

Boron-Containing Antibacterial Agents: Effects on Growth and Morphology of Bacteria Under Various Culture Conditions

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The effects of a number of synthetic antibacterial agents of the borinic acid and diazaborine types on the growth of enteric bacteria were examined. In minimal medium aerobic growth was immediately slowed; slow nonexponential growth continued for an extended period, and the cells remained viable. The effect was also seen in anaerobic cultures and was not antagonized by a number of common nutrients, vitamins, or growth factors. The response was modified by the addition of casein hydrolysate to the medium. This seemed to be a nutritional effect dependent principally on lysine and leucine. The modified response consisted of a period of abnormal growth lasting for at least one doubling time after the addition of the antibacterial agent. The turbidity at first increased more rapidly than that of the controls and was approximately equal to that of the controls after 30 min. However, the increase in bacterial mass (dry weight) was only 0.75 of the amount predicted by the change in turbidity. The viable count increased in proportion to the turbidity. Changes in the cell envelope were revealed by electron microscopy and by an alteration in the response of cells to lytic agents. After the period of abnormal growth cultures entered a premature stationary phase.

A number of boron-containing compounds studied in these laboratories and elsewhere (2) possess strong antibacterial activity specifically against the enteric group of gram-negative bacteria both in vitro and in animal infections. The formulas of the compounds used in the present work are shown in Fig. 1 together with their reference numbers, which are used throughout the paper. The compounds comprised two distinct chemical groups. One group was derived from disubstituted borinic acids. These were prepared as stable crystalline chelates with some suitable ligand. In solution the chelates dissociated, effectively producing the corresponding borinate ion; thus, in neutral solution ICI 65,468 and ICI 74,704 were present as salts of 2,2'-dithiazolyl borinic acid. The second group of compounds contained the diazaborine ring; they were solubilized for use by the formation of the sodium salt.

Although these two groups were structurally dissimilar, their microbiological and biochemical properties could not be distinguished qualitatively. Experiments carried out on both groups are reported here, and comparisons between them are made only to illustrate particular points.

The antibacterial action of these compounds presented certain unusual features. Under some

nutritional conditions the presence of the boron compounds led to an abnormal form of growth which suggested interference with the production of a cell envelope component.

MATERIALS AND METHODS

Bacterial strains and culture. The organisms used were *Escherichia coli* 198 (ATCC 11229), *E. coli* B laboratory strain, and *Proteus mirabilis* P1 (clinical isolate 1968) grown at 37°C on Davis and Mingioli minimal medium (1) modified to contain 0.5% glucose. In many experiments the minimal medium was supplemented with casein hydrolysate (Difco) (1% wt/vol, or as specified). Most cultures were grown in shaken capped tubes. For larger volumes 2-liter vessels were used with stirring and aeration. Anaerobic cultures were grown in O₂-free medium under pure N₂. Growth, usually ranging from 2 × 10⁸ to 12 × 10⁸ cells per ml, was measured nephelometrically and expressed as turbidity units. Cell weight was determined by filtration and drying on Millipore membranes.

Materials. The boron-containing antibacterial agents were prepared in these laboratories; their synthesis will be reported elsewhere. For tracer work ICI 65,468 was prepared with ¹⁴C label in positions three and four of both thiophene rings. Other chemicals were of the best analytical grades available.

Assay methods for boron-containing antibacterial agents. Compounds of the borinic acid series were converted to their fluorescent 8-hydroxyquinoline chelates (by the method of D. E. Case). The test

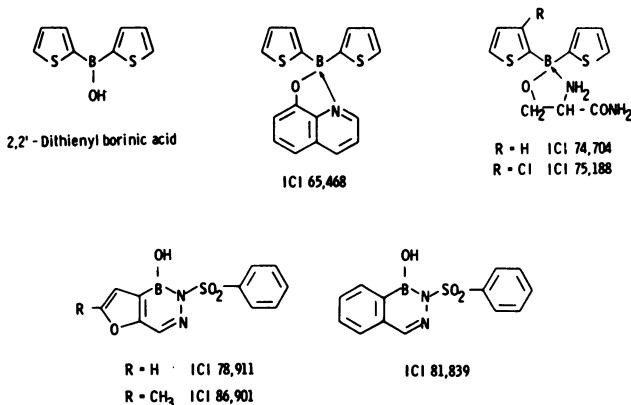


FIG. 1. Chemical structures of boron-containing antibacterial agents.

