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Rapid Report

Lipid vesicles penetrate into intact skin owing to the transdermal osmotic gradients and hydration force

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Gradients across the outer skin layers may result in fields enforcing a lipid flow into or through the intact skin surface provided that lipids are applied in the form of special vesicles. The osmotic gradient, for example, which is created by the difference in the total water concentrations between the skin surface and the skin interior, provides one possible source of such driving force. It is sufficiently strong to push at least 0.5 mg of lipids per hour and cm^2 through the skin permeability barrier in the region of stratum corneum. The lipid concentration gradient, on the contrary, does not contribute much to the lipid penetration into dermis. Occlusion, therefore, is detrimental for the vesicle penetration into intact skin.

It is becoming progressively popular to use different lipid formulations as a carrier, or even agent on its own merit, for the topical skin applications. Over one hundred cosmetic products claimed to contain lipid vesicles, liposomes, are being marketed in Europe. Some of these contain artificial lipids; others include lipids from a variety of biological sources, chiefly phospholipids.

The number of reports dealing with the therapeutic applications of lipid formulations is also permanently increasing. Products containing retinoic acid, tocopherol, diverse protein extracts, or antimicotics can already be bought. In the majority of cases, the active substance is incorporated into, or is applied together with, a lipid suspension in a hydrogel or, even more frequently, in an oil-in-water emulsion.

It is quite common to use such drug-containing preparations under occlusion, this is, under a water-impermeable enwrapping. This should increase the drug availability and activity, it is inferred. The recommended duration of a topical drug application with macromolecular carriers typically is on the order of hours or longer.

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Most manufacturers would have us believe that essentially any dermally applied lipid preparation is apt to have a set of wonderful properties: various advertisers insinuate that a treatment with the lipid vesicles is going to rejuvenile the skin by increasing its water content and by eradicating small wrinkles; treatments with liposomes are claimed to hyposensitize skin as well as to promote and prolong drug action. Similar statements have been made for several dermal lipid formulations, often without a sound scientific justification or clear identification of the supposed advantageous effects.

The real situation is more complicated and far less clear than the impressions one gets by reading such statements. To date, a consensus has not even been reached as to whether or not the dermally applied lipid vesicles really can penetrate into intact skin; and, if so, what is the cause of such penetration?

Here we show that some, but not all, lipid vesicles may indeed reach the deep skin layers and may even come far enough to get into the blood compartment. We, moreover, identify one possible, and probably the chief, reason for this penetration. And last but not least, we provide the evidence for the conclusion that elimination of the transdermal 'force of lipid dehydration', which occurs upon occlusive applications, may prevent the diffusion of lipid vesicles into intact skin.

Skin as a permeability barrier. Outer skin, epidermis, in humans, is normally 0.04 and in the extreme case up to 0.2 mm thick. It consists of a few dozens of kera-

