[Chem. Pharm. Bull. 24(2) 224—229 (1976)]

UDC 547.581.2'26.09 : 615.282.015.11 : 615.076.7

Selective Inhibition of the Germination of *Bacillus megaterium* Spores by Alkyl *p*-Hydroxybenzoates

Kenji Watanabe and Shigeshi Takesue

Faculty of Pharmaceutical Sciences, Fukuoka University[1]

(Received May 21, 1975)

The germination of *Bacillus megaterium* spores both in nutrient broth and in a germinant (L-alanine plus inosine in phosphate buffer) was inhibited by alkyl *p*-hydroxybenzoates at lower concentrations than those required for inhibiting the outgrowth of germinated spores. The inhibition occurred even when the drugs were added to the spore suspensions where germination had already begun. The degree of inhibition was proportional to the logarithm of alkyl *p*-hydroxybenzoate concentration present in the germination medium. The concentrations required to completely inhibit germination were 4.5 mM for methyl, 1.6 mM for ethyl, 0.52 mM for propyl, 0.80 mM for isopropyl, 0.22 mM for butyl and 0.22 mM for isobutyl *p*-hydroxybenzoate. respectively. Free *p*-hydroxybenzoic acid had no inhibitory activity. There was a linear relationship between the logarithm of germination inhibiting concentration and the number of carbon atoms in the alkyl chain. The inhibition of spore germination by alkyl *p*-hydroxybenzoates was reversible, since removal of the drugs from spore environment allowed germination to proceed. The possible mechanism of the inhibitory effect of alkyl *p*-hydroxybenzoates on spore germination is discussed.

Introduction

Alkyl *p*-hydroxybenzoates possess an antiseptic action on certain bacteria and fungi, and have been widely employed for preventing foods, drugs and cosmetics from spoilage. The detailed mechanism of their antimicrobial action, however, is not known. During the course of studies on the mechanism of germination of bacterial spores, we noticed that low concentrations of alkyl *p*-hydroxybenzoates inhibited the germination of *Bacillus megaterium* spores without affecting the outgrowth of germinated spores. Studies on this inhibition were thought to contribute to a better understanding of not only the nature of alkyl *p*-hydroxybenzoates as antiseptics but also the mechanism by which the process of spore germination is initiated by the germinants such as L-alanine. In this paper the inhibitory effect of alkyl *p*-hydroxybenzoates on the spore germination is described.

Experimental

Bacterial Spores——Spores of *Bacillus megaterium* ATCC 19213 were produced, harvested and cleaned by the procedure of Rode and Foster.[2] The cleaned spores, confirmed by phase contrast microscopy, were stored at 4° as a concentrated water suspension or as a lyophilized preparation. The concentration of viable spores was determined by serially diluting a given spore suspension and plating on nutrient broth supplemented with 1% agar.

Medium and Chemicals——The nutrient broth used was composed of 1% polypeptone, 1% meat extract and 0.3% NaCl, pH 7.2. Isopropyl and isobutyl ester of *p*-hydroxybenzoic acid, an analytical grade of Ueno-Seiyaku Co, were supplied by Dr. A. Murata. The other alkyl (methyl, ethyl, propyl and butyl) esters were obtained from Wako-Junyaku Co. Stock solutions of alkyl *p*-hydroxybenzoates were usually made of sterilized water.

Measurement of Germination——Germination of bacterial spores is characterized by the numerous changes such as loss of heat resistance, exudation of some cortex materials, onset of stainability, darkening

1) Location: *11, Nanakuma, Nishi-ku, Fukuoka, 814, Japan.*
2) L.J. Rode and J.W. Foster, *Proc. Natl. Acad. Sci. U. S.*, **46**, 118 (1960).

NII-Electronic Library Service

under a phase contrast microscope and decrease in optical density of spore suspensions.[3-5] In this paper, we used decrease in optical density of spore suspensions as the criterion of germination. In a standard procedure, the germination mixture of total 5 ml consisted of heat-activated spores (60° for 60 min in water) at a concentration of about 10^8 cells/ml and the germination initiator; 0.1 mM L-alanine plus 0.1 mM inosine in 3.3 mM phosphate buffer ($Na_2HPO_4+KH_2PO_4$), pH 7.5, which was abbreviated as 0.1 mM AIP in this paper. The mixture was incubated at 37° and at intervals the optical density of a spore suspension was measured in a Hitachi EPO-B photoelectric colorimeter fitted with a red filter. As a parameter of the kinetics of spore germination, we used the "extent of germination." It was calculated as percent decrease in optical density from the initial reading (OD_0) to the one (OD_t) at any time (t) during the incubation period by the formula, $(1-OD_t/OD_0)\times 100$. The percent inhibition of germination was then calculated as follows: Inhibition $(\%)=(1-I/C)\times 100$, where I is the percent decrease in optical density of the system with inhibitor and C that of control system. Phase contrast microscopy was also used to confirm germination; ungerminated spores were refractile and germinated ones darkened. When dormant spores were completely transformed to phase-darkened ones, the spore suspensions usually represented about 70% decrease in optical density.

