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Enhanced permeability of keratinized oral-mucosa to salicylic acid with 1-dodecylazacycloheptan-2-one (Azone). In vitro studies in hamster cheek pouch

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Summary

Enhancing effect of 1-dodecylazacycloheptan-2-one (Azone) on the permeability of keratinized oral-mucosa was investigated in vitro using hamster cheek pouch and salicylic acid as a model mucosa and a model compound, respectively. The in vitro permeability of hamster cheek pouch to salicylic acid was approximately 4.4-times higher than that of the abdominal skin, and the stratum corneum of the cheek pouch isolated by the trypsin-treatment showed the similar permeability to the full-thickness preparation of the cheek pouch. Azone-pretreatment enhanced the permeability of both the full-thickness preparation and the stratum corneum of the cheek pouch, and the magnitude of the enhancement was identical. The fluorescence polarization study using 1,6-diphenyl-1,3,5-hexatriene as a probe showed that Azone-treatment increased the fluidity of lipids extracted from the stratum corneum of the cheek pouch. The direct action of Azone during the pretreatment on the stratum corneum, a major barrier for drug permeation, was clarified.

Introduction

Oral-mucosal route is one of the advantageous drug delivery routes for systemic circulation that enable drugs to avoid both the exposure to gastrointestinal juices and the hepatic first-pass elimination (Bell et al., 1985; Hussain et al., 1986, 1987). The absorption of drugs from the oral cavity, in general, obeys the pH–partition hypothesis which is well explained by the passive diffusion mechanism (Anmo et al., 1968; Beckett and Moffat, 1969; Siegel, 1984; Kurosaki et al., 1986, 1987). However, there are two major disadvantages in systemic application of drugs to the oral mucosa. One is its relatively narrow area for the application compared with dermal one, and the other is its higher barrier property for permeation of foreign materials compared with other parts of alimentary canal such as small intestine. Therefore, the pharmaceutical approaches to facilitate drug permeation across oral-mucosa are important to develop the oral-mucosal dosage forms.

The oral-mucosa is composed of stratified squamous epithelium and is classified into 3 types according to the function: i.e. masticatory, lining

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and specialized mucosa (Berkovitz et al., 1978). There are regional variations in the epithelial thickness and the degree of keratinization (Squier and Johnson, 1975; Squier and Hall, 1985a and b) in relation to the mucosal function, and the regional differences may affect the permeability to drugs. Keratinized oral-mucosa is found in regions which are particularly susceptible to the stresses and strains resulting from masticatory activity; gingiva, dorsum of tongue, hard palate and transitional zone of lip are keratinized in human mouth (Berkovitz et al., 1978). We have developed a new experimental method for studying absorption processes across the keratinized oral-mucosa in vivo, using a hamster cheek pouch (Kurosaki et al., 1986), and recently reported that the ionic surfactants increase the permeability of keratinized oral-mucosa to salicylic acid (Kurosaki et al., 1988).

1-Dodecylazacycloheptan-2-one (Azone) is a smooth, oily, hydrophobic liquid, which has been reported to be capable of enhancing the percutaneous penetration of a variety of compounds (Stoughton, 1982; Stoughton and McClure, 1983).

In this study, we examined the effect of Azone on the permeability of hamster cheek pouch, a keratinized oral-mucosa, to salicylic acid by using an in vitro diffusion cell.

Materials and Methods

Chemicals

Azone was kindly supplied by Nelson Research (Irvine, CA) and was used as supplied. An emulsifier, polysorbate 20, was purchased from Tokyo Kasei Kogyo Co. (Tokyo, Japan). Trypsin (purified porcine pancreas, Type IX) was purchased from Sigma Chemical Co. (St. Louis, MO). A fluorescence probe, 1,6-diphenyl-1,3,5-hexatriene (DPH), was purchased from Nakarai Chemicals (Kyoto, Japan). All other chemicals were reagent grade products obtained commercially.

Animals

Male golden hamsters (100–130 g) were used.

Tissue preparation of cheek pouch

Hamsters were anesthetized with urethane (1.5 g/kg, i.p.). The cheek pouch was cleaned with saline. The tissue of the cheek pouch was excised with care and the inner surface of the tissue was cleaned from extraneous tissue by dissection. One specimen, approximately 25 mm^2, was immediately mounted in the diffusion cell.

