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The osmotic response of large unilamellar vesicles studied by quasielastic light scattering

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Large unilamellar vesicles of two phosphatidylcholines, one saturated (DMPC) and the other unsaturated (DOPC), prepared by the reverse-phase evaporation method were studied using the quasielastic light scattering technique. The accurate sizing obtained by this technique showed an osmotic response for the two kinds of vesicles when the salinity of the external medium was diluted. The elastic moduli of lipid vesicles bilayers in the liquid phase were then estimated according to the elasticity theory of spherical shells taking into account salt leakage data known from the literature.

Introduction

Understanding the mechanical properties of membranes would improve our knowledge of fundamental biological processes such as hemolysis, cell adhesion and fusion, cell destruction in microvasculature cell membrane rupture and fragmentation at swelling etc., all of which pertain to the stability of biological membranes [1–3]. However, for some time, many authors have realized that direct study of these natural membranes appeared to be complex and, therefore, simpler model artificial membranes known as phospholipid vesicles were intensively investigated. Although many efforts have been deployed to understand the structure of bilayers and their behavior [4–6], only a few have been concerned with the mechanical properties of vesicles [7–9] which were closely studied for cell membranes [10–13]. Our work reported here is an attempt to contribute to this point.

Indeed, the problem of swelling was raised as soon as the preparation of vesicles was well known [14,15], but the reports have been controversial: for small vesicles, Johnson and Butress [16] reported an osmotic insensitivity, whereas for large vesicles, Deamer and Bangham [15] noted an osmotic swelling. To our knowledge, the elastic modulus of vesicles bilayers has been investigated by only a few authors [7–9]. In most cases, the elastic modulus was estimated for isolated plane bilayers in such a manner that planar bilayers were compressed or broken down by an electric field [17–24].

In this work, by an accurate sizing we attempted to estimate the elastic modulus of vesicles

Abbreviations: DMPC, dimyristoylphosphatidylcholine; DOPC, dioleoylphosphatidylcholine; SUV, small unilamellar vesicles; LUV, large unilamellar vesicles.

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membranes expanded by an osmotic pressure gradient. We chose two phosphatidylcholines, one with saturated acyl chains (DMPC) and the other with unsaturated acyl tails (DOPC) because of their well-known structure, their importance and because of the known permeability of salt throughout the bilayers prepared from these lipids, these leakages are necessary in the computation of elastic moduli.

The method we used in this work is quasielastic light scattering. The advantage of this method for the characterization of vesicles has been well demonstrated [25–28]. It is quite suitable for the sizing of particles in the submicron range without alteration of the solution's quality, is very accurate and can give an indication on the polydispersity of the solution [29].

By this method, the vesicles translational diffusion coefficient, D, was measured and for unambiguous cases, the hydrodynamic radius determined by using directly the Stoke's-Einstein relation. In the case where there is a variation of D with concentration, generally following a linear law [16,30,31], corresponding to the attractive and/or repulsive interaction between the particles one has to extrapolate to infinite dilution (or zero concentration) to obtain the self-diffusion coefficient and then the hydrodynamic radius, but this procedure is straightforward. For this purpose, we carefully take this point into account by studying simultaneously the variation of the diffusion coefficient, D, versus salt dilution (which implies also a dilution of particle concentration) and versus pure dilution of particle concentration. The comparison between the two curves will then give the effect of salt generating the osmotic pressure. Consideration of the theory of spherical shells will allow us to estimate the elastic modulus.

