Structural Studies on Phosphatidylcholine–Cholesterol Mixed Vesicles†

George C. Newman and Ching-hsien Huang*

ABSTRACT: The homogeneous, single-walled phosphatidylcholine–cholesterol mixed vesicles were prepared by ultrasonic irradiation of egg phosphatidylcholine in the presence of various amounts of cholesterol in solution at 4° under a nitrogen atmosphere followed by molecular sieve chromatography on a Sepharose 4B column. Physicochemical studies performed on these systems including sedimentation velocity, diffusion, partial specific volume, intrinsic viscosity, and trapped volume measurements allowed estimation of the weight-average vesicle weight, the vesicle shape, and bilayer membrane thickness of the binary mixture of phosphatidylcholine and cholesterol. Vesicle hydration was calculated using two different methods and the agreement between them was excellent up to cholesterol concentration of 0.32 mole fraction. It was observed that the structural parameters change slowly with increasing cholesterol content up to around 0.3 mole fraction and a relatively abrupt structural alteration occurs above this cholesterol content. This abrupt structural change is consistent with the asymmetrical distribution of lipid composition between the inner and outer bilayer face.

Cholesterol is an amphiphilic molecule that appears to be present in all membranes of mammalian cells. Structurally, cholesterol is characterized by a relatively small hydrophilic 3β-OH group and a bulky fused ring system that is hydrophobic and stereochemically rigid and flattened. One functional role of cholesterol has been suggested to be to control the hydrocarbon chain fluidity of the lipid components of membranes (Ladbrooke et al., 1968). In recent years, many advances have been made in establishing the effects of cholesterol on the structural and motional properties of phospholipid acyl chains (Engelman and Rothman, 1972; Hinz and Sturtevant, 1972; Marsh and Smith, 1973; Gent and Prestegard, 1974); yet the effects of cholesterol upon phospholipid head group interactions have been largely neglected. The fact that a 3β-OH group is of critical importance for a sterol to produce permeability effects on phospholipid liposomes (Bittman and Blau, 1972; deKruyff et al., 1972) suggests that more attention should be placed on the interaction between the hydroxyl group of cholesterol and the phospholipid head group.

As a first step to investigate the phospholipid–cholesterol interaction in the polar head region, we have prepared phosphatidylcholine vesicles containing various mole fractions of cholesterol. In this communication, we report the physicochemical characteristics of the phosphatidylcholine–cholesterol binary system, and discuss the effects of cholesterol on the overall structure and hydration of the phosphatidylcholine bilayer in the form of small radius vesicles.

Experimental Section

Materials. The phosphatidylcholine was isolated from hen egg yolk employing two steps of silicic acid column chromatography (Litman, 1973). Purity of the preparation was checked by thin-layer chromatography (TLC) stained with Draggendorf reagent, chromic acid, or iodine (Skipski et al., 1962). Eventually, TLC plates were developed with ninhydrin reagent as well.

Ninhydrin development of thin-layer plates revealed the presence of primary amines in the isolated material. Since phosphatidylethanolamine had definitely been removed by the silicic acid procedure, the material was assumed to be lysophosphatidylethanolamine which moves with nearly the same R_f value on silicic acid adsorbants. Analysis of the sample showed that lysophosphatidylethanolamine was present as 0.3% of the total phospholipid. This contamination was made possible by the omission of the alumina column procedure of Singleton et al. (1965) and its substitution of a second silicic acid step. Evidently the silicic acid cannot completely separate phosphatidylcholine and the lysophosphatidylethanolamine. Thus it is strongly urged that the alumina column step be retained for future phosphatidylcholine isolation procedures.

Cholesterol was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, lot 3818. Two solvent systems, chloroform–ether (9:1) and benzene–ether (1:9), revealed a single spot moving from the origin on silica gel G thin-layer plates (Randerath, 1966). [4-^{14}C]Cholesterol was supplied by New England Nuclear, lot 640-275.

The buffer employed for all measurements was 0.1 *M* KCl–0.01 *M* Tris in glass-distilled, deionized water adjusted to pH 8.00. Tris-HCl and Tris base were obtained from Sigma Chemical Co., St. Louis, Mo. All other chemicals were of laboratory reagent grade.

Preparation of Phosphatidylcholine–Cholesterol Mixed Vesicles. Typically, 300 mg of phosphatidylcholine was dissolved in benzene solution containing an appropriate amount of cholesterol and 0.1 μCi of [^{14}C]cholesterol. The phosphatidylcholine–cholesterol mixture was colyophilized from benzene. Single-walled vesicles were prepared by ultrasonic irradiation of the colyophilized phosphatidylcholine–cholesterol mixture in an aqueous buffer solution followed by molecular sieve chromatography according to the

†From the Department of Biochemistry, University of Virginia, School of Medicine, Charlottesville, Virginia 22901. *Received March 31, 1975.* A preliminary account of this work was presented at the Biophysics/Biochemistry Meeting, Minneapolis, Minn., 1974. Portions of this paper are derived from the dissertation of G.C.N. presented in partial fulfillment of the requirements for a Ph.D. degree, University of Virginia (1974). This research was supported in part by U.S. Public Health Service Grant, GM-17452.

procedures described elsewhere (Huang, 1969; Huang and Thompson, 1974). The molecular sieve elution profile of the dispersion consisted of two distinct peaks (fraction I and II). For 50 mol % cholesterol, fraction I was significantly overlapped with fraction II. In this case, fraction II was pooled and reconcentrated and then rechromatographed on the Sepharose 4B column. This procedure always yielded a well-separated elution profile. Samples were collected from the trailing half of fraction II, all else being discarded. These fractions all showed a linear relation between absorption at 300 nm and lipid phosphorus, cholesterol, or total lipid. Lipid phosphorus was determined spectrophotometrically (Gomori, 1942). Cholesterol was determined by counting the decay of the tracer amounts of [4-^{14}C]cholesterol and relating that to the 0.1 μCi of ^{14}C originally lyophilized with the precisely weighed unlabeled cholesterol. Decay of ^{14}C and efficiency of counting were determined in a Beckman LS-233 liquid scintillation counter as monitored by an external standard and standardized quenching curve. All experiments were performed within 4 days of chromatography. It has been demonstrated previously that the vesicles are stable for at least this period of time (Huang and Charlton, 1972). Inspection of centrifuge data obtained in the present study also demonstrated that this is so.

