ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Dec. 1988, p. 1763–1768
0066-4804/88/121763-06$02.00/0

Vol. 32, No. 12

In Vitro Mechanism of Inhibition of Bacterial Cell Growth by Allicin

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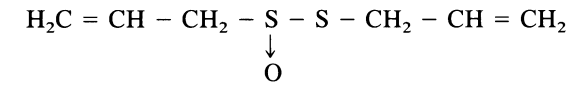
Received 31 May 1988/Accepted 31 August 1988

Diallyl thiosulfinate (allicin) is the agent found in garlic which is responsible for the antibacterial and antifungal activity of extracts of this plant. The effect of bacteriostatic concentrations of allicin (0.2 to 0.5 mM) on the growth of *Salmonella typhimurium* revealed a pattern of inhibition characterized by: (i) a lag of approximately 15 min between addition of allicin and onset of inhibition, (ii) a transitory inhibition phase whose duration was proportional to allicin concentration and inversely proportional to culture density, (iii) a resumed growth phase which showed a lower rate of growth than in uninhibited controls, and (iv) an entry into stationary phase at a lower culture density. Whereas DNA and protein syntheses showed a delayed and partial inhibition by allicin, inhibition of RNA synthesis was immediate and total, suggesting that this is the primary target of allicin action.

Natural products of plants have long provided a valuable source of pharmacological agents with important bioactivity (2). In addition, studies on the mode of action of a variety of drugs have provided insight into the processes of macromolecular synthesis and precursor metabolism. Garlic (*Allium sativum*) has traditionally been invoked as a protective agent against stroke, atherosclerosis, and coronary thrombosis and has also been implicated as an antibacterial agent. Over the years, this folk tradition has been examined in a more systematic manner. The extraction and organic synthesis of a potent antithrombotic agent, ajoene, has been reported by Block and co-workers (5, 6), and the overall chemistry of the allyl sulfur compounds has recently been reviewed by Block (4). The chemical responsible for the antibacterial and antifungal activity in garlic is diallyl thiosulfinate (common name, allicin) (24):



Allicin is not found in intact plants but is formed by the action of the enzyme alliin alkyl-sulfenate-lyase (EC 4.4.1.4) on the nonprotein amino acid *S*-allylcysteine *S*-oxide (alliin). In intact plants, the amino acid and the enzyme are stored in separate cellular compartments (15). However, upon injury to plants, barriers between these compartments are broken and the alliin lyase catalyzes the beta elimination of alliin to yield pyruvate, ammonia, and allylsulfenic acid, two molecules of which spontaneously react to form allicin. This enzyme has been purified from onion (*Allium cepa*) (20–22, 25) as well as from garlic (14, 18, 19).

One of the first reports on the antibacterial action of allicin was that of Cavallito and Bailey (8), who found that dilute allicin solutions inhibited the growth of both gram-positive and gram-negative microorganisms. A variety of studies have since confirmed the bioactivity of this compound, and in the 1970s several groups extended this work to an examination of medically important fungi (1, 3, 10, 16). A problem with many of these studies is that it was impossible to quantify the amount of active agent in a crude extract of garlic. In addition, the fact that such an extract is a complex mixture of many secondary plant products makes it impossible to elucidate the precise mechanism of action of inhibition. Although the organic synthesis of allicin has been described (23), Barone and Tansey (3) found that synthetic allicin contained large amounts of unreacted diallyl disulfide as well as at least nine other unidentified contaminants. In this report, we describe our studies on the pattern of inhibition of bacterial cell growth and macromolecular synthesis by allicin derived from the action of purified alliin lyase on synthetic *S*-allylcysteine *S*-oxide.

MATERIALS AND METHODS

Materials. Cystine, pyruvate, chicken heart lactic dehydrogenase, NADH, 2,4-dinitophenylhydrazine, pyridoxal 5′-phosphate, and *N*-ethylmaleimide were all products of Sigma Chemical Co., St. Louis, Mo. DEAE-Trisacryl M and hydroxyapatite-Ultrogel were purchased from LKB Instruments, Inc., Rockville, Md. Sephacryl S-300 was purchased from Pharmacia, Inc., Piscataway, N.J., and DEAE-cellulose was purchased from Whatman, Inc., Clifton, N.J. AG50W × 8 was obtained from Bio-Rad Laboratories, Richmond, Calif., and allyl bromide was obtained from Aldrich Chemical Co., Inc., Milwaukee, Wis. Radioactively labeled leucine and uridine were obtained from ICN Pharmaceuticals Inc., Irvine, Calif., and thymidine was obtained from Dupont, NEN Research Products, Boston, Mass. All other chemicals were obtained from VWR. *Salmonella typhimurium* 7004 was a gift of D. Bottstein.

