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The association of D-glucose with unilamellar phospholipid vesicles

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The equilibrium uptake of hydrophilic solutes, D-glucose and L-carnitine, by large unilamellar phospholipid vesicles composed of egg lecithin (PC), phosphatidic acid (PA), and various concentrations of cholesterol (Chol) has been measured. Calculation of the encapsulated volume of PC-PA and PC-PA-Chol vesicles, based on electron-microscopy data, agreed with the values directly measured by fluorescence techniques. Likewise, vesicle surface areas determined directly and from electron microscopy were in good agreement. Equilibrium uptake experiments by these well-characterized vesicles showed that glucose was taken up in excess of that amount predicted on the basis of the encapsulated aqueous volume. In contrast, the equilibrium uptake of carnitine can be predicted solely on the basis of the vesicle encapsulated volume. Each excess glucose molecule was found to be associated with from 7 to 5200 phospholipid molecules for 100 and 0.1 mM glucose, respectively. Uptake of glucose by PC-PA-Chol vesicles is independent of the cholesterol concentration and is similar to that observed in PC-PA vesicles. The cholesterol concentration independence and oil/buffer partitioning studies with octane and octanol, coupled with previous studies, strongly suggest that excess glucose is located in the vicinity of the phospholipid head group. A probable mechanism would have phospholipid, water and glucose all involved in the interaction rather than a competition between water and glucose for the phospholipid surface, as has been suggested in the literature.

1. Introduction

For many years there has been extensive interest in the active and passive transport of glucose and related polar nutrients through biological membranes and membrane models [1–3]. In most analyses of the passive transport of glucose, it is assumed that transport is membrane-controlled, and that association with the membrane bilayer is negligible [4–6]. In studies with phospholipid multilamellar vesicles, for example, it has been generally assumed that glucose is exclusively located in the aqueous compartments [4,7,8]. Recent studies of passive uptake of D-glucose, some amino acids and L-carnitine into intestinal brush border membrane vesicles [9] have indicated a rather rapid uptake of glucose relative to the other polar solutes. It became of interest, therefore, to explore the possibility that the equilibrium association of glucose with phospholipid bilayers was more significant than had been previously thought.

In the present work, large unilamellar vesicles (LUV), that were well-characterized with respect to surface area and internal volume were used to determine the equilibrium uptake of D-glucose and L-carnitine. Unilamellar vesicles were chosen rather than multilamellar vesicles (MLV) in order to mimic more closely studies with brush border membrane vesicles, since it has been reported for some systems that solute concentration in the various lamellae of MLV is not uniform [10,11] and thus in order to prepare a vesicle suspension with as uniform a particle size as possible, something more easily done with LUV.

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2. Experimental

2.1. Materials

Egg lecithin (PC), phosphatidic acid (PA), and cholesterol (Chol) were obtained from Sigma and were used as received. Their purities were checked by silica gel thin-layer chromatography (chloroform/methanol/acetic acid/water, 65 : 35 : 8 : 4) and confirmed to show a single spot. D-[^{3}H]Glucose and L-[^{3}H]carnitine were purchased from Amersham. D-Glucose and NaCl were reagent grade and were used as received from Fisher. Calcein, Mops, and DL-carnitine were purchased from Sigma.

The purity of the D-[^{3}H]glucose (0.01 mCi) was determined by silica gel thin-layer chromatography (EM reagent silica gel 60 F-254) employing three different solvent systems: *n*-butanol/pyridine/acetic acid (1 : 1 : 1), *n*-butanol/ethanol/water (104 : 66 : 30) and ethyl acetate/acetic acid/water (9 : 2 : 2). After developing, the plates were fractionated by freeing the silica from the backing, 1 cm at a time. ^{3}H radioactivity was then measured in each fraction. For each of the solvent systems employed, only a single radioactive spot was observed (data not shown). To identify the radioactive solute in the fractions, 10 μl of a 0.75 M solution of unlabeled D-glucose was also subjected to thin-layer chromatography in each of the above solvent systems. After developing, unlabeled D-glucose was visualized by charring with 50% sulfuric acid. For the unlabeled D-glucose, only a single charred spot was observed (data not shown). Further, the mobilities (R_f values) of the radioactive solute and the unlabeled glucose were found to be identical for a given solvent system, confirming the source of the radioactivity as D-[^{3}H]glucose.

