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Percutaneous Absorption of Butylparaben from Liposomes *in Vitro*

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Percutaneous penetration of ^{14}C-butylparaben (BP) from a liposome suspension was studied *in vitro* using a flow-through type diffusion chamber. The amount of ^{14}C-BP that penetrated through the skin of guinea pigs from liposome suspension decreased as the lipid content increased. This suggested that BP in the outer aqueous phase mainly contributed to the percutaneous penetration. However, ^{14}C-BP in liposomes was also demonstrated to participate in the penetration to a small extent. Mathematical analysis based on diffusion theory supported these findings.

Substantial entrapment of BP in liposomes was maintained during the period of the *in vitro* experiment (30 h), indicating that the free concentration of BP would be maintained in the donor region after the application of the liposome formulation to it. Furthermore, liposomes induced neither alteration of the barrier function of the stratum corneum nor denaturation of skin proteins *in vitro*. These findings are consistent with a contribution of direct BP absorption from liposomes at the skin surface.

Keywords——percutaneous penetration *in vitro*; liposome; butylparaben; mathematical analysis; barrier function

Introduction

Interactions between liposomes and cells have been widely investigated and can be classified into four categories[1]; *i.e.*, adsorption, endocytosis, fusion and exchange of lipids. Little work has been done, however, on the interactions between topically applied liposomes and the skin. The barrier function in the skin resides mainly in the uppermost layer, the stratum corneum,[2] which lacks nuclei and organella but contains keratin fibers; further, most of the phospholipids are replaced by less polar lipids.[3] These considerations suggest that interactions between liposomes and the skin should be different from those observed in cells.

Recent studies have revealed various effects of liposomes on drug absorption after topical application. Accumulation of triamcinolone acetonide in the skin[4] or "over-concentration" of sodium pyrrolidone-carboxylate in the stratum corneum[5] were reported after the topical application of liposomes. On the other hand, a great decrease in the percutaneous absorption of dihydrotestosterone was observed,[6] probably due to the entrapment in liposomes.

In our previous study,[7] the percutaneous penetration of butylparaben (BP) and dipalmitoylphosphatidylcholine (DPPC) from a liposome suspension was examined *in vivo* by autoradiography. It was found that ^{14}C-BP (carboxyl-^{14}C) penetrated through the skin, whereas ^{14}C-DPPC (dipalmitoyl-1-^{14}C) was scarcely detected in the body, suggesting that the liposomes themselves remained on the skin surface. However, autoradiography did not permit quantitative evaluation of the effect of liposomes on the drug penetration.

In the present study, the percutaneous penetration of BP from a liposome suspension was investigated *in vitro* with a diffusion chamber in order to ascertain whether liposomes increase or decrease drug penetration. The results of the diffusion experiments were analyzed based on

diffusion theory, and the effects of formulations were characterized in terms of parameters relating to diffusion in the skin and distribution of the drug between the vehicle and the skin. The effects of liposomes on the barrier function of the stratum corneum were also assessed from various viewpoints.

Materials and Methods

Chemicals——^{14}C-BP was purchased from Daiichi Pure Chemicals and ^{3}H-DPPC (2-palmitoyl-9,10-^{3}H) was obtained from New England Nuclear. Their specific activities were 1.94 mCi/mmol and 60.0 Ci/mmol, respectively, and they were employed without further purification. Egg phosphatidylcholine (egg PC) was purified from egg yolk according to the method described by Rhodes and Lea.[8] Other chemicals were of reagent grade and were used without further purification.

Preparation of Liposomes——Liposomes were prepared by the hydration method.[7] A lipid thin film in a round-bottomed flask was hydrated with 10 ml of water, vortexed for 10 min and then sonicated for 2.5 min under an atmosphere of nitrogen.

System compositions of liposome formulations tested in this study are listed in Table I. BP was added to chloroform solution before evaporation to the lipid film in system S—V or an aqueous solution of BP was added to empty liposomes in system W—Z. The specific activity of liposome suspension was 1 μCi/ml for ^{14}C-BP or 3 μCi/ml for ^{3}H-DPPC.

