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A Study on the Absorption Mechanism of Drugs through Biomembranes

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The effect of lipophilicity on the mechanisms of drug absorption through biomembranes was investigated employing HPLC system and the fluorescence technique. Human erythrocyte ghost membranes were used as a model biomembrane. A series of four parabens (methyl, ethyl, propyl, and butyl) and p-hydroxybenzoic acid were used as the model drugs for lipophilicities and their partition coefficients were measured in Sörensen's phosphate buffer solution (pH 5)/octanol system. Absorption amount of parabens through erythrocyte ghost membranes increased with an increase of lipophilicity resulted from the addition of methylene group to the n-alkyl chain of parabens. And the effect of parabens on the fluidity of ghost membrane also increased with an increase of their lipophilicities.

Key words: Human erythrocyte ghosts, Parabens, Lipophilicity, Membrane fluidity, Drug absorption

INTRODUCTION

A relatively large number of studies for transport of drugs through biomembranes have been performed using human erythrocytes as a model of biomembrane (Björn *et al.*, 1974; Sheetz and Singer, 1974; Wallace *et al.*, 1977; Jun *et al.*, 1980; Matsumoto and Ohsako, 1989a, 1989b, 1990). The erythrocyte membrane is one of the best studied biological membranes because the ready accessibility, ease of preparations and wealth of available information make this membrane a useful system for the evaluation of drug in biological membrane systems (Dodge *et al.*, 1963; Bjerrum, 1979; Loyter, 1980; Lieber *et al.*, 1982).

The process of drug absorption may be categorized as either passive diffusion or active transport. Passive diffusion is the major transmembrane process for most drugs. The driving force of passive diffusion is the concentration gradient that is the difference in drug concentrations on either side of the cell membranes. If administered drug concentration is constant, the permeability of biomembrane to the drugs may give the most great influence on drug absorption. Therefore, the most important factor to decide the bio-physical characteristics such as permeability of biomembrane may be membrane fluidity, as a whole, expressed as mobility of molecules in biomembranes.

In this study, the relationships between the effects of various parabens on membrane fluidity and their lipophilicities were investigated. And the correlations between the absorption amount of parabens through ghost membranes and their lipophilicities were also evaluated.

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MATERIALS AND METHODS

Materials

Liquid paraffin, 1,6-dipyrenyl-1,3,5-hexatriene (DPH), p-hydroxybenzoicacid (HBA) and n-alkyl p-hydroxybenzoates such as methylparaben (MHB), ethylparaben (EHB), propylparaben (PHB) and butylparaben (BHB) were purchased from Sigma Chem Co. (St. Louis, MO) and water was purified with a Milli-Q water system (Millipore, Bedford, MA, USA). Methanol and acetonitrile obtained from Merck Sharp Dom Res. Lab. (Westpoint, PA) were HPLC grade and all other reagents were of analytical grade. 1,3-di(1-pyrenyl)propane (py-3-py) was a gift from Dr. Yun, College of dentistry, Pusan National University.

Apparatus

Refrigerated centrifuge (Denley, BR 401, USA), ultra-

centrifuge (OTD-60B, Sorvall Ins., Dupont), and ultrasonic homogenizer (Type 4510, Ultrasonic Ltd.) were used for preparation of erythrocyte ghost membranes. UV/Vis-spectrophotometer (Model 930, Kontron, Swiss), HPLC system (Waters Co., Milford, MA) equipped with a solvent delivery system (Model 510), UV detector (Model 481), and data module (Model 730) and spectrofluorometer with polarizer (Model 25, Kontron, Swiss) were used for measurements of absorption amount of drugs through ghost membranes and fluidity of membranes, respectively. Scanning electron microscope (SEM; Jeol JSM-35 CF, Japan) and rheometer (Model Rheolab SM-HM, Physica, German) were used for the examination of the shape of erythrocyte ghost and the measurements of microviscosity of ghost membranes, respectively.

