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Some biochemical effects of isopropyl myristate and squalane on rabbit skin

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Synopsis

A biochemical method was employed to study the responses of RABBIT SKIN to ISOPROPYL MYRISTATE, SQUALANE, and DECANE. Skin samples excised with a Castroviejo keratotome were separated into lipid, TCA-soluble, RNA and DNA fractions after incubation with 40 μCi $Na_2H^{32}PO_4$ at 37°C for 1 hr. BIOCHEMICAL CHANGES in the epidermis with time were evaluated in terms of the changes in total phosphorus contents, specific activity and incorporated amount per unit DNA of each fraction. The results showed that decane damaged the skin so severely that the biosyntheses of lipids, RNA and DNA were reduced markedly for the first three days after application, but increased rapidly after that due to the repair. The effect of squalane was found to be weaker than that of isopropyl myristate, though both oils induced the stimulation of biosyntheses in the epidermis. The magnitude of the biochemical effects of the three oils on the skin was increased in the order of squalane, isopropyl myristate and decane, which was consistent with the results of macroscopic and histological observations. From the profiles of the effects it is postulated that the repairing processes are controlled by some feedback mechanisms.

INTRODUCTION

There are many studies on the biochemical changes in the skin caused by the percutaneous application of a chemical substance. Aso and Okazaki (1) measured the epidermal mitotic activity with ^{3}H-thymidine and the biosynthesis of epidermal lipids with ^{14}C-malonyl CoA and ^{14}C-mevalonic acid after the application of vitamin A acid on human psoriatic skin and normal guinea pig skin. Prottey et al. (2) studied the effects of soaps upon the metabolism of DNA and lipids of rat skin with the aid of ^{14}C-thymidine, ^{32}P-orthophosphate, ^{14}C-acetate and ^{14}C-glycerol. Mezei (3) investigated the effect of polysorbate 85 upon human skin in terms of the changes in the rate of ^{32}P incorporation into lipid, TCA-soluble, RNA and DNA fractions after incubating the skin sample in a medium with $Na_2H^{32}PO_4$. Stegman et al. (4) observed the inhibition of the effect of concanavalin-A on the epidermis of newborn rats by α-methyl-D-glucopiranoside in terms of the incorporation of ^{3}H-thymidine and ^{3}H-histidine.

On the other hand, there are few reports on the effects of the oils commonly employed

in cosmetics. Suzuki et al. (5) studied the permeability and skin irritation potential of five oils useful for cosmetics and found that isopropyl myristate showed histologically the severest irritation among the five oils although all were found to penetrate into the skin by microautoradiography. From their results, it was supposed that dynamic changes in the skin due to the cosmetic oils also could be detected biochemically. In this paper biochemical effects on Angora rabbit skin were studied by the application of isopropyl myristate. Effects of squalane were also studied since it induced only a minor irritation on the skin, although it was found to penetrate into the skin by microautoradiography (6). In addition, the effects of decane were studied as a positive control.

EXPERIMENTAL

Male Angora rabbits were used as experimental animals. The average weight of 60 rabbits employed in this study was 2.47 ± 0.19 kg.

Disodium hydrogen phosphate-^{32}P ($Na_2H^{32}PO_4$, 1.04 mCi/mg) was obtained from Daiichi Radioisotope Laboratory and diluted with water to 40 μCi/0.1 ml before use.

The hair of two 8 × 10 cm areas on the dorsal region symmetrical with a median line was removed with a hair clipper and an electric shaver (Braun) 1 day (d) prior to topical application. A 0.2-ml aliquot of squalane, isopropyl myristate or decane was applied to an 8 × 10 cm Japanese paper which covered one of the clipped areas, and was left in place for 1 hr. The clipped area on the opposite side of the rabbit served as an untreated control. After the removal of the paper, the rabbits were put back into the cages with polyethylene cangs to prevent disturbance of treatment site until sacrifice. At 2 and 6 hr, 1, 2, 3, 4, 5, 7, 10 and 14 d after the removal of the paper, both the treated and the untreated areas were wiped three times with cotton soaked in diethyl ether. Two animals were used for each period after application.

The test sites were observed macroscopically before excision. Five pieces of epidermis were then excised for each site with a Castroviejo keratotome with a 0.3-mm wedge. One of the pieces was fixed in formalin for histological evaluation. Eight test tubes were prepared for each period after application, half for the treated skin and the other for the normal. The test tube contained two pieces of epidermis, one from each animal. The epidermis samples in the test tube were weighed accurately, then incubated in a 4-ml aliquot of a medium (56 m*M* glucose, 5 m*M* KCl and 147 m*M* NaCl) with 40 μCi/0.1 ml $Na_2H^{32}PO_4$ at 37°C for 1 hr.

The fractions of lipids, TCA-soluble, RNA and DNA were isolated from the epidermis according to the method as shown in Figure 1 (7,8).

A 0.5-ml aliquot of each fraction was taken into a vial to which 10 ml of a scintilator (4 g PPO, 0.1 g POPOP, 333 ml triton X-100 in 667 ml toluene) (9) was added. The radioactivity was measured by a liquid scintilation counter (Aloka LSC-601).

The amount of total phosphorus of each fraction was measured according to the Bartlett method (10).

The contents of epidermal RNA and DNA were determined using calibration data derived from the solutions of yeast RNA and calf thymus DNA as standards, respectively.

