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Influence of Macromolecular Biosynthesis on Cellular Autolysis in *Streptococcus faecalis*

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The addition of several different antibiotics to growing cultures of *Streptococcus faecalis*, ATCC 9790, was found to inhibit autolysis of cells in sodium phosphate buffer. When added to exponential-phase cultures, mitomycin C (0.4 μg/ml) or phenethyl alcohol (3 mg/ml) inhibited deoxyribonucleic acid synthesis, but did not appreciably affect the rate of cellular autolysis. Addition of chloramphenicol (10 μg/ml), tetracycline (0.5 μg/ml), puromycin (25 μg/ml), or 5-azacytidine (5 μg/ml) to exponential-phase cultures inhibited protein synthesis and profoundly decreased the rate of cellular autolysis. Actinomycin D (0.075 μg/ml) and rifampin (0.01 μg/ml), both inhibitors of ribonucleic acid (RNA) synthesis, also reduced the rate of cellular autolysis. However, the inhibitory effect of actinomycin D and rifampin on cellular autolysis was more closely correlated with their concomitant secondary inhibition of protein synthesis than with the more severe inhibition of RNA synthesis. The dose-dependent inhibition of protein synthesis by 5-azacytidine was quickly diluted out of a growing culture. Reversal of inhibition was accompanied by a disproportionately rapid increase in the ability of cells to autolyze. Thus, inhibition of the ability of cells to autolyze can be most closely related to inhibition of protein synthesis. Furthermore, the rapidity of the response of cellular autolysis to inhibitors of protein synthesis suggests that regulation is exerted at the level of autolytic enzyme activity and not enzyme synthesis.

Growing cultures of a wide variety of bacterial species have the capacity to autolyze. In several instances, cellular autolysis has been shown to be initiated by an enzymatic attack on the rigid heteropolymer (peptidoglycan or mucopeptide) of the protective, bacterial cell wall. For a variety of reasons, participation of autolytic enzymes in surface growth and division has long been postulated (6, 7, 11, 16, 17), although their precise roles are not yet known. However, any role in a cell division cycle would require mechanisms of regulation and of close coupling to other events in the cell cycle. This regulation and coupling could be via control of a stage of autolytic enzyme biosynthesis, of autolytic enzyme activity, or via some effect at the level of substrate. It would seem likely that a coupling of autolysis to the biosynthesis of one of the informational macromolecules would best facilitate a linking of processes occurring within and without the permeability barrier.

With *Streptococcus faecalis*, ATCC 9790, it was shown by Pooley and Shockman (10) that inhibition of protein synthesis with chloramphenicol (50 to 100 μg/ml), tetracycline (10 μg/ml), or threonine deprivation was accompanied by a very rapid decrease in the ability of exponential-phase cells to autolyze. Ten minutes after chloramphenicol addition, cells autolyzed at about 20% of the control rate. However, isolated walls from both chloramphenicol-treated and threonine-deprived cultures were found to contain nearly the same level of active and latent (proteinase activatable) autolysin for many hours after treatment. This retention of autolysin activity suggested that both forms of the enzyme have a relatively long half-life and that some other mechanism rapidly inhibits the ability of the enzyme to lyse cells. At that time (10), a localized change in the wall substrate at nascent cross wall sites was postulated. These cross wall sites were also shown to be engaged in wall enlargement (5) and to be sites of localized wall dissolution (15).

It seems equally possible that the results of Pooley and Shockman (10) could be accounted for by an effect of a regulatory molecule of the autolytic enzyme system. While such a mole-

cule could control the rate of autolysin activity in intact cells, it would probably be lost during wall isolation procedures. A mechanism of regulation of the *activity* of the autolytic enzyme system would permit the rapid, precise, and reversible changes in activity required for postulated roles of such enzymes in the cell division cycle. Coupling of the activity or availability of such a regulatory molecule to the synthesis of one type of informational macromolecule could serve as a mechanism for the integration of the hydrolytic activity of the autolysin occurring outside of the cellular permeability barrier with those major cellular biosynthetic processes occurring within.