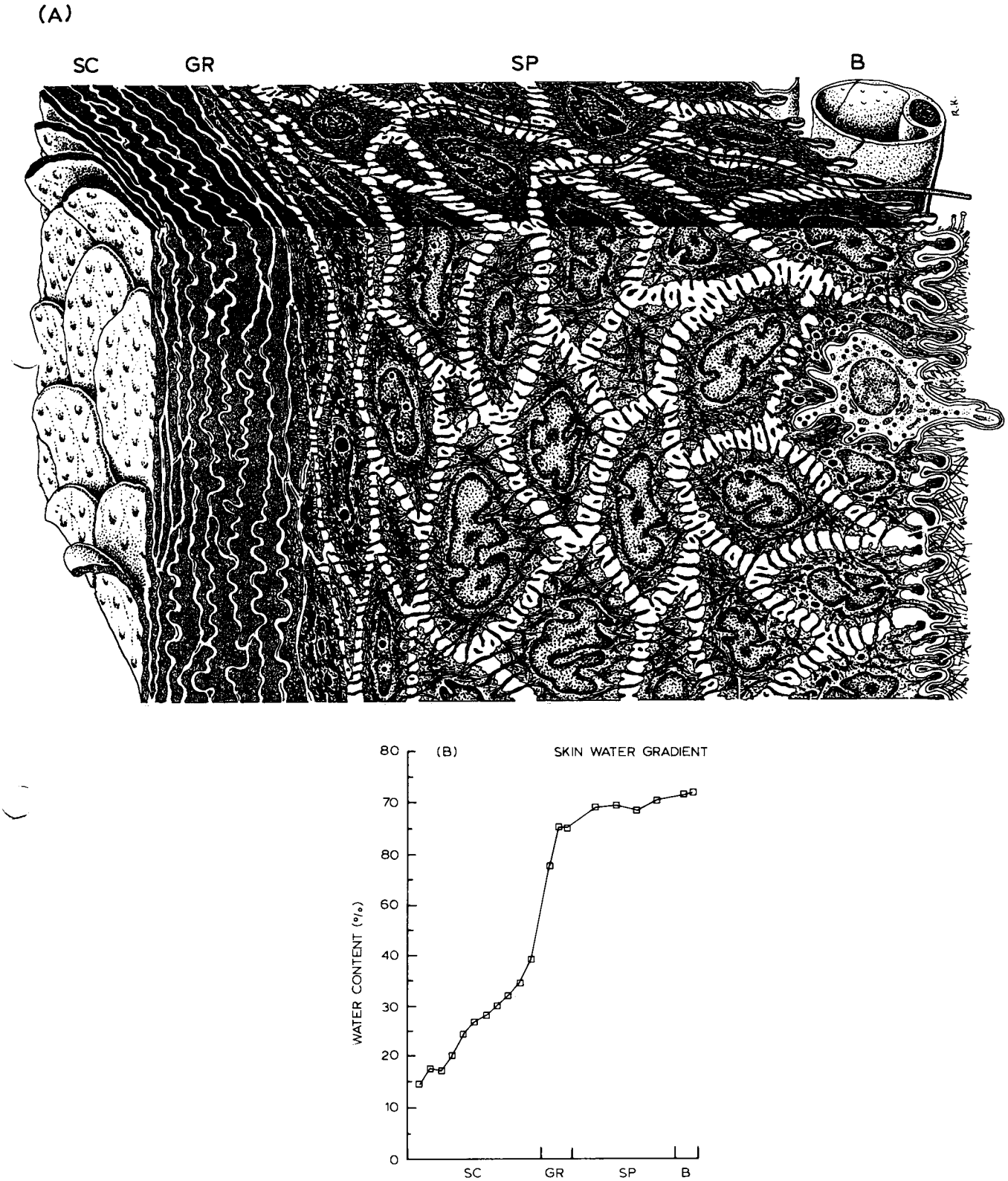

Fig. 1. (A) Schematic representation of human skin (from Krstić, 1986). Under several layers of keratinized, dead and flacid corneocytes, which comprise stratum corneum (SC), a narrow region of stem cells, stratum germinosum (GR), is located. This is followed by an extended basal region, stratum basale (SP). The outermost layer represents the primary permeability barrier; the innermost layer shelters nerve endings, blood capillaries (B), tiny lymph vessels, immunologically active cells, etc. (B) Water distribution profile in intact skin (from Ref. 10). Stratum corneum contains only small amounts; in the deeper skin layers, however, water concentration approaches that characteristic of other living body tissues.

tinized, horny epithelium layers. The next skin region, dermis or corium, is 10–20-times thicker. It shelters blood capillaries, certain glands, immunologically active cells, nerve endings, etc. The total skin width, which encompasses dermis and epidermis – called together cutis – measures between 0.5 and up to 2 mm [1]. Fig. 1A illustrates this arrangement schematically.

Epidermis is acid resistant and represents the first and main mechanical barrier between the external world and the body interior. Epidermis also prevents the loss of water and body fluids from the depth of the skin and restrains the uptake of detrimental agents from environment into body; simultaneously, epidermal layers hamper the penetration of the superficially applied drugs into the body interior.

In order to reach deeper skin regions and ensure a profound or even systemic drug action, the dermally applied agents can be transported through the largely dead cells (corneocytes) in the outermost epidermal layers, so-called horny layer or stratum corneum. Alternatively and more frequently, such agents can diffuse through the interstices between the horny cells in the outer skin region [2]. Shunt-penetration through the hair follicles and gland caverns is merely of marginal importance.

Interstices between the horny cells in stratum corneum are maze-like and narrow, their width being typically less than 0.1 μm. The effective lumen of the inter-cellular spaces in this region is further diminished by the quasi-lamellar lipid bodies located in the intercorneocyte space; ample biopolymeric networks also fill-out certain parts of this region. Most of the topically applied lipophilic or hydrophilic molecules, consequently, are highly efficiently sequestered from the deep skin regions. Indeed, it is the 'brick-and-mortar' architecture of stratum corneum just described which prevents the permeation of most molecules into intact skin; more deeply located skin layers are normally not an important obstacle to permeation [3].

