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Influence of novel percutaneous absorption enhancers, cyclohexanone and piperidone derivatives, on histopathology of rat skin

Danyi Quan [1,2], Kozo Takayama [1], Toji Mitsuzono [3], Koichi Isowa [3] and Tsuneji Nagai [1]

[1] *Department of Pharmaceutics, Hoshi University, Ebara-2-4-41, Shinagawa-ku, Tokyo 142 (Japan),*
[2] *College of Traditional Chinese Pharmacy, China Pharmaceutical University, Tongjia Xiang 24, Nanjing (China)*
and [3] *The Center of Japan Biological Chemistry Co., Ltd. Fukue Kaizu-cho, Kaizu-gun, Gifu-ken 503-06 (Japan)*

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Summary

The influences of 2-*n*-octylcyclohexanone, 2-*tert*-butylcyclohexanone, 1-*n*-dodecylpiperidone, 1-*n*-cetylpiperidone and Azone® on the histopathology of rat skins which were treated with gel ointments containing 1–10% of these enhancers were investigated in order to gain insight into the skin damage caused by the enhancer and its irritation activity. All of the enhancers at each concentration caused epidermal liquefaction and collagen fiber swelling to some extent. The skin damage by the enhancer increased with increasing concentration of enhancer. At 3% concentration of these enhancers, certain recoverabilities of damaged skin were observed within 5 days after removing the test ointments. At low concentration (1%), recoverabilities were significantly observed, especially in the cases of 1-*n*-dodecylpiperidone and Azone®. No direct correlation was found between the permeation enhancement and the skin irritation of the enhancer.

Introduction

One approach to the delivery of an effective dose of drug through the skin is to reduce temporarily the barrier function of the skin with the aid of penetration enhancers or accelerants (Okamoto et al., 1988; Bodde et al., 1989). Barry (1983, 1987) reviewed the ideal properties of a penetration enhancer and suggested that the desirable attributes should include immediate and full recovery of the normal skin barrier property. It is unlikely that any single material would possess such a formidable array of desirable properties. However, at the first stage of developing an acceptable and applicable penetration enhancer in transdermal drug delivery systems, the irritancy of a compound should be examined as well as its penetration enhancing activity.

In previous reports (Quan et al., 1989a, b) we investigated the promoting effects of three series of penetration enhancers on drug permeabilities. Among them, 2-*tert*-butylcyclohexanone, 2-*n*-oc-

Correspondence: D. Quan, Department of Pharmaceutics, Hoshi University, Ebara-2-4-41, Shinagawa-ku, Tokyo 142, Japan.

tylcyclohexanone, 1-*n*-dodecylpiperidone and 1-*n*-cetyl-piperidone showed marked enhancements of drug permeabilities. Hence, it should be determined whether the penetration enhancement is related to irritation or toxicity and whether their actions are reversible. Little is known about skin irritancy from a histopathological viewpoint (Lashmar et al., 1989). The aim of this investigation was to obtain insight into the skin irritancies of these four penetration enhancers by means of a histopathological method. This is very important, since the practical use of penetration enhancers requires careful balancing of their benefits and risks, i.e., penetration action and irritation. A histopathological study of Azone® is also discussed in this investigation in comparison with the others.

Materials and Methods

Materials

The penetration enhancers, 2-*n*-octylcyclohexanone, 1-*n*-dodecyl and 1-*n*-cetylpiperidones were prepared by the previously reported method (Quan et al., 1989b); 2-*tert*-butylcyclohexanone was obtained commercially from Aldrich Co.; Azone® (1-dodecylazacycloheptan-2-one) was generously supplied by Sumisho-Nelson Co., Ltd. Formaldehyde solution (formalin), paraffin wax (histo. prep.), hematoxylin and eosin were purchased from Wako Pure Chemical Industries Ltd. Indomethacin (IMC) was purchased from Sigma. Other chemicals and solvents were of reagent grade.

Preparation of gel ointment

The gel ointments were prepared as follows: a 2% carboxyvinyl polymer was first thoroughly dissolved in 39.5% of distilled water, and triethanolamine (about 2.5%) was then added to this gel solution in order to adjust the pH value to 7.0. Separately, 3% of IMC was dissolved in 50% of ethanol with 1–10% of enhancers. Both were well incorporated and stirred at room temperature to give a homogeneous gel ointment. The control sample had no enhancer in the gel ointment.

Irritation test and preparation of microscopic samples

Skin irritation tests were performed with five enhancers (2-*tert*-butylcyclohexanone, 2-*n*-octylcyclohexanone, 1-*n*-dodecylpiperidone, 1-*n*-cetylpiperidone and Azone®). The abdominal dosing sites of male Wistar rats (Saitama Experimental Animals Supply) weighing 180–200 g to which glass cells were attached were previously prepared and had an available surface area of 2.01 cm^2. Test gel ointments containing 3% of IMC and 1–10% of enhancers were placed in the cells which were covered with laboratory parafilm. Eight animals were used for each preparation. After 24 h, the application was removed and the residual formulation was washed away from the skin for each rat. Visual irritation assessments were made. Each application site was scored for erythema and eschar formation, and edema formation as evaluated according to the criteria of Draize (1959) as follows:

Skin reaction	Score
Erythema and eschar formation	
No erythema	0
Very slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate to severe erythema	3
Severe erythema to slight eschar formation	4
Edema formation	
No edema	0
Very slight edema (barely perceptible)	1
Slight edema (edges of area well defined by definite raising)	2
Moderate edema (raised approx. 1 mm)	3
Severe edema (raised more than 1 mm)	4

For each rat, the two scores for the 24 h reading (erythema and eschar formation, and edema) were added and divided by two to give a primary skin irritation score which was averaged for four rats.

Following the visual irritation evaluation, two of the dosing sites on the skin for each preparation were immediately taken from the rats each sample of which was about 1 cm^2. The other rats were maintained in cages with food and water freely available during the experiment and observed for the further recoverability test. Two of the application sites on the skin for each preparation were also separated from the rats 1, 3 and 5 days later. All of the separated skins were kept in 10% neutralized carbonate-buffered formalin for microscopic examination.

Microscopic study

Each skin was fixed in 10% neutral carbonate-buffered formalin for at least 24 h before routine processing and then cut vertically against the skin surface at the central region in 4 mm width. Each section was dehydrated using a graded series of ethanol solutions and embedded in paraffin wax. Tissues were divided into small pieces (about 3 μm in thickness) and stained with hematoxylin and eosin. All sections were examined by optiphoto light microscopy (Optiphoto, Nikon, Tokyo).

