Mechanisms for determining the time scales in vesicle budding

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(Received 29 November 1993)

Approximate calculations show that the dynamics of the shape transitions observed in vesicle budding upon increasing the temperature as well as during recooling cannot be explained based on friction alone. Two additional mechanisms, (a) lateral segregation of lipid impurities and (b) van der Waals attraction of bud and mother vesicle, appear to be involved. Experiments are suggested to test the mechanism of lateral segregation.

PACS number(s): 87.22.Bt, 47.15.Gf

I. INTRODUCTION

An increase in surface area and a smooth change in shape (from a sphere to a pear) was observed when the temperature of monolamellar giant vesicles made of lecithins with two saturated hydrocarbon chains [1] was slowly increased. At a certain temperature the vesicle was unstable in that the neck of the pear spontaneously shrank to submicroscopic diameter connecting the mother vesicle and a smaller bud. We call this spontaneous process budding. Upon decrease of the temperature a hysteresis was observed in that the shapes were different from those at the same temperatures during heating. When the recooling was performed slowly (on the order of 10 °C/h) the shape remained budded in most experiments with the bud continuously shrinking in diameter [2]. In a minority of these experiments an oscillation between an almost complete opening and a reformation of the neck occurred [2]. This kind of oscillation was consistently observed [1] upon "fast" recooling (about 1 °C/min).

A theoretical description of these effects should be based on a model in which single-layer bending as well as bilayer-couple bending [3] contribute to the elastic energy. Since the membrane is essentially symmetric its spontaneous curvature in single-layer bending may be approximated by zero. Such a model, called here the elastic bilayer-couple model, has been presented [4,5] and spontaneous transitions were predicted. Käs *et al.* [6], however, claimed that only a third-order term in bilayer-couple bending could explain the experimental findings. Further, van der Waals attraction of mother vesicle and bud has been suggested to play a role [6,7]. As a third mechanism a lateral segregation of lipid impurities was invoked [8]. In all models the dynamics of the spontaneous closure of the neck as well as of its opening were not considered.

This work was undertaken because it was felt that these processes are slower than expected on the basis of friction alone and that lateral diffusion may be responsible for the observed time scale. To support or reject these contentions characteristic times of vesicle shape changes are determined approximately and compared to the observed values. It is then proposed that in addition to the elastic bilayer-couple model both van der Waals interaction and lateral segregation of lipids are needed to explain the observations.

II. ESTIMATES

We account for three frictional contributions opposing the shape changes: (i) shear deformation of the lipid bilayer, (ii) shear flow of the liquids adjacent to the bilayer, and (iii) drag between the two lipid monolayers. In one case bending of the lipid bilayer is also considered. For an approximate determination of the respective power dissipation simplified kinematics are chosen which resemble the observed shape changes.

A. Spontaneous budding upon heating

1. Shape change

Figure 1 shows the geometry on which the estimate is based. Two spherical surfaces (radius R_m for the mother vesicle and radius R_b for the bud) are connected by a circular cylinder the radius (ρ) of which shrinks linearly with time:

$$\rho(t)=R_n\left[1-\frac{t}{t_{\mathrm{NC}}}\right] . \tag{1}$$

R_n is the radius of the cylindrical neck at the beginning of the spontaneous transition and t_{NC} is the time of neck closure. By comparison with Fig. 3(5) of Käs and Sackmann [1] we take $R_m=12$ μm, $R_b=5$ μm, and $R_m=4$ μm. The length (H) of the cylinder is chosen so as to make the total surface area of the vesicle equal to that of two complete spheres of radii R_m and R_b:

$$H(t)=h_m(t)+h_b(t) , \tag{2}$$

where

$$h_m(t)=\frac{R_m a_m(t)}{\rho(t)} , \tag{3}$$

and

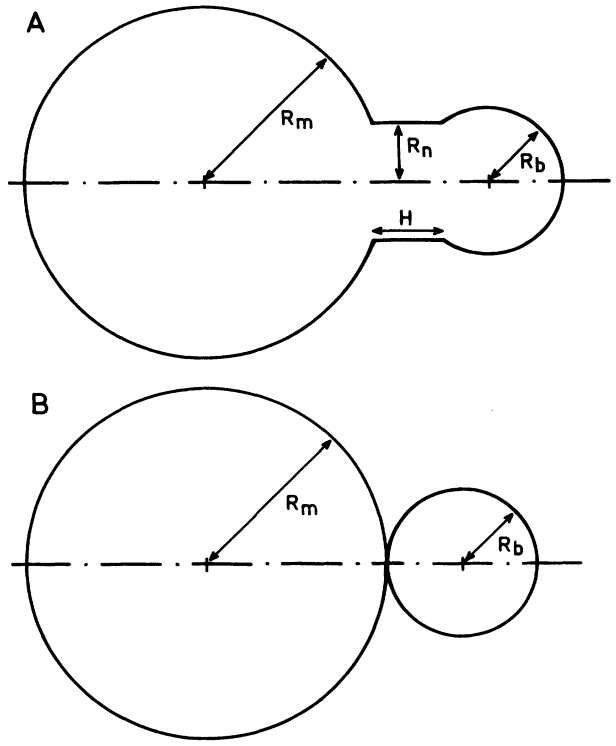

FIG. 1. Simplified geometry assumed for the spontaneous transition of a pear shaped vesicle to two buds connected by a submicroscopic neck; (A) shape before transition; (B) budded shape.

$$a_m(t)=R_m-\sqrt{R_m^2-\rho(t)^2}\ . \tag{4}$$

h_b and a_b are obtained by replacing the index m by the index b in Eqs. (3) and (4).

2. *Shear of the bilayer*

Two dimensional (2D) shear takes place in the cylindrical part of the membrane only. The power dissipation (P_l) due to shear of the lipid bilayer is a function of time. From Evans and Skalak [9] we get

$$P_l=4\eta_l(\dot{\rho}/\rho)^2 2\pi\rho H\ . \tag{5}$$

η_l is the surface viscosity for which we adopt from Waugh [10] a value of 5×10^{-6} dyn s/cm. The dissipated energy (E_l) is obtained by integration of P_l from zero to $t_{\rm NC}$. We get $E_l=2.1\times10^{-11}$ erg s/$t_{\rm NC}$.

3. *Shear flow of liquids*

3D shear takes place practically everywhere inside and outside of the vesicle. We subdivide the space into four regions. In each we use rather crude approximations which, however, preserve the typical character of the flow. Because of this crudeness it does not matter that our vesicle geometry does not preserve the vesicle volume.

