basic science. Consequently, such major enterprises must be examined outside the normal level-of-effort framework. And since the economics of choice do not help much in dealing with such matters, the politics of choice may govern—such factors, for example, as national scientific posture.

From all this one can gather that budget decisions affecting science are among the hardest to make. What has to be remembered is that decisions affecting research are frequently as opportunistic as those affecting other public investments, in the sense that they are made in a bargaining process that is common ground for all problems of choice. There can be no fail-safe procedure to eliminate risk in budgeting for science.

In the folkways of bureaucracy, the role of the Bureau of the Budget tends to loom bigger than life. Political and behavioral scientists have begun to probe this phenomenon, with results that threaten to expose us as human, after all. Still, the legends persist. One is made to feel that spine-chilling rites are being practiced in the quaintly Byzantine edifice next to the White House. This is largely our own fault, for taking to heart the counsel of anonymity. We suffer in silence and bury our dead quietly. Victories go uncelebrated, defeats unrecorded. Yet there is in the bureau's character a saving streak that recalls the opening line of *Scaramouche*—he was born with the gift of laughter and a sense that the world is mad.

As this is being written, the 1968 budget is before the Congress. Though its prose is sedate, one can hardly miss the signs of ordeal that marked its preparation. Painful choices are apparent, and the ends-means squeeze starkly visible. With all this, expenditures for R & D are budgeted to increase by one-half billion dollars, to a record high of more than $17 billion. Funds for development will be smaller, while outlays for research will be significantly greater than in the current fiscal year. It may not be an affluent growth pattern, but neither does it justify gloom and predictions of doom. In the marine sciences, in urban research, in basic science, in weather research, and elsewhere, some gains have been managed. Under less trying circumstances the outcome might have been considerably better for science, but, by any reasonable measure, the aggregate level of investment is massive and growing.

But size and trend are not indicators of balance or of social returns on investment. This is precisely why it is always open season when the budget sprints across the political horizon. As a moving target it brings out the sportsman in each of us.

Mechanisms of Enzymatic Bacteriolysis

Cell walls of bacteria are solubilized by action of either specific carbohydrases or specific peptidases.

Jack L. Strominger and Jean-Marie Ghuysen

During the 1920's, Fleming worked extensively with two types of bacteriolytic agents, one present in nasal secretions and other animal tissues (*1*) and the other secreted by a penicillium mold (*2*). It is a remarkable fact that the modes of action of these two types are now known to be fundamentally similar. Each brings about a loss of integrity of the cell wall of bacteria, and, as the consequence, the organisms are unable to survive. However, these two types of agents, one an enzyme (called lysozyme by Fleming) and the other an antibiotic (penicillin), bring about their effects on the integrity of the cell wall through entirely different mechanisms. Penicillin interferes with one of the terminal steps in synthesis of the bacterial cell wall, a cross-linking reaction catalyzed by peptidoglycan transpeptidase (*3*). Its effect on wall synthesis is highly selective, and no other metabolic processes in bacteria are known to be inhibited under conditions in which cell-wall synthesis is virtually totally blocked. Inhibition of cell-wall synthesis is also the mode of action of bacitracin, vancomycin, the ristocetins, and D-cycloserine. On the other hand, lysozyme is an enzyme which catalyzes the hydrolysis of a structurally important linkage in the cell wall, and, as a result, this complex polymeric substance is solubilized (*4*). In fact, lysozyme is only one of a class of enzymes that hydrolyze bacterial cell walls. Many of these enzymes are bacteriolytic; they lyse intact bacterial cells. Although a great deal of attention has been given to antibiotics, that is, substances of relatively small molecular size which are produced by some microorganism and kill others, comparatively less work has been done on another large group of bacteriocidal substances found in nature, the bacteriolytic enzymes.

The possible presence of bacteriolytic enzymes in bacteria and in animal tissues was recognized early in this century, and interest in this group of agents was revived during the 1920's when Fleming (*1*) conducted his extensive studies of the bacteriolytic enzyme in animals and Lieske (*5*) and Gratia and Dath (*6*) carried out similar studies of the bacteriolytic agents excreted by *Streptomyces* strains. The latter were shown to be enzymes by Welsch (*7*). Indeed, Gratia attempted to produce soluble antigens through the action of these bacteriolytic agents on microorganisms. During the next decade, lysozyme was crystallized from egg white and was shown to be a glycosidase. Its natural substrate is the polysaccharide of the bacterial cell wall (*8*).

Plants, as well as animal tissues, contain bacteriolytic enzymes. These enzymes are also produced by various microorganisms under many circum-

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stances. These include autolysins (intracellular enzymes capable of degrading the cell walls of the same organism in which the enzyme is found, as well as those of others), virolysins (produced by bacteria on infection with bacteriophages or found as constituents of the virus itself), extracellular enzymes (sometimes excreted in large amounts by bacteria), and spore lysins (presumably produced or activated at the time of spore germination).

The precise mechanisms by which a number of these enzymes hydrolyze bacterial cell walls have been discovered in recent years. Although the term "lysozyme" has generally been used to apply to all of these bacteriolytic enzymes, it is now apparent that a large number of hydrolytic mechanisms occur and that the term lysozyme should be restricted to that group of basic proteins which are thermostable and which catalyze the hydrolysis of the glycosidic linkage between acetylmuramic acid and acetylglucosamine. So far as is known, this linkage is found in all bacterial cell walls. In addition to lysozymes, a variety of other carbohydrases, both endoacetylmuramidases and endoacetylglucosaminidases, occur which can hydrolyze the polysaccharide of the bacterial cell wall. It has become apparent that peptidases can also hydrolyze cell walls. In fact, a large number of such enzymes have been discovered. The availability of carbohydrases and peptidases which hydrolyze cell walls has enabled the isolation of both the intact cell-wall polysaccharide and the cell-wall poypeptide which are cross-linked in an intricate net to form the cell wall. Both are freely soluble in water. From earlier studies on lysozyme it had been believed that the insolubility of the carbohydrate accounted for the insolubility of the cell wall, but it is now apparent that the insolubility is due to the cross-linking of the two polymers to each other.

