Chem. Pharm. Bull.
34(8)3415—3422(1986)

Preservative Activity and *in Vivo* Percutaneous Penetration of Butylparaben Entrapped in Liposomes

Hideo Komatsu,[a] Kazutaka Higaki,[b] Hirokazu Okamoto,[b] Kazuko Miyagawa,[b] Mitsuru Hashida[b] and Hitoshi Sezaki*,[b]

Pola Laboratories,[a] 27–1, Takashimadai, Kanagawa-ku, Yokohama 221, Japan and Faculty of Pharmaceutical Sciences, Kyoto University,[b] Yoshida Shimoadachi-cho, Sakyo-ku, Kyoto 606, Japan

(Received January 22, 1986)

Butylparaben (BP) was added to liposomes prepared by the hydration method from egg phosphatidylcholine (egg PC), cholesterol, and dicetylphosphate in a molar ratio of 4:2:1 for the purpose of antimicrobial preservation. Most of the BP was entrapped in liposomes near the polar surface of the lipid bilayers, and rapid equilibrium was observed in its distribution between liposomes and the outer aqueous phase.

The antimicrobial activities of liposomes containing BP were studied qualitatively against eight kinds of microorganisms. Although the liposome suspension without BP (empty liposomes) had no effect on the microorganisms, liposome suspensions with BP showed antimicrobial activities. The preservative effect was roughly proportional to the free concentration (not the total concentration) of BP.

Percutaneous penetration of liposomes containing BP after topical application was studied *in vivo* in guinea pigs by autoradiography. ^{14}C-BP in liposomes was observed to penetrate into the body, but no difference was found in its distribution pattern in comparison with that after administration as an ointment. On the other hand, ^{14}C-dipalmitoylphosphatidylcholine, a marker of egg PC, remained on the skin surface and was scarcely detected in the body, suggesting that the percutaneous penetration of liposomes themselves did not occur.

Keywords——butylparaben; liposome; preservative activity; percutaneous penetration; autoradiography; dipalmitoylphosphatidylcholine

Introduction

Liposomes have been widely studied as an injectable drug delivery system.[1)] In recent investigations, it has been shown that liposomes are also effective and advantageous as a topical drug delivery system.[2a–c)] However, there are several problems in the utilization of liposomes as a topical delivery system, one of which is the prevention of microbial contamination. Since microorganisms exist on the skin surface,[3)] preservation of a topical formulation is required unless it is to be used in a short time or is prepared *de novo*.

Butylparaben (BP) is one of the most effective preservatives employed widely in foods, drugs and cosmetics.[4)] Although BP is a lipophilic compound, it is also partitioned to water to a small extent. In micellar and emulsion systems, the free fraction of BP was reported to be effective against microorganisms[5)] but little is known about the preservative activity of BP in liposomes. Since liposomes can entrap both lipophilic and hydrophilic drugs in the lipid bilayer and in the inner aqueous phase, respectively,[6)] BP added as a preservative to liposomes would be located in three phases, *i.e.*, the inner aqueous, lipid bilayer and outer aqueous phases. Therefore, it should be interesting to study the preservative activity of BP in relation to its distribution in liposomes. The effect of empty liposomes on microorganisms is also of

interest, since the changes in the fluidity of the membrane induced by the exchange of cholesterol (Cho) between liposomes and cells[7] could have a cytotoxic effect.

Little information is available on the percutaneous absorption of liposomes[2b] and the entrapped drugs applied topically.[2c,d] It would be interesting to elucidate whether liposomes penetrate the skin intact or remain on the skin surface.[2b]

In this study, the interaction of BP with liposomes, the antimicrobial activity of liposomes containing BP and the percutaneous penetration of BP and phospholipid from liposomes were studied in order to characterize BP as a preservative of liposomes.

Materials and Methods

Chemicals——^{14}C-BP(carboxyl-^{14}C) with a specific activity of 1.94 mCi/mmol was purchased from Daiichi Pure Chemicals. ^{14}C-Glucose(U) and ^{14}C-dipalmitoylphosphatidylcholine (^{14}C-DPPC, dipalmitoyl-1-^{14}C) were obtained from New England Nuclear; their specific activities were 4.8 mCi/mmol and 112.0 mCi/mmol, respectively. They were employed without further purification.

Egg phosphatidylcholine (egg PC) was purified from egg yolk according to the method of Rhodes and Lea.[8] After purification, egg PC gave a single spot on a silica gel thin layer plate developed with chloroform, methanol, and water (65 : 25 : 4). Other chemicals were of reagent grade and were used without further purification.