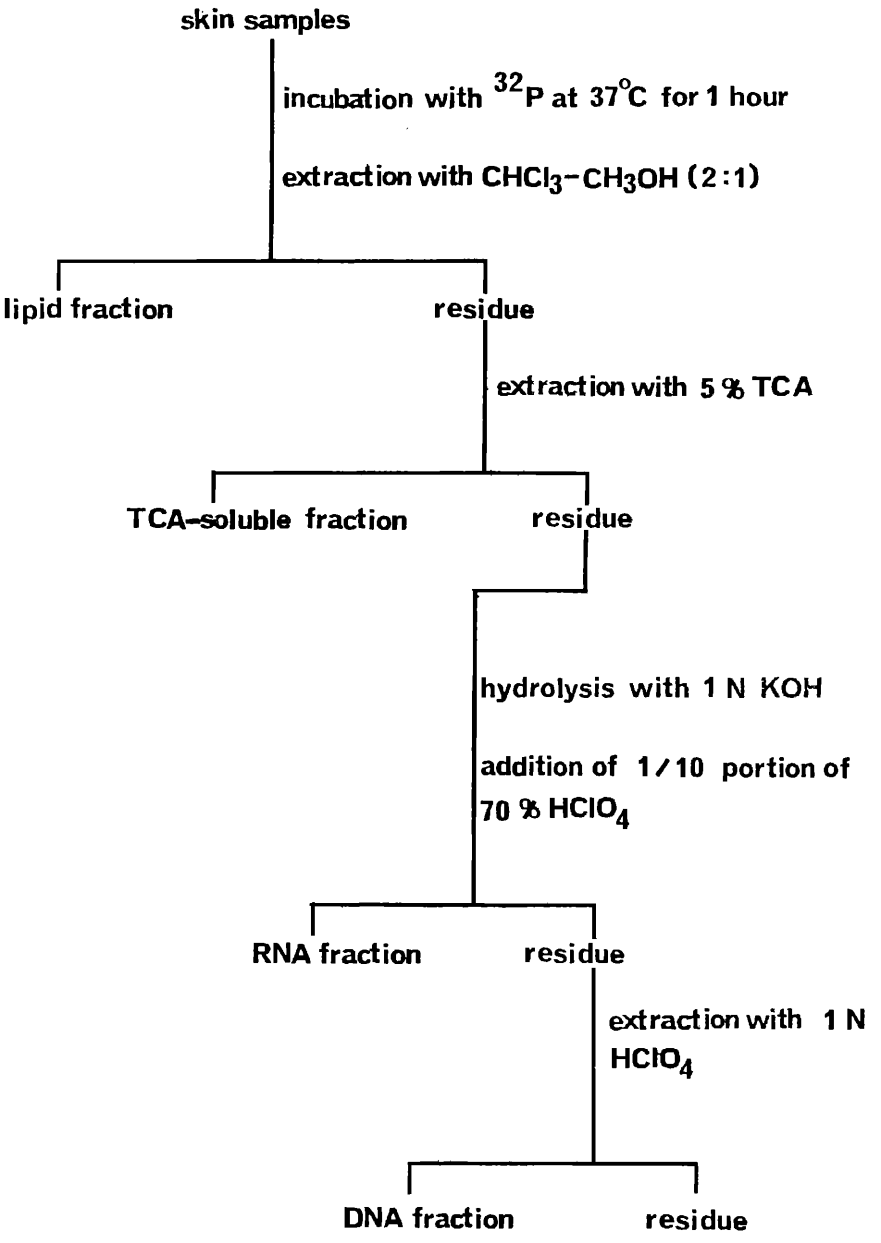

Figure 1. Separation method for isolation of lipids, RNA and DNA.

Standard solutions of RNA and DNA were prepared by dissolving yeast RNA and calf thymus DNA in 1 N KOH, respectively. A standard solution of phosphorus was prepared by dissolving purified KH_2PO_4 in distilled water. Calibration curves were drawn between the concentration and the optical density by the colorimetric method described by Bartlett (10). As shown in Figure 2, linear relationship was ascertained in all cases.

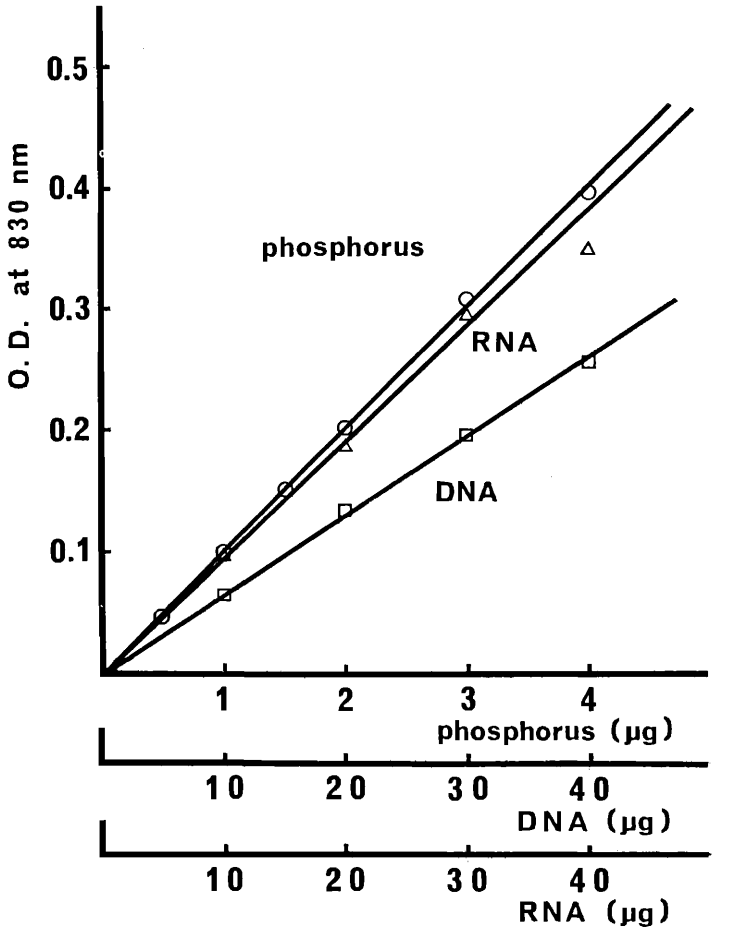

Figure 2. Phosphorus, DNA and RNA calibration data using the Bartlett method, optical density (O.D.) measured at 830 nm with a 1-cm light path.

RESULTS AND DISCUSSSION

PRELIMINARY EXPERIMENTS

At 24 hr after the application of isopropyl myristate on Angora rabbit skin for 1 hr, the epidermis samples from the treated and untreated sites were incubated for 0.5, 1 and 2 hr under the experimental conditions outlined above. Lipid, RNA and DNA fractions were separated and the radioactivity of each fraction was measured by the same procedures mentioned above. Figure 3 illustrates the result for the DNA fraction. The rate of ^{32}P incorporation into this fraction was nearly proportional to the incubation time and higher in the treated epidermis than in the untreated. Similar results were observed for the other two fractions. From these results, the incubation time of 1 hr was thought to be sufficient for the subsequent experiments.