solution (1 ml) containing the borinic acid (1 to 10 μ g) mixed with sodium acetate buffer (0.2 M; pH 4.6; 1 ml) was shaken with benzene (10 ml) and separated. Part of the upper layer (3 ml), dried over Na_2SO_4 , was mixed with 8-hydroxyquinoline (30 μ g) in benzene (1 ml). After 30 min, the fluorescence was measured at 500 nm with excitation at 400 nm (the fluorescence of 8-hydroxyquinoline was negligible under these conditions).

For the biological assay, a sterilized test solution was added to minimal medium, and falling twofold dilutions were made with the medium. Standards of the reference compound in the medium were made at close concentration intervals over the sensitive range. All tubes were inoculated with *E. coli* 198, and growth continued until uninhibited cultures reached about 5×10^8 cells per ml. Growth in all tubes was then measured nephelometrically. An accurate estimate of the antibacterial concentration could be made for that dilution of the test solution which allowed an intermediate level of growth falling near the steepest part of the standard growth response curve.

Lysis experiment. ICI 78,911 (36 μ M) was added to an exponential culture of *E. coli* 198 in minimal medium plus 1% casein hydrolysate. Growth was continued until the turbidity had doubled. A similar untreated culture was grown to the same turbidity. Cells from each culture were suspended in tris(hydroxymethyl)aminomethane buffer (0.12 M; pH 7.8) and placed in a recording spectrophotometer set at 578 nm. Lysozyme (55 μ g/ml) and sodium ethylenediaminetetraacetate (2.2 mM) were added. The decrease in optical density was recorded at 20°C.

Uptake of boron-containing antibacterial agents by bacteria. ^{14}C -labeled ICI 65,468 (16 μ M; 13.6 nCi/ml) was added to a culture of *E. coli* 198 growing exponentially in minimal medium plus 1% casein hydrolysate. When growth had almost ceased (turbidity increased 2.5 times) the cells were removed, washed, and disrupted, and the retained ^{14}C was counted in dioxan phosphor.

RESULTS

Effect of boron-containing antibacterial agents on the growth of gram-negative en-

teric bacteria in minimal medium. When an effective concentration of a boron-containing antibacterial agent was added to *E. coli* 198, *E. coli* B, or *P. mirabilis* growing exponentially in minimal medium, growth was checked without measurable delay (Fig. 2). Growth became linear for a period and gradually declined later. The linear growth rate was 25 to 30% of the rate prevailing at the time of addition.

A sufficiently high concentration of an antibacterial agent (e.g., ICI 78,911; 7 μ M) added to the medium before inoculation prevented measurable growth for several hours. As progressively lower concentrations were tested, a fairly abrupt change from inhibition to full growth was seen. At this point the growth rate typically rose from 10 to 90% of the control rate with a halving of the antibacterial concentration.

The effect of the boron-containing antibacterial agents was bacteriostatic, not bactericidal. When inhibited cells were washed and suspended in drug-free medium they grew at the normal rate after some initial delay. Inhibited cultures with an antibacterial agent still present continued to grow slowly. After many hours the growth rate sometimes increased, perhaps to the normal rate. This was not due to the selection of resistant cells, as the progeny were still fully susceptible, but to the gradual decomposition of the antibacterial agent.

Growth inhibitory effects were similar when the glucose in the medium was replaced by malate or succinate as the carbon source. The boron-containing antibacterial agents also caused the same type of inhibitory effects on *E. coli* 198 growing in minimal (glucose) medium under strictly anaerobic conditions.

Comparison of the antibacterial activity of different compounds. The susceptible growth response of *E. coli* 198 to limiting concentrations of a boron-containing antibacterial

agent was used for comparing the activities of different compounds. The activity was expressed as the concentration required to reduce growth to 50% of a control culture (the steepest part of the response curve). Selected results shown in Fig. 3 cover a 50-fold range of activities.