Tissue preparation of abdominal skin

The hair of the abdominal parts of the hamster was carefully shaved with a razor one day before the experiment. The full-thickness abdominal-skin was excised under urethane anesthesia and was cleaned from subcutaneous tissue and fat. One specimen, approximately 25 mm^2, was immediately mounted in the diffusion cell.

Separation of stratum corneum from the cheek pouch tissue

Stratum corneum was separated from the tissue preparation of cheek pouch according to the method for skin (Knutson et al., 1985). A segment of the cheek pouch preparation was placed in the lamina propria-side down on the filter paper saturated with 1% trypsin solution (pH 8.0) and was stood for 4 h at 37 °C. A sheet of stratum corneum was isolated from the tissue and was immersed in the saline. The sheet was then soaked with the trypsin solution and was incubated for 1 h at 37 °C. The resultant stratum corneum samples were then rinsed with the saline and used for both the drug permeation and the fluorescence polarization studies.

Drug permeation study

A glass cell having an available diffusion area of 1.13 cm^2 was employed. The design and the dimensions of the cell are shown in Fig. 1. The receptor compartment was filled with 18.5 ml of Krebs-Ringer phosphate buffer solution (pH 7.4). The tissue preparation was mounted in the cell with the stratum corneum facing upwards. On determining the permeability of isolated stratum corneum of the cheek pouch, a glass filter (GA-100, Toyo Roshi, Tokyo, Japan) was clamped under the stratum corneum as a support. It was previously confirmed that the permeation of salicylic

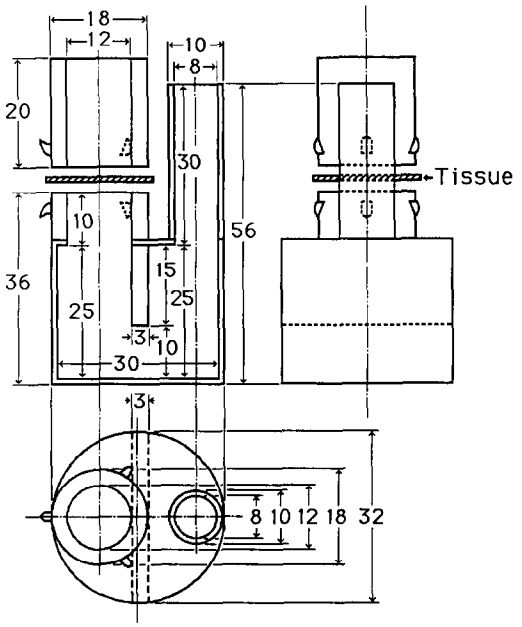

Fig. 1. Schematic representation of a glass diffusion-cell.

acid through the glass filter was fast enough not to underestimate the permeability of the stratum corneum. Salicylic acid solution (10 mM) was prepared with isotonic buffer solutions (citric acid–Na_2HPO_4 and NaH_2PO_4–Na_2HPO_4 for pH 3.0 and pH 6.0, respectively). One ml of the drug solution was applied to the donor compartment and the donor chamber was closed from the atmosphere with Parafilm (American Can Co., Greenwich, CT). Then, the whole system was placed in a thermoregulated chamber at 37 °C. The receptor compartment was stirred continuously at 300 rpm. At appropriate times, an aliquot (usually 1.0 ml) of the receptor fluid was sampled out and the same volume of the fresh Krebs-Ringer buffer solution was supplied to the receptor compartment. The concentration of salicylic acid in the sample was determined by a high pressure liquid chromatography (HPLC).

Pretreatment with Azone

To investigate the effect of in vitro pretreatment with Azone on the permeability to salicylic acid, the tissue and the stratum corneum preparations were pretreated with Azone-emulsion for 1 h in the diffusion cell. Azone-emulsion containing 5% (v/v) Azone and 0.1% (w/v) polysorbate 20 in isotonic NaH_2PO_4–Na_2HPO_4 buffer solution (pH 7.0) was prepared by sonication with a Sonicator 5210 (Ohtake Works, Tokyo, Japan). After washing the pretreated surface, in vitro permeation study was carried out similarly to the manner described above. As for the control study, the pretreatment with 0.1% (w/v) polysorbate 20 solution (pH 7.0) was also done.