Partition Coefficients

Sörensen's phosphate buffer solution (pH 5.0) and octanol were mutually saturated at 35℃ before use. The distribution coefficients of parabens were determined by dissolving paraben 1000 μg in 10 ml of pH 5 phophate buffer and shaking intermittently with 2 ml of octanol kept at 35℃ for 5 days to reach a distribution equilibrium. The phases were separated by centrifugation at 2500 rpm for 10 min, and the concentration in each phase was determined spectrophotometrically. The distribution coefficient was calculated by Eq. 1 (Huang *et al.*, 1983a; 1983b),

$$\text{Distribution coefficient (CD)} = \frac{(C_b - C_a)V_w}{(C_b)V_o} \quad (1)$$

where C_b and C_a represent the concentration of parabens in Sörensen's buffer phase before and after distribution, respectively; V_o and V_w represent the volume of octanol and Sörensen's buffer phases, respectively.

The partition coefficient was calculated from the distribution coefficient by Eq. 2;

$$\text{Partition coefficient (PC)} = (\text{DC}) \times \left(+ \frac{1}{\text{antilog}(\text{pH} - \text{pKa})}\right) \quad (2)$$

The pH of the buffered phase at 35℃ was not changed after distribution was complete. The pKa of parabens was 8.45 and that of HAB was 4.36.

Prepraration of Ghost Cells

Erythrocyte ghost membranes were prepared by a slight modification of the method described by Bjerrum *et al.* (1979). Heparinized blood drawn freshly from a human volunteer was centrifuged at 1630 g for 15 min at 4℃. After removing the plasma water and buffy layer containing leucocytes, the packed erythrocytes were washed three times with isotonic phosphate buffer solution containing 140 mM NaCl (pH 7.4, 20 mOsM isotonic phosphate buffered saline, PBS). After the final washing, the erythrocytes were hemolyzed with 10 mM phosphate buffer solution (pH 7.4, 20 mOsM phosphate buffer, PB) and the mixture was centrifugated at 20,000 rpm for 15 min at 4℃ to remove water soluble fragments. This process was repeated with PB two or three times. The resulting erythrocytes were resuspended in 9 volumes of PBS containing 0.1 mM of $MgCl_2$ and incubated for 30 min at 37℃ with slightly shaking in order to reseal the open erythrocyte ghost membranes by previously conducted hemolysis (Kohana *et al.*, 1991). Resealed erythrocyte ghosts were harvested by centrifugation at 4500 rpm for 30 min. The resealed ghost membranes then stored at 4℃ and used for experiments within 2 days.

Examination of Erythrocyte Ghosts by Electron Microscopy

Four volumes of ghost suspensions containing 100 μg of membrane protein per ml of PBS were diluted with one volume of 25% glutaraldehyde and, after incubation at 4℃ for 2 hr, the pre-fixed particles were sedimented in a bench centrifuge. Washing of sediment ghosts was carried out in the ependorff with pipetting PBS three times at 15 minute intervals and kept overnight. One volume of 2% OsO_4 then was added to four volumes of sediment ghost and, after incubation at 4℃ for 2 hr, the post-fixed particles were sedimented in a bench centrifuge again. Dehydration was carried out in the ependorff by exposuring the pellets for 10-15 min at 4℃ to ethanol solutions, its concentration increased consecutively (30, 50, 70, 80, 90, 95 and 100%). The pellet then was removed from the ependorff and replaced in the capsule of the critical-point dryer. Dehydration was completed perfectly during the critical-point drying process for SEM while aspirated liquid CO_2 gas into the tube of the capsule. The shape of erythrocyte ghost was observed by scanning electron microscope.

Transport of Drugs Through Ghost Membranes

Ghost cells (100 μg protein of ghost membrane/ml of PBS) were suspended in PBS containing 1 mM of each of various parabens. The ghosts suspension was incubated at 37℃ under slight shaking for the time indicated. Ghost cell suspensions then were centrifuged at 1500 rpm for 15 min at 4℃ and the supernatant containing the drug remained, that was not transported from the suspending medium into ghost cells, was deproteinized with acetonitrile and determined using HPLC. The HPLC system was used as the following conditions; column, Lichrospher 100 RP-18 (5 μm)

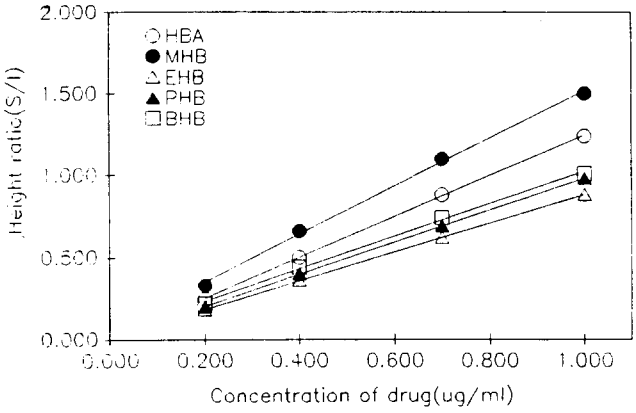

Fig. 1. Calibration curves of HBA and various parabens. S and I are peak heights of sample and internal standard, respectively.