Response of *E. coli* 198 to boron-containing antibacterial agents in supplemented medium. Attempts were made to antagonize the action of the boron-containing antibacterial agents by supplementing the minimal medium with vitamins, growth factors, and potential nu-

trients. Significant effects were observed only with amino acid mixtures, particularly with casein hydrolysate. When ICI 75,188 was added to an exponential culture of *E. coli* 198 growing in medium supplemented with 1% casein hydrolysate, instead of the immediate check in growth seen with cultures in minimal medium, the turbidity continued to increase more rapidly than in the controls (Fig. 4). After 1 to 1.5 doubling times the test and control cultures showed identical turbidities. This period has been studied in

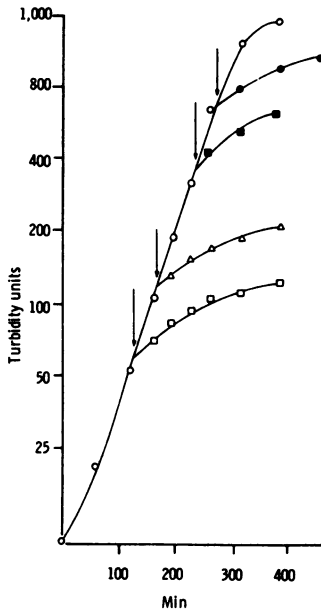


FIG. 2. Effect of ICI 78,911 on the growth of *E. coli* 198 in minimal medium with stirring and aeration. Control curve (○); growth curves for cultures receiving ICI 78,911 (36 μM) at the points shown by the arrows (other symbols).

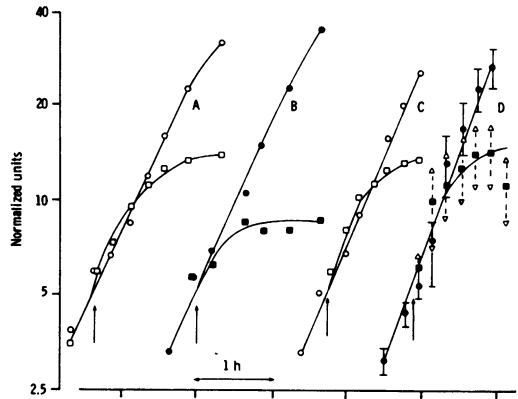


FIG. 4. Effect of ICI 75,188 on the bacterial dry weight and viable count of *E. coli* 198 grown in minimal medium supplemented with 0.5% casein hydrolysate. ICI 75,188 (32 μM) was added at points shown by the arrows. Turbidity measurements (A) compared with bacterial dry weight (B). Symbols: ○ and ●, controls; □ and ■, ICI 75,188 treated. Turbidity measurements (C) compared with viable counts (D). Symbols: ○ and ●, controls; □ and ◁---■---▷, ICI 75,188 treated. Normalized units on the ordinate represent turbidity units $\times 10^{-1}$; cell dry weight (micrograms per milliliter) $\times (4.1 \times 10^{-2})$; viable count 10^{-7} . In the viable count measurements the bars show 95% confidence limits. Cultures were stirred and aerated.

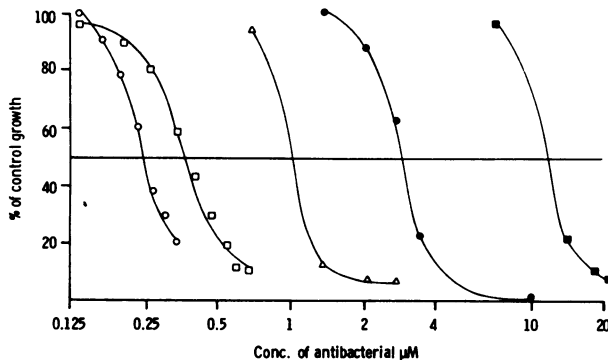


FIG. 3. Assay of antibacterial activity of different compounds. The concentrations of the added compounds are plotted against the growth of *E. coli* 198 relative to a control culture grown to the late exponential phase. Compounds: ○, ICI 75,188; □, ICI 74,704; Δ, ICI 86,901; ●, ICI 78,911; ■, ICI 81,839.

detail and will be referred to as parallel growth. Beyond this time the growth of the treated culture declined sharply, whereas the control culture continued to grow exponentially until the onset of the stationary phase at a much higher cell density.

