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Mechanism of Excretion of a Bacterial Proteinase: Factors Controlling Accumulation of the Extracellular Proteinase of a *Sarcina* Strain (Coccus P)[1]

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It has been known that the extracellular proteinase of Coccus P is found only in cultures grown in the presence of Ca^{2+}. It is now shown that this cation is required neither for synthesis, excretion, or activation of a zymogen nor as a prosthetic factor necessary for enzymatic activity. The only function of Ca^{2+} is to stabilize the active structure of the enzyme molecule, presumably by substituting for absence of *S-S* bridges. In the absence of Ca^{2+}, the excreted proteinase undergoes rapid autodigestion and, instead of the active protein, its hydrolytic products are accumulated in the culture fluid. In minimal medium and under conditions of enzyme stability [presence of Ca^{2+} and Ficoll (Pharmacia)], Coccus P accumulates the proteinase at a gradually reduced speed although the rate of cultural growth remains constant. It is shown that this decline in rate of accumulation is caused by the excreted proteinase itself, possibly acting on its own precursor emerging from the cell in a form susceptible to proteolytic attack and not amenable to Ca^{2+} protection. A proteinase precursor is actually demonstrable in a calciumless culture at the onset of the enzyme accumulation which follows Ca^{2+} addition. It is suggested that excreted proteins require an unfolded (or incompletely folded) structure to cross the cell envelope.

The proteinase excreted by a *Sarcina* strain (Coccus P) is found only in cultures containing Ca^{2+} ions (1), a feature common to proteinases of other bacteria (4, 12, 18) and to other excreted enzymes (14). Among the nontoxic divalent cations, Ca^{2+} is rather specific in this effect. Other ions such as Mn^{2+} or Mg^{2+}, the latter being present in all media as an indispensible growth factor, are ineffective. Addition of Ca^{2+} to the proteolytically inactive supernatant fluid of a calcium-free culture does not result in the appearance of the missing enzyme activity. The early assumption that Ca^{2+} was needed for enzyme synthesis or excretion (1) was challenged when the observation was made (5) that Ca^{2+} and not Mn^{2+}, Mg^{2+}, Sr^{2+}, or Ba^{2+} was needed for preventing irreversible loss of activity of several bacterial proteinases. In particular, in the case of the excreted proteinase of Coccus P, it was shown (17) that this irreversible inactivation is due to autodigestion occurring in the absence of Ca^{2+}. An antiwetting agent, Ficoll, delays this autodigestion, suggesting that the function of Ca^{2+} is to stabilize an already active form of the enzyme molecule rather than to act as a constituent of the prosthetic group required for activity.

It has also been observed that, when Coccus P is grown in a complex proteose peptone medium, the proteinase appears abruptly late in the growth of the culture. The sudden burst of activity was explained by demonstrating the presence of a zymogen which is activated autocatalytically (8). The late appearance of activity was accounted for when it was discovered that in minimal medium containing Ca^{2+}, Coccus P excreted the proteinase immediately at the onset of growth (9), but that addition of Casamino Acid hydrolysate delayed enzyme production for a length of time roughly proportional to the amount added (H. Ennis and L. Gorini, 1959, *unpublished data*). A similar amino acid effect was observed for other proteolytic bacteria (3, 13). It was assumed,

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therefore, that in the absence of amino acids an unrestricted proteinase production could be found. However, another deviation, from a constant relationship between amount of enzyme and amount of cells producing it, became evident by using minimal medium. The rate of accumulation of enzyme decreased gradually, long before exponential growth had slowed down (T. Heyman and L. Gorini, 1955, *unpublished data*). As yet, no explanation for this decline has been provided.

In this paper, in addition to studying the role of Ca^{2+} in enzyme production, we also analyze the kinetics of enzyme appearance and accumulation in minimal medium. It is found that Coccus P does not require Ca^{2+} for proteinase synthesis or excretion but only in so far as it is essential for enzyme stability. It is further found that the factor responsible for the decline in enzyme accumulation, observed under conditions in which enzyme inactivation or autodigestion is prevented, is the proteolytic activity of the enzyme itself. Thus, in addition to synthesis, excretion, activation, and stability, a novel element plays a role in controlling enzyme accumulation in the culture fluid.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used were wild type of Coccus P, the proteinase C-less CP10 mutant already described (17), and a lysine-requiring mutant which was obtained from the wild type by nitrosoguanidine mutagenesis (17) and selected by the penicillin technique (7).

Growth media. The liquid medium used was minimal medium, MMCP, described by Gorini and Lord (9). For low-calcium conditions (10^{-6} M), no external Ca^{2+} was added; for high-calcium conditions, the Ca^{2+} concentration was raised to 10^{-3} M as described before (17). Solid selective media were milk and casein plates as previously described (17).

The effect of Ficoll (Pharmacia) was tested at 5% concentration in the low-Ca medium, since it was already established (17) that 10% Ficoll did not affect bacterial growth and did not raise the Ca^{2+} concentration over the 10^{-6} M low-Ca level. To the high-Ca cultures, 5% Ficoll was generally added to reduce the loss of enzymatic activity resulting from prolonged shaking. However, in the chromatographic experiments, 5% Ficoll interferes with gel filtration of the concentrated cultural fluid. Therefore, the Ficoll addition was reduced to 1% which limits the loss of enzyme activity to no more than 10 to 20%. For the experiment in which the products of proteinase autodigestion in high- and low-Ca cultures were compared, no Ficoll was added to either culture. In all experiments, as samples were withdrawn from the cultures, $CaCl_2$ (to 10^{-3} M Ca^{2+}) or Ficoll (or both) was added when necessary to create conditions of maximal proteinase protection.

Growth conditions: flask cultures. The inoculation procedure was as previously described (17), and experiments were started when cultures reached a cell density of 2×10^8 cells/ml. Samples were withdrawn, and the culture growth was stopped as described for each experiment. Bacterial density was measured by optical density (OD) at 490 nm (17). One unit of OD corresponds to a bacterial density of 4×10^8 cells/ml.

