. If liposomes could be separated completely from the aqueous phase without changing the distribution of solute (e.g., by centrifugation), the four measurements could consist of weighing the supernatant and precipitate, and using a ^{14}C-labeled solute to determine the amount of solute in each phase.

We next consider three reasons why the actual situation in membrane suspensions is not quite so simple: trapped water in the precipitate, tritium exchange with the phospholipid, and nonsolvent water.

Complication 1: Trapped Water in the Precipitate. In practice, liposomes and water cannot be separated completely by centrifugation: some water (about 60% of the pellet by weight) is trapped in the liposome pellet. Thus, in Eq. (1) M_l must be corrected for the weight of trapped water, and N_{sl} must be corrected for the weight of solute in the trapped water. If M_o, M_p and M_q represent the weights of supernatant, precipitate and trapped water, respectively (i.e., $M_p = M_l + M_q$), and N_{so}, N_{sp} and N_{sq} represent the weights of solute in the supernatant, precipitate and trapped water, respectively, then Eq. (1) takes the form

$$K = \frac{M_o}{N_{so}} \frac{(N_{sp} - N_{sq})}{(M_p - M_q)}. \tag{2}$$

If all the trapped water had the same physical properties as bulk water, then the solute would have at equilibrium the same concentration in the trapped water and in the supernatant ($N_{sq}/M_q = N_{so}/M_o$), and Eq. (2) would

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1 K must be multiplied by ρ_l/ρ_w, where ρ is the density of each phase, to convert to the more usual partition coefficient on a volume basis. In our experiments ρ_l was 1.014 g/ml, the density of maximally hydrated lecithin (Lecuyer & Dervichian, 1969), while ρ_w was 1.005 g/ml, the density of an aqueous 150 mM/liter KCl solution. The conversion factor would therefore be 1.014/1.005 = 1.009 and would be the same for all solutes studied.

become

$$K = \frac{M_o(N_{sp} - N_{so} M_q/M_o)}{N_{so}(M_p - M_q)}. \tag{3}$$

Since Eq. (3) contains one more independent variable than does Eq. (1), one additional measurement is now needed – e.g., using tritiated water to determine the amount of water trapped in the precipitate.

To re-express Eq. (3) in terms of quantities that can be measured experimentally, we write

$$N_{so} = \delta_{bo}/\sigma_b = h_{bo}/\sigma_b \varepsilon_{bo}$$
$$N_{sp} = \delta_{bp}/\sigma_b = h_{bp}/\sigma_b \varepsilon_{bp} \tag{4}$$
$$M_o = \delta_{to}/\sigma_t = h_{to}/\sigma_t \varepsilon_{to}$$
$$M_q = \delta_{tp}/\sigma_t = h_{tp}/\sigma_t \varepsilon_{tp}$$

where subscripts b and t refer to carbon-14 and tritium, respectively, δ's are the radioactive disintegrations per minute of either isotope in the supernatant (subscript o) or precipitate (subscript p), h's are the corresponding radioactive counts per minute, ε's are the counting efficiencies expressed as fractions (counts recorded per disintegration) and σ's are specific activities on a weight basis (disintegrations per minute per gram of solute or of water). Substituting Eqs. (4) into Eq. (3) yields:

$$K = \frac{\delta_{to}\delta_{bp} - \delta_{bo}\delta_{tp}}{\delta_{bo}M_p\sigma_t - \delta_{bo}\delta_{tp}}. \tag{5}$$

We define the quantities C, the disintegrations per minute of carbon-14 or tritium in the supernatant or precipitate, per gram of supernatant or precipitate, by the equations

$$C_{bp} \equiv \delta_{bp}/M_p; \quad C_{bo} \equiv \delta_{bo}/M_o; \quad C_{tp} \equiv \delta_{tp}/M_p; \quad C_{to} \equiv \delta_{to}/M_o = \sigma_t. \tag{6}$$

Substituting Eqs. (6) into Eq. (5) yields

$$K = \left(\frac{C_{bp}}{C_{bo}} - \frac{C_{tp}}{C_{to}}\right) \bigg/ \left(1 - \frac{C_{tp}}{C_{to}}\right). \tag{7}$$

The denominator of Eq. (7) is $L \equiv M_l/M_p$, the grams of lipid per gram of precipitate. The numerator, denominator and K must all be positive or zero. The denominator can be zero only if no phospholipid is present, in which case the numerator is also zero.