Materials and Methods

Preparation of dispersions. The lipids were purchased from Sigma and other chemical reagents were from Merck and Aldrich. Small unilamellar (SUV) and large unilamellar vesicles (LUV) were prepared by two different methods. For the preparation of LUV, we used the reverse-phase evaporation method of Szoka and Papahadjopoulos [32]: 1.5–1 μmol of lipid (dimyristoyl- or dioleoylphosphatidylcholine, DMPC or DOPC) was dissolved in an organic solvent, generally a mixture of 0.5 ml chloroform + 3.5 ml diethyl ether. After complete dissolution, 2 ml of buffer containing 10 mM Tris-HCl/0.02% NaN_3/150 mM NaCl (pH 8.3) was added. The solution was then briefly vortexed and sonicated for 10 min by using a 60 W Bransonic B12 bath-type sonifier before being submitted to evaporation under reduced pressure in a rotative evaporator. A gel-like phase was observed as indicated in the paper of Szoka and Papahadjopoulos [32] before a clearance was observed. The solution was held for 3 h at a temperature well above the transition point, then centrifuged to eliminate dust and large aggregates and finally filtered through 0.45 μm Millex filters. This method gave well-stabilized DMPC and DOPC vesicles of a size in the range 1600–2000 Å with a polydispersity 0.15–0.25. The SUV were dispersed by the standard process of sonication [14,32]. 6 μmol of DMPC was dissolved in chloroform contained in a glass flask, the system was then left to evaporate moderately until a thin layer of lipid was formed on the wall of the flask. The flask was then heated to 35°C before 6 ml of a preheated buffer (as above) was added into it. The solution was then vortexed to be homogenized before being submitted to sonication treatment for 30 min.

For this purpose we used a Bioblock E 72 401 sonifier (with a tip of 3 mm in diameter) working at a power 30 W intermittently, every second, with a duty time of 0.9 s. During the sonication, the solution's temperature was maintained at 35 ± 0.1°C well above the transition point. After the solution became clear, an ultracentrifugation, $10^5 \times g$, was performed for 2×1 h followed by filtration with Millipore Millex 0.22 μm filters which completed the preparation of SUV. This method gave SUV of 600 Å in size.

To change the ionic strength, a buffer without NaCl but otherwise identical to the buffer used for the preparation of the vesicles was slowly added to avoid possible breaking of vesicles. All buffers or organic solvents such as chloroform and ether were filtered thorough 0.1 μm Millipore filters before being used to avoid dust contamination which can lead to errors in quasielastic light scattering measurements.

The lipid concentration, C, was controlled by the enzymatic method [33]. The lipid concentrations at the beginning of the measurements were in the range 20–40 μmol/l (or 15–30 $\mu g \cdot ml^{-1}$). These low concentrations were to avoid aggregation of the vesicles.

Electron microscopy. A control of the unilamellar properties of the prepared dispersions was done by freeze-fracture electron microscopy. The samples were sandwiched between two thin copper disks (Escaig, J. (1982) J. Microsc. 126, 221–229) and frozen without any cryoprotectant by plunging into liquid nitrogen at −210°C. They were fractured at −150°C under ultra high vacuum in a Reichert-Jung Cryofract CF 190 apparatus. The replica were observed with a Philips EM 300 electron microscope working with a magnification in the range of 33000–42000.

Quasielastic light scattering. The background of quasielastic light scattering has been thoroughly reviewed [29,34]. In a homodyne detection, the auto-correlation function of the photocurrent obtained from the scattered light can be expressed as

$$C(\tau) = A + B|g^{(1)}(\tau)| \quad (1)$$

where A and B are constants and $g^{(1)}(\tau)$ is the normalized correlation function. For non-interacting monodisperse particles $g^{(1)}(\tau)$ is a single exponential, $\exp(-\Gamma\tau)$. The inverse correlation time is related to the translational diffusion coefficient, D, by the formula, $D = \Gamma/q^2$ where q is the scattering vector which depends on the scattering angle θ, the incident wavelength, λ, and the solvent refractive index n via

$$q = (4\pi n/\lambda) \sin \theta/2$$

For polydispersed solutions, $g^{(1)}(\tau)$ is no longer a single exponential and the method of cumulants [35] is used to fit g to an average exponential and to calculate the normalized variance of the distribution of decay rate Γ around the Z-average $\bar{\Gamma}$. For each sampling time, one can expand the logarithm of $g^{(1)}(\tau)$ as

$$\ln g^{(1)}(\tau) = -\bar{\Gamma}\tau + \frac{K_2}{2\bar{\Gamma}^2}(\bar{\Gamma}\tau)^2 \quad (2)$$

The mean value of D can be deduced from the average $\bar{\Gamma}$ and the 'polydispersity factor' $K_2/\bar{\Gamma}^2$ gives the width of the distribution around $\bar{\Gamma}$.

