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Penetration of spin-labeled retinoic acid from liposomal preparations into the skin of SKH1 hairless mice Measurement by EPR tomography

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Summary

13-*trans*-Retinoic acid (vitamin A acid) was spin-labeled by forming its carboxylamide with 3-aminomethylproxyl. The spin-labeled derivative is slightly more hydrophobic than the original retinoic free acid (log $p = 1.35 - > 1.52$). The substance was incorporated into liposomes prepared from various phospholipids including an archaebacterial tetraether lipid, and from phospholipid mixtures. With these liposomes pharmaceutical gels were prepared. The penetration of the spin-labeled retinoic acid from these liposomal gel preparations into the skin of hairless mice was determined by means of EPR imaging and compared to that from a non-liposomal hydrogel and from a fatty ointment. A new EPR imaging of the penetration of the spin label into the skin of hairless mice is presented. The advantage of a liposomal gel preparation for the penetration of spin-labeled vitamin A acid is demonstrated.

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Abbreviations: 3DOCH, 3-doxyl-cholestane; MOSS, modulated simultaneous scan; MPL, main phospholipid; OHAP, oleoyl hydrolysed animal protein; PL 90, soy lecithin phospholipid; 5SASL, 5-doxyl-stearic acid; 16SASL, 16-doxyl-stearic acid; THF, tetrahydrofuran; TLC, thin-layer chromatography; VAA, vitamin A acid (= retinoic acid); VASL, vitamin A acid spin label.

Introduction

all-*trans*-Retinoic acid is an effective therapeutic agent, which has been used for many years in the topical treatment of comedogenic and papulo-pustular acne, because it loosens the adhesion of corneocytes and induces proliferation of the follicular epithelium (Orfanos et al., 1987). Therefore, its potential efficacy in the treatment of actinically damaged skin and photoaging has

recently been proposed (Weiss et al., 1988). On the other hand, irritation with redness and inflammation with scaling have been observed, followed by pustular eruption after 2–4 weeks of topical application (Orfanos et al., 1987). Topically applied, retinoic acid has proven to have the same effectiveness at 10-times lower concentrations from liposomal systems as compared to alcoholic solution. The diminution of the effective concentration in liposomal systems furthermore implies reduction of side effects (Meybeck et al., 1987). Previous studies on the percutaneous penetration of retinoic acid from various pharmaceutical preparations were accomplished with radiolabeling (Connor et al., 1985; Lehman et al., 1988; Franz and Lehman, 1989), however, no data exist on the penetration of the drug from liposomes. This paper reports on percutaneous penetration of spin-labeled retinoic acid from variously composed liposomes in comparison to traditional pharmaceutical preparations. The liposomal constituents were soybean lecithin (PL 90), cholesterol, a patented oleoyl/protein hydrolysate (OHAP), and the main phospholipid (MPL) from the archaebacterium *Thermoplasma acidophilum*. The latter is a tetraether lipid of membrane spanning extension which forms stable liposomes.

For detection the method of EPR imaging was used. EPR is a spectroscopic method for investigation of paramagnetic species in biological tissues.

EPR imaging allows spatial resolution of paramagnetic centers in different tissue planes, and combined with modulated field gradients this method provides the possibility for differentiation of EPR spectra in selected volume compartments.

Materials and Methods

Chemicals

13-*trans*-Retinoic acid (tretinoin) was obtained from BASF, Ludwigshafen (Germany). 3-Aminomethylproxyl spin label and *N*-ethylmaleimide were purchased from Sigma, Deisenhofen (Germany). Carbonyldiimidazole, Tris and cholesterol were products of Merck, Darmstadt (Germany). Soy bean lecithin (PL 90) was obtained from Nattermann, Köln (Germany) and Carbopol 940 from Goodrich, Neuss (Germany).