First we consider the flow within the cylinder. We introduce cylindrical coordinates with the z axis along the cylinder axis. The positive direction is into the bud. $z=0$ is chosen in such a way that the cylindrical surface and the spherical surfaces meet at $z=h_b(t)$ and $z=-h_m(t)$, respectively. For later use we conceptually divide the vesicle in two parts: the bud part ($z>0$) and the mother part ($z<0$). The flow velocity is split up in a purely radial (v_r) and a purely axial (v_z) component. For v_z a parabolic velocity profile is assumed. We set $v_z=0$ at $z=0$ and obtain from continuity

$$v_z=-\frac{4\dot{\rho}z}{\rho^3}(\rho^2-r^2) \tag{6}$$

and

$$v_r=\frac{\dot{\rho}r^3}{\rho^3}\ . \tag{7}$$

From the derivatives of these velocity components we get after volume integration (in r direction from 0 to ρ and in z direction from 0 to h_b) the power dissipation (P_{w1}) in the bud part:

$$P_{w1}=\frac{4}{3}\pi\eta_w\dot{\rho}^2\left[13h_b+8\frac{h_b^3}{\rho^2}\right], \tag{8}$$

where η_w is the viscosity of water. The total dissipation is obtained by adding the analogous contribution from the mother part. Integrating the sum from zero to $t_{\rm NC}$ yields the dissipated energy $E_{w1}=2.2\times10^{-11}$ erg s/$t_{\rm NC}$.

Second, we account for the flow in the body of the mother or bud, respectively. We approximate it by the flow of a point source in a wall. In spherical coordinates with the z axis normal to the wall and the origin at the location of the source, the radial velocity v_r is obtained from Happel and Brenner [11]:

$$v_r=\frac{3Q\cos^2\theta}{2\pi r^2}\ , \tag{9}$$

where θ is the angle measured from the z axis and Q is the flow emanating from the source. The other velocity components are zero [11]. In calculating the power dissipation (P_{w2}) the integration is carried from ρ to infinity. We get

$$P_{w2}=\frac{14}{5\pi}\frac{Q^2\eta_w}{\rho^3}\ . \tag{10}$$

After integration versus time we obtain the dissipated energy. The contributions from bud and mother add up to $E_{w2}=0.6\times10^{-11}$ erg s/$t_{\rm NC}$.

The flow within the spherical caps on both sides of the cylinder is assumed to continue in axial direction in the same fashion as it leaves the cylinder. We obtain for the dissipated energy $E_{w3}=1.0\times10^{-11}$ erg s/$t_{\rm NC}$.

Outside of the vesicle we use the flow of an infinitely long line sink. From continuity we get in cylindrical coordinates

$$v_r=\frac{\dot{\rho}\rho}{r} \tag{11}$$

the other velocity components being zero. To obtain the power dissipation we integrate in r direction from ρ to

infinity and in z direction from 0 to H. Integration versus time yields $E_{w4}=0.4\times10^{-11}$ erg s/t_{NC}.

4. Interlayer drag

It is obvious that interlayer slip always lags the shape change. We consider two extreme cases: (1) interlayer slip is as fast as the shape change (quasistatic equilibrium), and (2) interlayer slip is much slower than the shape change. We first treat quasistatic equilibrium. The direction of the z axis and the position $z=0$ are defined as in the beginning of Sec. II A 3. We denote the difference in actual surface area between the neutral surface of the bilayer and the neutral surface of the outer monolayer by γ. We call the distance of these surfaces δ and obtain to first order in δ for the bud part

$$\gamma_b=2\pi\delta\left[\left(\frac{R_b}{\rho}-2\right)a_b+4R_b\right] . \tag{12}$$

The value for the total vesicle is obtained by adding the contribution γ_m from the mother part. The time derivative ($\dot\gamma$) of this sum turns out to be positive for $t=0$, and to decrease almost linearly to a negative value at $t=t_{NC}$.

The total surface area of the neutral surface of the bilayer is constant (Sec. II A 1). Therefore a negative value of $\dot\gamma$ corresponds to an increase in surface area of the outer monolayer. We assume this increase to emanate from a line source on the surface of the cylinder at $z=0$ and to flow to both sides in proportion to the appended surface areas. We call the respective flows $\dot\gamma_b$ and $\dot\gamma_m$. Because of symmetry the flow velocity is along the surface generator on the cylindrical and along the meridians on the spherical surface. To preserve global bending the flow velocity at any value (z_a) of z must be proportional to the surface area beyond that value ($|z|>|z_a|$). This condition determines v_z on the cylindrical part and v_θ on the spherical part of the surface. We obtain in the bud part

$$v_z=\frac{\dot\gamma_b}{2\pi\rho}\left[1-\frac{z\rho}{2R_b^2}\right] \tag{13}$$

in cylindrical coordinates and

$$v_\theta=\frac{\dot\gamma_b}{4\pi R_b}\frac{1+\cos\theta}{\sin\theta} \tag{14}$$

in spherical coordinates (with $z=0$ at the center of the spherical surfaces and $\theta=0$ in the middle of the neck). The power (P_h) dissipated in hydrocarbon slip is obtained by integration over the surface

$$P_h=2b_h\int v^2 dA , \tag{15}$$

where the interfacial drag coefficient $b_h=5\times10^7$ dyn s/cm^3 is taken from Evans *et al.* [12]. v denotes the velocity of the outer layer which is equal and opposite to that of the inner layer. Integration versus time yields the dissipated energy (for the first case): $E_{h1}=0.1\times10^{-11}$ erg s/t_{NC} [13].

We now treat the case where shape change and interlayer slip are sequential events. For a conservative estimate (upper bound) of the dissipated energy we choose 1 nm for the neck radius in the budded state, the smallest possible value. The equilibration of the imbalance in surface area is assumed to occur with constant rate:

$$\langle\dot\gamma\rangle=\frac{\gamma(t_{NC})-\gamma(0)}{t_{IS}} . \tag{16}$$

Here, t_{IS} is the characteristic time of interlayer slip. Substituting $\langle\dot\gamma\rangle$ for $\dot\gamma$ in Eqs. (13)–(15) we obtain the dissipated energy: $E_{h2}=0.3\times10^{-12}$ erg s/t_{IS}. The numerical part is much smaller than that of E_{h1}. For an explanation remember that due to the zero passage of $\dot\gamma$ its integrated square is much larger than that of $\langle\dot\gamma\rangle$.

5. Characteristic times

To calculate characteristic times we need the elastic energy set free during the transition. We call the difference in energy between the (static) states before and after the transition ΔE_{tb}. The index t relates to "total" as opposed to partial quantities considered below. The index b indicates that the released energy is mainly stored in bending. Numerical results for ΔE_{tb} in the elastic bilayer-couple model are not available. For this reason we use $\Delta E_{tb}=\pi k_c$ as obtained from the spontaneous-curvature model [14]. We also disregard an influence of lateral segregation on ΔE_{tb}. k_c denotes the constant of bending elasticity of the bilayer. A typical value for lecithins with saturated hydrocarbon chains is 0.7×10^{-12} dyn cm [15]. It is in the middle of the wide range of published values determined from shape fluctuations and compares well with the values determined from tether pulling [16].

