

# Material Transport Across Permeability Barriers by Means of Lipid Vesicles

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## 1. Introduction

Nature is full of barriers. It was not before the formation of the first membraneous sacks that the self-replicating and catalytic molecules encapsulated in such vesicles have started to act as primitive creatures. The reason for this is that membranes present a barrier to the free diffusion of water soluble substances and thus enforce material localization. The very limited diffusion of the membraneous bodies through the partly filled space plays an important role in this as well.

Primordial membranes must have emerged from the pools of ubiquitous simple lipids which have been reported to exist even in the interstellar space. Most lipids being nearly insoluble in water, they tend to form membranes spontaneously. This is true for all basic amphiphiles<sup>1</sup> of the biological membranes at least, with the solubilities between  $10^{-10}$  and  $10^{-6}$  moles per litre. Biogenic lipids thus normally do not dissolve but rather disperse in the aqueous systems in the form of extended aggregates [1].

The most common form of the polar lipid aggregates in water are bilayers. These consist of two opposing hydrocarbon monolayers separated from the surrounding water by two layers of the polar lipid headgroups. It is this membrane core and the two interfaces which act as a diffusion barrier and/or as biochemical catalytic sites, depending on the type of the molecules added. Bilayers chiefly form closed spherules, so-called liposomes, with a radius much greater than the lipid dimensions, owing to the monolayer packing constraints [2, 3]. In the thermodynamic equilibrium vesicle radii are normally rather large,  $r_v \gtrsim 40$  nm.

Only the most polar lipids are soluble in water at concentrations higher than  $10^{-5}$  and up to  $10^{-2}$  moles per litre. When this solubility limit is exceeded, however, even the most hydrophilic lipids aggregate spontaneously. The resulting lipid micelles normally contain just a few dozen of molecules [5], by and large. Micellar shape depends on the precise distribution of the polar residues on each individual molecule [4]. Most lipid micelles are spherical or disk-like (cf. fig. 1) and correspondingly small. Their surface, consequently, is at least locally highly curved ( $r_m \lesssim 5$  nm) and resembles the 'edge' of an open lipid bilayer.

Addition of large amounts of the highly water-soluble lipids to the suspension of less polar lipids solubilizes the closed lipid bilayer. Lipid solubilization normally proceeds via the formation of mixed-micelles and other types of mixed aggregates [7]. The solubilization-inducing lipids, consequently, are also called surfactants or detergents, to stress this fact.

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<sup>1</sup> From the Greek, *amphi*, on both ends, and *philos*, loving; this is, containing a hydrophilic and a hydrophobic end.

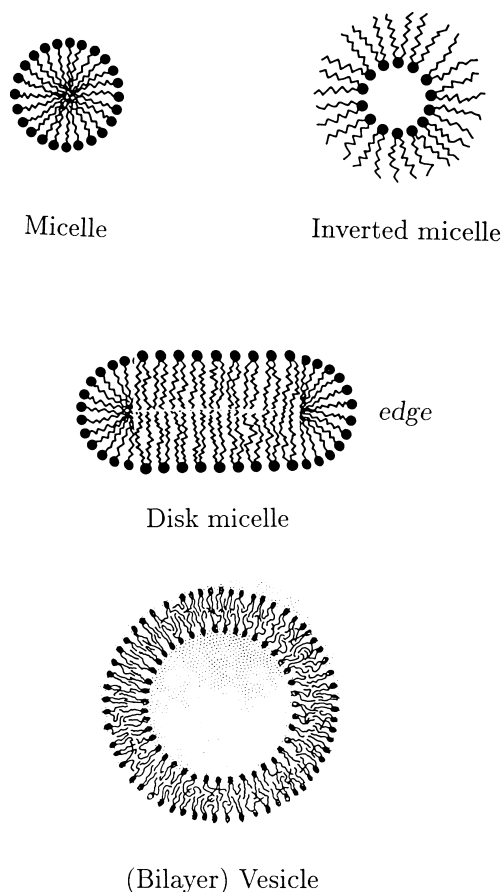


Fig. 1. Schematic representation of various types of lipid aggregates.

## 2. Lipid bilayers and vesicles

Lamellar lipid structures either contain a stack of multi-bilayers or a large number of single lipid bilayers in the form of closed vesicles, liposomes [8]. These are useful as a model of the biological membranes; they are also a convenient vehicle for the delivery of various agents in many systems (see the contribution of D.D. Lasic in this volume).

In order to be good agent carriers liposomes should fulfill several conditions [12]. Perhaps most importantly, liposomes should carry a high pay-load and ensure a well controlled agent-release or agent-transfer rate; some site-specificity is also often desirable. Liposomes for the biological applications, moreover, should be biocompatible and commercially affordable. This explains the preferred use of the biogenic lipids in many (bio)technological liposome applications; this is also the reason for the multi-component character of many common liposome preparations.

Agents, additives and stabilizers, or simply lipoidal impurities, as well as their effects on the properties of lipid vesicles, consequently, should be understood well before one tackles the problem of rational design of the useful lipid-derived agent carriers [13].

### 2.1. Mixed lipid bilayers

Different lipid molecules mixed in a single membrane will only form an uniform bilayer if their characteristics are reasonably compatible. Otherwise lateral phase separation will occur, sometimes in a time- and location-dependent manner [8].

Local composition and local curvature of lipid bilayers are normally coupled. Lateral non-uniformity in a lipid membrane may thus induce local membrane shape and vesicle shape transformations (see, e.g., [9–11]). The reverse is also true, of course: highly polar lipids with a relatively strong repulsion between the hydrophilic parts of the molecule tend to concentrate at the sites of high negative local curvature. This also pertains to all common surfactants, for example. Lipids with a predominantly hydrophobic character, on the contrary, are believed to accumulate in the membrane regions with a positive surface curvature or at the sites with a quasi-planar geometry. Enforced vesicle shape transformations in external gradients, therefore, are likely to go hand in hand with molecular rearrangements in the lipid bilayer. Both may change the characteristics of the entire lipid vesicle.

This can be understood qualitatively by considering the constraints that originate from the different effective molecular shapes of individual lipids (fig. 2) [2]. These shapes, as well as the resulting lipid organization in a bilayer, should always be treated in a self-consistent manner with allowance being done for the dynamic membrane effects.

### 2.2. Elasto-mechanic bilayer properties

The reason for this is that the elastic bilayer properties are governed by the system's capability to sustain or respond to a (dynamic) mechanical stress. The latter can vary along each molecule, between different membrane sites, and in the time.<sup>2</sup> Introduction of freely moving 'impurities' into a lipid bilayer therefore changes the elasto-mechanical properties of the resulting mixed lipid membrane [15]. This happens in a well defined and, to some extent, predictable manner.

To demonstrate this, one can consider the simplest elasto-mechanical description of the lipid membrane [16]. Bilayer free energy in such an approximation is taken to be proportional to the elastic membrane curvature modulus  $\kappa$  and to the squared sum of the principal membrane curvatures. The latter are identical to the corresponding inverse vesicle radii,  $C_i \equiv 1/r_{vi}$ . (For an ellipsoidal vesicle two such radii must be

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<sup>2</sup> With appropriate numerical procedures all characteristic elasto-mechanical parameters of the lipid bilayers, such as the volume and area compressibility moduli ( $K$  and  $K_A$ , respectively), the elastic and Gaussian curvature moduli ( $\kappa$  and  $\bar{\kappa}$ ), can be traced back to the (intra)bilayer stress profiles and their moments [14].

