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Relationship between the Latent Form and the Active Form of the Autolytic Enzyme of *Streptococcus faecalis*

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A 10-hr starvation of *Streptococcus faecalis* ATCC 9790 for the amino acids methionine and threonine results in cells which are resistant to autolysis and which contain greatly reduced quantities of both active and latent (proteinase activable) forms of the autolytic enzyme (an *N*-acetyl-muramide glycanhydrolase). Cell walls were isolated from cells harvested at various times during the recovery from such starvation and were assayed for active and latent forms of the autolysin. Within 10 min of recovery the latent enzyme began to increase. Only after 30 to 60 min did the active enzyme begin to increase; after a similar lag, the cells' proneness to lysis markedly increased. The intracellular localization of both forms of the autolysin was examined, using as an experimental tool the ability of added cell wall to bind autolysin. ^{14}C-lysine-labeled, inactivated cell walls were added to exponential-phase cells, which were then disrupted, and the mixed wall population was isolated. Measurement of the ^{14}C release during wall autolysis indicated that the active enzyme in the cells was not available for binding to the added ^{14}C-labeled walls and was therefore wall-bound in vivo. In contrast, up to 85% of latent autolysin activity was found to have been efficiently bound to the added ^{14}C walls. The results obtained suggest (i) cellular autolysis is a reflection of the level of active enzyme and not of latent enzyme, and (ii) autolysin is synthesized and mainly located in the cytoplasm as an inactive latent precursor (proenzyme) which is transported to sites on the cell wall associated with wall biosynthesis, where it becomes activated.

The autolysin of *Streptococcus faecalis* ATCC 9790, an *N*-acetylmuramide glycanhydrolase (EC 3.2.1.17; 15), was previously found to be almost exclusively associated with the cell wall fraction in both active and latent (proteinase activable) forms (3, 15). The latent form was found to be present in four to five times the amount of the active form (15). Active autolysin was preferentially associated with the recently synthesized portion of the wall, whereas the latent form appeared to be randomly located in the isolated, purified cell wall fraction (13). By using the fluorescent antibody labeling technique, it was shown that wall growth in *Streptococcus pyogenes* (2) and *S. faecalis* (R. Cole, *personal communication*) was localized at specific rings or bands at the coccal equator, the region where the new septum is formed. In rapidly growing, exponential-phase cultures, two new bands, located midway between the coccal equator and the poles, were often initiated before the previous cross wall was completed. Electron microscopy observations (14) of cell walls undergoing autolysis were consistent with both the autolysin localization experiments (13) and the fluorescent antibody experiments (2) in concluding that the active autolysin was mainly concentrated in the localized bands of wall synthesis. The association of active autolysin with areas of the wall in which active biosynthesis is taking place is consistent with a role for the active enzyme in providing new sites for the addition of the biosynthetic precursors to the existing wall peptidoglycan polymers. Until now, the random location of latent autolysin was more difficult to reconcile with a role in peptidoglycan biosynthesis.

In this work, we have concerned ourselves with the following: (i) the significance of the latent autolytic activity, (ii) the relationship of latent autolytic activity to the active enzyme, and

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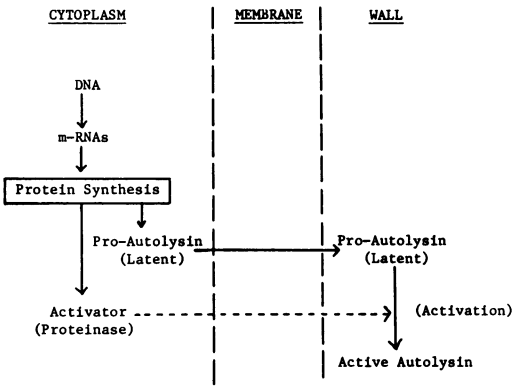

FIG. 1. *Possible relationship between the latent and active forms of autolytic enzyme. The latent form is shown as a precursor (proenzyme) of the active form. Activation of the latent enzyme, by means of an activating enzyme (proteinase), takes place after the enzyme has passed through the membrane and become bound to the cell wall.*

(iii) the meaning of the different locations of the two activities in the isolated cell wall fraction.