Complication 2: Tritium Exchange with the Lipid or Membrane. Eq. (7) tacitly assumes that all the tritium in the precipitate is in the form of tritiated water. If any hydrogens of the phospholipid or membrane exchanged for tritium of the water, the factor C_{tp}/C_{to} of Eq. (7) would overestimate the amount of the trapped water in the precipitate and underestimate the amount of phospholipid. For dimyristoyl lecithin the sole possibility for tritium exchange would arise from keto-enol equilibria involving the four methylene hydrogens on the two carbons adjacent to the two ester carbonyl groups. Since the rate constant for enolization of acetone is only 3×10^{-8} min^{-1} (Gould, 1959, p. 381) and is expected to be much lower for esters, the extent of tritium exchange resulting from enolization in an experiment lasting up to 200 hr may in practice be neglected for dimyristoyl lecithin.

The following correction formula may be used in systems where tritium exchange is not negligible. Properly, Eq. (7) should be replaced by

$$K = \left(\frac{C_{bp}}{C_{bo}} - \frac{C_{tq}}{C_{to}} \right) \Big/ \left(1 - \frac{C_{tq}}{C_{to}} \right) \tag{8}$$

where C_{tq}, the disintegrations per minute of tritium in the trapped water of the precipitate per gram of precipitate, is related to C_{tp} by the equation

$$C_{tp} = C_{tq} + \alpha L. \tag{9}$$

Here α is the disintegrations per minute of tritium per gram of lipid, arising from the exchangeable hydrogens of the lipid, and L is still the grams of lipid per gram of precipitate. Thus, L, instead of equalling $1 - C_{tp}/C_{to}$, is given by

$$L = 1 - C_{tq}/C_{to} = 1 - \frac{C_{tp}}{C_{to}} + \frac{\alpha L}{C_{to}}. \tag{10}$$

From Eq. (10),

$$L = (1 - C_{tp}/C_{to})/(1 - \alpha/C_{to}) = 1 - C_{tq}/C_{to} \tag{11}$$

or

$$\frac{C_{tq}}{C_{to}} = \left(\frac{C_{tp} - \alpha}{C_{to}} \right) \Big/ \left(1 - \frac{\alpha}{C_{to}} \right). \tag{12}$$

The inequality $\alpha/C_{to} \ll 1$ will generally hold for tritium exchange between water and phospholipid. For example, water has two exchangeable hydrogens per mole, dimyristoyl lecithin at most four, and their respective molecular weights are 18 and 678, so that an upper limit for α/C_{to} would be $(4/678)/(2/18) = 0.053$. Furthermore, the inequality $\alpha/C_{to} \ll C_{tp}/C_{to}$ holds for our experiments ($\alpha/C_{to} \le 0.053$, $C_{tp}/C_{to} \sim 0.6$). With these approximations

Eq. (12) becomes

$$\frac{C_{tq}}{C_{to}} \sim \left(\frac{C_{tp}-\alpha}{C_{to}}\right)\left(1+\frac{\alpha}{C_{to}}\right) \sim \frac{C_{tp}-\alpha}{C_{to}} + \frac{C_{tp}}{C_{to}^2}. \tag{13}$$

The corrected expression for K is finally obtained by substituting Eq. (13) into Eq. (8):

$$K = \left(\frac{C_{bp}}{C_{bo}} - \frac{C_{tp}-\alpha}{C_{to}} - \frac{\alpha C_{tp}}{C_{to}^2}\right) \Big/ \left(1 - \frac{C_{tp}-\alpha}{C_{to}} - \frac{\alpha C_{tp}}{C_{to}^2}\right). \tag{14}$$

The smaller the partition coefficient, the larger will be the relative discrepancy between the K values calculated from Eq. (7) and Eq. (14).

Complication 3: Nonsolvent Water. To correct for the solute in the trapped water in the precipitate (Complication 1), Eq. (3) assumes that solute is present at the same concentration in the trapped water as in the bulk supernatant. In fact, a significant amount of water is bound strongly to lecithin (Elworthy, 1961; Chapman, Williams & Ladbrooke, 1967; Levine & Wilkins, 1971) and is unavailable for dissolving solute (Huang & Charlton, 1971). Thus, Eq. (3) overestimates the correction for solute in the trapped water and underestimates K. In some cases Eq. (3) can yield negative values of K, a physical absurdity. For instance, in dimyristoyl lecithin liposomes at 55 °C, Eq. (3) yields $K = -0.36$ for sucrose.

