Mechanism and Factors Influencing the Veronal Inhibition of Bacterial Spore Germination

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The inhibitory effect of sodium 5,5-diethyl barbiturate (Veronal) on the L-alanine-induced initiation of germination of Bacillus subtilis spores was examined. Veronal reversibly inhibited the initiation of germination by a noncompetitive mechanism. The inhibition was time-independent and it took place whether L-alanine was or was not allowed to permeate the spore before the addition of the inhibitor. The concentration of the inhibitor and the pH of the initiation system were important factors determining the effectiveness of Veronal as an inhibitor. The magnitude of the inhibition increased linearly with decreasing pH at constant concentration and with increasing concentration at constant pH. These results suggest that the inhibition involves a permeability phenomenon related to the access of drug to the active sites in the spore and that the entry of Veronal into the spores is regulated by the concentration of undissociated molecule. At the physiologically important pH of 7.4, initiation with alanine in phosphate buffer at high spore densities (about 10^8 spores per ml) was 50% inhibited by 4 mM Veronal, and 8 mM Veronal inhibited initiation completely. L-Alanine initiation in tris(hydroxymethyl)amino-methane-hydrochloride buffer was completely inhibited by 5 mM Veronal. The inhibition could be partially reversed by the combined addition of D-fructose, D-glucose, and K^+. Possible reasons for the failure of otherwise inhibitory concentrations of Veronal to inhibit completely the L-alanine-induced initiation when a combination of fructose, glucose, and K^+ was present and a suggested relationship to two functional roles of L-alanine in the initiation of germination are discussed.

Bacterial spores are the result of a process (sporulation) of intracellular differentiation. The endospore is embryonic in function and contains all of the genetic information for the entire life cycle. Two fundamental steps occur sequentially in spore development: degradation of some pre-existing structures (initiation of germination) and synthesis of new cell components (outgrowth). Both initiation and outgrowth are probably governed by enzymatic reactions. Initiation terminates irreversibly the cryptobiotic state of the spore and results in a viable spore which has lost the typical resistance of the dormant spore. Initiation of germination takes place after the addition of physiological initiators such as simple sugars or amino acids. Their nature suggests that some enzymatic reaction is involved which eventually triggers the degradative series of reactions characteristic of initiation. The mode of action of any agent that produces specific effects on initiation of germination is of interest for both basic and applied research. Selective inhibition of triggering systems designed to examine the mechanism(s) of initiation of germination in spores has been little exploited, although experiments of this kind also have relevance to food microbiology. Inhibitors of initiation can be useful tools to prevent spoilage caused by the development of these typically resistant forms of bacteria.

The first report of the adverse effect of sodium 5,5-diethylbarbiturate (Veronal) on Bacillus subtilis spore development was recently published (10). Veronal does not have a general effect on the development of cultures of this organism. The drug inhibits selectively the phase of initiation of germination in complete medium and the L-alanine-induced initiation under conditions in which it does not inhibit outgrowth or cell division. Encouraged by these findings and by the paucity of inhibitors of initiation, we decided to explore further the effect of Veronal on initiation. The present report extends our observations on the inhibitory effect of Veronal to include some characteristics of the inhibition and discusses the
mode of action of Veronal on the L-alanine-induced initiation of germination of B. subtilis spores.

MATERIALS AND METHODS

Organisms and preparation of spores. Spores of B. subtilis ATCC 6051 were used in most of the experiments. B. subtilis var. niger strain globii (Microbiological Research Establishment, Porton, England) was also used in this study. The organisms were grown in a medium containing Nutrient Agar supplemented with MnSO₄·H₂O and NaCl to essentially complete and synchronous sporulation as previously described (8). After keeping the cultures for 3 days at 4 °C, the spores were harvested and washed extensively with deionized water. The clean spores were stored at 4 °C in concentrated suspensions and used within 1 week.

Assessment of initiation of germination. The initiation was judged by two methods: (i) the decrease in absorbancy of the spore suspensions and (ii) the loss of spore phase brightness under phase-contrast microscopy.

