論 文

Some Theoretical Considerations on Calorimetrically Determined Bactericidality of Antimicrobial Drugs and Its Concentration Dependence

Sandra Wirkner and Katsutada Takahashi

(Received April 3, 2000; Accepted April 22, 2000)

Based on the theory to characterize the bacteriostatic and bactericidal actions of drugs proposed by Takahashi,[1] further theoretical considerations were attempted on the bactericidality of a drug and its concentration dependence. An index term to show the bactericidality as a function of drug concentration was defined and the concentration dependence was graphically drawn on the basis of inhibitory parameters determined calorimetrically. The result was obtained that if the parameters characterizing the apparent cooperativity in drug actions obtained from the growth rate is different from those obtained from the growth retardation, the bactericidality changes largely depending on the drug concentration. The result was also found to be consistent with the observation for some drugs where the *SCI* plot defined previously[1] does not show a straight line, but exhibits an upward curvature.

1. Introduction

In one of the preceding reports from the present authors group it was shown that bacteriostatic and bactericidal actions of antimicrobial drugs can be qualitatively characterized by a calorimetric method.[2] It was found that these two actions are given as a change in pattern of growth thermograms that are observed with growing cultures of microbes in growth media containing various amounts of drugs.[2] Moreover, based on that finding, Takahashi made a proposal to introduce the "bacteriostatic/bactericidal index, *SCI*", an index term to show how a drug action is characterized in terms of a proportion of bacteriostatic action over the entire actions for a given drug and showed that it can be easily given by the slope of a plot when the specific growth activity μ_i/μ_m is plotted against the specific growth retardation $t_\alpha(0)/t_\alpha(i)$, both being obtained from the growth thermograms.[1] According to this proposal, a drug having purely bacteriostatic effect has a value of $SCI = 1.0$, whereas the one having purely bactericidal effect has a value of $SCI = 0$. It was also shown that many existing drugs have *SCI* values between 0 and 1.0, depending on the kind of their characteristic features related to the action mode on bacterial cells. Thus the method proposed enabled to characterize the nature of antimicrobial drugs in terms of the bacteriostatic and bactericidal actions and the values of *SCI* were actually determined and shown for various drugs.[1]

However, in the above method, only the apparent

Abbreviations used: SCI, bacteriostatic-bactericidal index / MIC, minimum inhibition concentration / butylparaben, *p*-hydroxybenzoic acid propyl ester / Q15, cis-isomer of 1-(-3-chloroallyl)-3,5,7-triaza-1-azoniaadamantane-chloride, N-(3-chloroallyl)-hexammonium chloride, Quarternium 15 or Dowicil 200 as a commercial name / IDU, imidazolidinyl urea, Germal 115 as a commercial name / Triclosan, 2,4,4'-Trichloro-2'-hydroxydiphenylether / DMH, 1,3-dimethylol-5,5-dimethylhydantoin, 1,3-bis-(hydroxymethyl)-5,5'-dimethyl-2,4-imidazolidinedione, Dimethyloldimethylhydantoin or DMDM hydantoin.

Table 1 Antimicrobial drugs and their inhibitory parameters used for the calculation.

drug	microbe	*SCI*	k_1	m_1	k_2	m_2	MIC_θ	ref.
butylparaben	*S. cerevisiae*	1.02	2005.33	1.629	752.90	1.442	0.0101 %	7, 9
sodium bennzoate	*Asp. oryzae*	0.74	0.0327	0.0587	0.0377	0.634	176 mM	8
Q15	*K. pneumoniae*	0.17	121.64	1.370	591.2	1.363	0.00926 %	2
IDU	*K. pneumoniae*	0.06	0.331	0.624	547.6	1.978	0.0413 %	2
Triclosan	*K. pneumoniae*	0.34	1.0610e7	3.64	1715.09	1.617	0.0100 %	9
DMH	*K. pneumoniae*	0.17	1410.95	2.281	29.52	0.953	0.0287 %	9

All of the parameters were determined from the growth thermograms observed for the growing culture of the each strain in medium containing the drugs. The parameters are defined in equations (2) and (3) and were determined by regression analysis either on the specific growth activity μ_i/μ_m or the specific growth retardation $t_\alpha(0)/t_\alpha(i)$. MIC_θ is the minimum inhibition concentration as determined from the specific growth retardation on the basis of the equation (5).

proportion of bacteriostatic action involved in the entire actions of an antimicrobial drug is given and it provides no information about its dependence on drug concentrations. In another word, *SCI* itself is the value derived with the assumption that the antimicrobial action of drugs is not a function of drug concentration.