Results and Discussion

Primary skin irritation test

Fig. 1 shows primary irritation scores induced by 24 h application of gel ointments containing 1–10% of enhancers. In the case of low concentrations of enhancers (1%), Azone® and 2-*n*-octylcyclohexanone showed the largest primary irritation, followed by 1-*n*-cetylpiperidone, while 2-

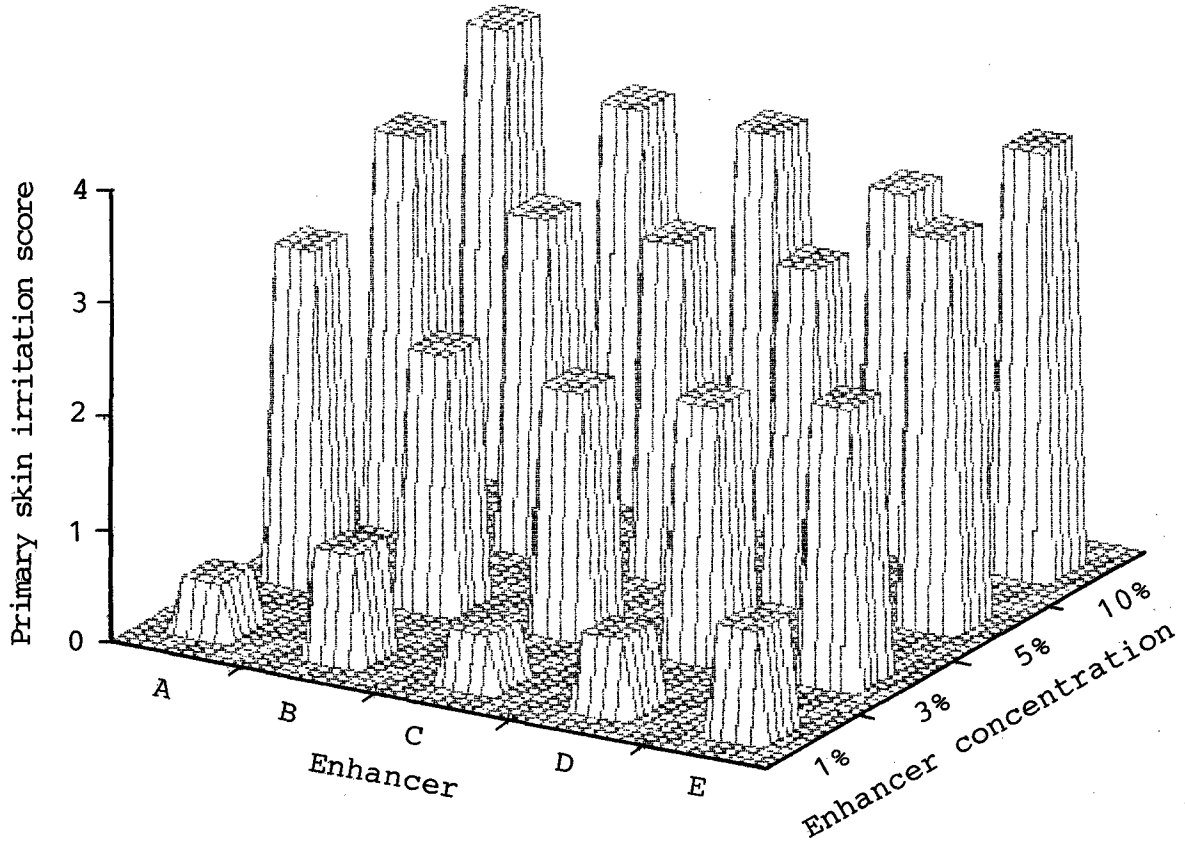

Fig. 1. Primary skin irritation scores of five enhancers examined at different concentrations at 24 h after application. (A) 2-*tert*-Butylcyclohexanone; (B) 2-*n*-octylcyclohexanone; (C) 1-*n*-dodecylpiperidone; (D) 1-*n*-cetylpiperidone; (E) Azone®.

tert-butylcyclohexanone and 1-*n*-dodecylpiperidone showed only slight irritancy on rat skin. When high concentrations of enhancers (3, 5, 10%) were used in the gel ointments examined, 2-*tert*-butylcyclohexanone produced the most severe irritation at each concentration. The skin irritations of the other enhancers at three concentrations are summarized in the following order: Azone® > 2-*n*-octylcyclohexanone = 1-*n*-dodecylpiperidone > 1-*n*-cetylpiperidone. No recoverabilities from erythema were observed in 5 days after removing the application in the case of high concentrations of enhancers (> 5%). The pustulations under the hard eschar induced by 10% enhancers were found to reach different extents after 3 days, especially for 2-*tert*-butylcyclohexanone.

Information concerning the chemical structure of the enhancer and skin irritancy is available in the literature (Aungst, 1989; Hadgraft and Guy, 1989; Ponec et al., 1989; Wong et al., 1989). It was reported that skin irritation produced by compounds with double bonds was much stronger than that produced by compounds with a saturated alkyl chain. In our cases, there seems to be no regular correlation between the chemical structure of the enhancer and the skin irritancy observed. Although the chemical structures of these five enhancers used had certain similarities, their skin irritation activities showed a number of differences. Considering the skin irritancy caused by 2-*tert*-butylcyclohexanone, the steric conformation of the enhancer seems to be an important factor in the skin irritation.

Histopathological study

The skin is a multilayered organ and has, anatomically, many histological layers. It is generally described in terms of three tissue layers: the stratified, avascular, cellular epidermis, the underlying dermis of connective tissue, and the subcutaneous fat layer. In addition, the highly vascularized dermis and the epidermis support several skin appendages: errine, apocrine, sebaceous glands, and hair follicles (Okum and Edelstein, 1976; Chien, 1982; Mehregan, 1986). In this study, the influence of each enhancer examined on the skin irritation and its barrier property will be discussed in detail in light of the above-mentioned three tissue layers with the aid of histopathological findings.

Influence of different concentrations of enhancers on skin damage. The results of the histopathological study with different concentrations of the enhancers are summarized in Table 1. All of enhancers caused significant epidermal liquefactions even when the lower concentration of enhancer was used. Only 1% of 2-*tert*-butylcyclohexanone was an exception. It was found almost to lack irritancy on rat skin. On the whole, when the concentration of enhancers used was more than 1%, 2-*tert*-butylcyclohexanone showed the most severe epidermal liquefaction; the others displayed no significant differences in their epidermal liquefactions. In the stratum papillare and reticulare of the dermis, collagen fiber swelling caused by each enhancer was observed. Comparing their actions on the dermis, it was clear that 2-*tert*-butylcyclohexanone was also the strongest among the five enhancers; both Azone® and 1-*n*-cetylpiperidone were weaker; 2-*n*-octylcyclohexanone and 1-*n*-dodecylpiperidone showed slight collagen fiber swelling only at high concentrations (> 5%). These five enhancers also showed different actions on the hypodermal tissue. Infiltration of inflammatory cells was found at each concentration of 2-*tert*-butylcyclohexanone or Azone®, respectively. In the case of 2-*n*-octylcyclohexanone and 1-*n*-dodecylpiperidone, when a concentration of more than 5% was used, very slight infiltration of inflammatory cells could be observed in the hypodermis. 1-*n*-Cetylpiperidone showed the weakest effect on the infiltration of inflammatory cells; only the high concentration (10%) resulted in slight infiltration. In general, with increase in the concentrations of enhancers, the infiltration of inflammatory cells in the dermis and hypodermis increased. In addition, the phenomenon of focal hemorrhage in the hypodermis was found at a high concentration of 2-*tert*-butylcyclohexanone. This phenomenon was not observed with the other four enhancers. From this result, it is also noted that the degeneration of skin appendages which was caused by these enhancers varied with the concentrations of enhancers examined. 2-*tert*-Butylcyclohexanone still had the strongest effect on the degeneration of skin appendages.

