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Studies on Fatty Acid Esters of Pyridoxine. II.[1] Percutaneous Absorption of Pyridoxine and Its Derivatives[2]

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Relations between the properties of pyridoxine and pyridoxine 3,4-diacylates and the changes of vehicles in percutaneous absorption were studied.

a) Depilated skin: PIN is absorbed more rapidly than PIN–HCl. While, PIN showed a rapid change in blood level, PIN–DK, –DIK gave high blood level 24 hours after the application of the drugs.

b) Normal skin: The normal skin cases for PIN, PIN–DK and –DIK showed the same blood level as depilated cases 8 or 24 hours after application of the drugs. The blood level of PIN did not decrease within 10 hours unlike the depilated skin.

It has been well recognized that vitamins play an important role in the living body. In dermatology and cosmetic chemistry, pyridoxine has been rather high-lighted in the maintenance of normal muco-cutaneous surfaces. Recently, the importance of pyridoxine has been increased with the development of study on the agent's biological function.[4] It was found that animals fed on diets deficient in pyridoxine showed marked abnormalities of the skin.[4,5] Thus, pyridoxine offers interesting applications in cosmetics and dermatologic ointments, since it is an important factor in combating several skin diseases.

An application of pyridoxine to ointments and cosmetics is accompanied by some difficulties, because of the agent's poor stability against light and heat with a resultant reddish color.[6] Furthermore, pyridoxine is considered to have difficulties of percutaneous absorption because of its high hydrophilic property.

Recently, relationship between the absorption of drugs and their physicochemical properties has been studied on the basis of oil–water partition. Modifications of water-soluble drugs into oil-soluble ones have been successfully developed for many drugs[7] as well as pyridoxine.[8]

Kobayashi and Ohta[9] have developed a new synthetic method of pyridoxine 3,4-diacylates which are stable against light and heat and soluble in oils.

Pyridoxine diacylates and triacylates proved to be less toxic to mice than pyridoxine hydrochloride and to have a vitamin B_6 activity to mice on oral administration.[10]

1) Part I: N. Mizuno, *Yakuzaigaku*, submitted.
2) This work was reported at the 14th Kinki Branch Meeting of the Pharmaceutical Society of Japan, Kyoto, November, 1964.
3) Location: *Toneyama, Toyonaka, Osaka.*
4) P. Holtz and D. Palm, *Pharmacol. Rev.*, **16**, 113 (1964).
5) S. Rothman, "Physiology and Biochemistry of the Skin," University of Chicago Press, Chicago, 1954.
6) E. Cunningham and E.E. Snell, *J. Biol. Chem.*, **158**, 491 (1945).
7) K. Yagi, J. Okuda and A.A. Dmitrovskii, *J. Biochem.*, **48**, 621 (1960).
8) T. Sakuragi and F.A. Kummerow, *J. Am. Chem. Soc.*, **78**, 839 (1956).
9) K. Kobayashi and H. Ohta, *C.A.*, **62**, 11825 *d* (1965).
10) *a*) T. Sakuragi and F.A. Kummerow, *Arch. Biochem. Biophys.*, **63**, 32 (1956); *b*) T. Sakuragi and F.A. Kummerow, *J. Nutr.*, **58**, 557 (1956); *c*) S. Shintani, F. Tanaka, M. Nakamura and M. Sato, *J. Vitaminol.*, **7**, 122 (1961).

The above derivatives revealed to be equally effective as vitamin B_6 in treating skin diseases.[11]

It has been well recognized that the biological activity of drugs varies considerably depending upon the process of preparation. The degree of percutaneous absorption changes considerably being subjected to the kind of vehicles and of physicochemical property of drugs employed. Thus, the duration of efficacy of the drug at a therapeutic concentration in skin may differ considerably with changes of vehicles used.

In the present report, relations between the properties of pyridoxine derivatives and the changes of vehicles in percutaneous absorption were studied on the basis of a physicochemical aspect.

Experimental

Materials

Pyridoxine 3,4-Diacylates——Pyridoxine and its diacylates employed in the present study are presented with some physicochemical constants (Tables I[12] and II).

