NATURE OF THE PRIMARY ACTION OF THE AUTOLYSIN OF *BACILLUS SUBTILIS*

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It has been observed (Nomura, 1955*a*) that when cells of several amylase-producing strains of *Bacillus subtilis* were grown aerobically in shaking cultures and then left without further aeration, a very rapid autolysis occurred. This phenomenon was called "anaerobic lysis." It is first recognized at the last stage of the log phase when amylase begins to appear in the medium, and can be observed thereafter until the cessation of amylase production. When cells are cultivated in media which are suitable to good growth but not to the production of amylase this phenomenon is not significant. Therefore, it appears that amylase production and anaerobic lysis take place under the same conditions.

Though experimental results clearly excluded the participation of free but carried bacteriophage in this phenomenon the present authors supposed some relationship existed between the anaerobic lysis of *B. subtilis* and a lethal synthesis of lysogenic phage. Attempts to demonstrate the existence of phage in the lysate failed (Nomura, 1955*b*). However, instead of a phage two factors were discovered in the filtered lysate; one of them is an antibiotic substance which specifically kills certain strains of *B. subtilis* and related species and is fairly thermostable ("killing factor"), whereas the other is the thermolabile autolytic factor ("autolysin") (Nomura, 1955*b*). Some properties of this autolysin were studied (Nomura, 1955*b*; Nomura and Hosoda, 1956). It was found to be nondialyzable, precipitated by saturated ammonium sulfate and to have a pH optimum between 6 and 8. It is active against *B. subtilis* and *Bacillus megaterium*, but not other bacteria such as *Bacillus cereus*, *Micrococcus pyogenes* var. *aureus*, *Sarcina lutea*, *Micrococcus lysodeikticus*, and *Escherichia coli*. While we could not rule out the possibility of bacteriophage, many results lead us to believe that this autolysin is a so-called autolytic enzyme and studies have been continued on this line.

The phenomenon of autolysis is very complex and the so-called autolytic enzymes seem to be composed of a group of different enzymes, each one of them acting upon a different component of the cellular structure. However, detailed studies of autolysis are relatively few and have been mainly confined to the pneumococci (Goebel and Avery, 1929; Dubos, 1937). Following the view of Dubos, Stacey and Webb (1948) believed that the autolysis of most gram positive microorganisms consists of two separate and distinct stages, namely, (a) the change from gram positive to gram negative state, and (b) the subsequent lysis of the gram negative cytoskeleton and they emphasized the importance of RNAase in the first step of bacteriolysis. However, Welsch (1949) who studied an autolytic enzyme of *Staphylococcus aureus* stated that this gram conversion reaction is not always necessary. On the other hand, besides the autolytic enzyme, many biologically produced substances have been known to lyse living microorganisms. Among them, lysozyme has been studied most extensively for the mechanism of its action. The lytic action of lysozyme was demonstrated to be due to the dissolution of the bacterial cell wall (Salton, 1952; Welshimer, 1953; Weibull, 1953) and this action seems to be due to hydrolysis of certain mucopolysaccharides contained in the cell wall (Meyer *et al.*, 1936). Considering the knowledge of bacteriolysis there seem to be two main ways of explaining the primary action of autolytic enzyme; either (1) the RNAase-like action on the gram positive ribonucleate complex, or (2) lysozyme-like action on the polysaccharide. From these points of view studies were undertaken on the nature and the primary action of autolysin obtained from *B. subtilis*.

MATERIALS AND METHODS

B. subtilis strain H, which has been used throughout the previous studies, was also used

in this investigation. This bacterium produces a large amount of amylase when cultivated in suitable media [e.g. Soy medium (Nomura, 1955*a*)] but little when cultivated in ordinary bouillon media.

The preparation of autolysin. This has already been described in the previous paper (Nomura and Hosoda, 1956). Usually the crude autolysate of this bacterium is saturated with ammonium sulfate and the precipitate is dissolved in water, dialyzed against running tap water and used as the autolysin preparation. Concentration of autolysin is expressed as the ratio of the volume of the autolysin preparation to the total volume of the reaction mixture.

