Effects of the Structures of Polyoxyethylene Alkyl Ethers on Uptake of Butyl *p*-Hydroxybenzoate by *Escherichia coli* and Its Antibacterial Activity

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The effects of the structures of non-ionic surfactants on the uptake of butyl *p*-hydroxybenzoate (BP) into *Escherichia coli* cells and its antibacterial activity were systematically studied using polyoxyethylene alkyl ethers (PAEs) possessing various oxyethylene and hydrocarbon chain lengths. The uptake of BP into bacterial cells in an aqueous PAE solution was proportional to free BP in an aqueous phase, depending on the structures of PAEs. The antibacterial activity of BP decreased in the presence of PAEs, whereas it was greater than that anticipated from free BP. However, only PAE with 12 carbons in the hydrophobic group caused unusual increases in the uptake and antibacterial activity of BP, and the surfactant was more extensively incorporated into bacterial cells, differing from other PAEs, which were much less incorporated.

The PAEs were thus concluded to increase the susceptibility of bacteria against BP due to direct interactions with the cells. Particularly, the PAE with 12 carbons in the hydrophobic group, which penetrated abundantly into the cells, might result in an increase in the fluidity of the cellular lipid matrix and a decrease in the resistance of drug permeation.

Key words butyl hydroxybenzoate; *Escherichia coli*; antibacterial activity; polyoxyethylene alkyl ether; drug susceptibility

p-Hydroxybenzoic acid esters (parabens) are widely used in pharmaceuticals and cosmetics as antimicrobial preservatives; they are inactivated in aqueous solutions in the presence of non-ionic surfactants.[1,2] As pointed out in many studies,[3-6] the inactivation of parabens by non-ionic surfactants is caused by a decrease in free parabens in an aqueous phase of the solution resulting from interactions between parabens and surfactant micelles. We reported that the antimicrobial activity of parabens in the absence of surfactants was related to the extent of paraben uptaken into bacterial cells.[7] The interactions between parabens and non-ionic surfactants were influenced by changes in micellar property due to the structures of the surfactants.[8] The uptake of parabens into microbial cells in the presence of non-ionic surfactants is thus likely to be influenced by the structures of the surfactants, because surfactant micelles and/or surfactant molecules may exert some effect on the cells. Little has been reported on the effects of non-ionic surfactants on the uptake of parabens into microbial cells.

In this paper, to clarify the effects of the structures of non-ionic surfactants on the reduction of the antibacterial activity of parabens, the relationship between the uptake of butyl paraben by *Escherichia coli* and the antibacterial activity in the presence of surfactants was systematically studied using polyoxyethylene alkyl ethers (PAEs), which possessed various oxyethylene and hydrocarbon chain lengths.

Experimental

Materials Butyl *p*-hydroxybenzoate (BP, Ueno Pharmaceutical Co., Tokyo) and PAEs (Nikko Chemicals Co., Tokyo), shown in Table 1, were of JP XIII and JSCI-II grade, respectively, and used without purification. All other reagents and solvents were of analytical reagent grade.

Determination of Minimum Bactericidal Concentration (MBC) of BP *Escherichia coli* NIHJ (*E. coli*) was incubated at 37 °C for 18 h in soybean-casein digest agar. *E. coli* cells scraped aseptically from the agar medium were suspended in sterilized distilled water and diluted to a prescribed cell concentration. The diluted cell suspension (0.1 ml) was inoculated in a 4.9 ml BP solution (a multiple of 7.24×10^{-5} to 1.09×10^{-3} M) with PAE (0 or 0.5 mg/ml). The cell numbers in the solution, determined by the plate count method using soybean-casein digest agar with lecithin and polysorbate 80, were always set at 5.1×10^5 cells/ml. After being kept at 25 °C for 1 d, the aqueous BP solution with the cells (0.1 ml) was inoculated in *ca.* 5 ml of soybean-casein digest broth with lecithin and polysorbate 80. The MBC of BP was determined after no bacterial growth in the broth was evident following inoculation at 37 °C for 2 d.