General Structure of the Bacterial Cell Wall Peptidoglycan

A discussion of the mechanisms of hydrolysis of cell walls by bacteriolytic enzymes requires a brief description of cell wall structure. Ghuysen, Strominger, and Tipper have provided a review of the structure of the cell wall (*9*). The bacterial substrate which is hydrolyzed by the bacteriolytic enzymes so far studied is a rigid peptidoglycan located within the cell wall and built up of polyacetylhexosamine and peptide chains. Schematically, the linear polysaccharide chains are composed of alternating β-1,4-linked *N*-acetylglucosamine (X) and *N*-acetylmuramic acid (Y) residues. Peptide chains substitute either all of the acetylmuramic acid residues in some microorganisms or some of them in others through *N*-acetylmuramyl-L-alanine linkages. The constituents of these peptide chains are almost always L- and D-alanine, D-glutamic acid, and one dibasic amino acid which is most often L-lysine or one of the isomers meso, DD-, or LL-diaminopimelic acid; but sometimes another dibasic amino acid is found. The structure of the peptide subunits of the peptidoglycan of the bacterial cell wall has been determined in several cases. With *Staphylococcus aureus*, the sequence acetylmuramyl-L-alanyl-D-γ-isoglutaminyl-L-lysyl-D-alanine occurs in the peptidoglycan. As a working hypothesis, one assumes that this acetylmuramyl tetrapeptide, or a similar peptide containing another dibasic amino acid, is the peptide subunit of all bacterial cell-wall peptidoglycans. The precursor of this structure is a uridine nucleotide, UDP-acetylmuramyl-L-Ala·D-Glu·L-Lys·D-Ala·D-Ala (*10*). The last amino acid of the pentapeptide is lost in a transpeptidation, the last reaction in cell-wall biosynthesis.

Cross-links occur between tetrapeptide subunits resulting in a network of two or three dimensions. So far as is known, the terminal carboxyl group of D-alanine and the ϵ-amino group of L-lysine or one of the amino groups of DAP are involved in these cross-links, either directly or by the means of additional peptide chains which are referred to as peptide bridges. The nature and the length of these cross-linking bridges vary according to the bacteria: pentaglycine in *Staphylococcus aureus* strain Copenhagen; tri-L-Ala-L-Thr in *Micrococcus roseus* R27; tri-L-Ala in another strain of *M. roseus;* di-glycine in *M. radiodurans* and mono-L-Ala in *Arthrobacter crystallopoietes.* L-Ornithine has also been shown to be the dibasic amino acid taking part in the peptide cross-links in *Micrococcus radiodurans.* No direct evidence of cross-links involving one of the amino groups of the unusual dibasic amino acids, hydroxylysine, diaminobutyric acid, or 2,6-diamino-3-hydroxypimelic acid, has been reported but analyses of the terminal amino groups in the relevant cell walls suggests that at least part of them are actually engaged in such cross-links. In all cases studied, D-glutamic acid is not a branching point in the peptide moiety. Its α-carboxyl group, however, is sometimes substituted, for example, as an amide (in the case of walls of *Staphylococcus aureus, Arthrobacter crytallopoietes, Micrococcus roseus, Streptococcus pyogenes,* and *Corynebacterium diphtheriae*) or a glycine residue (in the walls of *Micrococcus lysodeikticus*). Similarly, the "extra" carboxyl group of DAP which is not used in cross-linking may also be substituted by an amide, as found in *Corynebacterium diphtheriae.*

The tightness of the peptidoglycan network obviously depends on the frequency with which the polysaccharide chains are substituted by peptide subunits and with which these peptides are, in turn, interlinked. The extent of the peptide cross-linking can be measured by the terminal amino and carboxyl groups occurring in the peptidoglycan. Measurement of the groups present in one of the tightest peptidoglycans so far known, that of *Staphylococcus aureus,* shows that cross-linking gives rise to peptide-linked polymers whose average size does not exceed ten tetrapeptide subunits. Similarly the intact polysaccharide has been isolated from various bacterial cell walls. It is soluble in water and consists of chains of relatively small size, not exceeding about 12 disaccharide (X-Y) units.

Schematic Structure of Peptidoglycans from Cell Walls

In the peptidoglycan of *Staphylococcus aureus* (Fig. 1), almost all available chemical groups in the network are used in cross-links (*9*). All acetylmuramic acid residues are substituted by the tetrapeptide subunits which, in turn, are interlinked through D-alanyl-$(glycyl)_5$-N^{ϵ}-L-lysine bridges. However, about 7 percent of the peptide subunits are not cross-linked to any other unit. These nascent peptidoglycan units contain at their carboxyl terminal end an additional D-alanine residue as found in the cell-wall precursor UDP-acetylmuramyl pentapeptide. These nascent units also bear open pentaglycine chains. In the glycan, both acetylmuramic acid and acetylglucosamine are linked to each other by the 1,4-linkage. The β-configuration of the linkages from *N*-acetylglucosamine to *N*-acetylmuramic acid has been established. The β-configuration for the linkages from *N*-acetylmuramic acid to *N*-acetylglu-

cosamine is suggested by nuclear magnetic resonance studies, but further work is required to establish this conclusively. Finally about 60 percent of the *N*-acetylmuramic acid residues are substituted on carbon-6 by an *O*-acetyl group. The peptidoglycan of *Micrococcus roseus* resembles that of *Staphylococcus aureus* (*9*). In the former, however, the glycan moiety has no *O*-acetyl substituent. The tetrapeptide substitutes are identical to those found in the latter. However, about 25 percent of the peptide subunits are tripeptides, L-Ala-γ-D-Glu(αCONH$_2$)-L-Lys rather than tetrapeptides. These tripeptides are located at the carboxyl terminal end of the polypeptide polymer. The peptide bridges are, in the two strains so far examined, tri-L-Ala and tri-L-Ala-L-Thr.

