Transcellular Permeation of Nitrophenols through Newborn Rat Skin Epidermal Cells in Monolayer Culture

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The permeation of nitrophenols through epidermal cells from newborn rat skin cultured on type IV collagen-coated Millipore filters was studied under various conditions. The order of permeation through the cultured skin cells was found to be *p*->*m*->*o*-nitrophenol at both 10 and 37 °C. This order was the same as that of their affinities to isolated skin cells. The permeation of nitrophenols was not inhibited by the inhibitors of energy transduction 2-deoxyglucose and NaN_3. These results suggest that the permeation of nitrophenols across a cultured cell layer occurs by simple diffusion. The order of permeation of nitrophenols across newborn abdominal epidermis was exactly the opposite of that of their permeation across a cultured cell layer.

Keywords skin; epidermal cell; cell culture; nitrophenol permeation

Introduction

Examination of the permeabilities of various chemicals through the cultured skin cell layer, and comparison of these permeabilities with those through skin should provide useful information for understanding the mechanism of percutaneous absorption of bioactive compounds. Skin epidermal cells have usually been cultured on a type I collagen film either with or without 3T3 cells or extracellular matrices.[1] For permeation studies through the cultured cells, a confluent cell layer covering the whole surface of supporting material is necessary. However, in these cultured systems, it is not uncertain whether a confluent cell layer can be obtained or not. Recently we found that the viability and confluency of epidermal cells were greatly improved when cells were cultured on a type IV collagen film (Ohkura *et al.* submitted for publication). Thus, this cultured cell layer system is expected to be useful for studies on the permeability of chemicals.

As a first step of the permeability study, we characterized the permeation of mono-nitrophenols, such as *p*-, *m*- and *o*-nitrophenol, across the cultured skin cell layer covered on type IV collagen film as a supporting matrix, and their permeabilities were compared with those across the skin abdominal epidermis. We found that the transport of nitrophenols through the cultured cell layer was essentially independent of bioenergy and insensitive to temperature. Of the nitrophenols examined, *p*-nitrophenol permeated fastest and *o*-nitrophenol slowest through the cultured epidermal cell monolayer, and the order of permeations was completely the reverse that of their permeations across the abdominal epidermis. This paper describes these studies and discusses the mechanism of permeation of nitrophenols across the skin.

Materials and Methods

Reagents The sources of the reagents used in this study were as follows: Eagle's minimum essential medium (MEM), from Flow Laboratories, McLean, Scotland; fetal bovine serum (FBS), from Whittaker M. A. Bioproducts, Inc., Walkersville, U.S.A.; dispase, from Godo-Shusei Co., Tokyo, Japan; Millicell-CM (12 mm-diameter), from Millipore Products Co., Bedford, U.S.A.; and antitype IV collagen serum (rabbit), from Medac Co., Hamburg, Germany.

Preparation of Epidermal Cells Male Wistar rats 3 d-old were decapitated, and their skin was removed and cut into pieces. The pieces of skin were digested with dispase (1000 U/ml) overnight at 4 °C, then the epidermis was peeled off the dermis. The epidermal sheets were washed with Ca^{2+}- and Mg^{2+}-free phosphate buffered saline (PBS) and incubated in 0.25% trypsin–0.02% ethylenediaminetetraacetic acid (EDTA) solution at room temperature for 10 min. An epidermal cell suspension was obtained by dissociating the epidermal sheets by shaking them in the culture medium on ice.

Cell Culture Skin epidermal cells were suspended in Eagle's MEM buffered at pH 7.2 with 23.8 mM sodium bicarbonate and 20 mM Hepes (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) and supplemented with 10% FBS. The suspension (0.5 ml) was seeded on a type IV collagen-coated Millipore filter at an initial density of 1.2×10^6 cells per well, and the cells were incubated at 37 °C for 20 h in a CO_2-incubator.