Enhanced skin penetration. Skin surface is an especially effective barrier for the substances with a molecular weight higher than a few hundred Daltons *. Even for a small molecule, such as fluorouracyl, the total amount of drug penetrating through an intact skin seldom exceeds a fraction of a percent per hour and centimetre [4]. The decapeptide des-enkephalin-γ-endorphin, a somewhat greater molecule, crosses the skin permeability barrier at an even lower rate of just 1 pmol/h per cm^2. These minute values may be increased by the addition of skin fluidizers. But even in the presence of one of the most common such penetration enhancers, 1-dodecylazacycloheptan-2-one (azone), the increase is not more than by a factor of four [5].

The view thus prevails that the permeability of undamaged skin for most common drugs is too small for a successful therapy [6,7]; the greater is the drug molecule the more this is true. What is the reason for this?

After any dermal drug or carrier application, a concentration gradient is created between the application site and the skin interior. The magnitude of this gradient in molar terms inevitably decreases with the increasing molecular weight of the applied substance, for obvious reason. This means that the practical va' of a simple drug application will be severely restricted by the lowest concentration required for the desired effect and by the highest concentration achievable; the latter is normally determined by the molecular solubility and the drug toxicity constraints.

In the case of lipids, for example, skin surface concentrations higher than 0.3 mol/l, or some 20 weight%, at the time of application are difficult to obtain. This is owing to the difficulties of creating a highly concentrated, stable, and homogeneous lipid suspension [8,9]. For lipids, moreover, the high local concentrations of the native epidermal lipids tend to dissipate the lipid concentration gradients already near the very skin surface.

For small molecules with a mass $\leqq$ 1000 Da, surface densities on the order of 1 mmol cm^{-2} have been reported to create a picomolar transdermal flux per hour and unit area [4]. Lipid quantities higher than a few milligrams per cm^2, which is in the same concentration range, are difficult to apply. For phospholipids with a typical molar mass around 800 Da one would expect, in analogy with previous example, that transdermal lipid fluxes on the order of picomoles, or 0.1 ng cm^{-2} h^{-1}, should be feasible. This pertains to the case of monomer diffusion under optimal conditions. Lipid aggregates have effective molecular masses in the range of megadaltons, however; this implies that their concentration at the skin surface will inevitably be lower by orders of magnitude compared to that of free drugs or lipids. The concentration driven vesicle permeation, therefore, can be expected to be on the order of femtomoles and thus negligible. Transdermal lipid concentration gradients, consequently, can not explain the penetration of lipid vesicles across intact skin.

We wish to draw attention to the fact that another gradient is available in the skin under virtually all conditions. This is the humidity or water-concentration gradient. Epidermal surface is known to be relatively dry; normally it contains less than 15% water [10]. This

* The probability for most common drugs to penetrate the surface of intact skin, consequently, is only a few percent at the best. The greater is an applied substance, the lower is its skin permeability. Macromolecules and most kinds of particles, such as typical microorganisms, therefore do not pass through an intact stratum corneum at all [2,3].