In spite of rigorous standardization of all of the operations during the preparation of spores, it was difficult to obtain different preparations of spores from the same organisms with a complete optical stability. Independently of initiation, an “optical effect” occurred, whereby the absorbancy of a suspension of intact B. subtilis spores in NaH₂PO₄·K₂HPO₄ (phosphate), tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, or borate buffer decreased 0 to 8% in the first 10 to 15 min of incubation at 37 °C. Thereafter, the suspension remained optically stable. An initial limited spontaneous initiation of germination is not indicated, since all of the spores remained phase-bright and the inhibitory effect still prevailed upon addition of L-alanine plus suitable concentrations of Veronal. Optically stable suspensions were prepared by adding small amounts of the surface-active ester polyoxyethylene sorbitan monooleate (Tween 80). The presence of this compound also facilitated the handling of the spore suspensions. Although B. subtilis spore suspensions in deionized water or in buffers have a smooth, diffuse turbidity, they have the tendency to form a film on clean glass surfaces. The presence of Tween 80 overcomes this problem. Tween 80 is not hydrolyzed by B. subtilis spores or its extracts (9); it was experimentally verified in the present study that, at the concentration used (0.26 mM) in the initiation determinations, Tween 80 does not affect either the rates or the extension of the L-alanine-induced initiation (both in L-alanine alone or in L-alanine plus fructose, glucose, and K⁺) or its inhibition by Veronal. Consequently, as a rule, before use in each experiment, the spores were washed, uniformly resuspended, and diluted in 0.8 mM Tween 80-deionized water. The standardization of the spore suspensions and the determination of the spore concentration of the suspensions were accomplished as described previously (8). The percentage of reduction in absorbancy was a reliable indication of the percentage of spores which completed the initiation phase. This was verified in each experiment by phase-contrast illumination, judging from refractile spores and darkened spores. The transformation of a spore suspension (2 × 10⁸ spores per ml of initiation medium) of B. subtilis ATCC 6051 to a completely phase-darkened suspension represented a 68 to 70% reduction in absorbancy. A completely Veronal-inhibited spore suspension is optically stable, and the spores remained fully phase-bright.

For each assay of a duplicated experiment, fresh 1-ml portions of a cold spore suspension, containing approximately 6 × 10⁸ spores, in 30-ml nepheloflasks were supplemented with cold solutions of the desired components in buffer of suitable pH to a total volume of 3.0 ml. Veronal was diluted in water and adjusted to the desired pH with HCl before it was added to the initiation media. The initial absorbancy was measured on the cold system and again after the desired period of incubation in a water bath at 37 °C in the following way:

Each flask was vigorously shaken in a Vortex Jr. mixer, the side tube was wiped dry, and the contents were tipped in the tube before insertion in a Coleman Junior spectrophotometer. The absorbancy was measured at 660 nm with a 1-cm light path. Unless otherwise indicated, the period of incubation was 60 min.

The data are presented as per cent reduction in absorbancy (A) with the initial reading taken as 100%, or as per cent inhibition of initiation computed as follows: per cent inhibition = [(per cent reduction in A of control system) − (per cent reduction in A of system with Veronal)/per cent reduction in A of control system] × 100. pH determinations. The pH values reported here are those of the complete system after all additions and were read with a Beckman Zeromatic II pH meter, the calibration of which was checked before each assay was run. Stirring was continuous, and the pH was read allowing the electrode potential to stabilize.

Chemicals. L-Alanine used in these studies was of a chromatographically homogeneous grade and was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio. Veronal (National Formulary grade) was obtained from Fisher Scientific Co., Fair Lawn, N.J. Tween 80 was purchased from Atlas Powder Co., Canada Ltd., Branford. Other chemicals used were reagent grade from Fisher Scientific Co.