We have now examined the early effects of a series of agents on cellular autolysis and macromolecular synthesis. Concentrations selected to have reasonably specific inhibitory effects on deoxyribonucleic acid (DNA), ribonucleic acid (RNA), and protein synthesis were used. In this way we have been able to link the rapid inhibition of cellular autolysis to the inhibition of continued synthesis of one class of macromolecule.

MATERIALS AND METHODS

Growth of *S. faecalis*, ATCC 9790, on a chemically defined medium was followed turbidimetrically as described previously (14). In all cases experimental treatments were initiated after the culture had undergone at least 8 to 10 doublings in mass at a constant doubling time of 31 to 33 min.

Measurement of the effect of various inhibitors on DNA, RNA, and protein synthesis. The basis of the quantitative method used was the measurement of the change in the kinetics of incorporation into acid-precipitable material of fully equilibrated, radioactively labeled, specific precursors after addition of the inhibitor. In all cases labeled precursor was present for eight to ten doublings in mass before inhibitor addition, so that subsequent incorporation occurred at the same rate as the increase in mass of the culture. The radioactivity measurements are thus truly an index of the cellular rate of synthesis of that particular macromolecular species (12). The effect of inhibitors on the exponential rate of precursor incorporation can then be measured and quantitatively expressed as the percentage of the untreated control over any time interval.

For studies of DNA synthesis, media were supplemented with 15 μg of thymidine per ml and 1.5 μCi of thymidine-*methyl*-^3H per ml; for protein synthesis, media contained 20 μg of L-leucine and 0.1 μCi of L-leucine-^{14}C per ml. For RNA synthesis, media contained 20 μg of uracil and 0.5 μCi of ^{3}H-uracil per ml. All radioisotopes were purchased from New England Nuclear Corp., Boston, Mass. Specificity of the incorporation of ^{3}H-thymidine into DNA was checked by alkali treatment (0.3 M KOH for 16 hr at room temperature).

Antibiotics were added at a turbidity equivalent to a culture mass of 0.13 mg/ml (dry weight). At intervals, 0.5-ml samples were removed and pipetted into 5 ml of ice-cold 10% trichloroacetic acid. Acid precipitates were collected on glass fiber filters (Reeve Angel 984-H glass fiber ultradiscs, Hurlbut Paper Co., South Lee, Mass.) and washed three times with 10% trichloroacetic acid. The discs were transferred to vials, and precipitates were dissolved in 0.5 ml of solubilizer (NCS; Amersham/Searle Corp., Des Plaines, Ill.) for at least 30 min at room temperature, and 5 ml of a toluene base scintillator was added to each vial. Samples were counted in a Mark I liquid scintillation counter (Nuclear-Chicago Corp., Des Plaines, Ill.), and disintegrations per minute were calculated by using an external standard. The efficiencies were approximately 60% for ^{14}C and 25% for ^3H. In dual label experiments, data were corrected for overlap by using external standard channel ratios for each sample (16 to 24% ^{14}C into ^3H). All results from radioisotope incorporation studies are expressed either as percent of control incorporation or as percent inhibition (2). A fuller explanation of this treatment is given below and in the legend to Fig. 1.

Cellular autolysis. Samples of growing cells were pipetted onto ice at regular intervals. The cells were harvested on a nitrocellulose filter (0.65-μm pore size, 47-mm diameter; Millipore Filter Corp., Bedford, Mass.) and washed twice with 5 ml of ice-cold double-distilled water. The filters containing washed cells were immediately placed into screw-cap tubes containing 6 ml of ice-cold 0.01 M or 0.3 M sodium phosphate buffer, *p*H 6.7. The cells were rapidly suspended in the buffer by using a Vortex mixer and stored on ice for no more than 60 min. Cellular autolysis was determined, essentially as described previously, by following loss of turbidity of the cell suspension at 37 C (9). The results of cellular autolysis were computed as first-order reaction rates (in hours^{-1}) determined on the basis of the half-life of the reaction. The half-life was obtained from the steepest portion of the curve which usually showed exponential decay at a constant rate. In most cases, the data are then expressed as percent of the control rate.