Analytical methods

The sample solution was made acidic with 1 ml of concentrated HCl and salicylic acid in the sample was extracted with chloroform. An aliquot of the organic layer was evaporated and the residue was dissolved in 0.5 ml of the mobile phase for HPLC. The resultant was filtered through a 0.45 μm pore-size filter (Nihon Millipore Kogyo, Yonezawa, Japan). Salicylic acid concentration in the receptor compartment was determined by HPLC. A high-pressure liquid chromatograph (LC-5A, Shimadzu, Kyoto, Japan) equipped with UV-detector (SPD-2A, Shimadzu) operated at 236 nm was used in a reversed phase with a Inertsil ODS column (4.6 dia. × 150 mm, Gasukuro Kogyo, Tokyo, Japan). A mixture of methanol and 10 mM ammonium acetate aqueous solution (20 : 80 by volume) was used as the mobile phase at a flow rate of 1.0 ml/min.

Calculations of the cumulative amount of salicylic acid permeated (Q) and the apparent permeability coefficient (K)

The cumulative amount of salicylic acid permeated across the tissue, Q, was calculated by the following equation:

$$Q_n = V_R \cdot C_{R,n} + V_S \cdot \sum_{i=1}^{n} C_{R,i-1} \quad (1)$$

with $C_{R,0} = 0$, where V_R and V_S are the volumes of the receptor compartment (18.5 ml) and the sampling volume (usually 1.0 ml), respectively, C_R is the concentration of salicylic acid in the receptor compartment, and n is the subscript for the n^{th}

sampling. Q was plotted against time, and the permeation rate of salicylic acid was estimated from the slope of the linear portion of the profile. Assuming a passive diffusion mechanism, the permeation rate (dQ/dt) can be expressed as the following differential equation:

$$dQ/dt = K \cdot A \cdot (C_D - C_R) \quad (2)$$

where K, A and C_D are the apparent permeability coefficient, the diffusion area (1.13 cm^2) and the concentration of salicylic acid in the donor compartment, respectively. C_D at time t($C_{D,t}$) can be estimated by the following equation, assuming that the accumulation of salicylic acid in the tissue was negligible:

$$C_{D,t} = (D - Q_t)/V_D \quad (3)$$

where D and V_D are the dose (10 μmol) and the donor volume (1.0 ml), respectively, and t is the subscript for time t.

Extraction of lipids from stratum corneum of the cheek pouch

The stratum corneum was isolated from the cheek pouch as the same manner described above and the lipids were extracted as follows. Tissue samples were homogenized in chloroform–methanol (2 : 1 v/v). One ml of the solvent mixture was used for 10 mg of the tissue. The tissue homogenates were left overnight and then filtered through a fine mesh glass filter (GC-50, Toyo Roshi). The protein residue was rinsed with the same solvent mixture and the washings were combined with the filtrate. The filtrate was evaporated to dryness under nitrogen gas, followed by vacuum drying. The remains were redissolved in the solvent mixture and then centrifuged at 2000 rpm for 10 min. After removal of the insoluble materials, the solution of total-lipids extract was stored under nitrogen gas at $-20\,^\circ$C. The cheek pouches used for Azone-treated group were pretreated with Azone-emulsion for 4 h under urethane anesthesia in vivo. Then the lipids were extracted similarly from the tissue.

Fluorescence polarization study

A lipid-soluble fluorescent probe, DPH, was used to measure the fluorescence polarization which depends upon the rotation rate of the probe. Aqueous suspensions of DPH were prepared as described by Schachter and Shinitzky (1977) and diluted to 0.6×10^{-6} M suspension with Krebs–Ringer phosphate buffer solution. The chloroform–methanol solutions of total-lipid extracts from non-treated and Azone-treated stratum corneums were evaporated to dryness under nitrogen gas and were swollen in the diluted DPH-suspension (0.6 mg lipid/ml). The dispersions were then subjected to an ultrasonic irradiation with a Sonicator 5210 (Ohtake Works) at 100 W for 10 min under nitrogen gas stream in the ice-water. An aliquot of the sonicated dispersion was diluted with Krebs–Ringer phosphate buffer solution to the final concentration of 0.3 mg lipid/ml and the resultant was incubated at 37 °C for 4 h. Azone emulsion in the same buffer solution was prepared by the sonication at the concentration of 0.06 mg/ml and was used instead of the buffer solution for preparing Azone-added sample from the sonicated dispersion of the non-treated group. Thereafter, fluorescence polarization was measured at 360 nm and 430 nm for excitation and emission, respectively, by using a Hitachi MPF-4 spectrofluorophotometer equipped with polarizers and thermoregulated cells (Hitachi, Tokyo, Japan). The degree of polarization, P, was calculated according to the following equation:

$$P = (I_{VV} - C_f \cdot I_{VH})/(I_{VV} + C_f \cdot I_{VH}) \quad (4)$$

where I is the fluorescence intensity, and V and H are the subscripts for the vertical and horizontal orientations, respectively, of the excitation (first) and emission (second) polarizers. $C_f (= I_{HV}/I_{HH})$ is a correction factor (Azumi and McGlynn, 1962). The temperature in the cell was monitored with a thermistor.

Statistical analysis

Results were expressed as the mean ± S.E.M. The statistical analysis was carried out by the Student's t-test.

Results

Permeability of abdominal skin and cheek pouch of hamster to salicylic acid

The permeability of hamster cheek pouch, a model of keratinized oral-mucosa, to salicylic acid was measured and was compared with that of the abdominal skin at pH 3.0 using an in vitro diffusion cell shown in Fig. 1. The permeation profiles for the abdominal skin (Fig. 2) and the cheek pouch (Fig. 3) consisted of a lag phase followed by the steady-state permeation. The lag times of the permeation estimated by the extrapolations of the linear portions of these curves were approximately 4 h and 20 min for the skin and the cheek pouch, respectively. The apparent permeability coefficients calculated at the steady-state were approximately 0.18 mm/h and 0.8 mm/h for the skin and the cheek pouch, respectively. The stratum corneum layer of the cheek pouch, isolated by the trypsin-treatment, showed the similar permeability to the full-thickness preparation of the cheek pouch (Fig. 3). These results suggest that the permeability of the cheek pouch to salicylic acid is approximately 4.4-times higher than that of the abdominal skin and that the major permeation barrier of the cheek pouch exists in the stratum corneum layer likewise the skin.

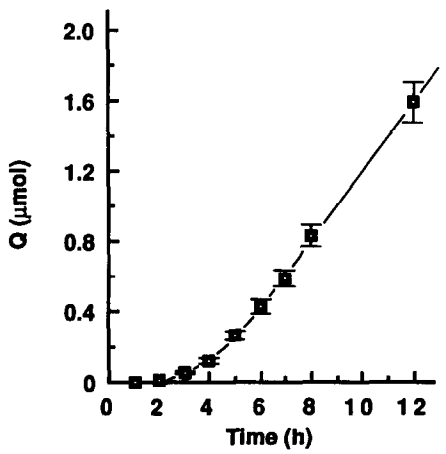

Fig. 2. Permeability of hamster abdominal skin to salicylic acid at pH 3.0. Cumulative amounts permeated across the skin, Q, were calculated by Eqn. 1 and are plotted against time. Dose of salicylic acid in donor side was 10 μmol. Results are expressed as the mean ± S.E. of 7 experiments.

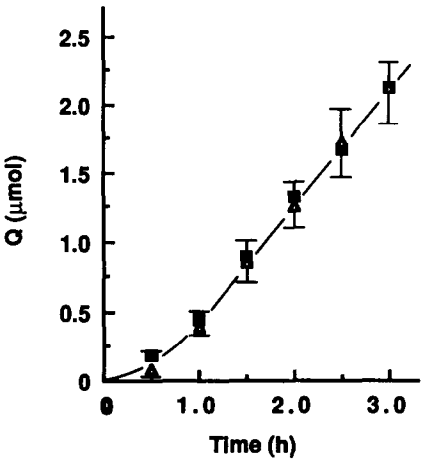

Fig. 3. Permeability of hamster cheek pouch to salicylic acid at pH 3.0. Cumulative amounts permeated, Q, across the full-thickness preparation (■) and isolated stratum corneum (△) are plotted against time. The dose and the calculation of Q were the same as described in Fig. 2. Results are expressed as the mean ± S.E. of 5 experiments. There are no statistically significant differences between two preparations.