Quasielastic light scattering measurements are made with a self-beating spectrometer. The light source is an Ar^+ Spectra Physics model 165 laser (wavelength 5145 Å). The sample is placed in a cylindrical cell immersed in an index matching liquid bath thermostated and the scattered light is observed with an EMI 9863 KB 100 photomultiplier tube. The scattering direction was determined by a system of two pinholes. The electrical signal is amplified and fed into a discriminator connected to a 64-channel K 7025 Malvern multidigital correlator. The autocorrelation function is then computed in a multibit mode and analyzed by using the method of cumulants on a CBM 3032 computer. The apparatus was calibrated with a 0.005 $g \cdot l^{-1}$ aqueous solution of polystyrene latex of 1090 Å in diameter. The accuracy is better than 1%.

Results and Discussion

Electron microscopy observation of unilamellar properties

Fig. 1 gives some features of the LUV prepared by the reverse-phase evaporation method. From the freeze fracture we can check that our large vesicles are almost unilamellar. It should be noted that in our figures obtained from the Philips EM 300 microscope, none of our vesicles is multilamellar. This quality is very important for the computation of the elastic modulus in a following section.

Variation of the apparent translational diffusion coefficient, D_a, versus ionic strength

The apparent variation of D_a of DMPC and DOPC large unilamellar vesicles at a temperature in the liquid phase versus the salinity, C_s, of the external medium is plotted as D_a curves in Fig. 2a and 3a. To vary the salinity, volumes of buffer without salt were added so that there was simultaneously a variation of the lipid concentration, C. These two quantities are represented on the abscissa. The possibility of a concentration dependence of the translational diffusion coefficient was well known in micelles [30], in emulsions [32] and in vesicles [16]. Hence, to extract the true effect of NaCl we studied simultaneously and in the same

Fig. 1. Electron micrograph of freeze-fractured preparations of DMPC large vesicles prepared by the reverse-phase evaporation method. From these micrographs the unilamellar property of the studied vesicles was controlled.

way the effect of lipid concentration by adding volumes of buffer containing 150 mM NaCl, i.e., the same salinity as in the vesicles solution. The pure concentration effect can be seen from the D_a^c curves of Figs. 2b and 3b.

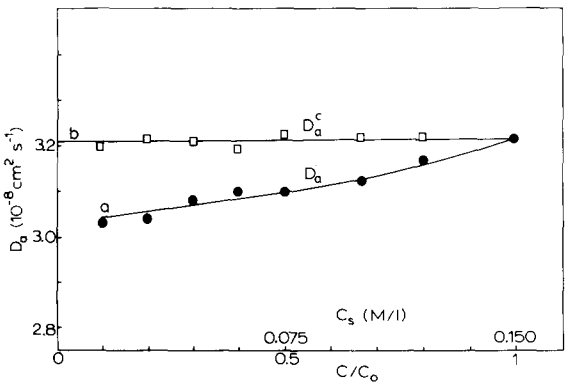

Fig. 2. Variations of the apparent translational diffusion coefficient D_a^c and D_a of DMPC large unilamellar vesicles versus (a) salt dilution (●) and (b) pure lipid dilution (□). All buffers were at 33°C (pH 8.3) and C/C_0 is the relative concentration. Each point is an average of ten measurements.

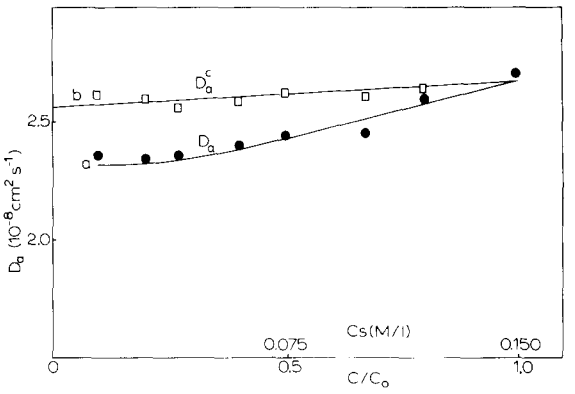

Fig. 3. Variations of the apparent translational diffusion coefficient D_a^c and D_a of DOPC large unilamellar vesicles versus (a) salt dilution (●) and (b) pure lipid dilution (□). All buffers were at 33°C (pH 8.3) and C/C_0 is the relative concentration with respect to the initial concentration. Each point is an average of ten measurements.