Antibiotics were obtained from the following sources: chloramphenicol, Parke Davis and Co.; puromycin, Lederle Laboratories Div., American Cyanamid Co.; 5-azacytidine, Calbiochem; mitomycin C, Nutritional Biochemicals Corp.; phenethyl alcohol, Eastman Organic Chemicals, Eastman Kodak Co.; tetracycline, Charles Pfizer & Co., Inc.; actinomycin D, Merck, Sharp and Dohme Research Laboratories; rifampin, Dow Chemical Co.

RESULTS

Effects of inhibitors of DNA synthesis on the synthesis of informational macromolecules and on cellular autolysis. The effects of mitomycin C (0.4 μg/ml) on the increase in mass and on the incorporation of thymidine, uracil, and leucine, when added to an exponentially growing culture, are shown in Fig. 1A

through D. At this concentration, mitomycin C inhibited the incorporation of thymidine into DNA (Fig. 1A) but had little or no effect on the incorporation of uracil (Fig. 1B) or leucine (Fig. 1C) or, for that matter, on the increase in mass of the culture (Fig. 1D). However, higher concentrations of mitomycin C (1.5 μg/ml) did significantly decrease incorporation of uracil into RNA and leucine into protein (*unpublished data*). To compare the degree of inhibition of synthesis of the various types of macromolecules with each other during exposure of cultures to inhibitors, results can be expressed relative to the untreated control rate (as the percent of incorporation by the untreated control culture over that time interval). When this is done (Fig. 2A), it can be seen that DNA synthesis is rapidly inhibited, whereas protein synthesis remains at the control rate. RNA synthesis (not shown) was only slightly affected (i.e., inhibited to only 60% of the control at 45 min). In the presence of mitomycin C (0.4 μg/ml), the ability of cells to autolyze remained the same as that of the control (Fig. 2A and 3) over the 45-min period of exposure. Similar results were obtained with phenethyl alcohol, another but somewhat less specific inhibitor of DNA synthesis (Table 1). Thus, it seems clear that significant inhibition of DNA synthesis was not accompanied by an inhibition of the ability of cells to autolyze. It is to be noted that the rate of incorporation of ^{3}H-thymidine into DNA paralleled the rate of mass increase (Fig. 1A, D, E, and H).

Effects of inhibitors of protein synthesis on the synthesis of informational macromolecules and on cellular autolysis. Figure 1 shows that within about 5 min tetracycline (0.5 μg/ml) severely inhibited incorporation of leucine into protein (Fig. 1G), whereas it had less