For one particular mole % cholesterol, cholesterol was also determined by dry weight determination and by colorimetric assay (Huang et al., 1974). The dry weight method gave cholesterol values which were higher than that determined by ^{14}C counting by a little more than 10%. The colorimetric assay employing *o*-phthalaldehyde (Rudel and Morris, 1973) agreed within experimental accuracy (about 1%) with the ^{14}C counting method, which, in turn, corresponded to the proportion of cholesterol originally colyophilized.

For a 30 mol % cholesterol preparation sonicated for 3 hr, a thin-layer plate was run to determine if any chemical degradation was being produced by prolonged sonication. The solvent used for development was $CHCl_3$/MeOH/H_2O/HOAc, 65:50:5:0.7. The plate was sprayed with potassium dichromate in H_2SO_4 and charred. It showed only two distinct spots which corresponded exactly with unsonicated cholesterol and lecithin standards. No other spots were observed.

Viscosity and Density Measurements. A single Cannon-Ubbelhode semimicrodilution viscometer with a shear rate of about 1500 sec^{-1} and a flow time for water of about 255 sec was used in all the viscosity measurements, both measurements of intrinsic viscosity of vesicle solutions, and measurements of the different suspending D_2O buffer media used in determination of the vesicle isodensity point. No kinetic energy correction was necessary.

When experiments making use of vesicle solution were performed, the viscometer cylinder was coated with poly-L-arginine type II-B in order to abolish the ζ potential of the glass (Y. Barenholz, personal communication). A 0.5-mg/ml solution of the poly-L-arginine in 0.05 M Tris at pH 7.0 was loaded into the cylinder and allowed to sit for 30 min. The viscometer was then cleaned in the usual manner.

The viscosity of the solution or medium relative to that of water, η_r, was calculated using the relationship $\eta_r = (td/t_w d_w)$ where t is the flow time and d is the density of the solution or medium. The subscript w represents the measurements with distilled and deionized water. Flow times determined with an electric timer were the result of a minimum of five experiments. The density for either vesicle solution or D_2O buffered medium was determined in duplicate on a Parr-Mettler cavity resonance type densimeter, Model DMA O2C, with temperature maintained at 20 ± 0.005°. The viscometer was maintained at 20 ± 0.02°. Flow time determinations for vesicle solutions involved in measurements of intrinsic viscosity were always made on two identical solutions.

The relative viscosity of 0.1 *M* KCl determined with this system was 0.999, in excellent agreement with the value of 0.998 obtained from the International Critical Tables. The intrinsic viscosity of each mole % cholesterol vesicle suspension studied was obtained from the plot of η_{sp}/c vs. c where η_{sp} is the specific viscosity and c is the lipid concentration in grams per 100 ml. Three points were used for each plot.

Sedimentation Velocity and Partial Specific Volume Measurements. The sedimentation velocity method was used to determine the sedimentation coefficient in aqueous buffer and the isodensity point of phosphatidylcholine–cholesterol vesicles suspended in buffered D_2O solutions (Huang and Lee, 1973). The sedimentation coefficients, s, for either aqueous or D_2O media of various densities were measured using a Beckman-Spinco Model E analytic ultracentrifuge equipped with schlieren optical system. The ultracentrifuge experiments were performed at 20° as controlled by the RTIC unit. The An-D rotor, operated at 42,040 rpm, was employed; a double-sector, capillary-type, synthetic boundary cell with a 12-mm optical path was used for most experiments. The relative amounts of vesicle solution and its corresponding supporting medium introduced separately into the two sectors of the centerpiece were so chosen as to allow the formation of the boundary near the center of the cell.

The heterogeneity parameter, p, was calculated for the dispersions containing various amounts of cholesterol with the following equation (Fugita, 1962):

$$\sigma^2(r_0/r)/2t = D + [(p^2\omega^4 r_0)rt/2] \quad (1)$$

where r is the distance from the center of rotation to the point of the maximum ordinate on the moving-boundary gradient curve at time t, r_0 is the distance at time zero, ω is the angular velocity of the rotor, and σ^2 is the square of the second moment of the gradient curve.

Since the vesicles were homogeneous with respect to size, and the schlieren patterns were highly symmetrical, s was calculated from the least-squares slope of a plot of t vs. log r where r is again the radial distance from the center of rotation of the centrifuge rotor to the point of the maximum ordinate on the schlieren peak at time, t. The sedimentation coefficients of the vesicles suspended in each percentage of buffered D_2O solution were first measured in duplicate at 5 or 6 total lipid concentrations, and were then extrapolated linearly by a least-squares treatment of the experimental data to obtain s^0, the value of sedimentation coefficient at infinite dilution. The individual value of s^0 obtained in a given buffered D_2O solution was multiplied by the relative viscosity, η_r, of the buffered D_2O suspending medium and the values of $\eta_r s^0$ were plotted against the respective density of the suspending buffered D_2O solution. At the zero value of $\eta_r s^0$, the density of the supporting D_2O solution, corresponding to the reciprocal of the specific volume of the phosphatidylcholine–cholesterol vesicles, is defined as the isodensity point (Huang and Lee, 1973).