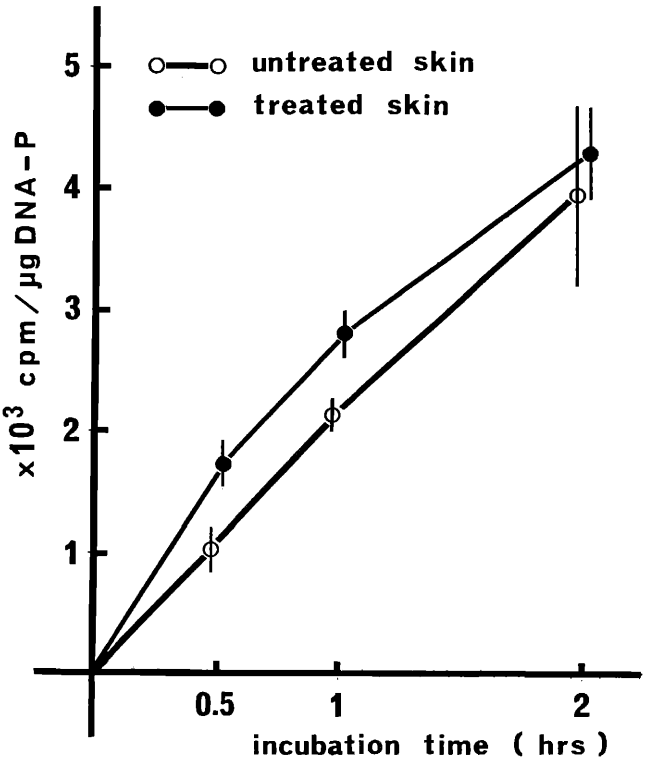

Figure 3. The effect of incubation time on the incorporation of ^{32}P into DNA. Results are expressed as counts per minute (cpm) per μg DNA-P. Each point represents a mean with standard deviation from three experiments.

MACROSCOPIC AND HISTOLOGIC OBSERVATIONS

Macroscopic observations indicated that squalane induced only minimal erythema at 2 and 6 hr after application but no changes in the skin surface in the following days. Isopropyl myristate caused distinct erythema until the second day and crusta and hemorrhage at 3-7 d, but the skin became normal at 10-14 d. After the application of decane hemorrhage and strong erythema appeared in the early stage, and crusta, disappearance of elasticity and cracks were observed at 3-7 d, but the repair of skin proceeded to be almost normal at 10-14 d. These gross observations indicated that the irritation potentials of squalane, isopropyl myristate and decane increased in this order.

Histological observations supported the macroscopic findings described above. Squalane only induced acanthosis until the third day and no histological changes were observed in the epidermis after that. Isopropyl myristate caused acanthosis and hyperkeratosis in the epidermis until the tenth day. Decane caused the severest changes including acanthosis, parakeratosis and crusta. Slight acanthosis was continued for 14 d (Figure 4).

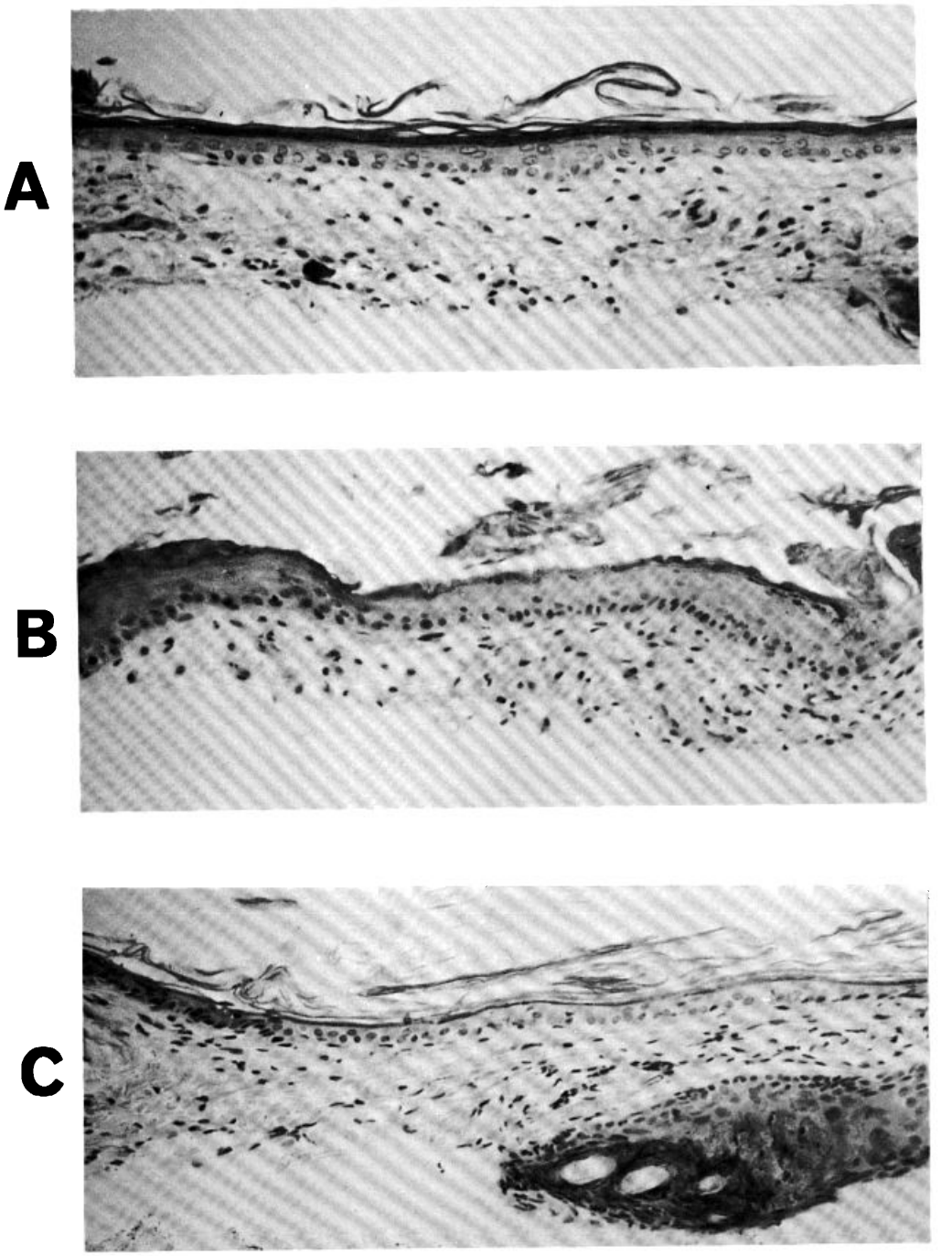

Figure 4. Light micrographs showing the histological feature in the skin of Angora rabbits after topical application of oils for 1 hr. The sections were stained with hematoxylin and eosin. A) isopropyl myristate, at 7 days, B) squalane, at 3 days, C) decane, at 14 days.