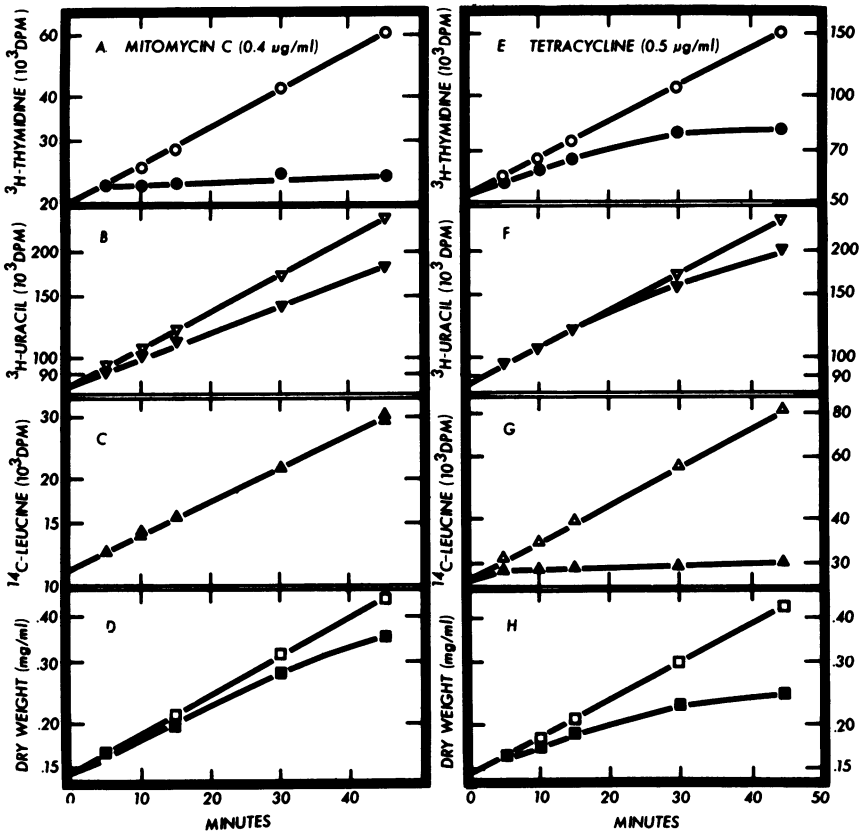

FIG. 1. *Effect of mitomycin C (0.4 μg/ml) and tetracycline (0.5 μg/ml) on macromolecular parameters. Open symbols, untreated controls; solid symbols, antibiotic-treated cultures. Effects of mitomycin C on DNA synthesis (A), RNA synthesis (B), protein synthesis (C), and cellular mass (D). Effects of tetracycline on DNA synthesis (E), RNA synthesis (F), protein synthesis (G), and cellular mass (H). Data from these and similar experiments with other inhibitors were compared by handling the data as follows. Incorporation of precursors from zero time to each sampling time of inhibited cultures was compared to that of untreated controls as percent of control incorporation (×100). Such data are presented in Fig. 2 and 4 and Table 1.*

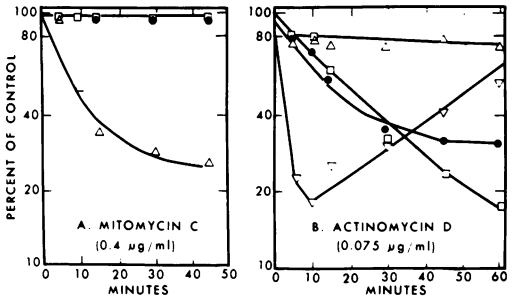

FIG. 2. *The effects of mitomycin C (0.4 μg/ml) (A) and actinomycin D (0.075 μg/ml) (B) on the biosynthesis of macromolecules and on cellular autolysis. RNA (▽), DNA (△), and protein (□). These parameters are expressed as percent of untreated control incorporation. Cellular autolysis (●) is expressed as the percent of the rate of autolysis of untreated cells at the sample time (see Materials and Methods).*

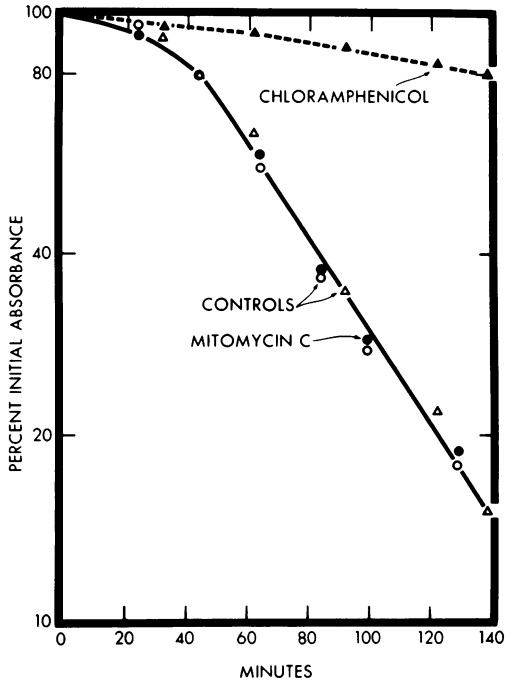

FIG. 3. *The kinetics of cellular autolysis after a 15-min exposure of an exponential-phase culture to mitomycin C (0.4 μg/ml) or chloramphenicol (10 μg/ml). Data are expressed as percent of initial absorbance. Open symbols, untreated controls; solid symbols, treated cultures. Chloramphenicol (triangles) and mitomycin C (circles).*