Preparation of *S*-allylcysteine *S*-oxide (alliin). Alliin was prepared by organic synthesis from L-cystine and allyl bromide, using the technique described by Freeman and Whenham (9). The final product was purified through a Bio-Rad AG50W × 8 column to give pure alliin as deter-

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TABLE 1. Purification of alliin lyase from *A. sativum*

Fraction	Vol (ml)	Protein (mg)	Activity (U)	Sp act (U/mg)	Recovery
1 (extract)	1,570	37,680	240,053	6.37	
2 (DEAE)	1,780	12,816	182,906	14.26	76%
3 (S-300)	560	1,680	78,848	46.93	33[a]
4 (hydroxyapatite-Ultrogel)	808	644	23,023	35.75	10[a]

[a] Based on total units in fraction 1. Since only 45% of fraction 2 was used in this purification, actual recoveries will be 2.2 times higher.

mined by paper chromatography (3). The final concentration of alliin obtained was 1.1 to 1.5 M, and this material was adjusted to pH 6.5 before use. Although this preparation gives both the *S*(+) and *S*(−) isomers, our studies with the garlic alliin lyase suggest that both are substrates for the enzyme.

Purification of alliin lyase. All phases of the preparation of alliin lyase were performed by using potassium phosphate buffers at pH 6.5 containing 10^{-5} M pyridoxal 5′-phosphate and 5% glycerol and were carried out at 4°C. One unit is the amount of enzyme needed to convert 1 μmol of substrate to product in 1 min at 25°C in a lactate dehydrogenase-coupled assay containing 4.33 mM alliin, 25 mM potassium phosphate buffer (pH 6.5), 67 μM NADH, and 15 U of lactate dehydrogenase in a total volume of 1.5 ml. The purification summarized in Table 1 is from an extract of 4,000 g of fresh garlic cloves. After peeled cloves were processed through a juicer, the extract was made up to 0.05 M potassium phosphate (pH 6.5), 10^{-5} M pyridoxal 5′-phosphate, and 5% glycerol by addition of 1/10 volume of a 10× buffer and centrifuged at 20,000 × *g* for 60 min. The supernatant fraction (fraction 1) was concentrated by ammonium sulfate precipitation at 50% saturation. The pellet was suspended in 0.05 M potassium phosphate buffer, dialyzed against this buffer, and then purified by negative chromatography on a DEAE-Trisacryl M column (5.5 by 10 cm). After precipitation by 50% ammonium sulfate, the pellet was resuspended and applied to a Sephacryl S-300 column (10 by 26 cm) equilibrated with 0.01 M potassium phosphate buffer. Activity eluted with the exclusion volume and was applied directly to a hydroxyapatite-Ultrogel column (4 by 8.4 cm). The column was washed with several volumes of 0.01 M phosphate buffer and with 0.07 M phosphate buffer, and the enzyme activity was then eluted with 0.20 M phosphate buffer. Sodium dodecyl sulfate-gel electrophoresis showed that the hydroxyapatite-Ultrogel eluate contained one major band migrating with an apparent molecular weight of 41,000. The enzyme used in allicin preparation was present at 25 U/ml.

Preparation of allicin. Allicin was prepared by the reaction of alliin with alliin lyase. Alliin lyase (6.25 U) was added to a total volume of 40 ml containing 13 mM alliin, 0.05 M potassium phosphate buffer (pH 6.5), 10^{-5} M pyridoxal 5′-phosphate, and 5% glycerol, and the mixture was incubated for 4 h. The extent of reaction was monitored by measuring pyruvate formation by the dinitrophenylhydrazine assay (13). Reactions ran to 90 to 95% completion at this concentration of alliin. Higher concentrations of alliin resulted in lower overall yields, with a maximal formation of 13 mM pyruvate (6.5 mM allicin). The allicin was concentrated by extraction of the reaction mixture with an equal volume of ethyl ether. This extraction was repeated, and the two ether phases were combined. The ether was removed with a stream of nitrogen, and the oily residue was dissolved in 4 ml of deionized water. The concentration of allicin, determined by the *N*-ethylmaleimide assay of Nakata et al. (17), was 35 mM.