Table I. Pyridoxines and Pyridoxine 3,4-Diacylates Employed

Compounds	mp (°C)	Formula	Mol. wt.
Pyridoxine 3,4-di-*n*-octanoate	69—71	$C_{24}H_{39}O_5N$	421.58
Pyridoxine 3,4-di-isooctanoate	liquid	$C_{24}H_{39}O_5N$	421.58
Pyridoxine	160	$C_8H_{11}O_3N$	169.17
Pyridoxine HCl	205—212	$C_8H_{12}O_3NCl$	205.64

Table II. Solubility of Pyridoxine 3,4-Dioctanoate

Solvents	M/liter at 37 (°C)	Solvents	M/liter at 37 (°C)
Chloroform	soluble	Propylene glycol	0.1601
Acetone	soluble	Mineral oil	0.0013
Oleyl alcohol	0.4533	Water	almost insoluble
Isopropyl myristate	0.1963		

Table III. Standard Cream Base

Isopropyl myristate	1 g	BC–5[b]	0.15 g
Cetyl alcohol	0.25	Liquid paraffin	1.35
Stearic acid	0.4	10% triethanolamine aq. soln.	1 ml
Paraffin wax	1	Pyridoxine derivatives	1%
BL–9[a]	0.2	Water to make	10 g

a) BL–9: poly(oxyethylene) lauryl ether *b*) BC–5: poly(oxyethylene) cetyl ether

Vehicle——The vehicle employed was a cream of oil-in-water type (Table III), which proved to have no irritative effects on human by the present authors' patch test. The content of pyridoxine hydrochloride (PIN–HCl) was 1%.

Pyridoxine (PIN) was incorporated as 1% of PIN–HCl into the cream and was neutralized to PIN with triethanolamine. Acylates were incorporated as 1% of PIN into the cream. PIN–diacylates, pyridoxine 3,4-di-*n*-octanoate (PIN–DK) and pyridoxine 3,4-di-isooctanoate (PIN–DIK) (supplied through the Nihon Surfactants Industries Co., Ltd.), were hydrolyzed in the presence of triethanolamine at elevated tempera-

11) G. Rocheggiani, *Seifen-Öle-Fette-Wachse*, **85**, 777 (1959); Y. Yasuda, *Japan. J. Dermatol.*, **73**, 487 (1963).
12) H. Ohta, *J. Soc. Cosmetic Chemists*, **16**, 349 (1965).

ture, but stable at room temperature. Thus, they were incorporated into the vehicles immediately before the experiment at room temperature.

Procedure

Percutaneous Absorption Test——The experimental animals were matured rabbits weighing 2.5—3.0 kg. The rabbit was lightly anesthetized with ether, and the abdominal area of a rabbit, 5×15 cm^2, was clipped with an electric clipper or depilated with Eba Soft Cream (6.5% of Ca thioglycolate in a cream base, Tokyo Tanabe Pharmaceutical Co., Ltd.) twenty-four hours prior to the application of pyridoxine cream. Before the application of pyridoxine cream, a check-up was made to assure little injury in the epidermis during clipping or depilation on gross observation and an electric conductivity measurement.[13] Being anesthetized with ether, the rabbit was restrained on a board on a surpine position. Six grams of cream, containing pyridoxine or its derivatives in the concentration described above, was spread uniformly over the clipped or depilated skin with a spatula. After application of the cream, one gram of blood was chronologically taken from the ear lobe by veni-puncture. The concentration of pyridoxine was assayed microbiologically with *Saccharomyces carlsbergensis* 4228.

Measurement of Electric Conductivity——To check any damage on skin during clip or depilation, an electric conductivity of the skin was measured in an ordinary manner.

Microbiological Assay of Pyridoxine——There are many methods to measure the amount of pyridoxine in pharmaceutical preparations and in living organisms. In general, a microbiological assay introduced by Atkin,[14] *et al.*[15] has been popular in measuring pyridoxine content in living organisms because of its high sensitivity. The method is also applicable to what has the same sensitivity for metabolites of pyridoxine, such as pyridoxine, pyridoxal, pyridoxamine and combined forms of vitamin B_6, by hydrolyzing with H_2SO_4.[16] However, the method is not applicable to diacylates because of their deficiency of microbiological activity of vitamin B_6.[10a,17] To assay the diacylates in blood, it is necessary to hydrolyze them to pyridoxine so that they may be active to microorganisms. To establish a standardized condition of hydrolysis, a study was made in acid and alkaline media with or without blood. It was found that the acylates in blood were completely hydrolyzed in the presence of 6 ml of 0.3N H_2SO_4 at 126.5° (20 pounds per sq. in.) for 1 hour without any loss of the vitamin B_6 activity for *Saccharomyces carlsbergensis*. Pyridoxine, pyridoxal, pyridoxamine