Measurement of the Minimum Inhibitory Concentrations of the Multiplication of Vegetative Cells——One tenth ml of overnight cultures were added to 10 ml of nutrient broth containing various concentrations of alkyl *p*-hydroxybenzoates, incubated in a Monod shaker-bath at 37° for about 20 hr, and the subsequent growth of cells was followed by observing the turbidity of cultures. In this case, since all alkyl *p*-hydroxybenzoates were slightly soluble in water, they were dissolved in 50% ethanol to obtain a high concentrations solution, and 0.2 ml of their ethanol solution was diluted 50-fold with the assay medium. Although the germination of *Bacillus megaterium* spores was somewhat inhibited by 1% ethanol, the vegetative growth was not affected by the same ethanol concentrations.

Results

Effect of Concentrations of Alkyl *p*-Hydroxybenzoates on Spore Germination

In order to test the possible inhibitory effect of alkyl *p*-hydroxybenzoates on the germination of *Bacillus megaterium* spores, the heat-activated spores were incubated with 0.1 mM AIP in the presence of various concentrations of butyl *p*-hydroxybenzoate and the optical density of spore suspensions was periodically measured. In a control without butyl *p*-hydroxybenzoate, the optical density of spore suspensions was decreased with time, and the decrease in optical density upto 70%, namely the completion of spore germination, was displayed within almost 15 min. On the other hand, when the drug was added, the spore germination was inhibited and the degree of inhibition at 15 min was proportional to the logarithm of the butyl *p*-hydroxybenzoate concentration. The spores inhibited completely from germination remained phase-bright. The spore suspensions containing various concentrations of butyl *p*-hydroxybenzoate were then serially diluted and plated on nutrient agar. The colony counts obtained were almost the same regardless of the concentration of butyl *p*-hydroxybenzoate to which the spores had been previously exposed. This result shows that butyl *p*-hydroxybenzoate has no sporocidal effect. Similar results were obtained for all the alkyl *p*-hydroxybenzoates studied; methyl, ethyl, propyl, isopropyl and isobutyl ester. However, the degree of germination inhibition by them at a fixed concentration was different. Fig. 1 shows the dependence of the inhibition of spore germination on the kind and concentration of alkyl *p*-hydroxybenzoates. From these data it was possible to obtain an extrapolated value for the minimum concentration of a given alkyl *p*-hydroxybenzoate required to completely inhibit germination. The calculated concentration was about 4.5 mM for methyl, 1.6 mM for ethyl, 0.52 mM for propyl, 0.80 mM for isopropyl, 0.22 mM for butyl and 0.22 mM for isobutyl *p*-hydroxybenzoate, respectively. Free *p*-hydroxybenzoic acid, not esterified, had no inhibitory effect on spore germination even at a concentration of 100 mM.

3) Y. Hachisuka, "Gahoh," Iwanami-Shoten, Tokyo, 1962, p. 88.
4) N.G. McCormick, *J. Bact.*, **89**, 1180 (1965).
5) G.W. Gould and A. Hurst, "The Bacterial Spores," Academic Press, London and New York, 1969, p. 397.

NII-Electronic Library Service

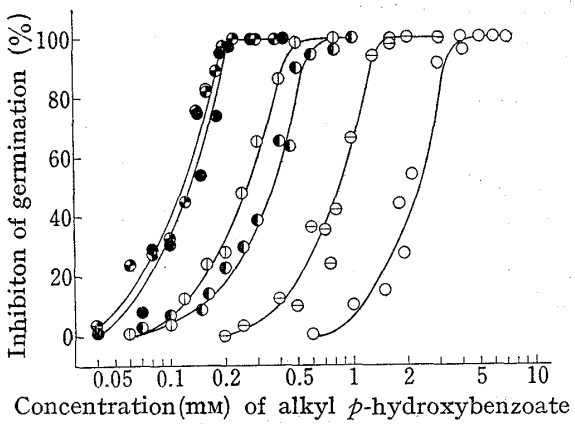

Fig. 1 Inhibition of Spore Germination by Various Kinds and Concentrations of Alkyl *p*-Hydroxybenzoates