Preparation of Liposomes——Liposomes were prepared by the hydration method[9] as shown in Chart 1. A lipid thin film was prepared in a round-bottomed flask by the evaporation of chloroform under reduced pressure. After addition of 10 ml of water, the mixture was vortex-mixed for 10 min and sonicated with a sonicator (20 kHz, 100 W) for 2.5 min under an atmosphere of nitrogen. BP was added to the lipids, to the water before vortexing or to empty liposomes as an aqueous solution. In the case of the preservative test, the pH of liposome suspension was adjusted with citrate–phosphate buffer.

System compositions of liposomes are listed in Table I. For the preservative test, liposomes containing various amounts of BP were also prepared based on the compositions of system T.

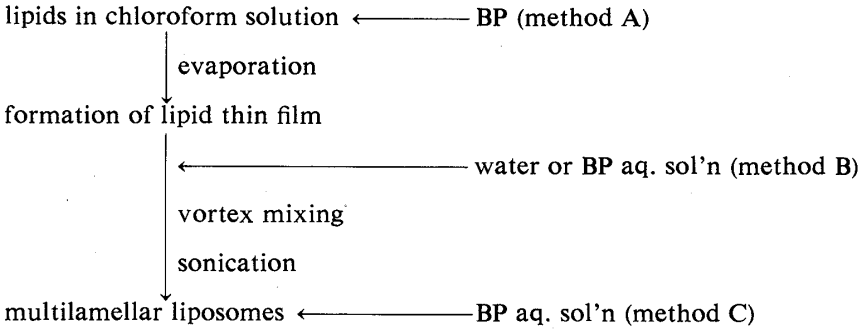

Chart 1. Preparation of Liposomes

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. Systems W—Z: method C.

Electron Microscopy——A liposome suspension (system T) was mixed with an equivalent volume of calf fetal serum (GIBCO), stained with 3.2% ammonium molybdate on a mesh and examined with a Hitachi HU 2000 electron microscope.[10]

Gel Filtration on a Sephadex G-25 Column——One milliliter of liposome suspension was loaded on a Sephadex G-25 column and eluted with water. Liposome fraction was determined by spectrophotometry (OD_{650}), and BP was measured by spectrophotometry (OD_{254}) or liquid scintillation counting.

Ultrafiltration of Liposome Suspension——About 1 ml of liposome suspension was centrifuged ($2000 \times g$) in a Micropartition System (Amicon MPS-1) for 20 min. The concentration of BP in the filtrate was measured by spectrophotometry or liquid scintillation counting.

If BP is partitioned between the liposome and outer aqueous phases based on simple partition equilibrium, Eq. 1 would hold;

$$C_{t0}/C_{f0} = 1 + K(LP)_0 \tag{1}$$

where C_{t0} and C_{f0} are the total and free concentrations of BP, respectively, $(LP)_0$ is the concentration of liposomes, and K is a constant. If the equilibrium is rapid, Eq. 1 would still hold after dilution of liposomes with water. Therefore,

$$\frac{C_{f0}}{C_f} = \frac{C_{t0}}{C_t} \cdot \frac{1+K(LP)}{1+K(LP)_0} \tag{2}$$

where C_t, C_f, and (LP) are the concentrations of total and free BP and of liposomes after dilution, respectively. If the dilution ratio, r, is defined as $r = C_{t0}/C_t = (LP)_0/(LP)$, Eq. 2 can be written as follows;

$$\frac{C_{f0}}{C_f} = r \cdot \frac{1+K(LP)_0/r}{1+K(LP)_0} = \frac{r+K(LP)_0}{1+K(LP)_0} \tag{3}$$

Interactions of BP with Fluorescent Probes——Perylene (0.25 μM),or 8-anilino-1-naphthalene-sulfonate (ANS, 50 μM) was added to a chloroform solution of egg PC, Cho and dicetylphosphate (DCP) (160 : 80 : 40 μM) and liposomes were prepared.[11] Aliquots of 4 ml of the liposome suspension thus prepared were placed in a cell in a Shimadzu RF-540 spectrophotometer. After adding an ethanol solution of BP, the fluorescence was measured. The excitation wavelength for the experiments with ANS was 381 nm and the emission wavelength was 470 nm. The experiments with perylene were performed at 413 and 473 nm, respectively. The effect of ethanol alone on the fluorescence was also measured as a control.