Uptake Measurement of BP and PAEs into *E. coli* Cells *E. coli* incubated at 37 °C for 18 h in soybean-casein digest broth was collected by the method described previously.[7] Collected *E. coli* cells were suspended in an aqueous BP solution (7.24×10^{-4} M) with PAE (0 or 1 mg/ml). A glass-stoppered centrifuge tube packed with a 6 ml cell suspension was shaken with a flask shaker (120 rpm, model TB-12T, Takasaki Kagaku Kikai Co., Saitama) at 25 °C for 60 min. The packed cell volume, determined by the method described previously,[7] and cell numbers in the suspension were 0.051 ml/ml and 1.2×10^{10} cells/ml, respectively. Following centrifugation ($1600 \times g$, 20 min) at 25 °C, the BP concentration in the supernatant was determined by HPLC.[7] No change in the BP concentration was observed by shaking for 15—120 min. No difference in the concentration of BP in the supernatant of the solutions prepared by distilled water or physiological saline was observed under the experimental conditions. The PAE concentration in the supernatant was determined using HPLC under the following conditions: gel-permeation column (Asahipak GS-510 7.6 × 500 mm, Asahi Chemical Industry Co., Tokyo); detection of refractive index (Shodex RI SE-51, Showa Denko Co., Tokyo); mobile phase (methanol : water = 99 : 1, v/v);

Table 1. Abbreviation of PAEs

PAE[a)]	Abbreviation[b)]
Polyoxyethylene 21 laury ether	$C_{12}E_{21}$
Polyoxyethylene 20 cetyl ether	$C_{16}E_{20}$
Polyoxyethylene 30 cetyl ether	$C_{16}E_{30}$
Polyoxyethylene 40 cetyl ether	$C_{16}E_{40}$
Polyoxyethylene 20 stearyl ether	$C_{18}E_{20}$
Polyoxyethylene 20 behenyl ether	$C_{22}E_{20}$

a) Numbers show moles of added oxyethylene units per molecule. *b*) C_nE_m represents the hydrocarbon (*n*) and oxyethylene numbers (*m*).

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flow rate of 1.0 ml/min at room temperature.

Ultrafiltration The above supernatant was ultrafiltered using an ultrafiltration unit (Ultrafree CL-LCC, Nihon Millipore Co.) at 25 °C.[7] The BP concentration in the ultrafiltrate, regarded as free BP in an aqueous phase of the cell suspension, was determined by HPLC. No PAEs in the ultrafiltrate were detectable by the phosphomolybdic acid method.[9]

Determination of Antibacterial Activity of PAEs The diluted cell suspension (0.1 ml) for the measurement of MBC was inoculated in a 4.9 ml PAE solution without BP, in which the concentrations of PAEs and the cells were 0, 0.5 or 1.0 mg/ml and 5.1×10^5 cells/ml, respectively. After the solution was allowed to stand for 1 and 2 d at 25 °C, living cell numbers in the solution were determined by the plate count method.

Data Treatments The concentration of free BP in an aqueous phase of the PAE solution (C_f) was determined according to Eq. 1[3]:

$$K_s = \frac{(C_t - C_f)/S}{C_f} \quad (1)$$

where K_s is the apparent partition coefficient of BP between PAE micellar and aqueous phases ($C_{12}E_{21}$, 1.63; $C_{16}E_{20}$, 2.23; $C_{16}E_{30}$, 1.69; $C_{16}E_{40}$, 1.57; $C_{18}E_{20}$, 2.42; $C_{22}E_{20}$, 2.28),[8] C_t is the total BP concentration in the PAE solution and S the concentration of PAEs.

The apparent concentration of incorporated BP in *E. coli* cells in the cell suspension (C_m) is determined by Eq. 2[7]:

$$C_m = (D_t - D_w)/V_m = (D_t - C_w(V_t - V_m))/V_m \quad (2)$$

where D_t and D_w are the BP amounts in the *E. coli* suspension and its supernatant, respectively, V_t and V_m the volumes of the cell suspension and packed *E. coli* cells, respectively, and C_w the BP concentration in the supernatant.