Assay of the lysis of cells. B. subtilis was grown in GBY medium (glucose, 0.5 per cent; peptone, 1 per cent; meat extract, 0.5 per cent; yeast extract, 0.2 per cent; NaCl, 0.2 per cent; pH 7.2) for 13 to 15 hr at 30 C with continuous shaking. Cells were harvested, washed with 0.2 per cent KCl, resuspended in phosphate buffer (M/25, pH 7.2) and incubated with the various additions at 30 C. Lysis was followed by measuring the optical density at 600 mμ ($O.D.^{600}$) in a spectrophotometer or by readings in a nephthelometer.

Analytical methods. Nitrogen was determined by a micro-Kjeldahl procedure; phosphorus by the method of Allen (1940), reducing substances by the method of Somogyi (1945), hexosamine by the method of Elson and Morgan (1933), and protease was assayed according to the method of Anson (1938) using casein as a substrate. Gram stain was performed according to the Hucker modification method (Conn, 1954).

Materials. Commercial preparations of testicular hyaluronidase and crystalline trypsin were used (Mochida Pharmaceutical Mfg. Co., Ltd., Tokyo). Crystalline pancreatic RNAase was kindly given by Mr. S. Naono of Osaka University and crystalline egg white lysozyme by Mr. H. Ono of Osaka University. Crystalline protease from *B. subtilis* was furnished by the Scientific Research Institute. This bacterial protease is very similar to that produced by *B. subtilis* strain H used in this investigation.

RESULTS

First the lytic action of the autolysin on this bacterium was compared with the actions of various known enzymes.

Action of protease on B. Subtilis. As already reported (Nomura, 1955*b*), the autolysin preparation lyses both living and heat-killed cells of *B. subtilis*, and it was found sometimes that the autolysin will lyse heat-killed cells even at a high dilution (e. g., when the final concentration of autolysin is $\frac{1}{400}$) where it does not show any lytic activity on the living cells. It has been known that protease can lyse heat-killed cells of bacteria but not living cells. Therefore the lytic action of autolysin on heat-killed cells is also supposed to be due to the action of protease contained in the preparation in addition to the enzyme concerned with its primary action on the living cells. The protease activity of the preparation was assayed using casein as a substrate. It was found that the autolysin preparation contains a quite active protease (86×10^{-5} units per ml). Accordingly, the lytic action of crystalline trypsin and crystalline protease from *B. subtilis* on heat killed cells (100 C for 10 min) was examined and compared with that of autolysin at concentrations exhibiting the same protease activity against casein. As shown in figure 1 the heat killed cells can be lysed by both trypsin and by protease from *B. subtilis*. However, in this case, the action of trypsin is the weakest and that of autolysin is stronger than that of crystalline subtilis protease at the low

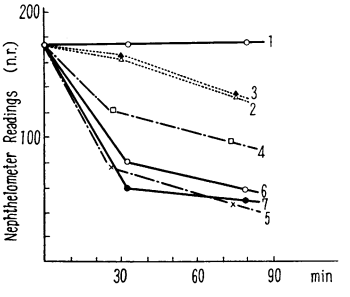

Figure 1. Action of the autolysin preparation, crystalline *Bacillus subtilis* protease or crystalline trypsin on heat-killed cells of *B. subtilis*. Composition of the reaction mixture: Washed cell suspension of *B. subtilis* H, phosphate M/96, and various enzymes as indicated. pH 7.0. Total volume 8.0 ml. Temp 30 C. Curve 1, control. Curve 2, trypsin 0.49 μg/ml or 0.56×10^{-5} units/ml. Curve 3, trypsin 4.9 μg/ml or 5.6×10^{-5} units/ml. Curve 4, *B. subtilis* protease 0.29 μg/ml or 0.56×10^{-5} units/ml. Curve 5, *B. subtilis* protease 2.9 μg/ml or 5.6×10^{-5} units/ml. Curve 6, autolysin preparation 1/160 or 0.56×10^{-5} units/ml. Curve 7, autolysin preparation 1/16 or 5.6×10^{-5} units/ml. Figures above show protease units.