severe and more delayed inhibitory effects on RNA (Fig. 1F) and DNA (Fig. 1E) synthesis and on the increase in cellular mass (Fig. 1H). The same concentration of tetracycline rapidly inhibited the ability of cells to autolyze. As shown in Fig. 4A, 15 min after tetracycline addition the rate of cellular autolysis was reduced to 5% of the control rate. When compared with the degree of inhibition of synthesis of informational macromolecules, significant inhibition of cellular autolysis was observed when little inhibition of DNA or RNA synthesis was seen (e.g., at 10 to 30 min when only protein synthesis was significantly inhibited). Similar results were obtained with chloramphenicol treatment at 10 μg/ml (Fig. 3 and 4B and Table 1), although, with this antibiotic, it was not possible to avoid inhibition of RNA and DNA synthesis and still observe rapid and profound inhibition of protein synthesis. Also, puromycin (250 μg/ml, Table 1) and 5-azacytidine (5 μg/ml, Fig. 4C and Table 1) both relatively selectively and rapidly inhibited protein synthesis and cellular autolysis 15 min after their addition, whereas their effects on RNA and DNA synthesis were significantly less.

TABLE 1. *Effects of inhibitors of protein, DNA, and RNA synthesis on cellular autolysis*[a]

Inhibitors of:	Percent of control at 15 min			
	DNA synthesis	RNA synthesis	Protein synthesis	Cellular autolysis
Protein synthesis				
Chloramphenicol (10 μg/ml)	39	50	17	16
5-Azacytidine (5 μg/ml)	80	100[b]	13	15
Puromycin (250 μg/ml)	76	78	4	13
Tetracycline (0.5 μg/ml)	64	100	7	5
DNA synthesis				
Mitomycin C (0.4 μg/ml)	29	76	100	99
Phenethyl alcohol (3 mg/ml)	62.5	28	50	75
RNA synthesis				
Actinomycin D (0.075 μg/ml)	75	25	60	57
Rifampin (0.01 μg/ml)	70	30	35	26

[a] The antibiotics are grouped according to primary target of activity, i.e., DNA, RNA, or protein synthesis. Values represent percent of control incorporation of ^{3}H-thymidine, ^{3}H-uracil, and L-leucine-^{14}C for DNA, RNA, and protein synthesis, respectively, at 15 min of exposure. Cellular autolysis is also expressed as percent of control. All calculations are as described in Materials and Methods and in the caption to Fig. 1.

[b] Determined as in Fig. 5.

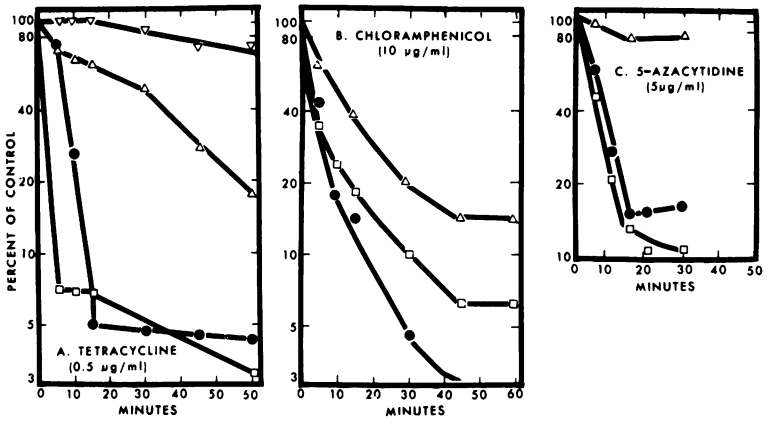

FIG. 4. *The effects of tetracycline (0.5 µg/ml) (A), chloramphenicol (10 µg/ml) (B), and 5-azacytidine (5.0 µg/ml) (C) on the biosynthesis of macromolecules and on cellular autolysis. The data have been expressed as in Fig. 2. Symbols are as in Fig. 2.*

