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論 文

Calorimetric Evaluation of the Antimicrobial Properties of 1,3-butanediol and 1,2-pentanediol on Various Microorganisms

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The inhibitory effect of 1,3-butanediol and 1,2-pentanediol against the growth activity of six microbial species in liquid media was quantitatively studied by using a 24-unit isothermal calorimeter. The addition of increasing amounts of any of the two alcohols into the growth medium produced distinct changes in the heat evolution curves observed with the calorimeter. These changes were analyzed by means of two parameters derived from the calorimetric curves: the value of the growth rate constant m and the retardation time t_α (the time required for the cultures to reach a certain level of heat production). Analysis of the observed values of the growth rate constant μ led to the determination of the concentration of alcohol which inhibits the growth of the microbes by 50 % (K_μ), as well as the concentration which completely prevents the growth (MIC_μ). Two parameters with similar meanings (K_θ and MIC_θ) were derived by analyzing the values of the retardation time t_α. It was concluded that the two alcohols, although used in cosmetics mainly for their properties as solvents, exert a significant antimicrobial activity. Also, 1,2-pentanediol was more inhibitory (values of MICm between 1.72 and 3.81 % w/v) than 1,3-butanediol (MIC_μ between 4.34 and 13.04 % w/v, depending on the microbial species).

Key words; 1,3-butanediol; 1,2-pentanediol; antimicrobial activity; calorimetric determination

1. Introduction

Besides the use of special ingredients with specific activities, the formulation of many kinds of cosmetics depends greatly on the use of basic solvents among which 1,3-butanediol (1,3-BD) occupies an important place. Recently, 1,2-pentanediol (1,2PD) has also become more and more used as a solvent in cosmetics. Although employed mainly for their properties as solvents, these two alcohols are of course expected to display certain antimicrobial properties in relationship with various microorganisms. In this context, for a better understanding of the role which these solvents can play both in preventing the spoilage of cosmetic products and in

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contributing to the general antiseptic action of some cosmetics, it has become of interest to quantitatively characterize their antimicrobial properties. In the present work an attempt was made to apply calorimetry to the study of the antimicrobial activity of 1,3-BD and 1,2-PD against 6 microorganisms. The results thus obtained and considerations related to the procedure itself are also presented.

2. Materials and Methods

The following microorganisms were used: *Staphylococcus aureus* 209P and MRSA OJ 51 (both from US Food and Drug Administration, Washington DC, USA); *Pseudomonas aeruginosa* (isolated from cosmetics at Kankohsha Co., Ltd.); *Candida albicans* IID 867 (obtained from the Laboratory of Culture Collections, Institute of Medical Science, Tokyo University); *Escherichia coli* IFO 3972 and *Aspergillus niger* (stock cultures of Mandom Co. Ltd, Central Research Laboratories, Osaka, Japan).

The sample of 1,3-butanediol was a reagent grade chemical obtained from Wako Pure Chemical Industries Inc., Japan. The sample of 1,2-pentanediol was produced by Dragoco Co., Ltd, Japan.

The bacteria were cultivated at 37 °C in brain heart infusion broth (BHIB) obtained from Nissui Seiyaku, Tokyo, Japan. *C. albicans* and *A. niger* were grown at 30 °C on glucose-peptone broth (GPB) with 20 g l^{-1} glucose, provided by Wako Pure Chemical Industries Inc., Japan. An inoculum was prepared by preincubating the microorganisms, on the same kind of medium used in the experiment, for 12-24 hours. The culture thus obtained was then properly diluted with distilled water so that the microbial count was approximately 10^6 cells ml^{-1}. In the case of *A. niger*, spores of the mature culture of the fungus were added to a flask containing sterile water and mixed thoroughly to make the inoculum as uniform as possible. The microbial concentration was checked using McFarland's method for turbidimetric approximation of microbial numbers (in the case of bacteria), by counting under microscope with a Thoma chamber (in the case of *C. albicans*) and with agar plates (for *A. niger*). In ordinary glass vials (30 ml volume) 5 or 10 ml of the growth medium were introduced and then autoclaved at 121 °C for 15 min. After cooling, the required amounts of 1,3-BD or 1,2-PD were pipetted in each vial. Finally, 1 ml (for yeast and fungus) or 0.1 ml (for bacteria) of the inoculum prepared as described above was added to each vial, and sets of 24 vials were introduced in the calorimeter.