For vesicles in aqueous buffer, in addition to the extrapolation to infinite dilution, the values of s^0 were reduced to values in water at 20°, $s_{20,w}{}^0$, using densities for the buffers

as determined by the methods described above, and the partial specific volume of the vesicles (Huang and Lee, 1973). These values of $s_{20,w}^0$ were employed in the Svedberg equation for calculation of the molecular weight.

Diffusion Measurements. A Beckman-Spinco Model E analytical ultracentrifuge equipped with schlieren optical system was used in these measurements. The temperature of the centrifuge rotor (An-D) was maintained at 20° by the RTIC unit during all diffusion experiments. Double-sector capillary-type, synthetic boundary cells equipped with sapphire windows were used for all of the experiments. In order to reduce sedimentation of the vesicle suspensions to a minimum, the suspending medium contained 5% D_2O by volume. The buffer and salt concentrations were maintained at the same values as in all other experiments. In addition, the rotor was operated at 9945 rpm. The apparent diffusion coefficient, D, was calculated by the maximum ordinate-area method from the following relationship (Ehrenberg, 1957):

$$D = (1/4\pi t)\,[A/kH_{max}]^2$$

where k is the magnification factor along the radial coordinate and t is the time in seconds elapsed between formation of the boundary and photographing the schlieren peak, A is the enlarged area of the gradient curve as measured on the photographic plate taken at time t, and H_{max} is the maximum height of that same enlarged curve. All enlarged photographic tracings were made in duplicate.

D was thus calculated at three total lipid concentrations with two experiments performed at each concentration, and linearly extrapolated to infinite dilution by the least-squares method to obtain D^0. These values of D^0 were, in turn, reduced to values in water at 20°, $D_{20,w}^0$. No zero time correction was made. However, since the $D_{20,w}^0$ has been obtained extremely accurately for pure phosphatidylcholine vesicles (2.03×10^{-7} cm^2/sec) (Huang and Lee, 1973) and $D_{20,w}^0$ as obtained by the method used here was slightly higher (2.09×10^{-7}), values reported here have been multiplied by (2.03/2.09) in order to normalize them to the more rigorous value.

Trapped Volume Measurements. In order to determine the trapped volume of the phosphatidylcholine–cholesterol vesicles, the vesicles were prepared by sonicating the mixed lipids in the presence of 0.5 M $K_3Fe(CN)_6$ solution containing 0.1 M KCl and 0.01 M Tris buffer at pH 8.0. After centrifugation, the dispersion was applied to a Sepharose 4B column following the same procedure as described earlier. As the lipid dispersions eluted from the Sepharose 4B column, the vesicles with trapped $K_3Fe(CN)_6$ were always completely separated from the free $K_3Fe(CN)_6$ which appeared at the position corresponding to the internal volume of the column. Because the vesicles present in aqueous buffer disintegrate in propanol–H_2O mixture (Tinker and Saunders, 1968), the trapped $K_3Fe(CN)_6$ was released from the vesicles by adding propanol (30–50% by volume) to the sample. The concentration of $K_3Fe(CN)_6$ released from the vesicles was determined from its absorbance at 420 nm. Assuming the original concentration of trapped $K_3Fe(CN)_6$ was the same as the bulk solution (0.5 M), it was then possible, after correcting the dilution factor due to the addition of propanol, to calculate the trapped volume term, TV_{exp}, from the absorbance at 420 nm. Inorganic phosphate and cholesterol concentrations were also measured from the same sample; the vesicle trapped volume in l/mol of total lipid was determined.

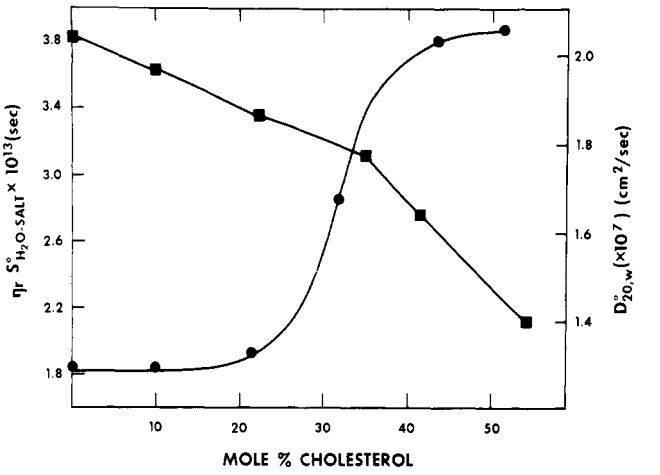

FIGURE 1: Plots of $\eta_r s^0$ and $D_{20,w}^0$ of phosphatidylcholine–cholesterol mixed vesicles vs. mole percent of incorporated cholesterol. Data obtained from the sedimentation velocity experiments; (■) results of the diffusion studies.

In order to calculate the grams of water *trapped* per gram of lipid, the following equation was employed: trapped water = $(TV_{exp})(\rho_{H_2O})/M_{lipid}$, where trapped water is expressed in grams of water per gram of lipid, TV_{exp} is expressed in l./mol of lipid, ρ_{H_2O} is the density of water (0.99827 g/ml), and M_{lipid} is the weight average molecular weight of lipid molecule defined as $M_{lipid} = xM_{PC} + (1 - x)M_{Ch}$, where x is the mole % phosphatidylcholine of the solution, M_{PC} is the molecular weight of phosphatidylcholine taken to be 775 g/mol, and M_{Ch} is the molecular weight of cholesterol taken to be 386.64 g/mol.