Effect of Azone on the permeability of hamster cheek pouch

Effect of Azone-pretreatment on the permeability of the cheek pouch to salicylic acid was in-

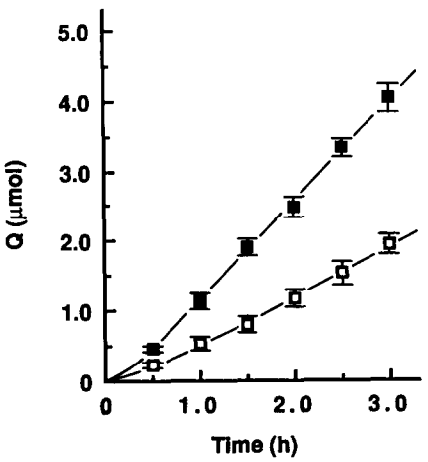

Fig. 4. Effect of Azone-pretreatment on the permeation of salicylic acid across full-thickness preparation of hamster cheek pouch at pH 3.0. Cumulative amounts permeated, Q, for Control (□) and Azone-pretreated (■) groups are plotted against time. The dose and the calculation of Q were the same as described in Fig. 2. Results are expressed as the mean ± S.E. of 3 experiments. All the Q values of Azone-pretreated group are significantly different ($P < 0.05$) from each Control value.

vestigated and the permeation profiles at pHs 3.0 and 6.0 are shown in Figs. 4 and 5, respectively. The permeability of the Control shown in Fig. 4 was not significantly different from that of the non-treated preparation (Fig. 3) at pH 3.0. The apparent permeation coefficient of the Control at the steady-state decreased from 0.8 mm/h to 0.8 $\times 10^{-2}$ mm/h as the pH value of the donor compartment changed from 3.0 to 6.0; i.e. as the unionized fraction of salicylic acid decreased from 50% to 0.1%. In either case, the permeability of the cheek pouch was enhanced significantly by the pretreatment with 5% Azone-emulsion for 1 h and the apparent permeation coefficients at the steady-state increased by approximately 2.4- and 8.4-fold at pH 3.0 (Fig. 4) and at pH 6.0 (Fig. 5), respectively, compared with the corresponding Control values. The lag times were not altered by Azone-pretreatment. Azone-pretreatment also enhanced the permeability of the stratum corneum of the cheek pouch (Fig. 6) and the magnitude of the enhancing effect was quite identical to that of the full-thickness preparation (Fig. 4). The results suggest that Azone mainly acts on the stratum corneum of the cheek pouch and causes the permeability enhancement.

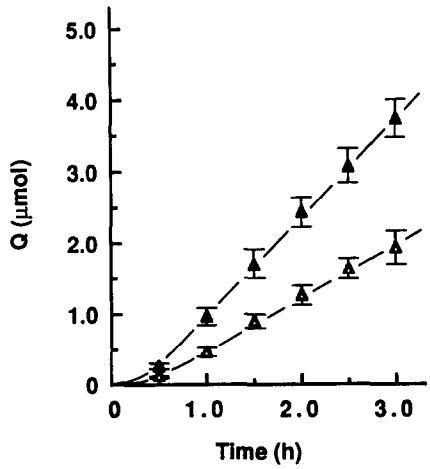

Fig. 6. Effect of Azone-pretreatment on the permeation of salicylic acid across stratum corneum of hamster cheek pouch. Cumulative amounts permeated, Q, of Control (△) and Azone-pretreated (▲) groups are plotted against time. The dose and the calculation of Q were the same as described in Fig. 2. Results are expressed as the mean ± S.E. of 4 experiments. All the Q values of Azone-pretreated group are significantly different ($P < 0.05$) from each Control value.