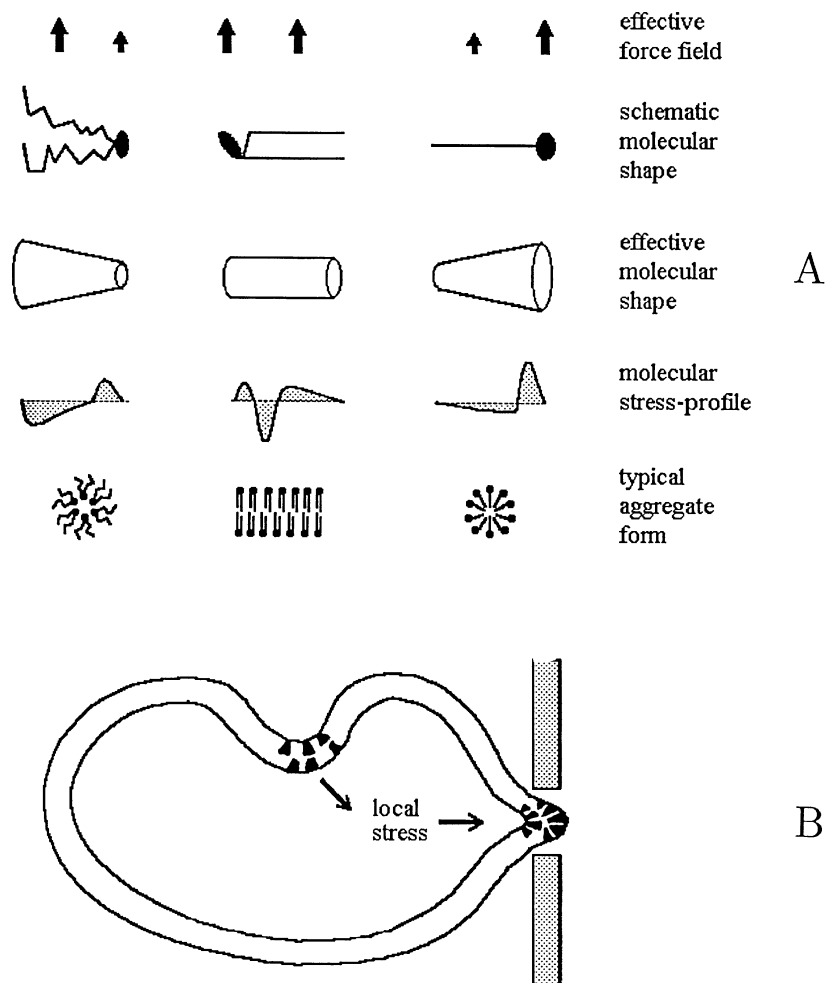


Fig. 2. A: Schematic representation of various types of lipids (second from top), with their main, mean effective force fields (top), effective molecular shapes (middle row), stress profiles (second from bottom) and typical aggregate forms (bottom); B: Schematic form of a vesicular lipid aggregate with the non-uniformly distributed membrane components at the sites of stress.

known and used,  $i = 1, 2$ .) This yields

$$G_{\text{uniform}}(\text{elastic}) = \frac{1}{2} \oint \kappa \left( \frac{1}{r_{v1}} + \frac{1}{r_{v2}} \right)^2 dA$$

where  $A$  is the vesicle area.

To obtain a related, approximate expression for the elastic energy of a two-component system one first should add an interaction term to the system's free

energy. In the simplest possible approximation this term can be written as [17]:

$$- \oint \Lambda_c \phi(s) [1/r_{v1}(s) + 1/r_{v2}(s)] dA.$$

It is taken to be proportional to the local density of the minor component  $\phi(s)$ , with  $s$  parametrizing the two-dimensional surface of the vesicle and the parameter  $\Lambda_c$  giving the strength of coupling between the local membrane composition and the local bilayer curvature. Furthermore, one should also allow for the intermolecular interactions in the mixed bilayers. In the simplest, Landau-like gradient expansion approximation, an expression of the form:

$$(1/2) \oint (a\phi(s)^2 + b|\nabla\phi(s)|^2) dA$$

is added to the free energy of the system [17],  $a$  and  $b$  being adjustable model parameters. This first yields

$$G_{\text{bi}}(\text{elastic}) = \frac{1}{2} \oint \left\{ \kappa \left( \frac{1}{r_{v1}(s)} + \frac{1}{r_{v2}(s)} \right)^2 - 2\Lambda_c \phi(s) \left( \frac{1}{r_{v1}(s)} + \frac{1}{r_{v2}(s)} \right) + a\phi(s)^2 + b|\nabla\phi(s)|^2 \right\} dA.$$

The partial summation over all  $\phi$ -configurations [17] then gives:

$$G_{\text{bi}}(\text{elastic}) \simeq \frac{1}{2} \oint \kappa \tilde{\delta} \left( \frac{1}{r_{v1}} + \frac{1}{r_{v2}} \right)^2 dA \quad (1)$$

with  $\tilde{\delta} \equiv 1 - \Lambda_c^2/a\kappa$ , on large scales. The elastic energy of a bi- or multi-component system can thus be mapped into the result for an uniform system [19].

Equation (1) suggests that the lateral membrane inhomogeneity changes the energetic cost of membrane bending, since  $\kappa$  is now replaced by  $\kappa\tilde{\delta} = \kappa - \Lambda_c^2/a$ . Mixed lipid bilayers thus may become extremely flexible, and form ultrasmall aggregates, if  $\Lambda_c^2/a$  becomes comparable to  $\kappa$ . This was inferred theoretically ( $\kappa\tilde{\delta} \rightarrow 0$  when  $\Lambda_c^2/a = \kappa$  [17]) without that it was known how such a situation could be achieved in practice. Based on the recent experimental data we have now postulated [18] that such a reduction of the membrane rigidity is particularly strong for the systems in which at least one membrane component has a strong preference for the highly curved surfaces. Metastable mixtures consisting of standard lipids and one or more edge-active membrane components (nearly) fulfill this condition. Consequently, such mixtures have a natural tendency to form ultraflexible membranes (see also further discussion).

Indeed, the recent experiments have shown that suitably optimized mixed and/or metastable lipid bilayers are potentially – but not necessarily – up to several orders of magnitude more flexible than standard or pure lipid membranes.

This can only be true locally, however, since the lipid vesicles would otherwise fall apart. The morphology of a typical lipid vesicle surface reflects this visually. In fig. 3A, for example, this surface is seen to be rather smooth and nearly spherical. This is certainly true for the standard unilamellar or bi-lamellar, relatively small liposomes, made of nearly pure phosphatidylcholine in the fluid lamellar phase, unless liposome aggregation has taken place. In contrast to this, the surfaces of mixed lipid vesicles consisting of phosphatidylcholine and bile salt are much less regular and spherical, when quenched from the fluid lamellar phase (cf. fig. 3B). Some vesicles even show well pronounced invaginations (see the arrows in fig. 3B). This suggests that bilayer surface for the latter type of lipid vesicles is excited more easily than for the former. Indeed, the elastic energy barrier for the bilayer surface fluctuations on the softened lipid vesicles has been estimated to be one to two orders of magnitude lower than for the standard liposomes. Whereas the average elastic energy of the optimized mixed vesicles is of the same order as thermal energy,  $\kappa \simeq kT$ , and locally may be much smaller than this,  $\kappa \ll kT$ , for the normal liposomes it is known that  $\kappa \geq 20kT$  [20]. This is also reflected in the corresponding spontaneous surface curvatures:  $r_{v0}(s) \ll r_{v0}$ .

Mixtures of the common polar and suitable edge-active lipids thus may form ultraflexible vesicles, so-called Transfersomes<sup>TM</sup> [18]. Such vesicles can attain much smaller local and even average radii of curvature than the standard, pure lipid, or non-optimized liposomes. This can be checked easily by measuring the equilibrium size of different populations of the lipid vesicles as a function of the concentration of the elasticity-increasing component in the system.

Vesicle radius at thermal equilibrium, in any such experiment, is observed first to decrease with the increasing relative concentration of any component with a higher capability to form or support edges. In this contribution such components are, consequently, called edge-active. Figure 4 illustrates this clearly for a series of phosphatidylcholine/bile-salt mixed membranes. At low concentrations of bile salt in the system the average value of the spontaneous vesicle curvature ( $\langle 1/r_{v0}(s) \rangle$ ) is smaller for the more highly doped membranes.

More specifically, from the data given in fig. 4 one concludes that a 10 w% phosphatidylcholine-bile salt mixture with a relative molar concentration ratio of approx. 3/1 should form particularly good transfersomes, since the radius of curvature of the vesicles made from such a mixture is at its minimum. Such lipid mixture is also close to, but not at, the phospholipid solubilization point. (For the soybean phosphatidylcholine vesicles solubilized by sodium cholate this point is reached at 1/1 mole/mole, as shown in fig. 4 by a vertical dotted line.) Transfersomal membranes which are relatively close to this transition point, consequently, are metastable to some extent.