(Merck, Cat. No. 50943); mobile phase, PB : methanol (7 : 3) for HAB; PB : methanol (1 : 1) for MHB and EHB; PB : acetonitrile (1 : 1) for PHB and BHB; flow rate, 0.9 ml/min; sensitivity, 0.005 AUFS; injected volume, 5 μl; detector, 254 nm of UV spectra. The drug concentrations of sample solutions were computed from the peak height corresponding to the calibration curve (Fig. 1) obtained from the standard solution.

Fluorescence Measurements

The incorporation of Py-3-Py was carried out by adding aliquots of a stocksolution of 5×10^{-5} M in ethanol to the ghosts suspension (100 μg protein of ghost membranes/ml of PBS), so that the final probe concentration was less than 5×10^{-7} M. The suspension was then incubated at 4℃ for 16 hr under gentle stirring with magnetic stirrer. Blanks, prepared under identical conditions without Py-3-Py, served as controls for the fluorometric measurements. The measurements were carried out with SFM 25 spectrofluorometer, equipped with a polarizer and a thermostatic cell holder. The excimer to monomer fluorescence intensity ratio (I'/I) was calculated from 430 nm to 362 nm signal ratio. The excitation wavelength was 338 nm. Parabens were dissolved in PBS and pH adjusted to 7.4. Solutions were immediately prepared before use. Parabens, regulating its concentration from 0.01 mM to 1 mM, were added directly to ghost membraranes suspended in PBS and the excimer to monomer fluorescence intensities were measured within 1 min at 37℃. Oxygen was removed by bubbling pure nitrogen through the suspensions for 15 min prior to the fluorescence measurements.

Microviscosity of Erythrocyte Ghost Membranes

The excimer to monomer fluorescence intensity ratio (I'/I) of Py-3-Py in erythrocyte ghost membranes can be translated into viscosity values by comparing these intensity ratios with those measured in an appropriate solvent or solvent mixture of known viscosity. For this purpose, white mineral oil was used as a reference system. The viscosity of mineral oil at the temperature range of 0℃ to 40℃ was determined using Physica rheometer. Py-3-Py was incorporated into white mineral oil with the same manner as performed in erythrocyte ghost membranes suspensions, and excimer to monomer fluorescence intensity ratio (I'/I) was also determined increasing temperature from 0℃ to 40℃ using SFM 25 spectrofluorometer. The calibration curve of $\ln\eta$ (viscosity) versus I'/I was obtained from both of plots of temperature (T) versus viscosity of mineral oil and temperature versus I'/I of mineral oil. The microviscosity of ghost membranes was evaluated from the calibration curve of $\ln\eta$ versus I'/I.

Fluorescence Polarization Measurements

In order to confirm the results of the intramolecular excimer fluorescence technique, the estimation of fluorescence polarization was also carried out using DPH, a lipid probe. DPH was dissolved in tetrahydrofuran and 0.5 μl of tetrahydrofuran solution per ml of PBS was added directly to the ghost suspensions (100 μg protein of ghost membranes/ml of PBS). The final probe concentration was 1 μg DPH/100 μg of membrane protein. The suspension containing DPH was shaken in the dark at 37℃ for 30 min. The blanks were prepared under identical conditions without DPH. Parabens were added to ghost suspensions containing DPH. The fluorescence intensity of DPH incorporated in ghost membrane was measured at various concentrations of parabens from 0.01 mM to 1 mM. The excitation wavelength for DPH was 362 nm and the emission wavelength was 430 nm. The fluorescence polarization (P) was obtained from intensity measurements using Eq. 3,

$$P=(I_{vv}-I_{hv}G)/(I_{vv}+I_{hv}G) \quad (3)$$

where G is a grating factor for the monochromator's transmission efficiency for vertically and horizontally polarized light. This value is given by the ratio of the fluorescence intensities of the vertical to horizontal direction. I_{vv} and I_{hv} are the intensities measured when the polarizer and analyser prisms are in the vertical or horizontal positions, respectively. This condition yields an equivalent fluorescence intensity (I) entering the monochromator irrespective of the orientation of the observation polarizer.