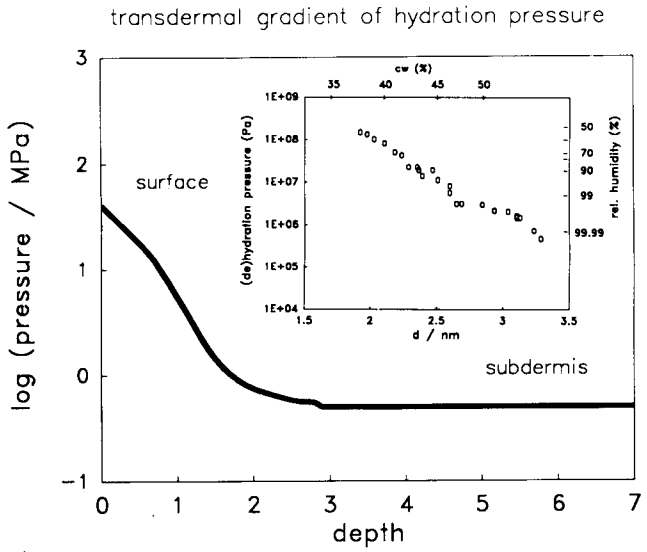

Fig. 2. Effective profile of the 'osmotic pressure', or pressure of lipid dehydration, in a typical skin section calculated from a combination of the data from Refs. 10 and 14. A pressure gradient of several hundred kPa (or over 20 bar) is likely to exist over the relatively dry outer skin layers and the skin permeability barrier. (Inset) Measured repulsion between the phosphatidylcholine multi-bilayers as a function of separation between lipid membranes (lower axis), effective concentration of water in the interbilayer region (upper axis) and ambient humidity (right vertical axis). (Data taken from Rand and Parsegian, 1981). Pressures as great as 1000 MPa (10000 atmospheres) can be created by exposing a lipid suspension to a dry atmosphere and pressures on the order of 100 MPa can be expected to exist under standard ambient conditions. The shown correlation between the interfacial separation and the total water content is somewhat arbitrary owing to the ambiguity associated with the assignment of zero-plane position.

is by a factor of 5 less than in the basal skin layers *. The transdermal water concentration gradient is relatively narrow, however. Fig. 1B shows that it only extends over the outer skin layers. The skin permeability barrier being also limited to the same skin layers, this perfectly suffices for the induction of a transdermal mass flow, however.

The inset to Fig. 2, taken from a seminal publication on hydration forces by Parsegian and Rand [11], illustrates the force between lipid bilayers as a function of the intermembrane separation (lower axis); the force dependence on the water concentration between the lipid bilayers (upper axis) or on the ambient humidity is also shown (right axis). This indicates, on the one hand, that lipid dehydration may impose an immense pressure on the lipid bilayers; in the limiting case, this may exceed 100 MPa (1000 atmospheres or $7 \cdot 10^{11}$ psi). (For a phosphatidylcholine preparation containing 25% of water the corresponding value is approx. 100 MPa, for example.) On the other hand, even quite small deviations in the relative water pressure relative to the saturation level are seen to suffice for depriving lipids of nearly 50% of their water of hydration [12,21].

Fig. 2 combines the information pertaining to the skin humidity gradient [10] with the osmotic force data [14]. It shows that an extended lipid membrane is likely to feel a significant pressure gradient between the skin surface and the deeper skin layers. The resulting pressure difference may create a force on the order of $F \cong \pi r_v^2 \cdot 10^5$ Pa $\approx 10^{-11}$ N per vesicle with a radius of $r_v = 60$ nm (or $\geqq 10^{12}$ N per mol of vesicles). Force of this strength should provide enough drive to push a large number of suitably devised lipid vesicles through the intact stratum corneum and into the epidermis, under appropriate conditions at least. This force, according to our estimates, is also sufficiently strong to push essentially all lipid vesicles into the outer skin layers – but not beyond stratum corneum. The general condition for such an osmotically driven mass flow is that the number and size of carriers is sufficiently high to bridge the skin surface and the deep skin layers as only then the strong overall transdermal and not just the much smaller local water concentration gradients are felt.

In order to check whether or not this hypothesis is correct we have determined the efficiency of permeation of various lipid vesicles into intact skin. We have used standard lipid vesicles, liposomes, and also a new type of lipoid carriers with an extraordinary high permeation capability, so called transfersomes. The latter were developed in our laboratory within the framework of our concept of rational membrane design and will be described in detail separately (Cevc, to be published).

To follow the fate of dermally applied transfersomes the distribution of radioactive tritiurated dipalmitoylphosphatidylcholine in various tissues and the appearance of radioactivity in the blood compartment were measured. For each experiment two NMRI mice were used. Animals with a mean body weight of 30 g were fed ad libitum, had a free access to water, and were kept in groups of three in standard laboratory cages. One to three days before each experiment an area of approx. 1×1 cm on the back of each experimental animal was plucked free of hair. Lipid suspension was then applied on this area by using a micropipette. The application site was either covered immediately with a small piece of wetted cotton followed by a gas-tight piece of plastics (occlusive application) or else the application site was permitted to dry out for approx. 30 min and was then covered with a piece of dry cotton material (open application).