The energy dissipated in the flows (2D in the lipid phase and 3D in the water phase) is obtained by adding up the respective contributions:

$$E_f=E_l+E_{w1}+E_{w2}+E_{w3}+E_{w4} . \tag{17}$$

In the first case (quasistatic equilibrium) t_{NC} is obtained from

$$\Delta E_{tb}=E_f+E_{h1} . \tag{18}$$

The friction due to bending of the lipid monolayers in neglected in Eq. (18). This is motivated by the results in Sec. II B 2. Substituting the numbers in Eq. (18) we obtain $t_{NC}=29$ s. This is the same order of magnitude as the observed value of 50 s [6].

The second case (successive occurrence of shape change and interlayer slip) requires that a spontaneous transition to a budded shape occurs without interlayer slip; i.e., the budded shape would be determined by a minimum in elastic energy stored in single-layer bending and *local* [3] bilayer-couple bending [17]. We denote the drop in energy that drives this (hypothetical) shape change by ΔE_{SC}. Its characteristic time is obtained from

$$\Delta E_{SC}=E_f . \tag{19}$$

The rest of the energy ($\Delta E_{IS}=\Delta E_{tb}-\Delta E_{SC}$) would be dissipated in interlayer slip with the vesicle shape not changing. Its characteristic time being much longer than

that of the shape change is equivalent to

$$\frac{E_{h2}t_{IS}}{\Delta E_{IS}} \gg \frac{E_f t_{NC}}{\Delta E_{SC}} . \tag{20}$$

According to our precondition ΔE_{IS} and ΔE_{SC} have the same sign. We therefore may rearrange and obtain

$$\frac{\Delta E_{SC}}{\Delta E_{IS}} \gg \frac{E_f t_{NC}}{E_{h2} t_{IS}} = 200 . \tag{21}$$

Using $E_f/E_{h1}=60$ and inequality (21) we can appreciate that the relative error made by calculating t_{NC} with Eq. (18) instead of Eq. (19) is less than $\frac{1}{60}$.

An estimate of the characteristic time of interlayer slip cannot be given. This would require the knowledge of ΔE_{IS} [3,18].

B. Neck opening upon recooling

1. Shape change

Two characteristic times, a fast and a slow phase of neck opening, can be appreciated from the graph in Fig. 2. The microscopic pictures show that in the fast phase the neck retains its wasp-waist shape and increases its diameter to almost the maximum value. In the successive slow phase, on the other hand, the neck becomes longer and changes from the wasp-waist shape to a much smoother, almost cylindrical shape.

The duration of the fast phase cannot be precisely measured since the neck is not perfectly in focus during the opening. Watching the videotape [19] in slow motion shows that most of the opening occurs within $\frac{3}{2}$ to 2 videoframes; i.e., within 60 to 80 ms. The time course of the shape change in the slow phase is quantified in Fig. 3. Its duration is between 2 and 3 s.

After the last opening the neck remains open. The fast and slow phases are similar to those in the transitory openings. It can be seen in Fig. 2 of Käs *et al.* [6] that after completion of the slow phase the neck becomes thicker and finally loses its constricted appearance. We will refer to this shape change as "neck disappearance." Experimentally a duration of 50 s was reported for the last opening phase [6].

2. Energy dissipation

For the fast phase we first use the analysis of Sec. II A. Qualitatively the initial and final shapes are as in Figs. 1(B) and 1(A), respectively. The values for the actual geometrical quantities are taken from Fig. 5(3) of Käs and Sackmann [1]: $R_n=2.3$ μm, $R_b=5$ μm, and $R_m=12$ μm, where R_n is now the radius of the cylinder at the end of the fast opening phase. Assuming that interlayer slip is in quasistatic equilibrium with the shape change we obtain $E_f+E_{h1}=1.4\times10^{-11}$ erg s$/t_{FO}$, where t_{FO} is the duration of the fast opening phase. For later use we note that $E_l=6.7\times10^{-12}$ erg s$/t_{FO}$.

Actually, the geometry of the neck (wasp waist) is quite different from the (cylindrical) shape used for this estimate. Since we will argue in Sec. III A that the characteristic times calculated based on friction alone are too small, we have to take care that our estimates of the dissipated energy are not too large. To this end we consider a geometry that starts from the same initial to a more appropriate final shape. In the following we describe the kinematics of the mother part in a cylindrical coordinate system as shown in Fig. 4. The initial ($t \le 0$) contour is spherical:

$$\rho_e = \sqrt{R_m^2 - z^2} , \tag{22}$$

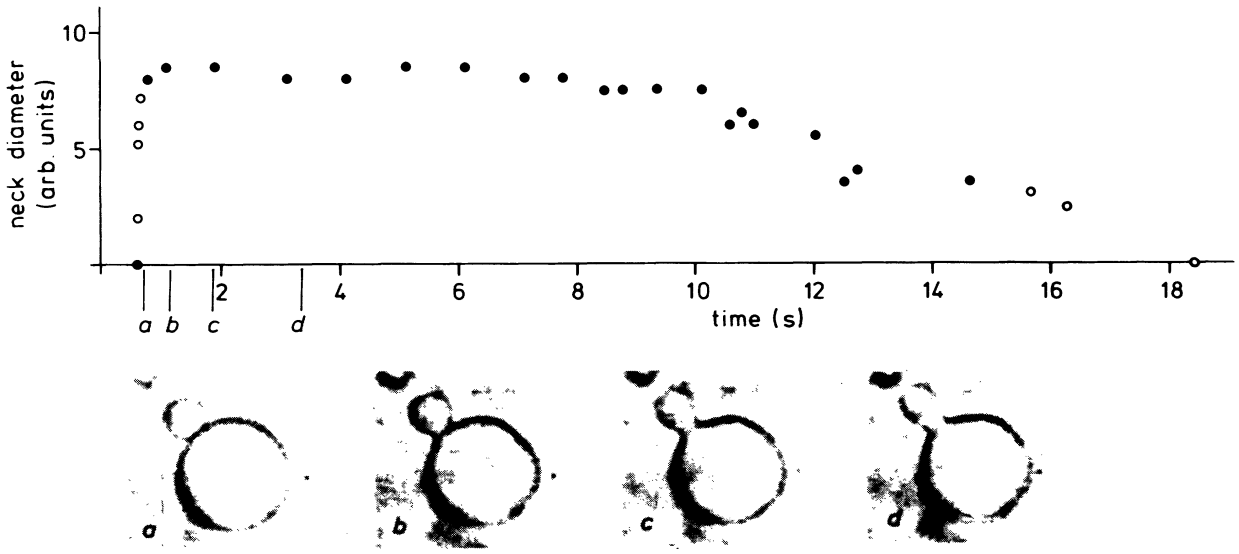

FIG. 2. Neck diameter vs time in a transitory opening of the neck [19]. The sphere enclosing the same volume has a diameter of 46 arbitrary units. Typical diameters are 25–30 μm [1]. Open symbols indicate that the measurement is uncertain because the neck was not in focus. The vesicle shape at selected times (indicated at the abscissa by *a*, *b*, *c*, *d*) is shown in micrographs.