RESULT AND DISCUSSION

Partition Coefficients

Lipophilicity is a molecular property, which can be

Table 1. Partition coefficients of parabens in octanol-Söson's phosphate buffer at 35℃

	HBA	MHB	EHB	PHB	BHB
Log PC	1.87	5.31	5.60	6.03	6.29

Each value represents the mean of three measurements.

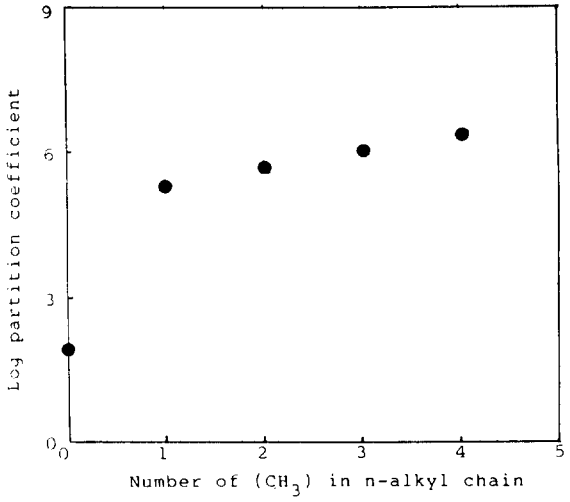

Fig. 2. Relationship between the octanol-Sösen's buffer partition coefficients and the length of n-alkyl chain, $(CH_3)_n$, of HBA and various parabens at 35℃.

factorized into two terms, one respresenting bulk or steric properties, while the other is related to electrostatic and polar properties (Van de Waterbeemd and Testa, 1987). Therefore, lipophilicity used in this report is expressed as a partition coefficient among the different lipophilicity descriptors. The partition coefficients of parabens were calculated by Eq. 2 from the distribution coefficients determined in the octanol-Söson's phosphate buffer system at 35℃. Table I also lists partition coefficients of parabens and shows that the lipophilicity of parabens increases with an increase in the length of n-alkyl chain, as shown in Fig. 2.

Erythrocyte Ghost Morphology

Erythrocyte ghosts obtained after the removal of the hemoglobin from the erythrocytes were resealed in PBS containing 0.1 mM of $MgCl_2$. The resealed white ghosts were examined by scanning electron microscopy. Figure 3 shows a scanning electron micrograph of the hemoglobin depleted, resealed ghost at their hemolytic volume. As shown in Fig. 3, erythrocyte ghost did not have any kind of crenatures or vagination. Ghost membrane was intact and its shape was spherical. Therefore, it was demonstrated that the hemoglobin depleted, open cell membranes were perfectly resealed by resuspending them in PBS with $MgCl_2$.

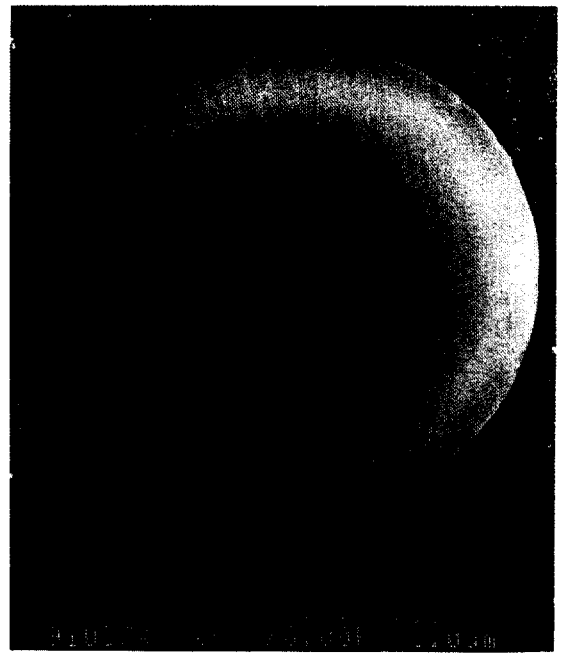

Fig. 3. Scanning electron micrograph (×6000) of a human erythrocyte ghost. Most of the hemoglobin were removed by repetitive osmotic hemolysis.