* Even after prolonged bathing the original state of humidity is nearly restored within less than an hour, the final water concentration in the outer skin strata after a full bath being frequently lower than prior to the skin exposition to an excess of water. This is owing to fact that the water-soluble moisturizers are washed-out during a bath [15].

flexible lipos
⇒ 0.1 mg lipid/h/cm^2 5×10^7 Pt for $r = 100$ nm
⇒ flux in range of few mg/h/cm^2

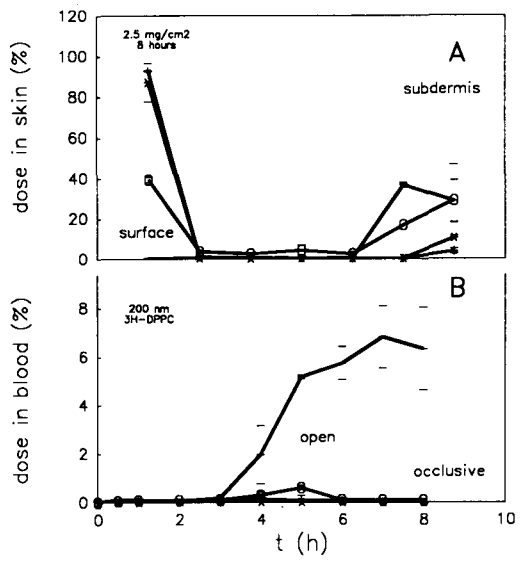

Fig. 3. (A) Distribution of the vesicle-associated radioactive lipid marker molecules in different skin layers after a dermal application of 2.5 mg lipid per cm^2 in an open (×, •) or occlusive patch (○, *). The first values correspond to the skin surface; the following 5 data points pertain to the consecutive strippings of stratum corneum layers and the last to the remaining, deeper skin tissues. All vesicles had a diameter between 130 and 230 nm. They were prepared by several different techniques and typically consisted of one or a few lipid bilayers. The major lipid component ($\geq 80\%$) was always soybean phosphatidylcholine (S100, Lipoid KG, Ludwigshafen); composition and relative abundance of other lipids was varied from preparation to preparation in a controlled manner. Tritium-labelled 1,2-dipalmitoyl-glycero-*sn*-phospho[*N-methyl*-^{3}H]choline (^{3}H-DPPC) was from Amersham Buchler (UK). (B) Serum concentration of the vesicle-associated radioactive lipid marker as a function of time after a dermal application in mice. After an occlusive application only negligible levels of ^{3}H-DPPC radioactivity are detected in the blood compartment; in contrast to this, an open application of the corresponding lipid suspension leads to markedly higher systemic radioactivity levels.

Blood samples ($\approx 20\ \mu l$) were taken at given intervals by a tail puncture; skin specimen were obtained by stripping the application area five times with a scotch-tape. The remaining skin was subsequently excised with a scalpel. 'Surface' values give the radioactivity count obtained from the covering material.

All manipulations on animals were done under general anaesthesia performed with a mixture of Ketavet and Rompun (0.3 ml per mouse of an isotonic NaCl solution containing 0.28% Rompun (Bayer) and 2.5 mg/ml Ketavet (Parke-Davis)).

Skin penetration by lipid vesicles. Results of animal studies with transfersomes applied under different conditions are summarized in Fig. 3.

After 8 h, $80 \pm 15\%$ of the dermally applied lipid material is recovered from the very skin surface when standard liposomes are used. Up to 25% of the total dose is associated with stratum corneum; we never find more than a few percent of the applied lipid material in the deeper epidermal layers, however, when standard liposomes are used.

A similar situation is encountered after occlusive transfersome applications. For transfersomal carriers of two different compositions (to be published), in a typical experiment, 93 and 87% of the total applied dose were found to reside at the very surface of stratum corneum even 8 h after dermal application. In contrast to the situation with standard liposomes, however, the remaining lipid mass was detected in the outer skin layers at concentrations of 0.5 to 1% per skin strip. This corresponds to an estimated total uptake of 0.05 mg per cm^2. Deeper skin regions in the application region contained 3.9 and 10% of the applied lipid mass, respectively.

The effectiveness of the skin penetration by suitably designed lipid vesicles was dramatically different in case of non-occlusive application. Transfersomal preparations left to 'dry' out at the skin surface were found to bring great quantities of applied lipids in the deeper skin layers; the radioactivity counts detected in stratum corneum were somewhat lower. Even in the worst case, less than 50% of the total applied dose remained confined to the very skin surface. Depending on the details of each particular transfersomal preparation and application, $30 \pm 10\%$ of the applied lipids were found in subdermis (not shown), the specific value in both present cases being 29% (cf. Fig. 3A).