RESULTS

L-Alanine initiation. Spores of the B. subtilis strain used required L-alanine to initiate germination. In addition to the inducer, some Bacillus spores require cations for initiation (5). Suspensions of intact spores (ca. 2 × 10⁸ spores per ml) of B. subtilis [50 mM phosphate, borate, and Tris-hydrochloride buffers (pH 7.4)] plus L-alanine (2 mM) completed the initiation phase and displayed a 65 to 71% reduction in the initial absorbancy at 660 nm within 60 to 120 min of incubation at 37 °C. Alanine alone was sufficient for initiation in Tris-hydrochloride buffer (without
the addition of cations), as is typical for the Marburg strain of *B. subtilis* (11).

Whereas heat activation is absolutely necessary for initiation of some *Bacillus* spores, others can germinate without it. However, in most *Bacillus* spores the rate of initiation is increased. With our spores, initiation was equally rapid in 50 mM phosphate buffer (pH 7.4) with 2 mM L-alanine whether the spores were or were not preheated in deionized water at 80 C for 15 min. Since “heat activation” was not required for maximal initiation rates, spores were not preheated.

**Inhibition of L-alanine initiation by Veronal.** Previous work (10) indicated that the addition of Veronal (10 mM) to a suspension of *B. subtilis* spores in phosphate buffer inhibits the L-alanine initiation. In the present work, experiments were undertaken to delineate conditions and characteristics of the inhibition. To explore the effect of buffers (pH 7.4) on inhibition, spore suspensions (ca. 2 x 10^9 spores per ml) were prepared containing three different concentrations of L-alanine (2 to 200 mM) in these buffers: 50 mM Tris-hydrochloride buffer plus 10 mM Veronal, 50 mM borate buffer plus 10 mM Veronal, and 10 mM Veronal-hydrochloride buffer. The suspensions were incubated at 37 C, and at intervals the absorbancy was measured. In all cases, the presence of Veronal completely prevented a decrease in absorbancy and the spores remained phase-bright during the experimental period (22 hr). Clearly, Veronal blocked the alanine-induced initiation independently of the buffer used. In this study, most of the experiments were carried out by adding Veronal to phosphate buffer. Thus, any significant effect resulting from a major change in the ionic strength of the system when Veronal was added could be excluded.

**Variation of Veronal inhibition with pH.** Information on the site of Veronal interaction with the spore resulting in inhibition of initiation may be obtained by studying the inhibition at different pH values. In the absence of Veronal, variations in pH values between 7.4 and 9.0 had little measurable effect on the L-alanine-induced initiation of *B. subtilis* spores (Fig. 1). However, the influence of pH on the Veronal inhibition was investigated by suspending the spores in a buffer containing L-alanine and Veronal at four pH values between 7.4 and 9.0. The inhibition exerted by Veronal was highly pH-dependent (Fig. 1). In the conditions of the experiment, the initiation was completely inhibited at pH 7.4, and an increase in pH was detrimental to the activity of the inhibitor. At pH 9.0, initiation occurred at almost the same rate as in the controls without Veronal. The fact that an increase in pH affects the dissociation of Veronal (pK_a = 7.4) suggests that this molecular variable may govern the inhibition.

An alternative which must be considered is that a change in pH not only affects the dissociation of Veronal but may affect the receptor site(s) of Veronal in the spore. To investigate this possibility, the variation of the inhibition with the concentration of Veronal was compared at different pH values.

**Variation of the inhibition with Veronal concentration.** The degree of inhibition increased linearly with increasing concentrations at a constant pH (Fig. 2). When the “variation inhibition” concentration of Veronal is compared at pH 7.4 and pH 7.9, the concentration of inhibitor that is equally active is roughly double at pH 7.9 than at pH 7.4. For example, at pH 7.4, 5 mM Veronal was equally inhibitory as 10 mM Veronal at pH 7.9.

Veronal was 50% ionized at pH 7.4 and 75% ionized at pH 7.9. Consequently, the concentration of unionized Veronal may be made the same at both pH levels by making the concentration of Veronal at the higher pH twice that at the lower pH. These results indicate that the pH dependence of the inhibition is mainly the result of ionization of Veronal and that the undissociated molecules,
rather than the Veronal anions, are responsible for the inhibition.

