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Nonsolvent Water in Liposomes

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Summary. In liposomes of dimyristoyl lecithin at 40 °C, a quantity of water equal to about 11.5 moles water per mole lecithin, or about one-third of the enclosed liposome water or one-fifth of the total pellet water, behaves as if it is unavailable for dissolving sucrose. This phenomenon represents permanent exclusion of sucrose, not simply a space that equilibrates slowly due to the low permeability of sucrose. The amount of nonsolvent water increases with temperature, and is similar to the amount of water bound to the phosphorylcholine groups as estimated by other methods. Nonsolvent water arises from a combination of the forces responsible for "salting-out" of nonelectrolytes from aqueous solutions by ions, and of steric effects adjacent to a surface. Measured liposome: water partition coefficients must be corrected for the effect of nonsolvent water.

In the preceding paper (Katz & Diamond, 1974*a* – abbreviated paper I) we described a method for measuring partition coefficients of nonelectrolytes between dimyristoyl lecithin liposomes and water. To determine the amount of ^{14}C-labeled solute dissolved in the lecithin, one measures the total amount of solute in the liposome phase and subtracts the amount of solute estimated as present in the trapped water of the liposome phase. However, one cannot assume that solute is uniformly present throughout the trapped water at the same concentration as in bulk supernatant water. Many lines of evidence show that some water is strongly bound to the polar head groups of lecithin and that this bound water differs in its physical properties from bulk water (Elworthy, 1961; Chapman, Williams & Ladbrooke, 1967; Lecuyer & Dervichian, 1969; Huang & Charlton, 1971; Levine & Wilkins, 1971; Rigaud, Gary-Bobo & Lange, 1972). Thus, we had to determine the effective amount of nonsolvent water in dimyristoyl lecithin liposomes in order to apply a correction to measured partition coefficients. This correction may be written in the simple form [Eq. (18),

paper I]

$$K = K' + f \qquad (1)$$

where K is the true partition coefficient, K' the measured partition coefficient calculated without a nonsolvent water correction, and f is the equivalent weight of the water that completely excludes solutes, per gram of lipid.

The present paper is concerned with the following questions: Does all the trapped water of the liposome dissolve solutes to the same extent as does bulk water? If not, how does the effective amount of nonsolvent water compare with the amount of water bound to lecithin? Does the nonsolvent water exhibit the same properties towards different solutes? To what extent does nonsolvent water affect measured partition coefficients?

The principle of the measurements consists of: (a) selecting a ^{14}C-labeled solute likely to be insoluble in the phospholipid itself (i.e., with a negligible partition coefficient), and determining the amount N_{sp} of this solute present in the whole liposome pellet; (b) using tritiated water to determine the total amount M_q of water in the liposome pellet; (c) calculating the amount of water N_{sp}/c_{sw} required to dissolve an amount of solute N_{sp} at the concentration at which the solute is present in the bulk supernatant (c_{sw}); and (d) calculating the amount of nonsolvent water as $f = (M_q - N_{sp}/c_{sw})/LM_p$, where LM_p is the weight of phospholipid in the pellet. The calculation tacitly assumes that some of the water of the liposome has bulk solvent properties and that the remainder of the liposome water completely excludes solute. This assumption is surely an oversimplification of the real situation, since different fractions of liposome water may form a continuous gradation between these extremes in solvent properties. However, this idealization would be a useful one for correcting partition coefficient measurements, if each fraction of liposome water had the same solvent effectiveness for all solutes studied. Physical measurements (Chapman *et al.*, 1967, pp. 454–457; Gary-Bobo, Lange & Rigaud, 1971, Fig. 2; Levine & Wilkins, 1971) suggest that most of the water either has bulk properties or else constant properties differing from bulk and that the idealization may be not too unrealistic.