Inhibition studies. Overnight cultures of *S. typhimurium* 7004 were grown in Luria broth (L broth) and diluted into 10 ml of fresh L broth medium in a Klett sidearm flask. Cells were grown with shaking at 37°C, inhibitors were added directly to the tube, and turbidity was monitored by using a Klett-Summerson turbidometer (green filter).

Macromolecular synthesis. The rates of protein, RNA, and DNA synthesis were determined in 2-min pulse-labeling experiments. Cultures were grown in M9 medium supplemented with 0.5% Casamino Acids, 0.2% glucose, and 2 mg of thymidine per liter. Pulse-labeling was carried out by transferring 200 μl of culture from the Klett tube at various times to a tube containing 0.20 μCi (in 20 μl) of L-[2,3,4,5-^{3}H]leucine (144 Ci/mmol), [5,6-^{3}H]uridine (44 Ci/mmol), or [*methyl*-^{3}H]thymidine (6.7 Ci/mmol). The tube was shaken at 37°C for 2 min, and labeling was halted by addition of 2 ml of ice-cold 5% trichloroacetic acid. Acid-precipitable counts were determined by filtering the material through Whatman GF/C filter disks, drying the disks, and counting in a liquid scintillation counter.

RESULTS

Inhibition of cell growth by allicin. Figure 1A shows the basic phenomena observed in the inhibition of cell growth by allicin. Addition of allicin to a final concentration of 0.3 mM (49 μg/ml) at a cell density of 35 Klett units (91-min time point) resulted in a continued increase in culture turbidity for approximately 20 min, followed by a complete inhibition of growth for 50 min. After this inhibition phase, logarithmic growth resumed but at only 55% of the rate of the uninhibited control. In addition, cultures exposed to allicin entered stationary phase at a cell density substantially lower than that of the control culture. Viable counts taken during the inhibition phase revealed an approximately 20% decrease in CFU per Klett unit, which indicated that the action of allicin at this concentration was predominantly bacteriostatic. After recovery from inhibition, viable counts taken at a Klett value of 88 were the same for allicin-treated and control cultures, which suggested that recovered cells were not different from control cells in light-scattering properties and that the same proportions of cells were capable of forming colonies in control and recovered cultures.

The effect of increasing the allicin concentration on bacterial growth is indicated in Fig. 1B. As the allicin concentration was increased from 0.2 to 0.5 mM, the duration of the inhibition phase increased, the rate of growth after inhibition decreased, and the cell density at which stationary phase was entered also decreased. However, the interval between allicin addition and the onset of growth inhibition remained relatively constant regardless of the allicin concentration. Although the duration of inhibition was proportional to the allicin concentration, there did not appear to be any simple linear or logarithmic relationship.

Allicin inhibition as a function of culture density. Inhibition characteristics were found to be highly dependent on the culture density at which allicin was added. Addition of 0.3 mM allicin to cultures at Klett values of 19, 36, 67, and 96 (Fig. 2) revealed a shorter duration of inhibition with more dense cultures, decreasing from approximately 95 min when allicin was added at a Klett value of 19 to as little as 20 min when it was added at a Klett value of 96. In contrast to the previous result, in which the secondary growth rate was