Results

Sedimentation Velocity and Partial Specific Volume. The analysis of the sedimentation coefficients for vesicles of all mole % cholesterol studied by the method of Fugita resulted in linear, horizontal slopes for plots of $\sigma^2 r_0/2rt$ vs. rt. The degree of contamination by multilamellar liposomes can be estimated, from the least-squares slopes for these plots, to be about 4% for all vesicle systems studied. The plots of s vs. $[\text{lipid}]_{total}$ were also linear and values of s^0 were obtained from the extrapolated least-squares intercept of zero concentration. These values of s^0 are plotted in Figure 1 as a function of mole % cholesterol. Values of $s_{20,w}^0$ are given in Table I. Values of ϕ, the effective specific volume, calculated from the isodensity point of lipid vesicles containing various concentrations of cholesterol are also shown in Table I. The values of ϕ are seen from this table to decrease slowly to about 22 mol % cholesterol but to fall off more rapidly thereafter.

Diffusion. Plots of D vs. $[\text{lipid}]_{total}$ were linear and errors in D^0, the extrapolated y intercept, were around 0.01×10^{-7} cm^2/sec for all vesicle systems studied. Values of $D_{20,w}^0$ are shown as a function of mole % cholesterol in Figure 1. The values are seen to vary linearly to 35 mol % cholesterol and then to decrease rapidly beyond that. The diffusion coefficient is inversely related to f, the frictional coefficient of the vesicle, by the Einstein–Sutherland equation:

$$D = kT/f \qquad (2)$$

where k is Boltzmann's constant and T is the absolute temperature (Svedberg and Pederson, 1940). The value of f is a function of the shape and volume of the hydrodynamic particle as well as the degree of rigidity.

Table I: Summary of Hydrodynamic Studies on Phosphatidylcholine–Cholesterol Vesicles.

Mole % Cholesterol	$s_{20,w}^0$ [a] ($\times 10^{13}$ sec)	$D_{20,w}^0$ ($\times 10^7$ cm^2/sec)	ϕ' [b] (ml/g)	Trapped Volume (l./mol)	r_0 (Å) [c]	M ($\times 10^{-6}$) [d]	No. of PC [e]/ Vesicle	No. of Chol [e]/ Vesicle	No. of Total [e]/ Vesicle
0.0	2.649 ± 0.184	2.03 ± 0.01	0.9848	0.154 ± 0.02	105.6	1.88 ± 0.22	2335	0	2335
7.5				0.152 ± 0.04					
9.5		1.96 ± 0.01			109.4				
10.0	2.648 ± 0.137		0.9847 ± 0.0009			1.94 ± 0.21	2298	255	2553
17.4				0.146 ± 0.02					
21.5	2.745 ± 0.102		0.9844 ± 0.0009			2.08 ± 0.19	2285	626	2911
22.5		1.86 ± 0.01			115.0				
28.7				0.149 ± 0.03					
31.9	3.985 ± 0.130		0.9836 ± 0.0006			2.90 ± 0.21	3031	1420	4451
35.0		1.77 ± 0.01		0.171 ± 0.02					
41.9		1.63 ± 0.01							
43,43.6	5.205 ± 0.128		0.9826 ± 0.0003			3.99 ± 0.21	3742	2822	6564
47.0				0.215 ± 0.03					
53,53.7		1.40 ± 0.01							

[a] Values of $s_{20,w}^0$ in Svedberg unit were calculated according to the equation: $s_{20,w}^0 = s^0\eta_T(1 - \phi'\rho_{H_2O})/(1 - \phi'_{buffer})$. [b] Values of ϕ', the effective specific volume, were calculated from the isodensity point of each vesicle system. [c] r_0, the vesicle outer radii, were obtained by assuming vesicle sphericity and using the Stokes–Einstein–Sutherland relationship, $D = (kT/6\pi\eta r_0)$. [d] M represents the weight average vesicle weights which were calculated from the Svedberg equation, $M = RTs_{20,w}^0/D_{20,w}^0(1 - \phi'\rho)$. [e] Values represent the calculated average number of molecules per vesicle.

Table II: Hydration Values for Phosphatidylcholine–Cholesterol Vesicles.

Mol % Cholesterol	f/f_0 [a]	β ($\times 10^{-6}$) [a]	Total Water [b]	Trapped Water [b]	Bound Water [b]
0.0	1.17		0.63	0.20	0.43
0.0		2.12 ± 0.05	0.61 [c]	0.20	0.41
10.0	1.20		0.72	0.20	0.53
21.5	1.23		0.85	0.21	0.64
28.8		2.09 ± 0.05	0.48 [c]	0.23	0.25
31.9	1.14		0.49	0.25	0.24
42.1		2.11 ± 0.04	0.37 [c]		
43.0	1.15		0.53		

[a] Calculated as described in the text. [b] Values in g_{H_2O}/g_{lipid}. [b] Hydration values calculated from intrinsic viscosity.

Weight Average Vesicle Weight. From the Svedberg equation, $M = RTs/D(1 - \bar{v}\rho)$, it is possible to calculate a weight average vesicle weight. Values of s and D are the values reduced to water at 20°, R is the universal gas constant, T is absolute temperature, ρ is the density of water at 20°, taken to be 0.99827 g/ml, and $\bar{v}$ is the partial specific volume. For the calculations performed here, ϕ, the effective partial specific volume in the presence of 0.1 M KCl, is used instead of $\bar{v}$. Values calculated in this manner, with the maximum error from all contributions, are given in Table I. Also presented in Table I are the average numbers of molecules in a vesicle which may be obtained from the weight average vesicle weights and the known mole % cholesterol. The value of pure phosphatidylcholine vesicle agrees with previously reported values (Huang and Lee, 1973). It is easily seen that these values show that up to 22 mol %, cholesterol is being added to phosphatidylcholine bilayers in a rather simple manner which does not significantly perturb the original number of lecithin molecules. It is also apparent that above this composition, the vesicles become much larger, the average weight of 43 mol % cholesterol vesicles being twice that of vesicles with less than 22 mol %. The weight average vesicle weight for 32 mol % cholesterol is seen to be intermediate but, as indicated by the heterogeneity parameter, comprised of only one population of vesicles.