TABLE 1

Histopathological findings of rat skins at 24 h after application of gel ointments containing 1–10% of enhancers

Histopathological findings	Enhancer:	1-*n*-Dodecyl-P				1-*n*-Cetyl-P				2-*n*-Octyl-C				2-*tert*-Butyl-C				Azone			
	Concentration:	1%	3%	5%	10%	1%	3%	5%	10%	1%	3%	5%	10%	1%	3%	5%	10%	1%	3%	5%	10%
Epidermis																					
Liquefaction		++	++	++	++	+	+	++	++	++	++	++	++	−	++	+++	+++	++	++	++	++
Dermis																					
Collagen fiber swelling																					
in stratum papillare		+	+	+	+	+	+	+	+	−	+	+	+	−	+	++	++	+	+	+	+
in stratum reticulare		−	−	±	+	+	+	+	+	−	−	+	+	−	+	++	++	+	+	+	+
Inflammatory cell infiltration																					
in stratum papillare		±	±	+	+	−	−	−	−	±	+	+	+	−	−	−	−	−	−	−	−
in stratum reticulare		−	−	±	+	±	±	+	+	−	−	±	±	−	−	−	±	±	+	++	++
Hypodermis																					
Collagen fiber swelling		−	±	+	+	−	−	−	+	−	±	+	+	−	+	+	++	−	±	+	+
Inflammatory cell infiltration		−	−	±	+	−	−	−	+	−	−	±	±	−	±	+	++	−	±	+	++
Focal hemorrhage		−	−	−	−	−	−	−	−	−	−	−	−	−	−	±	+	−	−	−	−
Skin appendages																					
Degeneration		−	+	+	++	−	±	+	+	−	+	++	++	−	++	++	+++	−	+	++	++

(−) No change; (+) very slight; (+) slight; (++) moderate; (+++) marked. P, piperidone; C, cyclohexanone.

To summarize, comparing the histopathological findings of all enhancers examined, the influences of these five enhancers on the skin irritancy and barrier property can be arranged as follows: 2-*tert*-butylcyclohexanone > Azone® > 2-*n*-octylcyclohexanone > 1-*n*-dodecylpiperidone > 1-*n*-cetylpiperidone. The microscopic results of these enhancers were in good agreement with those of the visual irritating evaluation. The results also revealed that these five enhancers had different degrees of accessibility into skin tissue, i.e., penetrating abilities into skin tissue. For instance, at the same concentration, not every enhancer showed infiltration of inflammatory cells in the hypodermis. This suggested that they had different abilities to penetrate into the deep layer of the skin and to damage selectively skin tissue. Furthermore, although 2-*tert*-butylcyclohexanone had a strong action on the hypodermis, it showed no penetration into the stratum papillare and reticulare of the dermis. This phenomenon might well be related to the physicochemical properties of the enhancer.

Reversibility study of rat skin after application.

A reversibility examination was performed using 1 and 3% concentrations of enhancers, since the high concentration of enhancer would lead to a strong irritation in each layer of skin. Figs 2–6 illustrate the histopathological behavior at different time courses using 3% of enhancers in the test ointments. At 24 h after application, each enhancer caused epidermal liquefaction, and no remarkable differences among the five enhancers were noted. The other microscopic findings were also similar at 24 h. At 1 day after removing the test ointment, 1-*n*-dodecylpiperidone and Azone® showed markedly more severe coagulative necrosis in epidermis and dermis than the others. At the same time, it was clearly seen that Azone® still caused hypodermal necrosis. The phenomenon of edema in hypodermal tissue was found with each enhancer, which suggested that the normal function of hypodermal cells and tissues might be inhibited. The obvious re-epithelization of the epidermis and eschar formation were observed at 3 days in the case of 1-*n*-dodecylpiperidone. Azone®, 1-*n*-cetylpiperidone and 2-*n*-octylcyclohexanone

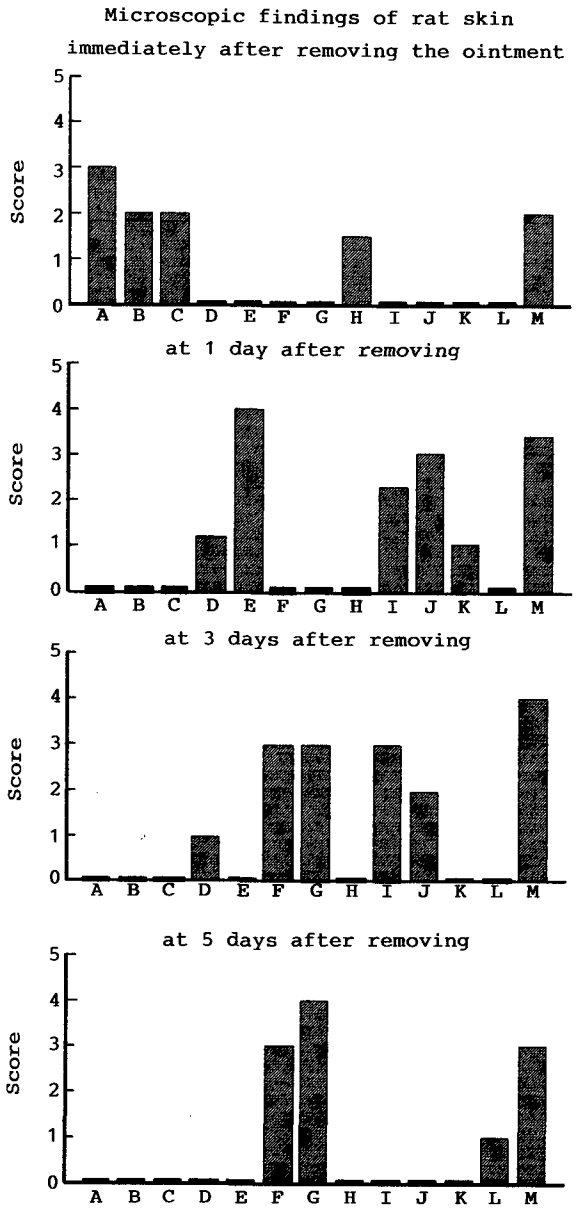

Fig. 2. Histopathological behavior at different time courses in 3% of 1-*n*-dodecylpiperidone. Score calculation is denoted by: 0, no change; 1, very slight; 2, slight; 3, moderate; 4, marked. Bar explanation are as follows: Epidermis: A, liquefaction. Dermis: B, collagen fiber swelling; C, infiltration of inflammatory cells. Dermis and epidermis: D, infiltration of inflammatory cells; E, coagulative necrosis; F, eschar formation; G, re-epithelization. Hypodermis: H, collagen fiber swelling; I, infiltration of inflammatory cells; J, edema; K, necrosis; L, granulation tissue formation. Skin appendages: M, degeneration.

showed re-epithelization of the epidermis at 5 days after removing the test ointment. Re-epithelization of the epidermis was not observed in the case of 2-*tert*-butylcyclohexanone until 5 days.