2.2. Production and characterization of vesicles

Large unilamellar vesicles were prepared according to the high-pressure extrusion method of Hope et al. [12]. Vesicles were prepared with egg lecithin and phosphatidic acid (PC-PA) in a mole ratio of 96 : 4 and also with cholesterol (PC-PA-Chol) in mole ratios of 96 : 4 : 10, 96 : 4 : 20, and 96 : 4 : 40. The various formulations are expressed in terms of mole ratios of the constituent lipids in accordance with the recommendations for standardization made by Papahadjopoulos [41]. In terms of mole fractions, the formulations can be expressed as 0.96 : 0.04 for the PC-PA vesicles and 0.87 : 0.03 : 0.09, 0.8 : 0.03 : 0.16 and 0.68 : 0.02 : 0.28 for the 96 : 4 : 10, 96 : 4 : 20 and 96 : 4 : 40 PC-PA-Chol vesicles, respectively. A stock solution of the appropriate ratio of lecithin, phosphatidic acid and cholesterol in chloroform was taken to dryness under a stream of nitrogen. Residual chloroform was removed by subjecting the film to a vacuum at 40 °C for 1 h. The lipid film was then hydrated with the appropriate buffer solution for 1 h at 40 °C. The buffer solution used throughout this work consisted of 0.16 M NaCl/0.01 M Mops at a pH of 7.5. Where indicated, the buffer also contained various concentrations of either carnitine or glucose, both unlabeled and tracer amounts of ^{3}H-labeled material. After hydration, the suspension was extruded a total of 10 times through a 0.1 μm pore size polycarbonate filter (Nucleopore) under a nitrogen pressure of 250 lb/inch^2. Silica gel thin-layer chromatography (chloroform/methanol/acetic acid/water, 65 : 35 : 8 : 4) of extracted lipids from stored samples showed no products of lipid degradation. Lipid phosphate determinations were performed by the method of Bartlett [13].

Negative-stain transmission electron micrographs were obtained by the technique of Bangham et al. [14]. A single drop of the vesicle suspension was applied to a Formvar-coated copper microscope grid and drawn off after 2 min. A single drop of freshly prepared 2% ammonium molybdate in buffer was applied to the grid and then drawn off. Electron micrographs were obtained using a JEOL 100 CX microscope operating at an accelerating potential of 60 kV. Photographs of the images were taken at a magnification of 36 000 or 58 000 ×. Examination of the electron micrographs clearly revealed that the vesicles produced were unilamellar. Since stereoscopic examination revealed that the vesicles on the grid had a thickness of 10 nm or less, and therefore that the spheres were distorted into a more oblate shape, the diameters measured could only be apparent

values. Therefore, for each image, the external diameter (in nm) of the vesicle while in suspension (d_e) was calculated from the apparent external diameter (d_a) obtained from the photograph by means of the following expression:

$$d_e = 0.579 d_a + 4.71 \quad (1)$$

This equation is derived in appendix A.

To have an independent check on the validity of the electron microscopy analysis, an alternative method of particle-size measurement was employed. The weight-average diameter of a PC-PA suspension at 1 μmol phospholipid/ml was determined by cumulant-moment analysis of quasi-elastic light scattering data. For comparison with the light-scattering results, the weight-average diameter (d_w) was calculated from the electron microscopy data by the use of

$$d_w = \left[\sum d_e^3 + \sum d_e (d_e - 8)^2\right] / \left[\sum d_e^2 + \sum (d_e - 8)^2\right] \quad (2)$$

This equation is derived in appendix B.

Knowledge of the vesicle diameter, bilayer thickness and cross-sectional area per phospholipid and cholesterol molecules permitted calculation of the specific internal volume and specific surface areas. The specific internal volume, in liters of encapsulated aqueous volume per mole of phospholipid (V_{sp}), was estimated as:

$$V_{sp} = 4/3\pi \sum \left[(d_e/2) - 4\right]^3 / M_t \quad (3)$$

where the value 4 (in units of nm) is taken to be the thickness of the bilayer, and the summation is over all vesicle images measured. The number of moles of phospholipid comprising the measured vesicles, M_t, was calculated from

$$M_t = \pi \sum d_e^2 / \left[N_{avo}\left(A_{pce} + (0.19R)\right)\right] + \pi \sum (d_e - 8)^2 / \left[N_{avo}\left(A_{pci} + (0.19R)\right)\right] \quad (4)$$

where N_{avo} is Avogadro's number, R the mole ratio of cholesterol in the vesicle suspension, and A_{pce} and A_{pci} the cross-sectional area of the phospholipid molecules in the outer and inner face of the vesicle membrane, respectively. The two sets of values employed for the cross-sectional area of the phospholipid molecules were $A_{pci} = A_{pce} = 0.60$ nm^2 [12] and $A_{pci} = 0.61$ nm^2, $A_{pce} = 0.74$ nm^2 [15]. The factor 0.19 is the cross-sectional area of a cholesterol molecule, assumed to be the same on both sides of the bilayer [15].