### *Transfersomes*

Figure 4 defines one special class of transfersomes. For different types of lipids or edge-active substances or even for different lipid concentrations the flexibility

Fig. 3. Cryo-electron microscopic picture of a commercial 1% suspension of standard phosphatidylcholine vesicles (liposomes, A) and of the ultraflexible lipid vesicles (transfersomes, B) of comparable total concentration. Standard liposomes are normally spherical and have a rather smooth surface, owing to the high energy cost of their surface excitations. In contrast to this, the transfersomal surfaces are non-spherical and full of undulations or even invaginations (arrows), owing to the high flexibility of the transfersomal membranes. (Cryo-microscopy by courtesy of Natterman Phospholipids.)

optimum is found for different membrane compositions. But one can always say

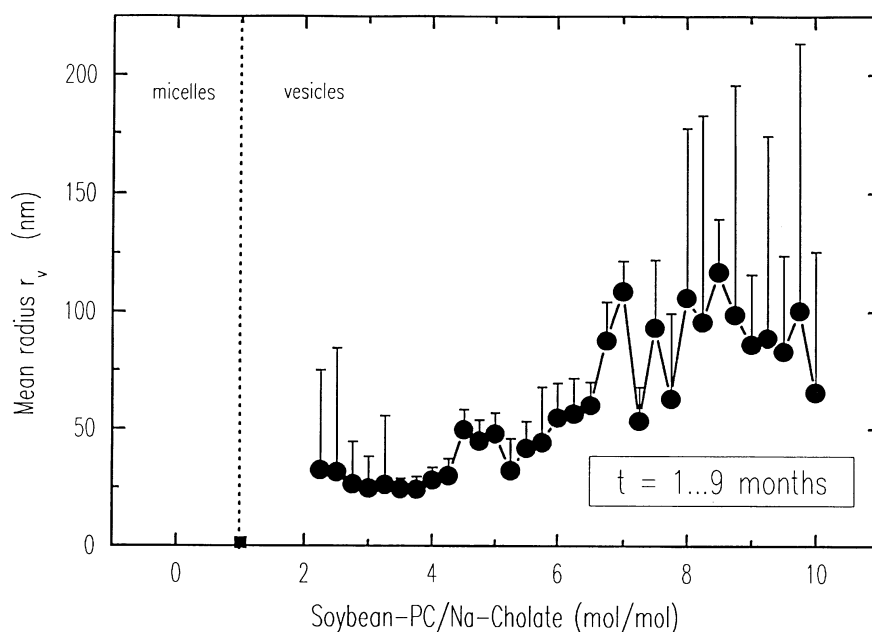


Fig. 4. Equilibrium radius of the mixed lipid vesicles made of phosphatidylcholine and sodium cholate as a function of the relative concentration of the latter, highly polar component. Increasing relative concentration of the edge-active component in the phospholipid bilayers reduces the energy cost for the membrane curving and thus diminishes the mean vesicle radius at the thermal equilibrium, averaged over a period of 9 months. The size of the vertical bars gives the standard deviation of  $\langle r_v(t = 1 \text{ month}) \dots r_v(t = 9 \text{ months}) \rangle$  and is thus indicative of the extent of vesicle size-growth over this period of time. Maximally flexible vesicles in the resulting formulations consist of soybean-phosphatidylcholine with the addition of approximately 30 mol% of sodium cholate.

that in the broadest sense of the word a transfersome is any body which is capable of getting through the permeability barrier. The proviso is that it also transfers its associated material between the application and the destination sites. More specifically, a transfersome is a lipid droplet of sufficient deformability to penetrate easily through pores much smaller than its own size. Most frequently, a transfersome is an ultraflexible mixed lipid vesicle in a (quasi)metastable state, which facilitates the formation of highly curved bilayers.

Transfersomes differ drastically in their elastic properties from the standard liposomes. In order to change the latter type of lipid vesicles into transfersomes one can incorporate, for example, edge-activators into the vesicular membranes. Such activators are often, but not necessarily, chosen from the class of surfactants. Different physico-chemical states of the host lipid molecules may also do the job. The preferred concentration range for such membrane 'additives' is normally between 0.1 and 99% of the concentration needed to solubilize the original lipid bilayer. Most frequently, optimum additive concentrations are between 10 and 60% of the solubilization concentration.

Transfersomes also differ in at least two basic features from the mixed micelles, with which they often share the same main components. Firstly, a transfersome is normally far greater than standard mixed lipid micelles, the corresponding sizes being  $r_{v0} > 50$  nm, and  $r_{m0} \gtrsim 3\text{--}5$  nm, respectively. (Transfersome diffusion is therefore always quantitatively different from that of the corresponding micelles.) Secondly, and more importantly, each vesicular transfersome contains a water-filled core whereas a micelle is just a simple fatty droplet. It is largely because of this aqueous inner lumen of the transfersomal vesicles that they incorporate and transport water soluble substances in addition to the amphiphilic and lipophilic substances in their surrounding lipid bilayers.

Transfersomes thus are material carriers with unprecedented properties. They are interesting, on the one hand, from the physical point of view as an example of the extremely elastic membranes. On the other hand, they are interesting as the promoters and mediators of the agent transport across a variety of permeability barriers, including animal and human skin, plant cuticle, and microporous membranes.

### 3. Ultraflexible lipid vesicles as material carriers

The density of the material flow through a permeability barrier is proportional to the force or pressure difference across this barrier,  $\Delta f$ , and to some characteristic barrier permeability parameter  $\mathcal{P}$  [8]:

$$j = \mathcal{P}_{\text{barrier}} \Delta f.$$

The reciprocal permeability value, so called barrier's resistance, moreover, is proportional to the exponent of the 'activation energy' for the permeation step,

$$\mathcal{P}_{\text{barrier}}^{-1} \propto \exp(\Delta G_{\text{act}}/kT)$$

in the simplest approximation [21].

For a lipid vesicle that is being driven through a hole smaller than its own diameter the activation energy largely stems from the cost of bilayer deformation. This cost can be estimated from any good theory of the membrane elasto-mechanics. In the approximation introduced in previous section one can assume, for example, that:

$$\mathcal{P}_{\text{barrier}}^{-1} \propto \exp(\Delta G(\text{elastic})/kT) \simeq 2\pi\kappa[(r_v/r_{\text{pore}})^2 - 1].$$

From eq. (1) it then follows that the minimization of the lipid bilayer rigidity, reflected in a small value of the parameter  $\tilde{\delta}$ , increases the rate of the vesicle penetration through the permeability barrier

$$j \propto \frac{\Delta f}{\exp(2\pi r_v^2 \tilde{\delta} \kappa / kT r_{\text{pore}}^2)} \propto \frac{kT}{\tilde{\delta} \kappa} \left( \frac{r_{\text{pore}}}{r_v} \right)^2 \Delta f \quad (2)$$

since the elastic energy, for all good transfersomes, is much smaller than the thermal energy,  $\tilde{\delta} \kappa \ll kT$ , as discussed previously. (The implicit assumption in eq. (2) is

that the deformed vesicle is leaky and thus not subject to any volume constraint. In light of the high concentration of the edge-active substances in transfersomes this is a justified approximation.) The flux of lipid vesicles driven by some constant transbarrier force or pressure gradient thus should increase quadratically with the relative pore size,  $r_{\text{pore}}/r_v$ . Moreover, it should decrease inversely proportionally with the effective bending rigidity of the lipid bilayers,  $\tilde{\delta}\kappa$ .

Equation (2) gives a simplified picture of the penetration of a real vesicle through a permeability barrier. This result neglects, however, the stress-induced bending rigidity changes, the corresponding modification in the *local* deformability of lipid bilayers, and other non-linear effects. Such effects are not always unimportant, however. Stress, for example, may promote the non-uniformity and thus the inhomogeneity of lipid membranes, especially at the maximally stressed sites. Local change in the lipid bilayer composition may also enhance vesicle metastability. All this is apt to decrease the value of  $\tilde{\delta}$ , probably in a dynamic way.

#### 4. Vesicle penetration through the artificial permeability barriers

All these model deficiencies notwithstanding, eq. (2) also has several merits. It explains, for example, why the vesicles made of a suitably designed lipid mixture, driven through a permeability barrier by an external force, are better permeants than standard liposomes, for all reasonable pore and vesicle sizes. This result, consequently, can be used to study the effects of membrane composition and the consequences of changing transbilayer gradients on the transport of vesicular permeants across the microporous artificial barriers.