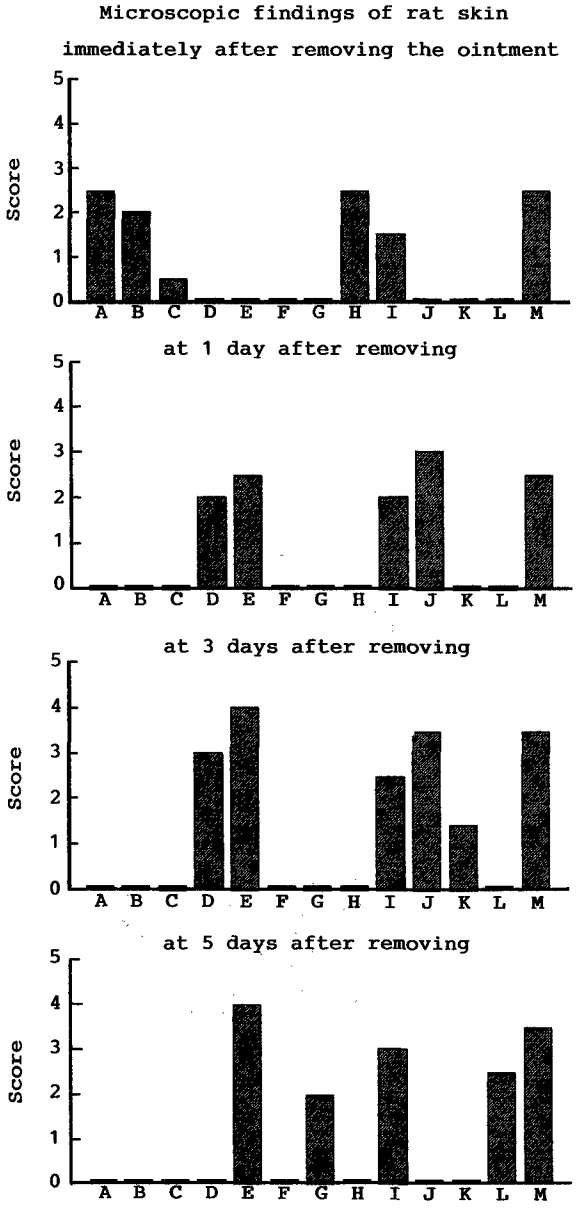

Fig. 3. Histopathological behavior at different time courses in 3% of 1-*n*-cetylpiperidone. Score calculation and bar explanation are the same as in Fig.2.

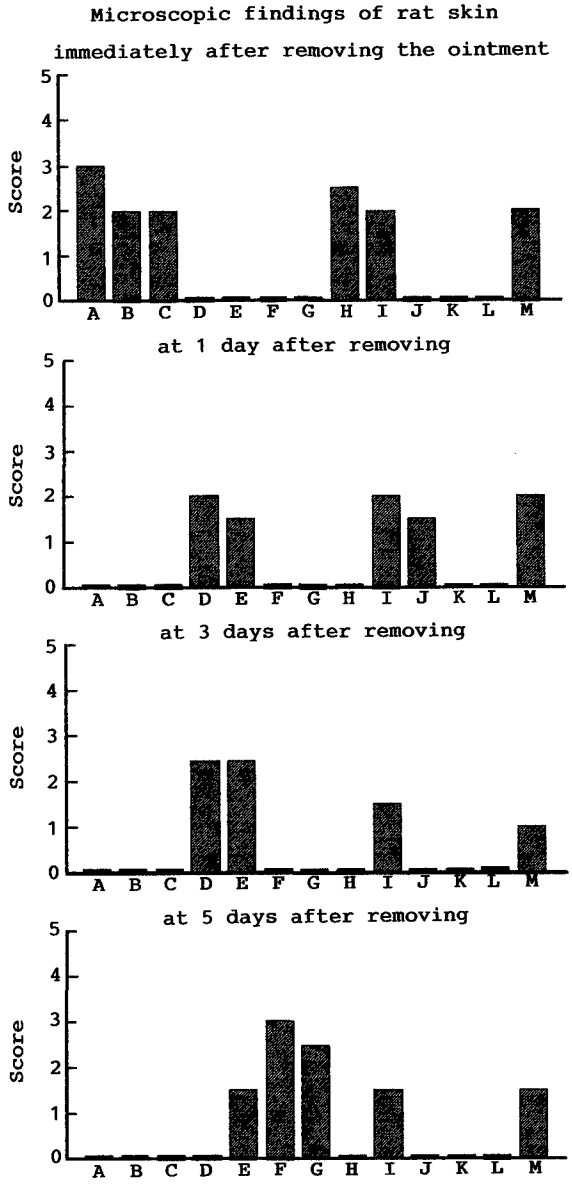

Fig. 4. Histopathological behavior at different time courses in 3% of 2-*n*-octylcyclohexanone. Score calculation and bar explanation are the same as in Fig. 2.

The four enhancers examined showed eschar formation at 5 days except for 1-*n*-cetylpiperidone. In general, necrosis is followed by formation of granulation tissue. We also observed this phenomenon in all of the enhancers at 5 days; of them,

Azone® and 1-*n*-cetylpiperidone displayed better formation of granulation tissue.

From these histopathological findings, we may conclude that 1-*n*-dodecylpiperidone and Azone® damaged the skin more severely than the other enhancers; although these two enhancers showed

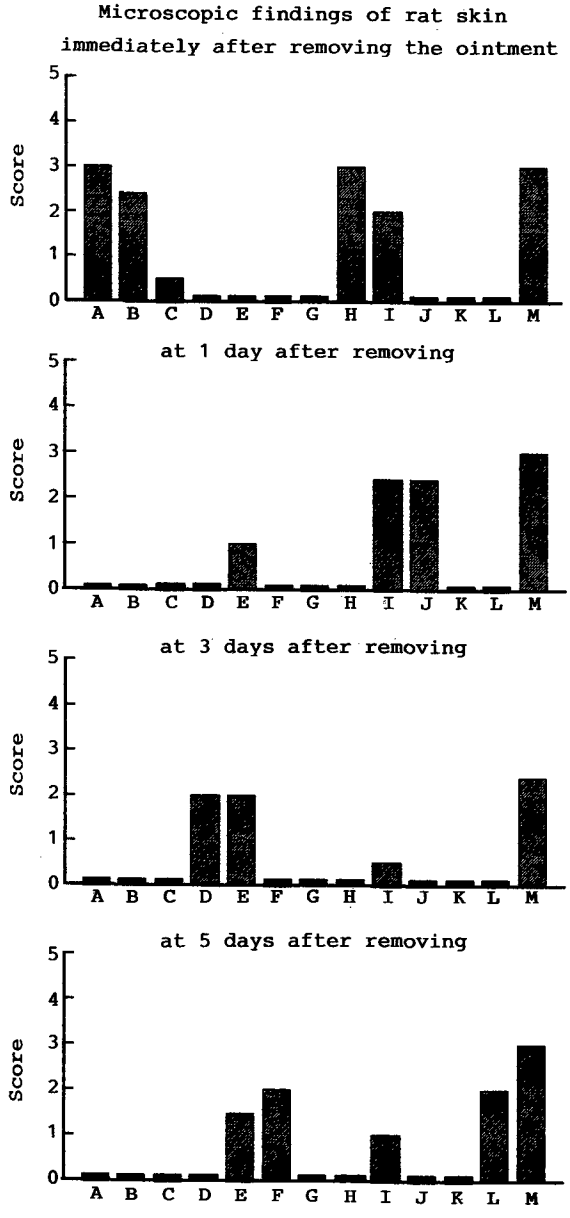

Fig. 5. Histopathological behavior at different time courses in 3% of 2-*tert*-butylcyclohexanone. Score calculation and bar explanation are the same as in Fig. 2.