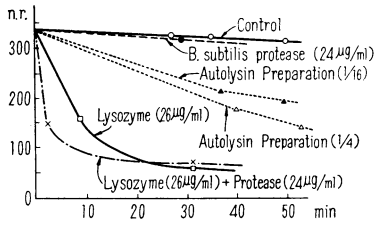

Figure 2. Action of autolysin, lysozyme protease on the living cells of *Bacillus subtilis*. Composition of the reaction mixture: Washed cells of *B. subtilis*, phosphate M/50, and various additions as indicated in the figure. pH 7.2. Total volume 8.0 ml. Temp 30 C.

concentration. These results can be explained by a difference in the specificity of trypsin and the bacterial enzyme or by the action of other enzymes contained in the autolysin preparation. It is clear that protease contained in the autolysin preparation can dissolve heat killed cells and might play some role in the lysis of living cells. Accordingly the possibility that protease might perform the primary lytic action on living cells was examined next. However, neither trypsin (59 μg/ml) nor protease from *B. subtilis* (15 μg/ml) exerted any lytic action on living cells (figure 2). Therefore, the primary action of autolysin appears to be due to enzymes other than protease.

Action of pancreatic RNAase on B. subtilis. The action of pancreatic RNAase on living cells of *B. subtilis* was examined in order to ascertain whether this enzyme is concerned in the primary action of the autolysin. No lytic action was observed when either RNAase (35 μg/ml) alone, or together with high concentration of bacterial protease (17 μg/ml), was added to the living cells; furthermore, no activating effect was recognized when RNAase (37 μg/ml) was added to the autolysin (1/18). After these experiments the cells were removed by centrifugation and the clear supernatant fluid was examined for ultraviolet absorbing compounds which might be liberated from the cells as the result of the RNAase action. The results were negative. The gram reaction of living cells after incubation with RNAase or RNAase plus protease remained positive.

A similar experiment was performed using a cell wall preparation (described below), instead of the living cells. Neither RNAase alone nor RNAase together with protease exerted any lytic action whereas autolysin was shown to lyse the cell wall preparation.

From the above results the primary action of autolysin seems to be different from that of RNAase. In these experiments pancreatic RNAase of animal origin was used. However, as reported (Nomura, 1956), this pancreatic RNAase is able to decompose the pentose nucleic acid of *B. subtilis* and, therefore, the possibility that the RNA of the cells forms the primary site of action but can be decomposed only by the bacterial RNAase under the experimental conditions seems to be ruled out.

Action of hyaluronidase. Capsular substances of group A and C streptococci are known to be hyaluronic acid and decomposed by hyaluronidases of both animal and microbial origin. In addition, according to recent studies by Warren and Gray (1954) a number of bacterial species contain hyaluronidase-sensitive polysaccharide inside the cell. Therefore the possibility that hyaluronidase-sensitive polysaccharide might exist outside the cells of *B. subtilis* and composes the primary site of lytic action was examined by observing the action of high concentrations of testicular hyaluronidase (200 v.r.u./ml, according to the method of McClean and Hale (1941)), either alone or combined with a high concentration of *B. subtilis* protease (73 μg/ml) on the living cells of this bacterium. No lytic action could be observed. Therefore, this possibility was excluded.

Action of lysozyme on the living cells. It is generally known that egg white lysozyme can lyse *B. megaterium* and *B. subtilis* in addition to *M. lysodeikticus* and *S. lutea*. As expected, the living cells of our amylase-producing strain H (*B. subtilis*) are also easily lysed with crystalline egg white lysozyme (figure 2). It can be also seen from figure 2 that the lysis progresses more rapidly when lysozyme is added together with the bacterial protease than when added alone. In contrast with living cells, the cells treated with formalin were lysed by neither lysozyme nor autolysin. According to Welshimer (1953) lysozyme lyses only the cell wall when acting upon the formalin treated cells of *B. megaterium*. Therefore, the apparent absence of lytic action of lysozyme and autolysin on formalin treated cells of *B. subtilis* may be explained in a similar way.