In the peptidoglycan of *Escherichia coli* B (Fig. 2) peptide cross-links are infrequent (*11*). Virtually all disaccharide units (X–Y) of the polysaccharides are substituted by tetrapeptide subunits, probably with the sequence L-Ala-D-Glu-meso-DAP-D-Ala. About 50 percent of those are cross-linked through direct D-Ala-meso-DAP linkages, the disaccharide units from two adjacent polysaccharide chains thus being paired. Peptide-linked dimers of two tetrapeptides are formed but larger polypeptide oligomers, such as those found in *Staphylococcus aureus* and *Micrococcus roseus*, do not occur. A random distribution of the disaccharide-peptide unit and of its dimer throughout at least a major part of the peptidoglycan has been proposed, resulting in a loose network of the type represented in Fig. 2. The glycosidic linkages have not been extensively studied; however, the data so far obtained are compatible with 1,4-linkages, as in the case of the walls of *Staphylococcus aureus* and *Micrococcus roseus*. The α-carboxyl group of glutamic and one of the carboxyl groups of DAP are unsubstituted.

The peptidoglycan of *Micrococcus lysodeikticus* (Fig. 3) has unusual properties. About half of the disaccharide units of the polysaccharide chains are free of peptide and form unsubstituted oligosaccharide segments containing up to eight *N*-acetylhexosamine residues. Most of the glycosidic linkages, if not all of them, are β-1,4-linkages. Short pentapeptides that are not cross-linked (tetrapeptides with an additional glycine residue on the α-carboxyl group of glutamic acid, that is, L-Ala-D-γ-Glu(α-Gly)-L-Lys-D-Ala) occur on acetylmuramic acid residues, or a cross-linked dimer of two pentapeptides links the disaccharide units of two adjacent polysaccharides, thus paralleling the structure encountered in the peptidoglycan from *Escherichia coli*. Larger peptide-linked oligomers (trimers or tetramers) also occur, but more extensive cross-linking with formation of even higher oligomers, as those found in both *Staphylococcus aureus* and *Micrococcus roseus*, does not occur. About 50 percent of the lysine residues are not engaged in cross-linking and have their ε-amino groups free.

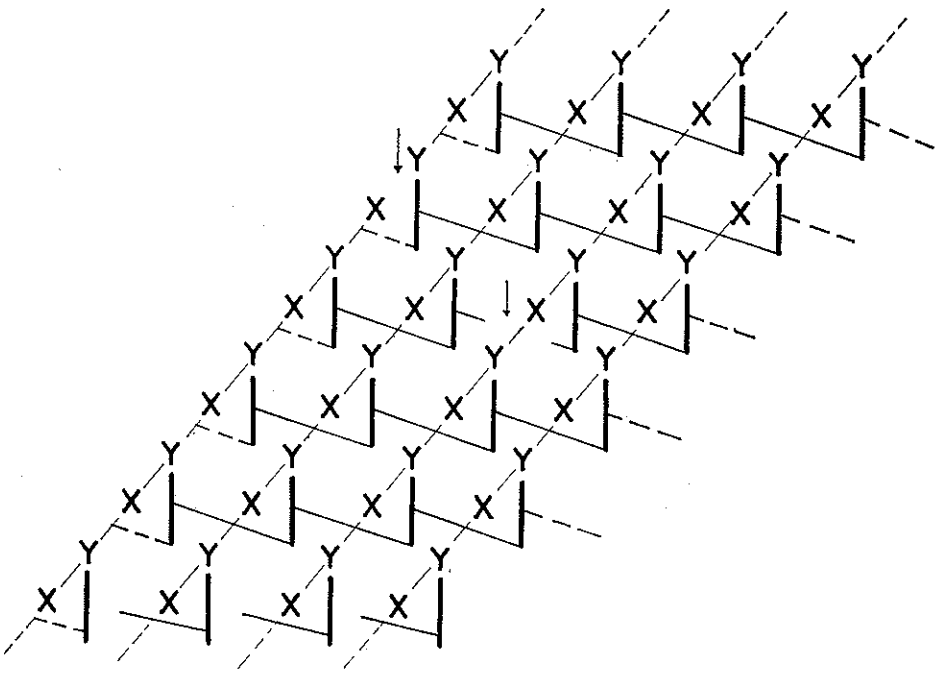


Fig. 1. Schemetic representation of the structures of the peptidoglycans of *Staphylococcus aureus* and *Micrococcus roseus*. These are tight networks. Note the large size of the polypeptide network. *X*, acetylglucosamine, and *Y*, acetylmuramic acid, are the two sugars in the polysaccharide. Light lines (———) are chemical bonds. Medium lines (———) represent the cross-bridge (pentaglycine in *S. aureus* or tri-L-alanyl-L-threonine in *M. roseus*). Heavy lines (▬▬) represent the tetrapeptide linked to acetylmuramic acid.