Table IV. Analytical Method of Pyridoxine and Its Derivatives in Blood

One g of blood + 6 ml of 0.055 N H_2SO_4
↓ 20 lb. (126.5°) for 1 hr
Cool to room temperature
↓
Add 1 ml of 2 N H_2SO_4
↓ 20 lb. (126.5°) for 1 hr
Adjust to pH 5.4 with NaOH
↓
Centrifuge at 9000 rpm for 7 min
↓
Separate the precipitated protein
↓
Make 50 ml with dist. water
↓
Take 0.5 or 1 ml (test solution)
↓
Table VI

Table V. Atkin's Culture Medium

Glucose	100 g
Casamino acid (Difco)	10 g
Inositol	50 mg
Nicotinic acid	5 mg
Calcium pantothenate	5 mg
Thiamine HCl	0.5 mg
Biotin	0.02 mg
Potassium citrate	10 g
Citric acid	2 g
KH_2PO_4	1.1 g
KCl	0.85 g
$CaCl_2 2H_2O$	0.25 g
$MgSO_4 7H_2O$	0.25 g
$MnSO_4 6H_2O$	0.005 g
$FeCl_3 6H_2O$	0.005 g
H_2O to make	1000 ml

13) I.H. Blank, R.D. Griesmer and E. Gould, *J. Invest. Dermat.*, **30**, 187 (1958).
14) L. Atkin, A.S. Schultz, W.L. Williams and C.N. Frey, *Ind. Eng. Chem.*, **15**, 141 (1943).
15) J.C. Rabinowitz and E.E. Snell, *Ind. Eng. Chem.*, **19**, 277 (1947).
16) D. Melnick, M. Hochberg, H.W. Himes and B.L. Oser, *J. Biol. Chem.*, **160**, 1 (1945).
17) S. Shintani, F. Tanaka, M. Nakamura and M. Sato, *J. Vitaminol.*, **7**, 182 (1961).

and their phosphates were naturally occuring compounds. They were mostly conjugated with high molecular weight protein or carbohydrate in a living body. Thus, it is necessary to hydrolyze the conjugated compounds and phosphates to possess their vitamin B_6 activity for the microorganisms.[18] The hydrolysis were generally performed with 4—8 ml of 0.055 N H_2SO_4 in 1 ml of blood at 126.5° for 1 hour. As a preliminary test, pyridoxal 5-phosphate was hydrolyzed with 0.3N H_2SO_4. A little loss in the biologic activity was observed. But pyridoxal was not decreased in its activity under the same condition of hydrolysis. Thus, the hydrolysis of the conjugated compounds and phosphates was performed with 0.055N H_2SO_4, then, the resulted compounds with 2N H_2SO_4.

The Microbiological Assaying Method of Pyridoxine is as Follows: The pretreatment of blood including hydrolysis is shown in Table IV. One gram of blood was hydrolyzed with 6 ml of 0.055N H_2SO_4 at 126.5° (20 pounds per sq. in.) for 1 hour, then cooled to room temperature and again the solution was hydrolyzed with 1 ml of 2N H_2SO_4 at 126.5° (20 pounds per sq. in.) for 1 hour, and neutralized with 1N NaOH to pH 5.4. The resulted solution was centrifuged at 9000 rpm for 7 min to separate protein in blood. The supernatant solution was diluted to 50 ml with water including water used for washing out pyridoxine adsorbed to protein. Five tenths or one ml of the above solution and 2.5 ml of Atkiń culture medium (Table V) were taken into a test tube, 16 mm in diameter, and diluted to 5.0 ml with water. The tube was covered with an aluminum cap. The solution was sterilized at 100° for 10 min and cooled to room temperature. Six thousandths ml of suspension of *S. carlsbergensis* 4228 was inoculated into the solution. Following incubation at 30° for 20 hours, the solution was steamed for 5 min and the growth of the yeast was measured turbidimetrically at 610 mμ. The suspension of *S. carlsbergensis* innoculated into the solution was prepared as described in Table VI.