##### 4.1. Pressure driven transbarrier flow of lipid vesicles

Figure 5 illustrates the results of one such investigation with standard vesicles, micelles, and transfersomes. It confirms the validity of (2) at least qualitatively. Furthermore, it indicates that the standard mixed micellar suspensions are pushed through a microporous barrier according to the simple mass-transfer law; the constancy of the effective barrier permeability for such suspensions as a function of the transbarrier pressure corroborates this conclusion (topmost line in fig. 5). Ordinary liposomes with a low admixture of the edge-active impurity get across the same barrier under comparable conditions at nearly unmeasurable rates. This is certainly true as long as the pore diameter is not greater than the lipid vesicle size ( $r_{\text{pore}}/r_v \gg 0.5$ , data not shown). Barrier permeability to such vesicles is also nearly pressure independent (lowest line in fig. 5).

Entirely different penetration behaviour is observed for the mixed lipid vesicles. Such transfersomal vesicles are much less sensitive to the pore-size limitation than are standard liposomes. The permeation of the former through the penetration barrier, moreover, exhibits a highly non-linear pressure dependence (middle curve in fig. 5). This is indicative of the limited validity of eq. (1), which only holds if  $\tilde{\delta}$  does not vary along the membrane surface. Mixed lipid transfersomes, consequently, cross the pores in permeability barriers extremely efficiently even if the transfersome radius

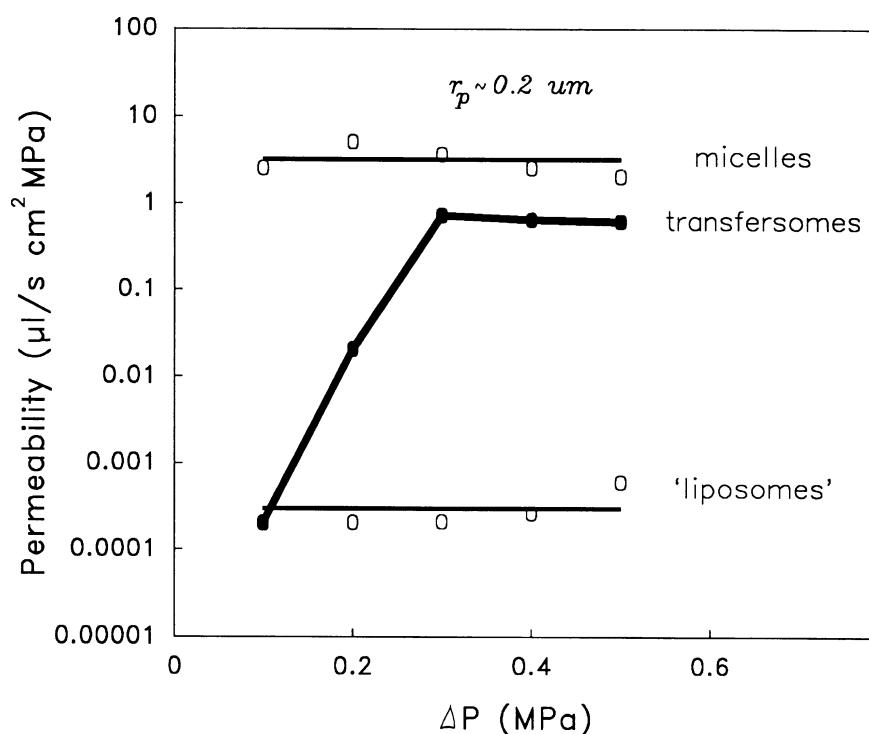


Fig. 5. Permeability of the microporous membranes with  $r_{\text{pore}} \sim 0.2 \mu\text{m}$  to different mixed lipid suspensions (2 w% total lipid concentration) as a function of the transmembrane pressure difference  $\Delta P$ . Membrane permeability is very high for the sufficiently stressed ultraflexible vesicles (transfersomes), which contain a relatively high concentration of the edge active substance ( $r_v \sim 0.6 \mu\text{m}$ , Na-Cholate  $\sim 15$  relative w%, middle data set). The same vesicles under low stress get through the pores only very inefficiently. High driving pressures, however, ensure that the maximum flux of such transfersomal suspensions is nearly as high as for the suspensions of much smaller mixed lipid micelles ( $r_v \sim 0.003 \mu\text{m}$ , Na-Cholate  $> 25$  relative w%, topmost data set). Nearly pure lipid vesicles, conversely, can hardly penetrate through the fine pores in a membrane (lowest data set). (Modified from ref. [22].)

is much greater than the pore size ( $r_v/r_{\text{pore}} \geq 0.25$ ). The proviso for this is that they are driven by a sufficiently strong pressure difference which causes the local membrane elasticity, and thus the value of  $\tilde{\delta}(s)$ , to adapt dynamically to the local stress. If so, the rate of transfersome penetration through a permeability barrier may be of the same order of magnitude as for the much smaller mixed lipid micelles ( $r_m/r_{\text{pore}} \leq 60$ ).<sup>3</sup> Mixed, ultraflexible vesicles consequently fully deserve the name transfersomes.

#### 4.2. Osmotically driven transbarrier flow of lipid vesicles

All polar lipids in a vesicle suspension attract some water [23]. This is due to

<sup>3</sup> The anomalous effect of the driving force on the efficiency of transfersome penetration through a permeability barrier indicates a non-Newtonian flow-behaviour.

the energetically favourable interactions between the hydrophilic lipid residues and their proximal water. Most lipid bilayers thus spontaneously resist to any externally induced dehydration. This necessitates the use of rather high pressures of approximately  $10^8$  Pa, perpendicular to the bilayer surface, to squeeze out the water molecules that are intercalated between the fully hydrated lipid bilayers [24]. Figure 6 illustrates this.

Dehydration pressure can be generated mechanically. Alternatively, an osmotic pressure can be used to dehydrate the lipid bilayers [25]. The magnitude of any (physico)chemically induced osmotic pressure is proportional to the logarithm of the diminished water activity coefficient in the bulk:  $P_{\text{osm}} = (RT/V_w) \ln a_w$ , where  $V_w$  represents the water molar volume [26]. If the net osmotic pressure on the lipid membrane is due to the physical lipid dehydration the pressure value is proportional to the logarithmic ratio of the initial (high) and the final (low) water concentration:

$$P_{\text{osm}} = (RT/V_w) \ln [c_w(\text{high})/c_w(\text{low})].$$

In both cases, an increased osmotic pressure causes some of the bilayer-associated water molecules to leave their binding sites and results in a partial system dehydration. Reversal, elimination, or reduction of the excess osmotic pressure, of course, leads to the rehydration of lipid bilayers [27].

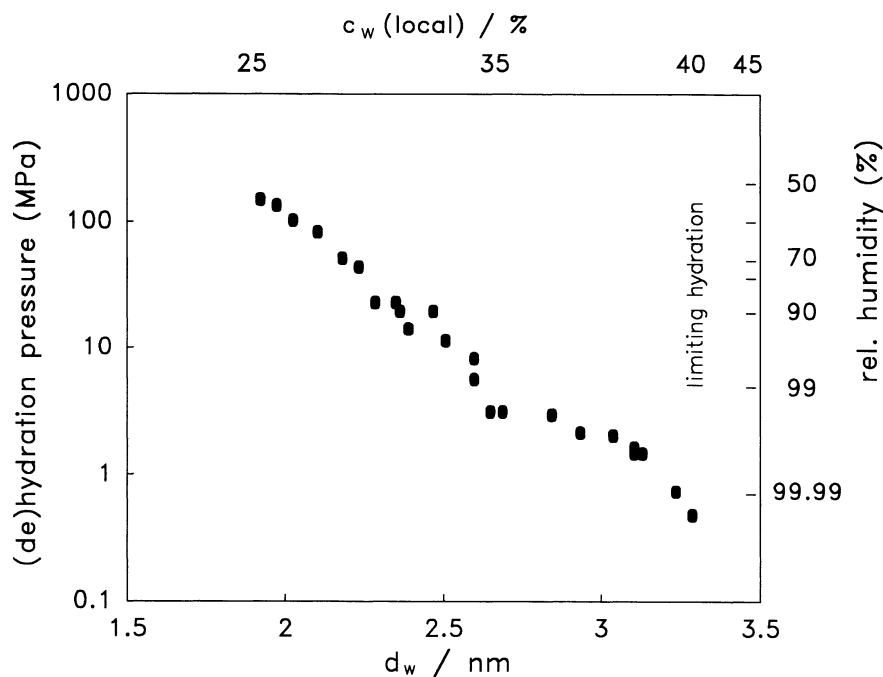


Fig. 6. Limiting hydration of and measured repulsion between the phosphatidylcholine multi-bilayers as a function of the separation between lipid membranes (lower axis), effective concentration of water in the interbilayer region (upper axis) and the ambient humidity (right vertical axis). (Data taken from ref. [25].)