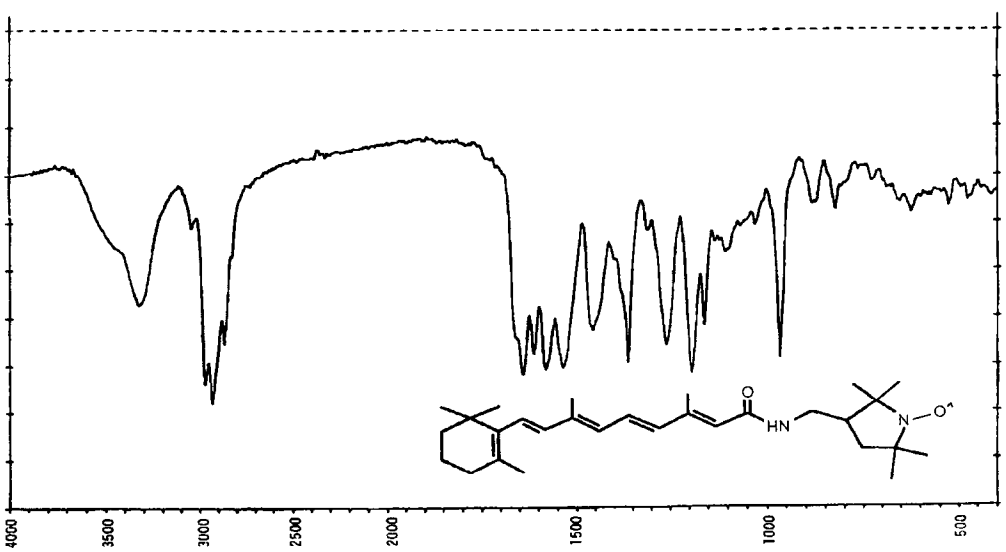

Fig. 1. Formula and infrared spectrum (IR scan 4000–400 cm^{-1}) of retinoic acid proxylamide. The formation of the amide was characterized by shifts of the bands in the 3300 cm^{-1} and in the 'fingerprint' (1650 cm^{-1}) areas.

Oleoyl hydrolysed animal protein (OHAP) was purchased from Henkel, Düsseldorf (Germany). All other chemicals were obtained from Merck or Sigma, all chemicals were the purest grade available.

The main phospholipid from *T. acidophilum* (MPL) was purified from the archaebacterium as described elsewhere (Langworthy, 1977). Growth of the bacterium has been described previously (Blöcher et al., 1984).

Spin labeling of retinoic acid (= vitamin A acid, VAA) was accomplished by synthesis of the carboxylamide with 3-aminomethylproxyl:

125.3 mg of retinoic acid and equimolar concentrations of carbonyldiimidazole (69.7 mg) were heated at 60°C for 30 min in 2 ml of absolute tetrahydrofuran (THF) in a 3 ml coloured glass reaction vessel under a nitrogen atmosphere. After addition of a solution containing 75 mg (= 5% molar excess) 3-aminomethylproxyl in 0.75 ml absolute THF the reaction vessel was closed under nitrogen with a septum and stirred at 60–70°C for 50 h. The volume of the reaction mixture was reduced with a stream of nitrogen to about 1 ml and the residual yellow solution was transferred to two preparative thin-layer chromatography (TLC) plates (SiO_2 60/2 mm, 20 × 20 cm, Merck, Darmstadt). The chromatogram was developed with diethyl ether in order to separate the spin-labeled retinoic acid from unreacted material. The former was removed from the plate by scraping, transferred to a glass column (d = 1 cm) and eluted from SiO_2 with ethyl acetate. The elution medium was removed, yielding 140 mg (= 71.8%) of *N*-(3-proxylmethyl)retinoamide (VASL).

The control of purity of the product was accomplished by means of TLC towards the original and intermediate compounds. The infrared (IR) spectrum of the spin-labeled amide is shown in Fig. 1.

Octanol/water partition coefficients (from 1 mM concentration) were determined photometrically according to a modified method of Papahadjopoulos et al. (1975) and by EPR spectroscopy according to Fuchs et al. (1989), the latter method only being possible for paramagnetic compounds. Octanol/buffer (50 mM phosphate, pH 7.0) partition coefficients are given in Table 1.

Preparation of liposomes and gels

Ethanolic solutions of the lipid and of spin-labeled retinoic acid were mixed in a suitable round flask. After removal of the ethanol, the remaining lipid film was dispersed in purified water yielding large multilamellar vesicles (LMV) loaded with the spin-labeled retinoic acid at 2 mM concentration. The LMVs were reduced in size and homogenized by means of a French Pressure Cell (four cycles; 20 000 lb/inch^2). Under these conditions, small unilamellar liposomes are formed with diameters less than 100 nm (Table 2).

The preparation of the MPL liposomes was accomplished as described above except for the buffer: 150 mM sodium chloride/10 mM sodium phosphate, pH 7.4.