BIOCHEMICAL STUDIES

Prottey et al. (2) employed a Castroviejo keratotome with a 0.2-mm wedge to take epidermal samples from rat and Mezei (11) excised the epidermis of rabbit with the aid of the keratotome with a 0.1-mm wedge. In the present study, however, a 0.3-mm wedge was used because of the difficulties of excising a uniform sheet of skin due to the hair on the skin re-growing during the period after application and changes in the skin surface caused by the oils. The skin sample obtained with a 0.3-mm wedge contained full epidermis and upper layer of dermis, but was found in a preliminary study to be the same as that obtained with a 0.2-mm wedge in terms of biochemical changes in the epidermis induced by the oil.

Phospholipids are the principal materials which constitute biological membranes including the cell membrane (12). It was suggested that changes in the rate of

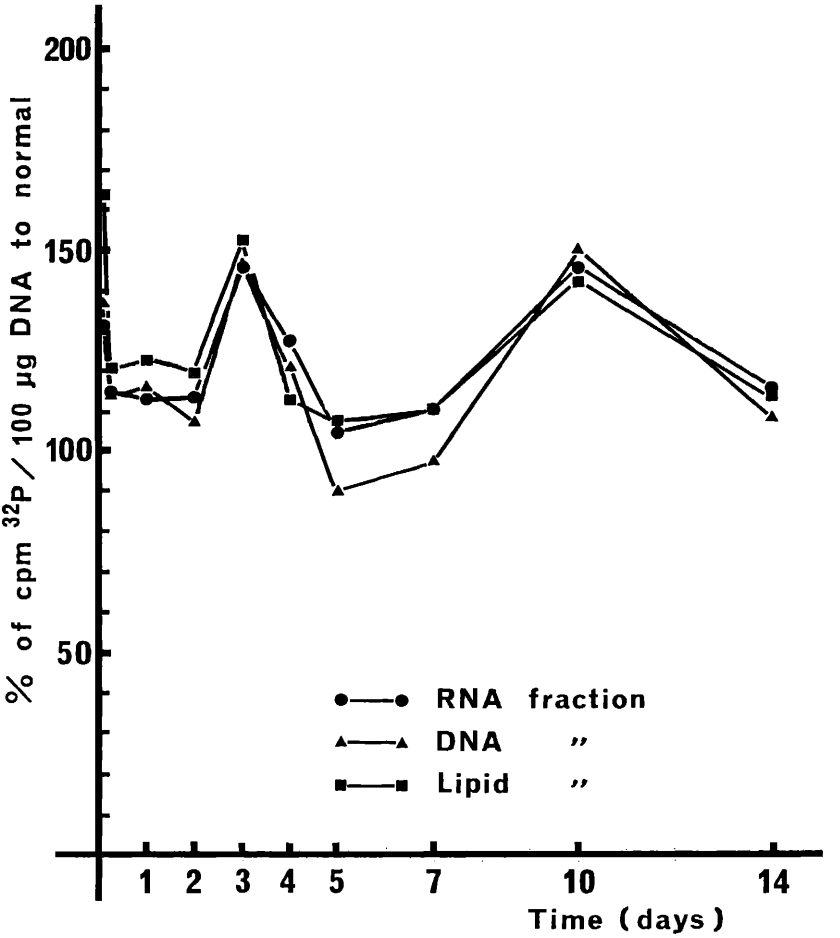

Figure 5. The effect of treatment with squalane on the incorporations of ^{32}P into RNA, DNA and lipid fractions. Results are expressed as percentage of cpm per 100 μg DNA as compared with the normal skin (100%). Each point represents a mean from four experiments.

phospholipid synthesis in the epidermis following the application of the oils on the skin were due to the reconstitution of the membrane damaged by the oils and the renewal of epidermal cells. As shown in Figures 5-7, the magnitude and pattern of the changes induced by each oil were different. Squalane induced mild changes as shown in Figure 5. Isopropyl myristate caused larger changes than squalane (Figure 6). Decane

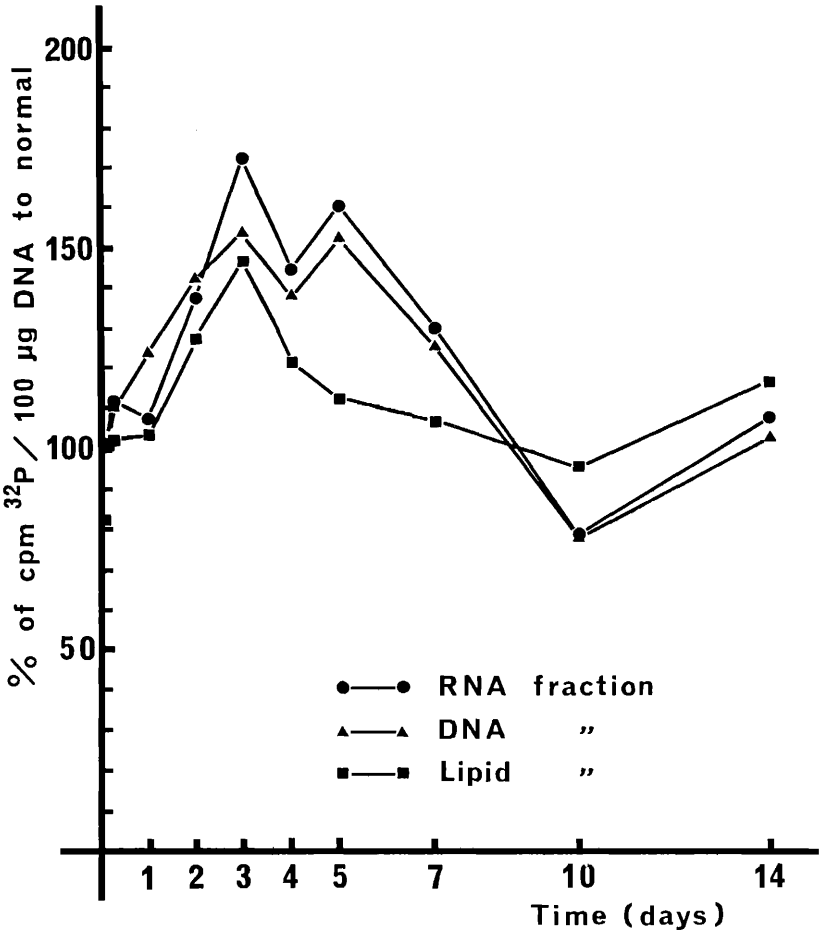

Figure 6. The effect of treatment with isopropyl myristate on the incorporations of ^{32}P into RNA, DNA and lipid fractions. Results are expressed as percentage of cpm per 100 μg DNA as compared with the normal skin (100%). Each point represents a mean from four experiments.

damaged the skin so severely in the early stage that biosynthesis of phospholipids was reduced to approximately 15% of normal at 3 d. The rate of biosynthesis increased after that due to repair, and exceeded that of normal (Figure 7). Although the changes in phosphorus content of the lipid fraction were not so large as those in the rate of ^{32}P incorporation, the trend of the effects of the three oils was found to be similar to that of ^{32}P incorporation.