Table VI. Microbiological Assay of Pyridoxine

Atkin's culture medium (2.5 ml) + test solution (1 ml) + water (1.4 ml)
↓ heat at 100° for 10 min
Cool to room temperature
↓ innoculate 0.1 ml of suspension[a] of *Saccharomyces carlsbergensis* 4228
Incubate at 30° for 20 hr
↓
Turbidity measurement at 610 mμ

a) preparation of *Saccharomyces carlsbergensis* 4228 suspension
Culture medium (1 ml) + water (1 ml) + pyridoxine HCl (10 mμg)
↓ heat at 100° for 10 min
Innoculate 1 loop of stocked *Saccharomyces carlsbergensis* 4228 in slant culture
↓ incubate at 30° for 24 hr
Centrifuge (3000 rpm)
↓
Wash the precipitate with physiological saline for 3 times
↓
Adjust to 40 ml with saline water
(suspension of *Saccharomyces carlsbergensis*)

The concentration of pyridoxine or its derivatives in blood obtained by comparing a standard growth curve for known amount of the same compound employed in a usual manner presented elswhere.[19] The concentration in blood was presented as a net increase of the compound by subtracting a natural level of vitamin B_6 in blood from a gross concentration.

Result and Discussion

Absorption of PIN-HCl and PIN through the Depilated Skin

Percutaneous absorption of PIN or PIN–HCl incorporrated into a cream was studied for the rabbit skin treated with a depilating cream (Fig. 1 and 2). Each plot in the figures represents a mean value and range of three tests.

18) H. Yamada, M. Tuji and K. Abe, *Bitamin*, 34, 452 (1966).
19) K. Matuo, *Nagoya Igaku*, 74, 700 (1957).

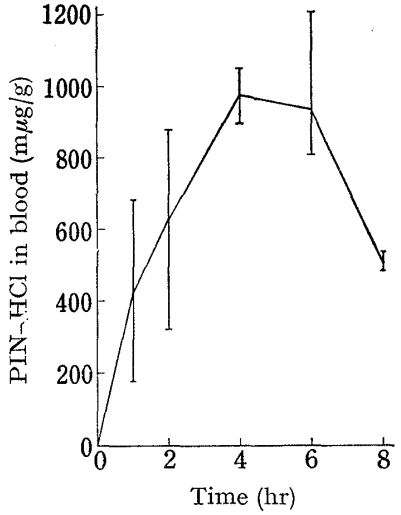

Fig. 1. Percutaneous Absorption of Pyridoxine (Pretreated with Depilating Cream)

Each plot represents a mean value and range of three tests.

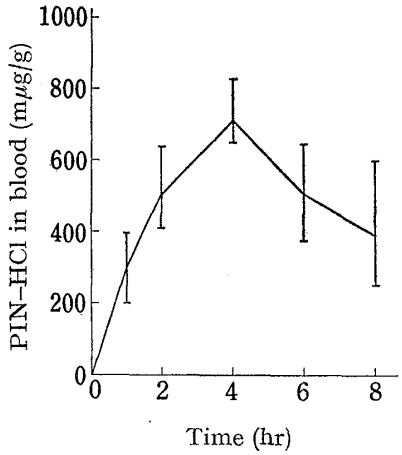

Fig. 2. Percutaneous Absorption of Pyridoxine hydrochloride (Pretreated with Depilating Cream)

Each plot represents a mean value and range of three tests.

In the present series of experiments, a natural level of vitamin B_6 in the rabbit blood was found to be 40—90 mμg/g in terms of pyridoxine hydrochloride quantitatively which was quite agreeable with the literature.[20]

After the application of the cream, the concentrations of PIN and PIN-HCl in blood increased within 15 min and reached maximum in 4 hours, then decreased rapidly.

It has been generally accepted that the unionized molecules were easily absorbed percutaneously, but not for the ionized ones.[21] Thus, a barrier for the percutaneous absorption was considered to have a lipophilic property. Solubility of PIN in chloroform was 248×10^{-6}M/liter at 37° and PIN-HCl in chloroform was 3×10^{-6}M/liter at the temperature. Thus, PIN was absorbed through lipid barrier in the skin more easily than PIN-HCl (Fig. 1 and 2).

