BIOCHEMISTRY OF THE ACTINOMYCETALES

III. Cell Wall Composition and the Action of Lysozyme upon Cells and Cell Walls of the Actinomycetales

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In the two previous papers of this series (Romano and Nickerson, 1956; Romano and Sohler, 1956), it was reported that cell wall preparations of a few representative species of the genus *Streptomyces* were lysed by lysozyme, while species of the genus *Nocardia* were resistant to the action of this enzyme. Furthermore, striking differences in the carbohydrate composition of the cell walls of these two genera were found. The major carbohydrate constituent of the lysozyme-sensitive Streptomyces walls was found to be a hexosamine. In contrast, the cell walls of the lysozyme-resistant Nocardia contained only small amounts of hexosamine, and the major carbohydrate constituents were arabinose and galactose. These differences in carbohydrate composition may explain to some extent the difference in susceptibility to lysozyme action, since this enzyme is believed to attack certain linkages of some amino sugar containing carbohydrates (Meyer *et al.*, 1936; Epstein and Chain, 1940).

A survey of a larger number of actinomycetes was conducted to determine the susceptibility of the cells of various species to lysis by lysozyme. The carbohydrate composition of the cell walls of some lysozyme-resistant Streptomyces was determined, and the amino acid composition of the isolated cell walls of a number of actinomycetes was investigated. In addition, some of the products resulting from the action of lysozyme upon susceptible cell walls were examined, in an effort to gain some insight into the mechanism of this reaction.

MATERIALS AND METHODS

Organisms. The organisms employed are listed in table 1. The number after the name of an organism refers to the strain number in the Institute of Microbiology culture collection.

Medium, cultural conditions, and the preparation of isolated cell walls. The organisms were grown, harvested, and cell walls were isolated as described in previous papers of this series, except that *Streptomyces lavendulae* was cultured for 48 hr, *Streptomyces roseochromogenus* for 72 hr, and Micromonospora for 48 hr. The time required for sonic disruption of the organisms was 5, 30, and 15 min, respectively.

Enzymatic lysis. Lysis of isolated cell walls by lysozyme was carried out as described in previous papers. The lysis of whole cells was conducted as follows: Cells which had been grown in shake culture for 48 hr were harvested by centrifugation and washed twice with distilled water. The cells were subsequently washed once with M/15 Sorensen's phosphate buffer, pH 6.6, containing 0.017 M sodium chloride. The washed cells were uniformly suspended in buffer by the use of a Tenbroeck tissue homogenizer, and the suspension adjusted to an optical density of 0.80. To a Klett tube containing 4.5 ml of the cell suspension was added 0.5 ml of a lysozyme solution containing 2 mg per ml of Armour's crystalline egg white lysozyme. A control in each instance contained the cell suspension and 0.5 ml of buffer in place of the lysozyme solution. The reaction was carried out in a water bath at 37 C, and optical density determinations at 540 mμ were made at suitable time intervals with a Klett-Summerson photoelectric colorimeter.

Analytical determinations and color reactions. Total reducing sugar, pentose, and hexosamine were determined as described previously. Uronic acid was detected by the napthoresorcinol reaction of Tollens and Elsner (1935) and the Percival and Ross (1948) modification of the Dische (1947) carbazole reaction. Total nitrogen was determined by the semimicro procedure described

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TABLE 1

Effect of lysozyme on actinomycete cells

Organisms Lysed by Lysozyme	Strain No.	Organisms Not Lysed by Lysozyme	Strain No.
Streptomyces		Mycobacterium	
S. alboflavus	3008	*M. phlei*	23
S. albus	3448	*M. smegmatis*	607
S. antibioticus	3435	Nocardia	
S. aureus	3484	*N. asteroides*	3599
S. bobiliae	3310	*N. convolutus*	3414
S. californicus	3312	*N. corallina*	3408
S. chrysomallus	3657	*N. farcinica*	3301
S. coelicolor	3442	*N. paraffinae*	3410
S. craterifer	3373	*N. polychromogenes*	3409
S. erythreus	3036	*N. rubra*	3639
S. flavus	3321	Streptomyces	
S. fradiae	3535	*S. aureofaciens*	3550A
S. fulvissimus	3665	*S. lavendulae*	3516
S. globosus	3736	*S. lavendulae*	3530
S. griseus	3475	*S. lavendulae*	3440-8
S. griseus	3492	*S. lavendulae*	3440-14
S. ipomoea	3476	*S. roseochromogenus*	3816
S. lipmanii	3331	*S. venezuelae*	3534
S. olivaceus	3335	*S. viridochromogenes*	3356
S. parvus	3686		
S. praecox	3374		
S. purpurescens	3660		
S. reticuli	3344		
S. rimosus	3558		
S. rimosus	3560 variant		
S. scabies	3649		
S. violaceus	3497		