Percutaneous Penetration *in Vitro*——The dorsal hair of a male guinea pig (Hartley Strain, average body weight 350 g) was removed carefully with hair clippers. The dorsal skin was excised after sacrifice and the subcutaneous fat was eliminated. The whole skin (epidermis and dermis) was employed for the experiment. The skin was set on a diffusion chamber of a flow-through type (Fig. 1).[9] The donor area was 3.14 cm^2, on which 2 ml of test solution was applied in contact with the epidermal side. The receptor volume was 4.4 cm^3 and the dermal side was continuously

TABLE I. System Compositions

System	Egg PC (mM)	Cho (mM)	DCP (mM)	BP (% w/v)
S	32	16	8	0.1
T	16	8	4	0.1
U	8	4	2	0.1
V	16/3	8/3	4/3	0.1
W	32	16	8	0.004
X	16	8	4	0.004
Y	8	4	2	0.004
Z	16/3	8/3	4/3	0.004

Systems S—V: method A. System W—Z: method C. Cho: cholesterol. DCP: dicetylphosphate.

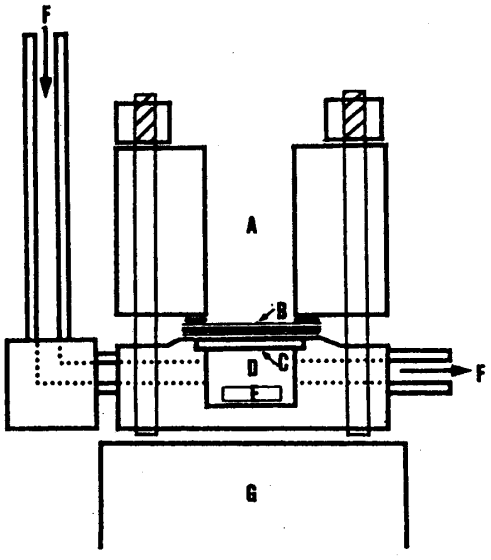

Fig. 1. Apparatus Used for the Measurement of Percutaneous Penetration *in Vitro*

A, donor compartment; B, skin; C, screen support; D, receptor compartment, E, stirring bar; F, flow of the receptor fluid; G, magnetic stirrer.

washed with saline at a constant flow of about 8 ml/h. Four experiments were carried out for each system at 37 °C. The receptor fluid was collected in fractions and the radioactivity was measured with a liquid scintillation counter.

Pretreatment of the Skin with Empty Liposomes——One milliliter of empty liposomes (system T without BP) was applied to the skin in the diffusion chamber. Twenty-four hours after the application, 1 ml of aqueous solution of BP (80 μg/ml) was added to the empty liposomes to make the composition the same as that of system X, and then percutaneous penetration of BP was measured for 30 h. As a control, percutaneous absorption of ^{14}C-BP from system X was studied.

Stripping of the Skin——After removal of the hair with hair clippers, about 10 × 10 cm of guinea pig dorsal skin was excised and the stratum corneum was stripped with Scotch tape® (3M No. 810) fifteen times successively. The surface of the skin acquired a glistening appearance and almost no horny cells adhered to the last few tapes. The stripped skin was mounted on the diffusion chamber and percutaneous penetration was measured.

Denaturation of Skin Proteins by Liposomes——A 50 mg portion of Hide Powder® (Wako) was shaken with 5 ml of test sample at 37 °C for 3 h. Sulfhydryl groups thus formed were reacted with 1-(4-chloromercuriphenyl azo)naphthol-2 (mercury orange). The complex thus formed was extracted with isoamyl acetate and determined by spectrophotometry (OD_{470}).[10]

Mathematical Analysis of Penetration of ^{14}C-BP——For analyzing the drug transfer in a simple manner, the skin was assumed to be a homogeneous plane barrier sheet. The total amount of penetrant Q which penetrates through the homogeneous membrane in time t from the donor solution at constant concentration (C) to the receptor phase under the sink condition is given by

$$Q=AKLC\left(\frac{Dt}{L^2}-\frac{1}{6}-\frac{2}{\pi^2}\cdot\sum_{n=1}^{\infty}\frac{(-1)^n}{n^2}\cdot\exp(-Dn^2\pi^2t/L^2)\right) \quad (1)$$

where A = area of application, L = thickness of membrane, K = partition coefficient of the drug between the skin and the vehicle, and D = diffusion coefficient.[11]

Based on Eq. 1, the diffusion coefficient (D) and partition coefficient (K) were determined by the least-squares method (damping Gauss–Newton method).[12] In this study, the value of A was 3.14 cm^2. In order to simplify the calculation, L was assumed to have a constant value of 0.1 cm.