Growth in a confined portion of the medium. A system similar to that used for concentration of toxins was employed. Bacteria were grown in a dialysis bag of 100-ml capacity, held rigid by insertion of a cylindrical grid, and suspended in 5 liters of MMCP. Air was bubbled through the liquid in the dialysis bag and in the surrounding flask, and the incubation was carried at 37 C. Under these conditions, bacteria can reach a density of 24×10^8 cells/ml before approaching stationary phase, as opposed to 6×10^8 cells/ml in a flask. The proteinase and other nondiffusible material excreted by the cells remain inside the bag so that their effect on the culture may be tested at much higher concentrations than in a flask.

Labeling the culture. ^{35}S-sodium sulfate or ^{14}C-leucine was used (New England Nuclear Corp.). The final concentration in the medium was 43 μg of ^{35}S-sulfate per ml at 13.6 Ci/mole or 21 μg of ^{14}C-leucine per ml at 11.9 Ci/mole. Samples were removed from the cultures as needed and chilled, and a 500-fold excess ^{32}S-sulfate or ^{12}C-amino acid was added. The supernatant fluid was separated from the cells as previously described (17).

Determination of trichloroacetic acid-precipitable radioactivity. The amount of excreted proteins is very small (17), and its separation as trichloroacetic acid-precipitable material is not possible without addition of a protein carrier. By means of a radioactive label, however, acid-precipitable radioactivity may be detected as early as 7 min after the addition of radioactivity (less than $\frac{1}{20}$ of the doubling time). One milliliter of the supernatant fluid was precipitated with 1 ml of 10% trichloroacetic acid in the presence of 0.02% albumin as a carrier. Albumin was added just before acid addition, and the sample kept at 4 C. The radioactive precipitate was left overnight, collected on a membrane filter (Millipore Corp.; 0.45-nm pore size), washed repeatedly with 5% trichloroacetic acid containing 0.25% unlabeled leucine or sulfate, and counted in a thin-window gas flow counter (Nuclear-Chicago Corp.).

Determination of radioactive, trichloroacetic acid-soluble metabolites. Sulfur-containing metabolic products, soluble in trichloroacetic acid, were determined in the trichloroacetic acid-treated supernatant fluid after the excess of ^{35}S-sulfate was removed by precipitation as $BaSO_4$. To a 5-ml sample of the supernatant fluid, 43 mg of ^{32}S-sodium sulfate and 5 ml of 10% trichloroacetic acid were added. Acid-precipitable material was eliminated by centrifugation and filtration, and the *p*H was restored to 7.0 with 5 N NaOH. A boiling solution of $BaCl_2$ (two times the stoichiometric amount) was added. The mixture was then boiled for 5 min and allowed to cool to room temperature. The $BaSO_4$ precipitate was filtered out, and the process was repeated once, omitting the acid addition. Radioactivity in the final supernatant fluid was measured in quadruple samples. A control experiment in which radioactive sulfate was added to sterile MMCP showed that under these conditions no radioactive sulfate remained in solution. The extent of absorption by $BaSO_4$ of nonsulfate radioactivity was determined in a reconstruction experiment. A known amount of a radioactive hydrolysate of the proteinase, obtained by digesting the heat-denatured enzyme with 100 μg of Pronase (Calbiochem) per ml, was

added, together with labeled sulfate, to the culture supernatant fluid. It was shown that 60 to 70% of the original hydrolysate counts remain in solution after precipitation of $BaSO_4$.

Gel filtration of labeled culture fluid. Gel filtration was performed on the supernatant fluid of cultures grown with ^{14}C-leucine. Samples (5 ml) of the culture fluid, separated from the cells by membrane filtration, were lyophilized either directly or after overnight dialysis against 1,000 ml of 0.1 M tris(hydroxymethyl) aminomethane (Tris)-hydrochloride buffer (*p*H 7.0) at 4 C, with one buffer change. ^{12}C-leucine (0.25 mg/ml) was added to the dialysis buffer. No appreciable enzyme activity was lost by lyophilization under these conditions. Chromatographic columns of Sephadex G-100 or G-100 Superfine (10 to 40 μm mesh) or G-200 (Pharmacia) were packed as previously described (17) in columns [60 cm (length) by 0.9 cm (internal diameter)]. The lyophilized samples were redissolved in 0.5 ml of 0.1 M Tris-hydrochloride buffer at *p*H 7, applied to the column, and eluted with the same buffer. The flow rate was kept constant at about two drops per minute with the use of a peristaltic pump, and 0.6-ml samples were collected. A concentrated solution of nonradioactive proteinase (800 units in 10 μliters) was added to the nonproteolytic samples, and the position of the enzymatically active peak was used as a reference. Samples (0.5 ml) of the chromatographic eluent were counted in a scintillation counter (Packard Tri-Carb, model 3375). Bray's scintillation fluid (2) caused the samples to precipitate in the cold. It was therefore replaced by a modification of a scintillation fluid used for sucrose fractionation (R. Garvin, Ph.D. Thesis, Montana State University, Bozeman, 1969) as follows: 1,018 ml of toluene, 900 ml of ethanol, 250 ml of tetrahydrofuran, and 6.4 g of Omniflur (New England Nuclear Corp.).

Enzyme assay. Proteinase activity was measured by milk coagulation as previously described (17).

Radioactive enzyme. Radioactive enzyme was prepared as already described (17).

Zymogens and enzymatically inactive derivative of proteinases. Commercially available chymotrypsinogen and trypsinogen (Worthington Biochemical Corp.) were used after removal of $MgSO_4$ by dialysis. Diisopropylfluorophosphate (DFP)-inactive enzymes were prepared as follows. To 6 ml of a solution containing 1 mg of chymotrypsin per ml or 2,000 units of Coccus P proteinase per ml (10^{-3} M Ca^{2+}), 1.2 ml of DFP (10^{-3} M) was added. The final molar ratio of DFP to chymotrypsin was 5 to 1. After 5.5 hr of incubation at room temperature, the solution was dialyzed three times against 1,000 ml of 0.1 M Tris-hydrochloride buffer, *p*H 7.2 (2×10^{-3} M Ca^{2+}), at 4 C for 8 hr each.

RESULTS

Susceptibility of proteinase and other cellular proteins to proteolytic attack. The study of excretion of a proteolytic enzyme is complicated by the fact that all cellular proteins are potential substrates. Moreover, some of the experiments described below were performed in the presence of trypsin or chymotrypsin. Therefore, the following facts were established beforehand.