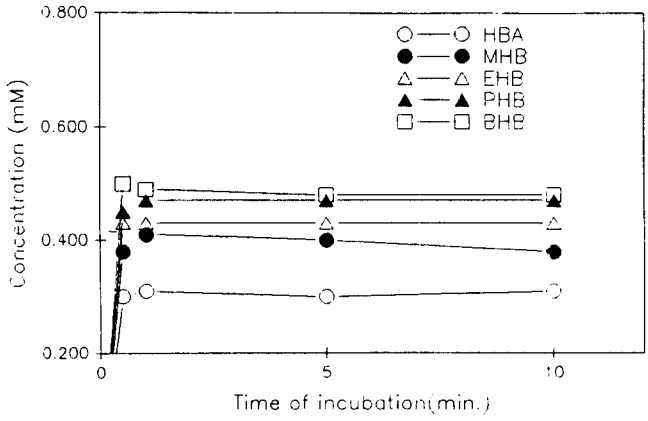

Fig. 4. Drug uptake by human erythrocyte ghosts from suspending medium of various parabens. The suspending medium containing 100 μg protein of ghost membranes/ml of PBS and 1 mM of paraben was incubated at 37℃.

Drug Transport Through Ghost Cell Membranes

Time course of inward transport of parabens from a drug-containing medium to the suspending erythrocytes was illustrated in Fig. 4. The rate of transport was very rapid and the partition equilibrium was attained almost within one minute as shown in Fig. 4. The transport of these drugs through ghost cell membranes from the suspending medium as measured in the present study may probably mean their partition between the cytosol fluid and the outer medium. These drugs

migrate rapidly between these two compartments in either inward and outward direction according to their concentration gradients. As shown in Fig. 4, the absorption amounts of various parabens were increased with an increase of their lipophilicities resulted from an addition of methylene group to the n-alkyl chain of parabens. From these results, it was found that absorption amounts of various parabens through ghost cell membranes had a direct relationship with their lipophilicities.

Effects of Parabens on the Fluidity of Ghost Membranes

Intramolecular excimer formation of Py-3-Py in viscous media such as phospholipid bilayers has been used to study the fluidity changes of biomembranes. Since it is a monomolecular process independent of concentration, the formation of aggregates can be avoided and the perturbation of biomembranes by a probe molecular itself can be minimized. Intramolecular excimer formation, due to association of an excited and an unexcited aromatic group incorporated in the same molecule, can be described by the excimer to monomer fluorescence quantum yield ratio, ϕ'/ϕ, which is proportional to the fluorescence intensity ratio, I'/I (Zachriasse *et al.*, 1980; 1982).

$$\frac{\phi'}{\phi}=\frac{K_f'}{K_f}\times\frac{K_a}{K_d+1/\tau_o'}=\frac{k'I'}{kI} \tag{4}$$

where K_f (monomer) and K_f' (excimer) are the fluorescence rate constants, K_a and K_d are the rate constants for excimer association and dissociation, respectively, and τ_o is the excimer life time in the absence of dissociation (K_d). k and k' are the spectral sensitivities at the wavelengths of the monomer and excimer fluorescence intensities, I and I', respectively. In the low temperature region, where

$$K_d \ll 1/\tau_o' \tag{5}$$

so that

$$I/I'=(k/k')(k_f'/K_f)K_a\tau_o' \tag{6}$$

In order to be applicable to fluidity investigation, the intramolecular excimer fluorescence probe must fulfil Eq. 5 for the low temperature region. As K_d is inversely proportional to K_η where stands for the viscosity, Eq.6 is most likely met in media of high vicosity and when the intramolecular excimer formation is accompanied by a high gain in free energy such as with Py-3-Py. Especially, it has been found with Py-3-Py that in highly viscous media I'/I decreases essentially linearly with increasing viscosity. This makes it possible to use Py-3-Py as a probe to determine effective viscosities in viscous media (Zachariasse, 1978).