The observation of growth was done by using a multiplex batch calorimeter capable of simultaneously monitoring 24 sample cultures. The calorimeter detects the heat evolved during the growth of the microorganisms and records its evolution in time under the form of a voltage signal. The recorded signal can be analyzed for the determination of the growth rate constant μ and another parameter called "the retardation time t_α", which in turn allow the determination of quantitative inhibitory parameters. A detailed presentation of the apparatus was previously made elsewhere.[1] At least two experiments were performed for each pair microorganism-alcohol, which thus provided at least 48 data points for the data analysis.

3. Results

Figure 1 shows some representative examples of the curves recorded as the calorimetric signal during the incubation in the calorimeter of the microbial cultures. The recorded signal can be considered, in a first approximation, to be proportional to the power evolved in each calorimetric unit during the microbial growth. In **Fig. 1** it can be seen, as a common characteristic, that increasing concentrations of 1,3-BD or 1,2-PD led to calorimetric signals with less steep initial portions and lower peaks. The growth thermograms recorded in the presence of inhibitors also required longer incubation times in order to reach the peak. These changes appeared to be related to the inhibitory effect of the 1,3-BD or 1,2-PD added to the cultures, and it was expected that their analysis could provide quantitative data to characterize this inhibitory effect.

Since a more detailed presentation of the data analysis procedures was already given in previous papers,[2-6] it will be presented here only briefly. From the growth thermograms (also named $g(t)$ curves) recorded as the calorimetric output during the microbial growth (**Fig. 1**), the so-called "actual heat evolution curves, $f(t)$" are computed using the relation:[2-4]

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

in which K is the heat conduction constant of the

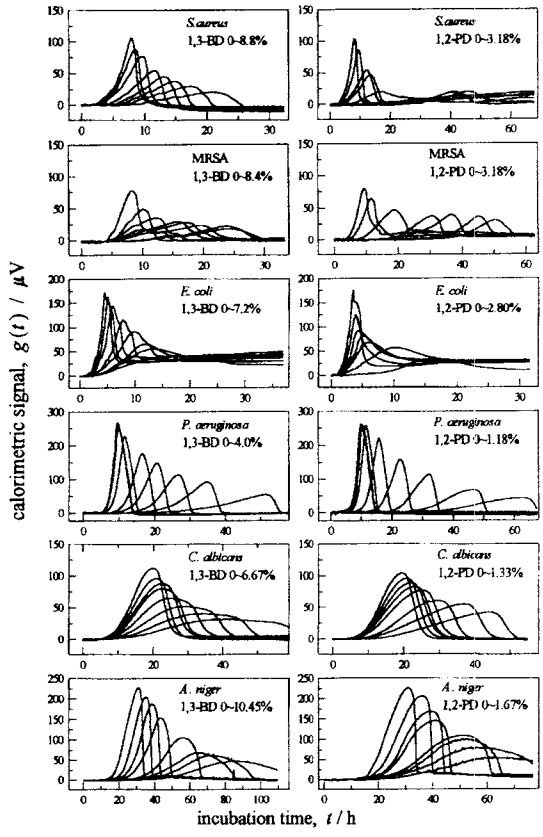

Fig.1 Representative examples of the growth thermograms recorded as the calorimetric output during the incubation of microbial cultures with addition of 1,3-BD or 1,2-PD in various concentrations.

calorimeter, and was determined in advance using the procedure presented elsewhere.[1]