Sucrose was chosen as test solute. Since the most important factor determining partition coefficients between uncharged solvents and water is the number and strength of hydrogen bonds formed between solute and water (Collander, 1949; Diamond & Wright, 1969*a*, *b*), and since sucrose can form approximately 20 hydrogen bonds, one might expect the partition coefficient of sucrose to be negligible. In the ether: water system $K_{sucrose}$

has been estimated as 1.1×10^{-6} (Collander, 1949), two orders of magnitude lower than that of any other solute we studied. The permeability of sucrose in lecithin liposomes is much lower than that of erythritol (Bangham, de Gier & Greville, 1967), the solute with the lowest partition coefficient that we were able to measure.

Methods

Methods were described in the previous paper (paper I). We reiterate that sucrose, like all other ^{14}C-labeled solutes used in this study, was used only at trace concentrations.

Results

If one forms dimyristoyl lecithin liposomes in a solution containing ^{14}C-sucrose and $^{3}H_2O$, permits them to equilibrate for 24 to 200 hr, and calculates the sucrose partition coefficient according to Eq. (7) of paper I, which ignores the possible existence of nonsolvent water, one obtains $K_{\text{sucrose}} = -0.31$ at 40 °C. This negative value is physically impossible and demonstrates the existence of nonsolvent water. That is, the precipitate-to-supernatant ratio of radioactivities (radioactive disintegrations per minute per gram) is higher for tritium than for sucrose ($C_{tp}/C_{to} > C_{bp}/C_{bo}$: *see* paper I, p. 73 for meaning of symbols), so that sucrose must be excluded from some of the trapped water of the precipitate.

The use of sucrose to determine the amount of nonsolvent water assumes that sucrose exclusion is an equilibrium phenomenon. However, the possibility of a nonequilibrium contribution to the exclusion of sucrose must be considered: sucrose might diffuse or permeate through liposomes so slowly that periods of 24 to 200 hr would be insufficient for equilibration, even though sucrose might equilibrate eventually with all the liposome water. This possibility was tested by two types of experiments.

The first experiment examined the permeation of sucrose into liposomes at 40 °C. Instead of including ^{14}C-sucrose as well as $^{3}H_2O$ in the original aqueous solution used to prepare liposomes, so that sucrose would already be present in the interlamellar water as the liposomes formed, we equilibrated liposomes in an aqueous solution containing $^{3}H_2O$ but no ^{14}C-sucrose. After 24 hr, ^{14}C-sucrose was added in trace concentrations, and the nonsolvent water (calculated as f in Eq. (17), paper I, assuming $K = 0$) was measured as a function of time. If sucrose had been completely impermeant, f would have been constant with time and equal to the total amount of water in the liposome. However, Fig. 1 shows that f decreases with time. Thus, sucrose is not completely impermeant, although its permeability is still much lower than that of the other solutes we studied. The total amount