The specific external surface area (A_{spe}) was determined from

$$A_{spe} = \pi \sum d_e^2 / M_t \quad (5)$$

and the specific internal surface area (A_{spi}) was calculated using

$$A_{spi} = \pi \sum (d_e - 8)^2 / M_t \quad (6)$$

The specific total surface area was taken as the sum of the specific external and internal surface areas.

The specific internal volumes of the vesicle suspensions were also directly measured by two different fluorescence techniques employing calcein. The calcein was purified as described by Ralston et al. [16].

In the first procedure, the in situ quenching method of Oku et al. [17] was employed. Vesicles were prepared at 5 and 10 μmol phospholipid/ml in buffer containing 1×10^{-6} M calcein. The fluorescence intensity of 0.1 ml of suspension was determined before (F_t) and after (F_{int}) the addition of 0.02 ml of 5×10^{-5} M cobalt citrate. Cobalt quenched the fluorescence of the unencapsulated calcein. The residual fluorescence intensity (F_{res}) was measured after the vesicles had been lysed by the addition of 0.02 ml of 10% (w/v) Triton X-100. The specific internal volume was then calculated via

$$V_{sp} = \left[(1.20F_{int} - 1.17F_{res})/(F_t - 1.17F_{res})\right]/P \quad (7)$$

where P denotes the phospholipid concentration of the suspension (in mol/l).

In the second fluorescence-based technique for the determination of internal volume (known as

the separation method), vesicles were prepared at 20 μmol phospholipid/ml in 1×10^{-3} M calcein, and the unencapsulated calcein was removed by Sephadex G-25 gel column filtration. Triton X-100 was added to the column eluate to a final concentration of 0.5% (w/v) to solubilize the lipid and release the encapsulated calcein. The fluorescence intensity of this solution was measured and the concentration of calcein determined by employing a standard plot of fluorescence intensity as a function of calcein concentration. Since the initial calcein concentration (C_i), final concentration (C_f) and final volume (V_f) were known, the initial volume (V_i) of the entrapped calcein was calculated by using the expression:

$$V_i = C_f V_f / C_i \quad (8)$$

To assess the validity of the electron microscopic analysis, the specific external surface area of PC-PA vesicle suspensions was directly measured by the uranyl titration method of Bangham et al. [18], later modified [19]. The method is based on the surface potential change of a monolayer of egg lecithin and phosphatidic acid at the air/water interface brought about by the electrostatic binding of uranyl ion to the head group of the lipid. The surface potential of the phospholipid monolayer (PC-PA, 96 : 4) at 60 $Å^2$/molecule was determined with a voltmeter (Orion 701-A) through a ^{241}Am air-ionizing electrode suspended over the monolayer and a reference electrode (Orion 900-100) in the subphase. Typically, 25 μmol vesicle phospholipid were added to the subphase.

2.3. Solute-uptake studies

Equilibrium uptake of solute by vesicles was accomplished by using two methods. In the first, the phospholipid was hydrated with buffer, followed by incubation of the vesicles with solute. The second procedure for attaining equilibrium uptake was to hydrate the phospholipid with buffer already containing the solute. It was shown that vesicles produced in this manner encapsulated the same amount of solute as was observed after incubation of vesicles with solute.

Separation of the vesicles from unencapsulated solute was accomplished by gel column filtration, as previously reported for various solutes [20,21] and for both large and small unilamellar vesicles [5,6,20]. Water-jacketed glass columns (1.1 × 20 cm) were silated prior to use and filled with hydrated Sephadex G-25 gel. The buffer and gel column were maintained at 25° C and the buffer elution rate was 1 ml/min. When a vesicle suspension was applied to the column, the lipid-containing fraction eluted between 7.5 and 9.5 min, just after the void volume. Samples of the eluate were assayed for phospholipid and for ^3H-labeled solute (Packard TriCarb scintillation counter). To confirm that complete separation was achieved by a single elution, the 7.5–9.5 min fraction was applied to a second, identical Sephadex G-25 gel column and fractions of the column eluate collected.

The equilibrium uptake of carnitine by PC-PA vesicles was examined as a function of carnitine concentration by incubation with 0.1, 1, 10 and 100 mM carnitine. In a second experiment, PC-PA vesicles were formed in the presence of 1 mM carnitine.

The equilibrium uptake of glucose by PC-PA vesicles was examined as a function of the phospholipid concentration. PC-PA vesicles were prepared at lipid concentrations of 5, 10 and 20 μmol/ml by hydrating the lipid with 1 mM D-glucose. The equilibrium uptake of D-glucose was examined as a function of the D-glucose concentration; PC-PA vesicles (lipid concentration, 10 μmol/ml) were prepared in buffer and combined with 0.1, 1, 10 and 100 mM D-glucose. The equilibrium uptake was also examined as a function of the cholesterol concentration. PC-PA-Chol vesicles of mole ratio 96 : 4 : 10, 96 : 4 : 20 and 96 : 4 : 40 (20 μmol lipid/ml) were prepared in buffer and combined with 1 mM D-glucose.