Antimicrobial Activity Test (Preservative Effect)——The test was performed according to the method described previously.[12] A test sample (liposome suspension) was mixed with a suspension of *Escherichia coli* (IFO[13] 3043), *Staphylococcus aureus* (IFO 3061), *Bacillus subtilis* (IFO 3024), *Pseudomonas aeruginosa* (IFO 3445), *Aerobacter aerogenes* (IFO 3320), *Aspergillus niger* (IAM[14] 3001), *Penicillium citrinum* (IAM 7316) or *Candida albicans* (IFO 0583). After incubation for up to 7 d at 32 °C, each mixture was transferred onto an agar plate. For *P. aeruginosa* and *A. aerogenes*, glucose was added to the agar; for *A. niger*, *P. citrinum* and *C. albicans*, Sabouraud medium was employed. Colonies were counted after incubation for 24 h at 37 °C for bacteria and after incubation for 48 h at 30 °C for fungi and yeasts. The preservative effect was evaluated in terms of the number of days of primary incubation necessary to achieve complete prevention of colony formation. The score was 8 when no colonies were found on the plate on day zero after the addition of the test sample. The score decreased with time; when colonies were still observed after 7 d, the score was 0.

Autoradiography——A liposome suspension (system T) was prepared to contain 10 μCi/ml of ^{14}C-BP or 5 μCi/ml of ^{14}C-DPPC. One milliliter of liposome suspension was soaked into gauze (4.5 × 6.5 cm) backed with Lumilar film® (Toray) and surgical tape, and applied occlusively to the dorsal skin of a guinea pig (Hartley strain, female, average weight 350 g) whose hair had been removed with hair clippers one day before application. At 6, 12, 24 and 48 h after application, the animal was sacrificed and whole body autoradiography was performed according to the Ullberg method.[15] Two animals were employed in each group. Microautoradiography of the skin was also done according to the method described previously.[15b]

Results

Interaction of BP with Liposomes

Figure 1 shows an electron micrograph of a liposome suspension (system T) with negative staining.[10] Multilamellar liposomes of 0.1—0.5 μm in diameter were dominantly observed. The inner aqueous volume of liposomes was determined with ^{14}C-glucose on a Sephadex G-25 column. The entrapped volume was calculated to be 4.7% from the area at the void volume.

The elution pattern of ^{14}C-BP in a liposome suspension (system T) from a Sephadex G-25

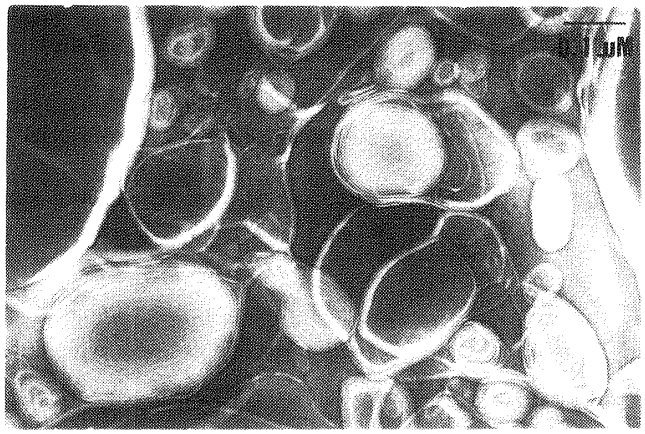

Fig. 1. Electron Micrograph of Liposomes Containing BP (System T)

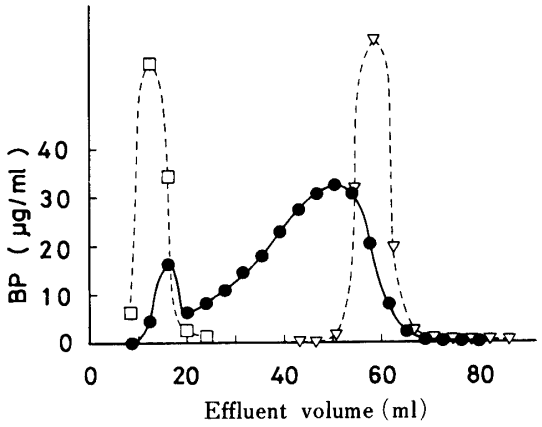

Fig. 2. Gel Filtration of Liposomes Containing BP (System T) on a Sephadex G-25 Column

●, BP in liposomes; ▽, BP in aqueous solution; □, liposomes (OD_{650}).