For this reason, in order to characterize the antimicrobial action of drugs more quantitatively, the theory presented by Takahashi[1] has been developed further and a new additional parameter, bactericidality σ, was defined to show the concentration dependence in the property of drug actions. The bactericidality σ actually obtained for various antimicrobial drugs will be shown as a function of the concentration.

2. Materials and Methods

Antimicrobial actions of drugs were calorimetrically studied using a multiplex batch calorimeter and the inhibitory parameters were determined on the basis of the method described in the previous works.[3-6] The antimicrobial drugs dealt with in the present paper for model calculation were: butylparaben, sodium benzoate, imidazolidinyl urea (IDU), Quarternium 15 (Q15), Triclosan and DM hydantoin (DMH). All of the calorimetric data for these drugs were taken from the works done by the present authors group.[2,7-9] The inhibitory parameters used for the calculation are summarized in **Table 1** together with their literature sources.

3. Results and Discussion

In **Fig.1** the *SCI* plot obtained for 6 different drugs, butylparaben, sodium benzoate, Q15, IDU, Triclosan and DMH, are shown. All of the data sets used in the plot

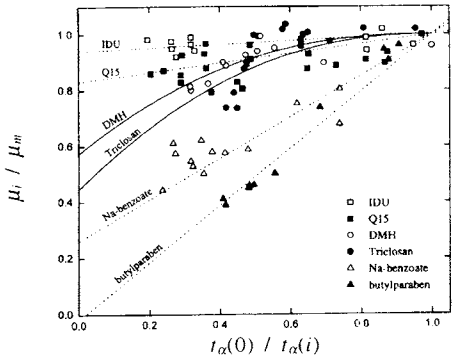

Fig.1 *SCI* plots made for the drugs: butylparaben, sodium benzoate, Q15, IDU, Triclosan, and DMH.

are reproduced from the calorimetric studies previously reported (see references given in **Table 1**). As can be seen in the plots, butylparaben have a slope of almost 1.0 with the indication that it has a strong bacteriostatic action. The *SCI* value of butylparaben actually obtained as the slope of the plot by regression analysis was 1.02.[1] Similarly sodium benzoate is found to have a *SCI* value of 0.74,[1] thus being also of bacteriostatic nature, but not as much as that of butylparaben. In contrast to the above two cases, the plots for Q15, IDU, Triclosan and DMH obviously have less steep slope. Although the data points are rather scattered, it is possible to determine the *SCI* value by regression analysis and they were obtained to be 0.17, 0.06, 0.34 and 0.17 for Q15, IDU, Triclosan and DMH, respectively.[1] This fact obviously indicates that the four drugs act bactericidally rahter than bacteriostatically and among them IDU exhibits almost 100 % bactericidality as evaluated from the *SCI* value.

From these results it may be concluded that the antimicrobial actions of Q15, IDU, Triclosan and DMH seem to be characterized by a strong bactericidal nature. However, there is also a distinct difference in the plots for Q15, IDU and those for Triclosan and DMH. While the data points of Q15 and IDU seem to fall fairly well on straight lines, the plots for Triclosan and DMH do not seem to show a straight line, but obviously have an upward curvature as shown by the solid lines. It seems probable to think that this feature with the upward curvature suggests the involvment of more than two antimicrobial actions having different potencies on microbial cells and that the two drugs, Triclosan and DMH, act rather bactericidally at a lower drug concentration range, while the bacteriostatic nature also gradually appears with increasing the drug concentrations. In another word the action pattern of Triclosan and DMH is assumed to change with their concentrations.