by Kabat and Mayer (1948). The Fiske-SubbaRow (1925) procedure was used for total phosphorus. Total sulfur and acetyl analyses were carried out by a commercial laboratory (Weiler and Strauss Microanalytical Laboratory, Oxford, England). The phosphate ester present in the cell wall was characterized by the barium salt fractionation procedure given in Umbreit *et al.* (1949) and by determination of acid lability on hydrolysis with 1 N HCl at 100 C (Leloir and Cardini, 1957).

Chromatographic and electrophoretic analyses. Chromatography for sugars was carried out by descending paper chromatography on Whatman no. 1 paper using the following solvent systems: ethyl acetate:pyridine:water (2:1:2), and *n*-butanol:acetic acid:water (4:1:5). The papers were irrigated with the solvent systems for 24 hr and 72 hr, respectively. Sugars were detected, after drying the papers, by spraying with aniline acid phthalate, according to the method of Partridge (1949).

Amino acids in the cell wall were determined by two dimensional paper chromatography after the cell walls had been hydrolyzed with 5 N HCl for 24 hr in a sealed tube at 100 C. Whatman no. 1 paper was used for the chromatograms, which were irrigated for 24 hr with phenol-ammonia in one direction and 18 hr with *n*-butanol:acetic acid:water (250:60:250) in the other direction. Amino acids were detected by spraying with 0.25 per cent ninhydrin in water-saturated butanol and heating at 100 C for 5 min.

The dinitrofluorobenzene (DNP) derivative of glucosamine was prepared and chromatographed according to the procedure of Kent *et al.* (1951). Paper electrophoresis of the DNP derivative was run on Whatman 3MM paper in 0.2 M borate buffer, pH 10, for 6 hr at a potential of 6.7 v/cm. A known sample of DNP glucosamine was run

as a standard for comparison in both the chromatography and electrophoresis.

Paper electrophoresis of the lysate fractions (resulting from lysozyme action) was carried out on Whatman 3MM paper in 0.2 M borate buffer, pH 8.6, and in 0.2 M acetate buffer, pH 5.6, at a potential of 6.7 v/cm for 6 hr. The peptides were located with the aforementioned ninhydrin spray. The hexosamine distribution was determined by cutting the paper strip into 2 cm bands. Each band was hydrolyzed with 2 ml of 2 N HCl. The hydrolyzates were neutralized with 2 ml of 2 N sodium hydroxide. The residual paper was removed by centrifugation and 2 ml of the supernatant was used for the hexosamine determination.

RESULTS

The results of the susceptibility of vegetative cells of actinomycetes to lysis by lysozyme are shown in table 1. Most of the Streptomyces tested were lysed by lysozyme, whereas strains of Nocardia and Mycobacterium were not. Lysis curves for a representative group of Streptomyces are shown in figure 1.

Cell wall preparations were made of two of the lysozyme-resistant Streptomyces, namely *S. lavendulae* and *S. roseochromogenus*. As was the case with whole cells of these species, the isolated cell walls of these organisms resisted dissolution by lysozyme. On the other hand, cell wall preparations of sensitive organisms were also sensitive to lysozyme. Thus, in all the cases examined, lysozyme-sensitive cells had cell walls sensitive to lysozyme, whereas resistant cells had lysozyme-resistant cell walls. It seems clear then, that the effect of lysozyme on actinomycetes is primarily upon the cell wall, as was shown to be the case for gram-positive bacteria by Salton (1954, 1956).

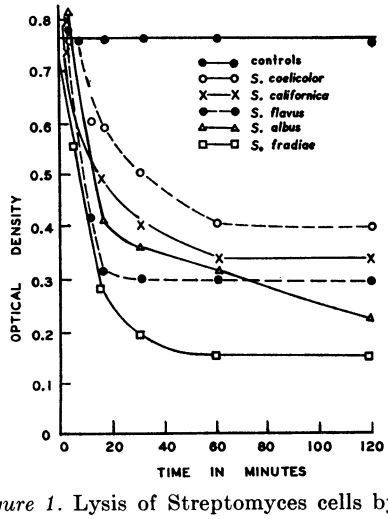

Figure 1. Lysis of Streptomyces cells by lysozyme, 100 μg per ml in M/15 Sorensen's phosphate buffer, pH 6.6, containing 0.01 M sodium chloride; reaction carried out at 37 C.