This is the reason why all polar lipid vesicles, in principle, would like to move from locations with a low water concentration to the sites with a higher water concentration. This gives all lipid vesicles a tendency to flow from the application site to some other location as long as the water concentration at the original site is lower than the so-called limiting water concentration, characteristic of the mobile lipid:  $c_w(\text{local}) \leq c_w(\text{excess})$ . The counter-directed water flow may, but need not, be important in this respect. If water concentration and its gradient in the system are kept constant such water flow may even be neglected completely.

Limiting hydration of each lipid is a function of the effective lipid headgroup polarity [28]. It is defined as the water concentration in a sample containing maximally hydrated lipid. For phosphatidylcholine, the value of this concentration amounts to approximately  $c_w(\text{excess}) \simeq 40$  w% of water; for the charged (and some strongly fluctuating) lipid bilayers the corresponding values may exceed 80–90%, however [8].

Equilibrium hydration pressure of the bilayer forming lipids thus changes with the logarithm of the limiting water concentration:  $P_{\text{osm}} \propto \ln c_w(\text{excess})$ . This permits the locomotion of lipid vesicles in a water-concentration gradient to be described by:

$$\begin{aligned} j &\simeq \mathcal{P}_{\text{barrier, v}} A_v \Delta P_{\text{osm}} \\ &= \mathcal{P}_{\text{barrier, v}} (RT/V_w) (\pi r_{v1}^2) \ln [c_w(\text{excess})/c_w(\text{local})] > 0 \end{aligned} \quad (3)$$

where it is assumed that the decisive action of the pressure difference is always along the small vesicle face, owing to the spontaneous vesicle elongation or orientation along the pore ( $r_{v1} \leq r_{v2}$ ).

Equation (3) suggests that the spontaneous lipid flow in an osmotic gradient should increase with increasing lipid polarity, since the latter determines the value of  $c_w(\text{excess})$ . Net lipid flow should also increase with the system's permeability to the moving lipid vesicles, which is described by the parameter  $\mathcal{P}_{\text{barrier, v}}$ . Increasing local water concentration should always decrease the osmotically driven vesicle flow, however.

If the permeability of a given barrier to the unilaterally applied lipid vesicles is estimated by means of eq. (2) one can rewrite expression (3) as:

$$j \propto \frac{kT r_{\text{pore}}^2}{\tilde{\delta} \kappa} \ln \frac{c_w(\text{excess})}{c_w(\text{local})}. \quad (4)$$

This summarizes several theoretical principles on which the rational design of ultraflexible agent transporters can be based.<sup>4</sup> It also gives several hints on the convenient and inconvenient modes for the vesicle application *in vivo* where each carrier is expected to pass through a series of osmotical different compartments characterized by a different osmotical pressure. Spontaneous motion of the standard liposome suspensions and of the transfersome preparations in the living skin may highlight some of these aspects.

<sup>4</sup> This result depends on several oversimplifications. It does not account, for example, for the dynamic changes in the vesicle shape and its effects on the driving force, for the spatial dependence of membrane elasticity modulus, for the dynamic variability of  $\tilde{\delta}$ , its sensitivity on the vesicle size, etc.

## 5. Vesicle penetration through the natural permeability barriers

For many years it was thought that it is impossible to bring large molecules through the biological permeability barriers, such as intact skin, owing to the very small pore-size in such barriers [30, 31]. Indeed, with the commonly known technologies only a few percent of the applied dose of large-molecules, at the best, could be brought across the skin without a hypodermic needle [32]. It was only very recently that a solution to this problem was found by the design of special ultraflexible lipid vesicles which can penetrate spontaneously through the permeability barrier of the intact stratum corneum [33]. How can such a transport of material be explained?

### 5.1. Skin as a permeability barrier

Outer skin, or epidermis, in humans, typically measures between 30  $\mu\text{m}$  and up to 0.2 mm in thickness. The next skin region, dermis or corium, is about 10–20 times thicker than the epidermis. This part of the skin is a shelter for the blood capillaries, lymph ducts, certain glands, immunologically active cells, nerve endings, etc. It is frequently the first goal of the dermally applied drugs as well as the place where such drugs can be brought into the blood circulation. Typical skin architecture is schematically illustrated in fig. 7.

In order to reach dermis, and potentially mediate a systemic action, any superficially applied agent must first pass through the topmost skin layer, so-called *stratum corneum*. This layer consists of a few dozen horny, keratinized epithelium layers [34]. The narrow interstices between the dead or dying keratinocytes in this region are filled with strongly hydrophobic lipid multilamellae. These lipid structures thus seal the interstices in the outermost skin layers nearly completely [35] and prevent the loss of the water and body fluids from the skin depth quite efficiently. Epidermis thus also restrains the uptake of the detrimental agents from the environment into the body, the residual transport proceeding normally through the, chiefly narrow, and irregular ‘pores’ in such a horny region.

### 5.2. Gradients and material flow across the intact skin

Material application at the skin surface creates a concentration difference  $\Delta c$  between the application site and the skin interior. Such gradient, in molar terms, inevitably decreases with the increasing molecular weight of the permeant, as long as the total applied material mass remains constant. This means that the highest molar concentration attainable or tolerable at the skin surface puts a limit to the net efficacy of the achievable material diffusion across the intact skin. This is seen from the expression for the calculation of diffusive flow density:  $j = \mathcal{P}_{\text{barrier}} \Delta c_{\text{sat}}$ . Limiting concentration  $c_{\text{sat}}$  is determined by the molecular solubility and the local toxicity constraints.

With lipid suspensions, for example, concentrations higher than 0.3 moles per litre, or some 25 weight%, are difficult to make and apply dermally. This is primarily due to the difficulty of creating a more concentrated, stable, and homogeneous lipid suspension at the skin surface [8, 12].