In order to understand the above situation more quantitatively further theoretical consideration was made as follows. From comparison of the two parameters, the specific growth activity and the specific growth retardation, we define here the bactericidal contribution relative to the bacteriostatic contribution (bactericidality) of a drug by the following equation;

$$\sigma = \{\mu_i/\mu_m - t_\alpha(0)/t_\alpha(i)\} / \{1 - t_\alpha(0)/t_\alpha(i)\} \quad (1)$$

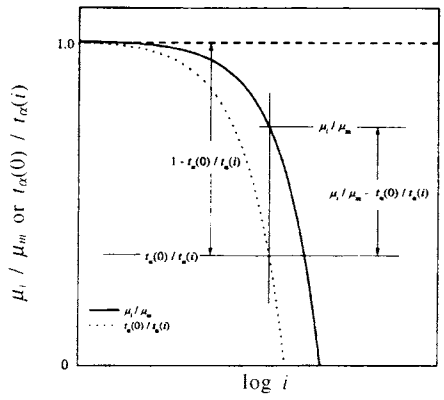

Fig.2 Graphic illustration of the bactericidality parameter. The raito of $\{\mu_i/\mu_m - t_\alpha(0)/t_\alpha(i)\}$ to $\{1 - t_\alpha(0)/t_\alpha(i)\}$ is defined as the bactericidality σ.

where the symbol σ denotes the bactericidality and μ_i/μ_m and $t_\alpha(0)/t_\alpha(i)$ denote the specific growth activity and the specific growth retardation, respectively and are defined by the following equations[3-6];

$$\mu_i/\mu_m = 1 - k_1 i^{m_1} \quad (2)$$

$$t_\alpha(0)/t_\alpha(i) = 1 - k_2 i^{m_2} \quad (3)$$

The meaning of σ given in equation (1) is that it is geometrically defined as the ratio of a segment $\mu_i/\mu_m - t_\alpha(0)/t_\alpha(i)$ to a segment $1 - t_\alpha(0)/t_\alpha(i)$ which are represented in model potency curves given in **Fig.2**.*

Using eqs. (2) and (3), it is possible to draw the potency curves for given sets of the parameters, k_1, m_1, k_2 and m_2. Examples are shown in **Figs.3(a)** and **3(b)**, where the parameters used for the calculations are summarized in **Table 2**. For convenience, the parameter set, k_2 and m_2, were kept constant and only the set of k_1 and m_1 were varied. In the calculation shown in **Fig.3(a)**, the parameter k_1 was taken as a variable and m_1 was set to be constant, while the curves shown in **Fig.3(b)** were drawn by taking m_1 as a variable and k_1 as the constant. Although the parameter values used for the calculation have no specific biochemical significance related to the action mechanism and they are the ones empirically determined on the basis of eqs. (2) and (3), one will see from **Fig.3** that the parameters, k_1 and k_2 , are related to the concentration range of drug action and that the parameter, m_1 and m_2, are related to the cooperativity in the drug action.

If we employ a definition of the minimum inhibition

---

* In the preceding paper,[1] the potency curves were drawn on the basis of equations

$$\mu_i/\mu_m = 1 / \{1 + (i / K_\mu)^{m_\mu}\} \quad (4)$$

$$t_\alpha(0)/t_\alpha(i) = 1 / \{1 + (i / K_\theta)^{m_\theta}\} \quad (5)$$

where K_μ and K_θ are the 50 % inhibitory drug concentrations dtermined from the growth rate constant and the growth retardation, respectively, and m_μ and m_θ are the parameters related to the cooperativity in the drug action evaluated from the growth rate constant and the growth retardation, respectively.[3-6] However, with these equations the knowledge about the so-called minimum inhibition concentration (MIC) can not be mathematically given. For this reason and since we are dealing with the MIC in the present paper, eqs. (2) and (3) which were defined to obtain the MIC value and to draw the MIC curve[3-6,10-12] were employed.