Here it must be admitted that the cream of PIN-HCl is emulsified excluding triethanolamine (Table III). Therefore, stearic acid incorporated into the cream is considered to increase a required HLB of the oil phase[22] as a result of excluding triethanolamine. Furthermore, the amount of surface-active agents in the cream is reduced and the HLB value of emulsifiers changes considerablly from the optimum HLB for emulsification of the cream. Thus, a poor emulsification and decreased stability were observed for the cream from which triethanolamine was excluded. To

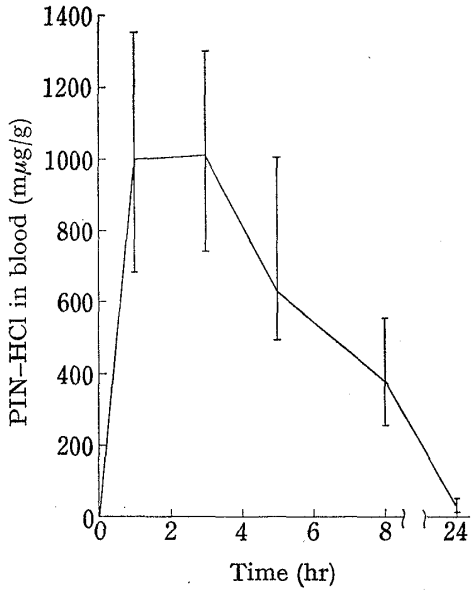

Fig. 3. Percutaneous Absorption of Pyridoxine (Triethanolamine is excluded from the Standard Cream, pretreated with Depilating Cream)

Each plot represents a mean value and range of three tests.

20) O. Frank, H. Baker and H. Sobotka, *Nature*, **197**, 490 (1963); H. Uetoko, *Nagoya Igaku*, **76**, 170 (1958).
21) S. Monash, *J. Invest. Dermat.*, **29**, 367 (1957); E.S. Stolar, G.V. Rossi and M. Barr, *J. Am. Pharm. Assoc.*, Sci. Ed., **49**, 144 (1960); E.S. Stolar, G.V. Rossi and M. Barr, *ibid.*, **49**, 148 (1960).
22) W.C. Griffin, *J. Soc. Cosmetic Chemists*, **1**, 311 (1949).

compare the difference of PIN and PIN–HCl for percutaneous absorption with the same cream, PIN was incorporated into a cream which was emulsified excluding triethanolamine of Table III (Fig. 3). The presence or absence of triethanolamine in a cream resulted in little change in the blood concentration of PIN (Fig. 1 and 3). The blood level of PIN decreased to normal level 24 hours after the application for the depilated skin. The same rapid decrease of PIN may be expected for PIN–HCl on depilated skin (Fig. 1 and 2).

Percutaneous Absorption of PIN-DK and -DIK through the Depilated Skin

Percutaneous absorption of pyridoxine 3,4-dioctanoate was studied for *n*- and isocotanoate (Fig. 4 and 5). The concentrations of the esters in blood increased gradually for 8—10 hours. The blood levels at 8 hours after a single application of a cream were 300 mμg/g for PIN–DK

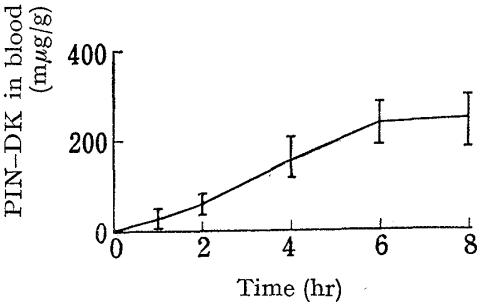

Fig. 4. Percutaneous Absorption of Pyridoxine 3,4-Di-*n*-dioctanoate (Pretreated with Depilating Cream)

Each plot represents a mean value and range of three tests.

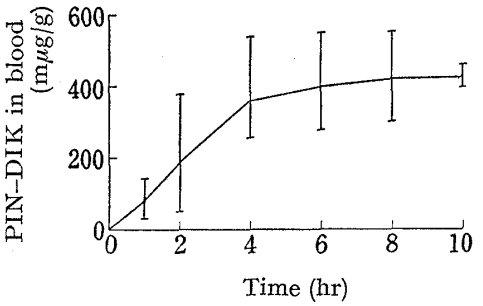

Fig. 5. Percutaneous Absorption of Pyridoxine 3,4-Di-isooctanoate (Pretreated with Depilating Cream)

Each plot represents a mean value and range of three tests.

and 400 mμg/g for PIN–DIK. While PIN and PIN–HCl showed a rapid change in blood level (Fig. 1 and 2), PIN–DK and PIN–DIK with a single application of cream followed by a constant blood level 8 hours after the administration. Thus, PIN–DK and PIN–DIK with their long standing blood level may be favorablly applied for dermatologic and cosmetic purposes.