Let us assume to a first approximation (*see* paper II) that, for a given molecular species of lipid, the amount of unavailable (nonsolvent) water is proportional to the weight of lipid and is independent of the solute used, though dependent on experimental conditions such as temperature. Then the amount of solute in the trapped water in the precipitate is not $N_{sq} = N_{so}M_q/M_o$, as assumed in Eq. (3), but $N_{sq} = N_{so}(M_q - fLM_p)/M_o$, where f is the grams of nonsolvent water per gram of lipid. Eq. (3) is thus replaced by Eq. (15):

$$K = \frac{M_o[N_{sp} - (N_{so}/M_o)(M_q - fLM_p)]}{N_{so}(M_p - M_q)}. \tag{15}$$

Substitution of Eqs. (4) into Eq. (15) yields:

$$K = \frac{\delta_{to}\delta_{bp} - \delta_{bo}\delta_{tp} + fLM_p\delta_{bo}\sigma_t}{\delta_{bo}M_p\sigma_t - \delta_{bo}\delta_{tp}}. \tag{16}$$

Substitution of Eqs. (6) and the relation $L = (1 - C_{tp}/C_{to})$ into Eq. (16) yields:

$$K = \left[\left(\frac{C_{bp}}{C_{bo}} - \frac{C_{tp}}{C_{to}}\right) \Big/ \left(1 - \frac{C_{tp}}{C_{to}}\right)\right] + f. \tag{17}$$

The quantity within the brackets is simply the partition coefficient uncorrected for nonsolvent water, as given by Eq. (7). Eq. (17) may be rewritten in the simple form

$$K = K' + f \qquad (18)$$

where K is the true partition coefficient and K' is the measured, uncorrected value.

Eqs. (17) and (18) show that the relative error f/K introduced by neglect of nonsolvent water is larger, the smaller the partition coefficient. Eq. (17) also shows that if one chooses a solute that is completely insoluble in the lipid ($K = 0$), f may be calculated from experimentally measureable quantities, as

$$-\left(\frac{C_{bp}}{C_{bo}} - \frac{C_{tp}}{C_{to}}\right)\bigg/\left(1 - \frac{C_{tp}}{C_{to}}\right).$$

If the correction due to tritium exchange with phospholipid (Complication 2) is not negligible, combination of Eqs. (14) and (17) will yield an equation that takes account both of tritium exchange and of nonsolvent water.

This treatment of the nonsolvent water oversimplifies the real situation. The simplifications, and the origin and measurement of the nonsolvent water, are discussed in detail in the following paper (paper II).

Materials and Methods

Preparation of Liposomes

All experiments were carried out on synthetic L-α-dimyristoyl lecithin obtained from Nutritional Biochemical Co. This neutral lipid was chosen because its chemical composition is uniform and well-defined compared to that of natural egg lecithin, and because all the hydrocarbon chains are saturated, so that there can be no changes of structure due to oxidation. The reported Chapman phase-transition temperature, above which the fluidity of the bilayer's hydrocarbon tails greatly increases, is near 23 °C (Chapman et al., 1967), so that the effect of a phase transition on partition can be conveniently studied. Partition coefficients measured with different batches of this lipid were found to be reproducible. Near the reported phase transition temperature a discontinuity in partition coefficients and in enthalpies and entropies of partition (papers III and IV) as well as in the nuclear magnetic resonance spectrum (see below) was observed as a function of temperature.

Liposomes were prepared by essentially the method described by Bangham, Standish and Watkins (1965). Briefly, lecithin was dissolved in chloroform (1 ml per 10 mg lecithin), and the solution, contained in a spherical flask, was evaporated to dryness on a rotary evaporator at 40 °C for 12 hr. To the lecithin was added an unbuffered aqueous solution (1 ml per 10 mg lecithin) of 0.15 M KCl containing tritiated water.

The pH of the aqueous solution was 5.5 because of equilibration with CO_2 in air and remained virtually the same after mixing with lecithin. The flask was stoppered, and the mixture was shaken in a water bath at 40 °C for at least 24 hr. Under these conditions the layer of lecithin that had been spread on the flask during rotary evaporation separated from the glass, and the suspension became turbid due to the formation of liposomes. Aliquots of this suspension were then transferred to smaller spherical flasks to which ^{14}C-labeled solutes were added. These flasks were restoppered and shaken in a water bath at the desired experimental temperature for at least a further 24 hr before the first measurements of partition coefficients were made.