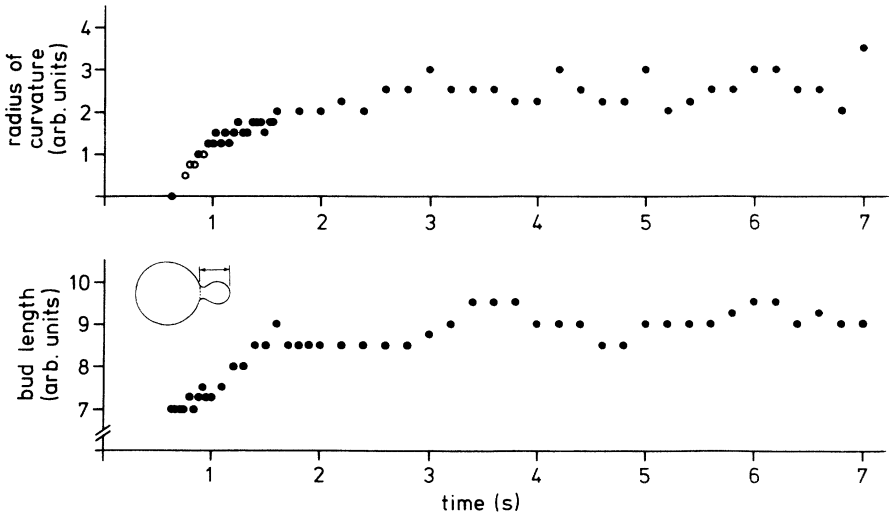

FIG. 3. Minimal meridional radius of curvature in the neck and length of the bud vs time in the same transitory opening as in Fig. 2 [19]. Open symbols indicate that the measurement is uncertain because the neck was not in focus. The definition of bud length is shown schematically.

where the index e relates to the observation (see Sec. II B 3) that the membrane is extended. For $0 \leq z < R_m$ the final (at $t = t_{FO}$) contour is given by

$$\rho_s = \rho_e + (R_m - \rho_e) R_n / R_m \ , \tag{23}$$

where the index s is derived from the observation (see Sec. II B 3) that the membrane is slack. For $z \leq 0$ the contour is not assumed to change; i.e., $\rho_s = \rho_e$. For $0 < t < t_{FO}$ the radial coordinate is assumed to change with constant velocity from ρ_e to ρ_s:

$$\rho(t) = \rho_e - (\rho_e - \rho_s) \frac{t}{t_{FO}} \ . \tag{24}$$

This deformation is within a few percent area conserving. With the origin of the coordinate system in the center of the bud and the positive z direction toward the neck, the description of the bud part is analogous. The contour of the whole vesicle at $t \leq 0$ and $t = t_{FO}$ is shown in Figs. 4(A) and 4(B), respectively. The power dissipation (P_l) due to shear deformation of the bilayer reads

$$P_l = 4\eta_l \int_0^{R_m} \left[\frac{\dot{\rho}}{\rho} \right]^2 2\pi R_m \, dz \ . \tag{25}$$

After adding the expression for the bud part and integration versus time we obtain for the dissipated energy $E_l = 10.8 \times 10^{-12}$ erg s$/t_{FO}$. Since this is appreciably larger than the estimate based on the geometry of Fig. 1 no other frictional contribution was estimated.

The slow phase is characterized by an elongation of the neck and a smoothing of the circular fold. For an approximate analysis of the 2D shear deformation we take as the initial shape that shown in Fig. 4(B) and assume the final shape of both bud and mother part to be composed of a spherical segment and a tangent truncated cone. The radii of the spherical segments are $f_m R_m$ and $f_b R_b$, and that of the neck is $f_n R_n$, where f_m, f_b, and f_n are numerical factors. The altitude of the cones follows from the condition of constant surface area. According to the pictures in Fig. 2 we choose $f_m = f_n = 1$ and $f_b = 0.89$. The corresponding shape is shown in Fig. 4(C). Its radial coordinate is called ρ_u as derived from "unfolded." Based on the condition of equal surface area we express ρ_u by the z coordinate of the corresponding location on the spherical shape shown in Fig. 4(A). The power dissipation is then given by Eq. (25). Since ρ_u and ρ_s are not very different we use for simplicity

$$\frac{\dot{\rho}}{\rho} = \frac{2(\rho_u - \rho_s)}{(\rho_u + \rho_s) t_{SO}} \ , \tag{26}$$

where t_{SO} is the duration of the slow opening phase. Substituting the numbers we obtain: $E_l = 4.3 \times 10^{-12}$ erg s$/t_{SO}$.

To account for the change in membrane curvature (in the slow phase) we consider the lipid monolayers as slabs (thickness $d/2$) of an isotropic material with a viscosity $\eta_h = 1$ dyn s/cm^2. This value is adopted from the so called microviscosity of the hydrocarbon moiety as obtained from spectroscopic measurements. To simplify the actual geometry we assume that a folded piece of such a slab becomes plane. The length of the fold is $2\pi R_n$ and the initial radius of curvature is χ. The dissipated energy is given by

$$E_{mb} = \frac{\pi^2}{48} \frac{\gamma_h d^3 R_n}{\chi t_{SO}} \ , \tag{27}$$

where the indices m and b indicate "monolayer bending." For a conservative estimate we substitute for χ the lowest values possible; i.e., 1 nm for the outer and 3 nm for the inner monolayer of vesicle membrane. We obtain for the energy dissipated in the bilayer 4×10^{-17} erg s$/t_{SO}$. This is neglected against E_l (of the slow opening phase).

For the neck disappearance we take the shapes shown in Figs. 4(C) and 4(D) as the initial and final shapes, respectively. The final shape is assumed to be composed of spherical segments and cones as well, however, $f_b = f_m = 1$ and $f_n = R_b / R_n$. The treatment is analogous to that of the slow phase. We obtain $E_l = 9.1 \times 10^{-12}$ erg s$/t_{ND}$, where t_{ND} is the duration of the neck disappearance.