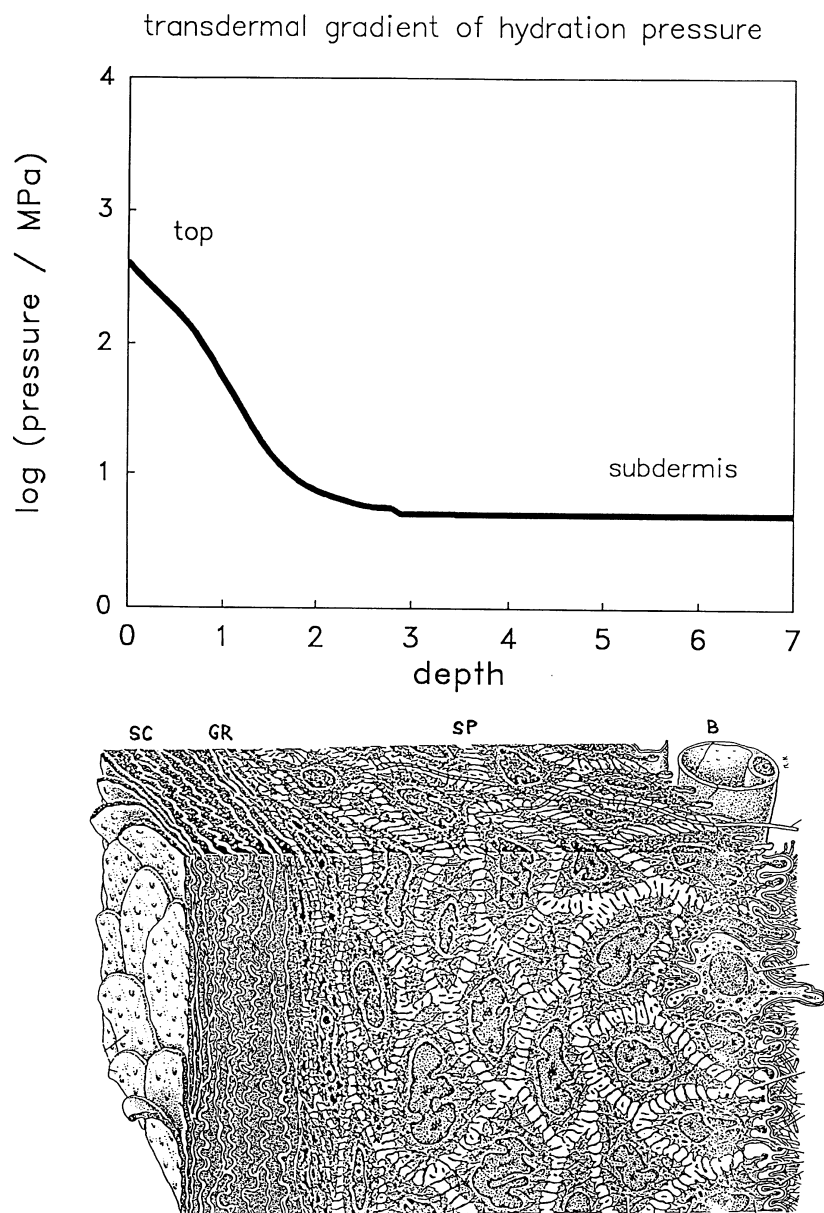


Fig. 7. Schematic representation of the transepidermal osmotic pressure profile (top) and of the micro-anatomy of the intact skin on a similar scale (bottom). To calculate osmotic pressure in the skin, water concentration data for the intact cutis were combined with the 'hydration force' data for the phosphatidylcholine multi-bilayers as a function of the ambient water concentration. SC denotes the *stratum corneum* region; GR denotes the *stratum granulosum* and SP the *stratum spongiosum*; B gives the position of the basal layer. The former two layers consist together the epidermis. (Modified from [33].)

For the small molecules with a mass around 1000 Da surface concentrations on the order of  $1 \text{ mmol cm}^{-2}$  have been reported to create a picomolar transepidermal flux per hour and square centimetre [36]. For the phospholipids with a molar mass around 800 Da one would expect, in analogy with this example, the transepidermal molecular flux to be of the order of a tenth of nanomole, or  $0.1 \mu\text{g h}^{-1} \text{ cm}^{-2}$ . This calculation has been done for the hypothetical case of a lipid monomer diffusion under the optimal conditions. Lipid vesicles with a much higher effective mass ( $\sim 5 \times 10^7 \text{ Da}$  for  $r_v \simeq 100 \text{ nm}$ ) under comparable conditions should give rise to a flux of just a few femtomoles of vesicles (or a few nanograms of lipid) per hour and unit area. Transdermal lipid concentration gradients, consequently, are poor promoters of the lipid vesicle transport across the intact skin.

In the normal skin, however, another gradient is normally available which provides a solution to this problem. This is the transepidermal humidity or water-concentration gradient.

Epidermal surface is known to be relatively dry. Normally it contains less than 15% water [37] which is by at least a factor of 5 less than in the basal skin layers. This causes the local activity coefficient of water to change by more than 75% across the outer skin layers. For all common polar lipids this suggests that water concentration in the outer skin region is significantly below the water concentration needed for the maximum lipid hydration ( $c_w(\text{local}) \leq c_w(\text{excess})$ ). Naturally occurring transepidermal water concentration gradients can thus drive appreciable numbers of lipid vesicles across the outer skin layers (cf. eq. (3)). (Such gradients also offer the advantage of not being diminished by the lipid aggregation into lipid vesicles, as can be seen from eq. (4).)

Enhanced water flow toward the skin surface, which results from the applied lipid concentration gradient, may change the kinetics of lipid vesicle penetration through the skin without affecting the real efficacy of such permeation. The reason for this is that the intra-dermal vesicle flow continues as long as enough water is lost at the skin surface to keep the decisive water concentration lower than the limiting water concentration. This is the case under most practically relevant conditions.

The top panel of fig. 7 illustrates the situation encountered in the normal human skin. It has been calculated from the measured skin humidity data [37] in combination with the appropriate ‘osmotic force’ data for the phosphatidylcholine bilayers [38] (see also fig. 6). From the illustrated pressure profile the maximum resulting force is estimated to be of the order of  $F \simeq \pi r_v^2 \times 10^5 \text{ Pa} \sim 10^{-11} \text{ N}$ , for a single vesicle with a radius of  $r_v = 60 \text{ nm}$ . This corresponds to a force of  $\geq 10^{12} \text{ N}$  per mole of vesicles.

The naturally available transepidermal osmotic gradient is thus sufficiently strong to push most of the lipid vesicles into the outer skin layers – but not necessarily through the entire *stratum corneum* [33]. For example, the available osmotic pressure difference across the normal skin is too low (cf. fig. 5) to deliver the permeation activation energy for the standard vesicles. Experiments with the dermally applied common liposomes vindicate this conclusion. In order to get large vesicles *into* the skin, membrane composition needs to be adjusted since only sufficiently flexible lipid vesicles can get between the intact horny layers into the dermis. Under optimal

conditions, however, the total lipid mass which can be transferred by means of transfersomes from the skin surface into the body may exceed 0.1 mg of lipid per hour and  $\text{cm}^2$ . This is identical to the human skin's own lipid mass density [33].

The efficacy and the depth of the transfersome penetration depends on the applied lipid dose. The duration of the carrier application also plays some role, of course. A 'row' of lipid vesicles, or a sufficiently extended transfersome, may directly connect the skin surface and the depth of the skin. The moving vesicle(s) then experience *total* rather than just the much smaller *local* transepidermal water concentration gradient. This ensures maximum material flux.

### 5.3. Spontaneous vesicle transport across the living skin

The conclusions of the previous section are supported by the directly measured data on the penetration of various types of lipid vesicles into the intact skin under osmotically different conditions.

Eight hours after the placement of the standard lipid vesicles, liposomes, on the intact skin surface,  $(80 \pm 15)\%$  of the totally applied lipid mass are recovered from the very skin surface. Up to 25% of the recovered lipid dose are associated with the upper *stratum corneum* layers and only a few percent are found in the dermis. A number of authors working with standard liposome formulations, prepared either from the common biological [39–43] or from the skin lipids [44, 45], have reached such a conclusion.

Occlusive application of the optimized ultraflexible lipid vesicles, transfersomes, yields similar results [33]. Water-tight skin wrapping causes a permanent lipid 'over-hydration',  $c_w(\text{excess}) \leq c_w(\text{local}) (\gtrsim 85)\%$  and prevents a significant lipid in-flow. Wet skin therefore retains at least 90% of its applied dose at the very surface or in the *stratum corneum* layers. The amount of the transfersome-derived material in the deeper skin layers after an occlusive application is only marginally higher than after the applications of standard liposomes for 8 h ( $\leq 5\%$ ).

An entirely different picture is obtained, however, if suitably designed, flexible lipid vesicles are used in an open, non-occlusive way [33]. Transfersomal preparations left to 'dry' out at the skin surface penetrate nearly completely into the skin. They transfer more than 85% of their material into or through the permeability barrier. Even in the worst case, less than 50% of the total applied lipid dose are found at the very skin surface. Depending on the details of each transfersome preparation and application used,  $(30 \pm 10)\%$  of the applied lipids are found in the subdermis (fig. 8).

Transfersomes are thus capable of transporting various molecules through the permeability barriers. They can act, for example, as transepidermal drug carriers after a percutaneous application and carry both water as well as fat-soluble agents to an appreciable depth in the skin. Transfersomal agent carriers may even reach deep body tissues. The kinetics, efficiency, and depth of the transfersome-mediated agent transport vary with the detailed carrier composition, application dose, and form. It is therefore possible to tailor the application characteristics within broad limits by using the rational membrane design.