Lysozyme is known to lyse the cell wall of

susceptible bacteria, and the possibility that the primary site of action of autolysin was also the cell wall was tested, using cell wall preparations isolated from this bacterium.

Preparation of the cell walls. Heat killed cells of *B. subtilis* can be dissolved easily by protease. The small amount of residual material is transparent, but retains the original characteristic morphology under the phase contrast microscope and is supposed, therefore, to consist mainly of cell wall. Thus protease resistant residue was prepared according to the following procedure, and used as the cell wall preparation.

Cells were grown in GBY medium at 30 C for 13 hr by shaking. After washing with 0.2 per cent KCl the cells were suspended in a small amount of distilled water, poured into 10 volumes of boiling water and heated for 10 min. After cooling, the cells were collected by centrifugation, washed and resuspended in M/15 phosphate buffer (pH 7.2) and treated with a mixture of crystalline trypsin and crystalline *B. subtilis* protease at a final concentration of 100 μg each per ml for 3 days at 30 C in the presence of toluol. After incubation the residue was collected by centrifugation, washed with buffer and resuspended in fresh buffer, treated with crystalline chymotrypsin (100 μg/ml) for an additional 2 days at 30 C. The residue was purified by differential centrifugation and washing and finally dried *in vacuo* in the frozen state.

White powder obtained in this way seems to consist of cell wall and adhering protease resistant material (cell wall preparation 1). It is almost transparent under the phase contrast microscope, stains well with Alcian Blue 8GN (Grula and Hartsell, 1954), and is highly resistant to the action of RNAase or protease. Analytical data: N, 6.2 per cent; P, 3.5 per cent; reducing substance liberated after acid hydrolysis (2N HCl, 4 hr at 100 C), 22.5 per cent as glucose; hexosamine liberated after acid hydrolysis, 5.2 per cent.

Cell wall preparation 1 was treated with 0.25 N NaOH for 2 days, then further digested with *B. subtilis* protease (100 μg/ml) for 1 day. These additional treatments removed most of the adhering material (preparation 2). Analytical data: N, 5.6 per cent; P, 0.67 per cent; reducing substance liberated after hydrolysis, 19 per cent as glucose; hexosamine liberated after acid hydrolysis, 8.6 per cent

Action of autolysin and lysozyme on the cell wall preparations. Cell wall preparations (preparation 1) were suspended in M/30 phosphate buffer (pH 7.2), mixed with autolysin solution, and the rate of dissolution at 30 C was followed by measuring the optical density change at 600 mμ. The autolysin dissolved the cell wall preparation. Crystalline lysozyme also dissolved the preparation. Preparation 2 was dissolved by both autolysin and lysozyme. In a similar experi-