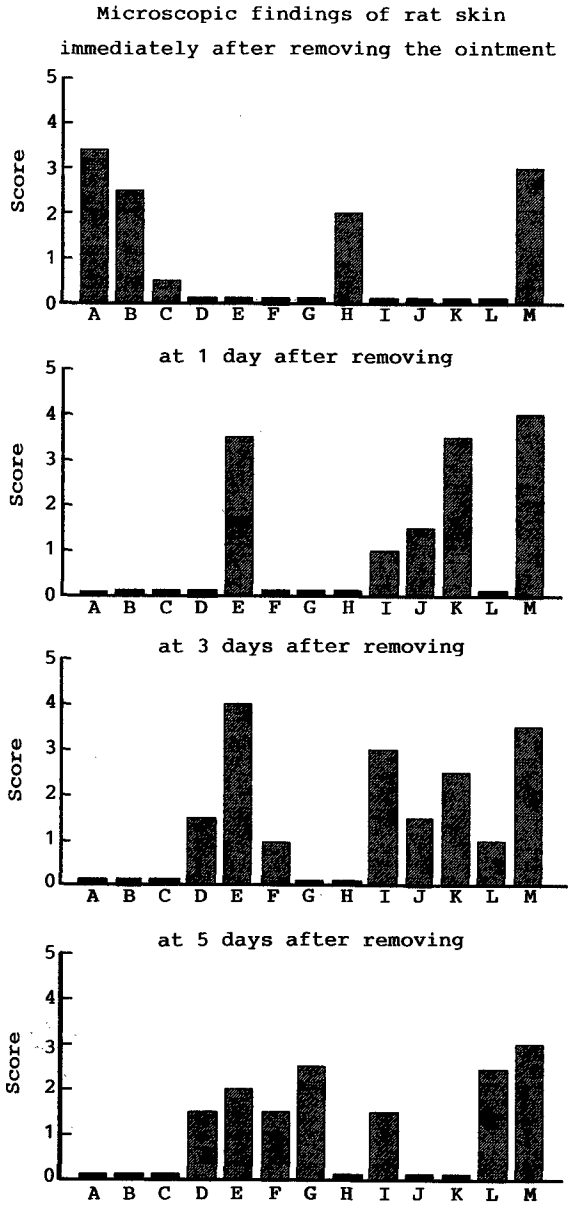

Fig. 6. Histopathological behavior at different time courses in 3% of Azone®. Score calculation and bar explanation are the same as in Fig. 2.

relatively severe skin damage at 1 day, their re-epithelizations of the epidermis (reparation function) were more rapid than those of the others. This may suggest that an appropriate skin damage may induce the rapid re-epithelization of the epidermis.

Possibly these two enhancers induced acute skin damage.

Figs 7–11 show the histopathological findings at 1% of the enhancers in order to estimate the reversibility of the damaged skin at low concentra-

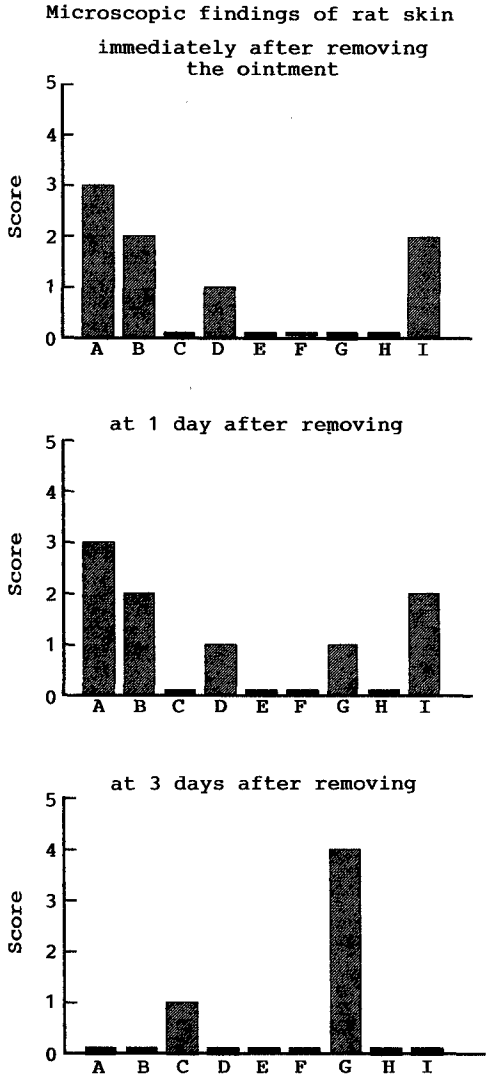

Fig. 7. Histopathological behavior at different time courses in 1% of 1-*n*-dodecylpiperidone. Score calculation is denoted by: 0, no change; 1, very slight; 2, slight; 3, moderate; 4, marked. Bar explanation as follows: Epidermis: A, liquefaction; B, collagen fiber swelling: C, acanthosis. Dermis and epidermis: D, infiltration of inflammatory cells; E, coagulative necrosis; F, eschar formation; cell; G, re-epithelization. Hypodermis: H, collagen fiber swelling. Skin appendages: I, degeneration.

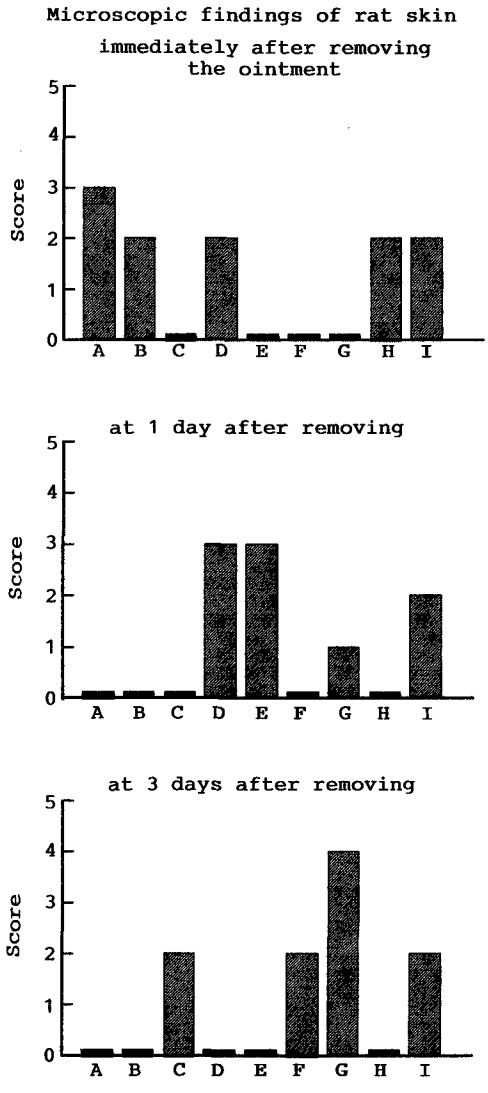

Fig. 8. Histopathological behavior at different time courses in 1% of 1-*n*-cetylpiperidone. Score calculation and bar explanation are the same as in Fig. 7.

tion. It is interesting to note that 2 -*tert*-butylcyclohexanone which had the strongest irritation at high concentration (> 3%) showed only very slight acanthosis of the epidermis (epidermal thickening) at 1 day after removing the test ointment; no further microscopic findings were observed until 5 days. As for the microscopic results of the other four enhancers, the epidermal liquefaction occurred to the same extent at 24 h for each enhancer; moreover, the accompanying col-

lagen fiber swellings of the dermis were found in the case of Azone®, 1-*n*-dodecyl and 1-*n*-cetylpiperidones; 1-*n*-cetylpiperidone still led to moderate coagulative necrosis and the infiltration of inflammatory cells in the epidermis at 1 day. Re-epithelization of the epidermis could be observed in 1-*n*-dodecylpiperidone and 2-*n*-octylcyclohexanone at 1 day, and in 1-*n*-cetylpiperidone and Azone® at 3 days. In addition,