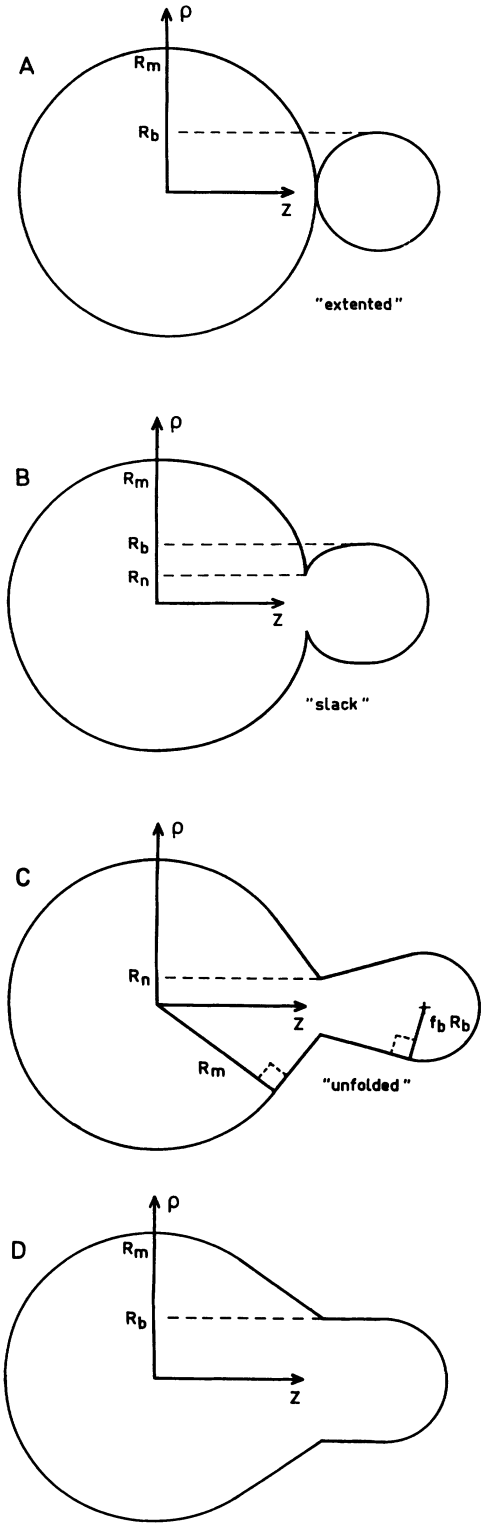

FIG. 4. Simplified geometry assumed for the opening of the neck; (A) budded shape before the transition where the membrane is extended due to isotropic tension; (B) shape after the fast phase of neck opening, the membrane is slack again; (C) shape after the slow phase of neck opening, the circular fold is smoothed out; (D) final shape after the last opening. The constriction of the neck is lost.

3. *Elastic energies*

For most of the time during recooling, membrane undulations are visible under phase contrast but disappear prior to the neck openings [6]. This indicates an increase in isotropic membrane tension (σ). Under reflection interference contrast which has a higher microscopic resolution, the undulations are visible during the whole experiment [2]. This shows that even the maximum isotropic tensions are caused by membrane undulations.

It can be appreciated from the videotape [19] that the undulations as observed under phase contrast reappear right after the fast phase. This indicates that the isotropic tension has returned to its low value. We denote the energy released in this relaxation by ΔE_{IT} and calculate following Käs *et al.* [6]

$$\Delta E_{\mathrm{IT}} = A \int_{\alpha_s}^{\alpha_e} \sigma \, d\alpha \ . \tag{28}$$

Here, A is the surface area of the vesicle and α denotes the relative decrease in projected surface area due to membrane undulations. The indices denote, as above, "extended" and "slack," respectively. Please note that α is always negative. The expression for σ is adopted from Helfrich and Servuss [20]. Substitution into Eq. (28) and integration yields

$$\Delta E_{\mathrm{IT}} = \frac{\pi kT}{8}\left(e^{8\pi k_c \Delta\alpha/kT} - 1\right)\left[1 + \frac{\sigma_s A}{\pi^2 k_c}\right] - \pi^2 k_c \Delta\alpha \ . \tag{29}$$

Here, kT denotes the thermal energy and $\Delta\alpha = \alpha_e - \alpha_s$. According to Käs *et al.* [6] $\Delta\alpha = 6.5\times10^{-3}$. By σ_s we denote the isotropic membrane tension prevailing when $\alpha = \alpha_s$. We take $\sigma_s = 2\times10^{-5}$ dyn/cm, the geometric mean of the range found by Kummrow and Helfrich [21] for unstressed vesicles. From Fig. 5 of Käs and Sackmann [1] we deduce $A = 2040\ \mu\mathrm{m}^2$ and obtain, from Eq. (29), $\Delta E_{\mathrm{IT}} = 14\times10^{-12}$ erg [22].

For the consecutive slow phase we use the same estimate as in Sec. II A 5 ($\Delta E_{tb} = 2.2\times10^{-12}$ erg). For the neck disappearance we use $\Delta E_{tb}/3$, a choice which is motivated by comparison to calculations of Seifert, Berndl, and Lipowksy [14] in the spontaneous-curvature model.

4. *Characteristic times*

From the equation $\Delta E_{\mathrm{IT}} = E_f + E_{h1}$ we obtain for the fast phase of neck opening $t_{\mathrm{FO}} = 1$ s. This is much larger than the observed value.

For the slow phase we calculated the energy dissipated in 2D shear only. To account for the other contributions we multiply by a factor 3, as adopted from the estimates made in Sec. II A. Equating this value with ΔE_{tb} gives for the slow phase of neck opening $t_{\mathrm{SO}} = 6$ s. This is the same order of magnitude as the observed value.

For the last phase we follow the same procedure and obtain $t_{\mathrm{ND}} = 37$ s. This is also the same order of magnitude as the observed value.

III. DISCUSSION

A. Deviation of estimates from experimental values

Our estimates for the duration of the first closure of the neck, the slow opening phase, and the neck disappearance seem to be in accordance with the experimental values (Sec. II A 5 and II B 4). The estimate for the fast opening phase, on the other hand, is larger by an order of magnitude than the experimental value. As an explanation for this discrepancy we propose the following.

To calculate the contribution of lipid shear to the energy dissipation we used a surface viscosity which has been specified as an upper bound of the actual value because of possible multilamellarity of the vesicles tested [10]. Unfortunately there are no other measurements. As an independent estimate [23] may serve: $\eta_h d = 4\times10^{-7}$ dyn s/cm, which is an order of magnitude below the value used in our estimate.

As for the contribution of water flow, an appreciable overestimate is not unlikely since the shear rates enter quadratically into the expression for the power dissipation and the real flows are certainly smoother than the ones assumed in our estimates [24].

For consistency we consider the estimates for the duration of the first closure of the neck, the slow opening phase, and the neck disappearance to be too large by an order of magnitude as well. This destroys the accordance with experimental observations as noted above. For this reason we suggest that the velocity of these processes is not determined by friction but by lateral diffusion of lipids in the plane of the membrane.

B. Hypothesis of lateral segregation

The hypothesis is based on the fact that commercial lipid preparations inevitably contain impurities. We propose the membrane in the budded shape to be heterogeneous in that certain kinds of these impurities are enriched in the neck region whereas in the nonbudded shape the membrane is assumed to be essentially homogeneous. In the following we refer to those impurities that become enriched as heterolipids. The lipid nominally used to prepare the vesicles is referred to as the principal lipid.

1. Origin and concentration of impurities

At first sight one might ask why synthesized lipids should not be pure. We quote two possibilities: (a) The substances used to synthesize the lipids are typically 99% pure, and (b) the synthesized lipids may degrade, e.g., by spontaneous hydrolysis.