Hydrodynamic radii and osmotic effect

The lipid concentration dependence of D_a^c reflects the effect of interactions between particles. Hence, the true self-translation diffusion coefficient, $D_{a_0}^c$, can be obtained only by using extrapolation to infinite dilution ($c \to 0$). The Stokes-Einstein relation is then used to determine the hydrodynamic radius, r_2, of the particles

$$D_{a_0} = k_B T/6\pi\eta r_2$$

where k_B is the Boltzmann constant and η the viscosity of the external phase at the temperature T.

The values of r_2 obtained in this way are given in Table I where one can also find the thickness of the bilayer at a temperature higher than the transition point. To determine the radii at various salinities, we proceeded as follows: taking the value of D_a at a given salinity, we know the corresponding lipid concentration, C; the curve (b) gave the value of D_a^c which would be the diffusion coefficient at the initial salinity (150 mM NaCl). On comparison with D_a^c, we obtain the diffusion coefficient D_a^c at the actual salinity and in extreme dilution

$$D_{a_0} = D_a \frac{D_{a_0}^c}{D_a^c} \qquad (3)$$

TABLE I

EXTERNAL SIZE OF DIMYRISTOYL- (SATURATED) AND DIOLEOYL- (UNSATURATED) PHOSPHATIDYLCHOLINE LARGE UNILAMELLAR VESICLES

The size was determined by the quasielastic light scattering method followed by extrapolation to zero concentration (Fig. 1b and Fig. 2b). Vesicles were prepared by the reverse-phase evaporation method. Buffer: 150 mM NaCl/10 mM Tris-HCl/0.02% NaN_3 (pH 8.3). The thickness values were taken from the literature (Ref. 26 for DMPC and Ref. 42 for DOPC). The accuracy of measurements is about 1%.

LUV	DMPC at 33°C	DOPC at 20°C
External radius (Å)	932	835
External area (10^{-21} m^2)	1.09	0.876
Thickness (Å)	34	42

For small vesicles, (data not shown) there is no difference between the D_a and D_a^c curves, D_{a_0} is constant and there is no observable swelling in diluting salt. Conversely, the situation is not the same with large vesicles. A decrease of D_{a_0} is well observed when diluting the salt of the external medium and, therefore, an osmotic response is evident.

The elasticity of the phospholipid vesicles

Theoretical background. From a theoretical point of view, when a hollow sphere of internal radius r_1, external radius r_2 and of which the constituent has an elastic rigidity (Young modulus) E, and a Poisson coefficient ν, is submitted to a stress generated by an internal pressure p_1 and a different external pressure p_2, any point at the distance r from the centre is displaced by a quantity U (given in Refs. 36 and 37)

$$EU = \frac{p_1 r_1^3 - p_2 r_2^3}{r_2^3 - r_1^3}(1-2\nu)r + (p_1 - p_2)\frac{r_1^3 \cdot r_2^3}{r_2^3 - r_1^3}\frac{1+\nu}{2r^2} \qquad (4)$$

For soft materials the value of ν can be taken as 0.5 and if the thickness e, of the hollow sphere is small in comparison with the radii, one can suppose that the displacement U is Δr_2 for every point of the hollow sphere, the formula can then be reduced to approximately

$$E = (p_1 - p_2)\frac{r_2^2(1-2e/r_2)}{4e\Delta r_2} \qquad (5)$$

Now in an osmotic problem, according to the Van't Hoff law [38] the osmotic pressure depends on the concentration of osmotic active molecules, p_1 varies with the internal volume and if there is leakage of these constituents by permeability of salt through the membrane, the determination of p_1 is somewhat complicated because the loss depends on time t and on the concentration C_{s2}, of the external phase [39]. Taking into account these considerations and assuming that (i) the inside salt concentration, C_{s1}, decreases following the law

$$dC_{s1}/dt = -k(C_{s1} - C_{s2}) \qquad (6)$$

during the leakage, (ii) the osmotic pressure p, is simply $p = 2RTC_s$ proportional to the concentration of salt and (iii) the osmotic response is much faster than the leakage, i.e., the radius of the vesicle varies immediately when a concentration gradient $(C_{s0} - C_{s2})$ is established at time $t = 0$ before the leakage takes place, this variation implies a modified value of C_{s0} into

$$C_{s10} \simeq C_{s0}\left(1 - 3\frac{\Delta r}{r}\right) \qquad (7)$$

and at any time t, during the leakage one has

$$C_{s1} - C_{s2} = (C_{s1} - C_{s2})_0 \times \exp(-kt) = (C_{s10} - C_{s2})\exp(-kt) \qquad (8)$$