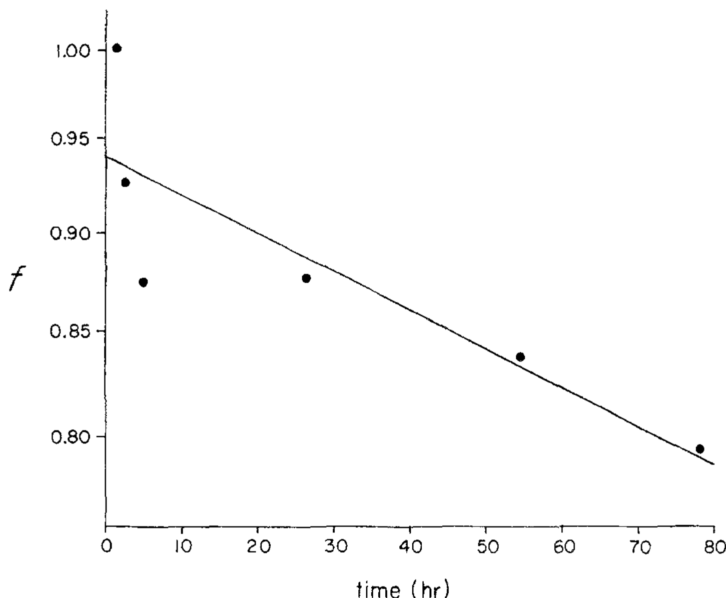

Fig. 1. Apparent nonsolvent water determined by ^{14}C-sucrose as a function of time, at 40 °C. The ordinate is f, the equivalent weight of water that completely excludes sucrose, per weight of lecithin, plotted on a logarithmic scale. From the ^{3}H and ^{14}C counting rates in the supernatant and precipitate, f was calculated as $-\left(\frac{C_{bp}}{C_{bo}}-\frac{C_{tp}}{C_{to}}\right)\Big/\left(1-\frac{C_{tp}}{C_{to}}\right)$ [*see* Eq. (17) of paper I]. Liposomes were prepared in water containing ^3H_2O, and at zero time ^{14}C-sucrose was added. At periodic intervals thereafter samples were drawn and centrifuged, and the ^{3}H/^{14}C ratio in the pellet and supernatant was compared. Each point represents an average of two values. The straight line was fitted through the points by least-mean-squares (without implying that this is the exact form of relation expected theoretically). The zero-time intercept gives approximately the amount of water that is enclosed within the liposome and that does not equilibrate very quickly with added sucrose. The slope means that sucrose slowly penetrates the liposomes

of water enclosed within the liposomes is the value of f extrapolated to zero time, or approximately 0.94 g water/g lipid. The value of f after 78 hr in the experiment of Fig. 1 is still 0.79, whereas the equilibrium value at the experimental temperature of 40 °C is 0.31 (Fig. 2). The experiment described in the next paragraph shows that when ^{14}C-sucrose is included in the solution used to prepare liposomes, f reaches equilibrium much more rapidly, within 12 hr. In the latter case equilibration is rapid because sucrose is initially present in the water between all lamellae, whereas in the former experiment equilibration is slow because sucrose must penetrate all the lipid lamellae to reach the innermost trapped water.

In the other type of experiment, the usual procedure was adopted of including ^{14}C-sucrose in the aqueous solution used for preparing the

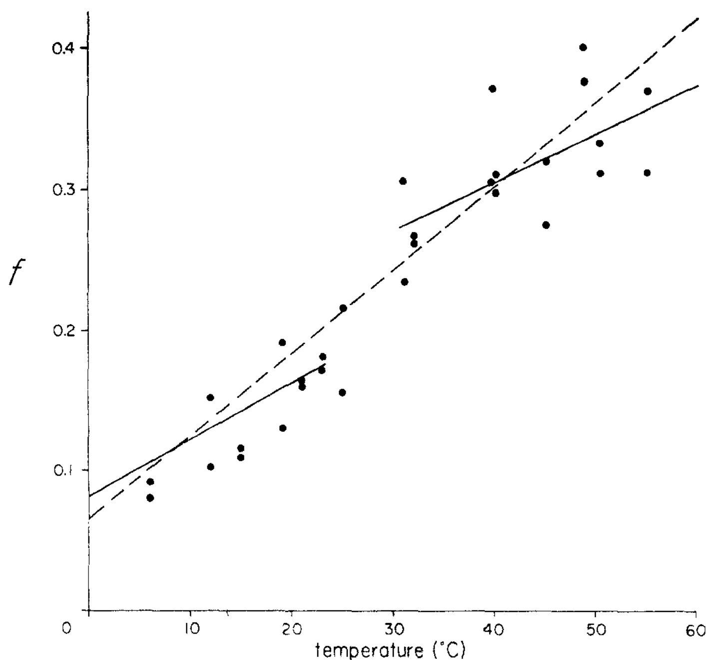

Fig. 2. Nonsolvent water determined by ^{14}C-sucrose, as a function of temperature. ^{14}C-sucrose was incorporated into the original aqueous solution used to prepare the liposomes, in contrast to the experimental procedure of Fig. 1. The ordinate is f, the equivalent weight of water that completely excludes sucrose, per weight of lecithin. f was calculated as in Fig. 1. The dashed straight line was fitted through all the points by least-mean-squares, while the two solid lines were fitted separately through the points at 6 to 25 °C and at 29 to 55 °C. Within the temperature range over which partition coefficients were measured (12 to 55 °C), the maximum deviation of the dashed line from the solid lines is 10% of the value given by the solid lines