Heat-activated spores (OD_0=0.35) were incubated at 37° in 0.1 mM AIP with butyl *p*-hydroxybenzoate of the concentrations shown in the figure. Extent of germination is that at 15 min.
alkyl group: —○—: methyl, —⊖—: ethyl, —◑—: propyl, —◐—: isopropyl, —●—: butyl, —◔—: isobutyl

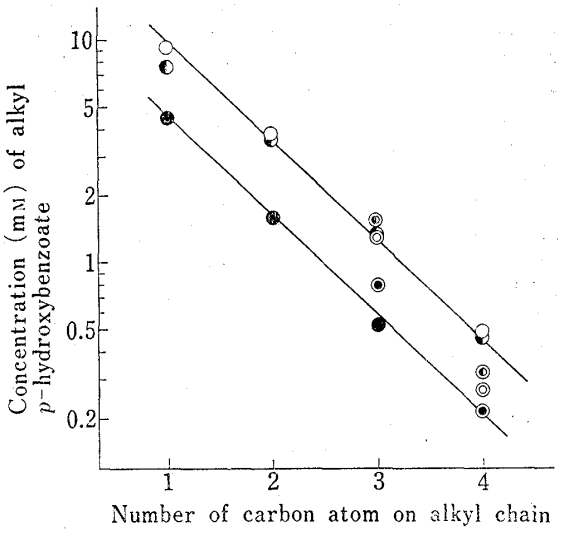

Fig. 2. Relationship between the Minimum Concentrations to Inhibit the Spore Germination, the Outgrowth of Germinated Spores and the Multiplication of Vegetative Cells and the Number of Carbon Atom in the Alkyl Chain of Alkyl *p*-Hydroxybenzoate.

symbols: —●—: germination, —◐—: outgrowth, —○—: cell multiplication
Double circles represent the respective isomer.

Effect of Alkyl *p*-Hydroxybenzoates on Post-germinative Development

The next feature was whether alkyl *p*-hydroxybenzoates at the concentrations for inhibiting germination would affect the process of outgrowth of germinated spores. When heat-activated spores were incubated with 0.1 mM AIP, rapid and complete germination occurred and almost all the spores had become phase-dark, but the germinated spores did not outgrow. Germinated spores thus obtained were collected, resuspended at the optical density of about 0.1 in nutrient broth of pH 7.2 with various concentrations of butyl *p*-hydroxybenzoate and incubated at 37° under shaking in a Monod bath. At intervals the change in optical density associated with outgrowth and subsequent division of vegetative cells was measured. At the completely germination-inhibiting concentration, butyl *p*-hydroxybenzoate inhibited partly the process of outgrowth. The presence of the concentration of 0.47 mM was needed to inhibit completely the outgrowth of germinated spores. The minimum concentrations of the other alkyl *p*-hydroxybenzoates for inhibiting outgrowth were all higher than those for inhibiting germination. It was 7.5 mM for methyl, 3.6 mM for ethyl, 1.4 mM for propyl, 1.6 mM for isopropyl and 0.32 mM for isobutyl *p*-hydroxybenzoate. These results indicate that the nature of germination and outgrowth are essentially different from each other.

The spore germination took place in this nutrient broth as in 0.1 mM AIP, and again the nutrient broth-induced germination was inhibited by these alkyl *p*-hydroxybenzoates at the concentrations inhibiting the AIP-induced germination. This result shows that there is no antagonising substances against alkyl *p*-hydroxybenzoates in the nutrient broth used and that alkyl *p*-hydroxybenzoates do inhibit spore germination at lower concentrations than those required to inhibit the outgrowth of germinated spores.

Relationship between the Germination Inhibiting Effect and the Alkyl Chain Length of Alkyl *p*-Hydroxybenzoates

Fig. 2 illustrates the relationship between the germination inhibiting concentrations of alkyl *p*-hydroxybenzoates in logarithmic scale and the number of carbon atom in the alkyl

NII-Electronic Library Service

chain. The logarithm of the concentrations decreased linearly with the increase in the carbon number of the alkyl chain, showing that the number of carbon atom in the alkyl chain possesses a relation to their toxic effects on spore germination. It was estimated from Fig. 2 that the inhibitory activity of alkyl *p*-hydroxybenzoates on germination increased ten times as about 2.3 carbon atoms were added to the alkyl chain. The minimum concentrations of respective alkyl *p*-hydroxybenzoate required for inhibiting both the outgrowth of germinated spores and the multiplication of vegetative cells were higher than the germination inhibiting concentrations as shown in the same figure. However, the slopes of their concentration-carbon number curve were almost the same as that of germination, although the branched isomers deviated a little from their respective theoretical curve.