Preparation of Type IV Collagen-Coated Millipore Filters Type IV collagen was prepared from bovine kidney cortex as reported previously.[2] Results of sodiumdodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and Western immunoblotting[3] with anti type IV collagen serum showed that the purity of the isolated type IV collagen was more than 99%. Type IV collagen dissolved in 0.1 M acetic acid at 3 mg/ml was diluted with ethanol to a final concentration of 0.06% (w/v). The solution was applied to 12 mm-Millipore filters (300 μl/well), and the filters were dried at room temperature under ultraviolet (UV)-light.

Permeation Across Cultured Cells The apparatus to monitor the permeability of nitrophenols across a cultured skin epidermal cell layer is depicted schematically in Fig. 1. A well with the epidermal cell monolayer on a type IV collagen-coated Millipore filter was placed in the end of a plastic cylinder with the surface coated and the cell layer facing inside of the cylinder; this cylinder was dipped into 50 ml of serum-free MEM, pH 7.4. Then 500 μl of nitrophenol solution in serum-free MEM (pH 7.4) was applied to the cell layer in a well. The level of solution above the collagen filter in the cylinder was kept exactly the same as that of the lower MEM solution. The concentrations of nitrophenols that permeated through the cell sheet were monitored as the absorbance changes of *p*-, *m*- and *o*-nitrophenols at their absorption maxima of 398, 340 and 412 nm, respectively.

Binding of Nitrophenols to Epidermal Cells A volume of 50 μl of

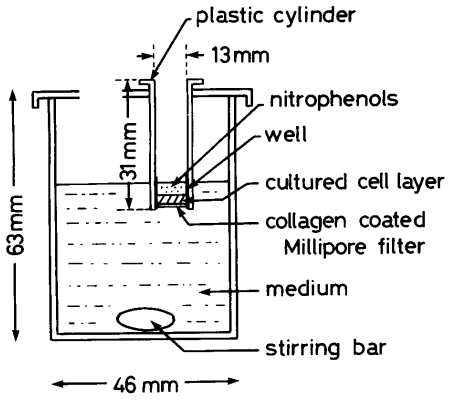

Fig. 1. Diagram of a Permeation Cell

epidermal cell suspension at 2.0×10^7 cells/ml in serum-free MEM without phenol red was added to 450 μl of nitrophenol solution in the same medium and the mixture was incubated at 0 °C for 1 h. Then the cell suspension was centrifuged for 3 min at 1000 rpm in a Kubota KS 5000-P centrifuge, and the amount of bound nitrophenol was determined from the difference between the initial and equilibrium concentrations of nitrophenols in the supernatant.

Permeation through Newborn Rat Abdominal Epidermis The abdominal skin sheet was excised from 3 d-old male Wistar rats with surgical scissors. Fat and other visceral tissue adhering to the skin were removed carefully from the under surface with forceps. The excised skin sheet was fixed with adhesive agent to the end of the plastic cylinder of the permeation cell shown in Fig. 1, with its outer surface facing the inside of the cylinder. Then 500 μl of 7.2 mM nitrophenol solution in serum-free MEM was introduced into the cylinder. The permeability of nitrophenols through the skin was monitored spectrophotometrically in a similar manner to that through the cultured cell layer.

Results

Transport of Nitrophenols through the Cultured Skin Cell Monolayer Light microscopic examination showed that epidermal cells cultured on type IV collagen supported by a Millipore filter became confluent 20 h after seeding, as shown in Fig. 2. The permeations of *o*-, *m*- and *p*-nitrophenol through this cell layer were examined in the permeation cell system shown in Fig. 1. The time courses of permeation of the nitrophenols through the cell monolayer at 37 °C are shown in Fig. 3A. As all the nitrophenols permeated very quickly at almost the same rate across a type IV collagen layer alone on the Millipore filter (closed circles in Fig. 3A), the collagen layer was only a very slight barrier to permeation, and the time courses of permeation of nitrophenols shown in Fig. 3A represent their actual rates across the skin cell layer.