Results and Discussion

Effects of Structures of PAEs on Antibacterial Activity of BP against *E. coli* Table 2 shows the MBC of BP against *E. coli* in the PAE solution and the C_f in its aqueous phase at the MBC determined using K_s according to Eq. 1 due to the negligible volume of the cells (10^{-5} ml/ml) in the PAE solution for the measurement of MBC. Added PAEs caused an increase in the MBC in analogy with many other studies.[1,2,4-6] Free parabens in an aqueous phase have generally been regarded as their effective form in surfactant solutions[4-6]; but the C_f at the MBC in the presence of PAEs (3.72×10^{-4}—5.50×10^{-4} M) was less than the MBC in the absence of PAEs (6.52×10^{-4} M). Thus, the antibacterial activity of BP in the PAE solution was greater than that anticipated from C_f, regardless of structural differences in PAEs. This may be attributed to an increase in the susceptibility of bacteria against BP, possibly due to direct interactions between PAEs and the cells. A decrease in the MBC with the oxyethylene chain length of PAEs (E_{20}—E_{40}) should be due to decreased interactions between BP and the surfactants.[8] No effect of the hydrocarbon chain length of PAEs (C_{16}—C_{22}) was found, and the MBC of $C_{12}E_{21}$ was significantly low.

Table 2. MBC of BP against *E. coli* and C_f at MBC in the Presence of Various PAEs

PAE	MBC[a] (M × 10^4)	C_f[b] (M × 10^4)
Free from PAE	6.52	6.52
$C_{12}E_{21}$	6.76	3.72
$C_{16}E_{20}$	10.6	5.02
$C_{16}E_{30}$	10.1	5.50
$C_{16}E_{40}$	9.41	5.27
$C_{18}E_{20}$	10.4	4.69
$C_{22}E_{20}$	10.9	5.07

a) The MBC was determined using aqueous BP solutions with a multiple of 7.24×10^{-5} M to 1.09×10^{-3} M in the presence of 0.5 mg/ml of PAEs, after inoculation of *E. coli* for 1 d. Data are the mean of three measurements. *b*) The C_f of BP was determined using K_s according to Eq. 1.[8]

Effects of Structures of PAEs on BP Uptake into *E. coli* Cells Table 3 shows the BP concentrations in the supernatant and its ultrafiltrate of *E. coli* suspension with PAEs. The difference in the BP concentration between the cell suspension and supernatant is assumed to be due to BP uptake into the cells.[7] Similarly, the difference in the BP concentration between the supernatant and the ultrafiltrate is attributable to the incorporation of BP into PAE micelles, because the concentration of PAEs is much greater than the critical micelle concentration reported for homologous surfactants,[3,10] and the complexes between BP and the micelles are impermeable passing through an ultrafiltration membrane.[3] Figure 1 shows plots of the C_m of BP in *E. coli* cells, determined according to Eq. 2, against C_f, determined by the ultrafiltration. A linear relationship between C_m and C_f indicates that the BP uptake into the cells is related to the free BP in the aqueous phase, regardless of the presence of PAEs with different structures, except for the BP-$C_{12}E_{21}$ system. A direct interaction between $C_{12}E_{21}$ and bacterial cells seems to con-

Table 3. Concentrations of BP in the Supernatant and Its Ultrafiltrate in an *E. coli* Suspension in the Presence of PAEs[a]

PAE	BP concentration (M × 10^4)[b]	
	Supernatant	Ultrafiltrate
Free from PAE	2.93±0.32	—
$C_{12}E_{21}$	4.07±0.50	1.73±0.14
$C_{16}E_{20}$	5.35±0.15	1.62±0.19
$C_{16}E_{30}$	4.96±0.34	1.88±0.14
$C_{16}E_{40}$	4.77±0.34	2.03±0.18
$C_{18}E_{20}$	5.23±0.39	1.68±0.11
$C_{22}E_{20}$	5.23±0.41	1.72±0.25

a) BP, cell and PAE concentrations in *E. coli* suspension are 7.24×10^{-4} M, 0.051 ml/ml and 1 mg/ml, respectively. *b*) Data are the mean±S.D. (n=4).