Effect of the Time of Addition of Alkyl *p*-Hydroxybenzoates on Spore Germination

At various times after the initiation of AIP-induced germination, butyl *p*-hydroxybenzoate of final 0.25 mM was added to the spore suspensions. As Fig. 3 shows, the addition of the drug led to an almost immediate inhibition of germination at any time. When the other alkyl *p*-hydroxybenzoates at the concentrations that inhibit completely the spore germination were used instead of butyl *p*-hydroxybenzoate, again such an immediate inhibition occurred. These results explain that all alkyl *p*-hydroxybenzoates penetrate rapidly to the sensitive sites of spore to inhibit the reactions leading to germination.

Reversibility of Alkyl *p*-Hydroxybenzoates Inhibition on Spore Germination

The results of preliminary experiment that all the spores stabilized with alkyl *p*-hydroxybenzoates were viable and, after effective dilution procedures, formed colonies on plating led us to imagine that the inhibition of spore germination by alkyl *p*-hydroxybenzoates might be reversible. Therefore, a study to examine this possibility was undertaken. After incubation of dormant spores in 0.1 mM AIP with butyl *p*-hydroxybenzoate of the germination inhibiting concentration for 30 or 60 min, the stabilized spores were freed from the drug by centrifugation and resuspended in 0.1 mM AIP to continue incubation. As Fig. 4 shows, the resuspended spores germinated promptly at the same rate as in a control in which the spores had not exposed to the drug from the beginning. Spores treated with butyl *p*-hydroxy-

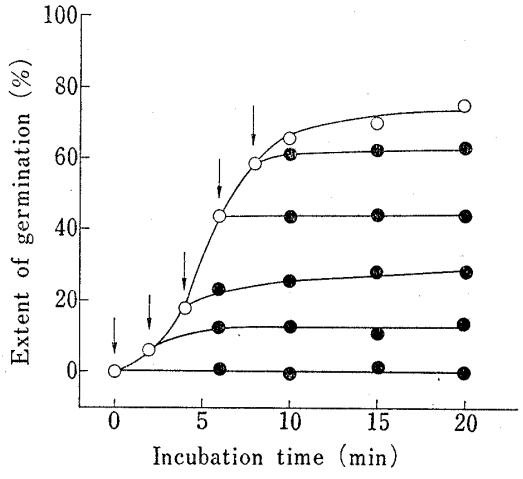

Fig. 3. Effect of the Added Time of Butyl *p*-Hydroxybenzoate on Spore Germination

Heat-activated spores (OD_0=0.33) were incubated at 37° in 0.1 mM AIP, and at the time indicated with arrow mark 0.25 mM butyl *p*-hydroxybenzoate (—●—) was added. Control (—○—) was without the drug.

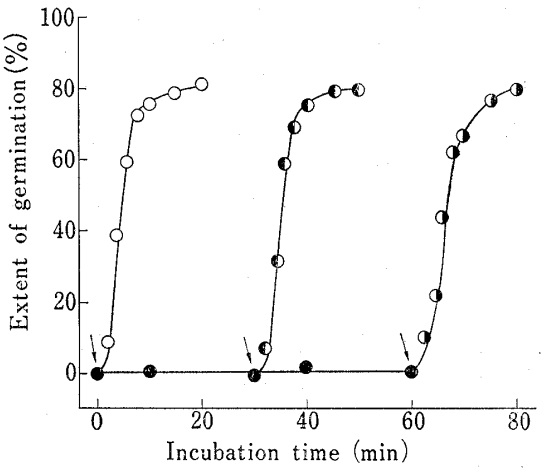

Fig. 4. Reversibility of the Effect of Butyl *p*-Hydroxybenzoate on Spore Germination

Heat-activated spores (OD_0=0.25) were incubated at 37° in 0.1 mM AIP with 0.22 mM butyl *p*-hydroxybenzoate (—●—). After 30 (—◐—) and 60 (—◑—) min incubation, the stabilized spores were collected and resuspended in the drug free 0.1 mM AIP to continue incubation. Control (—○—) was without the drug.

NII-Electronic Library Service

benzoate even for as long as 24 hr and inhibited from germinating by the drug was able to germinate normally when placed in the inhibitor-free medium, indicating clearly the reversibility of butyl *p*-hydroxybenzoate inhibition. Quite the same reversibility was observed when the other alkyl *p*-hydroxybenzoates of the germination inhibiting concentrations were used instead of butyl *p*-hydroxybenzoate. The inhibitory action of alkyl *p*-hydroxybenzoates on spore germination was thus of a reversible nature.