Carbohydrate composition. Analyses of cell wall preparations were made to compare lysozyme-sensitive and lysozyme-resistant cell walls. The results of analyses of cell wall hydrolyzates are shown in table 2. The hexosamine in the cell wall of *S. fradiae* has been shown to be N-acetyl glucosamine. The presence of glucosamine was established by paper chromatography and electrophoresis of the dinitrofluorobenzene derivative. Acetyl determinations on cell wall material revealed the presence of 3.5 per cent acetyl. This would account for the major portion of the glucosamine being acetylated. It is seen in this table that, in the case of lysozyme-susceptible cell walls, hexosamine accounts for the major portion of the sugar liberated by hydrolysis. The low amino sugar content of *S. lavendulae* and *N. rubra* probably explains the resistance of these cell walls to lysozyme. The cell wall of *S. roseochromogenus* was an exception, since it was found to contain a considerable amount of amino sugar. It differed from lysozyme-susceptible cell walls by having a higher carbohydrate content. In addition to hexosamine, a complex mixture of hexoses, principally mannose and galactose, was found to be present in the cell wall of *S. roseochromogenus*.

The cell wall of *S. lavendulae* was found to be more resistant to acid hydrolysis than was the case for other species examined. Whereas 2 N HCl hydrolysis at 100 C for 1 hr was sufficient for complete hydrolysis of cell walls of other species tested, with this organism hydrolysis was not complete after 4 hr under these conditions. Maximum liberation of reducing sugars was obtained by 5 N HCl hydrolysis at 100 C for 20 min; longer hydrolysis under these conditions resulted in some destruction of liberated sugars, as shown in figure 2. Hydrolysis curves of this type are often indicative of uronic acid-containing polysaccharides, since conditions required

TABLE 2

Carbohydrate composition of actinomycete cell walls; sugars liberated by hydrolysis

Organism	Total Reducing Sugar	Pentose	Hexosamine	Lysozyme Sensitivity
	%	%	%	
Streptomyces fradiae (3535*)	22.0	0	19.8	+
Streptomyces griseus (3492*)	21.6	0	19.9	+
Micromonospora (3452*)	15.9	1.8	14.8	+
Nocardia rubra (3639*)	18.1	10.5	2.6	−
Streptomyces lavendulae (3416†)	13.2	1.5	3.0	−
Streptomyces roseochromogenus (3816*)	27.2	1.2	14.8	−

* Hydrolyzed with 2 N HCl at 100 C for 2 hr.
† Hydrolyzed with 5 N HCl at 100 C for 20 min.

for the complete hydrolysis of glucosidic linkages involving uronic acids are often sufficient to cause the destruction of a considerable portion of the liberated uronic acids or accompanying sugars (Bell, 1955). A positive reaction for uronic acid was obtained with the naphthoresorcinol and carbazole tests, and paper chromatography of hydrolyzates using the solvent systems employed for sugars showed slow moving components corresponding to uronic acids. A positive identification of the particular uronic acid(s), or a reliable quantitative analysis, have not yet been obtained.

Amino acid composition. In contrast to the carbohydrate composition, the amino acid composition of the cell wall is qualitatively quite similar in all of the actinomycetes examined. Amino acids found by two dimensional paper chromatography of cell wall hydrolyzates are shown in table 3. Glutamic acid, alanine, and α-ϵ-diaminopimelic acid were found in major amounts in all of the cell walls studied. The presence of diaminopimelic acid was demonstrated by several criteria. Cystine and ethanolamine *ortho*phosphoric acid, which have R_f values similar to diaminopimelic acid, were excluded because on treatment of the hydrolyzate with hydrogen peroxide according to the method of Dent (1948), the amino acid pattern remained unchanged. This indicated that the diaminopimelic acid spot was not due to cystine, since cystine would oxidize to cysteic acid under these conditions. The absence of ethanolamine *ortho*-phosphoric acid can be established by electrodialysis of a sample of the hydrolyzate. Electrodialysis was carried out by placing a 1 ml sample of the hydrolyzate in a cellophane dialysis bag which was suspended between two carbon