Intrinsic Viscosity. The values of the intrinsic viscosity, $[\eta]$, for the 0, 28.8, and 42.1 mol % cholesterol vesicles studied are 0.0403 ± 0.004, 0.0367 ± 0.004, and 0.0338 ± 0.004, respectively. The intrinsic viscosity may be combined with the diffusion coefficient and weight average vesicle weight to calculate the value of β according to Scheraga and Mandelkern (1953):

$$\beta \equiv D[\eta]^{1/3}M^{1/3}\eta_0/kT \quad (3)$$

where M is the weight average vesicle weight, η_0 the absolute viscosity of the buffer, k the Boltzmann constant, T the absolute temperature, and β is a function of the shape of the equivalent volume of an ellipsoid of revolution, V_e. For spheres, $\beta = 2.12 \times 10^6$, theoretically the lowest possible value. As the shape of the particle deviates from sphere, β increases. The calculated values of β are also shown in Table II. It is clear that all three vesicle classes appear to be spherical.

Hydration. Lipid vesicles as solutes are normally hydrated in aqueous solution (Huang and Charlton, 1971). The volume occupied by the hydrated solute per grams dry weight of solute, v_h, is greater than that indicated by the partial specific column, $\bar{v}$, by an additional factor shown as follows: $v_h = \bar{v} + w/\rho$, where w is the grams of bound solvent of density ρ per gram of anhydrous solute (Yang, 1961). Based on the known vesicle weights and the reasonable assumption about the shape of the lipid vesicles, it is possible to obtain estimates of vesicle hydration, w, from hydrodynamic methods.

The first method consists of comparison of the experimentally determined frictional coefficient as obtained by diffusion measurements to the ideal frictional coefficient, f_0, for the situation in which the anhydrous mass is concentrated into a rigid, unhydrated sphere. It is possible to calculate f_0 by

$$f_0 = 6\pi\eta(3M\bar{v}/4\pi N)^{1/3} \quad (4)$$

Hence, the "frictional ratio" of Svedberg and Pedersen

(1940), f/f_0, can be calculated from eq 2 and 4 as follows:

$$f/f_0 = kT/D/6\pi\eta(3M\bar{v}/4\pi N)^{1/3} \quad (5)$$

Values of f/f_0 are given in Table II for vesicles containing various mole percents of cholesterol. However, f/f_0 is a compound of a hydration and a shape term and, following Oncley (1941), we may write

$$f/f_0 = (f/f_0)_s(f/f_0)_h \quad (6)$$

where $(f/f_0)_s$ accounts for effects of deviation from sphericity and $(f/f_0)_h$ contains effects due to hydration. The frictional ratio for hydration may be readily expressed in terms of the degree of hydration w, the grams of water bound per gram of lipid, as follows:

$$(f/f_0)_h = (1 + w/\bar{v}\rho)^{1/3} \quad (7)$$

Based on the assumption that the vesicles are spherical in shape so that $(f/f_0)_s = 1$ and the calculated value of f/f_0, values of the degree of hydration w can be calculated from eq 6 and 7 and are given in Table II.

Using the assumption of vesicle sphericity, it is also possible to calculate the outer radii of the various vesicles from diffusion measurements based on the Einstein–Sutherland equation ($D = kT/f$) and the Stokes' law ($f = 6\pi\eta r_0$). The calculated values, r_0, are given in Table I. Because the plot of $D_{20,w}{}^0$ vs. mole % cholesterol is linear up to 35 mol % (Figure 1), it was felt to be acceptable to calculate the value at every other mole % rather than at experimental points alone. Values are given up to 32 mol % cholesterol only, in anticipation of later results, and are shown in Figure 2. It is apparent from Figure 2 that vesicle radius is seen to increase monotonically with increasing concentration of cholesterol content. This result is in excellent agreement with the data obtained by a gel filtration technique (Gent and Pretegard, 1974).

A second hydrodynamic method which permits estimations of the shape and hydration of macromolecules employs the viscosity measurements. The intrinsic viscosity, $[\eta]$, can be related to the effective volume of a rigid equivalent ellipsoid of revolution, V_e, as follows (Scheraga and Mandelkern, 1953):

$$[\eta] = NV_e\nu/100M \quad (8)$$

where ν, the Simha coefficient, may be regarded as the viscosity increment which depends on the shape of the macromolecule. For spheres, the value of the shape coefficient, ν, is 2.5.

If the lipid vesicles are assumed to be spherical as suggested by the values of β (Table II), it is possible to calculate V_e from $[\eta]$. The radius, r_e, of each equivalent volume may be calculated for these spherical models of the hydrodynamic particle. The calculated values of r_e for vesicles containing 0.0 and 28.8 mol % cholesterol are 105.9 and 118 Å, respectively. Comparison with the values obtained from Stokes' law using diffusion measurements in Table I or Figure 2 reveals the remarkable agreement between the two analyses. However, comparison of the values r and r_e at 42 mol % cholesterol would reveal a difference of more than 4 Å. This is an indication that the assumption that the hydrodynamic particles are spherical does apply up to around 32 mol % cholesterol but clearly does not apply by 42 mol %.

By assigning $V_e = (M/N)(\bar{v} + w/\rho)$, it is possible to calculate hydration from the intrinsic viscosity data as suggested by Tanford and Buzzell (1956):

$$[\eta] = \nu(\bar{v} + w/\rho)$$

Values calculated in this manner are given in Table II.