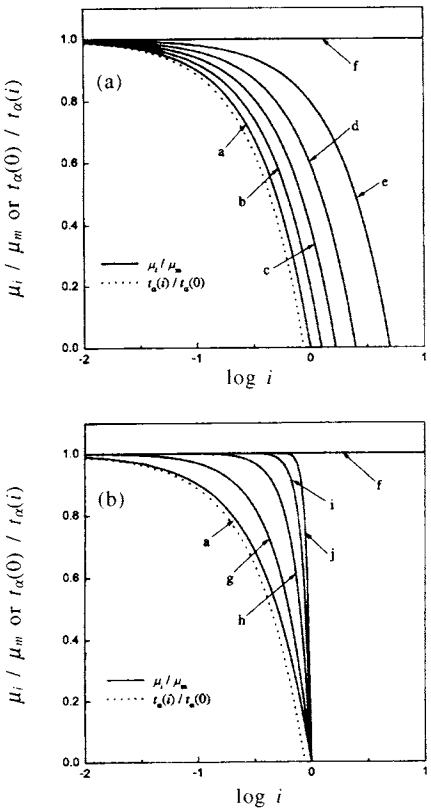

Fig.3 Model calculations showing the concentration dependence of either μ_i/μ_m {curves; a – f in (a) and a, f, g – j in (b)} and $t_\alpha(0)/t_\alpha(i)$ (dotted lines). The $t_\alpha(0)/t_\alpha(i)$ curves were drawn on the basis of eq. (2), while the curves a – j were drawn on the basis of eq. (3). All the curves correspond to those calculated for parameters sets given by notations a to j in Table 2.

concentration MIC to be the drug concentration at which the microbial activity becomes zero, it will be obvious from **Fig.3** that the intercept of the plots on the x-axes corresponds to the value of MIC. Consequently the MIC values are mathematically determined by the following equations[3-6,10-13]

$$\mathrm{MIC}_\mu = (1 / k_1)^{(1/m_1)} \tag{6}$$

$$\mathrm{MIC}_\theta = (1 / k_2)^{(1/m_2)} \tag{7}$$

where MIC_μ and MIC_θ are the minimum inhibition concentration as evaluated from the specific growth activity and the specific growth retardation, respectively. Since the value of MIC_θ is usually smaller than that of MIC_μ, we will consider hereafter the drug action only

Table 2 Parameters sets used for the theoretical calculation of bactericidality.

case	k_1	m_1	k_2	m_2
a	1	1	1	1
b	0.8	1	1	1
c	0.6	1	1	1
d	0.4	1	1	1
e	0.2	1	1	1
f	0.0	1	1	1
g	1	1.5	1	1
h	1	3.0	1	1
i	1	6.5	1	1
j	1	12.0	1	1

at a concentration range $0 < i < \mathrm{MIC}_\theta$.

For practical comparison of the above defined bactericidality parameters σ among various drugs, it will be convenient to use a reduced drug concentration i_{red} rather than the actual concentration i, where the reduced drug concentration i_{red} is defined by the following equation:

$$i_{red} = i / \mathrm{MIC}_\theta \tag{8}$$

Using eqs. (2) to (8), eq. (1) is rewritten as follows:

$$\begin{aligned}\sigma &= 1 - (k_1/k_2)(\mathrm{MIC}_\theta)^{(m_1 - m_2)}(i/\mathrm{MIC}_\theta)^{(m_1 - m_2)} \\ &= 1 - (k_1/k_2)(1/k_2)^{(m_1/m_2 - 1)} i_{red}^{(m_1 - m_2)}\end{aligned} \tag{9}$$

Eq. (9) indicates that using 4 experimentally determined parameters, k_1, m_1, k_2 and m_2, it is possible to predict the variation of σ as a function of drug concentration. Calculations were made for 10 given sets of parameters corresponding to the cases shown in **Table 2** (the case a to h) and **Fig.3** and the results obtained are shown in **Fig.4(a)** and **Fig.4(b)**. As can be understood from eq. (9), only when $m_1 = m_2$, the value of σ is independent on drug concentration i_{red} having a value of $1 - (k_1/k_2)$ as shown in **Fig.4(a)** and the larger the k_2 value compared to the k_1 value, the more is the bactericidality in the drug action. However, it should be noted that the above assumption that m_1 is equal to m_2 does not seem to be appropriate in actual case. In another word it is likely that in most cases the values of m_1 and m_2 are not identical, but they generally differ with each other. Model calculations made for the case $m_1 \neq m_2$ are shown in **Fig.4(b)** (case g to j). In contrast to the results shown in **Fig.4(a)**, the values of σ vary over a wide range depending on the reduced drug concentration i_{red}, when $m_1 \neq m_2$.