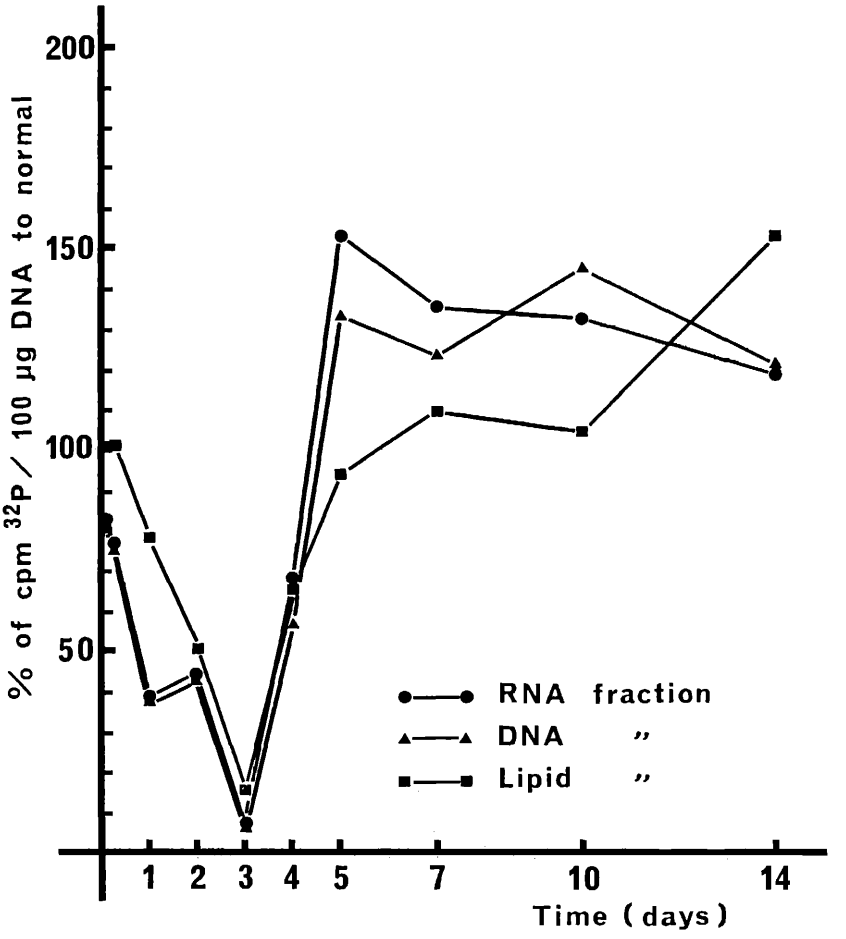

Figure 7. The effect of treatment with decane on the incorporations of ^{32}P into RNA, DNA and lipid fractions. Results are expressed as percentage of cpm per 100 μg DNA as compared with the normal skin (100%). Each point represents a mean from four experiments.

Prottey et al. (2) found that phospholipid metabolism in the epidermis of rats was stimulated in mildly irritated cases but diminished in severely irritated conditions when rats had been topically treated with soap solutions. They suggested that some systems required for the phospholipid synthesis were impaired by increased irritation. On the basis of their criteria, squalane and isopropyl myristate were judged to be mild irritants while decane was a severe one, results which were consistent with both macroscopic and histological observations.

Mezei (11) described an increase in the biosynthetic rate of epidermal phospholipids by the application of polysorbate 85 or polyethylene ether 96 on rabbit skin and suggested that this increase was due to repair of the biological membrane which was damaged by the surfactant. Some periodic changes in the biosynthetic rate could be seen in his data, which were also found in the present study.

Rohrschneider et al. (13) studied the changes in the metabolism of phospholipids in

the epidermis by the interaction of croton oil with the biological membrane when it was applied on the skin. They found two peaks of ^{32}P incorporation into phospholipids, the first occurred 4-6 hr after treatment while the second was observed at 2 d. They explained the two peaks of incorporation with time in terms of the difference in biosynthetic pathways of phosphatidylcholine by using (Me-^{14}C) methionine and (Me-^{14}C) choline. Although two peaks were found in the present study at 3 and 10 d for squalane, the mechanism suggested by Rohrschneider et al. was difficult to apply to this case since the patterns of the two peaks with time were different. It does appear, however, that the biosynthesis of phospholipids is regulated by some feedback mechanisms, especially when the irritation is mild.

Figure 8 illustrates the incorporated radioactivity into the TCA-soluble fraction per 100 μg DNA represented as the percentage against the normal. No trend was observed in the incorporated amount with time, since the TCA-soluble fraction contained various