For analyzing the participation of direct absorption of ^{14}C-BP from liposomes, the value of Q was divided into two fractions corresponding to the absorption from the outer aqueous phase (Q_{free}) and from the liposome ($Q_{liposome}$) as follows;

$$Q=Q_{free}+Q_{liposome} \quad (2)$$

The Q_{free} value for each formulation was calculated by using the value of ^{14}C-BP concentration in the outer aqueous phase determined by ultrafiltration.[7]

The concentration of BP in liposome particles ($C_{liposome}$) was calculated as

$$C_{liposome}=\text{entrapped amount of BP/volume of liposome particles } (V_{liposome}) \quad (3)$$

where the entrapped aqueous volume was employed as $V_{liposome}$.

Results

Percutaneous penetration of ^{14}C-BP from the liposome formulations listed in Table I was studied *in vitro* with a flow-through type diffusion chamber. Figure 2 shows the absorption of ^{14}C-BP from four liposome formulations with different lipid contents. In all cases, the percentage penetration was low and decreased with increase in the lipid content. The penetration of ^{14}C-BP from liposome suspension prepared by mixing ^{14}C-BP aqueous solution with empty liposomes at a final BP concentration of 40 μg/ml is illustrated in Fig. 3.

The changes in total and free concentrations of ^{14}C-BP in the donor phase were examined at various intervals after application of system T in the diffusion experiment. The concentration of ^{14}C-BP in the outer aqueous phase decreased very slowly in parallel with the total concentration, and 92.5% of the initial concentration was still maintained after 30 h.

In order to clarify the absorption mode of the drug from liposomes, percutaneous penetration of ^{3}H-DPPC incorporating into liposomes was examined; the results are illustrated in Fig. 4. The amount of ^{3}H-DPPC transferred through the skin increased with increase in the lipid content, but the degree of accumulation after 30 h remained in the region

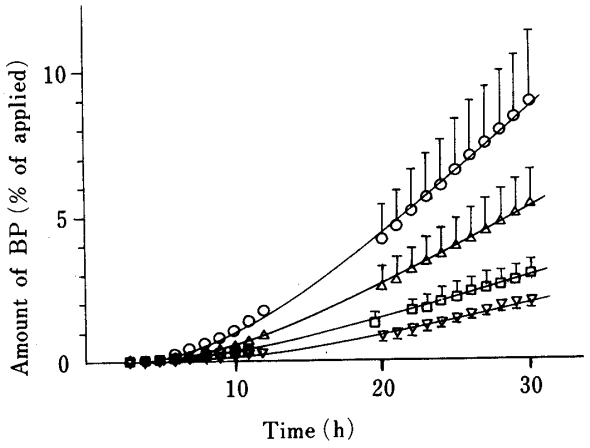

Fig. 2. Percutaneous Penetration of BP from Liposome Suspensions *in Vitro* (Mean ± S.D., $n=4$)

▽, system S; □, system T; △, system U; ○, system V.
The solid lines show the computer-generated curves based on Eq. 1.

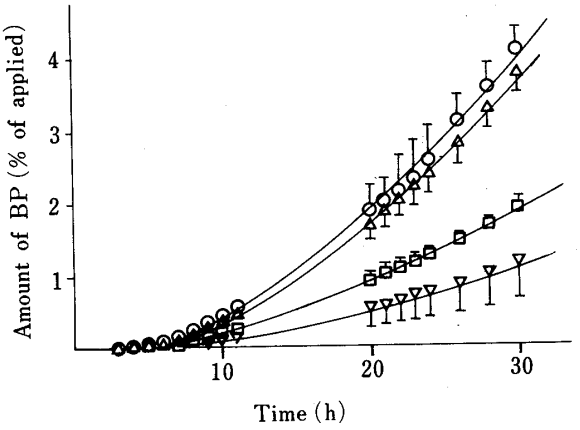

Fig. 3. Percutaneous Penetration of BP from Liposome Suspensions *in Vitro* (Mean ± S.D., $n=4$)

▽, system W; □, system X; △, system Y; ○, system Z.
Solid lines show the computer-generated curve based on Eq. 1.