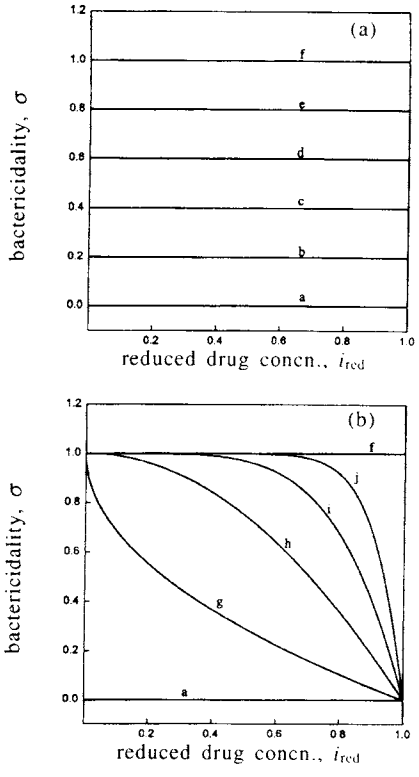

Fig.4 Bactericidality σ as a function of reduced drug concentration i_{rcd}. The lines, a – j, were calculated on the basis of eq. (9) by using the parameter sets with the notations a – j listed in Table 2, respectively. The each line corresponds to the potency curve of model calculations (a – j) shown in Fig.3.

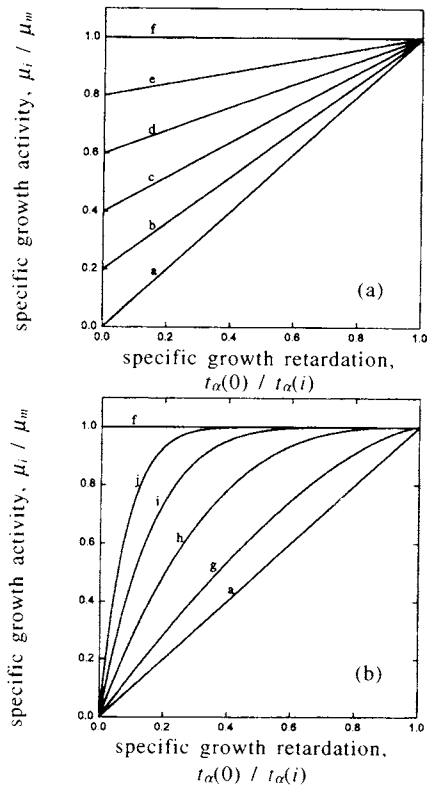

Fig.5 SCI plot of the model calculations made for the parameter sets with the notations a – j listed in Table 2. The each plot corresponds to the potency curve of model calculations a – j shown in Fig.3.

Thus it is reasonably assumed that the actual situation is more complicated with most drugs than what we are considering here and that the plot of σ against i_{rcd} does not give a straight line with a definite slope, but has a curvature, depending on the values of k_1, m_1, k_2 and m_2. The result shown in **Fig.4(b)** also indicates that the difference in the apparent cooperativity in drug action evaluated from the μ_i/μ_m and $t_\alpha(0)/t_\alpha(i)$ values plays an important role in characterizing the nature in antimicrobial actions.

Furthermore, the present consideration provides some more theoretical basis in the *SCI* plot proposed in the preceding paper[1]. Based on eqs. (2) and (3), we have the relation

$$\mu_i/\mu_m = 1 - k_1[(1/k_2)\{1-t_\alpha(0)/t_\alpha(i)\}]^{(m_1/m_2)} \qquad (10)$$

Eq. (10) is a general equation to show the relationship between μ_i/μ_m and $t_\alpha(0)/t_\alpha(i)$ and indicates that the *SCI* plot can be made by using the parameters k_1, m_1, k_2 and m_2 that are determined by regression analysis on eqs. (2) and (3).

In the case of a condition where $m_1 = m_2$, eq. (10) is rewritten as

$$\mu_i/\mu_m = -(k_1/k_2)\{t_\alpha(0)/t_\alpha(i)\} + (1 - k_1/k_2) \qquad (11)$$

Thus the plot of μ_i/μ_m versus $t_\alpha(0)/t_\alpha(i)$ gives a straight line with a slope of (k_1/k_2). The results of model calculation are shown in **Fig.5(a)** where the calcuation is made by using the parameter sets (a – f) given in **Table 2**. This fact indicates that, strictly speaking, the *SCI* value obtained as the slope of *SCI* plot is valid only when $m_1 = m_2$ and is equal to (k_1/k_2).