The *n*-octanol/buffer and *n*-octane/buffer distribution coefficients of D-glucose and L-carnitine were determined as a function of solute concentration. 5 ml D-glucose or L-carnitine in oil-saturated buffer (0.1, 1 or 10 mM) were combined with 5 ml buffer-saturated oil. After shaking for 7 days at 25° C, the aqueous and oil phases were analyzed for ^3H radioactivity. The distribution coefficients were calculated as the ratio of dpm in the oil phase to dpm in the aqueous phase.

3. Results

3.1. Vesicle characterization

Table 1 presents the calculated specific internal volumes of PC-PA and PC-PA-Chol vesicles, determined from the measurement of particle size for more than 400 diameters of each suspension. For the PC-PA vesicles, these results indicate that these figures are not strongly dependent on the value of the area/molecule employed. Also, increasing the cholesterol concentration in the membrane leads to only slight increases in the calculated internal volumes. Of importance for the interpretation of later results, the calculated specific internal volume of PC-PA vesicles made in the presence of 1 mM D-glucose was 1.50 l/mol phospholipid, not significantly different from that of vesicles made in the absence of D-glucose. The internal volumes determined by the fluorescence methods are also given in table 1. Within experimental error, the internal volumes of all vesicle suspensions studied directly by in situ quenching and by separation agree very well with those calculated from electron microscopy.

The weight-average diameters calculated from the electron microscopy data of the PC-PA and PC-PA-Chol vesicles made in glucose-containing buffer were 85 and 90 nm, respectively. The experimental values determined from quasi-elastic light-scattering studies for the same vesicle suspensions were 86 and 96 nm, respectively. This agreement indicates the validity of employing eq. 1 to calculate the external diameter of the vesicle in suspensions from transmission electron microscopic data. The similarity of the light-scattering results for vesicles made in the presence and absence of glucose confirms observations made in the electron microscopic studies that the presence of D-glucose has no effect on the structure of the vesicle.

Table 1

Specific internal volumes of PC-PA and PC-PA-Chol vesicles

Composition	Specific internal volume (l/mole of phospholipid)			
	In situ quenching [a]	Separation [a]	Calculated [b]	Calculated [c]
96:4	1.5±0.3	1.7±0.4	1.43	1.62
96:4:10	1.4±0.3	1.7±0.4	1.29	1.51
96:4:20	1.4±0.2	1.7±0.7	1.34	1.54
96:4:40	1.3±0.1	1.5±0.4	1.44	1.66

[a] Mean value ± S.E. for three determinations.

[b] Calculated from electron microscopy data with $A_{pce} = A_{pci} = 0.60$ nm^2/phospholipid molecule. The area per cholesterol molecule was 0.19 nm^2.

[c] Calculated from electron microscopy data with $A_{pce} = 0.74$, $A_{pci} = 0.61$ nm^2/phospholipid molecule.

The calculated specific external surface area of PC-PA vesicles was 2.1×10^9 cm^2/mol phospholipid for $A_{pci} = A_{pce} = 0.6$ nm^2/phospholipid molecule and 2.3×10^9 cm^2/mol phospholipid for $A_{pci} = 0.61$ nm^2 and $A_{pce} = 0.74$ nm^2. These values are quite close, indicating that the calculation is relatively insensitive to changes in the phospholipid head group area in the region studied. Similar results were also obtained for the calculated values of specific internal surface area. Increasing the cholesterol content in PC-PA-Chol vesicles led to only a slight increase (< 10%) in the calculated specific internal, external and total surface areas compared to PC-PA vesicles. The specific external surface area of PC-PA vesicles experimentally determined by uranyl titration was $(2.0 \pm 0.2) \times 10^9$ cm^2/mol phospholipid. Within experimental error, this value is identical to those calculated from electron microscopy data and, further, is approx. 56% of the theoretical specific total surface area of 3.6×10^9 cm^2/mol (the area covered by 1 mole of phospholipid at 0.60 nm^2/molecule). This indicates that approx. one-half of the available phospholipid is in the outermost-facing monolayer, confirming the conclusion of the electron microscopic study that the vesicles are indeed unilamellar.

3.2. Solute uptake

The elution profile of D-[^{3}H]glucose and phospholipid from the Sephadex G-25 gel column after the application of D-[^{3}H]glucose-loaded PC-PA vesicles showed good separation of the vesicles and unincorporated glucose (data not shown). Immediate re-elution showed no reduction in the value of moles of glucose per mole of phospholipid in the lipid fraction, confirming that all the

Table 2

Moles of solute taken up by PC-PA vesicles for various solute concentrations

The values represent pooled results for vesicles incubated in solute and vesicles made in the presence of solute. Phospholipid concentration was 10 μmol/ml.