An appreciable enrichment is only possible if the area fraction of the neck region is on the same order of magnitude as the mole fraction of the envisaged heterolipids. The neck diameter, when closed, is below optical resolution. The diffraction band of the membrane in the phase contrast pictures of Käs and Sackmann [1] is at minimum 0.6 μm wide. This means the surface area of a closed neck is smaller than $4\pi(0.3\ \mu\text{m})^2 \approx 1\ \mu\text{m}^2$. Consequently the upper bound for the ratio of the surface area of the neck region to that of the whole vesicle is 0.5×10^{-4}. At maximum the local mole fraction of heterolipids in the neck region is unity. This would require the global mole fraction to be 0.5×10^{-4}, which is in accordance with the occurrence of impurities.

2. Comparison to experiments

The mean distance (Δs) of heterolipids from a location in a plane at which they were concentrated at time $t=0$ is calculated according to

$$\Delta s = \sqrt{4Dt_d}\ . \tag{30}$$

Here, t_d is the diffusion time and D the diffusion constant for which we use the value 10^{-8} cm^2/s. For the first closure of the neck, which occurred during heating) we postulated the opposite process: the concentration of heterolipids at a location in a plane. Applying nevertheless Eq. (30) and substituting 50 s (the experimentally observed duration of the shape change) for t_d, we obtain $\Delta s = 14$ μm. This distance defines a drainage area for heterolipids into the neck region. In keeping with the hypothesis, it is smaller than typical vesicle dimensions. At face value, it indicates that an appreciable portion of all heterolipids in the vesicle membrane moves to the neck region.

As for the slow phase of neck opening we suggest its duration to be determined by a partial randomization of the heterolipids. Supposing that immediately after the opening they are concentrated in a band at the narrowest position of the neck we may estimate the widening of this band from Eq. (30). For $t_d = 2-3$ s we obtain $\Delta s \approx 3$ μm, which is in accordance with the observed neck dimensions.

A time interval of 20 s [6] was determined experimentally between a typical transitory opening of the neck and the successive closure. After subtraction of 2–3 s for elongation of the neck a time interval of 17–18 s remains for the closure. If, as suggested above, the distribution of the heterolipids at the beginning of neck closure is far from random, a smaller diffusion time than that calculated for closure during heating is indeed expected.

As to the neck disappearance, please note that this shape change occurred at constant temperature [6], i.e., at constant surface area. We suggest its duration to be determined by a randomization of the heterolipids over the whole vesicle surface. This is in accordance with the observation that the duration was the same as for the first closure of the neck.

3. Equilibrium distribution of impurities

We now ask what kind of mechanism might cause the postulated enrichment. We first discuss the decrease in elastic energy concomitant to a lateral segregation of heterolipids. For a convenient treatment of a heterogeneous membrane we use the deviatoric curvature ($\hat{c}$) instead of the Gaussian curvature [25]. In the neck region we neglect the mean curvature ($\bar{c}_n$) and assume $\hat{c}_n$ to be constant. Then the elastic energy density (e_n) reads [25]

$$e_n = 2\left\{\frac{k_c}{1.5}(0.5+2\tilde{\xi}\delta)\right\}\hat{c}_n^2 \ . \tag{31}$$

The expression enclosed by curly brackets constitutes an effective bending stiffness. We use this expression because it covers lipid species which, if present alone, would not form bilayers. Again, k_c is a typical bending stiffness of a lecithin with saturated hydrocarbon chains. As above we use the value 0.7×10^{-12} dyn cm. We divide this value by 1.5 because only the isotropic share of k_c enters here. According to Fischer [25] we use a value of 0.5 for the ratio of deviatoric to isotropic bending stiffness. $\tilde{\xi}$ denotes the spontaneous curvature of the single monolayers [26]. The more negative $\tilde{\xi}$, the smaller the effective bending stiffness. A value of zero is obtained at $\tilde{\xi}=-1/(4\text{ nm})$. We show that such values are not unreasonable.

Fitting of elastic constants of symmetric bilayers to a model accounting for the actual and spontaneous curvatures of the constituent monolayers gave values around $-1/(5$ nm) for polyunsaturated phosphatidylcholines [25]. Measurement of radii in the inverted hexagonal [27] or inverted micellar [28] phase gave values around $-1/(3$ nm) for phosphatidylethanolamines. Modei calculations of headgroup volumes indicate that the spontaneous monolayer curvature of diacylglycerol is still more negative [29].

We conjecture that the average molecular shape of the envisaged heterolipids is such that a hypothetical pure monolayer would have a strongly negative spontaneous monolayer curvature. The formation of the neck might then support a local enrichment of these molecules and vice versa. To calculate the energy gain per segregated heterolipid would require one to consider the time course of the strain distribution in the whole vesicle membrane during budding. For a rough approximation we assume that $e_n=0$ in the heterogeneous membrane and that the same neck is formed in the homogeneous membrane.

The homogeneous membrane consists basically of principal lipid. As such Käs and co-workers [1,6] used lecithins with saturated hydrocarbon chains. Their average shape is considered to be cylindrical [30]. We therefore substitute $\tilde{\xi}=0$ in Eq. (31). To calculate the elastic energy (ε_n) per molecule we multiply by 0.65 nm^2, a typical cross sectional area, divide by 2, the number of monolayers, and obtain $\varepsilon_n=k_c\hat{c}_n^2/6$. For a strong enrichment against the randomizing influence of the thermal energy ε_n should be five to ten times larger than kT. This in turn would require $\hat{c}_n$ to assume values which are not possible in a monolayer 2 nm thick.

We therefore conjecture that besides lateral distribution according to curvature preference, a molecular interaction exists, in that above a threshold value in deviatoric curvature the heterolipids attract each other. A similar mechanism was suggested by Seifert [31]. As mentioned, above, the transient from the essentially homogeneous to the heterogeneous membrane might be characterized by positive feedback between the change in membrane curvature and the segregation of heterolipids.

In equilibrium we expect the threshold curvature to separate a region in the neck in which the concentration of heterolipids is much larger than in the rest of the vesicle. This region may be stabilized by a line tension as introduced by Lipowski [32].

4. Additional forces

Equation (30) describes the average motion of heterolipids with time due to a gradient in their concentration. The additional force on a heterolipid due to a gradient in membrane curvature is not accounted for. To compare both forces rigorously would require study of the transients in shape change. For an approximate account we consider the budded shape as observed during heating and estimate the drop in energy ($\Delta\varepsilon$) upon lateral translocation of one heterolipid from the poles of the vesicle to a location just outside of the region in which the heterolipids are concentrated. We characterize these locations by indices p and j, respectively.

We denote the contribution which depends on the concentration of heterolipids by $\Delta\varepsilon_k$. The index k derives from the German spelling of concentration. For infinite dilution we obtain

$$\Delta\varepsilon_k = kT\ln(k_j/k_p) \ , \tag{32}$$

where k denotes the concentration of heterolipids. According to our hypothesis the neck region acts as a sink for heterolipids which in turn leads to a (negative) gradient in concentration of heterolipids going from the poles of the vesicle toward the neck. The ratio k_j/k_p is not known. It is, however, likely that it amounts to more than one order of magnitude. For a conservative estimate we use $k_j/k_p=0.1$ and obtain $\Delta\varepsilon_k=-10^{-13}$ erg.

