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Calorimetric Analysis of Antimicrobial Effect of *p*-Hydroxybenzoic Acid Alkyl Esters

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Calorimetric studies were conducted on the antimicrobial action of *p*-hydroxybenzoate alkyl esters (parabens), which are commonly used as preservatives in cosmetic products, and the parameters to quantitatively predict their actions on the microbial strains were determined. The result obtained was that their action on both *Saccharomyces cerevisiae* and *Klebsiella pneumoniae* became stronger as the number of carbon atoms in the alkyl chain increased. When comparing the affinity of the drugs to both microorganisms based on the drug concentration required to inhibit growth activity by 50%, a linear relationship was observed to exist between the number of carbon atoms in an alkyl chain and the affinity. In addition, the same drug exhibited a higher effect on *S. cerevisiae* than on *K. pneumoniae*, which may be due to the difference in the membrane structures of the microorganisms.

Key words : Preservative/Microbial calorimetry/Antimicrobial effect/Specific growth activity/Apparent binding affinity/Cosmetics and toiletries

INTRODUCTION

Like in food products, preservatives are added to in cosmetic products to prevent microbial contamination (Moral, 1992; Richardson, 1981; Tanaka, 1997). Moreover, antibacterial agents are contained in quasi-drugs and medical drugs in addition to preservatives with the aim of disinfecting the skin, and eliminating specific bacteria such as those causing acne, dandruff, and body odor (Charig et al., 1991; Hausteine et al., 1993; Kortig et al., 1991).

In using such preservatives and antibacterial agents in cosmetic products and quasi- or medical drugs, it is important to choose the most suitable kind of agent according to the characteristics of the product and purposes of its use (Corbett, 1992, 1997). However, actions of preservatives and antibacterial agents are not completely understood, and, at present, the selection of agents to be used is based

largely upon past experience. In recent medical drug development, knowledge of the relationship between the molecular design of drugs (drug design) and antimicrobial actions has become extremely important in the development of drugs which are effective against bacteria causing nosocomial infections (methicillin-resistant *Staphylococcus aureus*, MRSA, and vancomycin-resistant *Enterococcus*, VRE) (Hashimoto, 1996; Sasatsu et al., 1996; Takeda et al., 1996).

In order to help resolve of these problems, we investigated a method for qualitatively analyzing actions of antimicrobial drugs by microbial calorimetry. Microbial calorimetry is a method for monitoring the growth of microorganisms by determining the metabolic heat generated as a result of microbial growth from a non-destructive point of view (Antoce et al., 1996a; Takahashi, 1996). Since this microbial growth thermogram [$g(t)$ curve] accurately reflects the growth of microorganisms, changes in the patterns of growth thermograms obtained from the culture system to which a drug has been added can provide

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various kinds of information about the drug effects. Therefore, analyses of these changes by an appropriate method can provide a quantitative understanding of the drug effects which conventional methods cannot (Antoche et al., 1996b, 1997a, 1997b, 1998; Koumoto et al., 1996; Okuda et al., 1996). Accordingly, we investigated the antimicrobial actions of *p*-hydroxybenzoate alkyl esters (parabens) by microbial calorimetry. *p*-Hydroxybenzoate alkyl esters are widely used as preservatives in food and cosmetic products as well as in quasi- and medical drugs, have a broad antibacterial spectrum, and are considered to be very safe (Hatanaka, 1976; Matsuda et al., 1993; Richardson, 1981). To determine growth-inhibiting parameters for the actions of parabens, we obtained growth thermograms using a multiplex batch calorimeter (Takahashi, 1996) having 24 calorimetric units, in order to investigate the growth behavior of *Klebsiella pneumoniae* and *Saccharomyces cerevisiae* in the growth culture containing various concentrations of parabens. We then calculated the 50% growth inhibition concentration (K_5) on the basis of retardation in growth, using growth thermograms of *K. pneumoniae* and *S. cerevisiae* obtained from each drug sample. Then, we drew a drug potency curve according to the drug concentration dependency, and evaluated the drug effects.

Moreover, we determined the affinity of each drug to *K. pneumoniae* and *S. cerevisiae* microbial cells (changes in Gibbs free energy of binding) on the basis of the 50% growth inhibition concentration (K_5), and examined the morphology of molecules and drug effects. Methyl, ethyl, propyl, butyl, pentyl, and hexyl *p*-hydroxy benzoate were used as parabens.

MATERIALS AND METHODS

Devices and samples

For determining the metabolic heat of a microorganism, we used a multiplex batch calorimeter containing 24 calorimetric units (Bio Thermo Analyzer H-201, Nippon Medical & Chemical Instruments Co., Ltd., Osaka; abbreviated as BTA) which was previously reported by Takahashi (1996).