It seemed possible that the latent form was a biosynthetic precursor of active autolysin. Figure 1 presents a possible relationship of latent to active autolysin which has been used as a guide for these studies. In this scheme, autolysin is considered to be synthesized in the cytoplasm as an inactive precursor (proautolysin) which is activated by a native proteinase (activator) during, or more likely after, its transport through the cytoplasmic membrane to wall synthetic sites. Data have been obtained supporting the conclusion that protein synthesis is necessary for the activation step (*unpublished data*). Alternatively, it is possible that autolysin is synthesized and perhaps even transported to the wall sites as the active form. After its action at the wall biosynthetic sites, a portion, or perhaps all, of the active form might then be inactivated. This second scheme would imply that the activation of the latent form in vitro is fortuitous, but is easier to reconcile with a random location on the wall of the latent form.

The relationship of latent to active autolysin was investigated by observing, kinetically, the increases of latent and active enzyme which occur in cells recovering from a prolonged deprival of an amino acid which is not a component of the wall peptidoglycan. Deprival of one or more non-peptidoglycan amino acids results in cells that are highly resistant to autolysis (10, 11) and which contain reduced levels of both active and latent autolysin in their walls (3, 15). If latent autolysin is a precursor of the active form, as shown in the scheme (Fig. 1), upon recovery from starvation, the cellular content of latent autolysin should rise before that of the active form. In addition, at least some latent activity might be detected in a soluble form, before it becomes wall-bound. The latter possibility was examined by taking advantage of the high affinity of the wall substrate for autolysin (9, 13). The intracellular location of both autolysin forms in intact cells was investigated by adding inactivated walls to cells before disruption and measuring the ability of the added walls to compete with native walls for the binding of any unbound active or latent autolysin.

MATERIALS AND METHODS

Cell growth, amino acid starvation, and recovery from starvation. Cultures of *S. faecalis* were grown at 37 C as previously described (19) in a completely synthetic medium containing growth-limiting concentrations of both methionine (8.1 μg/ml) and threonine (8.8 μg/ml). These concentrations of the two amino acids resulted in their almost simultaneous exhaustion at a turbidity equivalent to 0.39 mg (dry weight) of cells per ml. The cultures were harvested by centrifugation 9 to 10 hr after the exhaustion of both amino acids, and the cells were suspended in prewarmed, complete growth medium (recovery medium) at an initial turbidity equivalent to 0.16 mg (dry weight) of cells per ml. These new cultures were incubated at 37 C and growth was observed turbidimetrically. At intervals, samples were removed, either to test the ability of the cells to autolyze or for the preparation of cell walls. This procedure was based on a method used to obtain synchronized cultures of *S. faecalis* (16).

Cell autolysis. All operations were carried out at 0 to 4 C unless otherwise specified. Samples (5 to 10 ml) of cells [approximately 0.2 to 0.4 mg (dry weight) per ml] were pipetted onto ice. The cells were rapidly harvested by filtration through a 47-mm membrane filter (0.65 μm pore size, Millipore Corp., Bedford, Mass.), and washed three times with 10 ml of distilled water. Cells were suspended in 6 ml of 0.01 M sodium phosphate (*p*H 6.7) by placing the filter in a 25 by 150 mm screw-capped tube and mixing vigorously with a Vortex mixer (Vortex Mixer Co., Lawrence, Mass.). Cell autolysis at 37 C was observed turbidimetrically as previously described (9).

Autolysis of *S. faecalis* in 0.01 M phosphate buffer is not usually linear (9), making it difficult to quantitate accurately and to compare the autolysis of cell populations at different stages of growth. Therefore, several ways of expressing autolysis were used. These included (i) the time taken to reach 50% of the initial cellular turbidity, (ii) the per cent of the initial turbidity which remained after a fixed time interval, and (iii) the rate of decrease in turbidity during the central, nearly linear, and most rapid portion of the autolysis curves. Although none of these methods is ideal, when their results were plotted against the time of incubation during recovery from amino acid starvation (e.g., Fig. 2 and 3), a rapid increase in the proneness of cells to autolyze was observed to occur at almost the same time. The results of cell autolysis presented below are expressed as in method iii above.

One unit of cell autolysis is defined as the loss of 0.001 optical density (OD) per hr. To compare the autolysis of different samples, results are expressed in units of autolysis per 0.1 OD of cell suspension. (One unit is then equivalent to a loss of 1% of the initial turbidity per hour.)