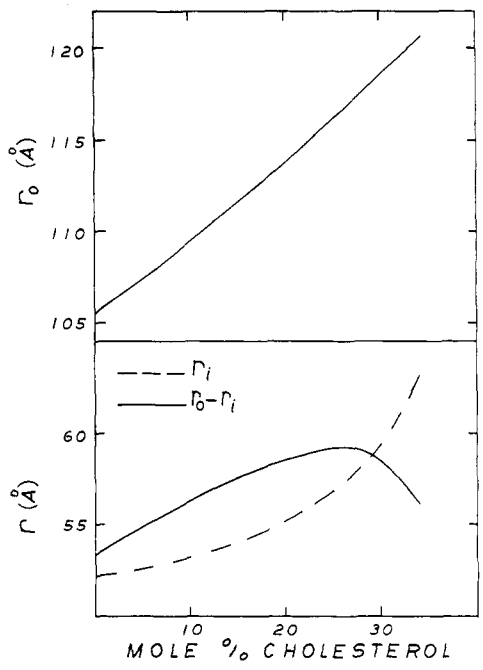

FIGURE 2: Plots of various calculated radii of phosphatidylcholine–cholesterol mixed vesicles vs. mole percent of incorporated cholesterol. The outer radius, r_0, is calculated from the diffusion coefficient, while the inner radius, r_i, is calculated from the trapped volume measurements. The apparent membrane thickness is taken as the difference between the outer and inner radius of the vesicle system, $r_0 - r_i$.

Comparison of these values with those obtained by diffusion measurements (Table II) shows that the agreement is excellent up to 32 mol % cholesterol, but poor at higher values. Clearly, the assumption of sphericity for the hydrodynamic particle is no longer valid above 32 mol %. Above this value, it is not possible to ascribe values for either hydration or shape.

The hydration obtained by either method described above is the entire hydration including water which is bound to the membrane and water which is merely trapped inside the internal solvent compartment. It is the bound water alone, however, which is of interest. Therefore, the column labeled "Bound Water" in Table II was calculated by subtracting the grams of water per gram of lipid calculated to be trapped inside the vesicle (as obtained in the following discussion of trapped volume) from the total hydration, w. This value of bound water is seen to increase from the initial value with no cholesterol present as the mole % cholesterol increases to 22 mol %. However, by 29 mol % there has been a sharp decline in bound water. This decline will be considered later in the Discussion section.

Trapped Volume and Estimation of Thickness. The determined values of trapped volume for each phosphatidylcholine–cholesterol vesicle system studied are shown in Table I. The result obtained with pure phosphatidylcholine vesicles is in excellent agreement with the published value of 0.16 l./mol of P_i, based upon ^{36}Cl flux measurements (Toyoshima and Thompson, 1975). The value is seen to increase rapidly above 28 mol % cholesterol. A first glance at these data is liable to give the false impression that the trapped volume compartment inside the vesicle is decreas-

ing around 20 mol % cholesterol. This is not the case. The trapped volume results reported in Table I are on a total lipid base. However, the total lipid per vesicle is also increasing and it turns out that the trapped volume *per vesicle* is a monotonically increasing function.

The trapped volume data may be used to obtain the quantity of water which is calculated to be inside the internal compartment. These values are shown in Table II.

The trapped volume data may also be used to estimate the thickness of the bilayer membrane and it is possible to follow changes in the thickness as a function of mole % cholesterol. The only method available from data presented in this study is to calculate the outer vesicle radius and subtract from it the radius of the internal solvent compartment. The outer radii have already been calculated from diffusion measurements. As previously stated, these values were calculated only at those mole % cholesterol which are known to form a spherical vesicle. It is similarly possible to transform the trapped volume data to a volume per vesicle and from this to calculate the "inner radius", r_i, of the bilayer. Again this is limited to spherical vesicles. Thus

$$r_i = [3TV_{exp}\,(\text{no. of molecules/vesicle})/4\pi N]^{1/3}$$

where TV_{exp} is the experimental trapped volume in units of l./mol of lipid, N is Avagadro's number, and no. of molecules/vesicle is taken from Table I. The values of r_i so calculated are shown in Figure 2. Finally, all that remains is to subtract r_i from r_0 to obtain the estimate of the membrane thickness, r_m. These values of r_m are also shown in Figure 2. The values of r_i were estimated at every other mole % cholesterol in order that the function $r_m = r_0 - r_i$ could be made more continuous. Since TV_{exp} vs. mole % cholesterol is not linear, the values estimated for r_i are not as accurate as the estimated values of r_0. Nevertheless, fairly reliable values can be obtained by piecewise connecting the six experimental points of TV. The behavior of r_0 and r_m ($r_0 - r_i$) as a function of mole % cholesterol up to 32 mol % is graphically summarized in Figure 2.

While consideration of the behavior of r_m is best left for the Discussion section, it is still pertinent to attempt to define here the contributions to the value of r_m. The most obvious contribution comes from the thickness of the hydrocarbon region of the bilayer itself. Also included will be the thickness of the polar head region and the bound water. On the outside of the vesicle "bound water" refers to water extending out to the shear layer of the hydrodynamic particle. On the inside of the bilayer, the "bound water" refers to water which is impermeable to the large $Fe(CN)_6^{3-}$ ion. In general, the polar head regions and their respective bound water will be overlapping.

Discussion

Experimental data presented in this communication show two distinctive characteristics for phosphatidylcholine–cholesterol mixed vesicles when the cholesterol content is increased. First, egg phosphatidylcholine vesicles with cholesterol content up to about 32 mol % appear to be spherical as evidenced by the comparative diffusion and viscosity measurements. Beyond 32 mol %, the vesicle is no longer a spherical object, possessing a small, but otherwise ill-defined, asymmetry. Second, from various plots of s^0, D^0, ϕ', and trapped volume vs. mole % cholesterol, it is evident that around 25–35 mol % cholesterol all the hydrodynamic properties related to the gross vesicle structure are abruptly altered. The calculated vesicle weight, shown in Table I, is seen to increase slowly as the mole % cholesterol approaches somewhere between 22 and 32 mol %, and then to increase rapidly as the cholesterol content reaches beyond 32 mol %.