Solute concentration (mM)	Moles of solute/mole of phospholipid [a]	
	Carnitine	Glucose
0.1	$(1.3\pm0.3)\times10^{-4}$	$(3.7\pm0.3)\times10^{-4}$
1	$(1.1\pm0.4)\times10^{-3}$	$(4.1\pm0.7)\times10^{-3}$
10	$(1.2\pm0.6)\times10^{-2}$	$(3.3\pm0.5)\times10^{-2}$
100	$(1.3\pm0.7)\times10^{-1}$	$(3.6\pm0.4)\times10^{-1}$

[a] Mean value ± S.E.

Table 3

Moles of D-glucose per mole of phospholipid taken up by vesicle suspensions of various composition

Composition	10^{-3} moles of D-glucose/mole of phospholipid
96:4 5 μmol/ml [c]	4.1 ± 0.7
10 μmol/ml	3.8 ± 0.7
20 μmol/ml	3.5 ± 0.2
96:4:10 [b]	4.3 ± 0.7
96:4:20	3.6 ± 0.5
96:4:40	4.1 ± 0.3

[a] Mean value ± S.E. for three determinations.
[b] Phospholipid concentration of all cholesterol-containing vesicles was 10 μmol/ml.
[c] Phospholipid concentration.

glucose in the lipid fraction was indeed associated in some manner with the vesicles.

The values of moles of L-carnitine/mole of phospholipid taken up by PC-PA vesicles for various D-carnitine concentrations are listed in table 2. For each order of magnitude increase in L-carnitine concentration, the uptake increases by a factor of about 10. Across the whole L-carnitine concentration range studied, the equilibrium uptake could be accounted for by only considering the internal volume of the vesicle suspension and the incubation concentration of L-carnitine. Thus, L-carnitine does not appear to associate with the phospholipid membrane in any measurable quantity.

The values of moles of D-glucose/mole of phospholipid taken up by PC-PA vesicles for various D-glucose concentrations are also shown in table 2. The results were shown to be independent of the method of attaining equilibrium uptake (data not shown) and so the pooled values are presented in table 2. Each order of magnitude increase in glucose concentration results in an increase in glucose uptake by a factor of about 10. However, unlike the results with L-carnitine, the magnitude of glucose uptake is about twice that predicted based on the internal volume and glucose incubation concentration. To investigate further this observation of excess uptake, the number of moles of glucose taken up per mole of phospholipid by PC-PA vesicles as a function of phospholipid concentration was studied. As shown in table 3, for 1 mM glucose the uptake of the sugar is independent of phospholipid concentration. The results in table 3 also indicate that the uptake of glucose from a 1 mM solution is independent of cholesterol concentration and, further, that the equilibrium uptake of glucose in PC-PA-Chol

Table 4

Distribution coefficients of D-glucose and L-carnitine at various concentrations for *n*-octanol/buffer and *n*-octane/buffer systems

	Distribution coefficients [a]		
	0.1 mM	1 mM	10 mM
D-Glucose			
n-Octanol	$(7.2\pm0.3)\times10^{-3}$	$(7.2\pm0.3)\times10^{-3}$	$(7.1\pm0.4)\times10^{-3}$
n-Octane	$(3.9\pm0.2)\times10^{-5}$	$(3.4\pm0.3)\times10^{-5}$	$(3.3\pm0.2)\times10^{-5}$
L-Carnitine			
n-Octanol	$(4.1\pm0.1)\times10^{-2}$	$(4.1\pm0.1)\times10^{-2}$	$(4.0\pm0.2)\times10^{-2}$
n-Octane	$(8.0\pm0.3)\times10^{-5}$	$(8.8\pm0.8)\times10^{-5}$	$(9.0\pm0.1)\times10^{-5}$

[a] Mean value ± S.E. for three determinations.

vesicles is similar in magnitude to that of PC-PA vesicles.

The *n*-octanol/buffer and *n*-octane/buffer distribution coefficients of D-glucose and L-carnitine, as a function of solute concentration, are presented in table 4. For both oils employed, the distribution coefficients of L-carnitine are slightly greater than those of D-glucose and are independent of the solute concentration employed.