As shown in Fig. 5, fluorescence measurements at 4°C, as a function of time after probe incorporation, shows that the excimer to monomer fluorescence intensity ratio reaches a constant value after approximate 10 hr. The initially higher value of I'/I may be caused by the fact that Py-3-Py is only very slightly soluble in water (less than 1×10^{-7} M) and may thus tend to form aggregates in aqueous solution. The incorporation time of 16 hr then reflects the time necessary for the complete transfer of the probe molecule from the aqueous phase into the membrane.

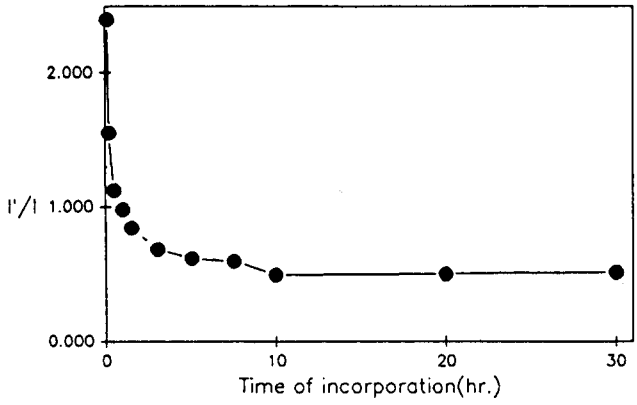

Fig. 5. Time dependence of the incorporation of Py-3-Py into human erythrocyte ghost membranes. The incorporation was carried out at 4°C under gentle stirring.

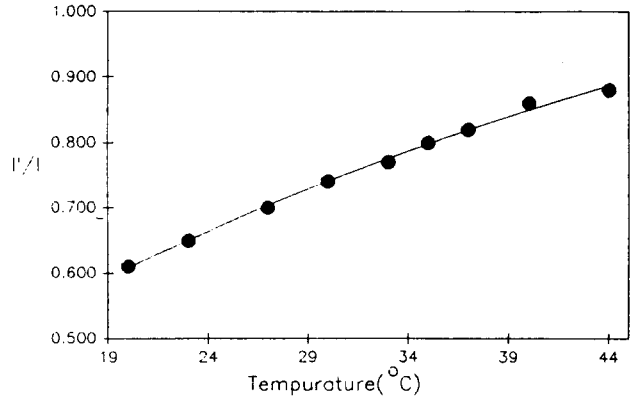

Fig. 6. Excimer to monomer fluorescence intensity ratio, I'/I, of Py-3-Py in erythrocyte ghost membranes as a function of temperature. I' was determined at 480 nm, while I was determined at 379 nm.

The excimer to monomer fluorescence intensity ratio, I'/I, where I' and I are measured at 480 nm and 379 nm, respectively, was increased strongly with increasing temperature, as can be seen from plots of I'/I of Py-3-Py versus temperature in Fig. 6. And, as shown in Fig. 7, it was found that the I'/I ratio of ghost membranes by various parabens increased with the increase of lipophilicity as rusulted from the addition of methylene group to the n-alkyl chain of parabens. It was

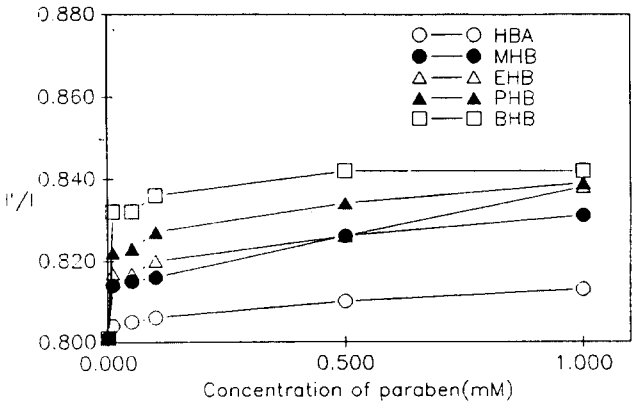

Fig. 7. Effect of various parabens on the excimer to monomer fluorescence intensity ratio, I'/I, of Py-3-Py incorporated into erythrocyte ghost membranes at 37°C. The intensities I' and I were determined at 480 nm and 379 nm, respectively.