(i) Proteinase C is stable under the experimental conditions we have used, i.e., incubation at 37 C with shaking for at least 2 hr in MMCP containing 5% Ficoll and 10^{-3} M Ca^{2+}, even in the presence of trypsin or chymotrypsin (Table 1). By testing with the CP10 strain, a proteinase C-negative mutant, it was also shown that cell growth does not affect proteinase C stability.

(ii) Other extracellular proteins, such as the inactive material which accumulates in the absence of Ca^{2+} (peak A, Fig. 1b), are stable to the action of proteinase C but are slowly digested by trypsin or chymotrypsin (Table 2). This accounts for the accumulation of peak A even in the high-Ca culture (Fig. 1a) where the proteinase C activity is high.

(iii) The cell envelope does not appear to be affected by proteinase C since addition of an excess of enzyme (600 units/ml) to a labeled culture did not affect growth and did not cause leakage of labeled trichloroacetic acid-precipitable proteins above the basal level of excretion normally occurring. An increased general leakage was expected if the cell envelope would be damaged by proteolytic attack.

(iv) No interaction is likely between excreted proteinase and proteins inside the cell, since the excreted enzyme does not reenter the cell as shown by the following experiment. Radioactive enzyme (2,000 counts per min per ml) was added to two growing cultures at 1.3×10^8 cells/ml and at 2.2×10^8 cells/ml, respectively. Growth was stopped after 30 min. The amount of radioactivity associated with the washed cells was negligible and was independent of the original cell density.

Chromatographic profile of excreted proteins. Two wild-type cultures, each labeled with ^{14}C-leucine, were grown in low- and high-Ca media, the latter containing 1% Ficoll, from 2×10^8 to 4×10^8 cells/ml. Contamination of the culture fluid by intracellular proteins was minimized by

TABLE 1. *Stability of proteinase to trypsin and chymotrypsin*[a]

Time (min)	Control (counts per min per ml)	+Trypsin (counts per min per ml)	+Chymotrypsin (counts per min per ml)
0	402	396	385
40		388	383
120	405	391	383

[a] To purified radioactive proteinase (40 units/ml, 400 counts per min per ml), 100 μg of trypsin per ml or 50 μg of chymotrypsin per ml was added. Ca^{2+} (10^{-3} M) and 5% Ficoll were present in all of the tubes, and the incubation was at 37 C with shaking. Samples were withdrawn at the indicated time, and 5% trichloroacetic acid was added. The trichloroacetic acid-precipitable radioactivity was determined.

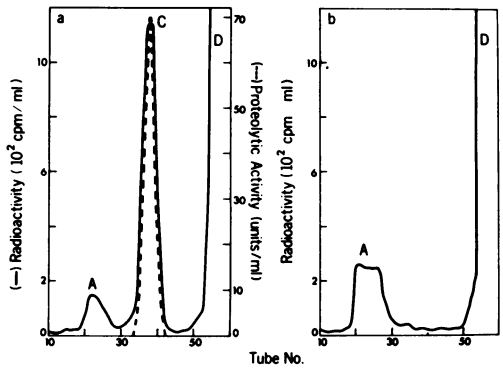

FIG. 1. *Elution profiles of cell-free supernatant fluids. To two wild-type cultures [10^{-3} M Ca^{2+} (a) and 10^{-6} M Ca^{2+} (b), respectively] radioactive leucine was added, and the cultures were grown for one generation. Samples (5 ml) were prepared as described in Methods and were chromatogramed on Sephadex G-100 superfine. Solid lines represent the radioactivity, and dashed lines represent the proteolytic activity of the fraction. (a) Proteolytic supernatant, (b) nonproteolytic supernatant.*

TABLE 2. *Sensitivity of proteins excreted in the low-Ca culture to trypsin, chymotrypsin, and proteinase*[a]

Time (min)	Control (counts per min per ml)	+Proteinase (counts per min per ml)	+Trypsin (counts per min per ml)	+Chymotrypsin (counts per min per ml)
0	148	136	147	155
40	126	146	100	87
120	120	141	72	56

[a] Wild-type low-Ca culture was grown with ^{14}C-leucine (final specific activity, 25 μCi/μmole) to 2 × 10^8 cells/ml. The culture fluid was separated from the cells and dialyzed. To 125 counts per min of this concentrate per ml, 340 units of the proteinase per ml, 100 μg of trypsin per ml, or 50 μg of chymotrypsin per ml were added. The experiment was completed as described in Table 1.

stopping growth after only one generation and by performing the analysis on 5-ml samples so that sterile filtration lasted less than 1 min. No proteolytic activity was detectable in the supernatant fluid of the low-Ca culture, whereas 100 units/ml was found in the high-Ca culture. The supernatant fluids were lyophilized immediately or after overnight dialysis at 4 C to eliminate excess ^{14}C-leucine. To test the role played by Ca^{2+}, which is known to be critical (17), the dialysis was performed under two conditions: low- and high-Ca concentrations. Figure 1 shows the nondialyzed supernatant fluids from the proteolytic (a) and the nonproteolytic (b) cultures; Fig. 2 shows the same supernatant fluids after dialysis.

The results presented in Fig. 1 confirm the earlier assumption (17) that the periplasmic proteinase B found in the supernatant fluid of cultures at the end of exponential growth was not due to active excretion. In fact, in the present experiment, there is no trace of proteolytic activity outside of the narrow peak C of radioactivity found in the high-Ca culture (Fig. 1a), which is missing altogether in the low-Ca culture (Fig. 1b). A peak A of nonproteolytic protein(s), digestible by trypsin or chymotrypsin but not by proteinase C (Table 2), is also found. It corresponds to the peak A found in the previous large-scale fractionation experiment (17) and indicates that proteolytically inactive protein material is excreted independently of the presence of proteinase C and Ca^{2+}. It may be noticed that peak A from the supernatant fluid of a nonproteolytic culture (Fig. 1b) is broader than that obtained from a proteolytic culture (Fig. 1a). Part of the material under this peak disappears after dialysis (Fig. 2b), in contrast with the corresponding material in the proteolytic culture. The nature of this additional amount of peak A material contained in the nonproteolytic culture is unknown. It may be an inactive form or aggregate of the proteinase C missing in calcium-free cultural conditions. The excess of free ^{14}C-leucine elutes in the region D at the end of the chromatogram, possibly masking other protein components.