The gels were prepared by strewing a mixture of carbopol/Tris onto the liposomal suspensions and after a swelling period a gel was obtained. The preparations were carried out and stored in coloured glass vessels under light protection.

The liposomal particle size was determined by means of laser light scattering in a Malvern Autosizer IIc from the dispersions and in the gel

TABLE 1

Partition coefficients of aminomethylproxyl spin lable, retinoic acid, and vitamin A acid spin label (VASL)

	Aminomethylproxyl	Retinoic acid	VASL
$P_{oct/buff}$	P_{332} = 1.8	P_{351} = 22.2	P_{332} = 33.3
Log P_{phot}	0.26	1.35	1.52
Log P_{EPR}	0.42	n.d.	2.75
Log $P_{HoffmannLaRoche}$		2.5	(personal communication)
Log P_{Hansch}		6.61	(Hansch, 1990)

TABLE 2

Characteristics of the liposomal gels

Lipid	% (w/w) in gel	Concentration of label (mM)	Particle size (nm)
MPL	2	2	95.6 ± 20.7
PL/chol.	10 (8:2)	2	48.7 ± 15.0
PL/OHAP	10 (8:2)	2	70.0 ± 23.0
PL/OHAP	10 (8:2)	20	44.8 ± 14.0

MPL, main phospholipid from *T. acidophilum*; PL/chol, soy lecithin/cholesterol; PL/OHAP, soy lecthin/oleoyl hydrolysed animal protein.

preparations. In the latter case, the gels had to be sufficiently diluted.

The final concentration of lipid was 100 mg/ml, with MPL being 20 mg/ml. The concentration of gel-forming substances was 1%. The preparations contained 2 mM of spin-labeled retinoic acid.

For comparison, a non-liposomal hydrogel preparation containing 1% of carbopol 940 and unguentum emulsificans aquosum DAB9 with the same amount of spin-labeled retinoic acid were used. The latter substance was incorporated into the gels from an ethanolic solution. These preparations contained 2 mM of the spin-labeled substance and 10% of ethanol.

For further measurements with higher resolution in EPR imaging (Fig. 5) two preparations containing non-therapeutic concentrations of 20 mM spin-labeled retinoic acid were prepared.

Animals

SKH1 euthymic hairless female mice (6–8 weeks old, weighing 30 g) were purchased from Charles River Wiga GmbH, Sulzfeld (Germany). They were fed by standard nutrition and had free access to drinking water. The animals were euthanized by CO_2 inhalation and decapitated. The skin was removed, rinsed with PBS and adhesive connective tissue was carefully scraped off. The skin was layered in a moisture chamber at 4°C and was used for EPR measurements within 6 h after excision. In order to inhibit spin label biotransformation the skin was pre-incubated with *N*-ethylmaleimide (NEM) at 30 mM concentration.

EPR imaging

The method of spectral spatial EPR imaging with modulated field gradient and simultaneous field scan (MOSS = modulated simultaneous scan) was applied. This technique allows two-dimensional resolution of paramagnetic signals in the skin. The first dimension is the spectral resolution of a paramagnetic species in a defined volume compartment and the second one displays the spectra as a function of location. Modulated magnetic field gradients can separate a small region within a sample which can be investigated spectroscopically by additional sweeping of magnetic field (Herrling et al., 1992). At a microwave frequency of 9.5 GHz (X-band), two-dimensional images are obtained from 0.6–0.8 mm thick skin biopsies by registration of the second derivative of the EPR spectra by means of an ERS 221 constructed in the Forschungsstelle für ortsauflösende Meßtechnik e.V. in Berlin.

Instrumental settings

Instrumental settings were as follows: B_0 field magnitude, 339.0 mT; microwave frequency, 9.5 GHz; microwave power, 50 mW; modulation frequency, 50 kHz (second derivative); modulation amplitude, 0.15 mT; gradient frequency, 70 Hz; field gradient, 4 T/m; scan range, 5 mT; spectral spatial matrix, 128×128; spatial resolution, 25 μm; scan time for 2D images, 4 min.