The value of C_{s20} is practically unaffected by the leakage because of the very small volume of vesicles in comparison with the external volume. In these circumstances, according to the relation of Eqn. 5, the Young modulus E, is then calculated from the strain $(\Delta r_2/r_{20})$ measured at time t_1 by the formula

$$E = 2RT\frac{r_{20}(1-2e/r_{20})}{4e}\left[(C_{s0} - C_{s2})\exp(-kt_1)\frac{\Delta r_2}{r_{20}} - 3C_{s0}\right] \qquad (9)$$

where r_{20} is the external radius before swelling, C_{s0} the internal salt concentration just before the external salinity was changed to C_{s2}. This change induced an immediate swelling which modifies the value of C_{s0} to C_{s10}, this correction is represented by the term $3C_{s0}$, in brackets. Moreover we have

assumed that the thickness, e, of the bilayer was not altered by the swelling.

Note that after time t_1, the internal salt concentration, C_{s1}, becomes $C_{s1}(t_1)$ which will play the role of C_{s0} when a new dilution C_{s2} is made. In our experiments it took 22.5 min.

The elastic modulus of phospholipid membranes. The most ambiguous question is that of the value of salt loss through the phospholipid bilayers. The data given in the literature are not yet adequate because the loss depends not only on the nature of the lipid but also on the temperature [40] and on whether the vesicles are uni- or multilamellar. For some phospholipids when there is no pore or transporter agents as drugs (gramicidin, amphotericin B etc.) the leakage of Na^+ and Cl^- was found to be very weak [41–43]. This is the case with egg phosphatidylcholine or dioleoylphosphatidylcholine [44,39], the order of k is 10^{-7} s^{-1} so, even after 1 day ($t = 10^5$ s), the loss of salt concentration is only 1%. However, Singer [40] reported a more drastic leakage in other pure synthetic lipid liposomes. According to this author, the leakage is maximum at the temperature of transition and, in most cases, the loss diminishes when the liposomes are brought far from the transition point. The loss, after 3 h, of Na^+ in DMPC liposomes was given as a function of temperature and can be used in our computation. We have estimated a value $4.5 \cdot 10^{-4}$ s^{-1} for k at 33°C. For DOPC vesicles, the measurements were made at 20°C, very far from the transition point (−22°C) the loss can thus be assumed as approximately the same as at 4°C and we used the value $1.4 \cdot 10^{-7}$ s^{-1} of k at 4°C reported by Schwarz [42].

Concerning the thickness of the bilayers, many values were reported for the liquid phase of the lipids at various temperatures and determined by various methods [4–6,26,42,44,45]. Our choices and references are given in Table I. With these values of k and of the thickness, the Young moduli of DMPC and DOPC large unilamellar vesicles were computed and are represented in Fig. 4.

It is emphasized that the elastic modulus, E, defined as above is somewhat a 'state' constant which depends on the state of the membrane. This point was made by Parsegian and co-workers in a study of the compressibility of plane bilayers [24].

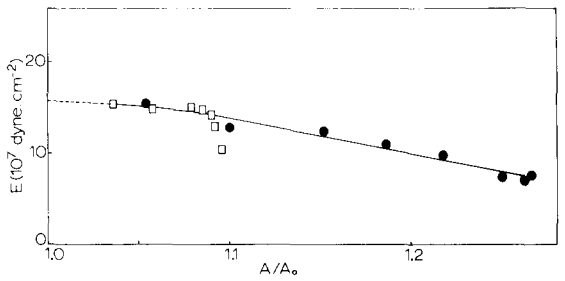

Fig. 4. Values of the elastic moduli of two phosphatidylcholine large unilamellar vesicles in buffers (pH 8.3) in various states of swelling (□) DMPC and (●) DOPC. The abscissa represents the ratio of the vesicle area, A, relative to the equilibrium area, A_0, when the osmotic pressures inside and outside are equal. The extrapolation to $A/A_0 = 1$ will give the elastic moduli at equilibrium. The range of size variation is narrower for DMPC vesicles than for DOPC vesicles, probably due to the more important salt leakage in DMPC lipid.