If the supernatant fluid from a proteolytic culture was dialyzed against high-Ca buffer (Fig. 2a), peaks A and C remained unchanged as expected, peak A being resistant to proteinase C and peak C itself being stable in the presence of 10^{-3} M Ca^{2+}. By contrast, the radioactivity of re-

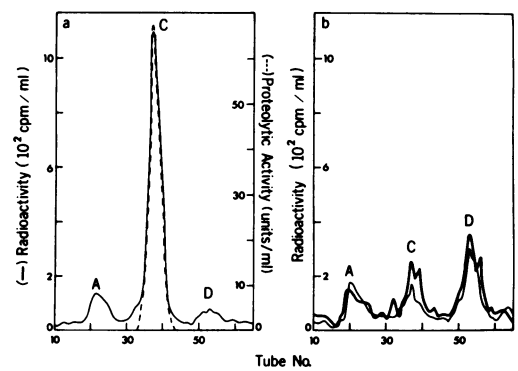

FIG. 2. *Elution profiles of cell-free supernatant fluids after dialysis in high or low-Ca buffers. The experiment was the same as for Fig. 1. Samples (5 ml) of proteolytic supernatant were dialyzed with high (a) and low-Ca (b, thick line), and the dialyzed concentrates were chromatographed as before. Thin lines in b are the result of dialysis of the nonproteolytic supernatant in high-Ca buffer. Dialysis with low-Ca buffer gave essentially the same profile.*

gion D was completely eliminated, confirming previous results (17) that there is no accumulation of inert proteins other than that or those present in peak A. When the proteolytic supernatant was dialyzed against low-Ca buffer (thick line, Fig. 2b), extensive autodigestion of proteinase C occurred, proteolytic activity disappeared completely, and a new radioactive profile was obtained, presumably corresponding to hydrolytic products of proteinase C. The results obtained after low- and high-Ca dialysis of the nonproteolytic supernatant fluid (Fig. 2b, thin line) were similar, showing not only that by raising Ca^{2+} concentration in a nonproteolytic cultural fluid no enzyme C activity is regenerated, which is expected, but also suggesting that the peaks obtained after dialysis of the nonproteolytic supernatant could have the same origin as those after dialysis of the proteolytic supernatant. This would agree with the assumption that proteinase C is excreted also in the low-Ca cultures but is rapidly degraded to inactive products.

Peptides in high- and low-Ca supernatants. Two parallel cultures of the wild-type strain in low- and high-Ca media were labeled with ^{35}S when the density reached 2×10^8 cells/ml. Accumulation in the supernatant fluids of trichloroacetic acid-precipitable and trichloroacetic acid-soluble metabolites containing ^{35}S was followed during the subsequent growth. Figure 3 shows that the rate of protein accumulation (Fig. 3a) was higher in the high-Ca than in the low-Ca culture but that this relationship was inverted in the case of accumulation of soluble metabolites (Fig. 3b). This result supports the assumption that the proteinase is excreted in both cases but that its subsequent fate is determined by the amount of Ca^{2+} present in the medium. In the high-Ca culture it accumulates as protein; in the low-Ca culture it is degraded. This result should be expected if the only effect of Ca^{2+} is to prevent audodigestion of the excreted proteinase.

In the low-Ca culture, which is enzymatically inactive, protein material accumulates at a low rate (Fig. 3a). This corresponds to the material (or part of it) eluting as peak A in the fractionation of both low- and high-Ca supernatant fluids (Fig. 1 and 2) and will be referred to as "basal accumulation." Since this protein(s) occurs in low-Ca and in high-Ca culture, the actual rate of proteinase accumulation should be obtained by subtracting this basal accumulation. The correctness of this assumption was confirmed by the observation that the proteinase C-less mutant, CP10, accumulated trichloroacetic acid-precipitable material at a similar "basal rate" irrespective of Ca^{2+} concentration.

The soluble metabolites containing sulfur

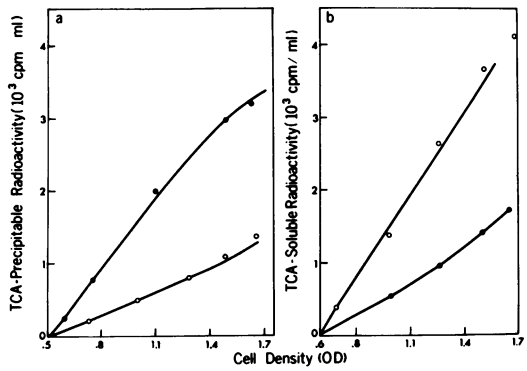

FIG. 3. *Accumulation of trichloroacetic acid-precipitable and trichloroacetic acid-soluble radioactivities in culture supernatant fluid. Wild-type culture was grown in high (●) or low-Ca (○) media, and ^{35}S-sodium sulfate was added at about 2×10^8 cells/ml. Samples were withdrawn periodically. Trichloroacetic acid-precipitable radioactivity (a) and trichloroacetic acid-soluble radioactivity (b) were determined.*

which accumulate at low rate in the high-Ca culture (Fig. 3b) cannot be due to proteinase action on itself or on some other protein since (i) autodigestion is prevented by the high Ca^{2+} concentration and (ii) it was found that the proteinase C-less mutant accumulated similar material at a slightly lower but still comparable rate, irrespective of Ca^{2+} concentration. Whatever is the origin of this material, the assumption seems valid that in a low-Ca culture the trichloroacetic acid-soluble material accumulating in excess of that found in a high-Ca culture consists of peptides originating from proteinase autodigestion. The notion that active proteinase is excreted in the absence of Ca^{2+} is thus supported.