Discussion

The development of bacterial spores into metabolically active vegetative cells is associated with a number of biochemical changes, and is known to occur in three steps. The first includes delicate activation changes by which the dormant spores become able to germinate, and the second, germination, includes specific degradative changes of some peripheral layers of spores. In complete medium, after germination, the spores enter the process of outgrowth which is characterized by the ordered synthesis of new macromolecules such as nucleic acid, protein and cell wall peptidoglycan. The process of outgrowth can, therefore, be inhibited by many common inhibitors used in biochemistry. On the other hand, specific inhibitors which act only on germination (degradative process) at low concentrations (physiological levels) are relatively few.[6-8] Parker[9] examined the effects of preservatives on both germination and outgrowth of *Bacillus subtilis* spores and found that the preservatives were fallen into two categories; inhibitors of germination and those of outgrowth. He showed that parabens, a commercial preservative agent containing methyl and propyl ester of *p*-hydroxybenzoic acid, belonged to the first category. However, the detailed studies on the inhibitory effect of parabens were not done. We have shown that homologous series of alkyl *p*-hydroxybenzoates is a strong but reversible inhibitor of the AIP-induced germination of *Bacillus megaterium* spores. The minimum inhibitory concentrations of these drugs against the outgrowth of germinated spores were all higher than those required for inhibiting germination. It is difficult at the present time to make clear whether or not their mode of inhibitory action on the germinative stage differs fundamentally from that on the post-germinative development. One of the approaches to gain an insight into their biological activities may be obtained from basic physicochemical consideration. Hirai[10] showed that the antimicrobial activities of alkyl *p*-hydroxybenzoates on *Zygosaccharomyces japonicus*, *Staphylococcus aureus* and *Bacillus subtilis* paralleled with the length of alkyl chain, and suggested that their biological activities could be explained to a certain degree by their characteristics such as lowering of surface tension, solubility in water and equilibrium between their "organic" and "inorganic" properties. The existence of a linear relationship between the logarithm of the minimum inhibitory concentrations of the multiplication of vegetative cells and the alkyl chain length has been proved by Oka[11] and Shibasaki[12] using several kinds of yeasts, molds and bacteria. We have obtained the same relationship in the case of spore germination. However, while in their cases the carbon number of alkyl chain needed to increase the activity ten times was from 3 to 4, in our case of the inhibition of spore germination the needed carbon number was smaller, being about 2.3. These findings suggest that hydrophobicity may play an important role more strongly in the case of spores. Generally, the documents on the effects of alkyl *p*-hydroxybenzoates on spore germination appear to have been less accumulated than those on their bacteriostatic

6) V. Vinter, *J. Appl. Bacteriol.*, 33, 50 (1970).
7) G.W. Gould, "Methods in Microbiology 6A," Academic Press, London and New York, 1971, p. 329.
8) G.W. Gould and G.J. Dring, "Spores V," American Society for Microbiology, Washington, D.C., 1972, p. 401.
9) M.S. Parker, *J. Appl. Bacteriol.*, 32, 322 (1969).
10) K. Hirai, *Yakugaku Zasshi*, 77, 1285 (1957).
11) S. Oka, *Bull. Agr. Chem. Soc. Japan*, 24, 412 (1960).
12) I. Shibasaki, *J. Ferment. Technol.*, 47, 167 (1969).

NII-Electronic Library Service

effects. The study on the inhibition of spore germination by alkyl *p*-hydroxybenzoates is thought to be useful not only to investigate the detailed properties of these drugs as antiseptics, but also to understand the mechanism of spore germination. Since their inhibitory action is of reversible nature, alkyl *p*-hydroxybenzoates may be loosely bound to spores. It can be suggested that the hydrophobic regions of alkyl *p*-hydroxybenzoates may be associated both with the binding of the drugs to spores and with their inhibitory action on spore germination. Recently, Murata and Shiroura[13] have found that alkyl *p*-hydroxybenzoates induce the premature lysis of J1 phage-infected cells of *Lactobacillus casei* and suggested that the principle of their actions may simply be a physical one as displayed by some surface active agents. In order to define the specific target of alkyl *p*-hydroxybenzoates on germination, this suggestion is very useful.

Acknowledgements The authors wish to express our appreciation to Dr. A. Murata of Saga University for his generous supply of isopropyl and isobutyl *p*-hydroxybenzoates. We are also grateful to Messrs. T. Tanigawa, M. Umeda, and T. Sakiyama for technical assistance in part of the experiment.

13) A. Murata and Y. Shiroura, *Nippon Nogeikagaku Kaishi*, **47**, 65 (1973).

NII-Electronic Library Service