The effect of casein hydrolysate on the inhibitory action of the boron-containing antibacterial agents was greatest at concentrations of 10 mg/ml but was detectable down to 0.1 mg/ml. In these experiments cells had been growing from the outset in supplemented medium, but even when casein hydrolysate was added at the same time as ICI 75,188 the inhibitory response was strongly modified (Fig. 5). If the addition of casein hydrolysate was delayed until growth inhibition was already established, the culture responded by an acceleration of growth to almost the control rate for about one doubling time before inhibition was reestablished. A mixture of 15 amino acids simulating the composition of casein hydrolysate produced similar effects, and the omission of any one of the component amino acids did not significantly reduce the response. Among many simpler mixtures of amino acids at corresponding concentrations, only those con-

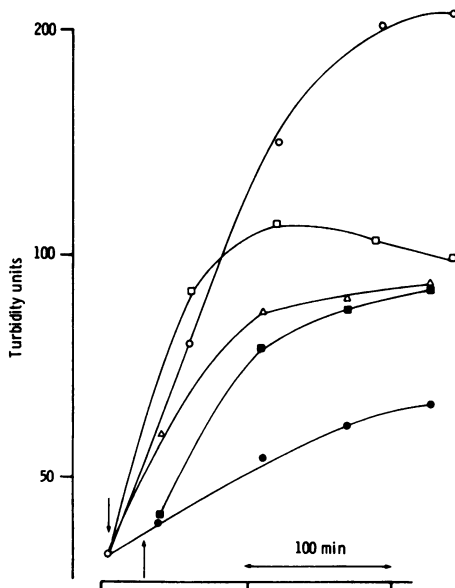


FIG. 5. Effect of ICI 75,188 on the growth of *E. coli* 198 in glucose-salts medium with a supplement of casein hydrolysate added at various times. ICI 75,188 (32 μ M) was added in the exponential phase at the point shown by the downward arrow. Casein hydrolysate (10 mg/ml) was added at the time of inoculation (\square), at the same time as ICI 75,188 (\triangle), and at 20 min after ICI 75,188 (upward arrow) (\blacksquare); no casein hydrolysate added (\bullet); control without ICI 75,188 (\circ).

taining both lysine and leucine modified the growth response to the boron-containing antibacterial agents. A mixture of these two amino acids as the sole supplement produced a marked effect, although less striking than that of casein hydrolysate.

Abnormal growth and morphology of *E. coli* 198 grown on medium containing casein hydrolysate after treatment with boron-containing antibacterial agents. When a boron-containing antibacterial agent was added to a culture of *E. coli* 198 growing exponentially in minimal medium supplemented with casein hydrolysate, turbidity readings increased twofold or slightly more before inhibition set in, but growth was abnormal. The increase in the dry weight of the bacteria was about 0.75 of the increase expected from the turbidity measurements (Fig. 4). Viable count measurements, however, agreed with the turbidity readings, showing a rapid doubling in cell numbers from the time of the addition of ICI 75,188 until inhibition began to become evident.

Cells grown under these conditions were shown by light microscopy to be longer, on the average, than the controls. They responded differently to negative staining with uranyl acetate, picking up a much larger amount of the reagent. Electron micrographs of cell sections showed the outer membrane to be more irregular and less heavily stained than in the controls. There was also some evidence of abnormal division. Cell walls prepared (3) from treated and control cells were compared by electron microscopy. The walls from normal cells showed the usual appearance of flattened envelopes, whereas those from treated cells were twisted and distorted; few were flat. The walls from treated cells also showed a greater tendency to clump together.

A further difference was in the response to lysis. A cell suspension from an exponential culture of *E. coli* 198 treated with lysozyme and ethylenediaminetetraacetic acid cleared in a few minutes through lysis (Fig. 6). A suspension of cells treated with a boron-containing antibacterial agent in the parallel growth condition showed only a small reduction in turbidity on similar treatment. Electron microscopy of the lysed cells showed that normal cells disintegrated to form sticky fibers which adhered to the walls of the vessel. Treated cells also broke up, but the fragments remained suspended, leaving a substantial turbidity.