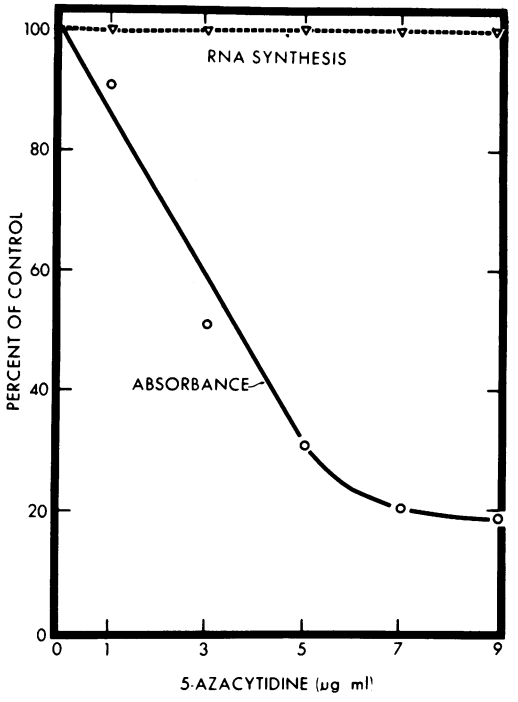

FIG. 5. *Effect of 66 min of exposure to various concentrations of 5-azacytidine on mass increase and RNA synthesis. Results are expressed as percent of control. Absorbance (O), RNA (▼). Because of the nature of the pyrimidine analog 5-azacytidine, with which uracil competes, RNA was determined by the orcinol reaction (1). At 66 min, the control yielded 0.127 mg of ribose per mg (dry weight) of cells.*

5-Azacytidine proved to be a most interesting, extremely selective, and, at specific concentrations, reversible inhibitor of protein synthesis. When added to exponentially growing cultures of *S. faecalis*, this pyrimidine analogue showed a dose-dependent inhibition of the increase in mass of the culture which, after a period of time, reversed itself, and growth resumed. As shown in Fig. 5, the extent of growth inhibition 66 min after addition of the compound was proportional to its concentration. Note also that at all concentrations tested RNA synthesis (in this case measured by the orcinol method [1]) was not significantly affected. 5-Azacytidine at 5 µg/ml (Fig. 4C) caused little inhibition of DNA synthesis, but resulted in very rapid inhibition of both protein synthesis and of the ability of cells to autolyze.

In contrast to the results observed with inhibitors of DNA synthesis, it seems clear that a variety of agents, at concentrations which selectively and rapidly inhibit protein synthesis, also result in a rapid decrease in the ability of cells to autolyze. In addition, as can be seen in Fig. 4, the rates of decay of the abilities to incorporate amino acids into protein and to autolyze closely approximate each other. For example, the half-times for cellular autolysis are 6, 4, and 5 min and for protein synthesis are, 2, 5, and 6 min for tetracycline, chloramphenicol, and 5-azacytidine, respectively.

Effects of inhibitors of RNA synthesis. We were unable to find concentrations of either of the RNA synthesis inhibitors tested (actinomycin D and rifampin) which would significantly inhibit the incorporation of uracil into RNA and not also inhibit protein synthesis. This is thought to be due to the close coupling of RNA and protein synthesis in *S. faecalis*. However, even with these inhibitors, quantita-

tive correlations could be made between degree of inhibition of protein, rather than RNA or DNA synthesis, and cellular autolysis. As shown in Fig. 2B, actinomycin D at 0.075 μg/ml inhibited RNA synthesis rapidly during the first 10 to 15 min, but the degree of inhibition reversed after that time. Protein synthesis inhibition occurred more gradually, and the decay in ability of cells to autolyze more or less paralleled inhibition of protein synthesis, rather than inhibition of RNA synthesis. DNA synthesis was not significantly inhibited. A similar correlation was observed with rifampin at 0.01 μg/ml (Table 1).