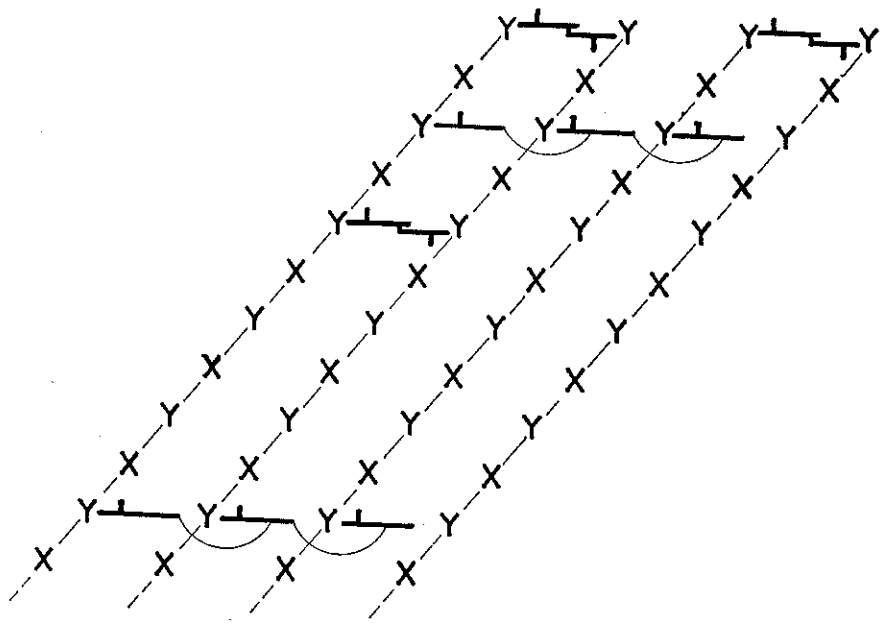


Fig. 2. Schematic representation of the structure of the peptidoglycan of *Micrococcus lysodeikticus*. This is a loose network in which peptide-linked dimers and trimers occur, and many acetylmuramic acid residues are unsubstituted. Some features of this structure are still unknown and this representation does not account for all of the available data. For symbols, see legend to Fig. 1.

Moreover, in this peptidoglycan, D-Ala-N^{ϵ}-Lys bonds interlink the peptide chains without any intervening peptide bridges. There are some very interesting unsolved problems in the petpide structure of the peptidoglycan of this organism.

Mechanism of Action of the Bacteriolytic Enzymes

The solubilization of peptidoglycan networks can be brought about by three classes of enzymes. These enzymes hydrolyze the polysaccharide chains (glycosidases), or they split the peptide cross-links (endopeptidases), or they cleave the junctions between polysaccharides and peptides (acetylmuramyl-L-alanine amidases). So far, no example of a bacteriolytic enzyme which splits within the tetrapeptide itself has been reported. Examples of each of these three classes of enzymes have now been studied (Table 1). Many of the peptidases which are bacteriolytic are entirely free of detectable carbohydrase activity and solubilize cell walls only by hydrolysis of peptide bonds. The long-held belief that the insolubility of the wall was due to the chitin-like glycan resulted from the fact that, for 20 years, the only characterized bacteriolytic enzymes were glycosidases. In fact, the glycan, like the polypeptide, isolated from the cell wall is a water-soluble polymer. The glycan, at least in *Staphylococcus aureus*, is in fact a substituted chitin (a water insoluble polymer of alternating β-1,4-linked acetylglucosamine residues found in crustacean shells, for example). However, in the cell-wall glycan every other acetylglucosamine residue is substituted by the D-lactic acid ether whose carboxyl groups are presumably responsible for the water solubility of this polymer. The insolubility of the wall is a property of the whole peptidoglycan and is due to the cross-linking of the polymers within it. So far as is known, the hydrolysis, in sufficient number, of any single chemical linkage within the cross-linked network can bring about solubilization of bacterial cell walls.

At least three factors are involved in the activity of the bacteriolytic enzymes (besides their intrinsic enzymatic specificity for chemical bonds hydrolyzed): the tightness of the network, the presence in the walls of nonpeptidoglycan components, and the chemical properties of the peptidoglycan substrate itself. Each of these factors are discussed separately.

Tightness of the peptidoglycan net. The number of bonds which have to be hydrolyzed in the peptidoglycan so that solubilization of cell walls can be induced depends on the degree of tightness of the net, or, in other words, on the frequency with which the peptide subunits are substituted on the polysaccharide chains and are, in turn, interlinked. For example, the digestion with the F_1 acetylmuramidase of 1 milligram of the wall fraction of *Micrococcus lysodeikticus* (which contains about 500 millimicromoles of disaccharide and disaccharide-peptide units) requires the appearance of only about 120 millimicromoles of reducing groups and yields soluble large oligosaccharide fragments (such as octasaccharides). Contrary to this, the solubilization of cell walls of *Staphylococcus aureus* and of *Micrococcus roseus* with the same enzyme is only completed when the whole polysaccharide is split into disaccharide units of which the number is then equivalent to the number of disaccharide-peptide subunits in the peptidoglycan. Similarly, when specific peptidases are used, splitting of only a small number of peptide linkages is required to solubilize the cell wall of *Micrococcus lysodeikticus*, while in the case of *Staphylococcus aureus* or *Micrococcus roseus* splitting of a large number of peptide subunits is required for solubilization (*12*, *13*).