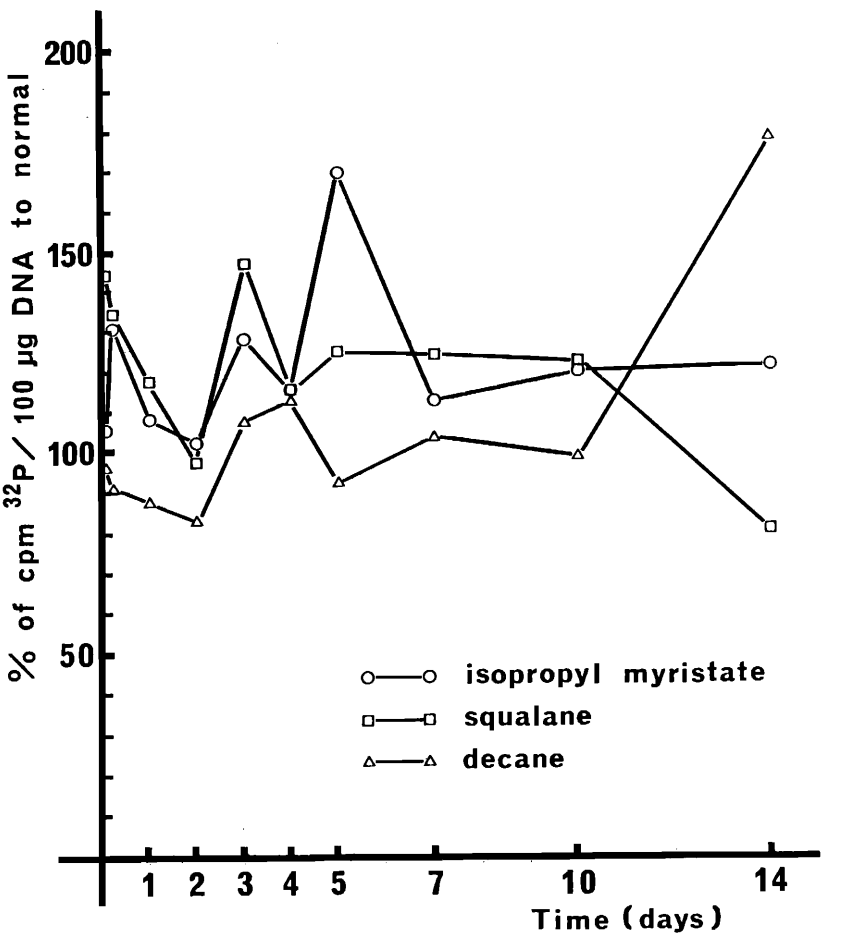

Figure 8. Incorporation of ^{32}P into TCA-soluble fractions of Angora rabbit skin treated with isopropyl myristate, squalane or decane. Results are expressed as percentage of cpm per 100 μg DNA as compared with the normal skin (100%). Each point represents a mean from four experiments.

precursors of phospholipids and nucleic acids so that the pattern of change appeared as the sum of the changes in the amounts of these precursors.

RNA is essentially requisite to the synthesis of proteins. It is well known that the amount of RNA is increased when the skin is damaged and the repairing processes begin (14). In the present study, changes in the rate of ^{32}P incorporation into RNA per 100 μg DNA induced by squalane showed a periodic pattern with two peaks (Figure 5). Those induced by isopropyl myristate were much larger as shown in Figure 6. In the case of decane (Figure 7), the biosynthesis of RNA was reduced markedly for the first 3 d, but increased rapidly after that; that is, it changed from below 10% of normal at the third day to over 150% at the fifth day. It was evident that it took a longer time to repair the skin damaged by the application of a more irritating substance.

It can be said that the increase in DNA synthesis is accompanied by the increase in proliferation in the epidermis (14); that is, the changes in proliferation of the epidermis

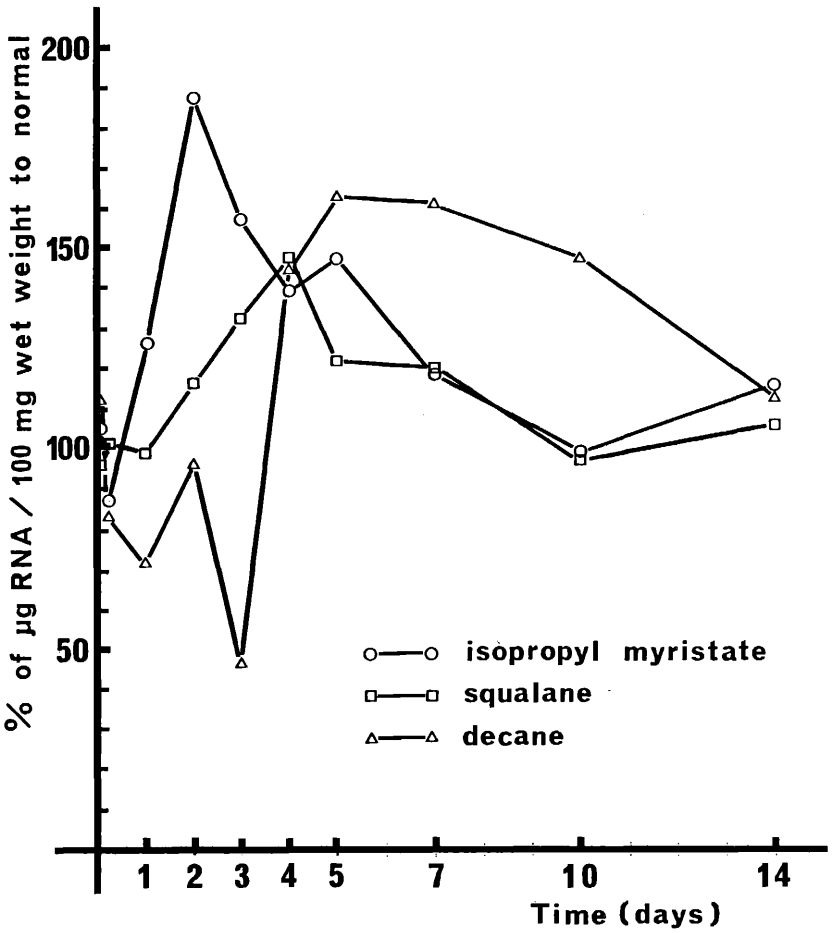

Figure 9. The effect of treatment with oils on the RNA content in the epidermis. Results are expressed as percentage of μg RNA per 100 mg wet weight of tissue as compared with the normal skin (100%). Each point represents a mean from four experiments.

or in the mitotic activity of the basal cells in the epidermis can be observed indirectly by the measurement of the changes in DNA synthesis. In the present study, the biosynthesis of DNA was evaluated in terms of the specific activity. Squalane induced two peaks of about 1.5 times that of the normal at 3 and 10 d (Figure 5). Isopropyl myristate induced a broad peak with two spikes at 3 and 5 d, whose levels were similar to those in the case of squalane (Figure 6). Decane induced a pattern of the biosynthesis of DNA similar to that found for phospholipid or RNA, indicating that this oil caused severe damage to proliferative cells in the epidermis (Figure 7).