We then consider the contribution that depends on the deformation of lipids due to a deviation of their spontaneous monolayer curvatures ($\tilde{\xi}$) from the actual curvature (c) of the membrane. We first estimate the increase in elastic energy ($\delta\varepsilon$) due to insertion of one molecule of heterolipid into a monolayer of the principal lipid. The drop in energy upon translocation of one heterolipid is then calculated according to $\Delta\varepsilon_c=\delta\varepsilon_j-\delta\varepsilon_p$. Here, the index c derives from "curvature."

The evaluation of $\delta\varepsilon$ is not trivial. If the values of $\tilde{\xi}$ for heterolipid and principal lipid are different an upper bound is obtained by assuming that each lipid species is deformed as if it were present alone. The real increase in elastic energy is lower because of a compensation in deformation between the two kinds of lipids.

We denote the spontaneous monolayer curvature of heterolipids by $\tilde{\xi}_i$, where the index is derived from "impurity." For a conservative estimate we use an extreme value: $\tilde{\xi}_i=-1/(2$ nm). At the poles of the vesicle we neglect $|c_p|$ against $|\tilde{\xi}_i|$ and obtain an estimate of the upper bound:

$$\delta\varepsilon_p = k_c(0.65\text{ nm}^2)\tilde{\xi}_i^2/1.5 \ . \tag{33}$$

To calculate the analogous contribution ($\delta\varepsilon_j$) next to the region of segregated heterolipids requires the knowledge of the local values of the mean curvature ($\bar{c}_j$) and the deviatoric curvature ($\hat{c}_j$). Except for the very unlikely case

that $\bar{c}_j$ and/or $\hat{c}_j$ are on the order of $|\tilde{\xi}_i|$ we may neglect $\delta\varepsilon_j$ against $\delta\varepsilon_p$ and obtain $\Delta\varepsilon_c \approx -\delta\varepsilon_p = -1.5\times10^{-13}$ erg.

Despite the conservative character of the estimate this is of the same order of magnitude as $\Delta\varepsilon_k$. Therefore including the driving force from elasticity would not severely change the values obtained from Eq. (30). In particular they would remain appreciably larger than those predicted based on friction alone. For the slow opening phase and the neck disappearance the reasoning would be similar.

5. *Comparison to theoretical models with a homogeneous membrane*

Based on the elastic bilayer-couple model Miao *et al.* [5] found discontinuous as well as continuous budding [33] when a temperature increase was modeled. For realistic values [34] of elastic constants discontinuous budding was found to be dominant. Below the temperature at which the (discontinuous) budding transition was predicted to take place, the budded configuration was found to have a lower energy than the nonbudded configuration and the energy barrier between both states was higher than thermal energies [5].

To bring our concept into accord with this model requires that the energy barrier is lowered in our potentially heterogeneous membrane so that the spontaneous transition from the metastable shape to the budded configuration takes place at temperatures below that predicted in a homogeneous membrane. Otherwise the shape change would be as fast as friction would allow and a segregation of lipids would not slow down the shape change. We will come back to this point in Sec. IV.

Besides the discrepancy between expected and observed time scales which is the main interest of this paper we present in Fig. 5 additional evidence for an inhomogeneous membrane. The pictures show the vesicle at selected times after the same transitory neck opening as in Fig. 2. It is striking that for about half a minute, mother and bud appear spherical but are separated by 2–3 arbitrary units which by comparison to published pictures [1] translate to about 1 μm. The diameter of the structure connecting mother and bud is below the resolution of the light microscope. Topologically, it must be like a cylinder connecting mother and bud since the neck opens again at about 5 min on the time scale of Fig. 5. A slender structure connecting two spheres is at stake to the predictions based on a homogeneous membrane [7,14,5] and supports our hypothesis of lateral segregation. In particular, a line tension, as mentioned in Sec. II B 3, might be involved.

One minute after neck opening the distance between mother and bud becomes submicroscopically small, probably due to the decrease in surface area.

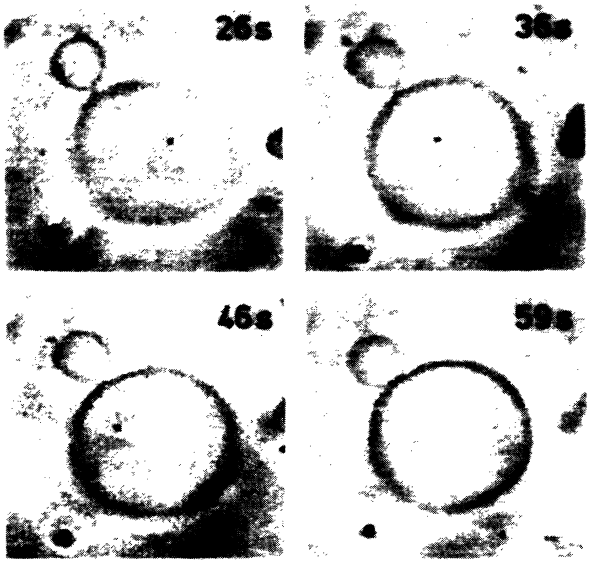

FIG. 5. Vesicle shape [19] after a transitory opening of the neck showing a distance between mother and bud. The time scale continues that in Fig. 2. Typical vesicle diameters are 25–30 μm [1].

C. Role of van der Waals interation

In Sec. II B 3 we calculated a value of ΔE_{it} much larger than Käs *et al.* [6]. This appears to question the evidence presented by these authors for the role of van der Waals interaction. We first argue that the assumption of Käs *et al.* [6] as to this role remains justified. Second, we suggest that the nice accordance with experimental results found by Käs *et al.* [6] is due to the compensation of two errors.

During slow recooling the neck (radius ρ) remained submicroscopically small in the majority of vesicles tested [2]. This can be explained by a dynamic equilibrium between the decrease in surface area and a volume flow of bud interior and a surface flow of bud membrane to the mother. We suggest these flows to be driven by the bending moments (in both single-layer and bilayer-couple bending) and isotropic tensions in the membranes of bud and mother.

Upon fast recooling all vesicles showed transitory neck openings. However, to study these openings with greater resolution Käs [2] switched from continuous (fast) cooling to a temperature decrease in steps of 0.1 °C. Each step lasted 5 min when the shape fluctuations as observed under phase contrast became visibly smaller and 15 min when shape fluctuations were no longer perceptible by eye. This corresponds to a minimum cooling rate of 0.4 °C/h which is much smaller than the slow (continuous) cooling rate (10 °C/h) and therefore shows that the neck radius (ρ_e) just prior to the transitory neck opening must have been much smaller than in dynamic equilibrium. Otherwise the neck would not have opened up. Further, the neck diameter decreased again to submicroscopic values although the temperature was further decreased. Both findings call for an additional force which for $\rho < \rho_e$ is responsible for reducing the neck radius below the value in dynamic equilibrium and which disappears at $\rho = \rho_e$.