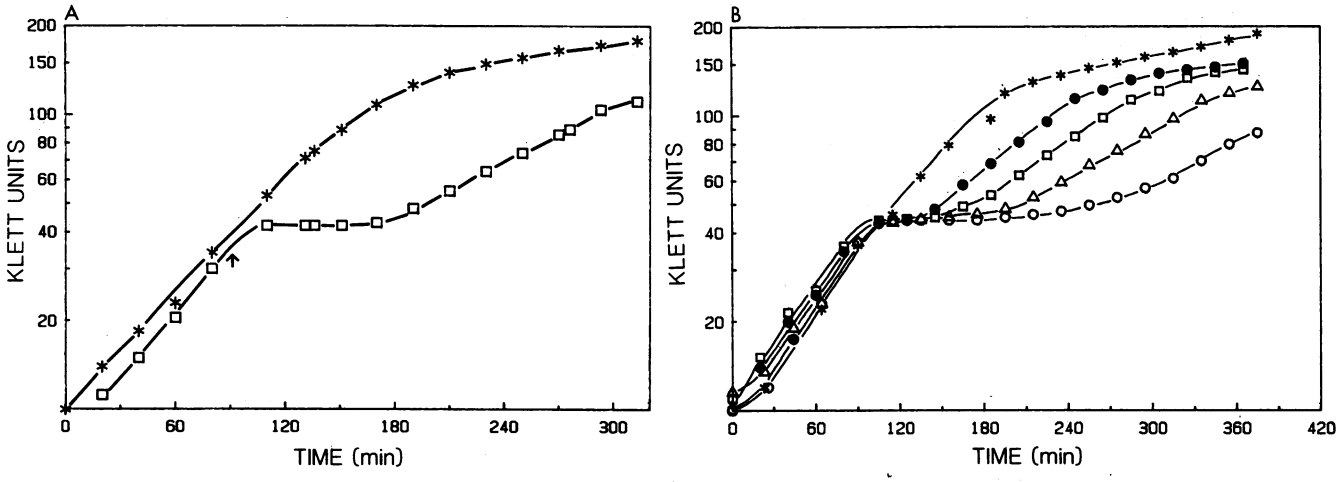

FIG. 1. (A) Growth curve of *S. typhimurium* 7004 in L broth at 37°C with no treatment (*) or with allicin added at 91 min to a final concentration of 0.3 mM (□). (B) Growth curve of *S. typhimurium* 7004 exposed to various concentrations of allicin. Allicin was added at 85 min to the 0.2 (●) and 0.3 (□) mM flasks and at 94 min to the 0.4 (△) and 0.5 (○) mM flasks. *, No treatment.

slower the longer the duration of the inhibition phase at a constant cell density, inhibition of a low-density culture resulted in a higher secondary growth rate even though it gave a longer growth inhibition phase than did a higher-density culture. The secondary growth rate decreased from 80% of the initial growth rate when allicin was added at a Klett value of 19 to 33% of initial growth when the addition took place at a Klett value of 96.

Temperature dependence of allicin inhibition. Examination of the effect of temperature on the parameters of allicin inhibition revealed that (i) the duration of inhibition was longer at lower culture temperatures, (ii) the resumed secondary growth rate, expressed as a percentage of the uninhibited growth rate, was the same for the 28, 32, and 37°C cultures, and (iii) the lag phase between allicin addition and onset of growth inhibition decreased as the temperature was increased (Table 2). The duration of the lag phase was calculated from the ratio of the Klett value during inhibition to that at the time of allicin addition. In an exponentially growing culture, this ratio will be equal to $2^{D/\tau}$ (7), where τ is the culture doubling time and D is the time between the addition of inhibitor and the onset of inhibition. To avoid effects of cell density, addition of allicin was made at a Klett value of 40 under all three conditions.

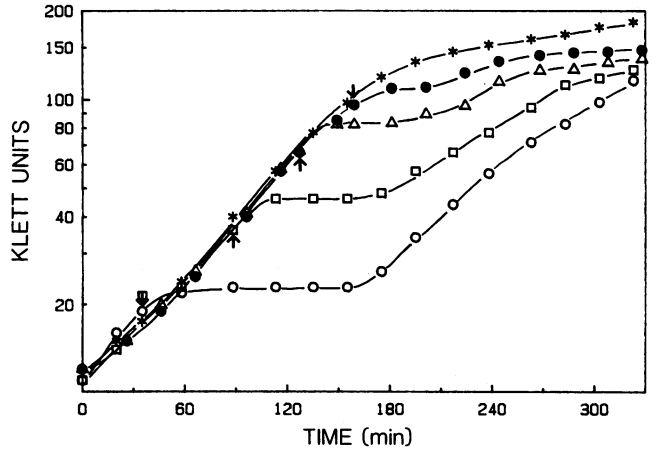

FIG. 2. Allicin inhibition as a function of culture density. Allicin (final concentration, 0.3 mM) was added to a growing culture of *S. typhimurium* 7004 at a Klett value of 19 (○), 36 (□), 67 (△), or 96 (●), and growth of the cultures monitored over time.