Strains of *E. coli* 198 resistant to boron-containing antibacterial agents. The occurrence of naturally resistant strains in *E. coli* 198 was shown by plating to be <1 in 10^8 , but resistant strains could be isolated by repeated subcultures in increasing concentrations of the anti-

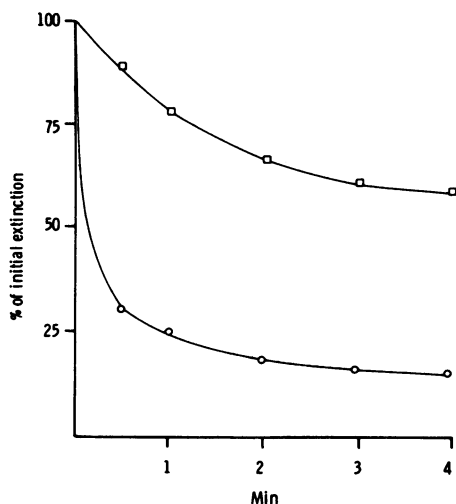


FIG. 6. Time course of lysis of *E. coli* 198. Cell suspensions were treated with lysozyme. Ethylenediaminetetraacetic acid was added at zero time. Control culture grown exponentially in minimal medium supplemented with casein hydrolysate (○); similar culture treated with ICI 78,911 (36 μ M) and allowed to grow for one doubling time (◻). Initial extinctions, 1.4 to 1.6.

bacterial agent. Resistant strains were readily obtained by mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, followed by selection on plates containing ICI 78,911 (360 μ M). Strains which grew well on unsupplemented medium containing ICI 78,911 (180 μ M) and retained their resistance on repeated subcultures were selected for further study. They showed cross-resistance to ICI 75,188, a boron-containing antibacterial agent of a different chemical group.

Uptake of boron-containing antibacterial agents by bacteria. The uptake of a borinic acid-type antibacterial agent was measured in *E. coli* 198 by the use of 14 C-labeled ICI 65,468 (16 μ M). Washed cells taken at the end of a period of parallel growth retained 75 ng of ICI 65,468 per mg (dry weight), i.e., about 74 μ M or 4.6 times the concentration in the suspending medium.

DISCUSSION

Observations on the effects of the boron antibacterial agents on the growth of bacteria revealed some clues as to their mode of action. Their inhibitory effects were demonstrable under strictly anaerobic conditions or when acids of the tricarboxylic acid cycle replaced glucose as a carbon source, so their effects are unlikely to involve the respiratory or glycolytic pathways.

The striking modification of growth response caused by supplementing the minimal medium with casein hydrolysate seems to be connected with the general nutritional state of the bacteria rather than with an action attributable to any single amino acid. The major contribution to this nutritional enhancement comes from lysine and leucine, compounds with dissimilar pathways of biosynthesis. The delay in the expression of inhibition might be attributed to the presence in the nutritionally favored cells of a reserve of an intermediate which could maintain cell growth until it was used up. However, the effect from adding casein hydrolysate was too rapid to permit a nutritional reserve to be built up. The nutritional condition of the cell more probably dictated its response to the boron-containing antibacterial agents. On minimal medium the antibacterial action was immediately reflected in the total cell growth. When the nutritional condition of the cell was improved by the addition of amino acids, some of the cell processes were able to continue unchecked for a time in the presence of the antibacterial compound. However, growth was abnormal. Cell mass did not increase in proportion to the rise in turbidity. Cells showed morphological changes mostly related to the cell envelope, and the behavior on lysis was altered. These changes progressively disturbed the cell metabolism until growth was no longer possible. Even under these conditions, however, the effect was reversible if the cells were removed to fresh medium, unless autolysis had set in.

Compounds of the dithiazolyl borinic acid series were, in general, more active than compounds of the diazaborine series in growth inhibition tests on minimal medium. (Unpublished results from these laboratories show that this difference in activity was much less apparent when the two groups of compounds were tested in vivo against infections in mice.) The most active compound described here was ICI 75,188, which inhibited the growth of *E. coli* 198 by 50% at 0.28 μ M; ICI 78,911, a typical compound of the other series, required a concentration of 3.1 μ M to produce the same effect.

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