Cell wall preparation. Samples, containing 15 to 80 mg (dry weight) of cells, were poured onto ice and harvested by centrifugation. Cells were broken by shaking 15 to 20 ml of cell suspension in distilled water with an equal volume of plastic beads (styrene divinylbenzene copolymer, 18 to 50 mesh, Dow Chemical Co., Midland, Mich.) in the Braun model MSK mechanical cell homogenizer for 10 to 15 min at 2,000 oscillations/min. The cell wall fraction was isolated and washed as previously described (15).

Competitive binding experiments. ^{14}C-labeled cell walls were isolated from exponential-phase cultures grown in the continuous presence of ^{14}C-L-lysine (Nuclear-Chicago Corp., Des Plaines, Ill.) at a specific activity of approximately 0.4 μc of L-lysine per mg as previously described (15). The autolytic activity of such walls (or of similarly prepared unlabeled walls) was inactivated by treatment with 2% sodium decylsulfate (SDS; 15). Known quantities of SDS-walls were thoroughly mixed with suspensions of unlabeled cells (or cells labeled with ^{14}C-lysine as above). The cell walls were then prepared from the mixtures of cells and inactivated walls as described above. Control mixtures were obtained by adding SDS-walls to cell wall fractions isolated from the cells broken in the absence of any added cell walls. The extent of binding to the added ^{14}C walls was determined by the release of ^{14}C from these walls during assay for active and total autolysin activities.

Wall lysis. Walls [0.2 to 1.0 mg (dry weight) per ml] were allowed to autolyze in 0.01 M phosphate buffer (*p*H 6.7) in the presence and in the absence of trypsin (1 μg/ml) as previously described (15). Autolysis of walls is usually linear, particularly in the presence of trypsin. One unit of autolysin has been defined as that causing a loss of 0.001 OD per hr (15). To compare the concentration (or specific activity) of autolysin in different samples of cell walls, results are expressed as units of enzyme per 0.1 OD of cell wall suspension [equivalent to 175 μg per ml (dry weight)]. A unit is then equivalent to a loss of 1% of the initial turbidity per hour. The concentration of active autolysin in a cell wall preparation was measured in the absence of proteinase. Total autolysin was measured in the presence of trypsin (1 μg/ml). The amount of latent autolysin present was obtained by subtracting active enzyme from total autolysin in the same wall preparation. A major difference in sensitivity of active or latent autolysin to inactivation during wall preparation was not observed. Precautions were taken to minimize such inactivation.

Radioactivity measurement. Release of "soluble" radioactivity (not sedimented at 30,000 $\times$ *g* for 30 min) during autolysis was measured as previously described (13). In most cases, scintillation gels were used for counting of radioactivity. The gels had the following composition: Naphthalene, 100 g; 2,5-diphenyloxazole, 7 g; 1,4-bis-2-(5-phenyloxazolyl)-benzene, 0.7 g; Cab-o-sil (a superfine form of silica; Cabot Corp., Boston, Mass.), 40 g; and dioxane to a total volume of 1 liter. After addition of sample (100 to 250 μliters) to 10 ml of the scintillation fluid, addition of 2 drops (about 0.1 ml) of 1 N NaOH and vigorous shaking for several seconds resulted in a transparent gel. Counting was performed in a Nuclear-Chicago Mark I scintillation counter at 10 C, at which temperature gels showed no tendency to freeze. At 5 C, samples tended to freeze; however, a significant change in count rate was not observed even in frozen samples.

The gel procedure gave stable counting rates over several days. This scintillation system was preferred to that described by Bray (1961) which had been used previously (15). When Bray's solution was used, a loss in counting rate of at least 10% over a period of 24 hr was observed. This was presumed to be due to settling of radioactivity to the bottom of the vial with a consequently less efficient conversion of β-emissions to scintillations, since swirling restored the original count rate. The background for ^{14}C in such gel preparations in glass vials was approximately 20 counts/min.