Effect of allicin on macromolecule synthesis. A direct measure of the effect of allicin on growing cells was obtained by following macromolecular synthesis as measured by incorporation of radioactive precursors into acid-precipitable material. RNA, protein, and DNA syntheses during inhibition were examined by using 2-min pulse-labeling with [^{3}H]uridine, [^{3}H]leucine, and [^{3}H]thymidine, respectively. The rate of protein synthesis as measured by counts per minute incorporated per Klett unit in a 2-min pulse decreased gradually during the logarithmic growth of the uninhibited culture, with a 2-min pulse at late log phase giving only 40% of the incorporation of a similar pulse in early log phase (Fig. 3). Addition of allicin gave no significant inhibition of protein synthesis in the first 7 min after addition (2-min pulses were started at $t = 0$ and $t = 5$ min), but by 10 min after allicin addition, protein synthesis was only 40% of the control value. Protein synthesis reached a minimum level of approximately 35% of the control value at 20 min and then recovered quickly, reaching uninhibited levels at 50 min, well before resumption of growth.

RNA synthesis, as measured by the incorporation of labeled uridine into acid-precipitable material, was much more profoundly inhibited by allicin exposure (Fig. 4). RNA synthesis was inhibited immediately, with uridine incorporation only 42% of the control value in the $t = 0$ pulse-labeling. At 5 min after allicin addition, RNA synthesis had decreased to 1% of that of the uninhibited control, and at 10 min it reached a minimum value of 0.6%. By 40 min after allicin addition, RNA synthesis was still only 6% of that of the control. By 80 min after allicin addition, RNA synthesis

TABLE 2. Effect of culture temperature on allicin inhibition

Temp (°C)	τ^a (min)	Lag phase (min)	Duration of inhibition (min)	Resumed growth rate (% of uninhibited rate)
28	68	17	110	55
32	55	12	90	56
37	48	10	78	56

[a] τ, Doubling time.

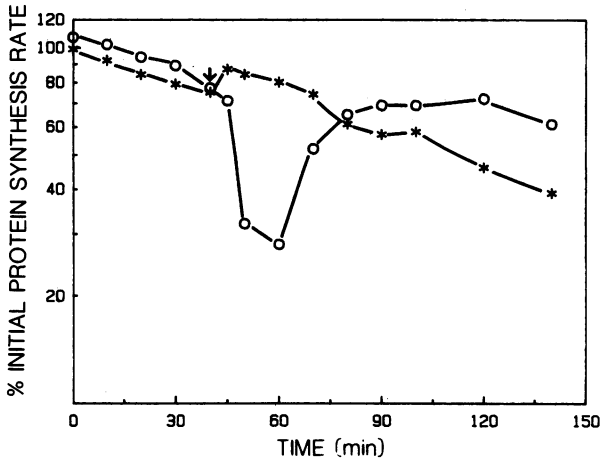

FIG. 3. Protein synthesis in control and allicin-treated cells. *S. typhimurium* 7004 was grown at 37°C in M9 medium, 200-μl portions were exposed to [^{3}H]leucine for 2 min at the times indicated, and acid-insoluble radioactivity was determined as described in the text. Symbols: *, control culture; O, allicin (0.3 mM) added at 40 min (arrow) to a culture of 58 Klett units. Data are calculated as counts per minute incorporated per Klett unit and are expressed as percentage of initial value.

had reached 73% of that of the control culture and growth had begun to resume. Incorporation of uridine, like that of leucine, decreased during exponential growth such that a 2-min pulse-label with the untreated control culture at the 220-min time point gave only 54% of the incorporation measured at the 80-min time point. However, unlike recovery of protein synthesis, which in the recovered cultures actually surpassed that of the uninhibited controls, recovery of RNA synthesis after allicin inhibition did not exceed that of the uninhibited culture and indeed reached only 88% of that of the uninhibited culture by 140 min after allicin inhibition.

DNA synthesis resembled protein synthesis in showing only a transient and partial inhibition in response to allicin.