Application of the gels and consecutive EPR imaging

6 mm biopsies of the prepared skin were incubated with the respective pharmaceutical preparations for 5–60 min. It is essential to ensure that the spin label-containing gels indeed penetrate through the stratum corneum and do not flow along the rim of the skin biopsy. After incubation, the gels were wiped off and the skin was cleaned with PBS in order to remove any residual spin-labeled retinoic acid from the skin surface. The biopsy was placed into a tissue cell and adjusted perpendicular to the magnetic field gradient. To obtain an image in a 128×128 array

the spectra of 128 points were measured for 128 successive spatial planes. Therefore, the zero plane of the modulated field gradient is shifted by a small distance which defines the spatial pixel resolution. For every plane an EPR spectrum was measured and all data were written into a two-dimensional 128×128 matrix and calculated on a PC.

Results

Characterization of the liposomal gels

Data of the investigated preparations and their lipid compositions are shown in Table 2.

The formation of small unilamellar vesicles from lecithins and from the tetraether lipid (MPL) with the French pressure cell method was demonstrated by freeze-fracture electron microscopy in the laboratory of Dr B. Sternberg, Jena, Germany. The results will be published in a forthcoming paper.

Order parameters of the liposomal membranes as obtained from the spin labels 5-doxyl- and 16-doxyl-stearic acids (5-SASL and 16-SASL) and 3β-doxyl-5α-cholestane (3DOCH) are listed in Table 3.

The EPR signals of the VASL from ethanolic solution and after incorporation into PL/OHAP liposomes are shown in Fig. 2 in order to demonstrate the immobilisation of the molecule within the membrane.

Penetration into the skin

Penetration profiles of the spin-labeled retinoic acid from different preparations are depicted in Fig. 3.

TABLE 3

Order parameter (S)

Lipid	5SASL	16SASL	3DOCH
MPL	0.848	0.258	0.932
PL 90	0.644	0.187	0.665
PL/OHAP	0.619	0.091	0.344

MPL, main phospholipid from *T. acidophilum;* PL 90, soy lecithin; PL/OHAP, soy lecithin/oleoyl hydrolysed animal protein.

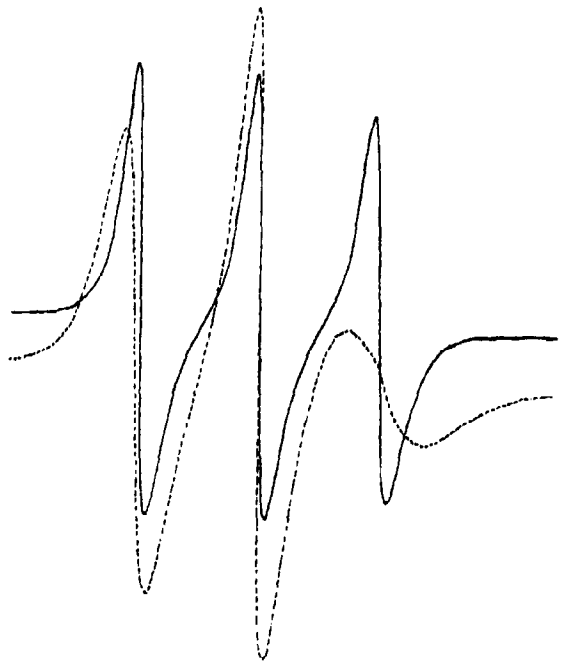

Fig. 2. The EPR signals (first derivative) of the retinoic acid spin label (VASL) in ethanolic solution (solid line) and after incorporation into PL/OHAP liposomes (dashed line).

The spin-labeled retinoic acid was administered at a concentration of 2 mM which is the generally used therapeutic level in non-liposomal preparations. The greatest amount of labeled drug penetrating into mouse skin was found to be from the liposomes composed of soybean lecithin/OHAP (oleoyl hydrolized animal protein), i.e., more than twice as much as compared to all the other preparations.

TABLE 4

The order of penetration after 45 min

Lipid/gel system	Arbitrary EPR signal intensities
Soybean lecithin/OHAP gel	(390)
Hydrogel	(166)
MPL gel	(149)
PL/chol gel	(141)
Ung. emuls. aquos.	(125)

After 60 min the EPR signal intensity from the MPL preparation was slightly higher (190) than that from the hydrogel (180) (not shown). The values in parentheses are arbitrary EPR signal intensities (Fig. 3)