RESULTS

Ability of cells recovering from amino acid starvation to autolyze. After the 10-hr starvation for methionine and threonine, cells autolyzed at a rate of 1 to 2 units compared with about 100 units for exponential-phase cells. During recovery from starvation, cells regained their ability to autolyze in a characteristic manner. In the experiment shown in Fig. 2, for example, the ability of

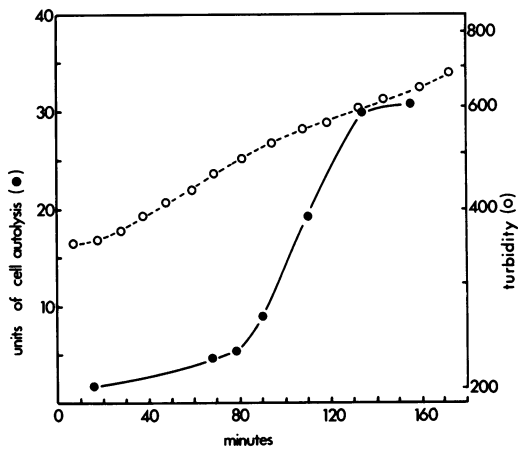

FIG. 2. *Increase in proneness of cells to autolysis during recovery from amino acid starvation. A culture starved 9 to 10 hr for threonine and methionine was harvested and suspended in complete medium. The culture turbidity* (○) *at 675 nm was observed. Adjusted optical density is turbidity adjusted to agree with Beer's law (18). Samples (5 ml) were removed at intervals from the culture and tested for their proneness to autolysis* (●). *(One unit of cell autolysis is equivalent to a loss of 1% of initial turbidity per hour.)*

cells to autolyze increased slowly during the first 80 min and then increased quite abruptly. Between 80 and 134 min, the cellular autolysis rate increased about fivefold whereas the turbidity increased by only about 20%. Similarly, in the experiments shown in Fig. 3, the cellular autolysis rate remained even lower (less than 1% of the rate for exponential-phase cells) until about 40 min, when it abruptly began its rapid rise. In nine other experiments, the abrupt increase in the ability of cells to autolyze was observed between 30 and 70 min after the beginning of recovery from amino acid starvation.

Sequence of appearance of active and latent forms of autolysin during recovery from amino acid starvation. During the recovery period, samples of cells were harvested and cell walls were isolated and assayed for active and latent autolysin. In a typical experiment (Fig. 3), walls from the zero time sample contained about five units of active autolysin and about 12 units of the latent form, about 25% and 15%, respectively, of their activities in walls from exponential-phase cells. The low value for active autolysin was maintained for about 30 min, after which it abruptly began to increase. This rise began about 5 min before that of the increase in sensitivity of cells to autolysis. During this first 30 min, while the activity of the active form was low and relatively constant, activity of the latent form increased, resulting in a doubling of latent autolysin to about 25 units at 30 min. The net result was an increase in the ratio of latent to active autolysin from slightly over 2 at the beginning of the recovery period to 4, at the time when active autolysin began to rise. [Four is the approximate ratio previously found (15) in walls from exponential-phase cells.] In two other similar experiments (*not shown*), the latent form increased well before that of the active enzyme and the abrupt rise in active enzyme occurred 33 to 36 min and 55 to 60 min after recovery from starvation, respectively. In both experiments, the abrupt rise in autolysis began within 8 min of the increase in active autolysin.

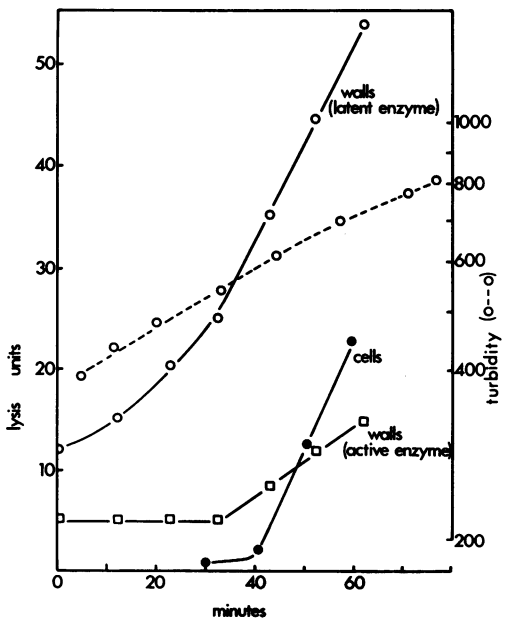

FIG. 3. *Increase in the levels of active and latent autolysin during recovery from amino acid starvation. Culture turbidity* (○) *and cell autolysis* (●) *were followed as in the experiment described in Fig. 2. Cell walls were isolated from samples (200 ml) withdrawn at various times during recovery, and assayed for their content of active* (□) *and latent* (○) *autolysin. Lysis units are expressed on the basis of cell or wall mass. (One unit is equivalent to a loss of 1% of the initial turbidity per hour.)*