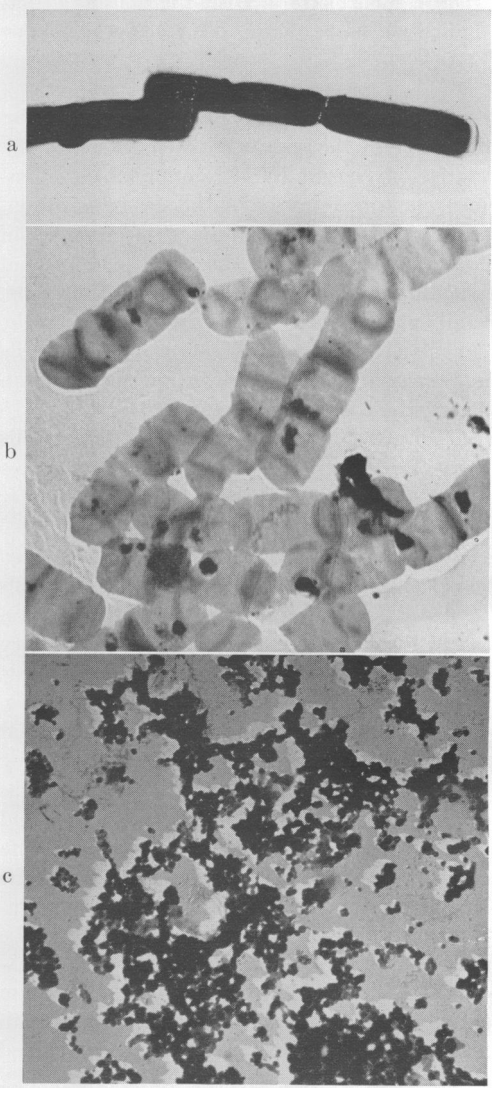

Figure 3. (a) Intact cells of *Bacillus subtilis.* (b) Cell walls of *Bacillus subtilis.* (c) The residue of cell walls after treatment with autolysin. Figures are electronmicrographs, × 7,000.

ment, the digested residue of the cell wall (preparation 2) was centrifuged at 13,000 rpm for 30 min after one night incubation in the presence of toluol. Only a small amount of residue was obtained. No residual cell wall could be seen under the electronmicroscope (figure 3b, c). Therefore it was established that this autolysin preparation contains an enzyme which dissolves the cell wall.

Formation of protoplasts. Weibull (1953) first demonstrated the formation of the protoplast of *B. megaterium* and explained the actual lytic effect of lysozyme on the whole living cells. As our strain of *B. subtilis* can be also lysed with lysozyme we attempted to obtain such protoplasts. First the cells were treated with lysozyme in various concentrations of sucrose. The apparent lytic effect was prevented in a concentration of 10 to 15 per cent of sucrose and a dilution of the suspending medium with water caused almost instantaneous clarification of the medium. Microscopic examination also confirmed that the effect of sucrose was the conversion of cells to protoplasts (figure 4). The formation of protoplasts are also observed in media containing 10 to 15 per cent polyethylene glycol (PEG, avg mol wt 400) instead of sucrose.

Action of autolysin in high concentrations of sucrose or polyethylene glycol. The dissolution of the cell wall by autolysin has been described above. If this process is the primary action of autolysin on the whole living cell, the formation of protoplasts by autolysin might be expected under suitable experimental conditions. Therefore, the effect of high concentrations of sucrose

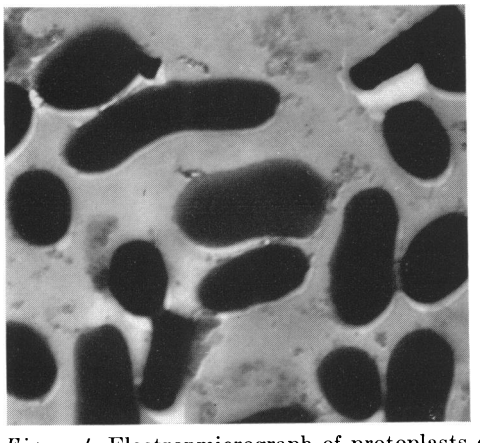

Figure 4. Electronmicrograph of protoplasts of *Bacillus subtilis*, × 8,400.

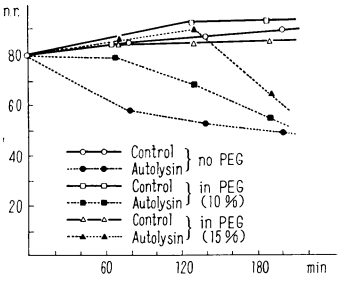

Figure 5. Effect of high concentration of polyethylene glycol on the action of autolysin. The composition of the reaction mixture: Washed cells of *Bacillus subtilis*, phosphate M/40, autolysin 1/8, and polyethylene glycol (PEG) as indicated in the figure. pH 7.2. Total volume 8 ml. The lysis was followed by taking the nephthelometer readings. Similar results were obtained when sucrose was used instead of PEG.