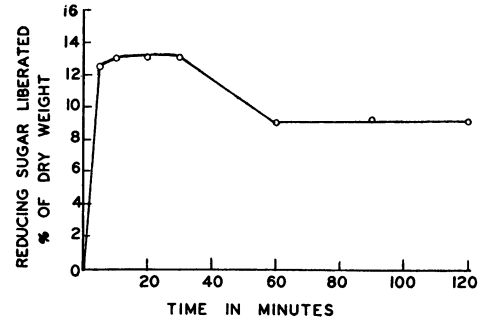

Figure 2. Rate of hydrolysis of *Streptomyces lavendulae* cell wall by 5 N HCl at 100 C.

electrodes in a cell through which 3 L of cold distilled water circulated. A potential of 45 v was applied with a resulting current flow of 0.2 amperes. Samples were removed at intervals and chromatographed for amino acids by two dimensional analysis. The rate of disappearance of the diaminopimelic acid spot was compared with the rate of disappearance of the glutamic acid spot, since these spots are normally of similar intensity on chromatograms. If the "diaminopimelic acid spot" were actually due to ethanolamine phosphoric acid, it would, due to its acidic nature, disappear as fast or faster than the glutamic acid spot in the presence of an electrical field. Finally, a sample of known diaminopimelic acid behaved identically with the material contained in the cell wall hydrolyzates. The presence of diaminopimelic acid in actinomycete cells has also been reported by Hoare and Work (1957) and in actinomycete cell walls by Cummins and Harris (1956*b*).

No sulfur-containing amino acids were found, although their presence cannot be entirely ruled

TABLE 3

*Amino acids found in hydrolyzates of actinomycete cell walls**

Amino Acid	*Streptomyces fradiae* (3535)	*Streptomyces griseus* (3492)	*Nocardia rubra* (3639)	Micromonospora (3452)	*Nocardia polychromogenes* (3409)
Alanine	++++	++++	++++	++++	++++
Arginine	+	+	+	++	++
Aspartic acid	++	++	++	++	++
Cysteic acid	0	0	0	+	0
Diaminopimelic acid	++++	++++	++++	++++	++++
Glycine	+	+	+	+	+
Glutamic acid	+++	++++	+++	++++	+++
Lysine	+	+	+	++	++
Phenylalanine, leucine, isoleucine	+++	++++	++	++	+++
Proline	0	0	0	0	±
Serine	+	+	+	+	+
Threonine	++	++	++	++	+
Tyrosine	+	0	0	+	+
Valine	++	+++	++	++	+
Hexosamine	+++	++	+	++	+

* ++++ = indicates large, intensely colored spot on chromatogram; +++ = indicates a fairly intense spot; ++ = is a small definite spot; + = is a faint spot; ± = doubtful; 0 = no spot was found, amino acid absent.

TABLE 4

Nitrogen, sulfur, and phosphorus content of actinomycete cell walls

Organism	Total N	Hexosamine N	Protein N*	Protein 6.25 × Protein N	Total S	Sulfur: Protein Ratio	Total P
	%	%	%	%	%		%
Streptomyces fradiae (3535)	6.7	1.5	5.2	32.5	0.2	0.62	1.3
Streptomyces griseus (3492)	7.1	1.6	5.5	34.4	0.2	0.58	2.7
Nocardia polychromogenes (3409)	8.4	0.2	8.2	51.2	0.3	0.59	0.5
Micromonospora sp. (3452)	8.1	1.2	6.9	43.1	0.4	0.93	1.3

* Protein N = total N − hexosamine N.

out, since considerable destruction of these amino acids can take place under the conditions of hydrolysis. However, if present at all, sulfur containing amino acids would represent a minor constituent of the cell wall protein, as evidenced by the low sulfur to protein ratio found (table 4). Proline also was not detected in any of the cell wall preparations investigated.

Nitrogen, phosphorus, and sulfur contents. Results of total nitrogen, phosphorus, and sulfur determinations are shown in table 4. Nocardia appear to contain a higher amount of protein than Streptomyces. The reverse is true with respect to phosphorus content. This latter finding may have some direct relationship with the observed differences in lysozyme-sensitivity. Meyer (1953) stated that the substrate for lysozyme is comprised of N-acetyl-D-glucosamine-alcohol-phosphate. The phosphorus and sulfur compounds occurring in these cell walls have not yet been positively identified, although the phosphorus appears to be esterified, since most of the phosphorus is found in the barium-soluble, alcohol-insoluble fraction upon barium salt fractionation, and only 50 per cent of the phosphorus in the cell wall is liberated by hydrolysis with 1 N HCl at 100 C for 15 hr. As was stated above, sulfur-containing amino acids were not found.