Cytoplasmic localization of latent autolysin in intact cells. The results presented above are in accord with the scheme in Fig. 1. The sequence of events strongly suggests that the latent form is a precursor of active autolysin. The high affinity of autolysin for the wall substrate (9, 13) called for a reexamination of the finding that almost all of both forms of the enzyme are located in the isolated cell wall fraction (13, 15). It was possible that the wall-bound location of autolysin was an isolation artifact. Only active or latent enzyme that is not already wall-bound in the intact cell is free to bind to (and hydrolyze) added wall substrate. Therefore, ^{14}C-SDS-walls, added to cells before disruption, would compete with "native" walls only for the binding of that portion of autolysin which was not wall-bound and, therefore, would be released, at least momentarily, during subsequent manipulations. ^{14}C-SDS-walls were unable to compete with "native", unlabeled walls for the active form of the autolysin, even when added before cell disruption. For example, a 30% loss of the initial turbidity of the wall mixture was not accompanied by a significant release of ^{14}C (Table 1, experiment I). This was almost identical to the results obtained with the control mixture. Assay for total autolysin indicated that, in contrast to active enzyme, the ^{14}C-SDS-walls successfully competed for the latent activity. When 30% of the initial turbidity of the wall mixtures had been lost, 18 to 80% of the total ^{14}C in the SDS-walls was found in the supernatant fluid (Table 1, total enzyme activity). In all experiments where the binding of total autolysin was measured, release of radioactivity from the ^{14}C-SDS-walls began almost immedi-

TABLE 1. *Release of radioactivity during wall autolysis from ^{14}C-labeled, inactivated walls mixed with intact cells before cell disruption*

Expt	Per cent ^{14}C-labeled wall in mixture[a]	Per cent release of ^{14}C during autolysis with							
		Active enzyme[b] at loss in turbidity of			Total enzyme[c] at loss in turbidity of				
		10%	20%	30%	10%	20%	30%	40%	50%
I	6	0	0	0	5	12	18	25	
Control[d]	6	0	0	0	0	0	1		
IIA	3	0	<5		16	33	52	78	100
IIB	8	0	<5		13	24	36	48	60
IIC	16	0	<5		25	54	80	100	
Control[d]	36	0	<10		0	3	7		

[a] Obtained from the specific activity of each wall mixture (dpm/mg) relative to the specific activity of the ^{14}C-labeled walls alone. The mass of a sample of each wall mixture was estimated from the turbidity measurement at 450 nm.

[b] Autolysis of walls in the absence of trypsin.

[c] Autolysis of walls in the presence of trypsin (1 μg/ml).

[d] Controls consisted of ^{14}C-SDS-walls mixed with unlabeled walls after cell disruption and wall isolation.

ately. In experiments IIA, IIB, and IIC (Table 1), ^{14}C release was more rapid than was the loss of turbidity. All of the ^{14}C present in the added SDS-walls was released when 50% of the turbidity in experiment IIA and 40% in experiment IIB had been lost. The ^{14}C-labeled walls added before cell disruption had, therefore, acquired a greater specific activity of total autolysin than the "native" walls. These results suggest that in competing for latent autolysin the added walls are more effective than the "native" walls by a factor of about 2.

The fraction of latent autolysin that is wall-bound in vivo. From the experiments described above, it was concluded that in the intact cells, at least part of the latent enzyme was not wall-bound. To obtain an estimate of the fraction of latent enzyme that was not bound, the following experiment was performed. Various amounts of unlabeled SDS-walls (from 0 to 56 mg) were each mixed before cell disruption with a constant amount of freshly harvested, exponential-phase cells (approximately 25 mg, dry weight) grown continuously in the presence of ^{14}C-L-lysine. The active and total autolytic activities that were bound to the ^{14}C-labeled walls in the isolated cell wall mixtures were assayed by observing the release of radioactivity during autolysis. The proportion of the ^{14}C-labeled walls in the isolated wall mixtures ranged from 7.5 to 100% of the total. The addition of all quantities of unlabeled SDS-walls before cell disruption failed to change significantly the rate of release of ^{14}C by the action of active autolysin (Fig. 4). This is consistent with the virtually exclusive wall-bound localization of