All solutes whose K's we determined were studied at trace concentrations. Lange, Gary-Bobo and Solomon (1974) have shown that K's of the solutes valeramide and isovaleramide in liposomes of egg yolk lecithin are independent of concentration up to at least 100 mM. Snart and Wilson (1967) demonstrated that K's of several steroid solutes in egg yolk lecithin liposomes are concentration-independent up to the solubility limit in water.

Most studies on the physical properties of lecithin liposomes have utilized either natural egg yolk lecithin or else various synthetic lecithins other than dimyristoyl lecithin. These studies have shown that lecithin liposomes are multilamellar structures in which lamellae of lipid, each approximately 25 to 40 Å thick and with the lecithin molecules arranged in a bilayer, are separated by layers of water of similar thickness (Papahadjopoulos & Miller, 1967; Bangham, 1968; Lecuyer & Dervichian, 1969; Levine & Wilkins, 1971; Williams & Chapman, 1971). There have been few studies specifically of dimyristoyl lecithin liposomes, but de Gier, Mandersloot and Van Deenen (1968, Table 1 and Fig. 3b) showed that liposomes of dimyristoyl lecithin exhibited osmotic behavior qualitatively similar to that of other lecithins, and that liposome permeability decreased drastically in a temperature range near the reported phase transition temperature of dimyristoyl lecithin. When examined by phase-contrast microscopy (through the kindness of Dr. J. M. Tormey), the dimyristoyl lecithin liposomes we studied were seen to range in dimensions from particles at the limit of resolution of the light microscope up to particles about 5 μ in diameter, with a general appearance similar to that described by Papahadjopoulos and Miller (1967, Fig. 2a) for liposomes of egg yolk lecithin. We do not know what fraction of the material is below the limit of microscopic resolution. The [13]C Fourier-transform nuclear magnetic resonance spectrum of our dimyristoyl lecithin liposomes after sonication was found to be virtually identical to the published spectrum of sonicated dipalmitoyl lecithin liposomes (Metcalfe, Birdsall, Feeney, Lee, Levine & Partington, 1971; Levine, Birdsall, Lee & Metcalfe, 1972), except that the transition temperature for line broadening is near 23 °C in the former and 41 °C in the latter, corresponding to the difference in the temperatures of phase transition (F.A.L. Anet & J. M. Diamond, *unpublished observations*). The spectrum of egg yolk lecithin liposomes is also similar (Oldfield & Chapman, 1971).

Addition of water to dry lecithin at temperatures above the phase transition of the lecithin results in formation of liposomes, but liposomes are not formed if water is added below the transition temperature (Chapman & Fluck, 1966). However, once formed, the individual liposomes remain unchanged in size on cooling through the transition temperature (Lee, Birdsall, Levine & Metcalfe, 1972). The partition coefficients that we measured were unaffected by cooling liposomes through the transition temperature and rewarming, and the same is true of nuclear magnetic resonance spectra and of other physical properties (Chapman *et al.*, 1967).

The fact that unsonicated lecithin liposomes are multilamellar requires that sufficient time be allowed in partition studies for added solutes to equilibrate with all lamellae. As will be discussed (p. 79; paper II, p. 91), we found that a 24-hr equilibration period was sufficient for the solutes we used.

Isolation of Liposomes

Four milliliters of an equilibrated liposome suspension were pipetted into each of two polycarbonate centrifuge tubes and spun down for 30 min at 25,000 rpm in a Spinco L-2 centrifuge with a sealed rotor. The resulting supernatant was optically completely clear. From each centrifuge tube, two samples of supernatant and then one sample of precipitate were taken up in micropipettes (Clay-Adams "Selectapette"). Each of the three samples as it was taken up was transferred as quickly as possible to a carefully tared scintillation vial, which was recapped before the next sample was taken. Each sample weight was in the range 60 to 130 mg, and sizes of the three samples from a given centrifuge tube were approximately matched in order to minimize variation in quench corrections during scintillation counting (*see* p. 81). The total transfer time, elapsed between removal of a centrifuge tube from the rotor and capping of the third scintillation vial, was approximately 3 min. During the time that the two supernatant samples were being transferred, the precipitate remained covered by the remaining supernatant (ca. 3.7 cc) in the centrifuge tube, to prevent evaporation of trapped water from the precipitate. After the six samples from the two centrifuge tubes containing a given liposome suspension had been transferred to vials, the vials were weighed. The vials had been presorted so that all three vials corresponding to a given centrifuge tube had the same weight within ± 10 mg, out of a total weight of approximately 16 g. This procedure minimizes any possible influence of differing vial geometries on scintillation counting efficiency or quench correction. Each vial was weighed twice as a tare and then weighed twice after addition of the sample. All weighings were made to ± 0.1 mg on a Mettler B5 H26 balance.