This permeation was hyperbolic with time. *p*-Nitrophenol permeated most rapidly, *o*-nitrophenol slowest, and *m*-nitrophenol at an intermediate rate. The amounts of *p*-, *m*- and *o*-nitrophenol that permeated in 2.5 h were 0.42, 0.37, and 0.28 μmol/cm^2, respectively. As the nitrophenols were initially present at 0.57 μmol/cm^2, 73.7% of the *p*-nitrophenol, 64.9% of the *m*-nitrophenol and 49.1% of the *o*-nitrophenol permeated through the skin in 2.5 h. The rates of permeation obeyed first-order reaction kinetics, as shown in Fig. 3B, and the apparent rate constants k of *p*-, *m*- and *o*-nitrophenol were 5.06×10^{-1}, 3.60×10^{-1} and 2.45×10^{-1} h^{-1}, respectively.

If the permeation of nitrophenols is mediated by a certain carrier system, the permeation should be saturable. To examine this possibility, we next measured the dependence of permeation on the initial concentration of nitrophenols. Figure 4 shows results on the permeation in 1 h at 37 °C of nitrophenols at various initial concentrations. The permeation of nitrophenols across a cultured skin layer increased linearly with increase in their initial concentrations at least up to 1.5 mM, and showed no saturation.

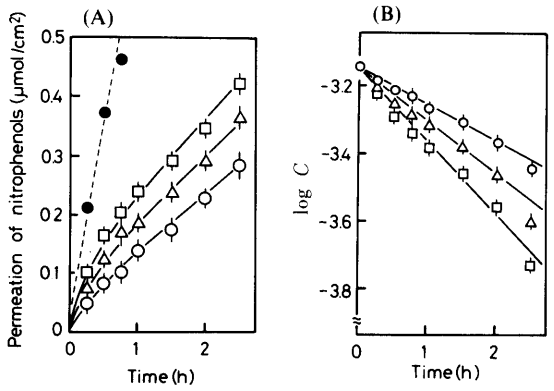

Fig. 3. A: Time Courses of Permeation of Nitrophenols through a Rat Epidermal Cell Monolayer at 37 °C

Results are means (vertical bars: ±S.E.) for three determinations.

B: Plots of the Permeation Data of Nitrophenols According to First-Order Reaction Kinetics

C is the Concentration (M) of nitrophenol in the donor phase of the permeation cell determined from the initial concentration inside the cylinder and that outside the cylinder, assuming that the amount of nitrophenol bound to the cell layer is smaller than that in the donor phase. □, *p*-nitrophenol; △, *m*-nitrophenol; ○, *o*-nitrophenol; ●, permeation of nitrophenols through type IV collagen without a cell layer.

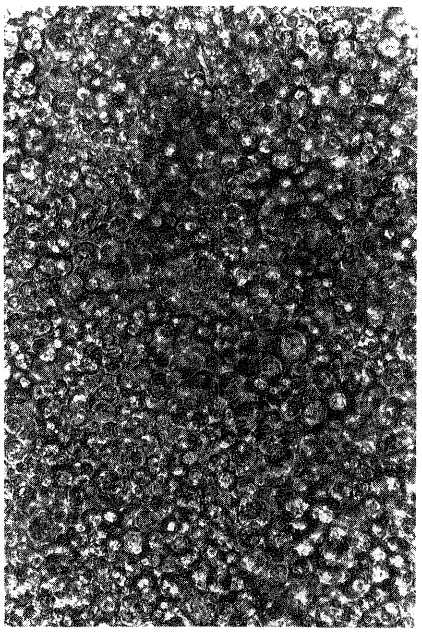

Fig. 2. Light-Micrograph of Rat Epidermal Cells Cultured for 20 h at 37 °C

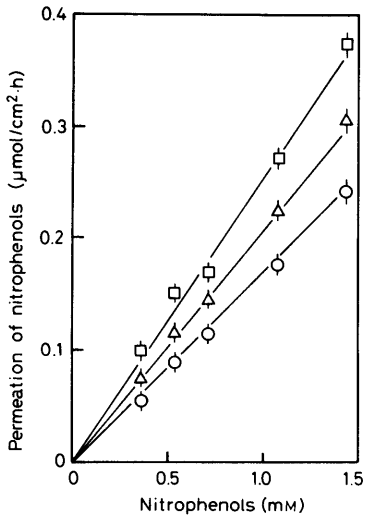

Fig. 4. Permeation of Nitrophenols at Various Initial Concentrations Across a Rat Epidermal Cell Layer at 37 °C

Results are means (vertical bars: ±S.E.) for three determinations. □, *p*-nitrophenol; △, *m*-nitrophenol; ○, *o*-nitrophenol.