Excretion of proteinase in the absence of Ca^{2+}. A culture of the wild-type strain was grown in low-Ca medium and, upon reaching a density of 2.4×10^8 cells/ml, was labeled with ^{14}C-leucine and immediately divided into four portions. (i) Ficoll (5%) was added, (ii) $CaCl_2$ was added to raise the Ca^{2+} concentration to 10^{-3} M, (iii) both Ficoll and $CaCl_2$ were added, and (iv) no additions were made. Growth was allowed to continue, and the increase of proteolytic activity and of trichloroacetic acid-precipitable radioactivity were compared in the four cultures. It is seen in Fig. 4 that the rate of both accumulations was initially very similar in cultures *a*, *b*, and *c*, culture *c* being slightly faster and culture *b* being the slowest. Subsequently, not only did the rate of enzyme accumulation in culture *a* gradually decline to zero, but also at the end of growth (not seen in Fig. 4), all enzymatic activity was lost, and the trichloroacetic acid-precipitable radioactivity reached the basal level found in the control

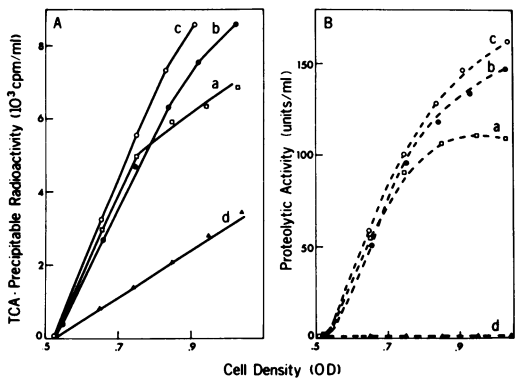

FIG. 4. *Effect of Ca^{2+} and Ficoll on kinetics of enzyme accumulation. To a growing wild-type low-Ca culture, ^{14}C-leucine was added at 2.4 × 10^8 cells/ml, and the culture was divided as follows: (a) 5% Ficoll, (b) 10^{-3} M Ca^{2+}, (c) 10^{-3}M Ca^{2+} + 5% Ficoll, (d) no addition. Samples were withdrawn at intervals. Trichloroacetic acid-precipitable radioactivity (A) and proteolytic activity (B) were determined.*

(culture *d*). These results are what one should expect if the proteinase was excreted at the same rate in all four cultures but accumulation depended on enzyme stability subsequent to excretion. Consequently, the proteinase should (and does) accumulate transiently in the Ficoll-containing low-Ca culture, in agreement with the results obtained with the cell-free system (17) which showed that autodigestion was only delayed but not stopped under the same conditions.

Kinetics of enzyme accumulation. In the experiment presented in Fig. 4, the cultures grew exponentially at least one generation beyond the time when the last sample was taken, and it is known (Table 1) that under the conditions realized in culture *c* no loss of activity occurs. Yet it may be shown that the specific rate of enzyme accumulation is not constant at any time during the exponential phase of growth. Initially, there is a period of acceleration particularly evident in experiments starting at a cell density lower than in that related in Fig. 4. It was found that the period of acceleration, even when it was pronounced, was noticeable only when accumulation was measured by increase in proteolytic activity and not when it was determined by increase of radioactivity. At high population density there is a decline in the specific rate of accumulation, detectable with either method of measurement. Notice that, in the radioactivity measurement, the net proteinase accumulation is obtained by subtracting the "basal" accumulation rate (curve c minus curve d in Fig. 4a). In contrast with proteinase C, the kinetics of "basal" accumulation was found strictly constant throughout all the exponential growth period. A detailed analysis of the complex kinetics peculiar to proteinase accumulation was performed.

Early phase. The initial kinetics of accumulation were studied by simultaneously adding Ficoll, Ca^{2+}, and ^{14}C-leucine to four parallel cultures at different cell densities. The experimental setting was the same as in culture *c* of Fig. 4, but the results are presented in a plot giving the differential rate of accumulation as a function of net increase in cell density (Fig. 5). When the proteinase was measured by enzyme activity, the differential rate of accumulation per unit increase in cell density was initially higher if Ca^{2+} was added at higher cell density. This produces an initial burst of enzyme accumulation followed by a leveling off to a lower level and finally by a gradual decrease irrespective of the initial cell density. By contrast, when the proteinase accumulation was measured by determining the increase of trichloroacetic acid-precipitable radioactivity, no burst was found at any initial cell density (insert, Fig. 5). This indicates that, at the moment of the simultaneous addition of the enzyme stabilizers (Ficoll and Ca^{2+}) and of the radioactive label, there was a transient excess of an

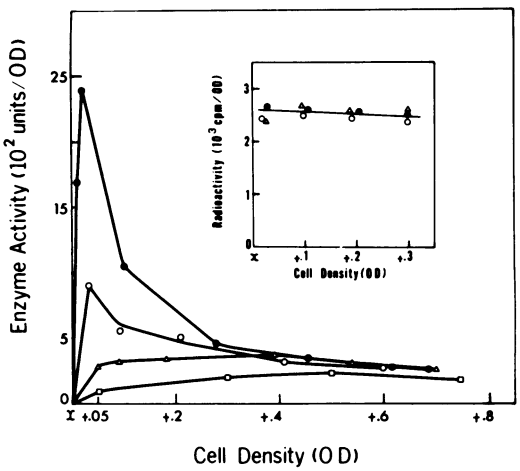

FIG. 5. *Effect of cell density on the kinetics of enzyme accumulation upon addition of calcium. Ca^{2+} (10^{-3} M), 5% Ficoll, and ^{14}C-leucine were added to four cultures at 0.8 × 10^8 cells/ml, 1.6 × 10^8 cells/ml, 2.8 × 10^8 cells/ml, and 3.9 × 10^8 cells/ml. The experiment was carried out as described in Fig. 4. The main figure shows the enzyme activity, and the insert shows the trichloroacetic acid-precipitable radioactivity. The abscissa is the cell density increase from the time of Ca^{2+} and Ficoll addition. X is 0.2 for □, 0.4 for △, 0.7 for ○, and 0.94 for ●. The ordinate represents units of proteolytic activity (or trichloroacetic acid-precipitable radioactivity) divided by the corresponding increase in cell density given in the abscissa.*

unlabeled precursor which would contribute to the enzyme activity but not to the radioactivity.