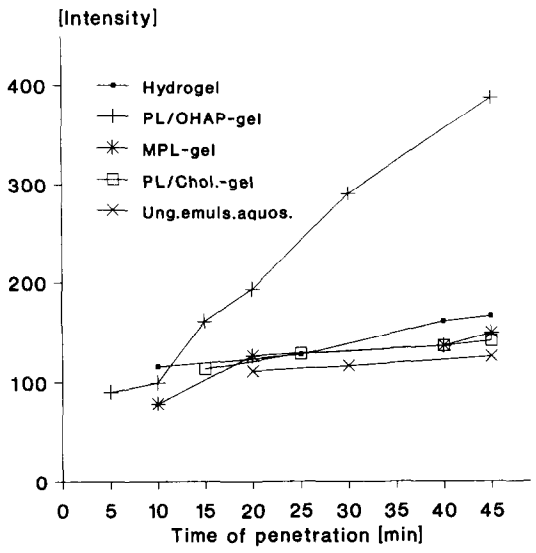

Fig. 3. Penetration profiles of spin-labeled retinoic acid (VASL) from 2 mM liposomal and non-liposomal preparations.

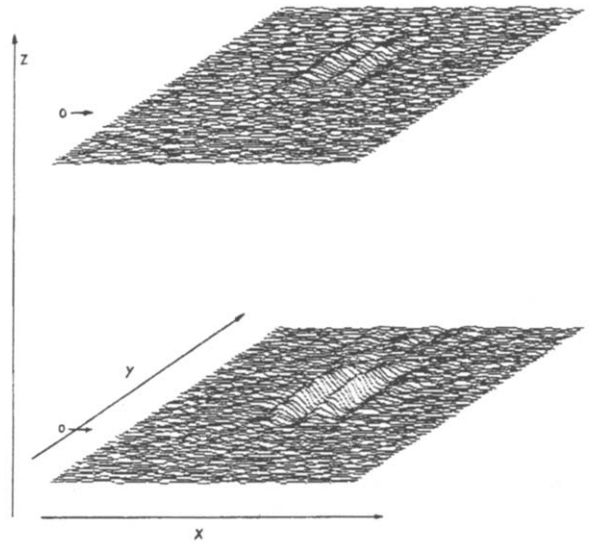

Fig. 4. Three-dimensional imaging of the penetration of retinoic acid spin label (VASL, 2 mM) from the liposomal PL/OHAP gel after 15 and 30 min. *x*-axis, magnetic field sweep; *y*-axis, depth of the skin; '0' denotes the epidermal surface; *z*-axis gives the concentration (signal height).