Time course of Veronal inhibition. At pH 7.4, the inhibition of initiation of spores by Veronal occurred rapidly, no incubation period with the inhibitor being required. Regardless of whether L-alanine was added to the spores (ca. 2 x 10^9/ ml) after 0, 15, 45, 60, or 120 min of incubation at 37 C with 5 mm Veronal in 50 mm phosphate buffer, the inhibition was 65%. The addition of Veronal to spore suspensions that had commenced the initiation with alanine resulted in cessation of initiation (Fig. 3). These results suggest that Veronal penetrates to sensitive sites of the spore at once.

Reversal of Veronal inhibition. Although the inhibitory action of Veronal on spore initiation is developed rapidly, there is a complete reversal of the inhibition when the inhibitor is removed. When intact spores were incubated at 37 C from 1 to 24 hr with 10 mm Veronal in 50 mm phosphate buffer (pH 7.4), washed twice with deionized water in the cold, and resuspended in 2 mm L-alanine in 50 mm phosphate buffer (pH 7.4), initiation of germination took place at the same rate as in the control spores. Veronal must be very loosely combined to its receptor(s) in the spore because it can be easily washed off these receptors, which then cease to register inhibition.

Kinetics of Veronal inhibition. Although not shown, the graphical determination of Dixon (3) and the Lineweaver-Burk plot indicated the kinetics of noncompetitive inhibition at both pH 7.4 and 8.0. As an example, the Lineweaver-Burk plot at pH 8.0 is shown in Fig. 4.

Reversal of Veronal inhibition by fructose, glucose, and K+. Glucose or fructose alone or a combination of d-fructose and d-glucose in 50 mm Tris-hydrochloride buffer plus K+ or in 50 mm phosphate buffer (pH 7.4) did not initiate germination of B. subtilis spores at 37 C during a period of 22 hr. The L-alanine initiation which was completely inhibited by 5 mm Veronal in Tris-hydrochloride buffer was not reversed by the addition of fructose and glucose. On the other hand, the L-alanine initiation which was completely inhibited by 5 mm Veronal in Tris-hydrochloride buffer plus K+ was partially reversed by the addition of fructose and glucose. Glucose or fructose alone had no effect (Fig. 5).

The inhibition was not reversed completely, indicating that the initiation in this system is also inhibited by Veronal. This was confirmed by increasing the Veronal concentration. The L-alanine initiation in the presence of fructose, glucose, and K+ was almost completely inhibited by 10 mm Veronal, and 20 mm Veronal inhibited the initiation completely.

Inhibition of L-alanine initiation of B. subtilis var. niger spores by Veronal. Suspensions of spores
Veronal to the initiation medium prevented a decrease in absorbance, and the spores remained phase-bright during 22 hr of incubation.

DISCUSSION

The first observation of a barbiturate blocking initiation of germination of bacterial spores in complete medium and in L-alanine resulted from the action of Veronal on B. subtilis spores (10). How Veronal blocks the initiation induced with alanine is difficult to explain unless some effect on enzyme reactions is involved. The dormant spore may not be triggered to initiate germination unless L-alanine penetrates the spore surface. Other studies (7) have indicated that the uptake of \(^{14}\)C-L-alanine occurred during initiation and that alanine became associated almost entirely with the spore protoplast. Initiation could be blocked by Veronal as a result of interference with alanine uptake. For example, Veronal could inactivate one of the components of the surface of the spore. The result would be a reduction in the supply of alanine to some intracellular enzyme. The observed data do not fit such a supposed mechanism. When Veronal was added to the spores after L-alanine permeation, inhibition occurred. Moreover, the initiation with L-alanine took place in the presence of otherwise inhibitory concentrations of Veronal when a combination of fructose, glucose, and K\(^+\) was added. These results indicate that L-alanine uptake does not determine the inhibition. Furthermore, the finding of a passive diffusion mechanism in spores, as opposed to active transport, for L-alanine and other solutes (1) favors this suggestion. Therefore, it is likely that Veronal does not act to prevent the uptake of the amino acid by the spore. Probably, Veronal has a direct effect on the sequence of reactions involved in the utilization of L-alanine. Regardless of which process takes place during inhibition, one could expect that Veronal must attach itself to specific intracellular sites to perform inhibition.