Parallel changes in the rate of ^{32}P incorporation were observed among phospholipids, RNA and DNA, in the present study as shown in Figures 5-7. Potten (15) suggested, however, that the synthesis of RNA was stimulated first, followed by the synthesis of DNA in the skin of mice by the plucking of the hair. Baird et al. (16) reported that the syntheses of RNA, DNA, and proteins were stimulated in this order when the mouse skin was topically treated by phorbol and its diesters. Although the same sequence of

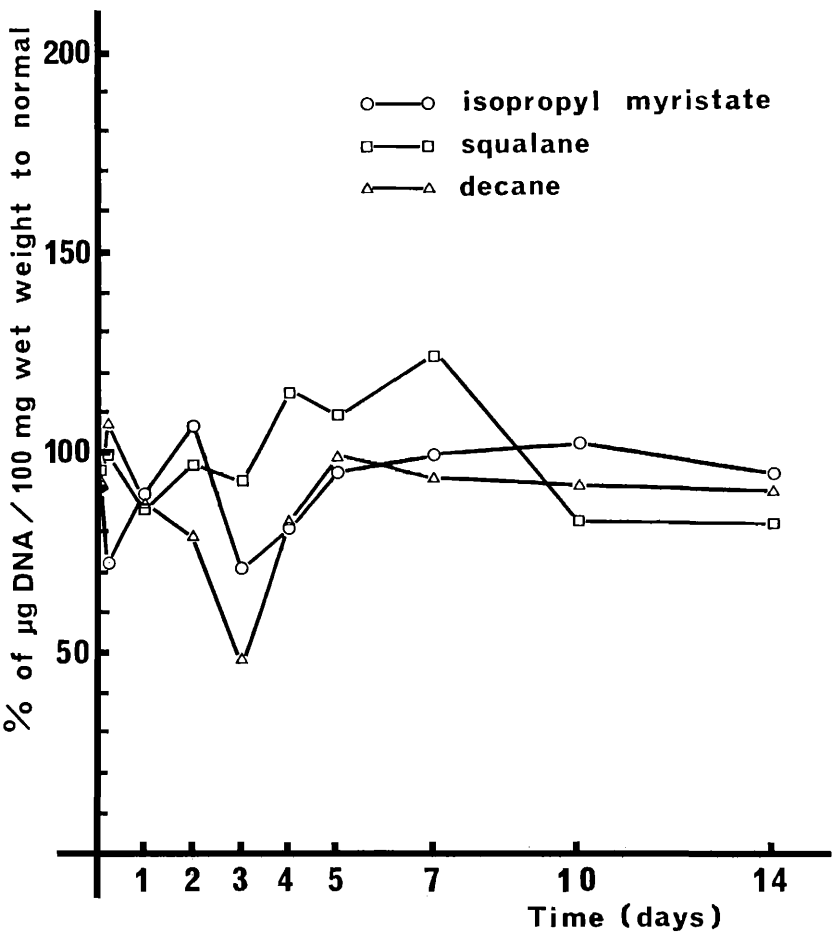

Figure 10. The effect of treatment with oils on the DNA content in the epidermis. Results are expressed as percentage of μg DNA per 100 mg wet weight of tissue as compared with the normal skin (100%). Each point represents a mean from four experiments.

metabolic changes in RNA and DNA was not found in the present study, it was suggested that the synthesis of RNA was more stimulated than that of DNA since Figures 9 and 10 showed greater changes in the amount of RNA per wet weight than those in the amount of DNA per wet weight, and Figure 11 showed a greater ratio of RNA to DNA than that of the normal. This stimulation was seen more clearly in Fig. 12, where the ratio of RNA-P per 100 μg DNA to the normal was plotted with time.

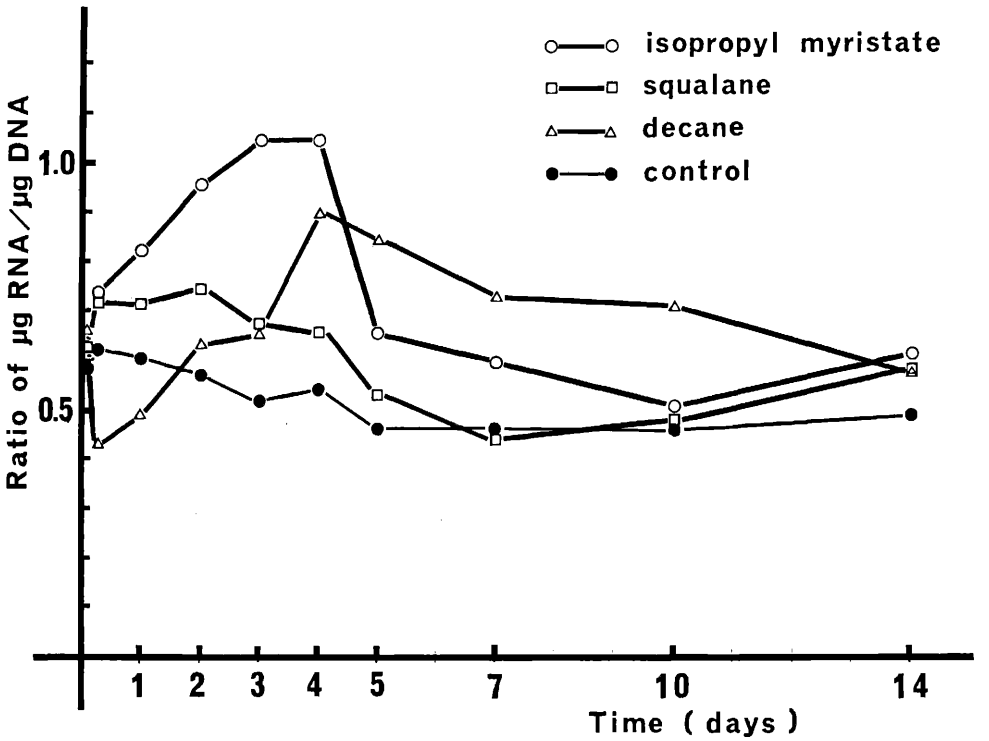

Figure 11. Comparison of the ratio of RNA to DNA in the epidermis between the oil-treated skin and the normal skin. Each point represents a mean from four experiments.