On the other hand, in the case of $m_1 \neq m_2$, the situation is quite different. Model calculations were made for the parameter sets (g – j) given in **Table 2** and the

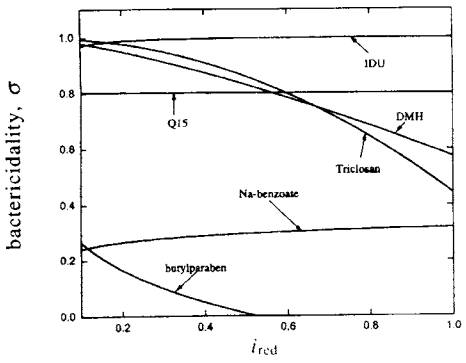

Fig.6 Bactericidality σ as a function of reduced drug concentration i_{red} obtained for the six drugs on the basis of eq. (9) by using the parameter sets listed in Table 1. The drugs are; butylparaben, sodium benzoate, Q15, IDU, Triclosan, and DMH.

results are shown in **Fig.5(b)** where the parameter k_1 was set to be equal to the parameter k_2 just for convenience. As can be known from **Fig.5(b)**, all of the plots do not show a straight line but are characterized by an upward curvature.

It would be clear from eq. (10) that the characteristic feature with upward curvature does not come from the condition $k_1 = k_2$, but stems from the condition $m_1 \neq m_2$. In fact, the *SCI* plot for Triclosan and DMH shown in **Fig.1** are the reflection of this matter and the solid lines are those actually drawn on the basis of eq. (10) by using the parameter sets given in **Table 1**.

Based on the consideration described above, the experimental results on actual drugs shown in **Fig.1** can be discussed more in detail in terms of the characteristic features of their bactericidality as a function of concentration. The variation of σ was calculated on the basis of eq. (9) and is presented in **Fig.6**. The calculation of σ was made by taking $i_{red} = 0.1$ as the lowest concentration, since no drug action is present at $i = 0$.

It can be seen that butylparaben has a lower value over a whole range of its concentration, indicating that its action is less bactericidal. Also it should be noted that at a concentration range $i_{red} < 0.5$, a slightly bactericidal contribution, though minor, is involved to a maximum extent of about 30 %. In total it may be concluded that butylparaben is of a strong bacteriostatic nature, being qualitatively consistent with the *SCI* value of 1.02 (**Table 1**) known from the *SCI* plot reported in the preceding paper.[1]

On the other hand, the values of σ for Q15, IDU, Triclosan and DMH are higher, indicating that all of them are of a strongly bactericidal nature. This result again coincides with the *SCI* values reported before (**Table 1**). In contrast to the above two cases, the value of σ for sodium benzoate is near around 0.2 and 0.3, being bacteriostatic but not as much as that of butylparaben. This result again agrees with the value of $SCI = 0.74$ (**Table 1**). Thus all the values of σ calculated for the 6 drugs on the basis of eq. (9) qualitatively agree with the *SCI* values obtained simply by comparing the specific growth activity with the specific growth retardation in the form of "*SCI* plot".[1]

However, the present finding shown in **Fig.6** provides more important aspect about the drug action. While with the 4 drugs, butylparaben, sodium benzoate, Q15 and IDU, the values of σ do not vary extensively over the concentration range, those for Triclosan and DMH decrease from 1 to 0.45 (Triclosan) and to 0.63 (DMH) with increasing their concentration. Thus both Triclosan and DMH acts rather bactericidally at low concentration range, but the bacteriostatic nature also gruadually comes out at higher concentration range, amounting to about 50 % at the concentration range near at their MIC_θ.

The action mechanisms of these two drugs are not known in detail and consequently it is not possible to explain this change in bactericidality in molecular terms. However, as discussed earlier, it is reasonable to consider that most antimicrobial drugs bind to more than one binding sites in microbial cells and that this would be especially the case with Triclosan and DMH. In that case, the present result is reasonably explained by the following manner; the drug binds to at least two binding sites and its binding to one of the binding sites having a higher affinity towards the drug causes the lethal effect, while the binding to the other site having less affinity affects to repress the metabolic activity and as a result the presence of the two binding modes with the different affinity leads to the variation of bactericidality with the concentration which is characterized by the bactericidal nature at lower concentration and the increasing bacteriostatic contribution at higher concentration.