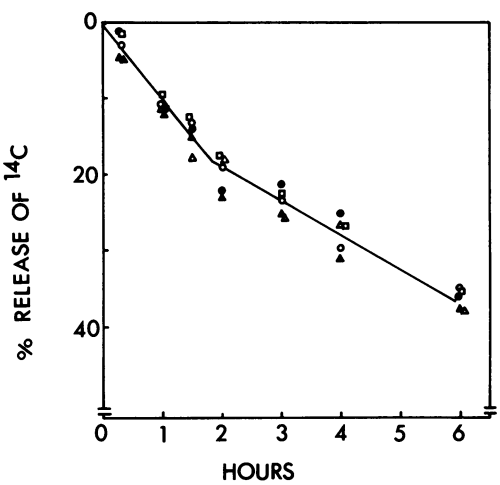

FIG. 4. *Ability of increasing quantities of unlabeled, SDS-walls to compete with ^{14}C-labeled, "native" walls for the binding of active autolysin. Samples (25 mg which contain about 6 mg of wall) of ^{14}C-L-lysine-labeled, exponential-phase cells were mixed with zero (●), 3.4 (△), 7.0 (▲), 21 (○), and 56 (□) mg of unlabeled SDS-walls. The ^{14}C-labeled cells plus SDS-walls were disrupted and the mixed wall fraction isolated. The kinetics of release of ^{14}C from the "native" walls due to the action of active autolysin is expressed as the per cent of total ^{14}C present in each sample. From the specific activity (dpm per mg) each wall mixture was found to contain the following percentage of ^{14}C wall: ●, 100; △, 56; ▲, 42; ○, 21; and □, 7.5. The relatively small differences between actual and theoretical percentages (100, 60, 46, 22, and 10%, respectively) were attributed to not quite complete disruption of the ^{14}C-labeled cells in some of the samples.*

active autolysin described above. In contrast, in the assay for latent autolysin action, the rate of ^{14}C release decreased as the fraction of unlabeled SDS-walls increased (Fig. 5) in a manner approximately proportional to the quantity of added, unlabeled wall present (Fig. 6). However, the curve for latent autolysin (Fig. 6) does not pass through the origin when extrapolated. The finite range (8 to 9 units) of ^{14}C release at 0% ^{14}C-labeled walls represents about 15% of the maximal rate (53 units) in the absence of added walls, and suggests that about 85% of the cellular latent autolysin was not wall-bound in the exponential-phase cells before cell disruption. These results are consistent with the scheme in