Non-peptidoglycan components. Walls of bacteria are never pure peptidoglycan, and the affinity of bacteriolytic enzymes for the sensitive linkages present in the peptidoglycan can be inhibited or abolished by other components of the walls. It is well known that egg-white lysozyme is inactive on cells, or the corresponding walls, of virtually all Gram-negative bacteria and of numerous Gram-positive bacteria such as *Corynebacterium*, *Propionibacterium* or *Clostridium* species in which the walls' nonpeptidoglycan components are polysaccharides, often associated with lipids and proteins. Sometimes a gentle physical dislocation of these components, by means of moderate heat, lyophilization, or detergents, may make the bacterial cells, or the corresponding walls, sensitive to the action of the enzyme. In other instances, these additional components must be completely or partially removed from the walls by more drastic procedures, such as hot formamide, hot phenol, or trichloroacetic acid. After this treatment the enzyme may then catalyze the breakdown of the residual stripped peptidoglycan (*14*). However, most of these procedures damage the peptidoglycan itself. Either they are hydrolytic to some extent (as in the case of trichloroacetic acid) or they modify some of the chemical groups in the peptidoglycan—for example, the formyl-

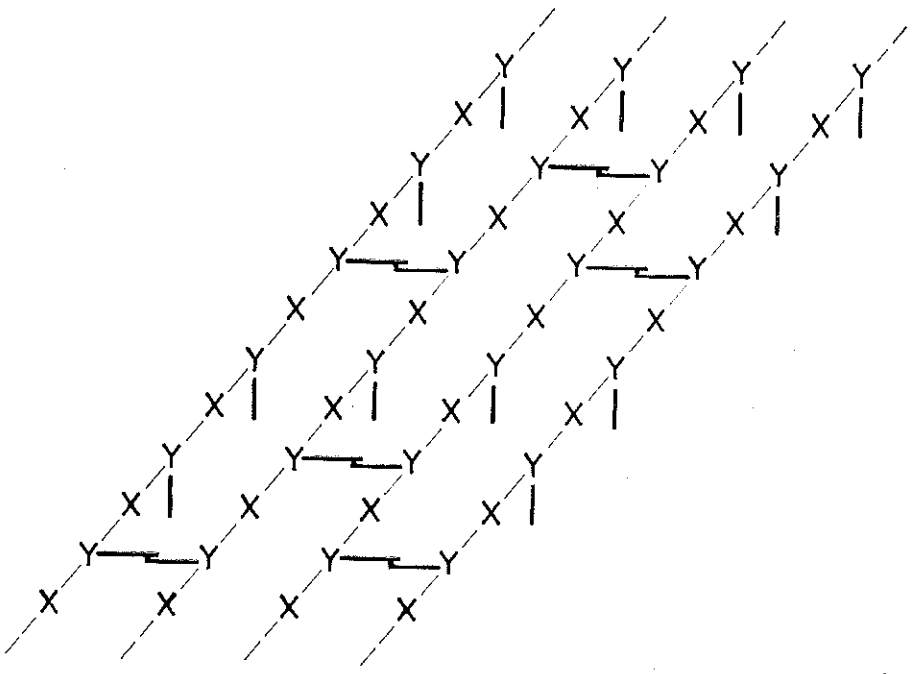


Fig. 3. Schematic representation of the structure of the peptidoglycan of *Escherichia coli*. This is a loose network in which all of the acetylmuramic residues are substituted either by tetrapeptide monomers or by peptide-linked dimers. For symbols, see legend to Fig. 1.

ation of the free amino groups by hot formamide (*15*). Whether or not these chemical modifications alone would have been sufficient to bring about susceptibility to enzymatic lysis in the residual cell-wall material is not known.

Substrate requirements of the bacteriolytic enzymes. Some lytic enzymes are likely to have strict substrate requirements with respect to the size of the polymer, the extent of peptide cross-linking, the frequency of peptide substitution on acetylmuramic acid residues, the net electrical charge, the presence of additional chemical groups, and others. Some examples are known. Acteyl groups that are ester-linked to the peptidoglycan in certain strains of *Micrococcus lysodeikticus* prevent the action of lysozyme (*16*). The same *O*-acetyl groups have been found in the wall peptidoglycan of *Staphylococcus aureus* strain Copenhagen (*9*), although, in this case, other structural properties of the peptidoglycan may also be responsible for the lysozyme-resistance of staphylococci. The B enzyme from *Chalaropsis* and the 32 enzyme and F_1 enzymes from *Streptomyces albus* G are endoacteylmuramidases, like egg-white lysozyme (see Table 1). However, they differ from lysozyme in that they can catalyze the hydrolysis of one of the sensitive linkages in cell walls of *Staphylococcus aureus* despite the presence of *O*-acetyl groups and the teichoic acid antigen (both of which are in part responsible for lysozyme resistance).

The *Streptomyces albus* G endopeptidase, which lyses cells of *Staphylococcus aureus*, opens polyglycine bridges through the splitting of D-Ala-Gly linkages, either in the walls or in the soluble peptidoglycan (*9*, *12*, *17*). The latter is obtained after lysis of the walls with an appropriate acetylmuramidase. During its lytic action on the cell walls the *Streptomyces albus* G endopeptidase seems no longer able to act when the size of the substrate is sufficiently reduced. Thus at completion of the digestion about 50 to 60 percent of the bridges are apparently opened when the enzyme solubilizes the walls, and 40 to 50 percent remain unopened. By contrast, when its substrate is the soluble peptidoglycan, only 15 to 25 percent of the bridges are opened and 75 to 85 percent remain

Table 1. Enzymes degrading peptidoglycans of bacterial cell walls. The endoenzymes listed are bacteriolytic with the exception of the glycosidase in lysostaphin, the acetylmuramyl-L-alanine amidase from *Streptomyces albus* G, and the nonlytic endopeptidase from *Streptomyces abus* G, which, when acting on walls of *Staphylococcus aureus*, require peptidoglycan solubilized by prior treatment of the walls with lytic enzymes. The endopeptidase and the acetylmuramyl-L-alanine amidase from *Escherichia coli* cells have not been obtained free of endoacetylmuramidase, and therefore it is not known whether they are lytic by themselves. The exoenzymes are also not bacteriolytic, and they remove terminal groups that are exposed as the consequence of action of endoenzymes.