liposomes, and the nonsolvent water was determined at 24-hr intervals beginning 12 hr after preparation. In three such experiments the change of f with time was $0.03 \pm 0.37\%$ per hr (average value $\pm$SEM), a rate not significantly different from zero. Thus, the time required for equilibration with most (79%) of the pellet water under these conditions must be short compared with 12 hr. The failure of sucrose to show any approach to equilibration with the remaining 21% of the pellet water in periods of up to 200 hr implies permanent exclusion of sucrose from this water.

Fig. 2 summarizes the results of all experiments in which ^{14}C-sucrose was incorporated into the original solution. Between 6 and 55 °C, f increases from approximately 0.085 to 0.36 g water/g lipid. There appears to be a discontinuity in f around 25 °C, although this conclusion cannot be considered certain because of the experimental scatter.

Discussion

Total Liposome Water

The total amount of water enclosed within the liposomes at 40 °C may be estimated from the experiment of Fig. 1 as the value of f extrapolated to zero time, approximately 0.94 g water/g lipid. From the results of Bourgès, Small and Dervichian (1967, pp. 160–161) on phase separation, one can calculate a value of 0.82 g water/g lipid for liposomes of egg yolk lecithin at an unspecified temperature (probably room temperature). When corrected for the differing molecular weights of dimyristoyl lecithin and egg yolk lecithin, both of these estimates yield 35 moles water/mole lecithin. The amount of water in the whole centrifuged liposome pellet is larger, 1.5 g water/g lipid. This value includes the water between separate liposomes as well as the water enclosed within the outer lamella of each liposome. Equilibration of the former water with ^{14}C-sucrose in the experiment of Fig. 1 would be limited only by the diffusion coefficient of sucrose in unstirred layers of free solution adjacent to the liposomes and would be expected to have a half time of a fraction of a minute. In contrast, equilibration of the enclosed water with ^{14}C-sucrose is limited by the need for sucrose to permeate through at least one lecithin bilayer, a much slower process. Thus, extrapolation of Fig. 1 to zero time, based on experimental values at 1.25, 2.5 and 5 hr yields the enclosed (interlamellar) water and does not detect the water between separate liposomes.

If one takes the area of a lecithin molecule at a bilayer: water interface as approximately 60 Å^2 (Lecuyer & Dervichian, 1969; Levine & Wilkins, 1971), one can calculate that 0.94 g enclosed water/g lipid at 40 °C corresponds to an 18-Å-thick water layer adjacent to each lecithin molecule, or a water layer of approximately 36 Å between lamellae. This estimate agrees with the value of 35 Å obtained for egg yolk lecithin (probably at room temperature) by Lecuyer and Dervichian (1969), whose estimate refers to the space lying between the glyceryl moieties of opposite-facing leaflets and occupied by the polar head groups as well as by water.

Amount of Unavailable Water

Comparison of the ^{14}C-sucrose space and the tritium space shows that at 40 °C about 0.31 g water/g lipid behaves as if it is unavailable for dissolving sucrose (Fig. 2). The remainder of the enclosed liposome water, or 67% of it, behaves as if it dissolves sucrose at the concentration present in

bulk water. The unavailable water represents 11.5 molecules of water per molecule of lecithin. Taking 10.5 $Å^2$ (Brunauer, 1945) as the area of a water molecule and 60 $Å^2$ as the area of a molecule of lecithin, one calculates that there are approximately two layers of unavailable water molecules per monolayer of lecithin.