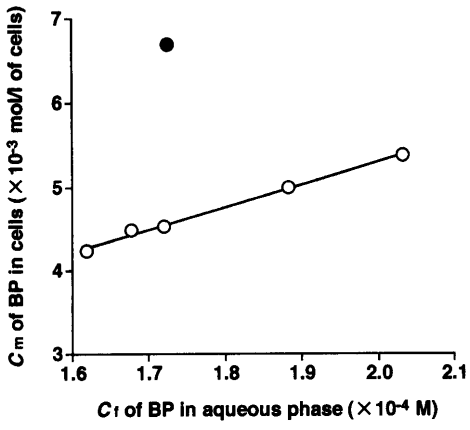

Fig. 1. Relationship between the C_m and C_f of BP in *E. coli* Suspension in the Presence of Various PAEs

Keys: ●, $C_{12}E_{21}$; ○, other PAEs. The C_f was determined by the ultrafiltration. BP, cell and PAE concentrations in *E. coli* suspension are the same as in Table 3. Each point is the mean of four measurements.

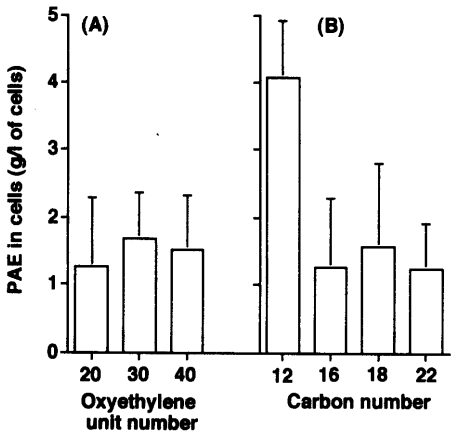

Fig. 2. Uptake of PAEs with (A) 16 Carbon Atoms in Hydrocarbon Chain and (B) 20 or 21 Oxyethylene Units into *E. coli* Cells

PAE and cell concentrations in *E. coli* suspension are the same as in Table 3. Each column is the mean and S.D. ($n=4$).

Table 4. Ratio of Living *E. coli* Cell Numbers in Aqueous $C_{12}E_{21}$ Solution in the Absence of BP[a)]

$C_{12}E_{21}$ (mg/ml)	Time after inoculation (d)	
	1	2
0	1.00	0.985
0.5	0.659	0.368
1.0	0.000[b)]	0.000[b)]

a) Data are the ratio of logarithmic cell numbers and the mean of three measurements. b) Cell number in the standing solution with extinct cells was dealt with 1.0 cell/ml.

tribute to a much greater C_m than that anticipated from C_f.

Interactions between $C_{12}E_{21}$ and *E. coli* Cells Figure 2 shows the apparent concentrations of incorporated PAEs in *E. coli* cells. The majority of PAEs were slightly incorporated into the cells (6—8% of the total amount), whereas $C_{12}E_{21}$ was found to be incorporated to a greater extent (about 20% of the total amount).

It is well known that many C_{12}-compounds with similar hydrophilic portions, such as fatty acids and amines,[11] anionic[12] and non-ionic surfactants,[13] caused the maximal transport of drugs across skin or gastric mucosa. Furthermore, the C_{12}-anionic surfactant exhibited a maximal hemolytic activity.[14] The exact mode of action of C_{12}-surfactants on biological membranes, however, remains unknown. As shown in Table 4, the living cell number of *E. coli* in the absence of BP decreased with an increase in $C_{12}E_{21}$, whereas other PAEs were inactive. The exceptional antibacterial activity of $C_{12}E_{21}$ may be attributed to the denaturalization of some proteins by penetrated surfactants and/or to damage of the cells by the solubilization of cell components. The small MBC of BP in the presence of $C_{12}E_{21}$ is attributable to a multiplier effect of a large amount of incorporated BP and the antibacterial activity of the surfactant. The penetrated molecules of the surfactant may reach the cellular lipid phase in bacterial cells by hydrophobic interaction and alter the fluidity in this phase. The enhanced uptake of BP into *E. coli* cells in the presence of $C_{12}E_{21}$ (Fig. 1) is possibly due to an increase in the fluidity of the lipid matrix by the surfactant.

Consequently, we concluded that PAEs increased the susceptibility of bacteria against BP due to a direct interaction between the surfactants and bacterial cells. Particularly, $C_{12}E_{21}$ molecules which penetrated abundantly into the cells might result in an increase in the fluidity of the cellular lipid matrix and a decrease in the resistance of drug permeation.

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