Enzymes	Type of linkage split
*Carbohydrases**	
Endoacetylmuramidases	
Plant and animal lysozymes (such as egg white) (*1*, *4*, *8*, *32*)	*N*-acetylmuramyl-*N*-acetylglucosamine (A)†
32 enzyme from *Streptomyces albus* G (*40*)	
F_1 enzyme from *Streptomyces albus* G (*13*, *21*, *22*, *41*)	
B enzyme from *Chalaropsis* (*42*)	
T_2 phage lysozyme (*43*)	
Autolysin from *Streptococcus faecalis* (*44*)	
Endoacetylglucosaminidases	
Streptococcal muralysin (*45*)	*N*-acetylglucosaminyl-*N*-acetylmuramic acid (B)†
Glycosidase in lysostaphin (*18*, *20*)	
Autolysin from *Staphylococcus aureus* (*46*)	
Exoacetylglucosaminidases	
Enzyme from pig epididymis (*47*)	
Enzyme from *Escherichia coli* autolytic complex (*48*)	
Acetylmuramyl-L-alanine amidases‡	
Enzyme from *Streptomyces albus* G (*25*)	*N*-acetylmuramyl-L-alanine (C)†
Autolysin from *Bacillus subtilis* (*24*)	
Enzyme from *Escherichia coli* autolytic complex (*48*)	
Autolysin from *Listeria monocytogenes* (*26*)	
Autolysin from *Staphylococcus aureus* (*46*)	
L_{11}, L_3, Lysostaphin and enzymes from *Myxobacterium* and *Sorangium* §	
Peptidases‡	
Endopeptidases (bridge-splitting enzymes)	
SA endopeptidase from *Streptomyces albus* G (*12*, *17*, *29*)	D-alanylglycine (D); and D-alanyl-L-alanine (E)
ML endopeptidase from *Streptomyces albus* G (*12*, *29*)	D-alanyl-N^{ϵ}-L-lysine (F)
MR endopeptidase from *Streptomyces albus* G (*12*, *29*)	L-alanyl-L-threonine (G) or L-alanyl-L-alanine
Endopeptidase from *Escherichia coli* autolytic complex (*11*, *31*)	D-alanyl-meso-DAP (H)
L_3 enzyme from *Streptomyces* (*30*, *49*)	D-alanyl-meso-DAP (H)
Lysostaphin (*18*, *34*)	Glycylglycine (I)
Nonlytic endopeptidase from *Streptomyces albus* G (*17*)	Glycylglycine (I)
L_{11} enzyme from *Flavobacterium* (*28*, *29*, *49*)	Glycylglycine (I) and D-alanylglycine (D)
Enzyme from *Myxobacterium* (*19*, *50*)	Glycylglycine (I) and D-alanylglycine (D)
Enzyme from *Sorangium* (*51*)	D-alanyl-N^{ϵ}-L-lysine (F)
Exopeptidases	
L-alanine and glycine aminopeptidase and D-alanine and glycine carboxypeptidases from *Streptomyces albus* G (*17*, *27*)	
D-alanine carboxypeptidase from *Escherichia coli* autolytic complex (*31*, *52*)	

* In addition to the enzymes indicated, many other enzymes which hydrolyze the carbohydrate polymer in bacterial cell walls have been found (*53*). However, these enzymes were discovered before methods were available for defining which of the glycosidic linkages was hydrolyzed. † Letters in parentheses refer to bonds hydrolyzed as identified in Figs. 4–7. The methods employed in our laboratories in elucidating the specificities of bacteriolytic enzymes have recently been described (*54*). ‡ A number of other enzymes have been identified which may lyse cell walls through hydrolysis of peptide linkages. Available data suggest that they are bridge-splitting enzymes and acetylmuramyl-L-alanine amidases, although the nature of the split linkage has not been completely defined (*55*). § Many of the bridge-splitting enzymes also hydrolyze the acetylmuramyl-L-alanine linkage. This hydrolytic activity is slower and is not complete until long after solubilization of cell walls. Therefore, the bridge-splitting activity is probably responsible for bacteriolysis and cell-wall solubilization.

unhydrolyzed. A second nonlytic endopeptidase may also be present in *Streptomyces albus* G, and this second enzyme hydrolyzes only the small soluble peptidoglycan fragments produced by the first endopeptidase; it is unable to hydrolyze the large insoluble peptidoglycan. Similar experiments with these enzymes have been carried out with cell walls of *Micrococcus roseus*.