Kinetics of recovery from 5-azacytidine inhibition. Because the effect of 5-azacytidine was highly selective for protein synthesis, advantage was taken of the reversibility of this inhibition with time in order to compare the rates of recovery of the ability of cells to autolyze with their ability to synthesize protein. As shown in Fig. 6, addition of 5 μg of 5-azacytidine per ml rapidly inhibited cellular autolysis, protein synthesis, and the increase in cellular mass. Approximately 20 min after 5-azacytidine addition, the ability of cells to autolyze began to increase, while little new protein was being made. Thus, it can be seen that between about 20 and 80 min after exposure to the antibiotic, the rate of increase in cellular autolysis was equivalent to a doubling time (T_D) of approximately 20 min, whereas the T_D for protein synthesis was approximately 80 min. The doubling time for the latter parameter returned to nearly the same rate observed before 5-azacytidine addition (T_D = 31 to 33 min) at about 80 min, when the ability of cells to autolyze had returned to the rate that was observed prior to antibiotic addition. After this time all further increases in protein, cell mass, and cellular autolytic activity occur in parallel, as would be expected in a balanced culture. Similar, although less clear-cut, results have been obtained from studies of recovery from chloramphenicol (10 μg/ml) and rifampin (0.01 μg/ml) inhibition. In all cases, cellular autolytic activity recovered earlier and more rapidly than protein synthesis.

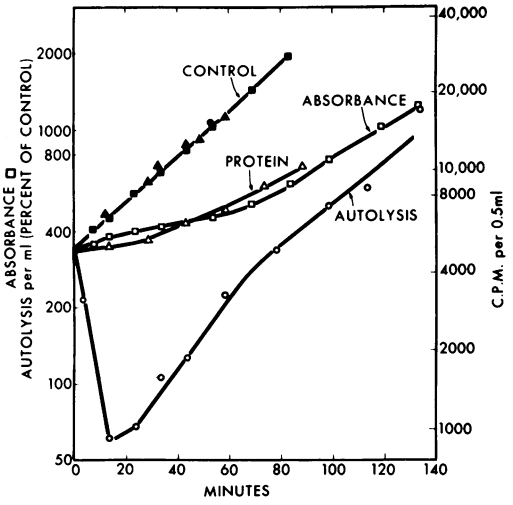

FIG. 6. *Reversal of the inhibition of protein synthesis and cellular autolysis after 5-azacytidine addition. At zero time 5-azacytidine (5 μg/ml) was added to an exponentially growing culture of Streptococcus faecalis (open symbols). Parallel, untreated controls (solid symbols) are also shown. Protein synthesis (triangles) was measured by the incorporation of fully equilibrated L-leucine-^{14}C. Absorbance of the cultures (squares) was followed and is expressed as 10^3 OD_{675}. At the times indicated, samples of cells were removed, and their ability to autolyze was determined (see Materials and Methods). In this case, the results of cellular autolysis (circles) are expressed as the fraction of the zero time rate multiplied by absorbance. This was done to express cellular autolysis in the same terms as the other parameters (i.e., per milliliter of culture).*

DISCUSSION

From these results it seems clear that there is a closer association of cellular autolysis with protein synthesis than with the biosynthesis of any other type of macromolecule. A relationship between RNA synthesis and cellular autolysis appears to be eliminated by the use of inhibitors such as puromycin, tetracycline, and 5-azacytidine which inhibited cellular autolysis and protein synthesis without significantly inhibiting RNA synthesis. Additionally, even in the presence of inhibitors of RNA synthesis, inhibition of cellular autolysis did not parallel the inhibition of RNA synthesis but, rather, more closely paralleled inhibition of protein synthesis. The correlation between inhibition of protein synthesis and cellular autolysis was perhaps most strikingly clear when inhibitors of protein synthesis were used. In all such cases examined, rapid and profound inhibition of protein synthesis was accompanied by a rapid and profound inhibition of cellular autolysis.