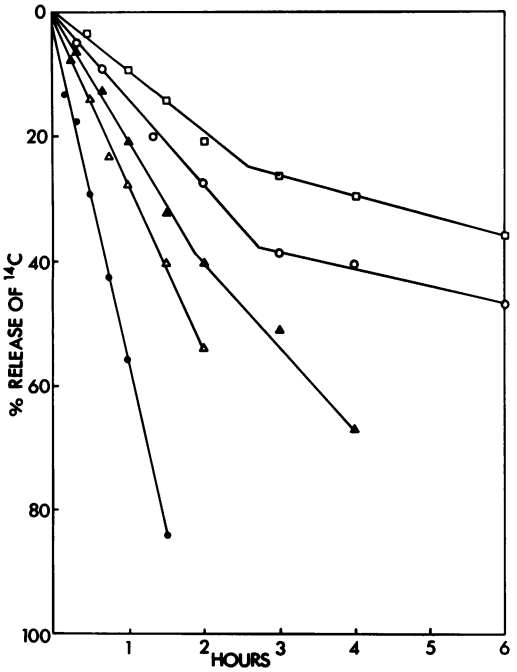

FIG. 5. *Ability of increasing quantities of unlabeled, SDS-walls to compete with ^{14}C-labeled, "native" walls for the binding of latent autolysin. Samples (25 mg which contain about 6 mg of wall) of ^{14}C-L-lysine-labeled, exponential-phase cells were mixed with zero (●), 3.4 (△), 7.0 (▲), 21 (○), and 56 (□) mg of unlabeled SDS-walls. The ^{14}C cells plus SDS-walls were disrupted and the mixed wall fraction isolated. The kinetics of release of ^{14}C from the "native" walls due to the action of latent autolysin is expressed as the per cent of total ^{14}C present in each sample. From the specific activity (dpm per mg) each wall mixture was found to contain the following percentage of ^{14}C wall: ●, 100; △, 56; ▲, 42; ○, 21; and □, 7.5. The relatively small differences between actual and theoretical percentages (100, 60, 46, 22, and 10%, respectively) were attributed to not quite complete disruption of the ^{14}C-labeled cells in some of the samples.*

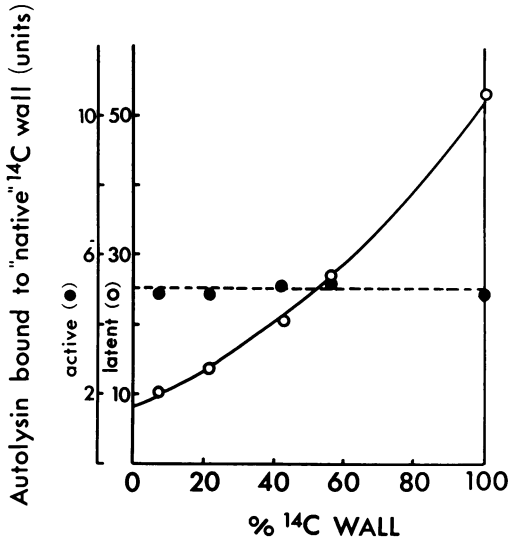

FIG. 6. *The relation between the proportion of ^{14}C-labeled walls and the specific activity of active (●) and latent (○) enzyme bound to ^{14}C walls in mixtures of ^{14}C-labeled and unlabeled cell walls in the experiment described in Fig. 5 and 6. Units of activity based on the per cent release of radioactivity are defined in an analogous way to units based on per cent loss of initial turbidity. [The release of radioactivity parallels the release of turbidity in the autolysis of uniformly labeled walls (11)].*

Fig. 1 in which the active autolysin is found only on the wall, whereas the bulk of latent enzyme is located in the cytoplasm.

In the experiments described in Fig. 4–6, the amount of both latent and active forms of autolysin was the same in all wall mixtures. Since the active form is bound to, and acts only on, the labeled wall substrate, the presence of unlabeled walls does not affect its action (^{14}C release). On the other hand, two populations of walls containing latent autolysin are present. The first population is the ^{14}C-labeled walls which contain "natively bound" activity plus latent activity which is bound during cell disruption. The second is the unlabeled added walls which contain only latent activity which is bound during cell disruption. The first activity was determined by ^{14}C release (Fig. 5 and 6). The concentration of latent autolysin averaged over the entire wall mixture in each tube (first plus second population) was determined from the rates of turbidity loss. In Fig. 7, the averaged level is compared with latent activity that is located only on the ^{14}C walls. The reciprocal of the rate of wall lysis by latent autolysin is plotted against the relative amount of added, unlabeled SDS-walls. The rate of hydrolysis of "native", ^{14}C-labeled walls rapidly

approaches a limiting value as available latent autolysin is diluted over a larger population of unlabeled walls. On the other hand, the rate of hydrolysis of the total population much more slowly approaches a limiting value. In other words, as the quantity of unlabeled walls available to compete with unbound enzyme increases, the fraction of latent autolysin previously bound to the ^{14}C-labeled portion of the population becomes a significantly greater factor in the release of ^{14}C than in the hydrolysis of the total substrate present. These results further support the conclusion that a small but significant portion of latent autolysin is wall-bound in the intact cell. When extrapolated, both lines in Fig. 7 intercept the ordinate between -0.4 to -0.8 mg of added wall per mg of ^{14}C-labeled wall, again suggesting that the added wall is approximately twice as effective as "native" wall in binding soluble latent autolysin.