Examples of this kind are also found among glycosidases. The endoacetylglucosaminidase from lysostaphin does not lyse walls of *Staphylococcus aureus* (*18*) but the intact polysaccharide, free of peptide, which is obtained by hydrolysis of these walls with the enzyme from *Myxobacterium* (*19*), is split into disaccharide units (Y-X) (*20*). This enzyme also lyses cells or cell walls of *Micrococcus lysodeikticus* (*18*) in which it is known that many acetylmuramic residues are unsubstituted by peptides. In contrast to this, the F_1 endoacetylmuramidase mainly (*21*, *22*) hydrolyzes linkages of acetylmuramic residues which are substituted by peptides; it is virtually inactive when the acetylmuramic residues are unsubstituted. Thus, it quantitatively hydrolyzes the polysaccharide in the cell walls of *Staphylococcus aureus* into disaccharide-peptide units, but it releases from the walls of *Micrococcus lysodeikticus* a mixture of unsubstituted disaccharide, tetrasaccharide, and octasaccharide (*23*). It has very little action on these isolated oligosaccharides or on the intact polysaccharide which has been isolated after digestion of the walls with the *Myxobacterium* enzyme. Lysozyme seems also to prefer a peptide-substituted acetylmuramic acid residue since the rate of hydrolysis by this enzyme of several cell-wall polysaccharides from which all the peptides have been removed is only a few percent of the rate of hydrolysis of the cell walls themselves (*19*, *24*).

The acetylmuramyl-L-alanine amidase from *Streptomyces albus* G requires solubilized peptidoglycan (*25*). By contrast, the amidase from the *Bacillus subtilis* autolytic system (*24*) is able to solubilize cell walls of *B. subtilis*. A similar observation has been made with the amidase from the autolytic system of *Listeria monocytogenes* (*26*). The amidases originating from other sources (Table 1) have not been obtained free of endoacetylglucosidases or are found in preparations which cleave other types of peptidoglycan linkages. Therefore, it is not known whether or not these amidases by themselves would be capable of lysing intact bacterial cell walls.

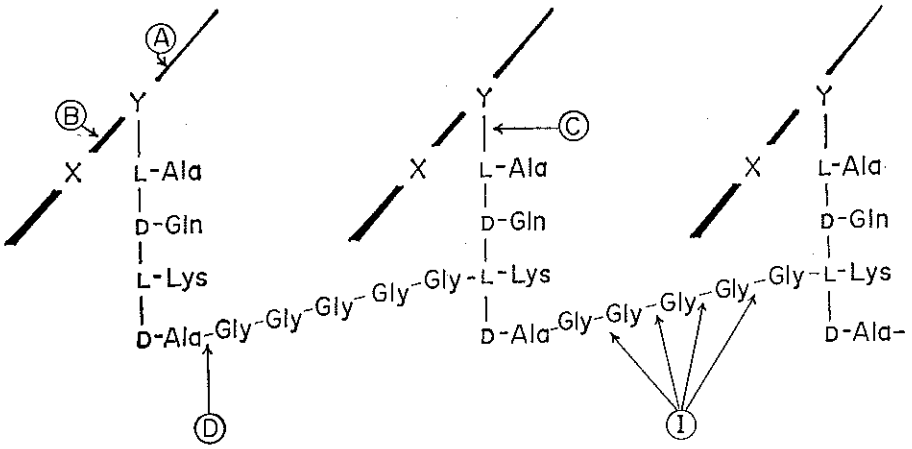


Fig. 4. A fragment of the peptidoglycan of *Staphylococcus aureus* showing points of cleavage by various enzymes (see Table 1).

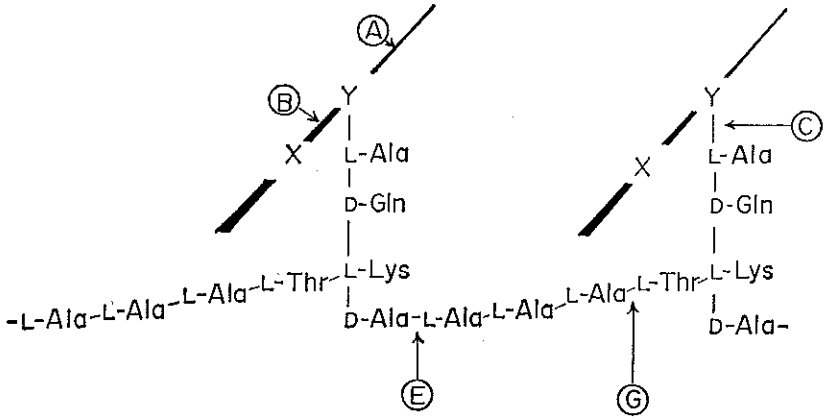
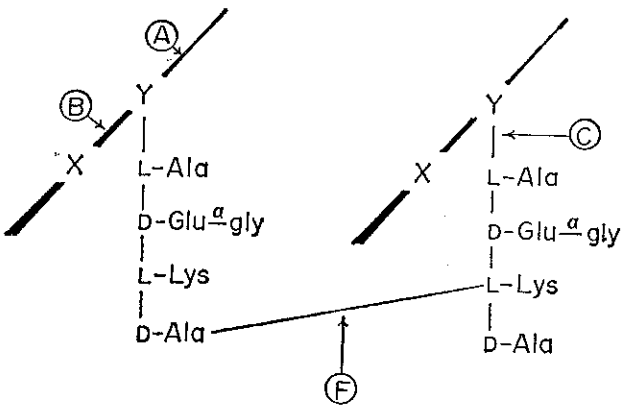


Fig. 5. A fragment of the peptidoglycan of *Micrococcus roseus* showing points of cleavage by various enzymes (see Table 1).