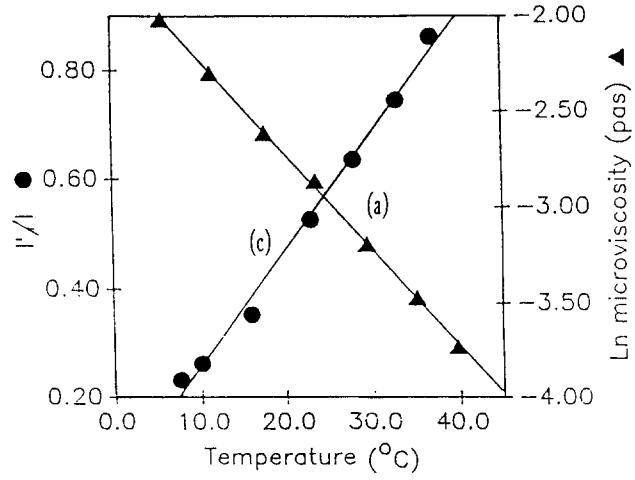

Fig. 9. The dependence of microviscosity of white mineral oil on the excimer-to-monomer fluorescence intensity ratio (I'/I). I' and I were determined at 480 nm and 379 nm, respectively.

also increased with the increase of concentration of various parabens. Thus, it appears that the effects of parabens on membrane fluidity depend mainly on both of the lipophilicity and the concentrations of parabens.

Microviscosity of Human Erythrocyte Ghost Membranes

The I'/I values of the probe molecules in the ghost cell membranes can be translated into viscosity values by comparing this intensity ratio with those measured in an appropriate solvent of known macroscopic viscosity. For this purpose, mineral oil was chosen as the reference solvent. The I'/I values of Py-3-Py were observed in mineral oil as a function of temperature. The

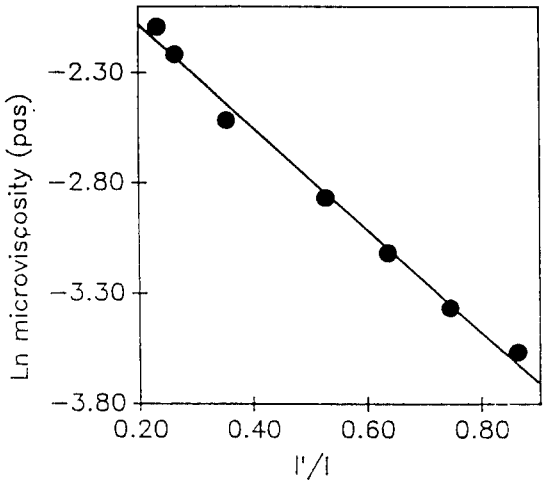

Fig. 8. The microviscosity and excimer-to-monomer fluorescence intensity ratio (I'/I) of Py-3-Py in white mineral oil as a function of temperature. I' and I were measured at 480 nm and 379 nm, respectively, and the viscosity was determined by Rheometer SM-HM.

viscosity of mineral oil was also measured by Rheometer SM-HM. Fig. 8 shows the viscosity and excimer to monomer fluorescence intensity ratio, I'/I, in mineral oil as a function of temperature. As can be seen from plots of I'/I versus temperature in Fig. 8, the intensity ratio increases with increasing temperature, while the viscosity decreases with increasing temperature. In Fig. 9, the plots of log (viscosity) versus I'/I were drawn by using the equations obtained from two curves in Fig. 8. The microviscosity of erythrocyte ghost membranes was determined from the calibration curve in Fig. 9 all over the concentrations used in this experiments. This methodology could be used since the fluorescence spectra of the pyrenyl probe are identical in both erythrocyte membranes and mineral oil. However, some values of microviscosity for ghost membranes were lower than that of mineral oil, so that those values were calculated from extrapolation of mineral oil data in Fig. 9. The apparent viscosity, 133 cP of erythrocyte ghosts at 37°C was also obtained from Fig. 9.