The action of lysozyme on the cell wall of S. fradiae. The action of lysozyme on isolated cell

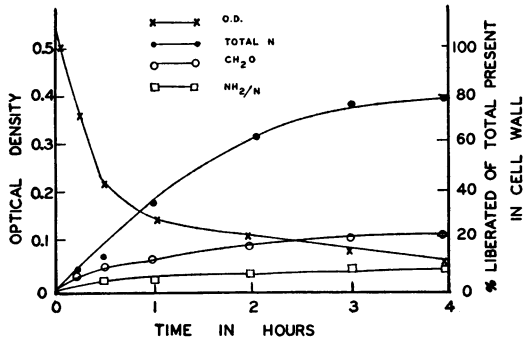

Figure 3. Action of lysozyme on the cell wall of *Streptomyces fradiae*. Lysozyme concentration 100 μg per ml; reaction carried out in M/15 Sorensen's phosphate buffer, pH 6.6, containing 0.017 M sodium chloride.

wall material was studied in an effort to gain some insight into the mechanism of the action of this enzyme, and to compare its action on actinomycetes to that reported by Salton (1956) on bacterial cell walls. The results of incubation of cell walls of *S. fradiae* with lysozyme are shown in figure 3. Concomitant with a decrease in optical density of the cell wall suspension, there was a liberation of 80 per cent of the total nitrogen of the cell wall. Twenty per cent of the total reducing sugar liberated by acid hydrolysis was liberated by lysozyme treatment, and 10 per cent of the total amino nitrogen was liberated. No free hexosamine or amino acids were found in the lysate.

The lysate was examined as to the nature of the products resulting from the dissolution of the cell wall material by lysozyme. After incubation of a cell wall suspension (containing about 1 mg cell wall material per ml) in the presence of lysozyme for 24 hr, 10 ml portions of the lysate were dialyzed against 1 L of distilled water for 48 hr. Dialyzates and the undialyzable fractions were lyophilized, as were aliquots taken before lysozyme action and before dialysis. These fractions were analyzed for hexosamine both before and after hydrolysis with 2 N HCl at 100 C. The results are shown in table 5. No free hexosamine was detected until after the lysate had been hydrolyzed. All of the hexosamine was found in the undialyzable fraction, indicating that the hexosamine was present in some form of polymer. However, hexosamine did not appear in the precipitate that formed when trichloracetic acid was added to the nondialyzable fraction of the lysate to make a final concentration of 8 per cent.

TABLE 5

Hexosamine distribution in various fractions of cell wall of Streptomyces fradiae after lysis by lysozyme

	Hexosamine
	μg
At zero reaction time	
Residue, hydrolyzed	2120
Supernatant, unhydrolyzed	0
Supernatant, hydrolyzed	0
After reaction of the cell wall with lysozyme for 24 hr.	
Residue, hydrolyzed	0
Supernatant, unhydrolyzed	0
Supernatant, hydrolyzed	2230
On dialysis of material which had been reacted with lysozyme for 24 hr.	
Dialysate	0
Undialyzable fraction, unhydrolyzed	0
Undialyzable fraction, hydrolyzed	2140
Trichloracetic acid fractionation of the undialyzable fraction	
TCA precipitate, hydrolyzed	0
TCA supernatant, hydrolyzed	2090

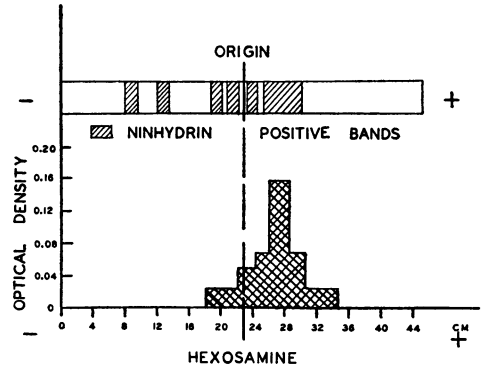

Figure 4. Electrophoretic analysis for ninhydrin positive material and hexosamine in the undialysable lysate fraction; conducted in 0.2 M acetate buffer, pH 5.6, for 6 hr at a potential of 6.7 v/cm.