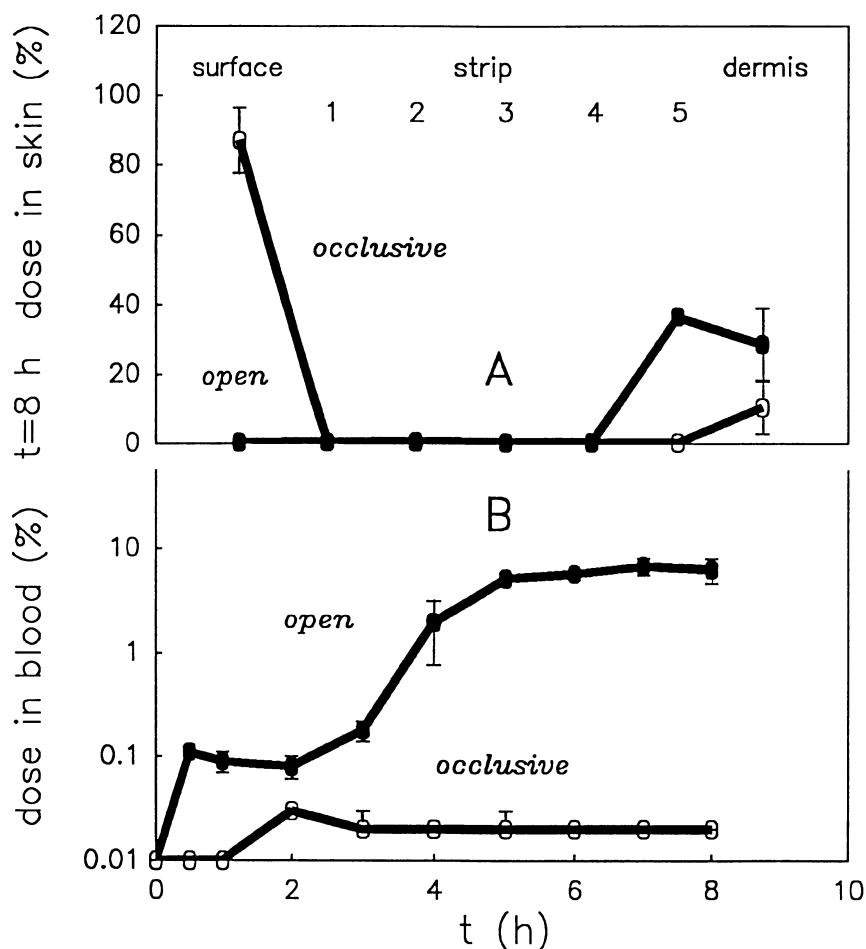


Fig. 8. Spontaneous penetration of transfersomes into the intact skin in the presence (closed symbols) or absence (open symbols) of the transepidermal osmotic gradient, corresponding to open and occlusive applications, respectively. Standard liposomes penetrate the skin as inefficiently as transfersomes under occlusion (not shown); neither ever get deep into the intact skin (the numbers in upper panel give the subsequent number of the corresponding strip). In contrast to this, the penetration of ultraflexible lipid vesicles (transfersomes) into such skin is highly efficient (panel A), unless the transepidermal osmotic gradient is eliminated by the skin occlusion (open symbols). The latter type of vesicles also guarantees appreciable systemic carrier concentrations (panel B). If not shown, standard deviations are smaller than symbols. (Modified from ref. [33].)

#### 5.4. Kinetics of transdermal vesicle transport in vivo

Kinetics of transfersome penetration through the intact skin is best studied in direct biological assays in which the vesicle-associated drugs exert their action directly under the skin surface. Local analgetics provide a very useful tool for this such investigations [46].

Figure 9 shows, for example, how dermally applied analgesic agents can be used to assess the kinetics of *stratum corneum* penetration by the carrier transfersomes. Lidocain-loaded transfersomes left to dry out on the intact skin reduce the animal's sensitivity to the local pain. Standard drug-carrying liposomes or simple lidocain solutions have no comparable effect. On the rats tails, for example, maximum analgesic effect is observed after 15 minutes of the transfersomal drug action, the characteristic onset and decay times being 11 minutes and 6.5 minutes, respectively. At later times, the drug-induced pain insensitivity decreases; animal's reaction time to the painful stimulus then becomes shorter again. However, a marked analgesic effect is still noticeable even after 30 minutes of the transfersomal drug action.

This shows that the permeation of analgesic transfersomes through the intact skin surface can delay the direct transferred drug action. The resulting lag-time is only short, however, around 10 minutes for the locally active substances. The pharmacokinetics of the transfersome-associated agents is thus probably often governed by the drug-distribution kinetics throughout the body and by the kinetics of the drug's biological action rather than by the speed of the carrier permeation through the skin.

The latter can be modified somewhat by changing carrier composition. Formulations with a very high or a very low concentration of the edge active components have been found experimentally to be less potent than the preparations with an intermediate lipid/surfactant ratio. This finding is in full accord with the hitherto discussed transfersomal characteristics and indirectly confirms the validity of eq. (4): preparations with only a small amount of admixed surfactant are too rigid to be properly active; preparations with a high concentration of surfactant contain micelles which are less sensitive to the osmotic stress than transfersomes.

## 6. Therapeutic efficacy of the dermally applied lipid vesicles

The most striking feature of all properly done transfersome applications is the high efficiency of material which they bring into the blood circulation. This transfer probably begins between the corneocytes at the skin surface then goes through the dermis and proceeds via the lymph. Within 30 min after a typical dermal transfersome application between 0.1 and 0.5% of the radioactively labelled lipids are found in the bloodstream. The biggest in-flow in mice is observed after 4 to 8 hours, however. At  $t = 8$  h, the resulting relative 'transfersome concentration' in the blood is between 0.6 % and, more frequently, 6...8%, depending on the transfersome composition and preparation. By this time, some 20–30% of the dermally applied lipid has also reached the phagocyte-rich organs, such as liver. Here, most of the lipid vesicles are taken up by the resident macrophages. In contrast, the blood concentration levels at  $t = 8$  h for the standard lipid vesicles, under comparable conditions, are 0.03 to 0.14% at the best. We have never found significant amounts of the exogenous lipids in the liver after the dermal applications of standard liposomes.

Their efficient penetration through the intact skin gives the transfersomes an appreciable therapeutic potential [47]. Figure 10 documents, for example, how the insulin-loaded transfersomes can be used to lower the blood glucose level without

using an injection needle. Typically, after any such transfersome application onto the intact mammalian skin the first signs of systemic hypoglycemia are observed after approx. 90–180 min, depending on the detailed carrier composition. This is a delay of 45–145 min relative to the onset of the subcutaneous insulin action. Maximum transfersome-mediated decrease in the blood glucose concentration is estimated to be approximately  $(35 \pm 10)\%$  of the effect of similar amounts of the subcutaneously injected transfersomal insulin.

All this proves beyond any doubt that transfersomes applied in an open patch or as an open droplet can get through the intact skin. This pertains to animals as well as to