Several other types of physical methods agree in demonstrating that a certain fraction of the interlamellar water has properties differing from those of bulk water. Levine and Wilkins (1971) showed by infrared absorption techniques that egg yolk lecithin at 23 °C in equilibrium with water vapor at 100% relative humidity binds 0.21 g water per g specimen, or 11.8 moles water per mole lecithin. The corresponding figure for nonsolvent water of dimyristoyl lecithin at 23 °C is 6.4 moles water/mole lecithin (calculated from Fig. 2). The infrared studies also showed that the bound water is associated with the polar head groups of the lecithin and that no bulk water is present in the lecithin at 100% relative humidity. The fact that the water bound by triglycerides is negligible compared to that bound by lecithin confirms that it is the polar head group that is responsible for binding (Elworthy, 1961)[1]. Much of the bound water must lie between adjacent polar groups (Lecuyer & Dervichian, 1969). Other possible manifestations of bound water are that a fraction of liposome water behaves as if it is osmotically inactive (Bangham *et al.*, 1967); that the effective diffusion coefficient of ^3H_2O in the interlamellar water of lecithin-water mixtures is an order of magnitude below the free-solution value until the thickness of the water layer exceeds the length of the polar head groups (Gary-Bobo *et al.*, 1971; Rigaud *et al.*, 1972); and that there is no transition at 0 °C in cooling or heating curves of lecithin-water mixtures, associated with bulk water freezing or ice melting, until the water content exceeds 0.25 g water/g lecithin (Chapman *et al.*, 1967). The amounts of water with distinctive physical properties as estimated by these methods are somewhat greater than the amount of nonsolvent water in dimyristoyl lecithin at the same temperature. The differences may be due partly to the different lecithins used (egg yolk lecithin or dipalmitoyl lecithin *vs.* dimyristoyl lecithin), partly to the different physical properties being measured.

The amount of nonsolvent water increases with increasing temperature (Fig. 2), as does the amount of bound water (Elworthy, 1961). This effect

1 While water partitioned into the hydrocarbon region of lecithin might in principle contribute to the nonsolvent water, this is likely to be of negligible quantitative significance, as indicated by the low solubility of water in bulk hydrocarbons (64×10^{-6} g water/cc hexadecane at 35 °C: Schatzberg, 1963) as well as by the low water binding to triglycerides.

may reflect the expansion in area of the lecithin bilayer with increasing temperature. In accord with this interpretation is the suggestive evidence for a small jump in the amount of nonsolvent water with increasing temperature around 25 °C (Fig. 2), near the estimated transition temperature between the solid and liquid-crystalline form of dimyristoyl lecithin (23 °C: Chapman *et al.*, 1967). At this transition temperature the fluidity of the hydrocarbon tails markedly increases, and the bilayer expands.

The study of bound water that is most nearly similar to ours with respect to the property detected is that of Huang and Charlton (1971). These investigators determined the sedementation velocity of sonicated, homogeneous, egg yolk lecithin liposomes in D_2O–H_2O mixtures of different composition and density. From the change in sedimentation velocity when KCl was added to the solutions, they could estimate how much liposome water behaved as if it excluded KCl. Their estimate at 20 °C corresponds to 1.6 water layers per egg yolk lecithin monolayer. From Fig. 2 the corresponding value we obtained at 20 °C for dimyristoyl lecithin, corrected to the value for water's molecular area used by Huang and Charlton, is about 1.2 water layers per lecithin monolayer. In view of the difference between the experimental methods as well as between the species of lecithin, the agreement of these two studies is satisfactory and strongly supportive of the reality of nonsolvent water.

Significance of Nonsolvent Water

In the Appendix we discuss the origin of nonsolvent water and the applicability of the correction based on sucrose to other solutes. It is shown that nonsolvent water probably arises from a combination of two well-known types of effects: the electrostatic effects responsible for "salting-out" of nonelectrolytes from aqueous solutions by ions; and steric effects near surfaces, as studied by workers on sedimentation velocities of macromolecules.