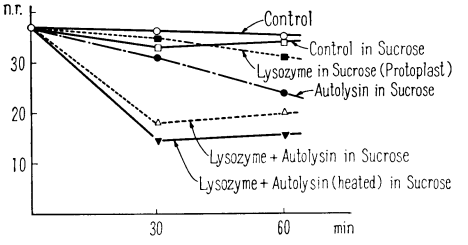

Figure 6. Action of the heated autolysin preparation on the protoplasts produced by the controlled treatment with lysozyme in sucrose. Composition of the reaction mixture: Washed cells of *Bacillus subtilis*, phosphate M/40, and the following additions as indicated in the figure. Autolysin preparation 1/8, lysozyme 6.2 μg/ml, sucrose 15%. Total volume 8.0 ml. pH 7.2. Heated autolysin preparation was prepared as follows: The autolysin solution was heated at 100 C for 10 min, coagulated protein was removed by filtration. Clear filtrate, adjusted to the original volume with water, was used as the heated autolysin preparation.

or PEG on the lytic action of autolysin was examined. As shown in figure 5 complete protection was observed during the first 1 to 2 hr but thereafter lysis occurred and the lytic action seemed to consist of two steps: (1) the dissolution of the cell wall, and (2) the disruption and lysis of the resulting protoplast. Autolysin preparations were heated at 100 C for 10 min to destroy the autolytic activity. Precipitated protein was filtered. This boiled solution was added to cells suspended in sucrose (15 per cent) together with the lysozyme solution. As can be seen in figure

6 complete lysis occurred while only protoplast formation was observed without boiled autolysin. The rate of dissolution of protoplasts by the boiled autolysin preparation was as fast as that by the nonboiled preparation. Both protease and RNAase were shown not to be able to dissolve protoplast under the experimental condition. From these results it was concluded that this autolysin preparation contains some heat stable substance(s) which causes the disruption of the protoplast, in addition to a heat labile enzyme which dissolves the cell wall, and that the primary site of action of autolysin is the cell wall.

Isolation of polysaccharide. The dissolution of the cell walls by autolysin may be supposed to be due to the hydrolysis of the polysaccharide contained in the cell wall as in the case with lysozyme. Therefore, the isolation of polysaccharide was attempted using a similar method to that of Meyer and Hahnel (1946).

Cells were grown in GBY medium at 30 C for 13 hr with shaking. The washed cells suspended in water were poured into 10 volumes of boiling water and heating was continued for 10 min. After cooling, the cells were centrifuged. In the light yellow supernatant the pentose nucleic acid was found (Nomura, 1956) but no polysaccharide could be obtained. Sedimented cells were treated with NaOH at a final concentration of 0.5 N and incubated for 12 hr at 30 C. The bulk of the cell materials was now dissolved and the resultant viscous solution was centrifuged in the cold. After washing with water the supernatants were combined and designated as alkaline extract I. The residual material was treated further with NaOH (1 N) at 30 C for 6 days. The supernatant obtained after centrifugation was named alkaline extract II. The residue of the second alkaline extraction was further extracted with alkali (2 N) at 30 C for 12 days and this third extract was named alkaline extract III. The isolation of polysaccharide was attempted from each of these extracts. The main preparations obtained were polysaccharide preparation A from alkaline extract I and polysaccharide preparation B from alkaline extract III. No significant amount of polysaccharide was obtained from alkaline extract II.

(a) Isolation of polysaccharide preparation A. The alkaline extract I was acidified with glacial acetic acid to pH 5.6 and the precipitate was removed. The clear supernatant was treated with 2 volumes of ethanol and stored in an ice box overnight. The precipitate was collected by centrifugation, washed with 80 per cent ethanol, dissolved in 10 per cent Na acetate solution and freed from the insoluble material by centrifugation. The clear supernatant was diluted with the same volume of water and shaken with a chloroform-butylalcohol mixture (V/V = 9:1) to remove protein. This process was repeated until no gel was formed on emulsification. To the aqueous solution finally obtained, $\frac{1}{10}$ vol of Zn acetate was added and the pH was adjusted to about 7.0. The precipitate was removed by centrifugation and the clear supernatant solution was acidified with acetic acid to pH 4 and precipitated with 2 vol of ethanol. The precipitate was collected after one night storage in the ice box, washed with 80 per cent ethanol, dissolved in 10 per cent Na acetate and reprecipitated with 3 volumes of ethanol. The final precipitate was dissolved in distilled water and dialyzed against frequent changes of distilled water in the ice box for 10 days and then freeze-dried. The white powder obtained was used as polysaccharide preparation A.