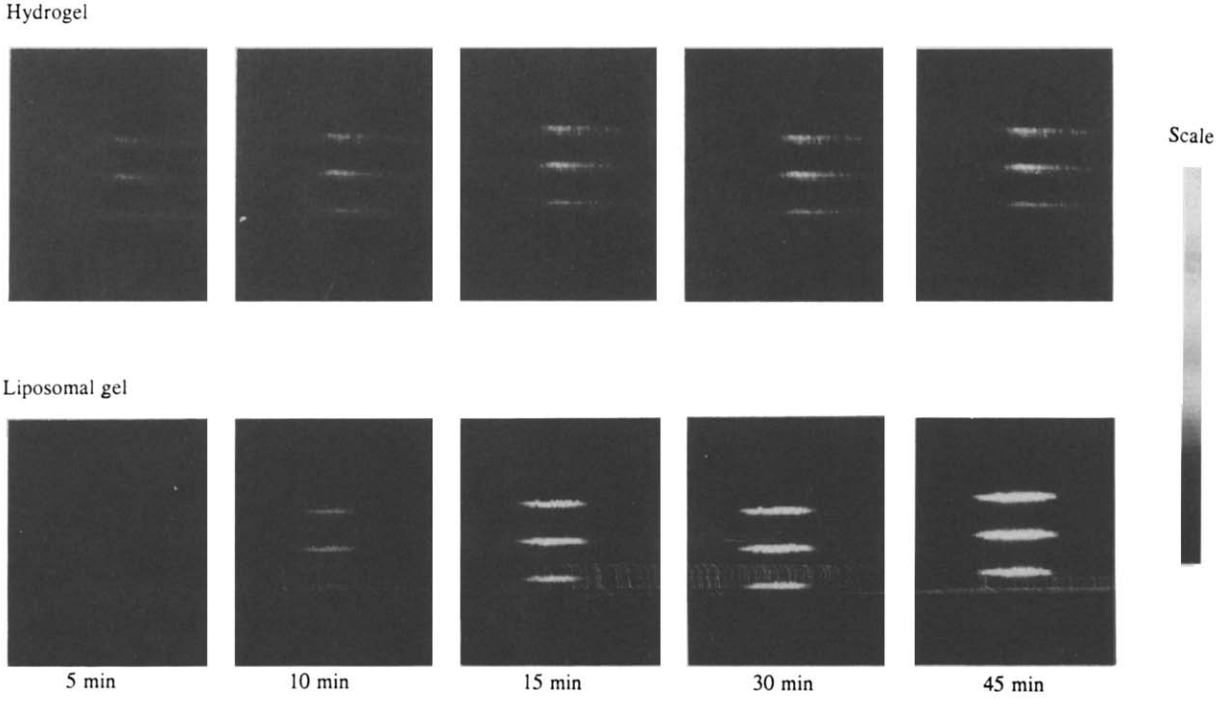

Fig. 5. Penetration of spin-labeled retinoic acid from the hydrogel (upper panel) and from the PL/OHAP-liposomal gel. The scale gives the correlation between the brightness of the signal and the concentration of the spin label (dark = background = zero concentration).

Imaging

Fig. 4 depicts the presentation as previously published for other spin labels (Fuchs et al., 1991). The h_{-1} bands are scarcely detectable in Fig. 4. In the two-dimensional presentation the amount of spin label penetrating into the skin can roughly be evaluated from the difference between the background and signal intensities. The three-dimensional presentation images the penetration into the skin (y-axis), whereas the z-axis denotes the concentration (= heights of the signal bands). The x-axis shows the magnetic field scan. Fig. 4 demonstrates the penetration of the spin label, 2 mM, from PL/OHAP liposomes after 15 and 30 min.

Penetration into the skin from the hydrogel and from the PL / OHAP liposomes, both preparations containing 20 mM spin label concentration

Fig. 5 presents the imaging with background tuning and scaling of the brightness of the signal bands (h_{+1}, h_0, h_{-1}). The upper series demonstrates the penetration of the spin label from the hydrogel after 5, 10, 15, 30, and 45 min. The lower series illustrates the penetration into the skin from the PL/OHAP liposomal preparation.

The penetration from the liposomal system is significantly more intensive than from the hydrogel, i.e., the signal intensity from the liposomal system is much greater. However, the depth of penetration does not appear to be increased as compared with the hydrogel (Fig. 5). This is also confirmed by the results shown in Fig. 4: there is a rapid penetration of spin-labeled retinoic acid into the stratum corneum and the epidermis, but obviously no further penetration into deeper layers within 45 min.

Discussion

Measurement of octanol / water coefficients

The photometric measurement of octanol/water coefficients according to Papahadjopoulos et al. (1975) had recently been applied with phenothiazines and tricyclic antidepressants (Freisleben and Zimmer, 1991). The equation $p = ((C_0 - C_x)/C_x) \cdot f^{-1}$ gives well-reproducible results. C_0 denotes the initial concentration in the buffer and C_x the final concentration in the buffer after shaking; f is the octanol/buffer volume ratio, if differing volumes are necessary.

For evaluation of the EPR signals the integrals of the area of the low field peak of the first derivative were divided by the respective amplification factors (= signal gain). The quotient (p) was calculated from these values.

The EPR method gives reproducible results as well, however, they do not correlate with the photometric determinations. Partition coefficients for spin-labeled tretinoin were one magnitude lower ($\log p = 1.5$) from photometry than from EPR spectroscopy ($\log p = 2.75$). From Hoffmann-LaRoche a value of $\log p = 2.5$ was given for tretinoin (personal communication) which may compare to our EPR value for retinoic acid proxylamide. If so, this means that retinoic acid proxylamide is slightly more lipophilic than retinoic acid itself. The same correlation was obtained from the photometric measurements (although at level one magnitude lower). Log p increases from 1.35 to 1.52 if retinoic acid is spin labeled.

Thus, in spite of the general differences between the two methods, the topic of interest in our context is evident. As could be expected, retinoic acid proxylamide is slightly more lipophilic than retinoic acid, however, the octanol/buffer partition coefficients do not differ drastically.