In practice, in the following paper (Katz & Diamond, 1974*b* – referred to as paper III) we apply to all solutes the f values obtained experimentally with sucrose (Fig. 2) and calculate in effect solute partition coefficients between the sucrose-excluding space of liposomes and bulk water. Even within the bilayer, solute distributions are certain to be nonuniform, with the more polar solutes distributed preferentially near the polar head groups, and a measured partition coefficient would represent an average value for the whole bilayer (Diamond & Katz, 1974 – referred to as paper IV). From partition measurements alone one cannot distinguish between polar solute

molecules adsorbed on the polar head groups and polar solute molecules inserted between the hydrocarbon tails at the end nearest the head groups.

It is useful to estimate the possible range of error introduced into partition coefficient determinations by uncertainty as to whether the nonsolvent water space of a given solute is identical to that of sucrose. The higher the bilayer:water partition coefficient of a solute, the lower the relative error. The worst error would arise for solutes that had free access to all the pellet water (i.e., $f=0$). From Eq. (1) one calculates that if, based on sucrose at 40 °C, one erroneously assumed an f value of 0.306, true K values of 0.1, 0.3, 1.0, 3.0 and 10.0 would correspond to measured values that overestimated K for such solutes by 306, 102, 31, 10 and 3%, respectively. If f were actually 0.215 (the value obtained from the pellet-to-supernatant ^{14}C ratio of glycerol on the assumption that glycerol is completely insoluble in lecithin), the corresponding overestimates would be 91, 30, 9, 3 and 1%, respectively. The striking effects that "freezing" the lecithin hydrocarbon tails produces on partition of solutes with K's as low as 0.1 to 0.4 (papers III and IV) suggest that K's of this size and larger are not subject to major artifacts caused by the assumed f value.

Appendix

The Origin of Nonsolvent Water

Since the amounts of bound water and of nonsolvent water are similar, and since the existence of bound water can be unequivocally attributed to interactions between water and the polar head groups, the phenomenon of nonsolvent water must be due in large part to interactions between water, the polar head groups, and nonelectrolytes. Ion-water-nonelectrolyte interactions also cause the complex phenomenon of salting-out (or salting-in) of nonelectrolytes by ions, a phenomenon that is similar to the "salting-out" of sucrose from liposome water by the polar head groups. Thus, the three types of forces responsible for salting-out (*see* Long & McDevit, 1952 and Bockris & Reddy, 1970 for further discussion) probably contribute to the origin of nonsolvent water, in addition to steric considerations operating near surfaces:

1. *Primary Hydration.* The presence of an ion reduces the amount of water available for dissolving any nonelectrolyte, by the amount of water removed from the solvent into the primary hydration shell of the ion.

2. *Secondary Hydration, or Ion-Dipole Forces.* The work required to replace a water molecule by a nonelectrolyte molecule at the same distance

from an ion equals the work to remove the water molecule to infinity minus the work required (= plus the energy released) to bring the nonelectrolyte molecule up from infinity. For distances beyond the primary hydration shells these work terms depend largely on ion-dipole forces and can be related to dipole moments or orientation polarizabilities. This effect tends to salt out nonelectrolytes for which the dipole moment is lower than that of water (since it requires net work against ion-dipole forces to replace water by the nonelectrolyte), and to salt in solutes with a higher dipole moment. The net effect of primary and secondary hydration operates to salt out almost all nonelectrolytes.