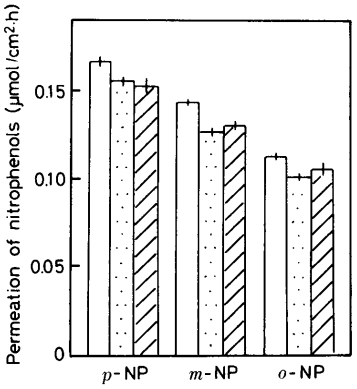

Fig. 5. Effects of the Inhibitors of Energy Transduction NaN_3 and 2-Deoxyglucose, and Temperature on the Permeation of Nitrophenols Across a Cultured Rat Epidermal Cell Layer in a 1 h Period

Initial concentration of nitrophenols: 0.72 mM. Open columns: permeation at 37 °C. Dotted columns: permeation at 10 °C. Hatched columns: permeation in the presence of 50 mM 2-deoxyglucose and 10 mM NaN_3 at 37 °C. Results are means (vertical bars: ±S.E.) for three determinations.

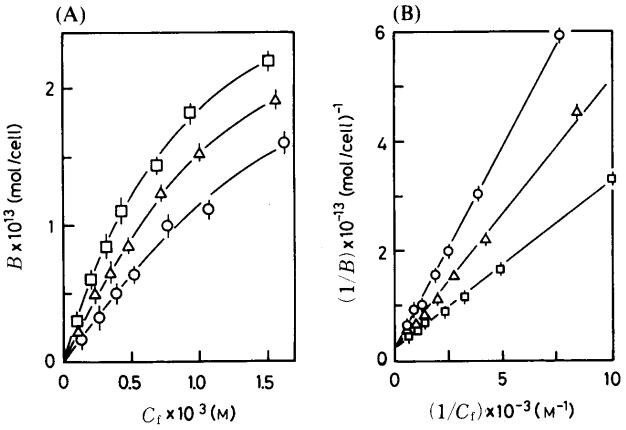

Fig. 6. Affinity of Nitrophenols to Suspended Epidermal Cells at 0 °C

(A) Adsorption isotherm. (B) Double reciprocal plots according to Eq. 1. *B*: bound nitrophenols per cell. C_f: concentration of free nitrophenols at equilibrium. □, *p*-nitrophenol; △, *m*-nitrophenol; ○, *o*-nitrophenol.

Effects of Temperature and Inhibitors of Bioenergy Transduction on the Permeation of Nitrophenols The above results suggest that the permeation of nitrophenols occurs by a simple diffusion process, and is not mediated by a certain carrier or dependent on biological energy. To confirm this, we examined the effects of temperature and inhibitors of energy transduction on the permeation, because the permeation supported by biological energy is inhibited in the presence of energy inhibitors, and that mediated by a carrier system is expected to be largely dependent on the temperature. We measured the permeation of nitrophenols without inhibitors at a lower temperature of 10 °C, and examined effect of the energy inhibitors NaN_3 at 10 mM and 2-deoxyglucose at 50 mM on the permeation at 37 °C for 1 h. As shown in Fig. 5, the permeations of all nitrophenols at 10 °C and those with the inhibitors at 37 °C were almost the same as those in the absence of energy inhibitors at 37 °C, indicating that the permeations were independent of biological energy and also insensitive to the experimental temperature.