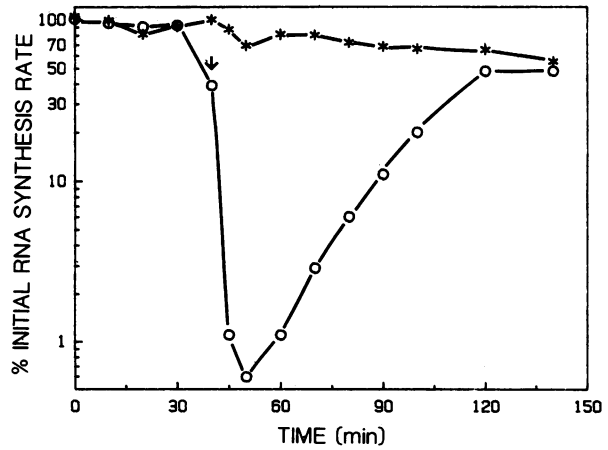

FIG. 4. RNA synthesis in control and allicin-treated cells. *S. typhimurium* 7004 was grown at 37°C in M9 medium, and 200-μl portions were exposed to [^{3}H]uridine for 2 min at the times indicated. Symbols: *, control culture; O, allicin (0.3 mM) added at 40 min (arrow) to a culture of 47 Klett units. Data are calculated as counts per minute incorporated per Klett unit and are expressed as percentage of initial value.

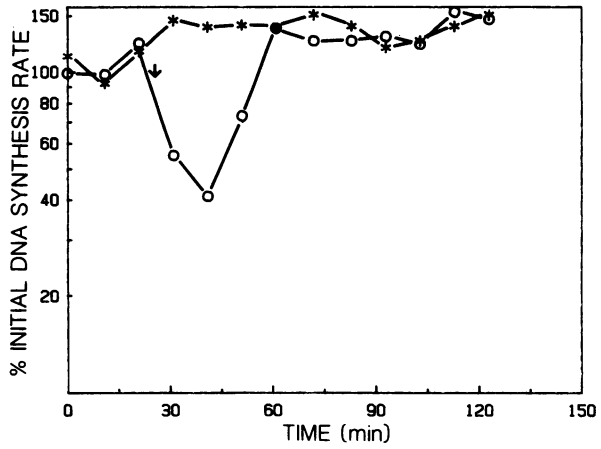

FIG. 5. DNA synthesis in control and allicin-treated cells. *S. typhimurium* 7004 was grown at 37°C in M9 medium, and 200-μl portions were exposed to [^{3}H]thymidine for 2 min at the times indicated. Symbols: *, control culture; O, allicin (0.3 mM) added at 25 min (arrow) to a culture of 47 Klett units. Data are calculated as counts per minute incorporated per Klett unit and are expressed as percentage of initial value.

Addition of allicin to the culture resulted in a level of thymidine incorporation 43% of that of the control at the 5- to 7-min pulse-label (Fig. 5). The pulse-label reached a minimum level of 32% of the control value at 15 min after allicin addition and was restored to 100% of the control value by 35 min after allicin addition, well before growth was resumed. Unlike the rates of protein and RNA syntheses, the rate of DNA synthesis in the control culture remained relatively constant over the entire growth range.

DISCUSSION

Previous work (3, 26) has ascribed the mechanism of allicin inhibition of cell growth to the inhibition of sulfhydryl-dependent enzymes. Wills (26), examining a variety of different enzymes for sensitivity to allicin, found that 11 of 15 enzymes inhibited by allicin were sulfhydryl reagent sensitive. In contrast, of 15 enzymes insensitive to allicin, only 3 were identified as sulfhydryl enzymes. In addition, both cysteine and glutathione protected against allicin inhibition. Earlier work by Small et al. (23) revealed that the thiosulfinate link (-SO-S-) was essential for bactericidal action. Wills (26) demonstrated that this functional group was also required for inhibition of succinic oxidase, with no inhibition observed with diallyl sulfide, diallyl disulfide, or diallyl sulfoxide. Barone and Tansey (3) also found that the anticandidal activity of allicin was inactivated by cysteine and dithioerythritol and suggested that the mechanism of action of allicin involved formation of mixed disulfides with protein sulfhydryl groups. Although it is likely that allicin can function as a general sulfhydryl reagent, our observations on the pattern of inhibition of bacterial growth and on the specific inhibition of macromolecular synthesis in allicin-treated cultures suggest that a more specific subset of cellular functions is inhibited by allicin in growing cells.