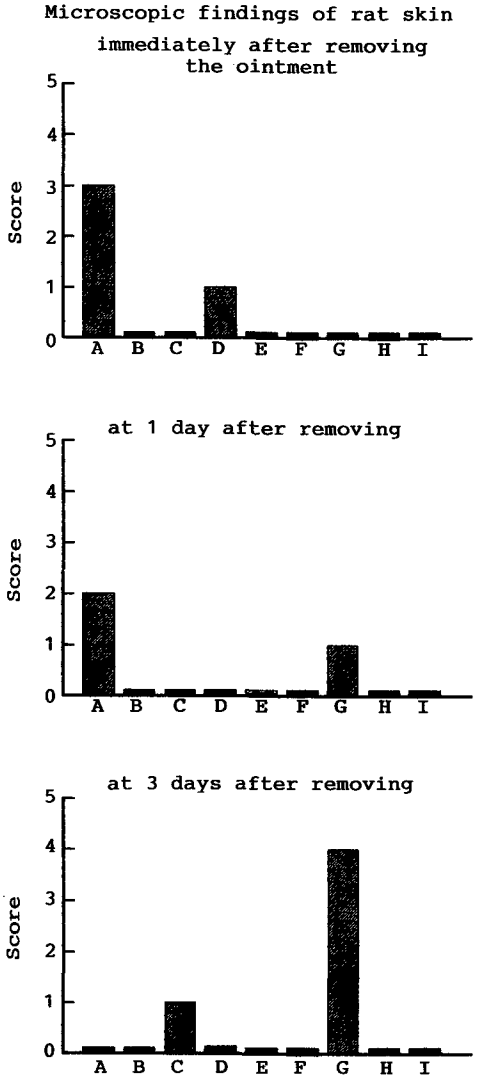

Fig. 9. Histopathological behavior at different time courses in 1% of 2-*n*-octylcyclohexanone. Score calculation and bar explanation are the same as in Fig. 7.

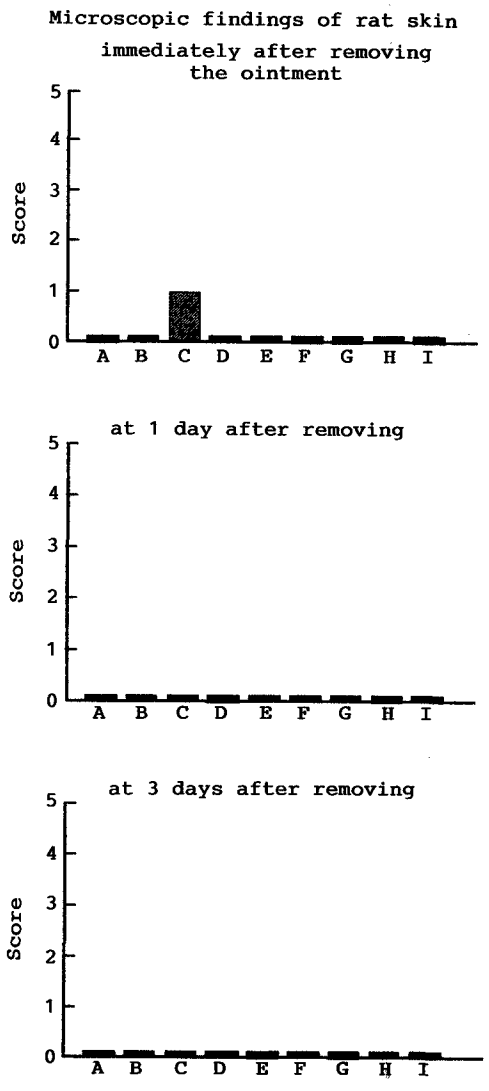

Fig. 10. Histopathological behavior at different time courses in 1% of 2-*tert*-butylcyclohexanone. Score calculation and bar explanation are the same as in Fig. 7.

Azone® caused stronger eschar formation and epidermal acanthosis than did the other enhancers.

This investigation of low concentration of enhancer can be summarized as follows: 2-*tert*-butylcyclohexanone showing temporary acanthosis of epidermis is considered to cause the weakest barrier damage to the skin; the others are listed in the following order: 2-*n*-octylcyclohexanone < 1-*n*-dodecylpiperidone < 1-*n*-cetylpiperidone < Azo-

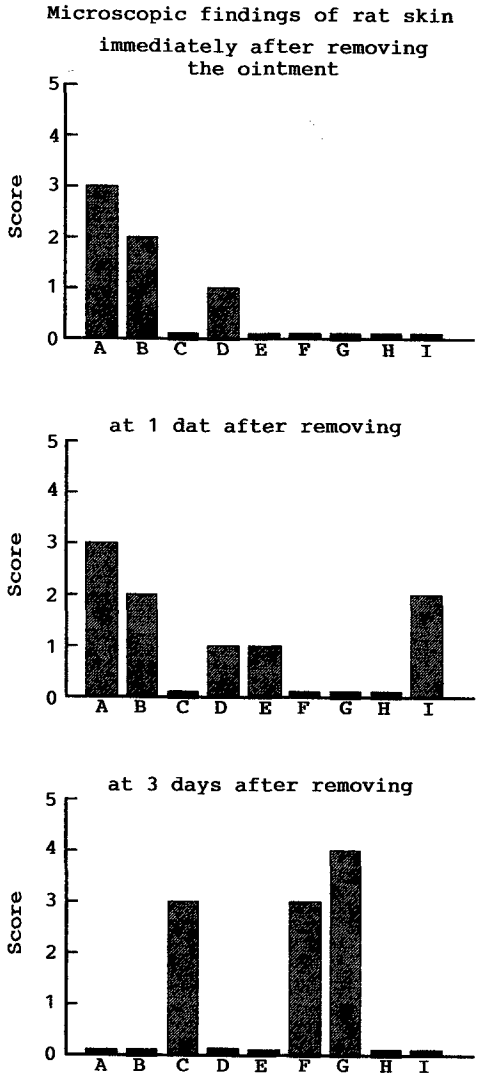

Fig. 11. Histopathological behavior at different time courses in 1% of Azone®. Score calculation and bar explanation are the same as in Fig. 7.

ne®. Thus, it can be seen that there is some correlation between the concentration of enhancer and its skin irritation. It is desirable that the enhancer at lower concentration shows both a definite enhancing effect and weak skin irritation.

Exemplification of microscopic photos. The microscopic photos of the histopathological findings are shown in Figs 12–15. Here, we selected only three groups of photos to illustrate the findings because of limited space. Fig. 12 shows microscopic results in the control sample which had no enhancer in the test ointment. It is clearly seen that the three layers of skin tissue (epidermis, dermis and hypodermis) showed no change at 24 h after application. Fig. 13 shows the microscopic appearance of rat skin when 1% of 1-*n*-dodecylpiperidone was used as penetration enhancer. Fig. 13a demonstrates liquefaction of epidermis, collagen fiber swelling and inflammatory cell infiltration in dermis, and degeneration of skin appendages. Similar results to those in Fig. 13a are observed in Fig. 13b. In addition, focal epidermal re-epithelization is evident in this photo. Fig. 13c demonstrates epidermal re-epithelization and acanthosis (epidermal thickening). Fig. 14 illustrates the microscopic appearance of rat skin when 1% of Azone® was used as penetration enhancer. Fig. 14a also shows liquefaction of epidermis, collagen fiber swelling and inflammatory cell infiltration in dermis, and degeneration of skin appendages. Fig. 14b shows liquefaction and slight coagulative necrosis of epidermis, inflammatory cell infiltration and collagen fiber swelling in dermis, and collagen fiber swelling of hypodermis. Fig. 14c shows re-epithelization and acanthosis of epidermis with eschar formation. Fig. 15 illustrates the microscopic appearance of rat skin after application of gel ointment containing 3% of 1-*n*-dodecylpiperidone. Fig. 15a demonstrates liquefaction of epidermis, collagen fiber swelling and inflammatory cell infiltration in dermis, and degeneration of skin appendages. Fig. 15b shows severe coagulative necrosis and inflammatory cell infiltration in dermis and epidermis. In addition, edema and inflammatory cell infiltration can be seen in hypodermis. In Fig. 15c, eschar formation under the necrosed epidermis and re-epithelization are observed. Re-epithelization of epidermis, eschar formation and granulation tissue formation of hypodermis are clearly seen in Fig. 15d.