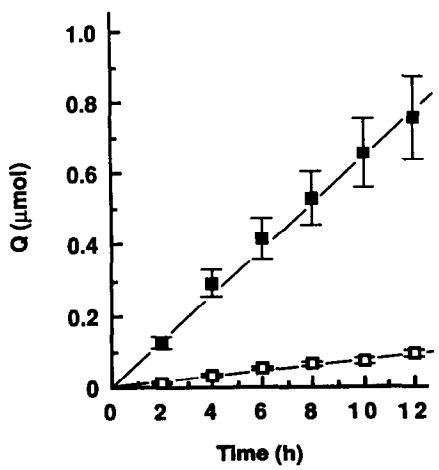

Fig. 5. Effect of Azone-pretreatment on the permeation of salicylic acid across full-thickness preparation of hamster cheek pouch at pH 6.0. Cumulative amounts permeated, Q, for Control (□) and Azone-pretreated (■) groups are plotted against time. The dose and the calculation of Q were the same as described in Fig. 2. Results are expressed as the mean ± S.E. of 4 experiments. All the Q values of Azone-pretreated group are significantly different ($P < 0.01$) from each Control value.

Effect of Azone on the fluidity of lipids extracted from stratum corneum of hamster cheek pouch

Fluorescence polarization studies with DPH were carried out to evaluate the effect of Azone on the fluidity of lipids extracted from the stratum corneum of the cheek pouch. As shown in Fig. 7, Azone-treatment increased the fluidity of the lipid extract of stratum corneum in the gel-phase. The phase transition observed around 50 °C in control group became obscure in Azone-treated group. This was also recognized when 10% (w/w) Azone was added to the lipid extract of Control group (Azone-added group). These results suggest that Azone molecules applied on the outer surface of the stratum corneum might be partially absorbed and localized in the stratum corneum layer, and

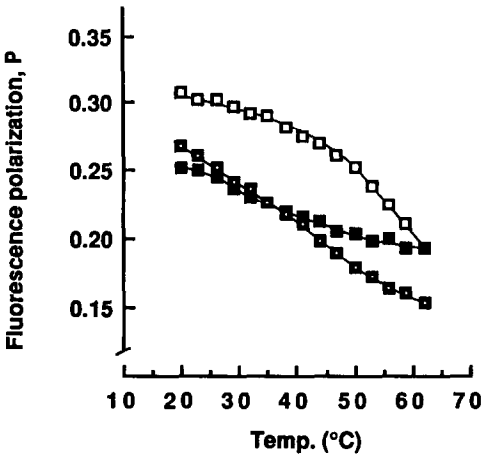

Fig. 7. Changes in the degree of fluorescence polarization, P, of stratum corneum lipids of hamster cheek pouch by Azone. The P values of Control (□), Azone-treated (■) and Azone-added (▣) lipids are plotted against the temperature examined.

caused the increased gel-phase fluidity of the lipids in the layer which acts as a major barrier against the permeation of salicylic acid.

Discussion

Drugs absorbed from the oral cavity can avoid both the exposure to the gastrointestinal juices and the hepatic first-pass elimination. It has been reported that the bioavailabilities of morphine (Bell et al., 1985), nalbuphine (Hussain et al., 1986) and naloxone (Hussain et al., 1987) were potentially improved by the oral-mucosal administration. It is generally recognized that drug absorption from whole oral-cavity obeys the pH–partition hypothesis (Beckett and Moffat, 1969, 1970; Achari and Beckett, 1982). However, the epithelial thickness and the degree of keratinization vary with the regions of the oral mucosa (Berkovitz et al., 1978). Keratinized oral-mucosa is similar to the skin in the morphological viewpont. Squier and Hall (1985a) investigated the in vitro permeability of porcine skin and keratinized and non-keratinized oral-mucosa to tritium-labeled water and horseradish peroxidase. They reported that skin was less permeable than oral mucosa and that the floor of mouth, a non-keratinized oral mucosa, was significantly more permeable than all other regions examined. Hamster cheek pouch is covered with the keratinized stratified squamous epithelium having a few stratum corneum layers. But the transepidermal water loss of hamster cheek pouch is approximately 10-times larger than that of hamster skin (Kligman, 1964). To investigate the absorption characteristics in the keratinized oral-mucosa, we developed an in vivo experimental method using a hamster cheek pouch as a model for the keratinized oral-mucosa. Thus, it was clarified that the absorption from the cheek pouch also depends on both the lipophilicity and the ionization of the test substance (Kurosaki et al., 1986). In the present study, we compared the in vitro permeability of the cheek pouch with that of the abdominal skin to salicylic acid ($pK_a = 3.0$) at pH 3.0, where the influence of the unstirred water layer adjacent to the mucosal surface of the cheek pouch on the absorption of salicylic acid is negligible (Kurosaki et al., 1987). The results showed that the permeability of the cheek pouch to salicylic acid is approximately 4.4-times higher than that of the adominal skin. In addition, it was suggested that the stratum corneum layer is the major permeability barrier of the cheek pouch likewise the skin (Figs. 2, 3).