The $f(t)$ curve resulted from the application of equation (1) has been shown to be well correlated with the curve of the microbial cell number during growth.[2-4, 6-10] It is therefore possible to calculate the growth rate constant μ by fitting the initial portion of the $f(t)$ curve with a simple exponential function. As shown in detail elsewhere,[2-4] another parameter, "the retardation time t_α", can be computed from the calorimetric curves. It represents the incubation time required for the $f'(t)$ curve (the time derivative of the $f(t)$ curve) to reach a certain level a selected in its initial exponential portion. If the values of the growth rate and retardation time observed in the absence of inhibitor are named, respectively, μ_m and $t_\alpha(0)$, and the corresponding values observed in the presence of inhibitor at concentration i are named μ_i and $t_\alpha(i)$, then we can calculate two specific parameters which describe the effect of various concentrations of inhibitors against the microbial growth. These are the parameter μ_i/μ_m or "the specific growth activity of the microorganism in the presence of the inhibitor at concentration i", and $t_\alpha(0)/t_\alpha(i)$ or "the specific growth retardation in the presence of the inhibitor at concentration i".

Figure 2 shows some representative examples of μ_i/μ_m and $t_\alpha(0)/t_\alpha(i)$ data, plotted against the concentration of 1,3-BD or 1,2-PD which was added as an inhibitor to the cultures. For better visibility, since all the data points obtained were situated in a limited range of concentrations (as indicated by the final results given in **Table 1** and **2**), only distinct examples were shown in **Fig.2**. The solid lines in **Fig.2**(a), also named "drug potency curves", are described by the previously reported equation:[2-4]

$$\mu_i/\mu_m = 1/\{1+(i/K_\mu)^{m\mu}\} \qquad (2)$$

where K_μ represents the concentration of inhibitor which reduces by 50 % the growth activity of microorganisms and μ_m is a numerical parameter related to the cooperativity in the action of the inhibitor. By fitting the μ_i/μ_m data points on the basis of equation (2) it is possible to calculate the values of K_μ and μ_m. Parameter μ_m is directly related to the steepness of the drug potency curve: the larger the value of μ_m, the steeper is the descent of the solid lines in **Fig.2**(a). Therefore, a larger value of μ_m indicates that a narrower range of concentrations is needed to reduce the specific growth activity μ_i/μ_m from 1 to 0.

A similar situation is shown in **Fig.2**(b), where the solid lines, also named "the drug potency curves drawn for the specific growth retardation $t_\alpha(0)/t_\alpha(i)$", were calculated on the basis of a similar equation:[2-4]

$$t_\alpha(0)/t_\alpha(i) = 1/\{1+(i/K_\theta)^{m\theta}\} \qquad (3)$$

In this case, K_θ signifies the concentration of inhibitor which doubles the time required for the microbial cultures to reach a certain level, and μ_θ is a parameter related to the cooperativity in the action of the inhibitor and has the same characteristics as the mm parameter discussed above.