Exposure of logarithmically growing *S. typhimurium* to allicin resulted in a consistent pattern of inhibition characterized by (i) a lag phase of somewhat less than 20 min between addition of allicin and onset of complete inhibition, (ii) an inhibition phase, and (iii) a resumed secondary logarithmic growth rate less than the original rate of growth. The

observation that the lag period between allicin addition and the onset of growth inhibition was independent of allicin concentration and culture density suggests that this interval is intrinsic to the mode of inhibition by allicin. The duration of this lag phase corresponds approximately to the D-period interval (the time between completion of chromosome replication and cell division) in the replication cycle model of Helmstetter et al. (11, 12), which suggests that cells which have completed DNA synthesis before addition of allicin can proceed through cell division. Inhibition of cells undergoing chromosome replication can be attributed to the almost total inhibition of RNA synthesis, since discontinuous DNA synthesis requires the action of RNA polymerase.

Although the duration of inhibition was proportional to the allicin concentration, there was no simple linear or logarithmic relationship. Except at high concentrations of allicin, cells were able to overcome the inhibition and resume growth, which suggests that they were able to metabolize the allicin to a noninhibitory compound. The duration of inhibition was not related to allicin stability, since separate studies on allicin stability (data not shown) indicated that this compound was stable in L broth medium for more than 4 h at 37°C. The inverse dependence of duration of inhibition on cell density (Fig. 2) also suggests either that the cells are able to metabolize the allicin or that the cells can essentially titrate the allicin with noncritical targets, thus decreasing the interaction with critical targets. Although allicin at these concentrations is bacteriostatic rather than bacteriocidal, the observation that the resumed growth rate is substantially lower than the uninhibited-culture growth rate suggests that the inhibited cells are not totally able to recover from allicin inhibition. The lower secondary growth rate could indicate either the presence of some unrepaired lesion in the cells or the depletion of limiting nutrients from the medium during the inhibition phase. Measurement of protein synthesis, however, supports the presence of some stable modification in the cells. In the uninhibited control cultures, protein synthesis was found to decrease gradually by 50% as the culture went from early-log-phase to late-log-phase growth. Although allicin-inhibited cells show a transient inhibition of protein synthesis, they also show a recovery to levels comparable to that in early-log-phase cells. If the inhibited cells had depleted the medium of limiting nutrients during the inhibition phase, we would expect that the resumed protein synthesis would reach levels no higher than those found in the uninhibited control culture. An alternate explanation for the slower growth rate is a deceased frequency of DNA initiation in the allicin-exposed cells due to a persistent lesion which resets the initiator protein mass/DNA ratio necessary for initiation of replication.

Further evidence for the specificity of allicin inhibition was derived from studies on macromolecular synthesis in allicin-treated cultures. RNA synthesis, as measured by 2-min pulse-label incorporation of labeled uridine into acid-precipitable counts, was inhibited immediately upon allicin addition, showing a 60% decrease in the first 2 min after addition. Within 5 min, uridine incorporation was inhibited by 99% and RNA synthesis was inhibited by more than 90% for approximately 40 min after allicin addition. Indeed, resumption of growth correlated most closely with resumption of RNA synthesis. In contrast, both protein and DNA syntheses showed less inhibition by allicin as well as a more transient inhibition. The onset of inhibition of both leucine and thymidine incorporation was slower than that of uridine incorporation, and both were maximally inhibited by only about 70% at 15 to 20 min after allicin addition. Unexpectedly, both showed a restitution to 100% of control levels by 40 min after allicin addition, at a time when RNA synthesis was still inhibited by more than 90%. This result was unexpected and could suggest that allicin can inhibit mRNA degradation as well as RNA synthesis, so that translationally active RNA is available when protein synthesis inhibition is released. Experiments with 0.40 mM allicin (data not shown) showed similar but prolonged patterns of inhibition of RNA, DNA, and protein syntheses. These effects on macromolecular synthesis also suggest that allicin is acting not as a general inhibitor of sulfhydryl-dependent enzymes but rather as a more specific but reversible inhibitor of RNA synthesis in the cells. Future work with isolated enzymes should reveal whether RNA polymerase is unusually sensitive to this agent and may prove of interest in the design of new inhibitors of this enzyme.

ACKNOWLEDGMENT

This work was supported by a Public Health Service biomedical research support grant from the National Institutes of Health to Tufts University.

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