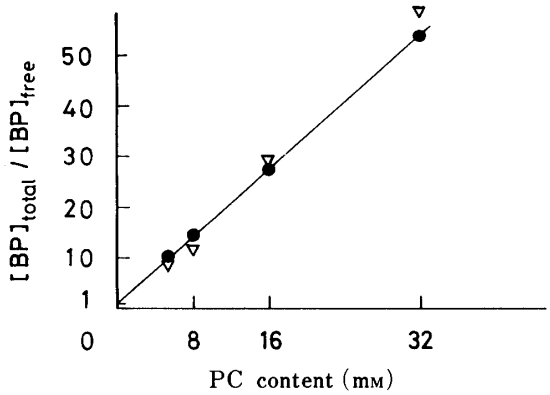

Fig. 3. Total-to-Free Ratio of BP in Liposome Suspensions

▽, systems S—V; ●, systems W—Z.

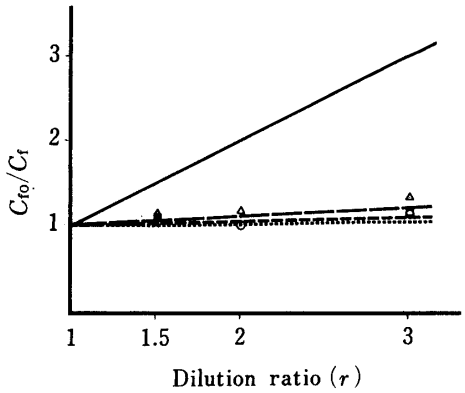

Fig. 4. Effect of Dilution of Liposomes with Water on the Free Concentration of BP

——, BP in aqueous solution; ------, system S, calculated; ▽, system S, found; – – – –, system T, calculated; □, system T, found; — —, system V, calculated; △, system V, found.

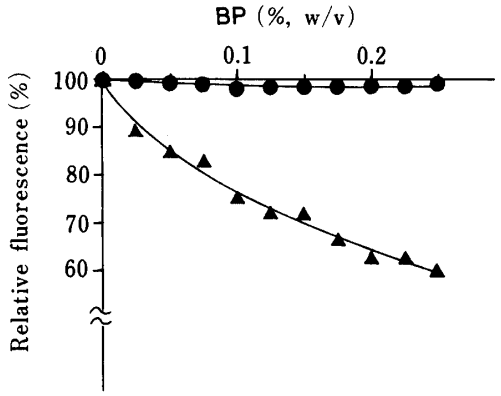

Fig. 5. Fluorescence of Perylene or ANS Incorporated in Liposomes in the Presence of BP

●, perylene; ▲, ANS.

column is shown in Fig. 2. ^{14}C-BP was eluted continuously from the void volume to the free ^{14}C-BP fraction. The same pattern was observed for liposomes with ^{14}C-BP prepared by methods B and C (Chart 1). Furthermore, when the eluted liposome fraction was re-

chromatographed on a Sephadex G-25 column, the same pattern was obtained.

The free concentration of BP in a liposome suspension was evaluated by using a Micropartition System® (Amicon MPS-1). Figure 3 shows the total-to-free ratio of BP concentrations *versus* lipid content in systems S—V and W—Z. There was a linear relationship with an intercept of unity on the *Y*-axis, suggesting that BP was distributed to liposomes based on a simple partition equilibrium according to Eq. 1. As was shown in the case of the interaction of BP with nonionic surfactants,[12] the free concentration of BP was low, and more than 90% of the applied BP was entrapped in liposomes.

Figure 4 shows the effect of dilution of a liposome suspension on the free concentration of BP as determined by ultrafiltration. The transfer of BP occurred very rapidly from liposomes to the outer phase to supplement the reduced concentration. The results are in good agreement with the values presumed based on the simple equilibrium relationship defined by Eq. 3.

The interaction of BP with fluorescent probes incorporated into liposomes is shown in Fig. 5. While BP did not reduce the fluorescence of perylene, a hydrophobic probe, it interacted with ANS, a marker which lies near the surface of the lipid bilayers.[11] Thus, BP was proved not to induce phase-transition of the interior hydrocarbon phase of lipid bilayers.