Percutaneous Absorption of PIN, PIN-DK, and PIN-DIK through the Normal Skin

PIN incorporated into a cream (Table III) was applied to the skin clipped with an electric clipper, denoted as normal skin (Fig. 6 and 7). The blood level of PIN increased gradually

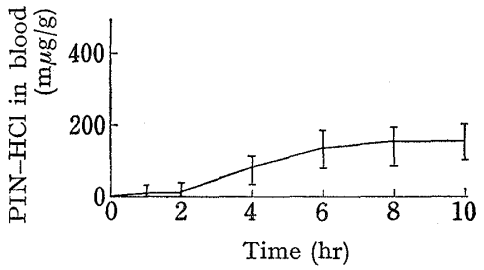

Fig. 6. Percutaneous Absorption of Pyridoxine (Normal Skin)

Each plot represents a mean value and range of three tests.

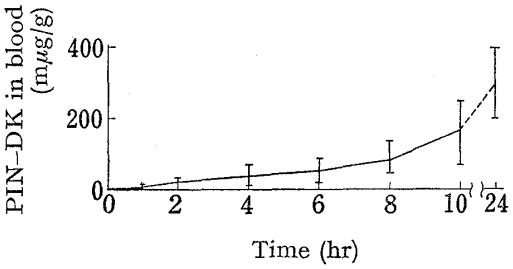

Fig. 7. Percutaneous Absorption of Pyridoxine 3,4-Di-*n*-octanoate (Normal Skin)

Each plot represents a mean value and range of three tests.

for 10 hours. Thus, it may be considered that the percutaneous absorption of PIN is markedly influenced by physiologic conditions of the skin.

Treherne[23] found that removal of the epidermis increased the permeability of rabbit skin by two to three orders. Many studies with a stripping technique have revealed the

23) J.E. Treherne, *J. Physiol.*, 133, 171 (1956).

location and physicochemical properties of the barrier membrane for percutaneous absorption.[24] Szakall also found that many lipid droplets were found in the barrier membrane.[25] And it has been known that the barrier membrane is permeable for substances which are freely miscible with cholesterol and phospholipids. Thus, the lipophilic substances penetrate with great ease through the skin, but not hydrophilic substances. Here, it may be considered that the barrier for percutaneous absorption plays an important role upon the absorption of PIN because of its poor lipid-solubility. Once the barrier shows no normal response to absorption, PIN is easily absorbed through the skin. Another finding was that PIN revealed a rapid excretion from the blood, which is a common defect for water-soluble drug.[26] For the normal skin, PIN in the blood was found to increase gradually for more than 10 hours after a single application of a cream. It is suggested that the amount of absorption of PIN is greatly reduced by the barrier of normal skin. Still the amount of absorption seems slightly higher than that of excretion. Thus, the limited percutaneous absorption of PIN keeps a blood level to be three times the normal level.

It may be considered that a blood level of a drug through percutaneous absorption is sustained in appropriate degree. For that purpose, it is required to control the absorption by modifying the rate of release of the drug from a preparation into the skin, and adjusting the amount slightly higher than that of excretion.

For PIN-DK, the difference of the effects between the normal and depilated conditions of skin upon percutaneous absorption resulted as a difference of increasing rate of blood levels. Here, it may be assumed that a barrier controls its absorption irrespective of its lipophilic properties (Fig. 4 and 7). The lipophilic property of PIN-DK is considered to be unappriate enough for percutaneous absorption (Table II) and/or its inadequate partition property between water and lipid. Thus, a poor permeation through the depilated skin was observed for PIN-DK.

For PIN-DIK, the absorption showed a delay for initiation of the drug penetration into blood (Fig. 8). The blood level started to increase 2 hours after application of a cream, and the level increased to 400 mμg/g in 10 hours as was the case with the depilated skin (Fig. 5).