Six commercially available antibacterial *p*-hydroxybenzoate alkyl esters (parabens) were used. The microorganisms employed in the study were our stock strain of yeast, *S. cerevisiae* no.9302, and *K. pneumoniae* which had been isolated from a cosmetic material. *S. cerevisiae* was incubated in a glucose peptone medium (Nippon Seiyaku, pH5.6-5.8) and *K. pneumoniae* was incubated in a brain heart infusion medium (NISSUI, pH 7.2).

Procedure

In the experiment, 5 ml of the glucose peptone medium or the brain heart infusion medium each containing a paraben at a specified concentration was placed in a 30 ml incubation vial which served also as a calorimetric vessel. 50 μ l of a cell suspension at 10^3 cells/ml was added and stirred, and each vial was placed in a calorimetric unit kept at 30°C to determine the heat evolution signals which result from growth.

RESULTS AND DISCUSSION

Figure 1 (I) shows the growth thermogram of the yeast, *S. cerevisiae*, incubated for 48 h in the medium containing propyl paraben at a concentration of 0 to 0.0168% (w/v). This thermogram will be referred to as the $g(t)$ curve. The ordinate represents the thermal electromotive force (μ V) determined by a thermal detector, and the abscissa represents the incubation time (h). The $g(t)$ curve shown in this figure exhibits changes in the pattern of the growth thermogram associated with changes in the propyl paraben concentration. As the propyl paraben concentration increases, the incubation time required for

FIG. 1. Growth thermograms (I) and growth curves (II) observed for the growing culture of *Saccharomyces cerevisiae* in brain-heart infusion medium containing various amounts of propylparabens. The 7 curves shown in each panel correspond from left to right to the concentration of propylparaben. a, 0; b, 0.0072; c, 0.0102; d, 0.0132; e, 0.0144; f, 0.0156; g, 0.0168% (w/v).

the peak of the microbial growth thermogram to be reached becomes longer, and the inclination at the beginning of the curve becomes smaller, suggesting inhibition of the microbial growth.

The $g(t)$ curve shows the heat evolution process associated with the microbial growth in the calorimetric unit. This, however, shows only apparent changes in heat, and the loss of the heat to the surroundings is also included in the results. When the heat conduction constant (Newton's cooling constant) of the calorimetric unit including the sample is denoted by K , the true amount of heat generated $[f(t)]$, corrected for the heat loss arising from heat exchange with the surroundings, can be obtained by the following equation (Takahashi, 1996).

$$f(t) = g(t) + K \int g(t) dt \quad (1)$$

In other words, the $g(t)$ curve obtained here can be converted into a calorimetrically obtained growth curve using a simple algorithm (Antoce et al., 1996, 1997a; Takahashi, 1996). The curve thus obtained is shown in Fig. 1(II) as the $f(t)$ curve. As is clear from this figure, the initial phase of the growth includes a portion which shows exponential growth, and this can be applied to the following exponential growth equation to obtain the growth rate constant μ (Antoce et al., 1996a; Hashimoto et al., 1982; Takahashi, 1996).

$$f(t) = AN_0 e^{\mu t} + BN_0 \quad (2)$$

In this equation, N_0 denotes the initial cell count, and A and B are constants. When the growth rate constant μ is used, the specific growth activity (μ_i / μ_m) can be obtained from the ratio of the growth rate constant, μ_i , at the drug concentration, i , to the growth rate constant, μ_m , at the drug concentration, 0. Thus, a drug potency curve can be drawn from the equation (3), and the drug potency characteristics can be quantitatively described (Antoce et al., 1996a, 1997a, 1997b).

$$\mu_i / \mu_m = 1 / \{1 + (i/K_u)m^u\} \quad (3)$$

In this equation, K_u denotes the mean value of the dissociation constant of the drug per site. While m_u can be regarded as the number of sites, it is also an exponent which expresses the synergism of drug actions. Accordingly, μ_i is given by a hyperbolic function as the m_u -th power of the drug concentration, i (Antoce et al., 1996b).

As shown in Figs. 1(I) and 1(II), the drug effects are expressed as changes in the patterns of the $g(t)$ and $f(t)$ curves, which imply the above-mentioned changes in the growth rate constant, and retardation in the growth.