3. *London Dispersion Forces (van der Waals Forces).* One must also compare the work done against very short-range London dispersion forces in substituting a nonelectrolyte molecule for a water molecule in an ion's primary hydration shell. These forces depend on the relative distortion polarizabilities of the nonelectrolyte and of water, and on the distortion polarizability of the ion. Distortion polarizabilities increase with molecular size. The larger the nonelectrolyte molecule compared to a water molecule, the larger a salting-in term this effect will produce. The larger the ion, the more likely this salting-in term is to equal or exceed the two previously mentioned salting-out terms. Since the tetramethyl ammonium ion is sufficiently large to salt in most nonelectrolytes and forms the ionic part of choline, the net effect of at least one of the two charged groups on the polar head group of lecithin is likely to be concentration rather than exclusion of solute. Where the ionic charge is part of a surface, as in lecithin bilayers, one would refer to the phenomenon as adsorption rather than as salting-in, but the underlying forces are the same.

4. *Steric Effects.* Consider two infinite plane surfaces separated by a distance $2d$, and let each molecular species i be idealized by a sphere of radius r_i. The centers of all molecules i must lie between planes separated by a distance of only $(2d-2r_i)$, since a center cannot be closer than r_i to a surface. For a solute larger than water, the ratio of solute molecules to water molecules in the space between the surfaces will, at equilibrium, be lower than the ratio in a free solution in contact with the interspace. If the volume of the space is measured with water itself, then the space will behave towards the solute as if there was a fraction of nonsolvent water given by

$$f=\frac{r_i-r_{H_2O}}{d-r_{H_2O}}. \tag{2}$$

This steric effect on equilibrium solute concentrations is the same as the effect giving rise to the first of the two factors in the Renkin equation

(Renkin, 1954) for restricted diffusion of solutes through narrow channels, except that the Renkin treatment is for a cylinder. (The second factor in the Renkin equation affects only solute mobilities and not equilibrium concentrations). Parallel plane surfaces should provide a reasonable approximation to the interlamellar space of liposomes, since solute exclusion will occur mainly in water layers whose radius of curvature is considerably greater than their thickness. The same steric effect is well known in studies on sedimentation velocities of macromolecules and expresses itself in an exclusion of bulky solutes from a water layer adjacent to the macromolecule's surface (Schachman & Lauffer, 1949). Since in our experiments $2d \sim 36$ Å, $r_{^3H_2O} \sim 1.65$ Å, $r_i \sim 2.4$ to 4.5 Å, the contribution of this steric effect to the origin of nonsolvent water may be significant. The actual effect in liposomes could be more complex than given by this picture, since the polar head groups are about 9 Å apart, extend from the bilayer surface for approximately 10 Å, and would provide further steric interference to the presence of solutes in the water between the head groups.

Thus, of the four effects likely to contribute to the phenomenon of nonsolvent water, the combination of the first two probably acts to exclude all the solutes we tested, the third acts to concentrate the larger solutes, and the fourth acts to exclude the larger solutes.

Table 1 presents two simplistic attempts to reconstruct the amount of nonsolvent water in terms of the first, second and fourth effect. Consider a space with an estimated total width (distance between glyceryl moieties) of the interlamellar space at 40 °C, 36 Å. *Theory 1* assumes that solute is completely excluded within a distance of 10 Å (the length of the polar head groups) of either wall by the first two effects ("bound water"), and that the steric effect applies to the space 16 Å wide between the tips of the polar head groups. *Theory 2* assumes that solute is completely excluded by the first two effects within 1.35 Å of either wall (this is the thickness of the first water layer bound to egg yolk lecithin at 40 °C, as calculated from the data of Elworthy, 1961, p. 5387), and that in addition the steric effect operates in the remaining channel width of 33.3 Å. Table 1 shows that theory 2 predicts the value of the sucrose-excluding space at 40 °C, while the prediction of theory 1 is too high.