Temperature Control

When measurements were to be made at a temperature other than 40 °C, the liposome suspension containing labeled water and solutes was allowed to equilibrate by shaking in a water bath at the desired temperature for 24 hr. The ultracentrifuge was also kept in a room whose temperature was held constant at the desired value. After centrifugation, the centrifuge tubes were kept in the rotor, whose large heat capacity made it effectively a constant-temperature environment for short periods, while the rotor was taken to another room for transfer of samples. Thus, the temperature of the suspensions was constant within ± 1 °C until samples were transferred to scintillation vials for weighing, at which point temperature no longer had any effect.

Controls Against Evaporation

Four types of controls show that any errors introduced into K determinations by evaporation of water or of solutes with low boiling points are negligible.

Evaporation of water would have revealed itself in an increase in counting rate for a nonvolatile ^{14}C-labeled solute, either in the liposome suspension from which aliquots were drawn for centrifugation, or else in the first to the second supernatant sample taken from each centrifuge tube after it had been removed from the rotor. In one experiment a liposome suspension containing ^{14}C-labeled sucrose was maintained at various temperatures between 25 and 55 °C for 200 hr, and each day two aliquots were drawn for centrifugation and two consecutive supernatant samples were drawn from each centrifuge tube. The standard error of the mean for counting rates of the 32 resulting supernatant samples was 0.09 % for ^{14}C and 0.10 % for ^{3}H.

From each liposome suspension, two 4-ml aliquots were routinely centrifuged in the same rotor, and after centrifugation one centrifuge tube remained in the rotor for

three additional minutes while samples were being transferred from the other tube. The difference between the K values calculated from the first and second centrifuge tube, divided by the average of the two values, was calculated for each solute at each temperature, and this ratio was averaged for each solute. The ratio (expressed as a percentage) ranged from $+5.2\%$ to -7.1%, with an average value of -0.2% for all solutes. There was no correlation between the boiling point of the solute and the magnitude or sign of this deviation. This observation also implies that any effect of centrifugal forces on K relaxes quickly after centrifugation or else is negligible.

Each scintillation vial was weighed twice after transfer of the sample and capping. The two weighings generally agreed to within the accuracy of weighing (0.1 mg).

Each set of scintillation vials was counted for at least 10 complete cycles in the scintillation counter, and the counting rates over all the cycles were averaged. No systematic trends in counting rates through the cycles were observed, and the replicate determinations for a given vial over all cycles agreed within a standard error of the mean of 0.3% or less.

Constancy with Time

In six experiments the partition coefficient of a given solute at a given temperature was compared at 12 hr and 130 hr, or else at 60 hr and 200 hr, after addition of the ^{14}C-labeled solute to the liposome suspension. In other experiments in which the effect of temperature on partition coefficients was studied, measurements of K were alternately made in the lower and upper parts of the temperature range. No time-dependent variation in K beyond the variation encountered between paired samples taken at the same time could be detected. This implies that the properties of the liposomes do not change for 200 hr after preparation, as already concluded by others from other measurements (e.g., Rigaud, Gary-Bobo & Lange, 1972). These results also imply that the time required for partition equilibrium to be achieved is short compared to 12 hr, even for the solutes tested with the lowest measureable partition coefficients and with the lowest permeabilities (except for sucrose). Half times for equilibration of most of the solutes studied are probably of the order of a few minutes (Bangham *et al.*, 1965, Fig. 13; Cohen & Bangham, 1972, Fig. 1). In practice, we allowed periods of at least 24 hr for equilibration of ^{14}C-labeled solutes. The only exception was in the case of sucrose, whose permeability coefficient is considerably lower than that of any other solute studied (*cf.* Bangham *et al.*, 1965, Fig. 13) and which also has the lowest partition coefficient, presumed to be undetectably low by our methods. When ^{14}C-sucrose was added to previously prepared liposomes, the equilibration period was found to be many days (paper II). Hence, ^{14}C-sucrose was incorporated in the initial aqueous solution added to the dry lipid spread on a spherical flask. Under these circumstances the sucrose is likely to have been present in the water between the liposome lamellae as the liposome was formed, and very short equilibration times are expected for lamellae and interlamellar aqueous spaces thinner than 100 Å. In fact, equilibration of ^{14}C-sucrose was found to be complete under these circumstances by the time of the first measurement at 12 hr (paper II).