Affinity of Nitrophenols to Skin Cells The binding of nitrophenols to skin epidermal cells was next examined:

Table I. Binding Parameters of Nitrophenols to Skin Epidermal Cells at 0 °C

	n (mol/cell)	*K* (M^{-1})
p-Nitrophenol	5.56×10^{-13}	6.49×10^{2}
m-Nitrophenol	5.56×10^{-13}	3.60×10^{2}
o-Nitrophenol	5.56×10^{-13}	2.40×10^{2}

Nitrophenols were incubated for 1 h with epidermal cells (1.0×10^6 cells) suspended in serum-free Eagle's MEM, and the binding parameter *n* (saturation binding of nitrophenols per cell) and *K* (affinity constant of the binding) were determined from the results in Fig. 6.

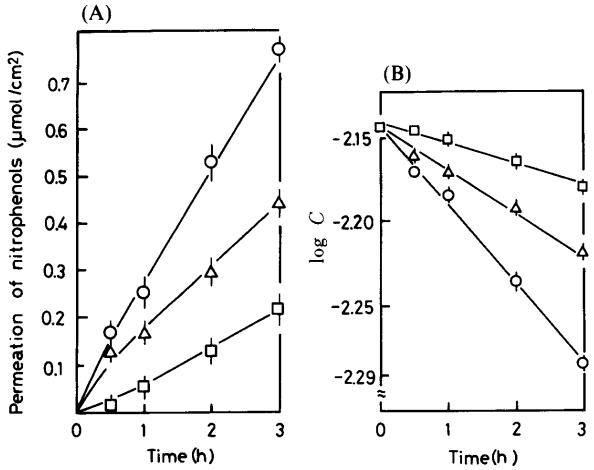

Fig. 7. Permeation of Nitrophenols through the Newborn Rat Abdominal Epidermis at 37 °C

(A) Time course of permeation. (B) Linear dependence of logarithm of concentration of nitrophenol in the donor phase (*C*) on the time of permeation. □, *p*-nitrophenol; △, *m*-nitrophenol; ○, *o*-nitrophenol.

Nitrophenols were incubated for 1 h with epidermal cells suspended in serum-free MEM and then the amounts of bound nitrophenols were determined. As shown in Fig. 6A, the binding of nitrophenols increased hyperbolically with increase in the concentration of free nitrophenols in the incubation medium. The binding was analyzed by a double-reciprocal plot according to the Langmuir adsorption isotherm, as shown in Eq. 1.

$$1/B = n \cdot K \cdot C_f/(1 + K \cdot C_f) \quad (1)$$

where *n* and *K* represent the saturation binding of nitrophenols per cell and the affinity constant of nitrophenols to the cells, respectively. C_f and *B* are the free concentration and the amount of bound nitrophenol per cell. The double-reciprocal plots are shown in Fig. 6B. The bindings of all three nitrophenols apparently accommodated well with Eq. 1. Values of the binding constants are summarized in Table I. The value of *n* was the same for all nitrophenols, and the order of the *K* values of *p*-, *m*- and *o*-nitrophenol was the same as that of their permeation across the cultured cell layer.

Permeations of Nitrophenols through Newborn Rat Abdominal Epidermis Next, we examined the permeations of nitrophenols through newborn rat abdominal epidermis in the permeation cell shown in Fig. 1 at 37 °C (Fig. 7A). These permeations were in accordance with first-order reaction kinetics, as shown in Fig. 7B, and the values of the first-order rate constants *k* were 2.53×10^{-2}, 6.22×10^{-2}, and

$10.82 \times 10^{-2}\,h^{-1}$ for *p*-, *m*- and *o*-nitrophenol, respectively. These values were smaller than those of permeations through the cultured skin cell layer. It is noteworthy that the order of percutaneous permeation was just the reverse of that through the cultured skin cell layer.

Discussion

In this study, we examined the permeability of nitrophenols across cultured skin epidermal cell layer, and the results were compared with that through newborn rat abdominal skin. First, we tried to determine whether the permeation of nitrophenols through a skin epidermal cell layer cultured on a type IV collagen-coated Millipore filter membrane is dependent on bioenergy or is mediated by a carrier system, and found that they permeated according to a simple first-order reaction. Because these permeations were independent of the inhibitors of energy-transduction 2-deoxyglucose and NaN_3, and insensitive to temperature, they were concluded to occur by simple diffusion, and not to require biological energy or a carrier. In contrast, we recently found that the low density lipoprotein (LDL) permeates across a cultured skin cell layer by the supply of bioenergy. This result will be reported elsewhere.