Two factors contribute to the different kinetics seen in the four cultures of Fig. 5. One is the amount of preformed precursor which obviously is proportional to the cell density. The other is the rate of precursor transformation into active enzyme, a process which is unknown and may occur in several discrete steps. In the attempt to collect more information about the factors controlling the overall activation process, the following experiment was performed. Ficoll and Ca^{2+} were added to two cultures growing in parallel containing the same amount of wild-type cells but possessing different cell density because, in one of them, the mutant CP10 was also present. Since CP10 produces neither proteinase C nor any precursor of it, the effect of cells not themselves contributing to enzyme formation on precursor activation could be studied. The rate of precursor activation was faster in the culture growing at higher cell density (Fig. 6). In a similar experiment, washed cells of a lysine auxotroph were added together with the enzyme stabilizers to a culture of wild-type cells growing in MMCP without lysine. Under these conditions, the lysine-negative cells are not contributing to growth, but they contribute to initial cell density. The kinetics of activation did not indicate a stimulation by the presence of nongrowing cells.

Late phase. By growing wild-type cells in a confined portion of the medium in a dialysis tube, it was possible to follow the increase of enzyme activity in exponential growth for 5.5 generations. As can be seen in Fig. 7 (lower curve), the enzyme increase is a continuously declining function of the increase in cell mass, as was already noticeable in Fig. 4 and 5. This cannot be attributed to the loss of enzyme after excretion, because the proteinase is stable under similar conditions in a cell-free solution (Table 1). Also, these declining kinetics cannot be due to a changed cell ability to excrete or activate a precursor because addition of inhibitors to protein synthesis, such as chloroamphenicol or puromycin (which do not affect enzyme activity), to an exponentially growing high-Ca culture stopped enzyme accumulation with no detectable lag. Similarly, radioactive label added to such a culture appeared without lag in the trichloroacetic acid-precipitable material. These facts point to the absence of any internal pool of proteinase or of an intermediate in cultures actively accumulating enzyme in their medium. It was concluded either that the rate of internal proteinase synthesis is not constant, which is unlikely in an exponentially growing culture, or that the cell growth causes the piling up of a factor which inhibits enzyme accumulation.

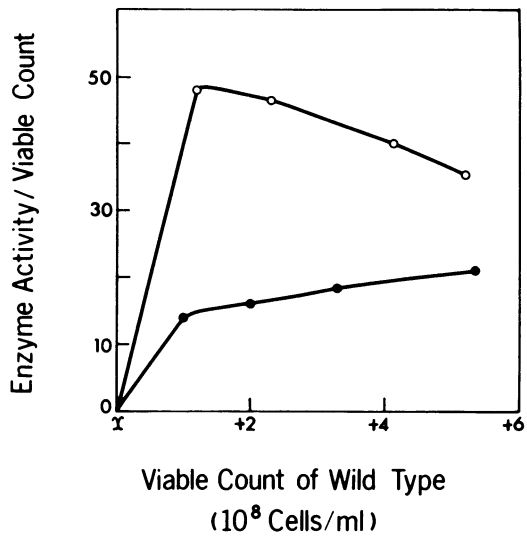

FIG. 6. *Effect of nonproteinase-producing mutant on the activation of the wild-type proteinase. To a growing wild-type culture at 2 × 10^8 cells/ml, 10^{-3} M Ca^{2+} was added, and the cells were divided into two equal portions, to one of which* (○) *2 × 10^8 cells/ml of growing CP10 mutant were added. Samples were withdrawn periodically, and proteolytic activity of the supernatant fluid was measured. The abscissa is viable count of the wild-type, determined on casein plates on which the wild type and the mutant may be distinguished (17). The ordinate is units of proteolytic activity divided by the corresponding increase in wild-type cell density given in the abscissa.*

A factor was indeed demonstrated to be released in the culture medium, because (i) inoculation of new cells in the supernatant of a partially grown culture resulted in a slowdown of the accumulation rate, and (ii) cells from dense cultures produced proteinase normally when centrifuged and resuspended in a fresh medium. The factor was thermolabile; autoclaved supernatant fluid did not inhibit when reinoculated with centrifuged cells. Finally, it was demonstrated that the factor was the excreted proteinase itself, because addition of concentrated crude proteinase (300 or 425 units/ml) to the medium before inoculation resulted in the accumulation kinetics presented in Fig. 7.

Other proteins were tested for an effect similar to that exerted by the proteinase. Inert proteins like serumalbumine or enzymes like alkaline phosphatase (which may be demonstrated as enzymatically active at the end of the experiment) were ineffective. By contrast, several proteolytic enzymes were found to be effective provided that they were in an active form. Thus, trypsin and chymotrypsin, which were demonstrated to be unable to attack the proteinase (Table 1), were

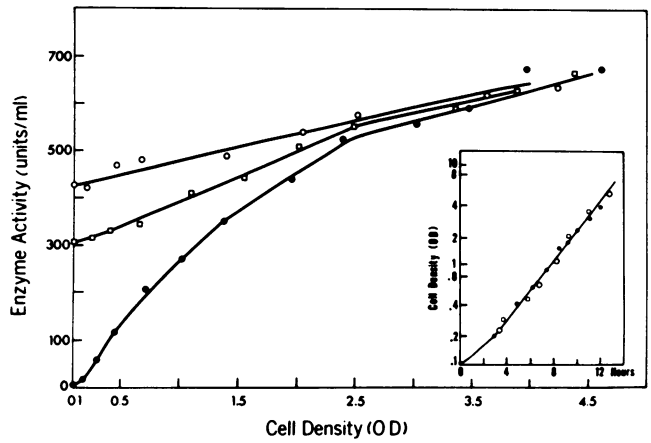

FIG. 7. *Effect of proteinase on its own accumulation. Cells were grown in a confined portion of the culture. The surrounding medium was 5 liters of MMCP containing 10^{-3} M Ca^{2+}. The wild-type strain was inoculated in a dialysis bag containing 100 ml of the same medium with addition of 0 (●), 300 (□), or 450 (○) units of concentrated crude enzyme per ml. Samples were withdrawn periodically, and proteolytic activity was measured. The insert shows bacterial growth as a function of time.*