Therefore, we have represented different values of E corresponding to various 'instantaneous vesicles areas' relative to the initial area under osmotic equilibrium (Fig. 4). The figure shows that by extrapolation to $A/A_0 = 1$, the value of E for DMPC and DOPC large vesicles prepared in the same conditions by reverse phase evaporation are in the order of $1.5 \cdot 10^8$ dyn · cm^{-2}. Table II situates this order in comparison with the results obtained from other kinds of bilayer prepared with some other lipidic materials and measured by different methods. Whereas there is a pronounced difference with the cell elastic modulus observed by Katchalsky et al. [10] or with that deduced from electric breaking down of planar glycerol monooleate bilayers measured by Alvarez et al. [18], the agreement is rather good as compared with the results for cell membrane obtained by Rand [11], Waugh and Evans [12–13] and for pure DPPC planar bilayers [17]. In particular, it is interesting to emphasize the good agreement of our results with the elastic moduli of vesicles prepared from DMPC or phosphatidylcholine observed by Kwok and Evans by micropipet aspiration, a quite different technique [7–9]. Their elastic area compressibility moduli can be converted in terms of the Young moduli, whose value is about $3.5 \cdot 10^8$ dyn · cm^{-2}. If there is a small difference between our results and theirs, we think that is due to the difference in curvatures and to

TABLE II

ELASTIC MODULI OF SOME BILAYERS

Lipid	Bilayer characteristics	Method	Area compressibility elastic modulus, K (dyn/cm)	Young elastic modulus, E(dyn/cm^2)	Ref.	Remarks
DMPC	vesicles	micropipet	125	$3.7 \cdot 10^8$ [a]	7,8	size: 15 μm,
Lecithin	vesicles	aspiration	140	$3.5 \cdot 10^8$ [a]	9	thickness $d = 40$ Å
DMPC	vesicles	osmotic		$1.5 \cdot 10^8$	Present	size 1600–
DOPC	vesicles	swelling			work	1800 Å liquid phase
Red blood cell	cell	micropipet	450	$4.5 \cdot 10^8$ [a]	12	$d = 100$ Å
Red blood cell	cell	aspiration	95	$3 \cdot 10^8$ [a]	13	
Red blood cell	cell			$10^6 - 3 \cdot 10^8$	11	
Erythrocyte cell	cell	Osmotic swelling		$2.4 \cdot 10^7$	10	
DPPC	planar bilayer	lateral pressure	18	$5.3 \cdot 10^7$	17	
GMO (glycerol monooleate)	planar bilayer	electric breaking down		$1.4 \cdot 10^9$	18	
GMO in n-octane	black lipid membrane	Electric breaking down		$1 \cdot 10^8$	19	

[a] value obtained by using the relation $K = Ed$, d is the thickness of the bilayer [3].

some extent to the permeability given in the literature and chosen for the computation. In a general context, the condition of preparation, the temperature (above or below the transition point) and the curvature would be important parameters influencing the elastic properties of the bilayers [46].

The values of E decrease slightly when the vesicle area increases by swelling. We noted that when using compression to decrease the plane bilayer area, the elastic modulus increases as observed by Parsegian et al. [24]. Moreover we observed a variation of vesicle area as important as 25% (DOPC vesicles) without noticeable breaking. The swelling in DMPC vesicles under the same salt concentration conditions is less pronounced. This may be due to the fact that the leakage in DMPC is more important than in DOPC vesicles. The temperature at which the measurements were made is very much farther from the transition point in DOPC than in DMPC vesicles which should be in a state where there are more dislocations. Another possible cause is that oleoyl tails each contain a 9-double bond so that adjacent unsaturated acyl chains interact more strongly than saturated chains [47] and, thus, DOPC vesicles resist better to the variation of the distance in the bilayer plane, i.e., to the variation of the vesicles area.

In summary, using the quasielastic light scattering technique we have obtained an accurate determination of the osmotic response and estimated the elastic moduli of large unilamellar phosphotidylcholine vesicles. The non destructive character and the quickness of the measurement are important in the investigation of biological materials.

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