Radioactive Counting Techniques

Tritiated water was obtained from Calatomic; ^{14}C-labeled ethanol, n-butanol, t-butanol, cyclohexanol, glycerol, urea and ethyl acetate from New England Nuclear Corporation; ^{14}C-labeled methanol and butyramide from American Radiochemical Corporation (Sanford, Florida); ^{14}C-labeled n-propanol, isopropanol, ethylene glycol and benzyl alcohol from DHOM (North Hollywood, California); ^{14}C-labeled erythritol

from Amersham/Searle; and ^{14}C-labeled benzoic acid from International Chemical and Nuclear Company (Irvine, California). All ^{14}C-labeled solutes were used only in trace concentrations (2 to 200 μM/liter).

To each sample of precipitate or supernatant in a scintillation vial was added 3 ml distilled water plus 10 ml of a scintillation fluid (Packard Instagel) to gel the mixture. A Packard Tri-Carb 3375 scintillation counter with external standard ([241]Am plus [226]Ra) was used. Counting efficiencies for ^{14}C and ^{3}H were approximately 60% and 0.1%, respectively, in one channel ("carbon channel"), and 6% and 20%, respectively, in the other ("tritium channel"). Background count rate was in the range 8 to 30 counts per min for both channels. Samples were left in the counter for at least 12 hr before counting began, to minimize errors caused by chemiluminescence. To minimize statistical counting errors, the amounts of tracers were chosen such that the activity in each sample was approximately 20,000 counts per min for ^{14}C and 100,000 for [3]H. Samples were counted for a total of at least 400,000 (^{14}C) or 2,000,000 (^{3}H) counts. The statistical counting error was thus approximately 0.1%. However, there are two other sources of counting errors. To minimize these sources of variation and to minimize the effect of accumulation of small errors on final partition coefficient values, we adopted certain variations in conventional double-label counting procedures.

First, there are slight fluctuations or drifts in the sensitivity of the scintillation counter that become apparent if the same sample is counted repeatedly. The effect of this fluctuation was minimized by counting precipitate and supernatant samples from the same centrifuge tube in immediate sequence, by including eight standards in each counting cycle, and by counting all samples and standards for at least 10 cycles. Each precipitate sample was always immediately preceded by one corresponding supernatant sample and immediately followed by the other corresponding supernatant sample. Counting of the eight standards was spaced at equal intervals through each counting cycle. It should be re-emphasized that the sole counting value used to calculate a partition coefficient [Eqs. (7), (17)] is the *ratio* of ^{14}C or ^{3}H radioactive disintegrations between supernatant and precipitate, C_{bp}/C_{bo} and C_{tp}/C_{to}, and absolute values of counting rates are not used. The standard error of the mean for the repeated counting of a given sample in either channel was generally 0.1 to 0.3% of the mean value.