The marked pH dependence of the inhibition disclosed in the narrow range of pH studied suggests a permeability phenomenon related to the access of Veronal to the active sites of the spore. At pH 7.4, Veronal seemed to pass as an uncombined molecule, with equal ease in both directions, across spore boundaries and to exist in a loose form within the spore. Since L-alanine and Veronal in aqueous solution have different molecular properties, the entry of these compounds into the spore would be controlled by different factors. Whereas there is a barrier to Veronal at pH 9.0, L-alanine passes this barrier easily. A variation in pH from 7.4 to 9.0 is accompanied by changes in the electrochemical properties of the spore surface and the Veronal
molecule. Douglas (4) suggested that at pH values above 7.0 the aqueous pores of the B. subtilis spore surface are lined with negative charges. At pH values above 7.0, the entry of Veronal anions would be prevented, whereas neutral Veronal molecules would pass unhindered. The failure of 10 mM Veronal to inhibit L-alanine initiation at pH 9.0 may well be related to an impermeability of the initiation occurred.

For example, molecular dissociation is one of the main factors affecting the depressant action of barbiturates upon such diverse systems as fertilized Arbacia eggs (2) and isolated cardiac tissues (6).

In contrast to Veronal, the unsubstituted compound, barbituric acid, is not inhibitory (10). However, this conclusion may now be justified from the information available, because it is possible that barbituric acid (pK = 3.8) at the pH used (pH 7.4), which is much above the pK, is unable to penetrate the spore readily. At near-neutral reaction barbituric acid is almost completely ionized, and the concentration gradient is so low that the rate of entrance in the spore is very slow and equilibrium can only be achieved slowly.

The initiation with L-alanine alone in Tris-hydrochloride buffer plus K⁺ was completely blocked by 5 mM Veronal; however, when a combination of fructose and glucose was added, initiation occurred. Wax, Freese, and Cashel (11) showed that L-alanine initiates germination of spores of B. subtilis by entering two metabolic pathways. The products of one pathway, which is inhibited by D-alanine or by elevated temperature, can also be derived from the combination of fructose, glucose, and K⁺. It is plausible that these two metabolic pathways for L-alanine initiation (11) are available in our spores. The second pathway that requires glucose, fructose, and K⁺ is less sensitive to Veronal. However, the steps involved in either metabolic pathway of L-alanine initiation have not been elucidated, and any postulated mechanism must be able to explain the effect of Veronal. It remains possible that the combination of glucose, fructose, and K⁺ protects the inhibitory action of small amounts of Veronal by interfering with the absorption of Veronal on the vital binding sites.

Preliminary experiments to be published later have shown that Veronal also impairs the capacity of B. subtilis ATCC 6051 spores to initiate germination in amino acids other than L-alanine. This unspecificity of the inhibition may suggest an extremely basic mechanism capable of being initiated by several amino acids. The L-alanine dehydrogenase activity of dialyzed B. subtilis ATCC 6051 spore-free extracts was unaffected by 80 mM Veronal (unpublished data). However, experiments with mutants devoid of L-alanine dehydrogenase (11) have indicated that neither of the reactions leading to L-alanine initiation of B. subtilis spores is catalyzed by alanine dehydrogenase (or only to a small extent).

Some other points of interest emerge from the experiments described in this paper. Initiation of germination with amino acids may be used as a simple model system in physiological studies on the effects of barbiturates. The relative instability of stored spore suspensions is a major difficulty in work with spores. Barbiturates may have practical value as inhibitors of spore initiation, both in spore preparation procedures (harvesting, cleaning, and storage) in which it is necessary to keep initiation at a minimum and as a means of suppressing germination of spores in food at optimal conditions for germination. Undoubtedly, some of the 2,000 barbituric acid derivatives remain potentially useful inhibitors of spore development.

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LITERATURE CITED