Antimicrobial Activity of Liposome Suspensions Containing BP

The antimicrobial activities of liposome suspensions (pH 6.5) on eight species of microorganisms are shown in Table II. The empty liposomes showed almost no effect, indicating that the empty liposomes themselves have no antimicrobial activity. On the other hand, liposome suspensions containing BP showed antimicrobial activities on several species of microorganisms. When the lipid content was constant, the activity increased with increase in the amount of BP added to the system. On the other hand, when the amount of BP in the system was constant, the activity increased with decrease in the lipid content. Table II also shows the free concentration of BP in each liposome suspension. A direct relationship between the free BP concentration and the antimicrobial activity (preservative effect) is suggested.

Autoradiographic Studies on the Topical Application of Liposome Suspension

Whole body autoradiograms of guinea pigs are shown in Figs. 6 and 7. After topical application of a liposome suspension (system T) containing ^{14}C-BP, radioactivity was observed densely in the urinary bladder and small intestine as well as at the site of application. At 24 h after application, high radioactivities were found in the gall bladder, small and large intestines, and feces (Fig. 6).

On the other hand, no radioactivity could be found in the body after topical application of a liposome suspension (system T) containing ^{14}C-DPPC (Fig. 7). Only the site of

TABLE II. Antimicrobial Activity (Preservative Effect) Scores of Liposome Suspensions

System	BP_{total} (%)	BP_{free} (%)	A.n.	P.c.	C.a.	B.s.	E.c.	S.a.	P.a.	A.a.
T	—	—	0	0	0	0	0	1	0	0
T	0.02	0.0013	0	0	0	0	0	1	0	0
T	0.05	0.0019	0	0	1	0	0	3	0	0
T	0.10	0.0039	0	0	3	6	0	3	0	0
T	0.15	0.0060	1	4	6	7	0	3	0	0
U	0.10	0.0072	1	6	6	7	0	3	0	0
V	0.10	0.0110	6	6	7	7	0	6	0	0

Abbreviations: A.n. = *Aspergillus niger*, P.c. = *Penicillium citrinum*, C.a. = *Candida albicans*, B.s. = *Bacillus subtilis*, E.c. = *Escherichia coli*, S.a. = *Staphylococcus aureus*, P.a. = *Pseudomonas aeruginosa*, A.a. = *Aerobacter aerogenes*.

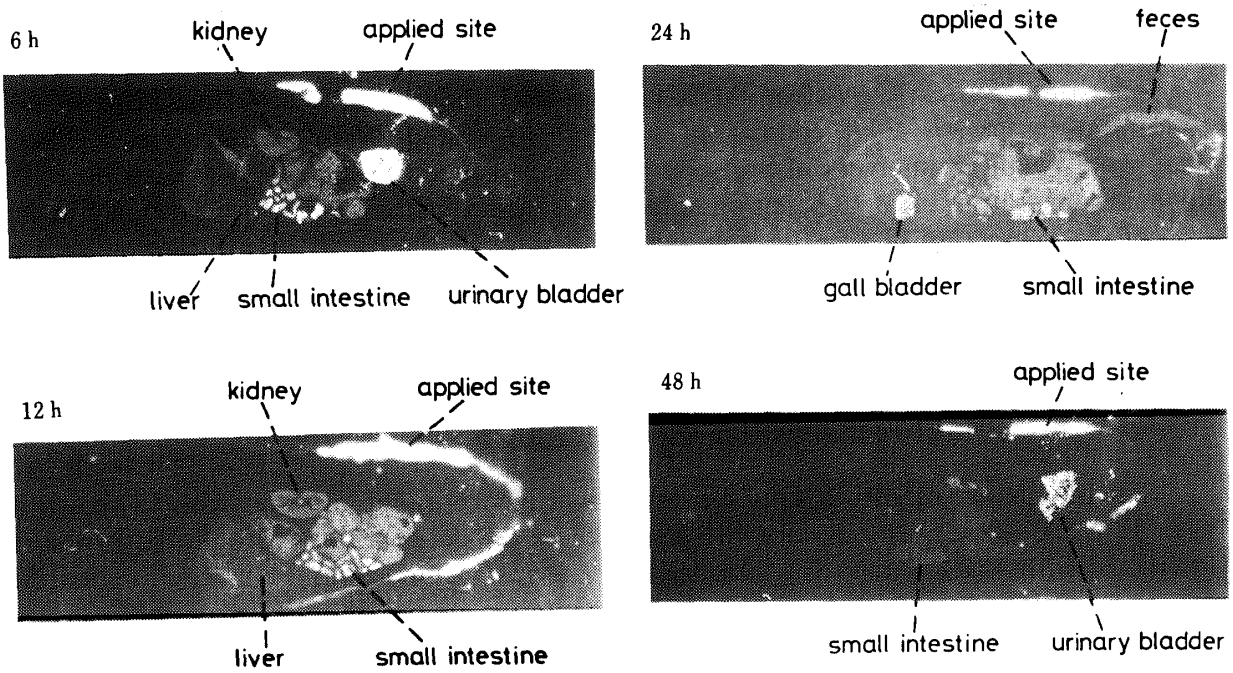

Fig. 6. Whole-Body Autoradiograms Showing the Distribution of Radioactivity in Guinea Pig after Topical Application of ^{14}C-BP in a Liposome Suspension (System T)