Azone is well known to enhance the percutaneous penetration of a variety of compounds (Stoughton, 1982; Stoughton and McClure, 1983). However, it was also reported that the vehicle composition of the formulation influences the enhancing effect of Azone on the dermal penetration (Wotton et al., 1985; Sheth et al., 1986). Pretreatment study is useful to avoid the interactions between Azone and the vehicle containing drugs. It has been demonstrated that the pretreatment of skin with Azone is effective to enhance the skin permeability to many drugs (Chow et al., 1984; Sugibayashi et al., 1985; Sheth et al., 1986). Sugibayashi et al. (1985) reported that the enhancing effect of Azone on the transport of 5-fluorouracil (5-FU) across the stripped skin is much smaller than that across the full-thickness skin. In addition, the same authors demonstrated that Azone has a significant effect on neither the epidermis-to-dermis transport of 5-FU across the stripped skin nor the dermis-to-stratum corneum

transport across the full-thickness skin (Morimoto et al., 1986). From these results, they concluded indirectly that Azone mainly affects the stratum corneum of the skin. Because of the presence of hair and sweat ducts, the barrier function of the skin stratum corneum must be lost when one separates this layer to investigate the drug permeation. Therefore, it is impossible to evaluate the effect of enhancers on the permeability of skin stratum corneum layer precisely. However, in the present study, we could separate the stratum corneum layer from the cheek pouch with intact barrier-function by the trypsin-treatment. Furthermore, we could reveal the enhancing effect of Azone on the permeability of stratum corneum layer directly (Fig. 6). The results shown in Figs. 4 and 6 strongly suggest that Azone mainly acts on the stratum corneum of the cheek pouch and causes the permeability enhancement. Comparing the result shown in Fig. 5 with that in Fig. 4, it is suggested that Azone-pretreatment enhances the permeability of cheek pouch more to polar substances than to non-polar ones.

Intercellular lipids are assumed to play an important role in maintaining the barrier function of stratum corneum. The degree of fluorescence polarization of DPH provides an index of the microviscosity of the hydrophobic region of the lipids (Schachter and Shinitzky, 1977). Knutson et al. (1985) reported that an endothermic peak of the lipids extracted from stratum corneum of the human skin was observed around 60 °C by differential scanning calorimetry (DSC). In their study, however, the beginning of the endothermic peak, which indicates the lower critical temperature of the lipid thermotropic transition, was vague. In the present study, the degree of fluorescence polarization, P, of the lipids extracted from the non-treated cheek pouch gradually decreased with increasing the temperature and the slope clearly steepened above 50 °C (Fig. 7). This means that the lipid phase-transition begins at above this temperature. The polarization profile of Azone-treated group shown in Fig. 7 clearly suggests that the fluidity of the lipid extract of stratum corneum was increased by Azone-treatment at physiological temperatures. This tendency was also recognized when Azone was added to the lipid extract of control group (Azone-added group). Though the exact relations between the degree of the lipid fluidity assessed by the fluorescence anisotropy and the permeability of the barrier layer consisted of lipids is still not elucidated, it is inferred that during the pretreatment Azone molecules probably penetrate into intercellular lipid layers of the stratum corneum and enhance the drug permeation across keratinized oral-mucosa.

In conclusion, we could clarify that: (1) the permeability of hamster cheek pouch, a keratinized oral-mucosa, to salicylic acid is approximately 4.4-times higher than that of the abdominal skin; and (2) Azone acts directly on the stratum corneum of the cheek pouch where the major barrier function exists, resulting in the enhancement of permeability. The usefulness of the stratum corneum isolated from the cheek pouch with intact barrier function for studying the in vitro permeability of the stratum corneum layer was additionally noted.

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