The observed slow change in the physical parameters upon addition of relatively small amounts of cholesterol to egg phosphatidylcholine vesicles can be attributed to the decrease in mean molecular area and the simultaneous increase in average chain length of phosphatidylcholine, or the so-called cholesterol condensing effect (Lecuyer and Dervichian, 1969). Two lines of evidence are consistent with this interpretation. First, the calculated apparent bilayer thickness ($r_0 - r_i$) shown in Figure 2 is seen to increase with increasing cholesterol content up to 28 mol %. Second, the corresponding vesicle weight can be accounted for by the addition of cholesterol to a roughly constant number of phosphatidylcholine molecules (Table I). Moreover, based on the X-ray data of cholesterol condensing effect on the planar bilayer system, the maximum amount of cholesterol molecules which can be incorporated into vesicles with a constant amount of phosphatidylcholine molecules can be calculated to be about 31 mol % (Huang et al., 1974). The experimentally determined concentration is somewhere between 21.5 and 31.9 mol % (Table I). In view of the difference between the experimental methods as well as the bilayer systems, the agreement of these values is satisfactory and supportive of the cholesterol condensing effect.

The abrupt structural change as revealed by all the hydrodynamic parameters at cholesterol content around 25–35 mol % may be related to the reorganization of the already condensed membrane components. In a previous paper, we observed that at about 30 mol % of incorporated cholesterol there is a marked increase in the concentration of phosphatidylcholine in the outer bilayer face relative to the inner face (Huang et al., 1974). Presumably, a fine balance between the small radius of the vesicle curvature and the differential cross-section area of the polar and nonpolar portions of the lipid molecules in the membrane is a major driving force for this compositional asymmetry (Thompson et al., 1974, Berden et al., 1975). The observed increase in the trapped volume of the internal aqueous compartment above 28 mol %, for example, may be attributed to the preferential distribution of cholesterol in the inner half of the vesicle bilayer. This increase in internal aqueous volume may be accentuated by the fact that the hydrophilic hydroxyl group of cholesterol is smaller in terms of molecular volume and also less hydrated than the polar head group of phosphatidylcholine; hence, the marker, $Fe(CN)_6^{3-}$ ions, used for measuring the trapped volume can come closer to the membrane surface leading to the observation of a relatively larger internal volume. This increased internal volume as likely caused by the preferential packing of cholesterol in the inner surface of the bilayer at a cholesterol content higher than 26 mol % can, in turn, explain the calculated decrease in the apparent membrane thickness at the corresponding higher cholesterol content in the vesicle bilayer.

Hydration of the 3β-OH group of cholesterol associated with the phospholipid–cholesterol interaction has been recently emphasized by deKruyff et al. (1973). Calculations based on our hydration data indicate that the water binding capacity of phosphatidylcholine–cholesterol vesicles increases with increasing cholesterol content up to 0.22 mole fraction, and then falls off at higher cholesterol content (Table II). The study of bound water that is comparable to our data with respect to the amount detected is that of Rigaud et al. (1972). By comparison with pure phosphatidyl-

choline bilayers, these investigators also observed a decrease in bound water for a 1:1 molar ratio of phosphatidylcholine-cholesterol multilamellar system. Interpretation of these results is not straightforward, since water molecules may not only bind to the head groups of the bilayer via hydrogen bondings, they may also be accommodated in the kink region within the hydrocarbon core of the bilayer (Träuble, 1971). Addition of cholesterol decreases the phospholipid chain mobility (Keough et al., 1973), and, hence, reduces the time-averaged kink formation. Consequently, there is less room available in the hydrocarbon core to accommodate water molecules. On the contrary, the phospholipid head group-head group separation is increased when cholesterol is incorporated and therefore there is more space available in the polar head region for water molecules to undergo hydrogen bondings. Our hydration data represent the net effect of cholesterol on the polar and nonpolar region of the phosphatidylcholine bilayer.

In discussing the effect of cholesterol on the polar head region of phosphatidylcholine bilayers, we have assumed that increase in hydration through hydrogen bonding interaction is self-evident, but the problem is actually quite subtle. There are several quite distinct sites in the polar head region of the phosphatidylcholine-cholesterol bilayer where hydrogen bonds to water can occur. First of all it should be borne in mind that phosphatidylcholine molecule has no hydrogen bond donor atom. The anionic phosphate oxygen and the carbonyl oxygen atoms of phosphatidylcholine molecule are potential sites for hydrogen bonding with water molecules, but they are hydrogen bond acceptors. The hydrophilic 3β-OH group of cholesterol has been suggested to hydrogen bond the ester >C=O oxygen of the phosphatidylcholine molecules in the bilayer (Brockerhoff, 1974; Yeagle et al., 1975). Addition of cholesterol to the bilayer may, therefore, release some bound water molecules at the site of carbonyl oxygens resulting in dehydration. However, the increased head group-head group separation of phosphatidylcholine molecules upon addition of cholesterol can weaken the dipolar interaction between the trimethylammonium group of one molecule and the phosphate group of an adjacent molecule (Newman, 1974; Yeagle et al., 1975); hence, more water molecules can hydrogen bond to the deshielded phosphate oxygens. In addition, water molecules may also undergo hydrogen bonding to the cholesterol oxygen, while cholesterol is donating its hydroxyl proton to the neighboring phospholipid carbonyl group (Brockerhoff, 1974). Clearly, the contribution of anionic phosphate oxygen and cholesterol oxygen is to increase the amount of bound water. The present hydrodynamic studies cannot distinguish the various hydration and dehydration processes that might occur in the polar head region of the bilayer, but hopefully, the observed nonlinear relation between the amount of bound water and the cholesterol content in the phosphatidylcholine-cholesterol vesicle will stimulate future studies pointing toward an understanding of the interaction between the hydroxyl group of cholesterol, the phospholipid head group, and water.