(b) Isolation of polysaccharide preparation B. Polysaccharide (preparation B) was isolated from the alkaline extract III by a method similar to that described in the previous section, except that Zn treatment was omitted.

These preparations (A and B) can be easily dissolved in water and show high viscosity in solution. Total reducing sugar and total hexosamine were determined after acid hydrolysis (2 N HCl, 4 hr at 100 C). Results are presented in table 1.

Action of autolysin and lysozyme on polysaccharide preparation A. Crystalline lysozyme was added to the solution of polysaccharide prepa-

TABLE 1

Analytical data on the polysaccharide preparation isolated from Bacillus subtilis

	Preparation A	Preparation B	Preparation C
Free reducing sugar*	<0.1%	<0.2%	
Total reducing sugar*	58%	42%	59%
Total hexosamine	0.46%	16.4%	2.0%
Total reducing sugar: Total hexosamine	126:1	2.6:1	30:1

* As glucose.

TABLE 2

Action of the autolysin and lysozyme on polysaccharide isolated from Bacillus subtilis

Expt. No.	Substrate	Enzyme		Time of Incubation (at 30 C)	Total Reducing Sugar of the Polysaccharide Added (as Glucose)	Reducing Sugar Liberated (as Glucose)	
			Conc.				Per cent of the amount of total reducing sugar of the polysaccharide added
				hr	*mg*	*μg*	
1	Polysaccharide B	lysozyme	44 μg/ml	4	6.8	650	9.6
				22	6.8	570	8.4
2	Polysaccharide B	autolysin	1/25	39	6.8	202	3.0
				62	6.8	222	3.3
3	Polysaccharide C	autolysin	1/25	48	12	63.4	0.53
	Polysaccharide C	autolysin	1/25	72	5.9	25	0.42
	Polysaccharide C	lysozyme	40 μg/ml	72	2.3	28.4	0.86

pH of the reaction mixture 7.2. Temp 30 C.

ration A at a final concentration of 98 μg/ml or 10 μg/ml. No reducing substance was liberated even after incubation for 72 hr at 30 C (less than 0.1 per cent of the total reducing sugar of polysaccharide added). The action of autolysin on this polysaccharide was also examined in a similar way at a final concentration of 1/25. In this instance, no appreciable increase in reducing groups was observed after 72 hr at 30 C (less than 0.02 per cent of the total reducing sugar of polysaccharide added).

Action of autolysin and lysozyme on polysaccharide preparation B. (a) Action of lysozyme. The results of the experiment presented in table 2 show that lysozyme at a final concentration of 44 μg/ml liberated about 9 per cent of the total reducing sugar of the polysaccharide within 4 hr and this amount seems to be the limit of decomposition.

(b) Action of autolysin. From the experimental results shown in table 2 it is clear that the autolysin preparation contains an enzyme which decomposes polysaccharide preparation B and liberates the free reducing groups. The amount of liberated reducing groups is approximately 3 per cent of the total reducing sugar of the polysaccharide added although the exact value of the degree of decomposition must await further experiments.

Isolation of polysaccharide (preparation C) from cell wall preparation and its decomposition by autolysin and lysozyme. Polysaccharide preparation B which was demonstrated to be decomposed by both autolysin and lysozyme was supposed to be present in the cell wall. Therefore isolation of polysaccharide from cell wall preparations was attempted.

A cell wall preparation 1 was treated with 2 N NaOH for 5 days at 30 C. Insoluble material was removed by centrifugation. Polysaccharide was isolated from this supernatant essentially in the same way as in the case of preparation B. Analytical data for this material (preparation C) are shown in table 1 and the results of experiments on the action of autolysin and lysozyme on this material are presented in table 2. It is clear that this polysaccharide preparation is also decomposed by both autolysin and lysozyme and liberates free reducing groups. Owing to the small amount of substance, exact values of the degree of decomposition were not determined, but they seem to be smaller than those in the case of preparation B. No fractional extraction with alkali was performed in the course of isolation of this material and consequently this preparation C seems to contain an inert polysaccharide such as preparation A in addition to the true substrate such as preparation B. These situations can be understood from the hexosamine contents of three preparations (table 1) which explains the low degree of decomposition of preparation C.