From the above consideration we believe that the theory developed here can be employed to characterize

the inhibitory action of various antimicrobial drugs. Furthermore it should be noted that the discussion made above is applicable not only to the antimicrobial actions of chemicals, but also to various culture conditions which affect on the growth behavior of various microbes. Such the conditions are, for example, UV and gamma-ray irradiations, electric pulse, electronic beam and so on. Much information will be obtained when the theory shown here is applied to such the systems including the industrial application for the practical control of the microbes.

Acknowledgment

One of the authors (S. Wirkner) has been financially supported by the Japanese Ministry of Education, Science, Sports and Culture (#981022).

References

1) K. Takahashi, *Netsu Sokutei* **27**, 170-178 (2000).
2) F. Okada, A. Kobayashi, N. Fujiwara, and K. Takahashi, *Biocontrol Sci.* **4**, 35-39 (1999).
3) O.-A Antoce, N. Pomohaci, V. Antoce, H. Fukada, K. Takahashi, H. Kawasaki, N. Amano, and T. Amachi, *Biocontrol Sci.* **1**, 3-10 (1996).
4) K. Takahashi, *J. Antibac. Antifung. Agents* **24**, 313-320 (1996).
5) O.-A Antoce, V. Antoce, K. Takahashi, N. Pomohachi, and I. Namolosanu, *Thermochim. Acta* **297**, 33-42 (1997).
6) O.-A. Antoce, V. Antoce, K. Takahashi, N. Pomohaci, and I. Namolosanu, *Am. J. Enol. Vitic.* **48**, 413-421 (1997).
7) F. Okada, A. Kobayashi, N. Fujiwara, N. Arimoto, and K. Takahashi, *Biocontrol Sci.* **4**, 67-73 (1999).
8) F. Okada, A. Kobayashi, N. Fujiwara, and K. Takahashi, *Biocontrol Sci.* **3**, 79-85 (1998).
9) F. Okada, PhD Dissertation, Osaka Prefecture University, March, 1999.
10) O.-A. Antoce, V. Antoce, N. Mori, S. Yasui, A. Kobayashi, and K. Takahashi, *Netsu Sokutei* **25**, 2-8 (1998)
11) A. Aono, K. Takahashi, N. Mori, H. Shimizu, A. Kobayashi, N. Fujiwara, and F. Okada, *Netsu Sokutei* **26**, 2-8 (1999).
12) O.-A. Antoce, V. Antoce, and K. Takahashi, *Netsu Sokutei* **24**, 206-213 (1997).
13) O.-A. Antoce, N. Mori, H. Shimizu, S. Yasui, and K. Takahashi, *Netsu Sokutei* **27**, 112-117 (2000).

要　旨

先に高橋によって提案された微生物の増殖サーモグラムを用いて抗微生物薬剤作用における静・殺菌性を定量的に把握する理論[1]を基本に，これをさらに発展させて，薬剤が持つ殺菌性の薬剤濃度依存性について考察した。熱測定法で導いた薬剤作用パラメータを用いて殺菌性の薬剤濃度依存性をあらわす式を導き，種々のパラメータの組合わせのもとで殺菌性がどのような濃度依存性を示すかをモデル計算で示した。その結果，増殖の遅れならびに増殖速度から評価した薬剤作用の協同性を表わす2種の指数に違いがある場合に，殺菌性に大きな濃度依存性があることが判った。また，その結果を用い，前報[1]で提案した静・殺菌指数プロットにおいて，薬剤により直線性を示さないものがあることを合理的に説明することができた。

Sandra Wirkner
Lab. of Biophysical Chemistry, Div. of Applied Biochemistry, Graduate School of Agriculture and Biological Sciences, Osaka Prefecture Univ., TEL/FAX. 0722-50-0525, e-mail: sandra@biochem.osakafu-u.ac.j
研究テーマ：微生物増殖活性に対する各種の物理的要因の影響の解析
趣味：テニス

高橋克忠　Katsutada Takahashi
Lab. of Biophysical Chemistry, Graduate School of Agriculture and Biological Sciences, Osaka Prefecture Univ., and Lab. of Non-destructive and Non-Invasive Analysis, Keihanna Veterans Circle, TEL/FAX. 0722-50-0525, e-mail: ktakahas@biochem.osakafu-u.ac.jp
研究テーマ：生化学反応の熱力学，生物細胞の増殖活性計測
趣味：テニス，音楽（声楽）