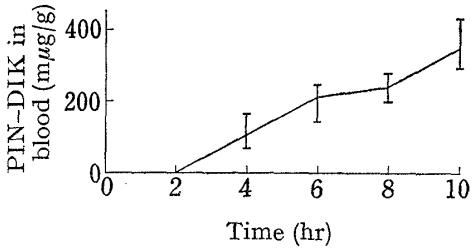

Fig. 8. Percutaneous Absorption of Pyridoxine 3,4-Di-isooctanoate (Normal Skin)

Each plot represents a mean value and range of three tests.

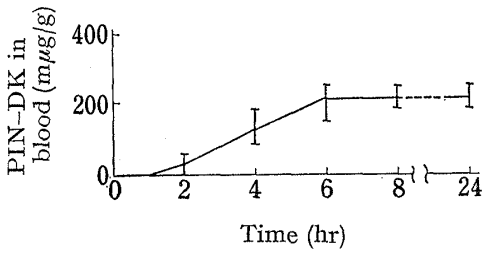

Fig. 9. Percutaneous Absorption of Pyridoxine 3,4-Di-*n*-octanoate (Triethanolamine is incorporated in PIN-DK cream, pretreated with cream base for 8—12 hours.)

Each plot represents a mean value and range of three tests.

The effect of pretretment of the cream on an electric resistance of the skin and on absorption of PIN-DK was studied (Fig. 9). The effect of pretreatment with the cream was revealed to be considerable on absorption. The time of plateau of blood concentration in depilated skin and pretreated one was faster than in normal skin. A similar relationship was

24) S. Monash, *J. Invest. Dermat.*, **29**, 367 (1957); S. Monash and H. Blank, *Arch. Dermatol.*, **78**, 710 (1958); F.N. Marzulli, *J. Invest. Dermat.*, **39**, 387 (1962); T. Tregar, *J. Soc. Cosmetic Chemists*, **13**, 145 (1962); J. Wolf, *Z. Mikroskop. Anat. Forsch.*, **47**, 351 (1940).
25) A. Szakall, *Arch. Dermatol. u. Syphilis*, **194**, 376 (1952).
26) B.B. Brodie, C.A. Hogben, *J. Pharm. Pharmacol.*, **9**, 345 (1957).

observed qualitatively for electric resistance changes between the three different conditions of skin. Thus, it may be concidered that the skin of rabbits is considerablly feeble for the cream base which has little effect on human skin during authors' patch test. The main reason is considered to be the influence of long term contact with surface-active agents, alkaline solution of triethanolamine, and/or water moisture, resulting a partial deformation of the skin barrier. Because the pretreated skin and the depilated skin was reddish. But, the alkaline condition is considered to have least effect on change of skin condition because of the same absorption degree between Fig. 1 and 3 which are the results employing creams of with and without triethanolamine. Thus, it must be admitted that the observed blood levels are influenced by a gradual deformation of a skin barrier during long term contact with a cream, and the level is reflected partially by an enhanced permeation as a result of deformation of the barrier, which is enhanced in the period of later period of the experiments.

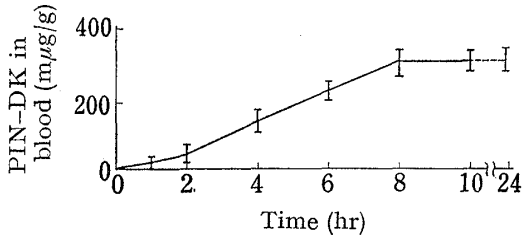

Fig. 10. Percutaneous Absorption of Pyridoxine 3,4-Di-*n*-octanoate (Morpholine is incorporated in PIN-DK cream, pretreated with cream base for 8—12 hours.)

Each plot represents a mean value and range of three tests.

It is known that a cream emulsified with amine soap is various in its appearance, stability and tactile feeling depending upon the kind of amine contained. According to the authors' experience, some compounds having a carbonic acid group are different in their biological activities depending upon the kind of amine contained; so are in the degree of irritation on patch test.

It was demonstrated that the use of morpholine resulted in much quicker absorption with continuous higher blood level than the use of triethanolamine (Fig. 10).

Acknowledgement The authors are deeply indebted to Dr. A. Hiraoka for much helpful advice in the microbiological assay of pyridoxine. Nihon Surfactants Industries Co., Ltd. supplied the pyridoxine 3,4-diacylates.