The dotted lines in **Fig.2**(a) and (b), also named

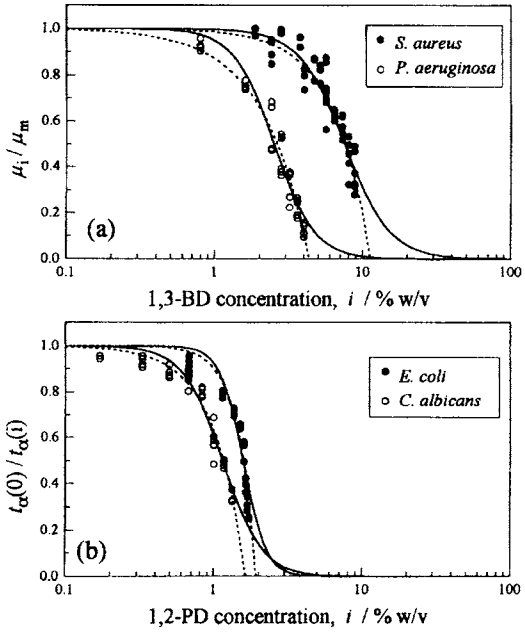

Fig. 2 Representative plots of μ_i/μ_m versus concentration (a) and $t_\alpha(0)/t_\alpha(i)$ versus concentration (b) which are used for the determination of inhibition parameters. The solid lines in (a), also called "drug potency curves calculated on the basis of mi/mm" , were drawn as the best fitted curves on the basis of equation (2). The solid lines in (b), which are the drug potency curves calculated on the basis of $t_\alpha(0)/t_\alpha(i)$, were fitted to the experimental data on the basis of equation (3). If an imaginary horizontal line is drawn at $\mu_i/\mu_m = 0.5$ or $t_\alpha(0)/t_\alpha(i) = 0.5$, it intersects the drug potency curves in a point which corresponds, on the horizontal axis, to the 50% inhibitory concentration K_μ or K_θ, respectively. The dotted lines in plots (a) and (b) are "MIC curves" fitted to the experimental data on the basis of equations (4) and (5), respectively. Their point of intersection with the horizontal axis provides the concentration which totally inhibits growth (MIC_μ and MIC_θ, respectively) .

"MIC curves", were drawn, respectively, on the basis of the previously reported equations (Antoce *et al.*, 1996a):

$$1 - \mu_i/\mu_m = k_1 i^{m1} \quad (4)$$

$$1 - t_\alpha(0)/t_\alpha(i) = k_2 i^{m2} \quad (5)$$

which were derived assuming that the loss in growth activity ($1 - \mu_i/\mu_m$ or $1 - t_\alpha(0)/t_\alpha(i)$) is proportional to, respectively, the m_1-th and m_2-th power of the concentration i of inhibitor added to the cultures. From these equations, two new parameters can be calculated:

$$MIC_\mu = (1/k_1)^{1/m1} \quad (6)$$

$$MIC_\theta = (1/k_2)^{1/m2} \quad (7)$$

Parameter MIC_μ represents the concentrations of inhibitor which completely prevents the growth of the microorganism; in other words, it is the concentration which makes $\mu_i = 0$ and thus $\mu_i/\mu_m = 0$). Parameter MIC_θ is the concentration of inhibitor which prevents the microbial culture from reaching a given level α; in other words, it is the concentration which makes $t_\alpha(i)$ infinite and thus $t_\alpha(0)/t_\alpha(i) = 0$. Both MIC_μ and MIC_θ have, therefore, the meaning of "minimum inhibitory concentration".

The equations 2-7 presented above were used for fitting the experimental data μ_i/μ_m and $t_\alpha(0)/t_\alpha(i)$ obtained from the growth thermograms recorded during the growth of the 6 studied microbes in the presence of various concentrations of 1,3-BD or 1,2-PD. The quantitative parameters obtained, which describe the inhibitory effect of 1,3-BD or 1,2-PD against the growth of these microbial strains, are given in **Tables 1** and **2.**

4. Discussion

As seen in **Fig.1**, the growth thermograms recorded in the presence of increasing concentrations of 1,3-BD were almost identical, in their general appearance, to those recorded in the presence of 1,2-PD, for all the 6 microorganisms studied. In contrast, growth thermograms recorded for different microorganisms were distinctively different in pattern, in agreement with previous results which suggested the possibility of using calorimetry for the identification of microbial strains.[11]

The scattering of the μ_i/μ_m and $t_\alpha(0)/t_\alpha(i)$ data in **Fig. 2** and the size of the standard error of the parameters given in **Tables 1** and **2** indicate that the reproducibility of the growth thermograms recorded varied from one strain to another, but could be considered acceptable for most experiments.

Apparent contradictions can be seen in **Tables 1**

Table 1 Parameters determined calorimetrically for the characterization of the inhibitory effect of 1,3-butanediol (1,3-BD) added to the microbial cultures. Bacteria were grown at 37 °C in BHIB; yeast and fungus were grown at 30 °C in GPB. The precision is given as the standard error.