Comparison of permeation enhancement and skin irritation activity of enhancers

As shown in Table 2, 1-*n*-cetylpiperidone had the greatest enhancing activity on the percutaneous absorption of IMC in vitro (Quan et al., 1989a, b, 1990) and the stronger skin irritancy.

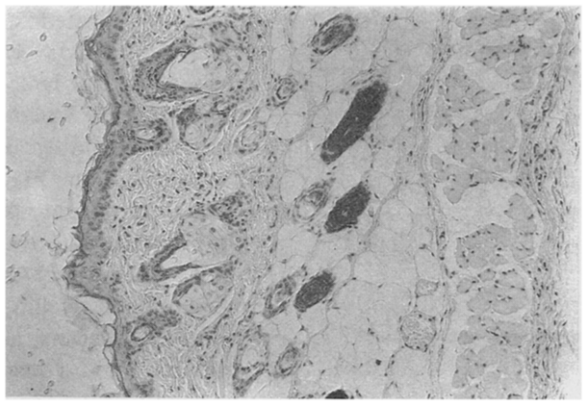

Fig. 12. Microscopic photo of rat skin at 24 h after application of gel ointment containing no enhancer. H&E × 100.

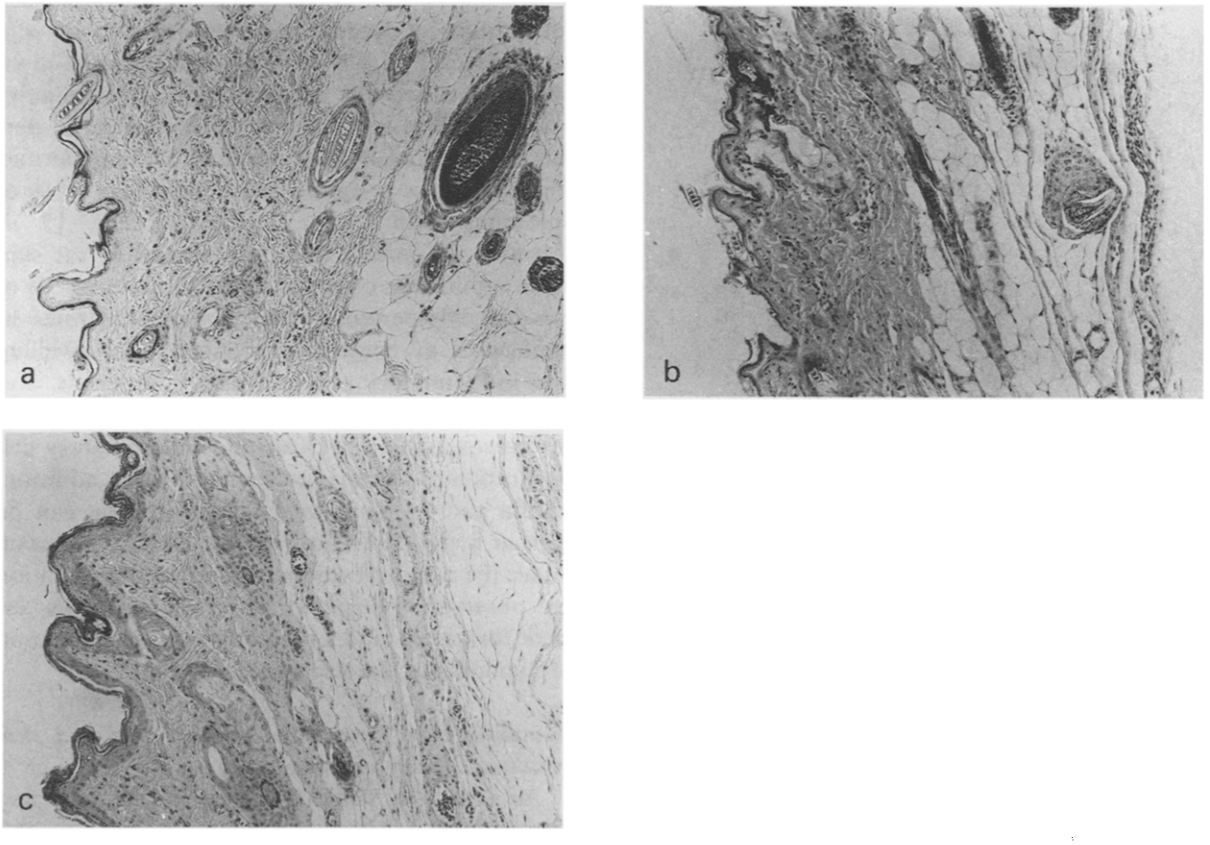

Fig. 13. Microscopic photos of rat skin after application of gel ointments containing 1% of 1-*n*-dodecylpiperidone. H&E × 100. (a) At 24 h after application. (b) At 1 day after removing the test ointments. (c) At 3 days after removing the test ointments.