The second additional source of counting error arises from differences in quenching among different samples. This complex problem was minimized as follows. Through each counting cycle were distributed four standard vials with equal disintegration rates of ^{14}C but different quench values, and four standard vials with equal disintegration rates of ^{3}H but different quench values. For each of the two sets of four standards in each of the two counting channels the efficiency of counting ^{14}C or ^{3}H was plotted against the efficiency of counting the external standard, and four calibration curves (one for each isotope in each channel) were obtained by determining the unique third-order polynomial that passed through the four standard points (Fig. 1). Ideally, one would like to work in the most nearly horizontal portion of the calibration curves, where ^{3}H or ^{14}C efficiency is most nearly independent of quench as determined by the external standard. The quench of the samples can be varied by adding water to them. However, the selection of the amount of water to add must represent a compromise among several considerations: the four calibration curves differ in the quench value at which the slope is a minimum; with too little water, fluctuations in readings are expected as the counting mixture composition is approached at which phases separate; and quench values for samples should be as close as possible to the quench value for some calibration point, to minimize the error arising from fitting a calibration curve. The compromise chosen was to add 3 ml of water, which, combined with the precipitate or supernatant samples (all in the weight range 60 to 130 mg), brought the quench value of all samples into a quench range of width 0.02. The actual quench range for samples in a given experiment
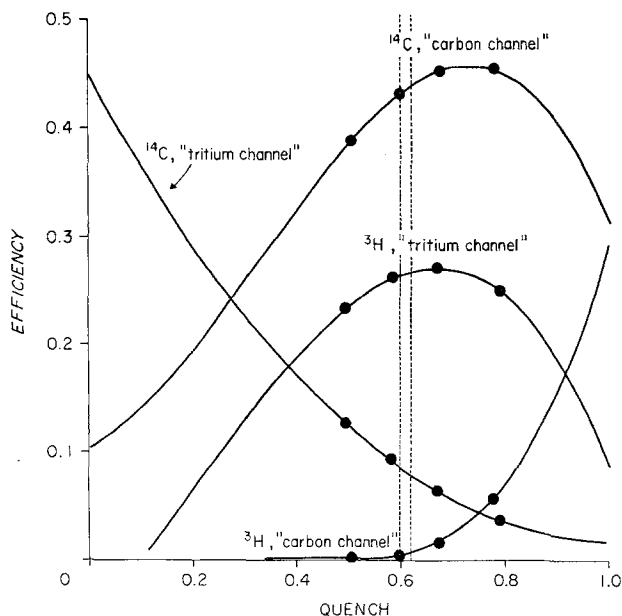
Fig. 1. Calibration curves for ^{14}C or ^3H counting efficiency as a function of quench. The efficiency of counting ^{14}C or ^3H in each of two channels (ordinate) was determined as a function of degree of quenching, by means of four standard vials containing ^{14}C or four standard vials containing ^3H but with different degrees of quenching. Quench is defined as the efficiency of counting an external radioactive standard with the same ^{14}C or ^3H standard vials in place. The curves are the unique third-order polynomials that pass through each set of four calibration points. The vertical dashed lines indicate the quench range within which all experimental samples fell

varied from 0.58–0.60 to 0.62–0.64, depending on the position of the calibration curve for the particular experiment. The quench values for precipitate and supernatant samples from the same centrifuge tube were made as nearly equal as possible, by making the weights of related precipitate and supernatant samples as nearly equal as possible within the range 60 to 130 mg. Since the amount of added water (3 ml) greatly exceeded the amount of sample, and since the quench values of a given triplet of precipitate and supernatant samples fell within a quench range of width 0.004, the sensitivity of total quench to small differences in sample size was small.

To improve the accuracy of the quench correction, we determined calibration curves for every group of samples counted simultaneously, by means of the standards counted at the same time. The error caused by drift in the calibration curves was estimated as follows. From Fig. 1 it can be estimated that a total quench range of 0.60 to 0.62 within one experiment, as encountered experimentally, corresponds to a variation in counting efficiency of up to 3%. However, the quench range among any given triplet of corresponding sample vials (one precipitate and two supernatant vials) was only about one-fifth of the total quench range. The ratio of calculated disintegration rates for hypothetical vials with maximally differing quenches of 0.60 and 0.62 was calculated for different experimental calibration curves. Differences in the value of this ratio for different calibration curves were always less than 0.04%. Hence the error in the ratio

of disintegration rates for matched precipitate and supernatant vials, due to drift in calibration curves, is less than one-fifth of this or 0.01%.

Thus, for each sample at least 30 counting numbers were obtained: the quench value determined by external standard, and the counting rate in each of two channels, for at least 10 cycles. All subsequent data extraction, including calculation of calibration curves, was carried out on an APL computer, to obtain the disintegrations per min of ^{14}C and of ^{3}H in each sample

Discussion

Comparison with Other Studies

The most extensive previous study of K's in membrane suspensions is that of Seeman, Roth and co-workers, based on methods developed by Metcalfe, Seeman and Burgen (1968), Kwant and Seeman (1969), and Seeman, Roth and Schneider (1971), and further used by Seeman (1969), Machleidt, Roth and Seeman (1972), Roth, Seeman, Åkerman and Chau-Wong (1972), and Roth and Seeman (1972). These workers applied their method to the determination of K's in three types of biological membranes (erythrocytes, synaptosomes and sarcoplasmic reticulum). Their method seems in principle equally applicable to liposomes, just as our method is in principle equally applicable to biological membranes. Basically, the method of Seeman, Roth and co-workers consists of adding a known amount of ^{14}C-labeled solute to a membrane-water suspension, centrifuging down the membranes, counting the supernatant (but not the membranes), and calculating the amount of solute in the membranes as the total amount of solute added minus the amount adsorbed to the glass vessels minus the amount left in the supernatant. Our method basically consists of adding ^{14}C-labeled solute to a membrane-water suspension, centrifuging down the membranes, and counting both the supernatant and the membranes, but not using for calculations the total amount of solute initially added.