Micro-organism	μ_m	K_μ % w/v	MIC_μ % w/v	μ_θ	K_θ % w/v	MIC_θ % w/v
S. aureus	2.81 ±0.24	7.49 ±0.16	11.27 ±0.38	2.84 ±0.15	7.15 ±0.10	10.94 ±0.17
MRSA	1.71 ±0.20	5.67 ±0.22	12.84 ±1.08	2.82 ±0.31	6.85 ±0.16	10.80 ±0.48
E. coli	7.73 ±1.82	6.43 ±0.13	7.74 ±0.26	2.43 ±0.22	6.28 ±0.14	9.95 ±0.45
P. aeruginosa	3.18 ±0.25	2.50 ±0.05	4.34 ±0.08	3.71 ±0.18	2.94 ±0.03	4.52 ±0.07
A. niger	2.87 ±0.20	8.23 ±0.14	13.04 ±0.47	2.16 ±0.09	7.47 ±0.10	13.70 ±0.22
C. albicans	2.55 ±0.16	6.22 ±0.12	9.24 ±0.27	3.11 ±0.12	5.91 ±0.06	8.36 ±0.09

Table 2 Parameters determined calorimetrically for the characterization of the inhibitory effect of 1,2-pentanediol (1,2-PD) added to the microbial cultures. Bacteria were grown at 37 °C in BHIB; yeast and fungus were grown at 30 °C in GPB. The precision is given as the standard error.

Micro-organism	μ_m	K_μ % w/v	MIC_μ % w/v	μ_θ	K_θ % w/v	MIC_θ % w/v
S. aureus	2.81 ±0.45	1.86 ±0.10	3.81 ±0.26	5.25 ±0.41	2.10 ±0.03	3.49 ±0.10
MRSA	2.27 ±0.22	1.58 ±0.06	3.54 ±0.22	3.48 ±0.27	1.66 ±0.04	2.97 ±0.07
E. coli	12.47 ±1.84	1.53 ±0.02	1.78 ±0.03	5.57 ±0.62	1.57 ±0.02	1.92 ±0.03
P. aeruginosa	3.95 ±0.23	0.76 ±0.01	1.23 ±0.01	3.98 ±0.17	0.84 ±0.01	1.28 ±0.01
A. niger	2.53 ±0.31	1.52 ±0.05	2.32 ±0.14	2.21 ±0.17	1.52 ±0.03	2.42 ±0.09
C. albicans	2.71 ±0.19	1.15 ±0.02	1.72 ±0.05	3.25 ±0.21	1.14 ±0.02	1.63 ±0.04

and **2** when both the 50% inhibitory concentration (K_μ or K_θ) and the MIC (MIC_μ or MIC_θ) are considered. For example, MRSA is less resistant to the action of 1,3-BD than *E. coli* from the viewpoint of K_μ (5.67 and 6.43 %, respectively). However, from the viewpoint of MIC_μ, MRSA (12.84 %) is much more resistant than *E. coli* (7.74 %). The reason for this is that the drug potency curves which were obtained for the two situations mentioned above have very different steepness, as can be understood from the values of μ_m. The drug potency of 1,2-BD is much steeper in the case of E. coli (μ_m = 7.73) than for MRSA (μ_m = 1.71). For any inhibitor of microbial growth there is only a certain range of concentrations for which the inhibitory action can be measured; if the inhibitor is present in extremely low concentrations then there is no inhibitory effect observed, and at concentrations beyond MIC the effect is already maximum. This observation allows the definition of a so-called "range of effective concentrations". It can be stated then, in our case, that the inhibitory action of 1,3-BD is characterized by a range of effective concentrations which is much narrow for *E. coli* than for MRSA. Similar situations can be found regarding the parameters determined on the basis of $t_\alpha(0)/t_\alpha(i)$; an example is shown in **Fig. 2**(b), where the drug potency curve (solid line) determined for *E. coli* is much steeper (m_θ = 5.57) than the one obtained for C. albicans (m_θ = 3.25, **Table 2**).