Systemic availability of lipid vesicles. A striking feature of an open transfersome applications on murine skin are the relatively high doses found in the blood circulation. Already 30 min after a dermal application moderate blood levels, on the order of 0.1 to 0.5%, of the radiactively labelled lipids are detected. The biggest increase in the exogenous lipid concentration in the blood compartment normally is observed between and 6 h after a dermal transfersome application. After 8 h we measure transfersome blood-dose levels of 0.6 and 6.8%, respectively, when preparations with a high penetration capability are used. The highest systemic values observed after occlusive applications in the present case were 0.14 and 0.03%, respectively.

Our study of the biological fate of the lipid vesicles which can penetrate through intact skin under various conditions has shown that transdermal lipid transport may occur spontaneously provided that the vesicles are exposed to a dehydration force resulting from an osmotic gradient between the skin surface and the deeper skin tissues.

Chemical gradients, which upon a topical lipid application are created between the skin surface and more deeply located skin strata, do not provide a driving force strong enough to take macromolecules through the skin permeability barrier. In our case, the practical insignificance of such gradients was inferred from the following observations and considerations.

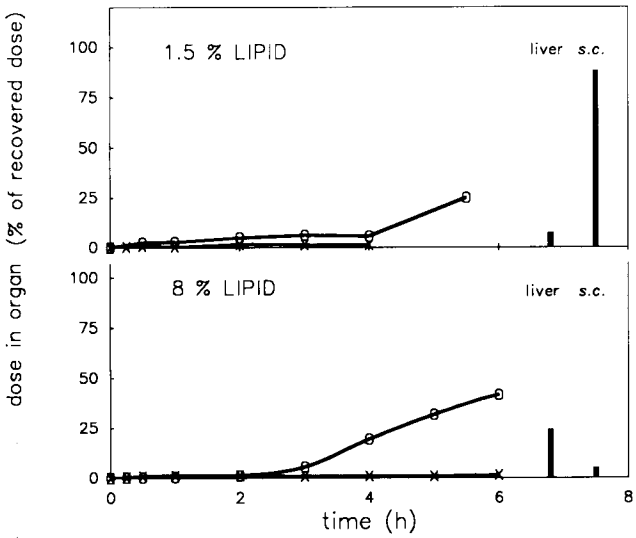

Fig. 4. Systemic availability of the vesicle-associated lipids as a function of the time after a dermal application of 8 or 1.5 mg cm^{-2} of lipids in the form of transfersomes (upper curves) or standard liposomes (lower curves). Results represent means of two independent experiments with NMRI mice; tritium-labelled phosphatidylcholine was used as a marker throughout. Within less than 8 h after a dermal drug-carrier application a significant proportion of the applied transfersome dose, but not of standard lipid vesicles, has passed the skin permeability barrier. Concentration changes affect the overall transport efficiency and the time of onset of permeation but influence only little the speed of transfersomal carrier penetration. Absolute values are given in relative units and therefore are higher than those shown in Fig. 4. Experimental details are given in the legend to Fig. 3A.

If the transdermal drug or carrier gradients, Δc, were important, one would expect material flow into the skin, j, to be proportional to the total applied nass, since $j \propto \Delta c$. Measurements illustrated in Fig. 4 suggest, however, that the speed of lipid penetration into skin, once it has started, depends only little on the starting lipid concentration at the skin surface. The uptake of dermally applied lipid is similarly rapid for both lipid concentrations tested; only the lag between the application time and the first appearance of lipid markers in the blood circulation are concentration dependent. A more detailed account on this will be published separately.

In agreement with this we observe that an elimination of the transdermal hydration gradients is detrimental for the lipid transport across an intact skin; in fact, occlusive application abolishes the penetration of lipid vesicles altogether. At the best, a few percent of the radioactively labelled lipids are recovered in the outer skin layers after an occlusive vesicle application and serum concentrations of the exogenous lipids never exceed 0.1%. Occlusive application or topical use of the concentrated hydrogels thus are likely to prevent drug action in the skin depth whenever drugs have been applied on the skin surface with the aid of lipid vesicles.