The correlation between protein synthesis and cellular autolysis cannot be due simply to the inhibition of synthesis or of activation of the autolytic enzyme. The observed, rapid decrease in the ability of cells to autolyze would require not only inhibition of further synthesis of enzyme protein but also a half-life of less than 5 min for previously made enzyme molecules. However, both active and latent auto-

lytic enzyme activities were somewhat unexpectedly found to have extremely long half-lives, exceeding several cell generation times (10). Similar levels of both active and latent autolysin activities were found in isolated walls from exponential-phase, chloramphenicol-treated, and threonine-deprived cells (10). This observation is inconsistent with regulation at the level of autolysin activation.

The very rapid recovery of cellular autolysis and slower recovery of protein synthesis, approximately 20 min after 5-azacytidine inhibition, is not consistent with a model based on regulation at the level of enzyme synthesis. Instead, the data obtained during reversal of the 5-azacytidine inhibition are more consistent with a rapidly reversible effect on autolytic enzyme *activity*. This hypothesis is further supported by the observation of a return to a balanced increase in cellular autolytic activity proportional to the rate of increase of cellular mass when the activity reached about the same level as that present at the time of antibiotic addition.

On the basis of these results we have postulated the presence of a substance which is capable of inhibiting the activity of the autolytic enzyme at its exocellular, cell wall site. By some unknown mechanism, this inhibitory substance would become more available for reacting with the autolysin upon inhibition of protein synthesis. Evidence for the presence of a low-molecular-weight compound in *S. faecalis* which can inhibit cellular autolysis has recently been obtained (M. Sayare, L. Daneo-Moore, and G. D. Shockman, Abstr. Annu. Meet. Amer. Soc. Microbiol., p. 48, 1972). The existence of such a regulatory compound would be useful to the cell in order to rapidly and precisely control lytic enzyme activity during the cell division cycle (6).

Evidence for the lack of a coupling of inhibition of DNA synthesis and decay of cellular autolysis in the *S. faecalis* system was also obtained. These results appear to rule out a model for the regulation of cellular autolysis activity based on DNA synthesis, but not on the initiation of DNA replication. Our findings contrast with those of Schwarz et al. (13) who found a correlation between autolytic activity and DNA synthesis in *Escherichia coli* and whose results may be explained by any of several possibilities.

Schwarz et al. (13) did not report on the specificity of the relatively high concentration of mitomycin C (10 μg/ml) or of the temperature-sensitive DNA synthesis mutant used. In light of our results, it seems possible that, in both cases, some inhibition of protein synthesis might have occurred and have been responsible for the effects on cellular autolysis. It is perhaps equally, if not more, likely that the mechanism of regulation of cellular autolysis and surface growth in the rod-shaped *E. coli* differs from that of the coccal-shaped *S. faecalis* (6).

Recent evidence suggests that a major role for bacterial autolytic enzymes is the hydrolysis of bonds in selected areas of the wall in order to permit morphogenetic changes during the cell cycle; this may include changes in cell shape, cell division, and the final step of separation at a completed cross wall to yield two daughter cells (6, 11). Evidence includes the finding that an autolytic defective mutant of *Bacillus licheniformis* tends to form chains (4) as well as the ability of either *Bacillus subtilis* autolysin or hen egg-white lysozyme to considerably reduce the chain length of *B. subtilis* growing at 48 C (3). Such evidence, that the final stages of cell division depend on the activity of autolytic enzymes, prompted Paulton (8) to suggest that, in *B. subtilis*, "a variation in their [autolytic enzymes] rate of synthesis or activity could be responsible for the unicellular or filamentous structure."

Autolytic enzyme systems are quite likely regulated at several levels. Candidates for regulatory steps include autolysin synthesis, proteinase activation, transport of enzyme or activator (or both), and substrate effects (6). Regulation at any of these levels would probably be too slow or gradual to provide the necessary variations postulated for a role in the cell division cycle. However, control of the activity of the autolysin system, coupled to protein synthesis, provides the required speed and reversibility.

ACKNOWLEDGMENTS

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