The van der Waals force between the two closely apposed spherical surfaces of mother and bud is a good candidate for the additional force because it has a small range which is likely to be exceeded while the neck diam-

eter is submicroscopically small. For $\rho < \rho_e$ the neck radius would then be determined by a balance of forces from membrane elasticity and van der Waals attraction. At $\rho = \rho_e$ the membrane would spontaneously approach a new equilibrium shape according to bending and isotropic elasticity of the membrane alone. This would explain why the neck opens (a) so quickly and (b) to such a large diameter.

As for the second point, Käs *et al.* [6] argued that the neck would open when the elastic energy stored in the membrane due to the increased isotropic tension becomes equal to the energy of the van der Waals interaction and showed that estimates of both quantities are equal. Such an energy compensation principle requires that the elastic energy stored in isotropic tension is exclusively spent to increase the distance between mother and bud. This is at least not obvious. We conjecture that this is the second error which compensated the first one noted in Sec. II B 3.

A puzzling question remains to be addressed. Why, in the majority of cases during slow (continuous) recooling, did van der Waals interaction not have the effect postulated during fast (continuous) recooling although the isotropic tension in the membrane must have been lower. As an explanation we propose the steric repulsion between fluctuating membranes to be larger during slow recooling than during fast recooling, thus keeping mother and bud at a distance larger than the range of the van der Waals attraction.

IV. SUGGESTION OF EXPERIMENTS

The hypothesis of lateral segregation could be tested by the following experiments. We suggest adding diacylglycerol (DAG) to the lipid used to prepare vesicles. Above a threshold value in molar fraction of the added molecules this might decrease the drainage area and consequently the duration of neck closure during heating. By a similar argument a decrease in the duration of the other diffusion-limited processes can be rationalized.

It was argued in Sec. III B 5 that the presence of heterolipids should lower the energy barrier between the metastable and the stable vesicle shape. Therefore above another threshold value in molar function of DAG a decrease in the temperature of the spontaneous transition during heating might be observable.

Besides adding DAG other lipids characterized by $\tilde{\xi} < 0$ could be used for doping. This might show the influence of molecular shape and/or interaction of these molecules.

A change in the principal lipid is expected to change the observations as well. Using lipids with increasing unsaturation would decrease the difference in spontaneous monolayer curvature between added lipids and the principal lipid species. This should decrease all effects of lateral segregation.

ACKNOWLEDGMENTS

I am indebted to the following persons: Dr. J. Käs, TU Munich, for communicating unpublished details of his experimental work, for sending a typescript of his paper before publication, and for providing a video recording of a typical experiment; Dr. C. W. M. Haest, RWTH Aachen and Dr. U. Seifert, KFA Jülich, Germany, for helpful discussions; and Dr. U. Seifert in addition for sending a typescript of his paper before publication.

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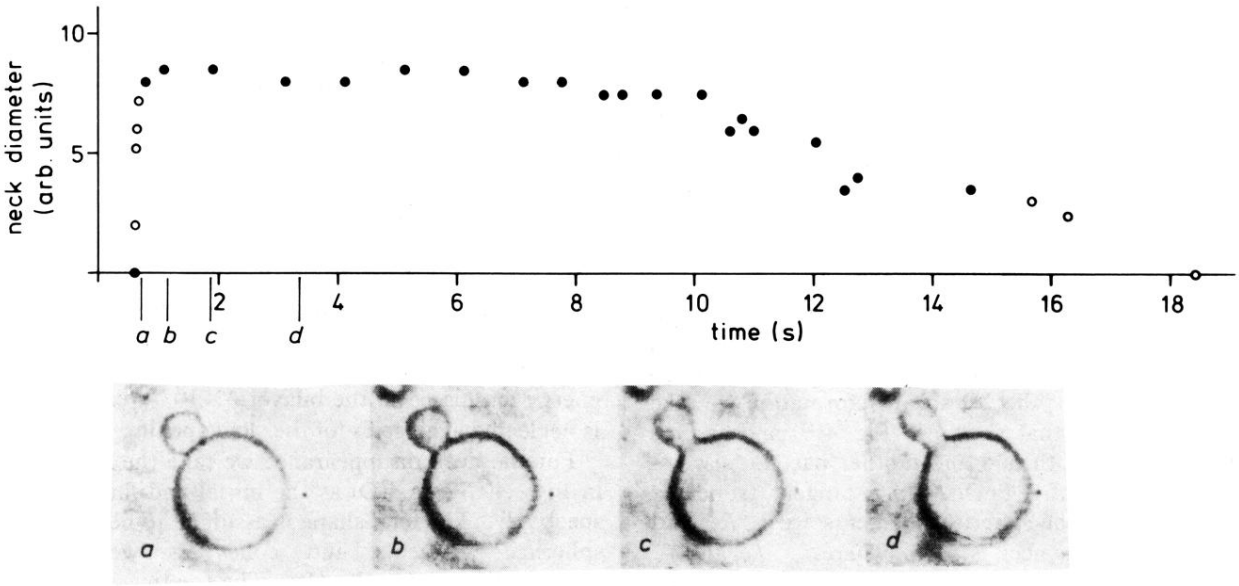

FIG. 2. Neck diameter vs time in a transitory opening of the neck [19]. The sphere enclosing the same volume has a diameter of 46 arbitrary units. Typical diameters are 25–30 μm [1]. Open symbols indicate that the measurement is uncertain because the neck was not in focus. The vesicle shape at selected times (indicated at the abscissa by *a*, *b*, *c*, *d*) is shown in micrographs.

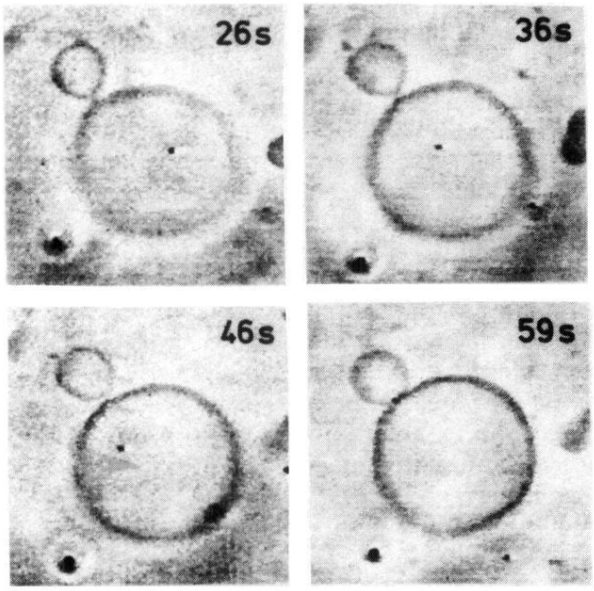

FIG. 5. Vesicle shape [19] after a transitory opening of the neck showing a distance between mother and bud. The time scale continues that in Fig. 2. Typical vesicle diameters are 25–30 μm [1].