The ratios of the permeations of *p*- and *m*-nitrophenol to that of *o*-nitrophenol, determined from the first-order rate constants of the permeations, were about 2.1 and 1.5, respectively. Because these values were in good accordance with the ratios of the affinity constants of *p*- and *m*-nitrophenol to that of *o*-nitrophenol of 2.7 and 1.5, respectively, the binding of nitrophenols to the cell layer seems to be of primary importance for their permeation. The partition coefficient between octanol and water, P_{oct}, is widely used as a useful parameter expressing the hydrophobicity of a bioactive compound. The values of log P_{oct} of *p*-, *m*- and *o*-nitrophenol are reported to be 1.96, 2.00 and 1.77, respectively.[4] The differences between these values are very small but definite, and are due to the differences in their electronic natures. At present it is not clear why *p*-nitrophenol permeated across the cultured skin cell layer fastest, and *o*-nitrophenol slowest. Possibly the difference in these permeations is, at least in part, due to the steric and electron-withdrawing effects of a NO_2 group on the formation of a hydrogen-bond between the phenolic OH and the carbonyl moiety of phospholipid molecules, as suggested for the permeation of phenols through liposome membranes.[5]

The permeations of nitrophenols through the newborn rat abdominal epidermis that consists of stratum corneum and epidermal cells was shown to be smaller than those through the cultured epidermal cell layer, and the order of permeations through epidermis to be just the reverse that of their permeations through the cultured skin cell layer, in accordance with the permeation across hairless mouse skin where that of *p*-nitrophenol was less than that of *o*-nitrophenol.[4] These results suggest that the limiting process in percutaneous absorption of nitrophenols is their permeation through the stratum corneum.[6] However, it is not clear at present why the order of permeabilities of nitrophenols across a cultured skin layer was different from that across the epidermis. It is possible that the interaction of nitrophenols with the stratum corneum, where keratins are the main constituents, is different from that with epidermal cells. Examination of this possibility would be important for understanding the mechanism of percutaneous absorption of bioactive compounds including nitrophenols.

The present study indicated that the cultured epidermal cell layer confluently covering the whole surface of type IV collagen matrix is very useful for examining permeation of bioactive compounds across epidermal cell layer. This cultured cell system could also be very beneficial for research on the action of bioactive compounds on epidermal cells, their metabolism in these cells, and the effect of culture conditions on the viability and confluency of the cultured cells.

References

1) *a*) K. Yoshizato, A. Nishikawa, and T. Taira, *J. Cell Sci.*, **91**, 491 (1988); *b*) J. G. Reinwald and H. Green, *Cell*, **6**, 331 (1975); *c*) E. Tinois, M. Faure, P. Chatelain, and D. Schmitt, *Arch. Dermatl. Res.*, **279**, 241 (1987).
2) T. F. Kresina and E. J. Miller, *Biochemistry*, **18**, 3089 (1979).
3) H. Towbin, T. Staehelin, and J. Gordon, *Proc. Natl. Acad. Sci. U.S.A.*, **76**, 4350 (1979).
4) W. E. Jetzer, A. S. Huq, N. F. H. Ho, G. L. Flynn, N. Duraisway, and L. Condie, Jr., *J. Pharm. Sci.*, **75**, 1098 (1986).
5) H. Miyoshi, H. Maeda, N. Tokutake, and T. Fujita, *Bull. Chem. Soc. Jpn.*, **60**, 4357 (1987).
6) *a*) H. Durrheim, G. L. Flynn, W. I. Higuchi, and C. R. Behl, *J. Pharm. Sci.*, **69**, 781 (1980); *b*) G. L. Flynn, H. Durrheim, and W. I. Higuchi, *ibid.*, **70**, 52 (1981).