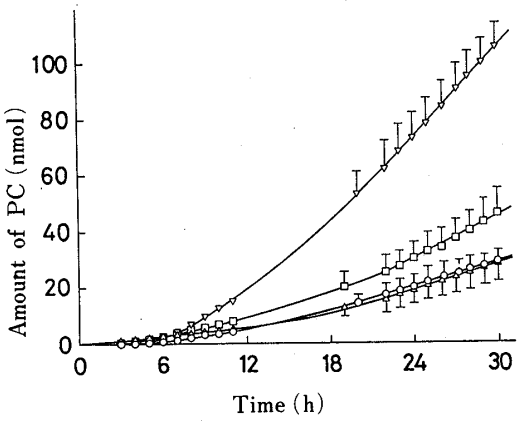

Fig. 4. Percutaneous Penetration of PC from Liposome Suspensions *in Vitro* (Mean ± S.D., $n=4$)

▽, system S; □, system T; △, system U; ○, system V.
The cumulative transferred amounts of ^{3}H-DPPC were 0.16% of the initial dose for system S, 0.14% for system T, 0.18% for system U, and 0.27% for system V, respectively.

of 0.14% to 0.27% of the initial dose for all formulations. Thus the transfer of ^{3}H-DPPC remained at a very considerably lower level than that of ^{14}C-BP. In the case of system T for example, where 96% or 1.92 mg of BP was entrapped in 2 ml of liposomes, only 0.14% of DPPC penetrated in 30 h. Therefore, the contribution of absorption from liposomes as a whole, if any, to the total absorption of BP (58.6 μg for 30 h) was calculated to be only about 4.6%.

Figure 5 shows the effect of pretreatment with empty liposomes on the subsequent penetration of ^{14}C-BP from liposomes. After 24 h pretreatment with liposomes, ^{14}C-BP penetrated the skin with almost the same pattern as in the case without pretreatment.

The percutaneous permeation of ^{14}C-BP applied in the form of liposome suspension was also determined in stripped skin. As shown in Fig. 6, the penetration of ^{14}C-BP was faster in stripped skin than in intact skin.

Figure 7 shows the effect of liposome treatment on the liberation of SH groups from Hide Powder® (Wako). Although sodium dodecyl sulfate, a positive control, liberated more SH groups than the buffer, polysorbate 80 solution did not significantly increase the amount. On the other hand, liposomes showed slightly less liberation of SH groups than that induced by the treatment with buffer.

In order to examine precisely the percutaneous permeation of ^{14}C-BP from liposomes,

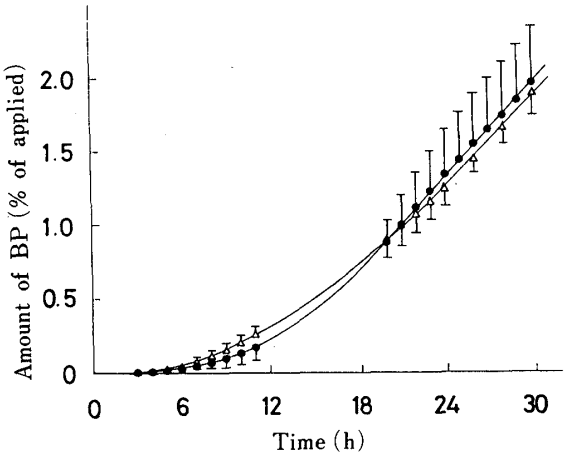

Fig. 5. Effect of Pretreatment with Empty Liposomes on the Percutaneous Penetration of BP from Liposome Suspension (System T) *in Vitro* (Mean ± S.D., $n=4$)

●, pretreatment; △, control.

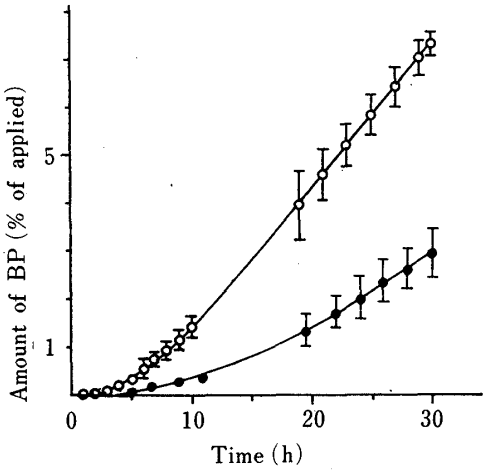

Fig. 6. Percutaneous Penetration of BP from Liposome Suspension (System T) through Stripped Skin *in Vitro* (Mean ± S.D., $n=4$)

○, stripped skin; ●, intact skin.