4. Discussion

At all concentrations of glucose, phospholipid and cholesterol studied, a greater number of moles of glucose per mole of phospholipid were taken up than could be accounted for by the association of glucose with the vesicle solely within the internal volume. The difference between the observed uptake and the predicted value is defined as the excess uptake. In order to be confident that a real excess uptake of glucose was being measured, possible sources of error were evaluated. One possible explanation for the observation of excess uptake would be the incomplete separation of the incubation medium from the vesicles by the gel filtration method. However, the results of the double elution experiment showed that the number of moles of glucose per mole of phospholipid in the lipid fraction, after the second elution, was unchanged from those determined after the initial elution. If a single elution down the gel column was insufficient to separate the incubation medium from vesicles (thus resulting in an excess), a second elution would have resulted in significantly reduced total number of moles of glucose per mole of phospholipid. As it was, however, the double elution experiment showed that adequate separation was attained by a single elution down the gel column. In addition, in comparing the total number of moles of carnitine taken up by PC-PA vesicles with that amount of carnitine that would be expected to be taken up based on the internal volume of the suspension, no difference was observed. Since the gel filtration was adequate to separate unencapsulated carnitine from vesicles, the procedure probably was adequate for the separation of external glucose from vesicles.

It could be argued that glucose may have induced a structural change in the vesicles, resulting in a larger internal volume and, therefore, that a greater value of the total number of moles of glucose was taken up per mole of phospholipid. Such changes might include, for example, an increase in the area per phospholipid molecule or the fusion of a number of vesicles to produce a larger vesicle. However, no difference in the calculated weight-average diameter from electron microscopy data and quasi-elastic light scattering of vesicle suspensions made in the presence and absence of glucose was observed.

A third possible source of error would be the presence of impurities in the D-[^{3}H]glucose. An impurity that would selectively associate with the lipid bilayer, either by partitioning into the hydrocarbon core or by electrostatically binding to the surface, would lead to uptake of the ^{3}H label that would not accurately reflect the uptake of the pure, unlabeled glucose. Experimental evidence argues against the existence of such impurities. Thin-layer chromatographic analysis resulted in the detection of only a single radiochemical solute, D-[^{3}H]glucose. In addition, the results of the *n*-octanol/buffer and *n*-octane/buffer distribution experiments, i.e., very low distribution coefficients, do not suggest the existence of a hydrophobic radiochemical solute (table 4). The possibility of electrostatic binding is also not supported by experimental evidence. Both Hauser and Dawson [39] and Ohki [40] found that increasing the ionic strength of the incubation medium resulted in a dramatic lowering of the binding of cationic local anesthetics (compounds known to have a very high affinity for phospholipid bilayers). These findings were explained, in part, as being the result of electrostatic shielding of the phospholipid surface. In this study, the binding of D-[^{3}H]glucose to PC-PA and PC-PA-Chol (96 : 4 : 40) vesicles was found to be independent of NaCl concentration up to 0.4 M (data not shown). Thus, a charged contaminant capable of binding to phospholipid head groups appears unlikely.

The location of the excess glucose associated with the lipid portion of the vesicle can be discussed in terms of two extreme cases, uniform partitioning into the nonpolar portion of the bi-

Table 5

Apparent membrane/buffer partition coefficients and surface coverages for PC-PA vesicles for various D-glucose concentrations

[D-Glucose] (mM)	Partition coefficients [a]	Surface coverage (molecules/cm^2) [a]
0.1	2.6 ± 0.2	$(3.2 \pm 0.6) \times 10^{10}$
1	3.2 ± 0.1	$(4.1 \pm 0.8) \times 10^{11}$
10	2.2 ± 0.2	$(2.7 \pm 0.6) \times 10^{12}$
100	2.6 ± 0.2	$(3.2 \pm 0.3) \times 10^{13}$

[a] Mean value ± S.E.

layer and association with the outer and inner phospholipid/water interfaces. With a knowledge of vesicle physical characteristics, it was possible to calculate membrane/buffer apparent partition coefficients by dividing the concentration of glucose associated with the bilayer by the concentration of glucose in the aqueous phase. Such apparent coefficients for PC-PA and various PC-PA-Chol vesicles are given in tables 5 and 6, respectively. All values of the apparent partition coefficients are independent of glucose concentration, are much greater than the distribution coefficients obtained for octane/buffer and octanol/buffer, and are independent of the presence or absence of cholesterol. The values relative to octane and octanol, as well as the independence of cholesterol concentration, strongly suggest that the major site for the location of glucose is not in the more interior portions of the bilayer, but rather at the interface in the vicinity of the polar groups. In the former case, it is well recognized that, for solutes that partition into lipid bilayers, the inclusion of cholesterol results in a sharp drop in the apparent partition coefficient [22–24]. It should also be noted that the *n*-octanol/buffer and *n*-octane/buffer distribution coefficients for carnitine listed in table 4 are slightly higher than those of glucose. Therefore, if the oil/buffer distribution coefficients of glucose were the sole indicators of the ability of a molecule to be taken up into a bilayer, then carnitine uptake by PC-PA vesicles should also be expected to show a significant excess. Since the data listed in table 2 clearly show that PC-PA vesicles do not take up a measurable excess carnitine, localization of excess glucose in the bilayer core cannot be an important factor. The conclusion that glucose is not partitioning into the nonpolar portion of bilayers to any significant extent does not go so far as to say that D-glucose cannot partition into the bilayer whatsoever. The data presented in table 2 as well as literature reports [4–6] confirm that glucose can indeed pass through a phospholipid bilayer, so some dissolution of glucose in the membrane must take place. The above analysis, however, does argue against glucose dissolving in a bilayer to the extent that such a mechanism could totally explain the observed excess uptake.