On the other hand, the discrepancies between different methods for determination and evaluation leaves skepticism towards the methods applied and especially towards absolute values published so far in the literature. The highest value we found was $\log p = 6.61$! for retinoic acid (Hansch, 1990). Hence, comparison of the different methods and new evaluation by means of a greater series of compounds appear necessary. However, this would be beyond the scope of this paper and should be the topic of a separate investigation.

From the lipophilic properties of spin-labeled retinoic acid it can be expected that it will insert into or at least associate with the hydrophobic liposomal membrane (Wasall et al., 1988).

EPR imaging

A recent publication on the comparison and evaluation of the penetration from liposomal systems into the skin was carried out with one-dimensional EPR imaging (Gabrijelcic et al., 1990). A hydrophilic spin label was used as a model compound which is not a pharmaceutical drug. In our case, vitamin A acid was the matter of investigation in different systems, which makes the study more closely related to pharmacology.

Furthermore, the above authors used the skin of pig ears. This is another animal model apart from the skin of hairless mice, both being suitable as models for human skin. From their 1D EPR imaging data, Gabrijelcic et al. calculated diffusion constants for the water-soluble spin label applied in their study. They demonstrated that water-soluble compounds need encapsulation into liposomes from 'fluid' lipids in order to be taken up appreciably into skin.

A lipophilic substance such as vitamin A acid will exhibit some degree of penetration into skin from almost any liposomal or non-liposomal gel or ointment preparation. The series determined for penetration demonstrates that liposomes from the soybean lecithin/OHAP mixture enhance the penetration of the incorporated drug more than 2-fold as compared to all other preparations. The lowest penetration occurs from unguentum emulsificans aquosum DAB9 which is certainly due to the relatively high content of vaseline which is known to be a poor vehicle for penetration into skin.

If the penetration from a conventional modern gel (i.e., hydrogel) is taken as 100%, that from the classical ointment unguentum emulsificans aquosum DAB9 will be only 75% and that from the PL/chol liposomal gel only 86%. The penetration from the PL/OHAP liposomal gel will then be over 230%. The penetration from MPL liposomes is about 90% of that from the hydrogel after 45 min and reaches that from the hydrogel after 60 min (103%).

Correlation between the penetration and the order parameters of the liposomes demonstrates that the system with greatest penetration is also the most fluid one which can be seen especially from 3-doxyl-cholestane: the shape of the spectrum is almost isotropic, similar to a solvent spectrum. On the other hand, the very high order parameters of the MPL liposomes reflect very rigid membranes in the polar region (3DOCH, 5SASL), however, the penetration from these liposomes is not worse, or even better than from PL 90 liposomes where the order parameters are lower. Hence, the order parameters correlate with the penetration only for the differences between PL/OHAP liposomes and all the others, but not with the penetration among the latter. Moreover, the size of the particles does not correlate with the penetration.

Conclusion and outlook

(i) An improved imaging of a drug into skin from liposomal and non-liposomal pharmaceutical preparations has been presented. This imaging will – after some further computer calculations – also provide quantitation of the concentration and the depth of penetration according to the scale of brightness in Fig. 5. This opens up the possibility of EPR tomographic presentation as is known from other physical tomography.

(ii) The use and advantage of an archaebacterial tetraether lipid may be questioned in this context. This lipid was applied for several reasons. Firstly, liposomes from this lipid are suitable to study the penetration of intact liposomes into the skin and to locate their decay. This is presently under investigation by means of different methods, i.e., EPR and NMR spectroscopy and electron microscopy. Secondly, tests are being conducted in order to ascertain whether membrane-spanning tetraether lipids can be suitable for stabilization of conventional liposomes, or are even advantageous over cholesterol in this respect.

(iii) It has been demonstrated that OHAP liposomes are advantageous over conventional hydrogel or ointment preparations in intra(epi)dermal administration of a lipophilic drug. The spatial resolution indicates that the liposomally applied compound penetrates at a high concentration into a defined and limited depth within 45 min. From the non-liposomal preparation in Fig. 5 the spin-label signal reaches only a considerably lower intensity, but penetrates at least as far as

the spin-label signal from the OHAP liposomes. Lasch et al. (1991) reported that "intact liposomes are confined to the horny layer and do not penetrate deeper". From the data it can be concluded that retinoic acid penetrates rapidly and intensively to a defined depth of the skin in intact OHAP liposomes. There, the compound may be slowly released from the liposomes (Guy et al., 1983), or further liposomal penetration is reduced. These mechanisms should be the scope of further investigations.

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