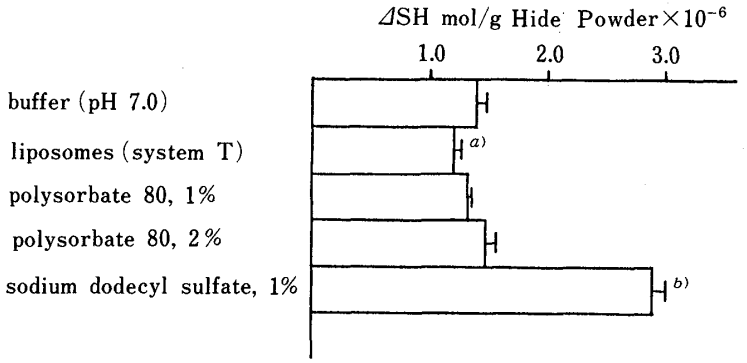

Fig. 7. Denaturation of Hide Powder by Liposome or Polysorbate 80 Solutions (Mean ± S.D., $n=4$)

a) ($p<0.02$) and *b*) ($p<0.001$) indicate significant differences from the buffer.

the results of the diffusion experiments shown in Figs. 2 and 3 were analyzed by fitting to Eq. 1. Table II summarizes the parameters for various liposome formulations obtained by the least-squares method. As shown in Figs. 2 and 3, the computer-generated curves describe the data very well. The parameters summarized in Table II were calculated based on the total concentration of ^{14}C-BP in liposome formulations. All formulations gave similar values of D, while the value of K decreased with increase in the lipid content.

For evaluating the contribution of the direct transport of ^{14}C-BP entrapped in liposome particles, the amount of penetrant transported by this route (Q_{liposome}) was calculated by subtracting the amount of penetrant from unentrapped ^{14}C-BP (Q_{free}) from the total amount of penetrant (Q) for each set of data. In the calculation of Q_{free}, the parameters $K=4.0$ and $D=2.1\times10^{-4}$ cm^2/h were employed based on the data reported previously,[9b] when percutaneous absorption of BP was measured up to 24 h with experimental and animal conditions consistent with those used in the present experiment. Table III summarizes the parameters for liposomally entrapped BP which were calculated by fitting Eq. 1 to the Q_{liposome}–time data. Compared with the K value of free BP (4.0), K values between the skin

TABLE II. Determination of Diffusion Coefficient (D) and Partition Coefficient (K) of Percutaneous Absorption of BP by Computer Simulation

System	BP_{total} (μg/ml)	BP_{free} (μg/ml)	D (cm^2/h, $\times 10^{-4}$)	K
S	1000[a]	15.3	1.125±0.158	0.721±0.026
T	1000[a]	40.0	1.535±0.147	0.622±0.016
U	1000[a]	71.4	1.528±0.135	1.134±0.027
V	1000[a]	132.0	1.626±0.182	1.708±0.053
W	40[b]	0.80	1.619±0.341	0.211±0.012
X	40[b]	1.56	1.467±0.159	0.424±0.013
Y	40[b]	2.84	1.304±0.189	1.003±0.039
Z	40[b]	3.78	1.333±0.240	1.044±0.050
T[c]	1000[b]	40.0	3.073±0.122	0.615±0.010

a, *b*) Liposomes were prepared by adding BP before hydration *a*) or by adding BP to empty liposomes *b*). *c*) Percutaneous absorption was measured with stripped skin.

TABLE III. Determination of Diffusion Coefficient (D) and Partition Coefficient (K) of Percutaneous Absorption of BP by Computer Simulation

System	$D_{liposome}$ (cm^2/h, $\times 10^{-5}$)	$K_{liposome}$
S	9.329±1.315	0.0752±0.0027
T	11.735±1.540	0.0273±0.0009
U	11.670±1.399	0.0267±0.0008
V	12.195±2.122	0.0268±0.0012
W	6.709±0.707	0.0507±0.0016
X	7.355±1.047	0.0272±0.0011
Y	6.625±1.094	0.0404±0.0020
Z	5.028±1.190	0.0403±0.0036

and liposomal particles were very low, while the D values were not so much reduced.

Discussion

It has been considered in general that the effective fraction of a drug for percutaneous penetration is that which exists in the outer aqueous phase free from entrapment in micelles and emulsions.[13] Therefore, addition of excess surfactant over the critical micellar concentration (cmc) increases the entrapment of a drug and results in a decrease in the percutaneous penetration of the drug.[14] In our previous study on the percutaneous penetration of BP from micellar solutions,[12b] the amount of penetration of ^{14}C-BP did indeed decrease with increase in the amount of surfactant added. However, it was suggested that BP in the micelles contributes in part to the total penetration of BP.