While the $f(t)$ curve indicates time courses of heat evolution during the microbial growth, the differential value, $f'(t)$, is considered to express the growth activity as measured from the metabolic heat at each

instant of incubation time based on the caloric generation. Here we define that the incubation time required for the microbial activity, $f'(t)$, to reach a certain value α , in the absence of the drug is expressed as $t_\alpha(0)$, and that the incubation time required for the same activity level to be reached at the drug concentration i , is expressed as $t_\alpha(i)$. Then, the specific growth activity based on retardation in the growth is expressed by the ratio $t_\alpha(0)/t_\alpha(i)$ (Antoce et al., 1996a, 1996b, 1997a, 1997b, 1998a). The specific growth activity based on retardation in the growth thus defined can be treated in the same way as the microbial activity based on the growth rate constant. The following equation, which is similar to equation (3), can be written for $t_\alpha(0)/t_\alpha(i)$,

$$t_\alpha(0)/t_\alpha(i) = 1 / \{1 + (i/K_\alpha)m_\alpha\} \quad (4)$$

where the parameters m_u and K_u were renamed as m_α and K_α , respectively (Antoce et al., 1996a).

Equations (3) and (4) suggested that both the specific growth activity based on the growth rate constant (μ_i / μ_m), and the specific growth activity based on retardation in growth ($t_\alpha(0)/t_\alpha(i)$) can be mathematically given by the same function pattern, and that the drug potency curve can be drawn not only from μ_i / μ_m , but also from $t_\alpha(0)/t_\alpha(i)$ using equation (4) (Antoce et al., 1996a; 1996b). However, it should be noted that the specific growth activity based on the growth rate constant does not completely match with the specific growth activity based on retardation in growth. For example, if a drug works bacteriostatically, then only the retardation in growth will be observed while the growth rate constant μ would be unaffected (Okada et al., 1998). Thus the discrepancy between the two parameters μ_i / μ_m and $t_\alpha(0)/t_\alpha(i)$ reflects essential differences related to the mechanism of action of the drug (parabens in the case of this study), as well as errors associated with optimization. In reality, all kinds of drugs exert bacteriocidal actions at concentrations higher than a certain level, and often completely inhibit the growth of microorganisms. Furthermore, if we look at the antimicrobial action through the growth rate constant, then the effect of bacteriocidal actions will be disregarded. In other words, $t_\alpha(0)/t_\alpha(i)$ appears to be more appropriate in the case of drugs having both bacteriostatic and bacteriocidal actions. Therefore, we used the specific growth activity based on retardation in growth, $t_\alpha(0)/t_\alpha(i)$, in discussing and comparing antimicrobial actions of parabens in this article.

Figure 2(I) shows the potency curves for six parabens as measured from retardation in growth of *S. cerevisiae*, including the experimental data of propyl paraben obtained from Figs. 1(I) and (II). The ordinate in Fig. 2 represents the specific growth

activity based on retardation in growth, $t_a(0)/t_a(i)$, and the abscissa represents the logarithm of the drug concentration. Plots represent experimental data and solid lines represent regression curves, *i.e.*, drug potency curves. As is evident from this figure, the drug potency curves for parabens represented as $t_a(0)/t_a(i)$ shows the shift to the left in the order of methyl, ethyl, propyl, butyl, pentyl and then hexyl. This indicates that the antibacterial activity is the highest for methyl, followed by ethyl, propyl, butyl, pentyl and then hexyl. Figure 2(II) shows the drug potency curves as measured from retardation in growth of *K. pneumoniae*. Similarly to the results with *S. cerevisiae*, the stronger antibacterial effects were observed when the alkyl chain was longer, but changes in drug potency curves were not as marked as those for *S. cerevisiae*. These findings suggest that, although the

antibacterial effects become stronger when the alkyl chain is longer, the increase in the antibacterial effects is not as significant as that observed with *S. cerevisiae*. The drug potency curves are shown only for methyl, ethyl, and propyl parabens in this figure, because the data of drugs having an alkyl chain longer than butyl could not be obtained due to limitations in the solubility (Hatanaka, 1976). The results obtained with *S. cerevisiae* and *K. pneumoniae* have revealed that changes in the pattern of the growth thermogram become more evident if drug potency curves are drawn.

When drug potency curves are drawn, the drug concentration inducing 50% growth inhibition (K_0) should be determined simultaneously (Antoce et al., 1996a, 1997a, 1997b; Takahashi, 1996). K_0 is an inhibition parameter representing the effective drug

FIG. 2. The specific growth activity as a function of drug concentration. Drugs are *p*-hydroxybenzoate alkyl ester. a, methyl ester; b, ethyl ester; c, propyl ester; d, butyl ester; e, pentyl ester; f, hexyl ester. (I) *Saccharomyces cerevisiae*, (II) *Klebsiella pneumoniae*.

concentration at which the growth activity is reduced by half, and is equivalent to the median concentration of the drug potency curve. The values of K_0 thus obtained are summarized in the fourth column of Table 1.

In addition to the parameter K_0 , it is also possible to obtain the minimum inhibition concentration from the drug concentration dependency of the growth rate (Antoce et al., 1996a). If we define that the reduction of the growth activity by a drug is expressed as $(1 - t_a(0)/t_a(i))$, and that it is regarded as being in proportion to the m_i -th power of the drug concentration, i , we can obtain the following equation

$$1 - t_a(0)/t_a(i) = k_i i^{m_i} \quad (5)$$

where, k_i is a proportional constant.