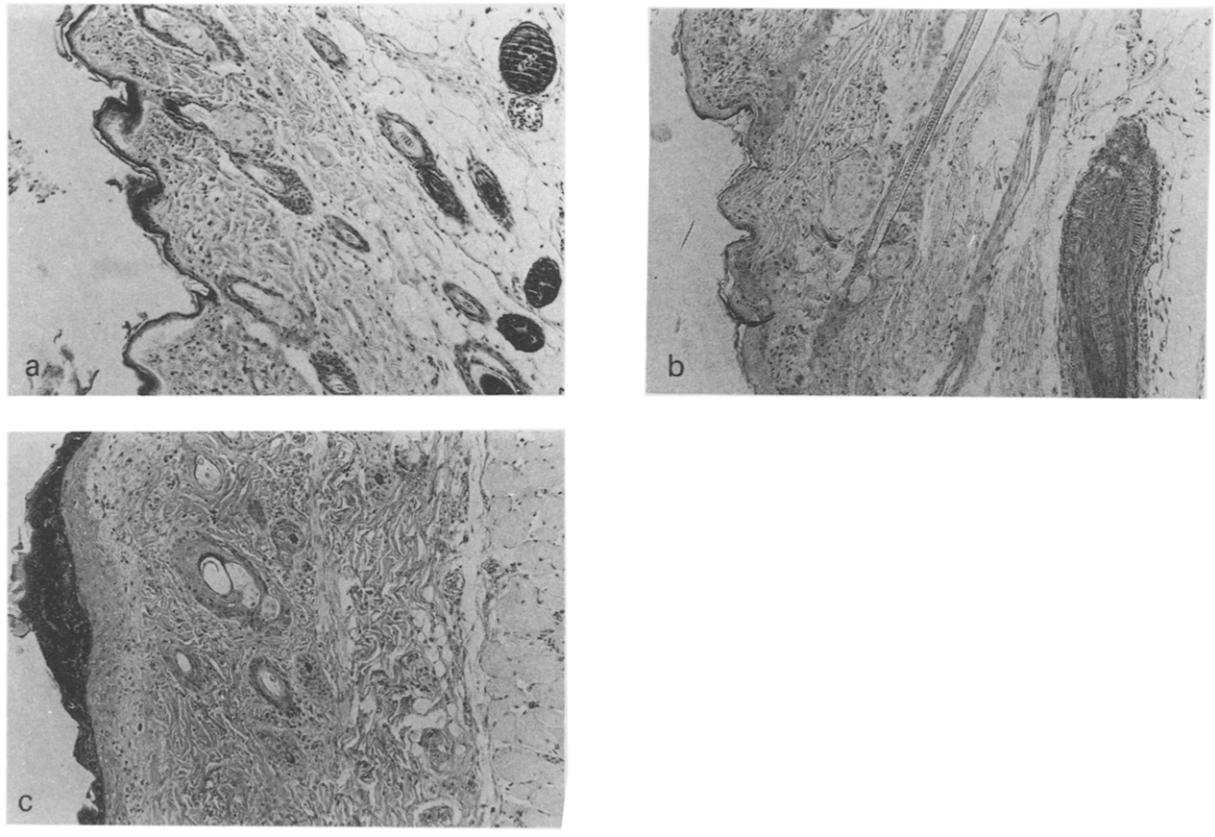

Fig. 14. Microscopic photos of rat skin after application of gel ointments containing 1% of Azone®. H&E × 100. (a) At 24 h after application. (b) At 1 day after removing the test ointments. (c) At 3 days after removing the test ointments.

Azone® showed the most severe skin irritation, but its activity as an enhancer was the lowest among these five enhancers. From the results, no direct relationship between permeation enhancement and skin irritation activity of enhancers was observed. It is well known in the dermatological literature that irritated skin is more permeable to applied chemicals and is more reactive to them. The reasons why 1-*n*-cetylpiperidone was less irritating than Azone®, but more enhancing than Azone®, are not clear. Probably, it should be attributed to many other factors, e.g. the physicochemical properties of the drug and enhancer used.

TABLE 2

Relationship between the skin damage indices and the permeabilities of IMC induced by 3% enhancers

Enhancer	Permeability ($cm/h \times 10^{-3}$)	Skin damage index
2-*tert*-Butylcyclohexanone	4.01 ± 1.07	0.8
2-*n*-Octylcyclohexanone	4.43 ± 0.34	0.8
1-*n*-Dodecylpiperidone	3.93 ± 0.71	1.1
1-*n*-Cetylpiperidone	25.8 ± 2.64	1.4
Azone®	3.41 ± 0.49	1.9

The data of permeabilities of IMC were cited from our previous paper. Skin damage indices were averaged by 5 days.

Conclusions

The histopathological studies of these five enhancers, 2-*n*-octylcyclohexanone, 2-*tert*-butylcyclohexanone, 1-*n*-dodecylpiperidone, 1-*n*-cetylpiperidone and Azone®, can be summarized as follows:

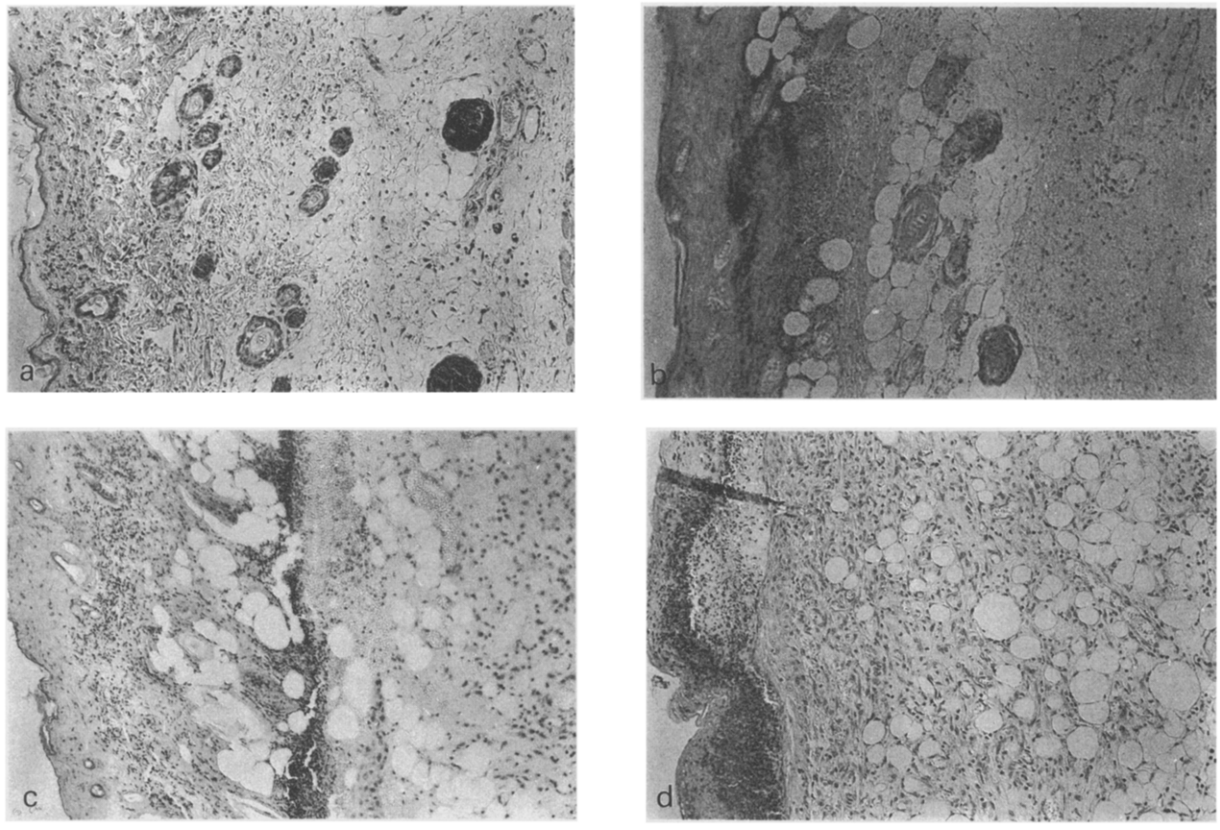

Fig. 15. Microscopic photos of rat skin after application of gel ointment containing 3% of 1-*n*-dodecylpiperidone. H&E × 100. (a) At 24 h after application. (b) At 1 day after removing the test ointments. (c) At 3 days after removing the test ointments. (d) At 5 days after removing the test ointments.

(1) With an increase in the enhancer concentration, the extent of skin damage will increase, and this effect will penetrate into the deep layer of skin tissue.
(2) These five enhancers examined in the present study showed a definite reversibility of the skin to a different extent. Comparatively, at a low concentration of enhancer, the reversibility of the irritated skin was more rapid than that at high concentration.
(3) Although Azone® and piperidone derivatives showed more severe skin damage than the others, their skin reparabilities, especially re-epithelization, were quicker than those of the others.
(4) From the results of this investigation, it is difficult to show a clear relationship between the permeation enhancement and the skin irritation activity of an enhancer.

It appears that much work is still required to screen some desirable penetration enhancers which minimize the skin irritation potential and optimize penetration enhancement.

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