In this study, similar results were obtained for liposome suspension. The percentage of BP which penetrated the skin was reduced with increase in the lipid content of the liposome formulation (Fig. 2). This suggested that BP in the outer aqueous phase mainly contributed to the penetration. Since the equilibrium in distribution of BP between liposome particles and the aqueous phase is attained rapidly,[7] the percentage of penetration of ^{14}C-BP was also decreased by the addition of empty liposomes to an aqueous solution of ^{14}C-BP (Fig. 3). However, the amount of penetrated ^{14}C-BP when liposomes were applied was larger than that expected from the concentration of free ^{14}C-BP: an aqueous solution of ^{14}C-BP is thought to

show lower penetration at the same free drug concentration.[9] This finding suggests that BP in liposome particles does contribute to the total absorption of ^{14}C-BP from liposomes.

Mathematical analysis yielded more precise information about these problems. As shown in Table II, K values obtained from cumulative penetrant amount (Q)–time data decreased with increase in the lipid content in liposomes. In addition, an almost linear relationship was observed between the Q and K values. These results suggested that the percutaneous penetration of ^{14}C-BP from liposomes was mainly affected by the partitioning of BP from the vehicle to the skin. Increase in the lipid content led to a decrease of apparent partitioning by enhancing the retention of BP in the vehicle. Subtraction of Q_{free} from total Q revealed that a significant amount of ^{14}C-BP was absorbed by some route(s) different from that of free BP in the outer aqueous phase: in the case of system T, for example, total Q value after 24 h was $40.0 \pm 1.0\,\mu$g while the corresponding Q_{free} was $20.6 \pm 1.6\,\mu$g.[9b] Thus, the amount of ^{14}C-BP absorbed from liposomes ($Q_{liposome}$) was estimated to be $19.4 \pm 1.9\,\mu$g. The diffusion parameters listed in Table III suggest that ^{14}C-BP in liposome particles penetrated the skin in the same manner regardless of the lipid content.

In general, vehicle components of topical dosage forms can affect the drug absorption at two stages; *i.e.*, 1) partitioning of the drug from the vehicle to the skin, which depends on the thermodynamic parameters of the drug in the vehicle, and 2) diffusion of the drug in the skin barrier. Figure 4 shows that DPPC penetrated the skin to a very small extent, and the amount increased with increase in the lipid content, which was inversely related to the BP penetration. This suggests that the co-penetration of BP with DPPC (liposomes) is not a plausible explanation for the increased penetration of BP, so that the effect of liposomes on the diffusion stage of BP should be negligible. The results of mathematical analysis supported this, since the variation in D values was relatively small compared with that in K values in various formulations. On the other hand, liposome formulations tested in the present investigation were found to be stable during the diffusion experiment. Based on these findings, it was concluded that discussion should be focused on the partitioning of the drug between liposome particles and the skin.

To confirm this conclusion, the effect of liposomes on the barrier function of the skin was further examined from several viewpoints. The "barrier" of the skin is considered to reside almost entirely in the stratum corneum, and drug penetration was reported to be enhanced by several treatments which denature the stratum corneum.[15] Figure 5 indicates that a 24 h pretreatment of the skin with liposomes induced only a small change in the penetration of ^{14}C-BP. Figure 6 also shows that liposomes did not induce the destruction of the barrier as stripping did. In addition, liposomes caused no denaturation of skin proteins, as assessed by measuring the increase in sulfhydryl groups caused by the unfolding of the protein[10] (Fig. 7). These results indicated that liposomes had no effect on the stratum corneum and induced no changes in the barrier function of the skin.

Recently, Ganesan *et al.* analyzed the percutaneous absorption of drugs applied with liposomes.[16] In their report, two kinds of penetration routes, *i.e.*, transport from the outer aqueous solution and direct transport from liposomes were proposed for the hydrophobic drugs, progesterone and hydrocortisone. Since BP is a hydrophobic compound which still retains water solubility to some extent, as shown in the previous report,[7] it is plausible that BP penetrates into the skin through these two routes, like progesterone and hydrocortisone.

In conclusion, entrapment of BP in liposomes reduced the concentration of free BP, resulting in a decrease in the percutaneous absorption. However, liposomes also acted as a drug carrier onto the skin surface, resulting in the direct transfer of BP from liposomes to the skin, though to only a small extent. No alterations of the barrier function of the stratum corneum were observed as a result of the *in vitro* topical application of liposomes. These results suggest that liposomes would be advantageous as a topical drug delivery system.

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