Table 6

Apparent membrane/buffer partition coefficients and surface coverages for PC-PA-Chol vesicles for various cholesterol concentrations

D-Glucose concentration was 1 mM.

Composition	Partition coefficient [a]	Surface coverage (10^{11} molecules/cm^2) [a]
96:4:10	3.4 ± 0.2	4.2 ± 0.7
96:4:20	2.5 ± 0.2	3.0 ± 0.6
96:4:40	3.2 ± 0.1	3.5 ± 0.7

[a] Mean value ± S.E.

The second possible location for the excess glucose molecules taken up by the vesicles is at the membrane surface, in the vicinity of the polar groups. In a PC-PA vesicle suspension, an enormous surface area is available since, at 0.60 nm^2/phospholipid head group, each mole of phospholipid has a total surface area of 3.6×10^9 cm^2. The apparent surface coverage, in molecules of glucose per cm^2 of phospholipid surface, is presented in table 5. For each 10-fold increase in incubation medium glucose concentration, the apparent surface coverage increases by a factor of about 10. The values of the apparent surface coverage of glucose with PC-PA-Chol vesicles are presented in table 6. Comparing the results to those for 1 mM glucose in PC-PA vesicles, it is clear that the presence of cholesterol in the bilayer does not have a significant effect on the apparent surface concentration of glucose. A molecular model indicates that, at most, a glucose molecule would occupy a surface area of approx. 76 Å^2. This converts to a monolayer coverage of 1.3×10^{14} molecules/cm^2. Thus, the data in tables 5 and 6

indicate that, even at the highest glucose concentration employed, the observed excess glucose uptake represents less than 20% of monolayer coverage.

Support for the location of glucose at the lipid/water interface in the presence of phospholipid polar groups can be found in a number of studies of sugar-phospholipid interactions, using such techniques as ESR [25] and NMR [26]. Crowe et al. [27] provided direct evidence for a sugar-phosphate interaction, in that the 'dry-state' spectrum of dipalmitoylphosphatidylcholine exhibited a shift in the P = O asymmetric stretch band when glucose was present. In addition, the glucose OH stretch bands were shifted in the presence of the lipid.

In aqueous solution, the phosphatidylcholine group is known to be highly hydrated [28]. Using a variety of techniques, it has been shown that from 12 to 20 total waters of hydration are associated with each phosphatidylcholine molecule [28–30]. Between eight and ten of these may be in a primary hydration shell with the remainder in a secondary shell. While the conclusion that distinct states of water exist near phospholipids (tight or loose association) has been challenged [31], water interactions with phospholipid are still considered an important factor governing the structure of phospholipid bilayers [28,31,32].

To obtain a better appreciation of the magnitude of the excess glucose uptake observed in this study with respect to phospholipid hydration, the number of molecules of phospholipid per molecule of excess glucose for the various glucose concentrations was calculated by taking the reciprocal of the value of the excess moles of glucose per mole of phospholipid from table 5. The results are presented in table 7. For 100 mM glucose, each excess glucose molecule interacts with about seven molecules of phospholipid. For 0.1 mM glucose, each excess glucose molecule interacts with over 5200 molecules of phospholipid. If each phospholipid molecule is hydrated by 12 water molecules, this would result in one glucose molecule for 84 molecules of water in the case of 100 mM glucose and over 62 000 water molecules in the case of 0.1 mM glucose.

Table 7

Molecules of phospholipid per molecule of excess D-glucose for PC-PA vesicles at various D-glucose concentrations

[D-Glucose] (mM)	Molecules of phospholipid per molecule of excess D-glucose [a]
0.1	5160 ± 950
1	410 ± 80
10	60 ± 10
100	7 ± 1

[a] Mean value ± S.E.