The principal difference is that our method determines the amount of solute in the membranes more directly, whereas the method of Seeman, Roth and co-workers determines this amount indirectly as the amount that is not found in the supernatant or adsorbed on the glass. The latter method therefore requires that K be sufficiently high, and/or that the ratio of the amount of membrane to the amount of supernatant be sufficiently high, that the fraction of the total amount of solute that is not in the supernatant or adsorbed on the glass is appreciable. Since the former method does not depend on changes in supernatant concentration, the amount of membrane used can be insignificant compared to the amount of supernatant, and K need only be sufficiently high that the amount of solute in the membranes is a significant fraction of the amount present in the trapped water of the

pellet. The former method therefore has advantages if the amount of membrane is small or if the solute is one with a low K. In practice, 13 of the 16 solutes that we selected for study in lecithin had K values of less than 4 (paper III), while 14 of the 17 solutes whose K's in erythrocytes were tabulated by Roth and Seeman (1972) had K values of 4 or greater. Two further important differences are that Seeman, Roth and co-workers do not correct for the effect of nonsolvent water (*see* paper II), and that their method but not ours requires measurement of the amount of solute adsorbed to the glass vessels. Both of these differences are significant for solutes with low K's but become progressively less important with increasing K. In addition, Seeman, Roth and co-workers determine the amount of membrane in the sample from the dry weight, while we determine it from the pellet wet weight minus the weight of pellet water as calculated from the ^{3}H activity. The patterns of the actual experimental results which Seeman, Roth and co-workers obtained for erythrocytes agree well with those that we obtained for lecithin (*see* paper IV for detailed comparisons).

Other authors have measured K's, generally of solutes with high K's such as steroids, in egg yolk lecithin liposomes and in erythrocyte membranes (Hoyes & Saunders, 1966; Ohtsuka & Koide, 1969; Lange, Gary-Bobo & Solomon, 1974; Snart & Wilson, 1967; Heap, Symons & Watkins, 1970; Heap, Symons & Watkins, 1971). The latter three groups of authors used an equilibrium dialysis method, in which dialysis sacs containing membrane suspensions are suspended in solutions containing the radioactively labeled solute whose K is to be determined. The amount of solute in the membranes is calculated from the radioactivity in the sac at equilibrium, minus the radioactivity in sacs containing buffer without membranes. Since the membranes are not separated from the sac water and constitute only a small fraction of the sac volume ($< 10\%$, generally $< 2\%$), the equilibrium dialysis method requires that K be sufficiently high that much or most of the solute in the sac be in the membranes. Otherwise, the correction to be subtracted for radioactivity in the sac water becomes an appreciable fraction of the total sac radioactivity. For example, suppose that one is studying a solute with $K \sim 5$ and has a membrane suspension that is 2% membrane, 98% water. Then $(100)(98 \times 1)/[(2 \times 5) + (98 \times 1)] = 91\%$ of the solute in the sac is in the water, and this must be subtracted from the total solute in the sac in order to estimate the solute in the membranes. However, if one centrifuges the suspension as discussed in the present paper to obtain a pellet that is 40% membrane, 60% water, the correction for solute in the pellet water is only $(100)(60 \times 1)/[(40 \times 5) + (60 \times 1)] = 23\%$ of the total solute (actually, 19% when one considers the nonsolvent water correction as

measured in paper II). In practice, the solutes studied by the equilibrium dialysis method have generally been ones with K values of 30 to 4,500, several orders of magnitude higher than K's of the solutes we studied. Studies by the equilibrium dialysis method have also neglected the non-solvent water correction, but this correction is negligible for solutes with K's high enough to be measured with accuracy by the equilibrium dialysis method.

Thus, it appears that the methods described in the present paper, in which the membranes are isolated, their solute content is determined directly, and a correction is made for nonsolvent water, become advantageous in studies of solutes whose K's are not high.

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