When a sample of the undialyzable fraction of the lysate was subjected to paper electrophoresis, in acetate buffer, 6 ninhydrin-positive bands were obtained. Hexosamine determinations made after cutting the paper strips into 2-cm bands showed that the broadest ninhydrin band corresponded to the hexosamine peak, as shown in figure 4.

It thus appears that lysis by lysozyme resulted in a liberation of a number of complex substances, as has been shown to be the case in bacteria (Salton, 1956). In contrast to Salton's findings, however, all of the hexosamine remained in the undialyzable portion, whereas Salton found that lysozyme treatment of various bacterial cell walls resulted in the liberation of a dialyzable "small fragment" containing N-acetyl glucosamine and an unidentified amino sugar.

DISCUSSION

The cell walls of actinomycetes are similar in several respects to the cell walls of a number of gram-positive bacteria. The cell walls of Nocardia, in particular, resemble the cell wall of Corynebacteria. Corynebacteria, according to Cummins and Harris (1956*a*), have arabinose and galactose as the major carbohydrate cell wall constituents, and glutamic acid, diaminopimelic acid, and alanine as the major amino acids present. These same constituents were found in the cell walls of all of the Nocardia examined. This finding could very well indicate a close relationship between the Corynebacteria and the Actinomycetales.

The "bacterial nature" of actinomycete cell walls is also evident from their mucoid characteristic. The mucoid nature of the cell wall is primarily evident in those cell walls which are lysed by lysozyme. Reference has already been made to the mucoid nature of the lysozyme substrate. The analytical data presented indicate that cell walls of actinomycetes are composed mainly of carbohydrate and protein or peptides.

The products resulting from the action of lysozyme on the cell walls of gram-positive bacteria and Streptomyces are similar in some respects. Salton (1956) reported that free amino acids and free amino sugars are not released by the action of the enzyme on the cell walls of *Micrococcus lysodeikticus*, *Bacillus megaterium*, or *Sarcina lutea*. The same situation was found with *S. fradiae*. Both in the case of the bacteria, and in the case of *S. fradiae*, a complex mixture of peptides is released by the action of the enzyme. Salton found that with bacteria a disaccharide composed of two amino sugars was one of the products released by the action of lysozyme. In the case of *S. fradiae*, all of the amino sugar was accounted for in the undialyzable fraction and a disaccharide was not found in the dialyzate. This was the principal difference which was found between the action of lysozyme on bacterial and on actinomycete cell walls.

The presence of N-acetyl glucosamine and a phosphate ester in the cell wall of *S. fradiae* may explain the susceptibility of these walls to lysozyme action. As noted previously, Meyer (1953) has stated that the substrate for lysozyme is comprised of N-acetyl-D-glucosamine-alcohol-phosphate. High phosphate contents were also found in *S. griseus* and Micromonospora, whereas the phosphate content of Nocardia was considerably lower. The low phosphorus and amino sugar content of the Nocardia walls may explain the resistance of these walls to lysozyme action.

The bacterial character of the actinomycete cell wall is further evident in the ease with which the wall is hydrolyzed and by the solubility of the wall in hot alkali. These properties indicate the absence of β-linked polysaccharides such as chitin, cellulose, and glucan, which are commonly found in yeast and fungi.

SUMMARY

The bacterial nature of the actinomycete cell wall was demonstrated by a number of criteria. In amino acid and carbohydrate composition the walls of species of the genus *Nocardia* are very similar to the walls of corynebacteria. Arabinose, galactose, glutamic acid, diaminopimelic acid, and alanine are the principal constituents found in these walls. In general, the actinomycetes resemble the gram-positive bacteria in that their amino acid composition is somewhat limited, and in that many of them are susceptible to lysis by lysozyme. Proline and sulfur-containing amino acids were generally not found in the cell walls of actinomycetes. The action of lysozyme is primarily upon the cell wall of susceptible organisms. The substrate for lysozyme seems to involve the presence of a considerable amount of hexosamine, as well as the possible presence of a phosphate ester. The lysis of the cell wall results in the release of a complex mixture of peptides, but free amino acids or free amino sugar are not released. Because of the complexity of the breakdown products resulting from the action of the enzyme, the nature of the linkage attacked by the enzyme could not be determined.

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