Conversely, lipid vesicles applied in an open patch or an open droplet can penetrate both into and through the intact outer skin layers. Depending on transfersome composition and dose applied, in our hands between 50 and 90% of the dermally deposited lipids are found beyond the level of stratum corneum; furthermore, between 1 and 10% circulate in blood 8 h after the beginning of an application. Notwithstanding this, it is possible to reduce the penetration of the lipoidal drug carriers through an intact skin even in the case of open applications provided that transfersomes with a diminished penetration capability are used.

We believe that lipids in the transfersomal form are transported through the intact skin chiefly in the form of lipid aggregates, such as vesicles. Several lines of evidence speak in favor of such conclusion. Firstly, it is unlikely, that during a period of 8 h an appreciable proportion of lipids would be degraded by the skin cells. The characteristic life-time of lipid-turnover in phagocytes is on the order of 6 h so that in an experiment lasting for 8 h too little time is available for the massive liposome degradation and subsequent monomeric skin permeation in the metabolically far less active outer skin cells. Secondly, the permeation effectiveness of the lipid-detergent mixed micelles is lower than in the case of transfersomes (not shown). Thirdly, some of the lipid-associated radioactivity after a dermal application is recovered in the liver which is known to accumulate particles and vesicles but not simple lipid molecules (to be published).

These findings, taken together, vindicate our conclusion that the lipid capability for a spontaneous skin penetration via lipid vesicle transport results primarily from the transdermal hydration gradients [10] and from the high lipid hydration energy [16]; the fact that lipids form extended structures or aggregates also plays some role. Lipid hydrophilicity leads to xerophobia, the tendency to avoid dry surroundings, and causes carriers sitting near or at the skin surface to resist to dehydration. In order to remain maximally swollen transfersomes near the skin surface thus try to follow the local hydration gradients and thereby get into deeper – and better hydrated – skin strata. This causes transfersomal carriers to retract from the relatively dry skin surface and to get into more humid regions in the deeper skin layers. Spontaneous drug-carrier and drug transport in the desired direction, from the dry skin surface toward the better hydrated skin interior, may result from this.

A prerequisite for the success of directed locomotion of dermally applied carriers is that the driving force is greater than the carriers-resistance to the penetration through an intact skin. Such resistance mainly arises from the need of a carrier particle to pass

through the series of narrow, lipid filled interstices in the stratum corneum region. A vast majority of liposomal preparations – including all standard liposome formulations tested in our laboratory – do not fulfill this criterion. On the contrary, standard lipid vesicles tend to clog the few transport pathways in the outer skin layers after having penetrated the top layers of stratum corneum.

Standard vesicles made from phosphatidylcholine of higher than 90% purity, for example, in our laboratory were repeatedly found to be nearly incapable to overcome the skin permeability barrier (cf. Fig. 4, lowest curves). After a dermal application of such drug carriers we routinely recover nearly all of the lipid-associated radioactivity in the cotton-cloth covering the application site; the residual dose of up to 25%, but normally less than 10% (corresponding to 0.25 $mg\,cm^{-2}$), is found in the outer stratum corneum layers, by and large. Similar observations have been made by other authors who have studied penetration of dermally applied traditional liposome formulations prepared from the common biological [17,18] or skin lipids [19,20]; typically, only a few percent of the applied lipids in such cases were found to have penetrated the skin to any depth and have mediated some biological activity.

The energetic cost of drug-carrier penetration through an intact skin depends on the carrier as well as on the skin characteristics. Skin with a partly damaged or very thin stratum corneum may be better suited for the topical drug applications. This should hold for the majority of hydrophilic or lipophilic therapeutics applied in the form of transfersomal preparations. Wet or strongly macerating skin, on the other hand, can be expected to be less suitable for the drug transport by using lipid vesicles. We believe, however, that it is possible to compensate for this variability at least partly by using rational membrane design. By using such conceptual approach optimal types of transfersome and/or appropriate application dose and form can be determined, however.

In summary, transfersomes, but probably only a few or no other types of lipid vesicles, can be used for the noninvasive percutaneous delivery of drugs into deep skin regions. Transfersomes provide a drug developer with a freely movable carrier which has its own, non-metabolic means of locomotion through the partly hydrated skin layers. To date, we have used transfersomal carriers for the transport of insulin [21], corticosteroids (to be published), and analgesics [22] through the intact human skin. It remains to be seen how fast our approach will find its way into medical practice.

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