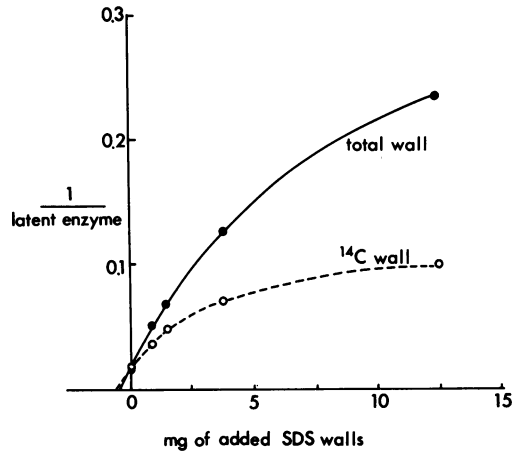

FIG. 7. *The effect of an increasing proportion of added unlabeled inactivated cell walls upon the reciprocal of the concentration of latent activity bound to ^{14}C wall (○) compared with the concentration bound to the total cell wall population (●). The experimental details of the preparation of the cell wall mixtures are described in the legends to Fig. 5 and 6.*

DISCUSSION

The sequence of events which occurred when cells were allowed to recover from starvation for threonine and methionine is consistent with the scheme shown in Fig. 1. The concentration of the latent form of autolysin increased first. In fact, the content of this form began to rise from the beginning of the recovery period (Fig. 3). A two- to fourfold increase in latent form occurred before an increase in the active form was detected. Both the concentration of active autolysin in the cell wall fraction and proneness of cells to autolyze did not increase from their minimal levels until after 30 to 60 min of recovery, when both began to rise almost at the same time. The almost instantaneous increase in both suggests that cellular autolysis is directly related to the level of active, but not to that of the latent, autolysin present in the cell. Although the rate of cellular autolysis increased 10- to 20-fold, walls isolated from such cells showed only a 3- to 4-fold increase in active enzyme. An explanation for this discrepancy may be related to the fact that cultures of this organism possess greatly thickened walls after prolonged starvation for certain amino acids (8). A relatively high concentration of active autolysin in a limited area of cell wall may explain a larger increase in the cell's proneness to autolyze relative to the increase in active enzyme, since only a few sensitive bonds in the rigid wall need to be broken to result in cellular dissolution.

The lack of the ability of walls added before cell disruption to compete for binding of the active form is consistent with the previous observation that almost all of this form of the autolysin is wall-bound in intact cells at sites of new wall synthesis (13). On the other hand, the ability of walls added before cell disruption (but not after) to compete for the bulk of the latent autolysin indicates that the latent form was located elsewhere in intact cells. The earlier finding of all of the latent form in the cell wall fraction randomly located on the isolated wall (13) appears to be due to random binding during cell disruption.

From the experiments shown in the table, it was concluded that the added SDS-walls were more effective in binding latent autolysin by a factor of about 2. It is interesting to note that the intercepts obtained by extrapolating the lines in Fig. 7 gave a similar figure for the relative effectiveness of native and added cell walls in competing for unbound latent enzyme. The explanation may lie in the fact that at the beginning of cell breakage the relative concentration of added SDS-wall to native cell wall is much higher than when breakage is complete. It is also possible that cell walls cleaned with detergent (SDS) possess a greater number of autolysin binding sites.

Our results appear to be best explained by the series of events presented in Fig. 1 where autolysin is synthesized in the cell cytoplasm as a soluble inactive precursor (proenzyme or zymogen). Without invoking the examples of the mammalian digestive enzymes, various zymogens have been reported in bacteria. These include the proteinase of a group A streptococcus (4). It has also been proposed that histidase and tryptophanase in *Escherichia coli* are made as inactive precursors which are activated by limited pro-

teolysis (13). The suggestion has been made that such situations may be more widespread than is currently appreciated (6). Our results support the idea that most of the proenzyme is not bound to the walls but that, when required by the biosynthetic processes, proenzyme is transported to specific sites on the wall where it is then bound and activated by a native proteinase. It is possible that the proenzyme is first activated and then bound to the walls. However, the fact that a small but significant portion of the latent enzyme appears to be bound to the cell wall in the intact cell makes this less likely. The cellular control of this potentially lethal enzyme appears then to rest both in the cellular location of the proenzyme and the necessity of proteinase activation of the wall-bound precursor. It seems highly probable that in growing cells one or more steps in this process are linked to the wall (and cell) biosynthetic process. This connection is currently being investigated.

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