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Characterization of Dog Small Intestinal Fucolipids with Human Blood Group H Activity†

Edwin L. Smith, John M. McKibbin,* Karl-Anders Karlsson, Irmin Pascher, Bo E. Samuelsson, and Su-Chen Li

ABSTRACT: Fucolipids with human blood group H activity were isolated from several dog small intestines. On the basis of mass spectrometry, periodate oxidation, enzyme degradation, methylation, and immunologic studies the following structure is proposed: Fucα(1→2)Galβ(1→4)Glc-NAcβ(1→3)Galβ(1→4)Glc-ceramide. The ceramide was shown by mass spectrometry to contain hydroxyhexadecanoic acid and phytosphingosine as major constituents.

Fucolipids with human blood group H activity have been isolated from human erythrocytes (Yamakawa et al., 1965; Hakomori and Strycharz, 1968; Hakomori, 1970; Koscielak et al., 1973; Stellner et al., 1973a; Gorniak and Koscielak, 1972; Watanabe et al., 1974), human pancreas (Hakomori, 1970), dog small intestine (Hiramoto et al., 1973), pig stomach mucosa (Slomiany et al., 1974), and adenocarcinoma of the gastrointestinal tract (Hakomori et al., 1967). Complete structures for the H-active fucolipids which have been reported included a ceramide tetraglycoside in pig stomach mucosa (Slomiany et al., 1974), and a ceramide pentaglycoside (Koscielak et al., 1973; Stellner et al., 1973a) and heptaglycoside (Koscielak et al., 1973) in human erythrocytes. H-active fucolipids with eight or more sugar residues per molecule have been described in human erythrocytes (Stellner et al., 1973a; Hakomori et al., 1972; Watanabe et al., 1974), but their complete structures have not been determined. Structure studies on the dog small intestinal H-active fucolipid are reported in this paper.

Experimental Procedures

Materials. Forssman hapten was isolated from dog small intestine by methods in use in this laboratory (Vance et al., 1966; Smith and McKibbin, 1972). Lacto-*N*-fucopentaose I was a gift from Dr. Ginsburg of the National Institutes of Health. Melibiose, lichenin, and laminarin were purchased from K and K Laboratories, Inc., Plainview, N.Y. Sodium taurocholate was purchased from Nutritional Biochemicals Co., Cleveland, Ohio. β-*N*-Acetylhexosaminidase and β-galactosidase from Jack bean and α-galactosidase from fig ficin were prepared according to the procedure reported previously (Li and Li, 1972).

Isolation and Purification of Fucolipids. The procedure used to isolate the fucolipids of dog whole small intestine has been described previously (Vance et al., 1966; Smith and McKibbin, 1972). All isolated fucolipids were found to be homogeneous when tested on thin-layer plates coated with silica gel G or H in three solvent systems: a neutral (system A, chloroform–methanol–H_2O; 65:35:8), a basic (system B, chloroform–methanol–concentrated NH_4OH; 40:80:25), and an acidic system (system C, chloroform–methanol–H_2O–glacial acetic acid; 65:35:4:4 or system D, 55:45:5:5) (Smith and McKibbin, 1972). These systems have been shown to separate intestinal fucolipid mixtures into various components. Additional evidence of purity was obtained by analysis of the intact methylated and intact methylated and reduced fucolipids using thin-layer chromatography and mass spectrometry.

Determination of Sugars. Fucolipids (250 μg) were analyzed for sugar after hydrolysis with 0.5 ml of 2.5 *N* HCl for 6 hr at 100–105° in an air oven; 0.25 μmol of arabinose was added to samples and standards as an internal standard. The hydrolysates were extracted with 1 ml of $CHCl_3$; the aqueous phases were lyophilized and reduced with 1.0 ml of 2% sodium borohydride. The borohydride was decomposed with 0.2 ml of 6 *M* acetic acid and the sample lyophilized. One-milliter portions of 2% HCl in methanol were added to the residue and dried with filtered air after each portion. The residue was then acetylated with 1:1 pyridine–acetic anhydride in a boiling water bath for 3 hr. The alditol acetates of the samples and sugar standards were analyzed in a Beckman GC-65 gas chromatograph using a 6-ft column (column A) of 0.2% ethylene glycol succinate + 0.2% ethylene glycol acetate + 1.4% XE-60 on Gas Chrom P (100–200 mesh, Applied Science Labs). The temperature program was 150° for 1.5 min with 2.5°/min increase until a final temperature of 220°, which was held for 25 min; helium gas flow was 60 cm^3/min.

† From the Department of Biochemistry, University of Alabama—Birmingham, Birmingham, Alabama 35294 (E.L.S. and J.M.M.), the Department of Medical Biochemistry, University of Göteborg, Fack, S-400 33 Göteborg 33, Sweden (K.-A.K., I.P., and B.E.S.), and the Department of Biochemistry, Tulane University, Delta Regional Primate Research Center, Covington, Louisiana 70112 (S.-C.L.). *Received March 24, 1975.* Supported by University of Alabama Medical Center Faculty Research Grants 82-6111 and 82-6117, American Cancer Grant IN-66K and National Science Foundation Grant 6B 43571, a grant from the Swedish Medical Research Council (03X-3967) and by National Institutes of Health Research Grant NS 09626, and National Science Foundation Grant BMS 74-03500.