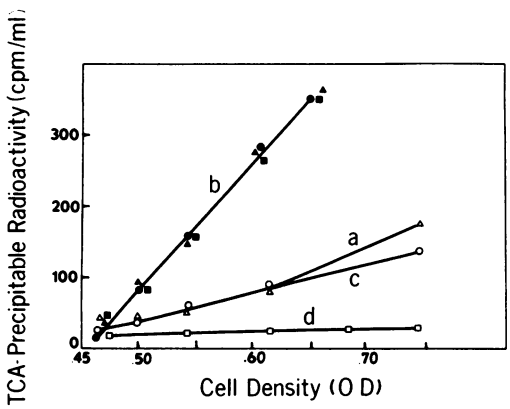

FIG. 8. *Effect of proteolytically active and inactive chymotrypsin on proteinase accumulation. ^{14}C-leucine was added to a growing wild-type culture at 1.8 × 10^8 cells/ml. The culture was divided into the following portions: (a) 10^{-3} M Ca^{2+} and 7 μg of chymotrypsin per ml (△), (b) 10^{-3} M Ca^{2+} alone (●) or with 50 μg of DFP-inhibited chymotrypsin per ml (▲) or 50 μg of chymotrypsinogen per ml (■), (c) no addition (○), (d) 10^{-3} M Ca^{2+} and 50 μg of chymotrypsin per ml (□). Samples were withdrawn periodically and trichloroacetic acid-precipitable material was determined.*

found to be inhibitory, whereas their zymogens or the DFP-inhibited enzymes were ineffective. Figure 8 presents a typical experiment performed with chymotrypsin. A low-Ca culture of the wild-type strain, labeled with ^{14}C-leucine and reaching a density of 2.4 × 10^8 cells/ml, was divided into several subcultures to which the following additions were made: (curve a) 10^{-3} M Ca^{2+} and 7 μg of chymotrypsin per ml; (curve b) 10^{-3} M Ca^{2+} alone or with DFP-chymotrypsin or chymotrypsinogen; (curve c) no addition; (curve d) 10^{-3} M Ca^{2+} and 50 μg of chymotrypsin per ml. Growth was allowed to continue, and the increase of trichloroacetic-precipitable radioactivity was followed. It was found that culture *c* showed the basal accumulation only, as expected (because no Ficoll and Ca^{2+} were present as stabilizers); cultures *b* showed the maximal rate of accumulation, indicating that inactive forms of chymotrypsin are ineffective; in culture *a* 7 μg of active chymotrypsin per ml inhibited transiently the proteinase accumulation. The escape from inhibition as growth continued was similar to the kinetics observed in Fig. 7 with addition of proteinase. Finally, when a larger amount of chymotrypsin was used, as in culture *d*, the basal accumulation itself disappears in agreement with the fact (Table 2) that peak A is digested by chymotrypsin when used in large amounts.

DISCUSSION

The medium of cultures of Coccus P in early exponential phase of growth, quickly separated from the cells, was found free of the periplasmic proteinase B which is one of the nonexcreted proteins leaking out more readily (17). Therefore, all of the proteins found in the chromatograms presented in Fig. 1 should be considered released through a cell boundary which is intact and physiologically functioning. The following conclusions confirming preliminary indications (17) can be drawn: (i) the entire amount of proteolytic activity existing in the supernatant fluid of a culture grown in high-Ca is found under only one narrow

peak (peak C) of radioactivity; (ii) this peak is absent from the proteolytically inactive supernatant fluid of a culture grown in low-Ca; (iii) a peak (peak A) consisting of a protein (or an unresolved group of proteins) proteolytically inactive is found in both low-Ca and high-Ca supernatant fluids. It is clear therefore that the excretion of the protein(s) eluting under peak A does not require the presence of Ca^{2+}. Figure 2 suggests that Ca^{2+} is not needed for the excretion of proteinase C either. It is seen in fact that, if Ca^{2+} is dialyzed away from the supernatant fluid of a high-Ca culture, all of the proteolytic activity is lost overnight at 4 C and the peaks appearing in the elution profile become similar in amount and location to those obtained from the dialyzed supernatant fluid of a low-Ca, proteolytically inactive culture. A more direct indication that proteinase C is excreted irrespective of Ca^{2+} presence is the finding that the ratio of ^{35}S peptides to ^{35}S proteins is low in the fluid of a high-Ca culture, whereas it is high in that of a low-Ca culture (Fig. 3). This suggests that in low-Ca cultures the proteinase is absent but that its split products are present instead. Definitive evidence was obtained by showing that the fluid of a culture grown in the presence of calcium-free Ficoll is proteolytic. It is found (Fig. 4) that the rates of accumulation of proteolytic activity or of trichloroacetic acid-precipitable radioactivity in a high-Ca or in a 5% Ficoll culture are initially the same. In cultures containing Ficoll alone, the proteinase accumulation slows down much earlier than in those containing high-Ca, whereas the highest accumulation is obtained in cultures containing both Ficoll and Ca^{2+}. These results indicate that addition of Ficoll, without simultaneous addition of Ca^{2+}, only delays the autodigestion of the enzyme which would otherwise occur more rapidly. Therefore, the function of Ficoll and Ca^{2+} during growth is no more than that of protectors already observed in a cell-free solution.

It is concluded that addition of Ca^{2+} to a calcium-free culture neither changes the rate of proteinase synthesis or excretion, nor provides the prosthetic factor for an inactive form of the enzyme. The only effect of Ca^{2+} is to arrest the degradation of the enzyme molecule after its release, thereby permitting accumulation of active enzyme in the culture medium.