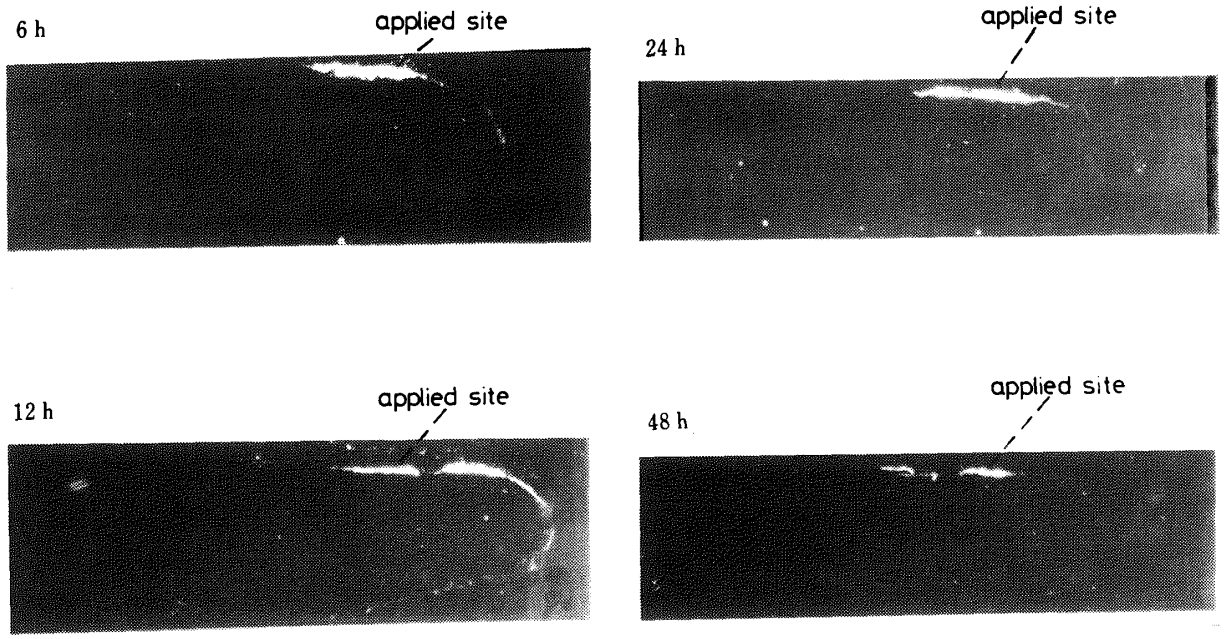

Fig. 7. Whole Body Autoradiograms Showing the Distribution of Radioactivity in Guinea Pig after Topical Application of ^{14}C-DPPC in a Liposome Suspension (System T)

application showed radioactivity even after 48 h.

Microautoradiograms of the skin 6 h after topical application of a liposome suspension (system T) are shown in Fig. 8. The silver grains on the film produced by β-ray exposure intruded into the inner skin in the case of ^{14}C-BP, whereas they remained mainly on the skin surface in the case of ^{14}C-DPPC.