Fluorescence Polarization

Fluorescence polarization has long been utilized to measure molecular rotational diffusion (Shinitzky *et al.*, 1971; 1976; 1978). When applied to membranes, the rotational motions of some of the probes have often been compared to the measurements made in oils of known viscosity, and quantified in terms of "microviscosity". DPH is the most widely used probe, because it partitions very favorably into membranes, has an intense fluorescence, does not appear to bind to proteins and is sensitive to the membrane physical

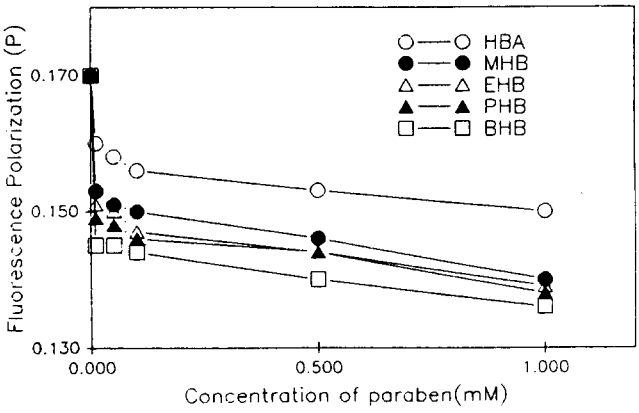

Fig. 10. Effects of various parabens on the fluorescence polarization(P) of DPH in erythrocyte ghost membranes. Fluorescence measurements were performed at 37℃.

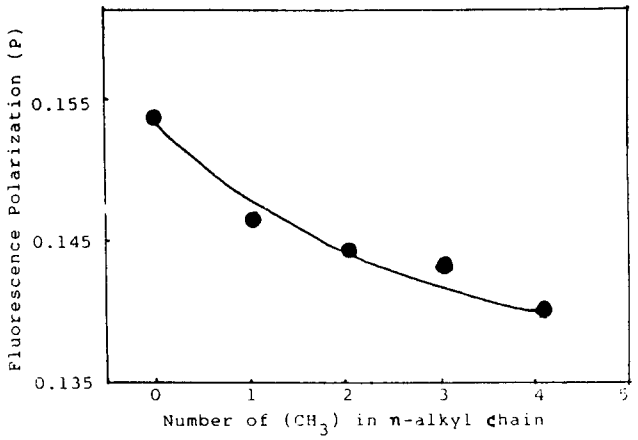

Fig. 11. Fluorescence polarization(P) of DPH in erythrocyte ghost membranes containing 0.5 mM paraben as a function of number of methylene group in n-alkyl chain of paraben, $(CH_3)_n$. Fluorescence measurements 8were performed at 37° C.

Key: 0, HBA; 1, MHB; 2, EHB; 3, PHB; 4, BHB.

state. In fluorescence polarization, polarized light preferentially excites those fluorophores whose molecular axes are orientated in a particular direction with respect to the plane of polarization. If the fluorophore remains immobile during its excited life time (10^{-8} sec), then the fluorescent light will also be highly polarized. If, however, the fluorephore rotates during its fluorescent life-time, then the resulting fluorescence will become less highly polarized. The polarization of fluorescence thus act as a conventient index of the extent of the molecular rotation during its excited life-time.

The effects of various concentrations of parabens on the fluidity of human erythrocyte ghost membranes were investigated by measurements of the fluorescence polarization of DPH incorporated in lipid bilayer of ghost membranes at 37℃. As shown in Fig. 10, the results were consistent with those obtained from the excimer formation method by Py-3-Py. From Fig. 10, it was known that the fluorescence polarization of DPH in erythrocyte ghost membranes decreased by the addition of parabens and the decreasing ratio of the fluorescence polarizations by various parabens decreased in proportion to an increase of lipophilicity resulted from the addition of methylene group to the n-alkyl chain of parabens. Figure 11 shows a decrease of fluorescence polarization in erythrocyte ghost membranes by various parabens at 37℃. It was confirmed substantially from these results that the fluidizing effect of parabens on erythrocyte ghost membranes increased with an increase of their lipophilicities.

CONCLUSION

In order to elucidate the mechanisms of drug absorption through biomembranes, the effects of lipophilicities on human erythrocyte ghost membranes were investigated employing HPLC system and the fluorescence probe technique. p-Hydroxybenzoic acid and methyl-, ethyl-, propyl-, and buthylparaben were used as model drugs for lipophilicities and the following results were obtained:

1) Absorption amount of parabens through erythrocyte ghost membranes increased with an increase of their lipophilicities by the addition of methylene group to the n-alkyl chain of parabens.

2) The effect of parabens on the fluidity of ghost membranes increased with an increase of their lipophilicities.

3) From these results, it was suggested that the increase of drug absorption through biomembranes along with an increase of their lipophilicities would be resulted from the modulation of fluidity of biomembranes.

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