Under our experimental conditions, the action of Ca^{2+} and Ficoll is immediate, and the resulting proteinase protection is an all or none phenomenon. Addition of these two substances provided, therefore, an easy way for starting enzyme accumulation at any chosen time during cultural growth. By adding simultaneously ^{14}C-leucine, it was possible to follow enzyme accumulation by either one of two determinations: enzyme activity or trichloroacetic acid-precipitable radioactive material. Comparison of the two kinetics obtained in this way lead to the individuation of an intermediate state of the enzyme protein preceding the excreted, finished enzyme. It was found in fact that accumulation starts with an initial excess of a precursor which is unlabeled and therefore must have been formed before ^{14}C-leucine addition. This finding coincides with the previous observation (17) that proteinase C activity is present in lysates of low-Ca cells although their surrounding medium is devoid of any demonstrable proteinase C. It might be that we are dealing with a precursor in the process of being excreted but still associated with the cells and therefore protected against the adverse conditions of a medium without Ca^{2+} (and Ficoll). The existence of a zymogen has been demonstrated previously in the supernatant fluid of cultures in complex media containing proteose-peptone or other incompletely digested proteins (8). Activation of this zymogen was found to be autocatalytic (8) but delayed in these media by the presence of one or more inhibitor peptides. In minimal medium this inhibitor is missing, and all attempts to isolate the zymogen from the supernatant of a high-Ca culture actively producing the proteinase have failed. Whether the precursor demonstrable at the moment of transition from a low-Ca to a high-Ca condition is the zymogen itself or an earlier form of the proteinase molecule remains to be seen. Even supposing that one or several intermediate steps separate the precursor presently found from the finished enzyme, it is conceivable that the kinetics of zymogen activation should be the limiting factor in the overall reaction. Zymogen activation is known to be autocatalytic (8); however, in a calcium-free culture, the life of the finished proteinase is so short that the activation reaction should be extremely slow. This would account for the presence of a small pool of precursor in a nonproteolytic, low-Ca culture. The hypothesis implies that the activation reaction itself does not require Ca^{2+}, a fact already shown in the conversion of trypsinogen to trypsin (6). It appears, however, that the overall process is more complex than a zymogen activation because it does not depend solely on precursor and enzyme concentrations. It is found in fact (Fig. 6) that in the presence of a proteinase C-less mutant (CP10), which contributes to cell density but not to proteolytic activity, the rate of precursor activation per unit of proteolytic cells present at the onset of the proteinase accumulation is a direct function of the whole mass of cells, irrespective of whether they produce the enzyme. This suggests that the cell envelope pro-

vides a factor needed for precursor activation. A similar function for the cell wall was demonstrated in the activation of a precursor of *Streptococcus* A proteinase (11). In that case, the cell wall was active in vitro, whereas in Coccus P growth seems to be necessary since the presence of nongrowing cells is ineffective.

After the brief initial period of accelerated precursor activation, the rate of accumulation of proteolytic activity does not become constant but decreases steadily throughout the exponential phase of growth (Fig. 7). This is contrasted by the kinetics of accumulation of the inert protein(s) which is always a constant function of the cell growth. By adding purified proteinase C at the beginning of the culture (Fig. 7), it was discovered that the inhibitor of the accumulation is the proteolytic activity of the proteinase itself because DFP-proteinase does not inhibit. By measuring the accumulation of trichloroacetic acid-precipitable material after addition of Ca^{2+} and Ficoll as enzyme stabilizers and of radioactive ^{14}C-leucine as radioactive label, it is possible to study the effect of other proteolytic enzymes which do not attack the proteinase in a cell-free system (Table 2). It is found that trypsin and chymotrypsin (but not trypsinogen, chymotrypsinogen, or DFP-enzymes) inhibit the accumulation in a fashion similar to that of the Coccus P proteinase. In short, the proteinase accumulation is inhibited by proteolytic activity rather than by a specific protein, although not all of the proteolytic enzymes possess the inhibitory action. Among the inactive proteases are, for instance, papain and carboxypeptidase, which indicate that some substrate specificity is required.

The mechanism by which proteolytic activity may counteract proteinase accumulation is a matter of speculation. Three main possibilities are seen. (i) Proteolytic enzymes act at the level of protein synthesis either directly, by reentering the cell, or via a diffusible product of their action outside (e.g., peptides and amino acids); (ii) they act at the level of excretion by damaging the excretory apparatus located at the outside region of the cell boundary; (iii) they act on the emerging enzyme molecule which, lacking a completed tertiary structure, may be susceptible to proteolytic attack. The first possibility can be excluded because the proteinase does not reenter the cell (Table 2), and no inhibitor, other than the proteinase itself, is found even by several-fold concentration of the supernatant fluids obtained under any of our experimental conditions.

According to the second possibility, the proteinase does not accumulate in the medium because it is not excreted. However, no indications are found that the general permeability of the cell is increased, even by addition of proteolytic enzymes at concentrations 10 times higher than those sufficient for inhibition; i.e., the slope of the curve of "basal" accumulation (Fig. 8) never increases. Thus, it is hard to see how the same factor which leaves the cell boundary intact could specifically destroy the excretory apparatus. Furthermore, the proteinase does not accumulate inside the cell at any time since the use of inhibitors of protein synthesis such as chloramphenicol or puromycin does not reveal an intracellular pool of proteinase or a precursor. Thus, if a loss of ability to excrete is postulated, one should also postulate the existence of a control of protein synthesis specifically coupled with its excretory apparatus. It should be concluded that if damage to the excretory apparatus is assumed, it would indeed have to be very specific in several directions.

According to the third possibility, the peptide chain of Coccus P proteinase crosses the membrane unfolded (or partially folded) and assumes further folding past the wall, the latter contributing to the process of transformation into the enzymatically active form. Only when the appropriate tertiary structure is achieved does Ca^{2+} play the stabilizing role which in other proteins is played by the *S-S* bridges. It is conceivable that Ca^{2+} has no effect on the stability of the unfolded peptide chain emerging from the cell which therefore may be highly susceptible to proteolytic attack even in the presence of Ca^{2+}. This unfolded precursor, which may still be associated with the cell envelope, could (but not necessarily) be an intermediate form preceding the zymogen.

In conclusion, our experimental results are in favor of the following general picture. The proteinase excreted by Coccus P contains no, or not more than one, cysteinyl residue, and is protected against autodigestion by Ca^{2+} ions (17). This indicates that Ca^{2+} electrostatic bridges replace the missing *S-S* covalent bridges in securing the proper folding, as has been suggested for example in the case of α-amylase (10). Once the enzyme is stabilized by Ca^{2+}, it no longer is susceptible to proteolytic attack. The action of the proteinase on its own accumulation must therefore be exerted on a different substrate. The target of this activity is an intermediate in which the peptide chain is exposed to proteolytic attack. Lack of a rigid tertiary structure was already proposed as a necessary condition for enabling a protein to cross the bacterial cell envelope (15). This was suggested by the fact that cross-linking cystinyl residues are generally absent in enzymes excreted by bacteria. The different susceptibility to proteolytic attack observed in this system offers an experimental support to the hypothesis that a pro-

tein emerging from a bacterial cell could indeed be unfolded or incompletely folded. An analogous hypothesis is suggested by the observation recently reported (16) that the penicillinase of *Bacillus licheniformis* exists in a different conformational form, depending on whether it is free or cell-bound.

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