Many studies on the changes in the metabolism of RNA and DNA due to the stimulated reaction of skin have been reported in the literature. Argyris (17) measured the amount of RNA in the epidermis at various intervals after the abrasion of the backs of mice and found that the amount of RNA per g epidermis was increased markedly with a peak of 3.43 times that of the normal at 4 d, concluding that an increase in the amount of RNA was in response to damage. However in the present study, as shown in Figure 9, the amount of RNA did not reach a level of twice as much as normal, which indicated that the effects of the oils in this study were apparently much weaker than that of abrasion. Raick and Burdzy (18) studied the increase in incorporation of a labelled precursor into skin RNA by a single application of a hyperplastic agent, ethylphenylpropiolate, on the mouse skin, which was consistent with the results of the present study where acanthosis or hyperplasia was observed along with an increase in ^{32}P incorporation into RNA. Hamilton and Potten (19) studied the proliferative changes in epidermal cells after plucking the hair of mice by measuring the amount of

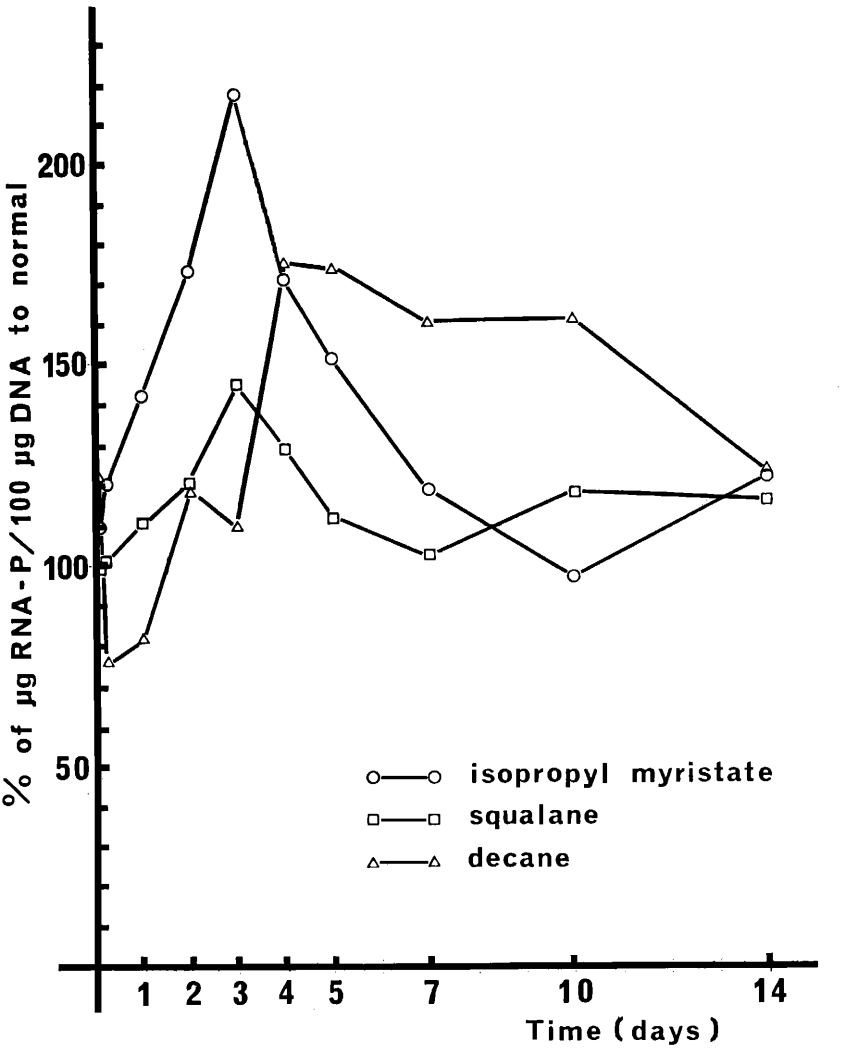

Figure 12. The effect of treatment with oils on the phosphorus content of RNA fraction. Results are expressed as percentage of μg RNA-P per 100 μg DNA as compared with the normal skin (100%). Each point represents a mean from four experiments.

incorporation of ^{3}H-thymidine into DNA. They found a small peak at 1.5 d, a high plateau at 4.5-8 d, a high peak at 10 d, a small peak at 14 d and a slight decrease of the amount of incorporation at 18-20 d, as compared with the normal. The maximum amount of incorporation was about 7 times the normal. Although the effect of plucking was shown to be more severe than that of the oils in this study, the up-and-down pattern of the incorporation of ^{3}H-thymidine into DNA was similar to that of ^{32}P-phosphate in the present study where periodic changes were observed in the rate of ^{32}P incorporation into DNA and the peaks remained about 1.5 times the normal in all cases (Figures 5-7). From these results, some feedback mechanisms (20 and 21) were presumed in the proliferative and differentiative processes in the epidermis, since

several investigations have suggested the existence of control mechanisms and substances like chalones (20, 22), cyclic nucleotides (22, 23) and others (20, 24).

Figures 4-12 indicated that the effects of squalane, isopropyl myristate and decane on rabbit skin increased in this order, but the irritation potentials of these oils were found to be significantly weak as compared with those of carcinogens (16, 25) or physical damages like plucking (19) and abrasion (17). In terms of the ratio of RNA to DNA, the oils in the present study increased the ratio in rabbit skin to twice the normal at best, while methylcholanthrene (25) or physical abrasion (17) induced an increase of 3-4 times in the epidermis of mice. The magnitude of the effect of vitamin A acid, when topically applied on the ear skin of guinea pig daily for 6 d (23), was found to be similar to that of these oils in terms of the stimulation of DNA synthesis.

Skin is a metabolically vigorous tissue, with continuing multiplication of cells in the basal layer and exfoliation of cornified cells in the stratum corneum. It possesses a great ability for healing the damaged epidermis by a highly active regeneration process. When chemical or physical treatments are applied to the skin, therefore, significant changes in proliferative activity will occur biochemically even though irritation potentials are weak as indicated by squalane. This finding indicates that care should be given to selection of cosmetic ingredients, even though they show low irritation potentials macroscopically or histologically, since cosmetics are applied repeatedly on the skin for many years.

CONCLUSION

A very sensitive method was employed to study the changes in the epidermis caused by cosmetic oils. In our previous paper (5), it was found that histological changes in the skin induced by a chemical irritant after percutaneous absorption bear no relation to the fate of the irritant in the skin. In the present study, more subtle changes in epidermis could be detected biochemically, especially for squalane which caused changes in ^{32}P incorporation periodically even after histological changes disappeared.

The magnitude of the biochemical effects for the three oils in this study was found to be weak in comparison with other chemical and physical irritations and to increase in the order of squalane, isopropyl myristate and decane, which was consistent with the results of macroscopic and histological observations. Some feedback mechanisms were suggested to control the processes of repairing the damaged skin.

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