DISCUSSION

It has been demonstrated that autolysin can dissolve the cell wall and decompose the poly-

saccharide isolated from the cell. Therefore, as concluded from previous studies, autolysin itself obtained after anaerobic lysis of this strain can not be considered as bacteriophage, but rather must be an autolytic enzyme. However, as already emphasized (Nomura, 1955*b*), the phenomenon of anaerobic lysis itself might have a relation with some sort of lethal biosynthesis in this bacterium and the possibility of the synthesis of lysogenic bacteriophage can not be ruled out.

From the experimental results described it may be reasonable to conclude that the primary action of autolysin is the hydrolysis of polysaccharide contained in the cell wall and, as a result of this action, the structure of the cell wall is destroyed, the permeability barrier is disrupted and finally the dissolution of the cytoplasmic material occurs and this causes the visible lysis. RNAase and protease may play a secondary role in this system. Thus the primary action of autolysin on the living cells can be visualized as being analogous to the action of lysozyme on a cell wall carbohydrate substrate. Both agents were shown to decompose the same preparation of polysaccharide which shows high hexosamine content though the specificity of the susceptible bacterial species is greatly different. However, the degree of hydrolysis of this polysaccharide (preparation B) seems to be different. The difference in the specificity of both agents may be explained either by the difference in the specific linkage hydrolyzed in the same polysaccharide substrate or by the difference in the polysaccharide substrates which might be contained together in a preparation in an impure state. The final solution of this problem must await further experiments.

Studies on the nature of so-called autolytic enzyme are few and the direct action of autolytic enzyme on the cell wall is first demonstrated in this paper. The primary action is concluded to be on the cell wall, possibly on a polysaccharide contained in it. This conclusion seems to contradict the statement of Stacey and Webb (1948). However they also assumed an enzyme which splits the linkage between RNA and polysaccharide to cause the primary reaction before the hydrolysis of RNA proceeds, although they emphasized the importance of this gram converting reaction. Dubos also observed the liberation of reducing sugars from hyaluronic acid of other origins by his enzyme preparation (Meyer *et al.*, 1937). Therefore, in these cases too, the primary action of the autolytic enzyme might be the hydrolysis of polysaccharide and the resultant destruction of the cellular structure might cause the reaction with RNAase and the conversion to the gram negative state. The conversion of gram reaction with RNAase does not seem to be a primary and obligatory reaction in the action of autolytic enzyme in our system.

Recently Greenberg and Halvorson (1955) reported an autolytic substance produced during the sporulation of *Bacillus terminalis*. The autolysin in our studies is produced under conditions where little spore formation can be recognized, and therefore both substances seem to be different. Yet the properties of both substances show some similarity. Furthermore, both agents are produced in the phase when multiplication of cells is finished and abnormal biosynthesis such as spore formation in one instance, and amylase production in another, takes place. Although Greenberg and Halvorson did not report the substrate of their autolytic substance, its action may also be the dissolution of the cell wall, such as is the action of our autolysin, and may be responsible for the liberation of the free spore.

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SUMMARY

The action of several known enzymes on the cells of *Bacillus subtilis* strain H was examined and compared with the action of the autolysin produced by this bacterium. Besides autolysin, only lysozyme has an action on the living cells.

Cell wall preparations were isolated from *B. subtilis* and found to be dissolved by both autolysin and lysozyme.

The effect of high concentrations of sucrose or polyethylene glycol on the action of autolysin was examined. Visible lysis of cells occurred only after a fairly long lag period and it is likely that the dissolution of living cells by autolysin is effected by an attack on the cell wall. Polysaccharides were isolated from the cells and one preparation was shown to be decomposed both by autolysin and by lysozyme, and to liberate free reducing groups. Polysaccharide substrates

of these enzymes were also isolated from the cell wall preparations.

From these results it may be concluded that the primary action of autolysin is on the cell wall; probably on the polysaccharide contained in it.

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