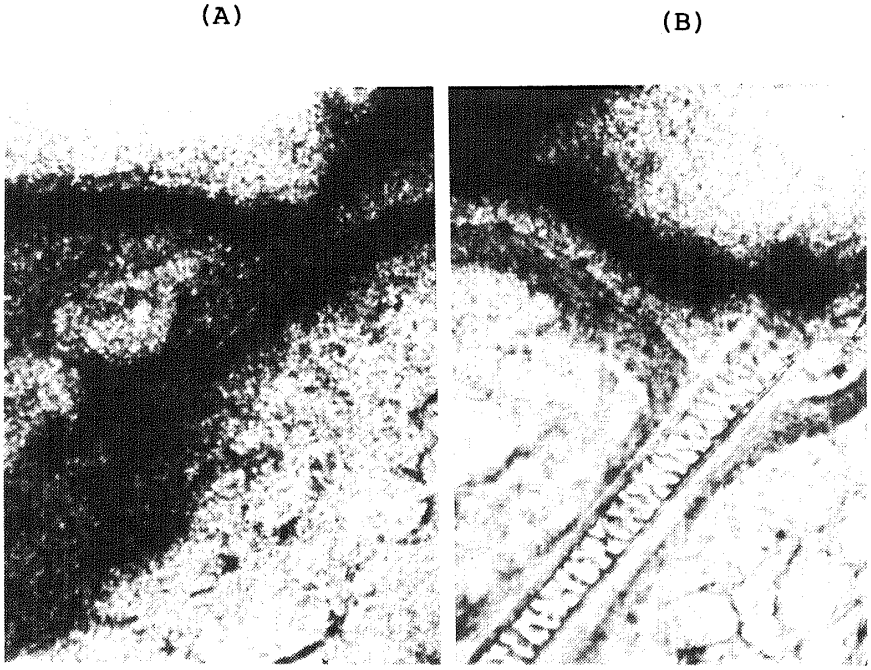

Fig. 8. Microautoradiograms Showing the Distribution of Radioactivity 6 h after Topical Application of ^{14}C-BP (A) or ^{14}C-DPPC (B) in a Liposome Suspension (System T)

Discussion

In the present investigation, a liposome formulation incorporating BP as a preservative agent was characterized from the viewpoints of pharmaceutical properties, antimicrobial activities, and *in vivo* disposition. The formation of multilamellar liposomes in the presence of BP was ascertained by electron microscopy (Fig. 1). The pattern of gel filtration (Fig. 2) suggested that BP was equilibrated so rapidly between liposome particles and the outer fluid that BP entrapped in liposomes could not be obtained separate from the free BP. Since the isolation of BP in liposomes was impossible, whole liposome suspension was employed in the present investigation.

The total-to-free BP concentration ratio linearly increased with increase in the lipid content (Fig. 3), suggesting that BP was entrapped in liposomes based on the lipid/water partition equilibrium. The results of the dilution experiment with liposome suspension (Fig. 4) also suggested that the partition of BP rapidly reached equilibrium. Rapid equilibrium in the distribution of a lipophilic drug into liposomes was also observed in the case of a mitomycin C prodrug; equilibrium was reached within a few minutes (unpublished data). The results of the experiment on the interaction of BP with fluorescent probes (Fig. 5) suggested that BP exists near the surface of lipid bilayers, like imipramine in liposomes.[11a)]

It is plausible that empty liposomes should have antimicrobial activity since the exchange of Cho between cells and liposomes[7)] might result in damage to the microorganisms. However, in fact the empty liposomes showed no such activity.

On the other hand, it was found that the free concentration of BP (not the total concentration) was important in the preservation of liposomes, as with emulsions and micellar solutions[12)] (Table II). In other words, liposomes seemed to inactivate BP by entrapping it in the lipid bilayers.

Autoradiography is an advantageous technique to determine and compare the routes of absorption or the distribution patterns of radiolabelled compounds.[15)] Although autoradiog-

raphy of liposomes which had interacted with cells or tissues has been reported,[16] there has been no investigation by autoradiography after topical application of liposomes. Whole-body autoradiograms obtained in this study indicate that ^{14}C-BP but not ^{14}C-DPPC penetrated into the body (Figs. 6 and 7). Therefore, ^{14}C-BP can be considered to penetrate the skin barrier separately from liposomes, which remain at the skin surface.

The distribution pattern of radioactivities after topical application of ^{14}C-BP in a liposome formulation was almost the same as that obtained after application of ^{14}C-BP in ointment.[15c] In both cases, ^{14}C-BP was excreted through the kidney to the urinary bladder or through the liver and gall bladder to the small intestine. It appears that liposomes do not affect the disposition of ^{14}C-BP in the body.

Microautoradiograms also indicated that ^{14}C-BP penetrated the skin while ^{14}C-DPPC was located mainly on the skin surface (Fig. 8). In the case of ^{14}C-labelled oils such as ^{14}C-isopropylmyristate and ^{14}C-triolein, radioactivity was found densely in hair follicles and sebaceous glands.[15b]

In this study, neither disposition of ^{14}C-DPPC in the sebaceous glands nor transfollicular penetration was observed, suggesting that liposomes or DPPC had little affinity for the sebum or sebaceous glands. The effect of liposomes (whether they enhance or suppress the penetration of BP) could not be assessed by autoradiography, and will be discussed in the following paper on the basis of *in vitro* experiments.