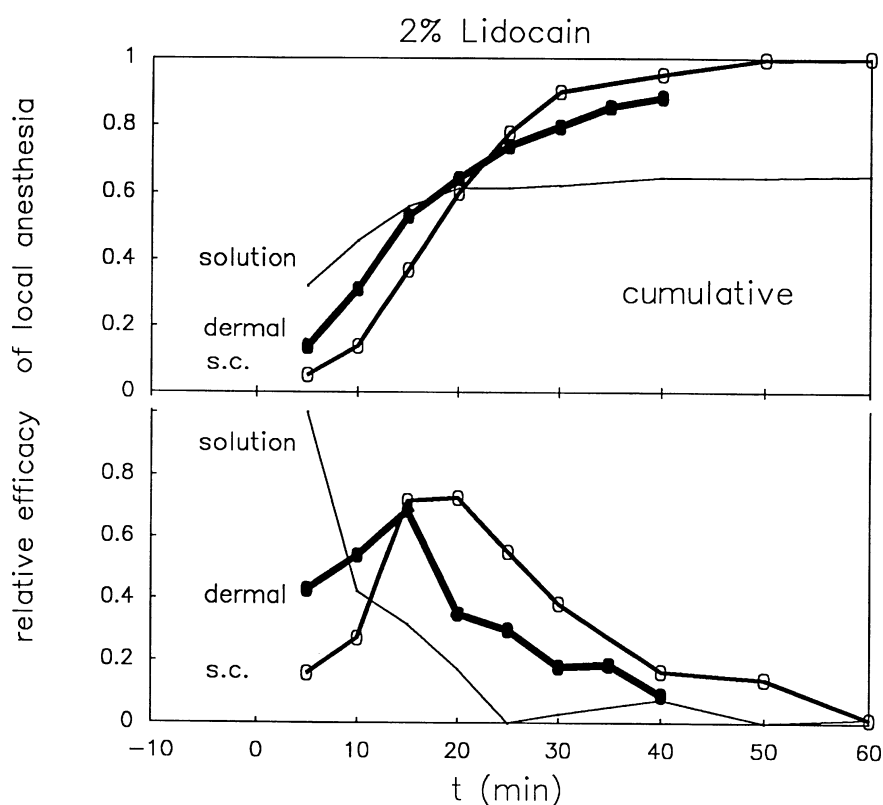


Fig. 9. Kinetics of penetration (bottom) and cumulative action (top) of the ultraflexible analgesic vesicles through the intact skin of rats tails, as measured in a local pain suppression assay. The three curves labelled by (i) solution, (ii) dermal, and (iii) s.c. stem from the epicutaneous of (i) a simple lidocaine solution, (ii) an epicutaneous application of the analgesic transfersomes, and (iii) from the subtraction of (i) from the results measured after a subcutaneous injection of the corresponding transfersome suspension. Dermally applied transfersomes containing a local anesthetic lidocaine (thick lines, ●) suppress the local pain sensitivity maximally after approximately 15 min. Their cumulative biological action (upper panel, ●) is even higher than that of the subcutaneous injections of comparable amounts of the free drug (thin lines). It is lower, however, by approx. a factor 2 than that of the subcutaneously injected analgesic transfersomes (○), which is used as the unit. Dermally applied analgesic transfersomes have a comparable efficiency as the vesicle-associated injected drug (○). (Modified from ref. [46].)

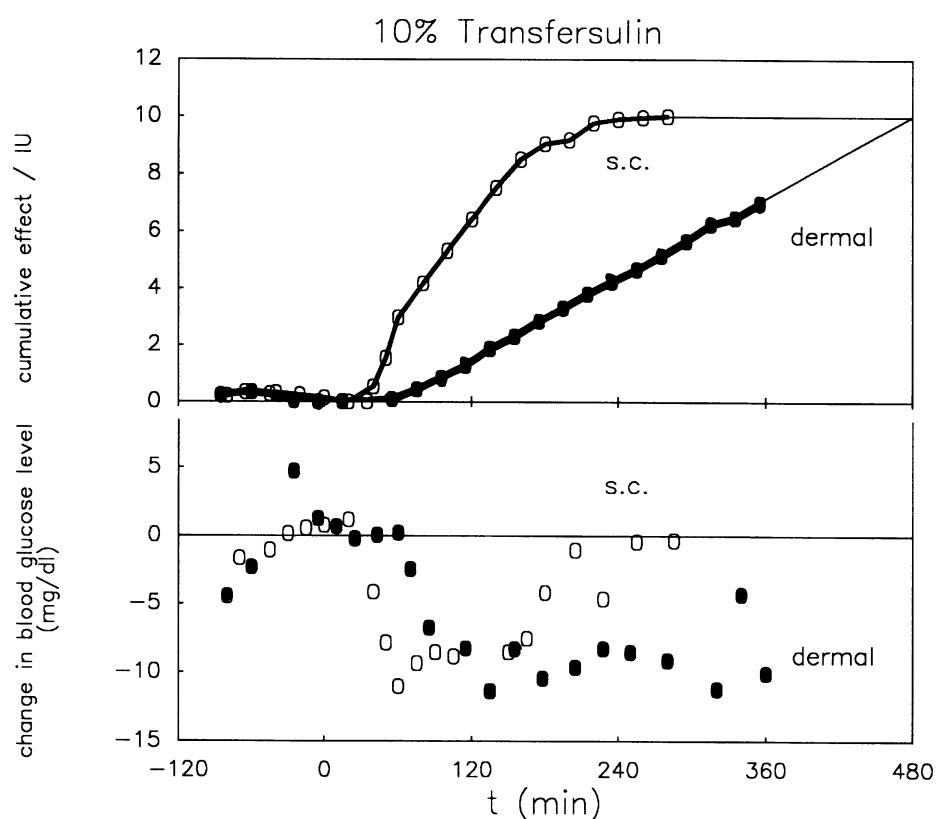


Fig. 10. Hypoglycemic efficiency of the transfersomal insulin, Transfersulin<sup>TM</sup> (5% lipid, 35 IU/ml) in human experiments as a function of time. The two sets of data denoted by (i) s.c. and (ii) dermal correspond to a subcutaneous Transfersulin injection and to an application of the same preparation on the intact skin surface, respectively. Subcutaneously injected Transfersulin (○, 0.115 IU/kg) begins to lower the blood glucose level after 45 min, as does standard insulin solution (not shown). Insulin applied on the intact skin surface in the form of ultraflexible mixed lipid vesicles, transfersomes (●, 0.23 IU/kg), is therapeutically active with an additional lag time of approximately 45 min (bottom panel). Its cumulative therapeutic effectiveness per international unit of insulin is comparable to that of the subcutaneous Transfersulin injections (top panel); for some preparations it may even exceed 100% (not shown).

humans but not to all types of the lipid vesicles. Depending on the composition and the applied transfersomal dose, between 50 and 90% of the deposited lipid material get across the normal skin within 8 hours. At the end of this period, between 1 and 10% of the applied lipid dose is still in the blood circulation.

## 7. Conclusions

In order to penetrate efficiently through the permeability barriers lipid vesicles should

be flexible enough to ensure that the energetic cost of their elastic deformation in the barrier pores is smaller than the work which can be delivered by the penetration-driving force. Normal lipid vesicles do not fulfill this requirement unless their size is comparable to or smaller than the pore diameter. High vesicle penetration rates, consequently, can only be achieved by using ultraflexible transfersomes.

The simplest transfersomes consist of a common phospholipid with an admixture of some edge-active membrane component. The latter ideally should soften the lipid membranes and catalyze the metastability of the combined system. Mixtures of common phospholipids, such as phosphatidylcholine, and of standard surfactants, such as bile salts, can guarantee the desired properties, for example. The elasticity of the corresponding optimized mixtures, consequently, may locally be up to 2...3 orders of magnitude higher than that of the pure phosphatidylcholine bilayers. The average vesicle elasticity is much higher, of course,  $\kappa\delta \geq kT$ . (If the elastic energy of a transfersome would be smaller than the thermal energy, vesicle suspension would become unstable.) This is the reason why the pressure-driven penetration of the appropriate mixed lipid vesicles through artificial permeability barriers can be nearly as high as for the much finer micellar suspensions whilst the colloidal stability of the suspended transfersomes is at least as high as for the standard liposomes.

Transepidermal lipid concentration gradients drive only minute material flow across the skin ( $\leq 1 \text{ ng h}^{-1}\text{cm}^{-2}$ ). Such gradients are thus of little practical value. It is predominantly the water-concentration gradient in the upper skin layers which can push noticeable amounts of the superficially applied lipid vesicles into the dermis. Owing to their surface hydrophilicity all polar lipid vesicles are prone to move in a water concentration gradient from the sites of the low water concentration toward water-rich sites. In order to get through the intact skin, however, such vesicles should also be ultraflexible. Natural or artificial hydration gradients, consequently, can drive appreciable amounts of the highly deformable lipid vesicles through the skin. This explains why transfersomes but not standard liposomes can be used for the transepidermal agent delivery. It also suggests that occlusion of the intact skin surface prevents any vesicle penetration into the depth of the skin.

The efficacy and the depth of lipid vesicle penetration into the intact skin, consequently, are strongly affected by the lipid bilayer composition. They also depend on the applied carrier dose. Standard vesicles made from the nearly pure phosphatidylcholine at any applied dose can only bring insignificant amounts of material across the skin permeability barrier. Transfersomes applied at a small dose also get across the skin rather inefficiently. Only ultraflexible vesicles with an optimal composition and applied in sufficiently high quantities can transport up to 95% of their associated lipid mass into and through the intact horny layers. Transfersomes, consequently, can replace hypodermic needles. This offers a new method for the non-invasive drug delivery in human and animal therapy.

Transfersomes thus offer an elegant, well defined, and generally applicable means for the transportation of various agents across artificial and natural permeability barriers. Such ultraflexible lipid vesicles, consequently, may become an important tool in human and animal medicine, dermatology, cosmetics, biology, biotechnology, agrotechnology and in other applied fields in the future.

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