According to the equation (5), the drug concentration at which the growth activity is completely lost can be obtained by the following equation (Antoce et al., 1996a, 1997a, 1997b; Takahashi, 1996).

$$i_c = (1/k_i)^{1/m_i} \quad (6)$$

The two unknown values (k_i and m_i) can be determined by the regression analysis on equation (5) and i_c can be calculated by equation (6). Needless to say, the value of i_c can be regarded as the minimum inhibition concentration by the nature of the problem. In the fifth column of Table 1 are given the values of MIC determined by the above procedure. In order to compare the antimicrobial activity of parabens more quantitatively, their apparent affinity to microbial cells were determined.

On the basis of the above-mentioned 50% growth inhibitory concentration, K_0 , the apparent affinity of parabens to microbial cells (A), or in other words, the changes in the Gibbs free energy of binding, was obtained using the Gibbs equation (Nitta et al., 1971; Nitta et al., 1996)

$$A = RT \ln K_0 \quad (7)$$

where R is the gas constant and T is the absolute tem-

perature. The values obtained are shown in the last column of Table 1. It is obvious that the apparent affinity increases with increasing numbers of alkyl carbons in both actions against both *S. cerevisiae* and *K. pneumoniae*.

Figure 3 shows plots of the drug affinity to the two microorganisms obtained. As is evident from the figure, an extremely favorable linear relationship is noted between the number of carbons in the alkyl chain and the drugs affinity. Moreover, differences in the antibacterial potency appears to be mainly attributable to the carbon chain. The increase in the inhibitory actions of parabens against microbial cells in association with increases in the number of carbons in the alkyl chain seems to strongly imply that hydrophobic interactions with cells are highly responsible for this.

Moreover, from the slope of the plots, the degree of affinity of drugs to cells was estimated to be 2.1 kJ/mol per carbon in the case of *S. cerevisiae*, and 1.1 kJ/mol in the case of *K. pneumoniae*. It was shown by various investigators in literature that among microbes, there is a large variety in membrane structures and their composition (Kabara, 1984). The discrepancy obtained above seems to be most reasonably explained by the differences in the membrane structures of *S. cerevisiae* and *K. pneumoniae*, and it may be assumed that the higher inhibitory effect on a microorganism cell observed with a paraben having a larger number of carbon atoms in its alkyl chain is attributable substantially to its hydrophobic interaction with the cell.

We have discussed the qualitative analysis of the potency of antimicrobial drugs in this study, citing parabens, which are commonly used as preservatives in foods, cosmetic products and medical drugs, as an example. It is clear that highly reliable information can be provided by the present method based on the measurement of microbial metabolic heat. As is rep-

TABLE 1. Fifty % growth inhibitory concentration (K_0 /mM), minimum inhibitory concentration (MIC₅₀/mM) and apparent binding affinities (A /kJ mol⁻¹).

<i>p</i> -Hydroxybenzoate alkyl ester	Strain	K_0 /mM	MIC ₅₀ /mM	A /kJ mol ⁻¹
Sodium salt	<i>S. cerevisiae</i>	112.98	206.21	5.5
	<i>K. pneumoniae</i>	69.70	126.15	6.7
Methyl	<i>S. cerevisiae</i>	4.47	6.69	13.6
	<i>K. pneumoniae</i>	6.85	18.48	12.6
Ethyl	<i>S. cerevisiae</i>	2.26	3.34	15.4
	<i>K. pneumoniae</i>	5.02	9.80	13.4
Propyl	<i>S. cerevisiae</i>	0.88	1.29	17.7
	<i>K. pneumoniae</i>	2.77	4.28	14.9
Butyl	<i>S. cerevisiae</i>	0.39	0.66	20.0
Pentyl	<i>S. cerevisiae</i>	0.20	0.29	21.5
Hexyl	<i>S. cerevisiae</i>	0.07	0.11	24.2

FIG. 3. Relationship between the number of alkyl carbons in *p*-hydroxybenzoate alkyl ester and the apparent drug affinity to the microbial cell.

resented by Fig. 3, examination of the details about structure-activity relationship is also possible. In light of the results presented here, we believe that this technique is effective not only for accurate evaluation of drug potency, but also for new approaches of molecular design (Okada et al, 1996b, 1998) and even the evaluation of synergistic effect of drugs (Antoce et al, 1998b). We also believe that the usefulness of analyses of antimicrobial actions by the calorimetric method in development of medicines will be recognized even more strongly in the future and that the methodology described here will contribute to introduce a new insight into the study of antimicrobial drugs.

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