One interesting question which might be addressed concerns the reason why glucose, a very polar molecule and one that is strongly hydrogen-bonded to water, should passively distribute from water to a phospholipid/water interface. Johnston et al. [33] have attributed the observation of an apparent interaction between sugars and phospholipids to an entropically driven process whereby sugars replace structured water associated with the head group of the phospholipid. However, unless the interaction between glucose and phospholipid is stronger than the respective hydration energies of glucose and phospholipid, such a mechanism seems highly unlikely. It seems more realistic, because of the strong and unique hydration properties of glucose [34–36] and phospholipid [28], that water remains at the interface and forms part of a ternary system, acting as a 'bridge' between the glucose and phospholipid. In a sense, we can think of glucose as being 'solubilized' in the polar portions of the bilayer, just as many polar molecules are solubilized in a variety of reverse micelles or microemulsions [37,38]. Whatever the mechanisms involved, this study has demonstrated for the first time that the effective 'partitioning' of glucose into a phospholipid bilayer is much greater than had been previously thought. In view of the importance of glucose and other sugars in biological systems, it would seem important to probe quantitatively the nature of this apparent interaction, particularly the role of hydration.

Appendix A

Stereoscopic-pair transmission electron microscopic images of vesicles revealed that the height

of the particles was less than or equal to 10 nm. Since the diameters of the images were in excess of this value, it was concluded that the spherical vesicles in suspension must flatten and spread slightly when applied to the electron microscope grid. Eq. 1 was derived to estimate the true external diameter (d_e) from the apparent external diameter (d_a).

The following analysis assumes that: (1) the vesicles in the suspended state are spheres while those on the grid are oblate ellipsoids; (2) the volume of the membrane lipid for a spherical vesicle is equal to that of the corresponding ellipsoid; (3) the membrane thickness of 4 nm is constant for both shapes; (4) the height of the vesicle on the grid is 10 nm.

For an ellipsoid, the volume (V) is expressed by

$$V = (4/3)\pi abc \tag{A1}$$

where a, b and c are the three principal radii. In the case of vesicles on a grid, radii a and b are identical and the volume of the ellipsoid becomes

$$V = (4/3)\pi a^2 c \tag{A2}$$

The volume of the membrane (V_{em}) can be calculated as the difference between the volume of the outer and inner ellipsoids, as defined by the outer and inner membrane surfaces. This can be expressed as

$$V_{em} = (4/3)\pi\left(a_o^2 c_o - a_i^2 c_i\right) \tag{A3}$$

where subscripts o and i refer to the outer and inner membranes, respectively. By assumption 4, c_o is 5 nm and by assumption 3, $a_i = (a_o - 4)$. By combining assumptions 3 and 4, c_i is found to be 1 nm. Substituting these values into eq. A3 and rearranging,

$$V_{em} = (4/3)\pi\left(4a_o^2 + 8a_o - 16\right) \tag{A4}$$

where a_o is the external radius of the vesicle as measured from the electron micrograph.

The membrane volume of a spherical vesicle (V_{sm}) can be calculated in a similar manner and is given by

$$V_{sm} = (4/3)\pi\left(12r_o^2 - 48r_o + 64\right) \tag{A5}$$

where r_o is the radius of the vesicle in suspension.

Setting V_{em} equal to V_{sm} (by assumption 2) and canceling,

$$\left(4a_o^2 + 8a_o - 16\right) = \left(12r_o^2 - 48r_o + 64\right) \tag{A6}$$

The apparent radius, a_o, obtained from the electron micrograph is substituted into eq. A6 and the roots of the right-hand side calculated. Only the real, positive root is accepted as the true external radius (r_o). When the true external diameter ($2r_o = d_e$) is plotted as a function of the apparent external diameter ($2a_o = d_a$), a linear relation is observed. Least-squares linear regression analysis for the best-fitting line results in

$$d_e = 0.5799 d_a + 4.7126 \tag{A7}$$

which is eq. 1.

Appendix B

B1. Calculation of the weight-average vesicle diameter from transmission electron microscopy data

The weight-average vesicle diameter (d_w) is defined as

$$d_w = \sum w_i d_e / \sum w_i \tag{B1}$$

where w_i and d_e represent the mass and external diameter, respectively, of the i-th particle. The mass of the lipid is obtained from

$$w_i = M_r m_i \tag{B2}$$

and

$$m_i = n_i / N_{avo} \tag{B3}$$

where M_r is the molecular weight of the lipid, m_i the number of moles of lipid in the i-th particle, n_i the number of lipid molecules in the i-th particle and N_{avo} Avogadro's number.

The number of lipid molecules in each vesicle can be calculated from the diameter by

$$n_i = \left(\pi d_e^2\right)/0.6 + \pi\left(d_e - 8\right)^2/0.6 \tag{B4}$$

The first term on the right represents the number of lipid molecules in the outer monolayer, the

second term denoting that in the inner monolayer. This expression assumes that the bilayer is 4 nm thick and that each lipid molecule occupies a surface area of 0.6 nm^2. Combining eqs. B1–B4, and canceling,

$$d_w = \left[\sum d_e^3 + \sum d_e (d_e - 8)^2\right] / \left[\sum d_e^2 + \sum (d_e - 8)^2\right] \quad \text{(B5)}$$

which is eq. 2.

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