Acknowledgement The authors wish to thank Ms. Tamiko Nakamura for her technical assistance in the antimicrobial activity tests.

References and Notes

1) R. L. Juliano and D. Layton, "Drug Delivery Systems," ed. by R. L. Juliano, Oxford University Press, Inc., New York, 1980, pp. 189—236.
2) *a*) H. E. Schaeffer and D. L. Krohn, *Invest. Ophthalmol. Vis. Sci.*, **22**, 220 (1982); R. E. Stratford, Jr., D. C. Young, M. A. Redel and H. L. Lee, *Int. J. Pharm.*, **13**, 263 (1983); *b*) R. M. Handjiani-Vila, A. Ribier, B. Rondot and G. Vanlerberghie, *Int. J. Cosmet. Sci.*, **1**, 303 (1979); M. G. Ganesan, N. D. Weiner, G. L. Flynn and N. F. H. Ho, *Int. J. Pharm.*, **20**, 139 (1984); *c*) M. Mezei and V. Gulasekharam, *Life Sci.*, **26**, 1473 (1980); *idem*, *J. Pharm. Pharmacol.*, **34**, 473 (1983); *d*) A. J. M. Vermorken, M. W. A. C. Hukkelhoven, A. M. G. Vermeesch-Markslag, C. M. A. A. Goos, P. Wirtz and J. Ziegenmeyer, *ibid.*, **36**, 334 (1984).
3) T. Nakamura, T. Suzuki and M. Makino, *Fragrance J.*, **62**, 85 (1983).
4) B. Croshaw, *J. Soc. Cosmet. Chem.*, **28**, 3 (1977).
5) T. Shimamoto, Y. Ogawa and N. Ohkura, *Chem. Pharm. Bull.*, **21**, 316 (1973); M. Yamaguchi, Y. Asaka, M. Tanaka, T. Mitsui and S. Ohta, *J. Soc. Cosmet. Chem.*, **33**, 297 (1982).
6) C. G. Knight, "Liposomes: From Physical Structure to Therapeutic Application," Elsevier/North-Holland Biochemical Press, Amsterdam, 1981, p. 391.
7) M. Inbar and M. Shinitzky, *Proc. Natl. Acad. Sci., U.S.A.*, **71**, 2128 (1974); B. Bloj and D. B. Zilversmit, *Biochem.*, **16**, 3943 (1977).
8) D. N. Rhodes and C. H. Lea, *Biochem. J.*, **65**, 526 (1957).
9) D. W. Deamer and P. S. Uster, "Liposome Preparation: Methods and Mechanisms," in "Liposomes," ed. by M. S. Ostro, Marcel Dekker, Inc., New York, 1983, p. 27.
10) A. D. Bangham and R. W. Horne, *J. Mol. Biol.*, **8**, 660 (1964).
11) *a*) J. Römer and M. H. Bickel, *Biochem. Pharm.*, **28**, 799 (1979); *b*) D. H. Hayness and H. Staerk, *J. Mem. Biol.*, **17**, 313 (1974); *c*) D. R. Ferguson, E. K. Mathews, M. D. L. O'Connor and A. D. Schuz, *Biochem. Pharm.*, **30**, 1613 (1981); *d*) J. Slavik, *Biochim. Biophys. Acta*, **694**, 1 (1982).
12) H. Komatsu and M. Suzuki, *J. Pharm. Sci.*, **68**, 596 (1979).
13) Institute for Fermentation, Osaka.
14) Institute of Applied Microbiology, Tokyo.
15) *a*) S. Ullberg, *Biochem. Pharmacol.*, **9**, 29 (1962); *b*) M. Suzuki, K. Asaba, H. Komatsu and M. Mochizuka, *J. Soc. Cosmet. Chem.*, **29**, 265 (1978); *c*) M. Suzuki and H. Komatsu, *Cosmet. Toilet.*, **94(6)**, 59 (1979).
16) J. E. Saffitz, R. W. Gross, J. R. Williamson and B. E. Sobel, *J. Histochem. Cytochem.*, **29**, 371 (1981); H. Onodera, G. Takada, K. Toda and R. J. Desnick, *Tohoku J. Exp. Med.*, **140**, 1 (1983).