The five solutes we studied with the lowest ether: water or olive-oil: water partition coefficients were sucrose, erythritol, glycerol, ethylene glycol and urea, whose molecular sizes decrease in that order. Their pellet-to-supernatant ^{14}C ratios increase in that order. For urea, the one with the highest ratio, the ^{14}C activity in the pellet is still just small enough that one could account for it by assuming that all the pellet water is available to urea and

Table 1. Ratio of nonsolvent water to enclosed ^3H_2O space in liposomes, at 40 °C

Test solute	Theory 1	Theory 2	Experiment
Sucrose	0.89	0.36	0.33
Erythritol	0.81	0.28	0.26
Glycerol	0.78	0.24	0.22
Ethylene glycol	0.76	0.23	0.155
Urea	0.76	0.23	0.055

The numbers are the nonsolvent water for the indicated solute (as calculated from Eq. (17), paper I, assuming $K=0$) divided by the enclosed ^3H_2O space of liposomes (0.94 g water/g lipid, from Fig. 1) – i.e., the fraction of the ^3H_2O space that is unavailable to each solute. Minimum cylindrical molecular radii were taken as 1.65 Å for ^3H_2O, 2.41 Å for urea, 2.45 Å for ethylene glycol, 2.77 Å for glycerol, 3.2 Å for erythritol and 4.5 Å for sucrose (Schultz & Solomon, 1961; Gary-Bobo, DiPolo & Solomon, 1969; Sha'afi, Gary-Bobo & Solomon, 1971). The last column gives the experimental results (paper III). The remaining columns give the results of two theoretical calculations: theory 1, assuming complete solute exclusion from a 10-Å layer at either edge of the 36 Å-wide interlamellar space, and applying Eq. (2) to the remaining layer ($2d=16$ Å) in the center of the interlamellar space; theory 2, assuming complete exclusion from a 1.35-Å layer at either edge and applying Eq. (2) to the remaining layer ($2d=33.3$ Å). Neither theory accounts completely for the experimental results.

that no urea partitions into the lipid. One might therefore be tempted to interpret these measurements as meaning that the lecithin:water partition coefficients of all these solutes are negligible and that the nonsolvent-water becomes increasingly available to these solutes as their molecular size decreases, due to diminishing importance of the steric effect. Table 1 tests this hypothesis by applying each of the two above-mentioned theories to each of these five solutes. Theory 2 correctly predicts the observed exclusion of the three larger solutes but overestimates for the two smaller solutes; theory 1 overestimates for all solutes. Theories 1 and 2 may be considered as assuming a lower possible limit and an upper possible limit, respectively, for the bound water. Each theory overestimates exclusion for at least some of the five solutes, so it seems fair to conclude that significant amounts of at least the smaller ones of these solutes are either partitioned into the lipid or else adsorbed.

The lipid solubility of these solutes is far from negligible: K_{ether} is 430 times higher for urea, and 4,800 times higher for ethylene glycol, than for sucrose. The permeability of egg yolk lecithin bilayers to these five solutes (Vreeman, 1966; Bangham *et al.*, 1967; Gallucci, Micelli & Lippe, 1971) is in the approximate sequence of their pellet-to-supernatant ^{14}C ratios in our experiments, suggesting that differences in these ratios may reflect differing partition into the bilayer, not just differing distribution spaces in the inter-

lamellar water. The partition coefficients for erythritol, glycerol, ethylene glycol, and urea in dimyristoyl lecithin, calculated using the f value for sucrose, agree reasonably with values predicted by extrapolating the curve for solutes with higher partition coefficients, which are less subject to error from uncertainties in f (paper III). The possibility therefore remains that the measurements for these four solutes do indicate finite partition into the bilayer, as an alternative to an interpretation in terms of adsorption.

Thus, it is likely that some water excludes all solutes, that some additional water excludes the larger solutes, and that some solutes are adsorbed to the surface of lecithin, but the relative contributions of these effects cannot at present be firmly assessed. We note finally that it would be unjustified to apply to liposome water the exclusion pattern for various nonelectrolytes in some other well-studied system, such as salt solutions (Long & McDevit, 1952, Figs. 1–22) or the nonsolvent water of hemoglobin (Gary-Bobo, 1967). The pattern in each system is complex, the patterns differ greatly from system to system, and solute exclusion decreases with temperature in salt solutions and in hemoglobin but increases with temperature for sucrose in the interlamellar water of lecithin.

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