It is therefore clear that any of the parameters μ_m, K_μ, or MIC_μ taken alone does not completely describe the effect of the inhibitor over its entire range of effective concentrations. While the value of K_μ "fixes" the midpoint of the drug potency curve (the solid lines in **Fig.2**), the value of μ_m characterizes the "steepness"

of the drug potency curve and the "relative width" of the range of effective concentrations. Here it is necessary to stress the "relative" aspect, because simply knowing the m_μ or m_θ value cannot indicate that a range of effective concentrations is wide or narrow. The values of m_μ and m_θ are meaningful only for the purpose of comparison; for example, when comparing the effects of a drug on two different microorganisms. Finally, it should be noted that the MIC curve is also necessary, because the drug potency curve is not suitable for the determination of a MIC-type parameter.

As shown above, two kinds of parameters were determined for each experiment: one on the basis of the specific growth activity μ_i/μ_m and another from the specific growth retardation $t_\alpha(0)/t_\alpha(i)$. This fact can appear redundant, and also may create difficulties in interpretation, when the difference between the two kinds of parameters is significant. Usually we recommend taking into consideration, for practical purposes, the values of K_μ and MIC_μ . Depending on purpose, it is also possible to consider the highest value of the two similar parameters. For example, in the cases when K_θ is larger than K_μ , it is K_θ which can be thought to describe better the 50 % inhibitory concentration. In practice, there are also situations when K_μ and MIC_μ cannot be determined. This occurs when the inhibitory activity of the drug is mainly bactericidal, which means that while the number of viable microorganisms at the beginning of the experiment is affected proportionally to the amount of inhibitor added, the later growth of the culture proceeds approximately with the same rate and thus μ_i/μ_m remains relatively constant. The drug potency curves obtained in such cases have a specific pattern, characterized by a parallel shift towards longer incubation times, but with similar steepness in their initial portion. In such situations the values K_θ and MIC_θ, still possible to obtain, remain as the only alternative to quantitatively characterize the inhibitory effect of the added drug.

Analysis of **Fig. 2** and **Table 1** and **2** allows the conclusion that 1,2-PD was more inhibitory than 1,3-BD for all the microorganisms tested. This is in agreement with previously published results[12,13] which show that the inhibitory efficacy of alcohols increases with their chain length. The chain length of the alcohols is directly related to their hydrophobicity and thus to their capacity of modifying the properties of the cellular membranes. *P. aeruginosa* was the least resistant to both alcohols tested as inhibitors, and this was true from the viewpoint of both the 50% inhibitory concentration (K_μ, K_θ) and the minimum inhibitory concentration (MIC_μ, MIC_θ). Both *S. aureus* and the strain of MRSA also showed relatively high resistance to the action of both alcohols tested. *A. niger* showed remarkable tolerance to 1,3-BD, while being less resistant than other species to the action of 1,2-PD.

Precise knowledge on the inhibitory action of 1,3-BD and 1,2-PD, which are mainly employed for their qualities as solvents, may prove useful for the design of smaller proportions of other antimicrobials and preservatives which are currently used in cosmetics. Future work is intended on the application of the same procedure for the study of synergistic and antagonistic interactions which may occur between solvents such as 1,3-BD and 1,2-PD and other special ingredients which are commonly used in cosmetics.

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要　旨

熱測定法により，1,3-ブタンジオールおよび1,2-ペンタンジオールの種々の微生物の増殖活性に対する影響を定量的に解析した。試験に用いた微生物は，*E. coli*, *S. aureus*, *MRSA*, *P. aeruginosa*, *C. albicans*, *A. niger*の6種であった。上記の薬剤を種々の濃度で含む培地を調製し，その中での6種の微生物の増殖サーモグラムを観測して，それぞれの条件における増殖速度パラメータを既報の方法で求めた。さらにその薬剤濃度依存性を解析して50％増殖活性抑制濃度，100％増殖活性抑制濃度を数値解析により決定した。いずれのパラメータも微生物種により差があるものの，すべての微生物種に対する作用は，1,3-ブタンジオールに比べて1,2-ペンタンジオールの方が強く，これらの薬剤の抗微生物作用は主としてアルキル基の疎水性の寄与によるものであると推測した。

【国際会議のお知らせ】

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