Bacteriolytic Enzymes as Tools for Structural Studies

From the foregoing it follows that digests of bacterial cell wall are extremely variable with respect to the size of fragments and the extent of the newly formed chemical groups (reducing, COOH- and NH_2-terminal groups) produced according to the type of bacterial wall submitted to the digestion and according to the type of lytic enzyme used. Examples have been given of endoacetylmuramidases that split the same type of chemical linkage, but whose activity depends on other aspects of peptidoglycan structure. Thus, the type of fragment produced from the cell wall depends both on the particular enzyme selected and the wall used. Difficulties in interpreting the structure of a peptidoglycan on the basis of the fragments liberated through its enzymatic solubilization can be anticipated if only a fraction of the sensitive links have been actually split. Structural studies of a peptidoglycan can therefore be approached only after thorough study of the kinetics and of the extent of the lysis. If enzymatic hydrolysis is stopped before a particular linkage undergoing hydrolysis is completely split, a needlessly complex mixture of fragments results. It should be emphasized that total solubilization of cell wall is not necessarily the indication that hydrolysis of all sensitive linkages has occurred.

As an example, the following sequential degradation of the cell walls of *Staphylococcus aureus* strain Copenhagen greatly contributed to the elucidation of its structure (*9*, *17*, *20*). Through the action of the *Streptomyces* 32 or F_1 or the *Chalaropsis* B endoacetylmuramidases, all the glycosidic links of *N*-acetylmuramic acid to *N*-acetylglucosamine were opened.



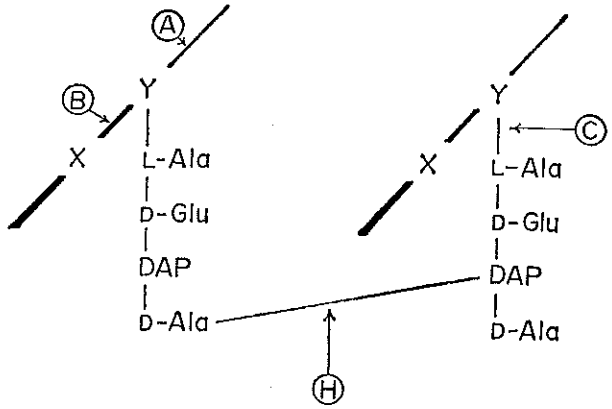


Fig. 6 (left). A fragment of the peptidoglycan of *Micrococcus lysodeikticus* showing points of cleavage by various enzymes (see Table 1). Fig. 7 (right). A fragment of the peptidoglycan of *Escherichia coli* or *Corynebacterium diphtheriae* showing points of cleavage by various enzymes (see Table 1).

Subsequent hydrolysis with the *Streptomyces* acetylmuramyl-L-alanine amidase quantitatively yielded the disaccharides *N*-acetylglucosaminyl-β-1,4-*N*-acetylmuramic acid and *N*-acetylglucosaminyl-β-1,4-*N*,6-*O*-diacetylmuramic acid which were isolated and characterized. In a third step, the two disaccharides were cleaved to the free *N*-acetylhexosamine residues by an exo-β-1,4-*N*-acetylglucosaminidase isolated from pig epididymis.

In a parallel degradation, polysaccharide chains were liberated from cell walls through hydrolysis by the *Myxobacterium* enzyme. These chains contained on the average about twelve disaccharide units. Subsequent treatment with the endoacetylglucosaminidase from lysostaphin quantitatively yielded the isomeric disaccharides, *N*-acetylmuramyl-*N*-acetylglucosamine and its *O*-acetyl derivative. The glycosidic links again were shown to be 1,4- and probably have the β-configuration. Thus, both of the disaccharides which comprise the polysaccharide chain were isolated and characterized, as was the intact polysaccharide.

In order to obtain the small peptide linked to acetylmuramic acid, cell walls were solubilized through the action of the *Streptomyces albus* G endopeptidase, which opened the polyglycine bridges at their amino end. Treatment with the *Streptomyces* aminopeptidase then liberated all of the glycine from these opened bridges. Subsequent treatment with the endo-*N*-acetylmuramidase hydrolyzed the polysaccharide and yielded the disaccharide-peptide subunits. The peptide itself was then obtained after hydrolysis with the *N*-acetylmuramyl-L-alanine amidase and shown to be mainly the tetrapeptide, L-Ala-D-γ-Glu(α-$CONH_2$)-L-Lys-D-Ala. This degradation was also applied to cell walls of *Micrococcus roseus* R27 and similarly yielded disaccharide, free L-Ala and L-Thr residues in a ratio of 3 to 1 (from the bridges) and a mixture of the above tetrapeptide and of the tripeptide L-Ala-D-γ-Glu(α-$CONH_2$)-L-Lys (*12*, *27*).

Specificity of the Bacteriolytic Enzymes

The polysaccharide moieties of the peptidoglycans from many bacterial species can be degraded by endoacetylmuramidases or by endoacetylglucosaminidases provided that the properties of the substrate—native walls, chemically modified walls, stripped peptidoglycan, or intact polysaccharide (free of peptide)—fits with the substrate requirements of the glycosidase. Among the glycosidases so far studied, F_1 acetylmuramidase has the broadest lytic spectrum, lysing cells or solubilizing the corresponding walls of a great variety of Gram-positive bacteria, such as *Staphylococcus* sp., *Lactobacillus* sp., *Corynebacterium* sp., *Propionibacterium* sp., *Bifidobacterium* sp., and *Clostridium* sp. Although much comparative biochemistry still needs to be done (for example, it is not known whether all of the glycosidic linkages are always the same), it can be postulated that the polysaccharide moieties of the peptidoglycans are rather uniform in composition and in structure.

Unlike the glycosidases, endopeptidases have very limited activity spectra. Endopeptidases exert their splitting effect within the specific interpeptide cross-links, and the limited range of hydrolytic activity of these enzymes is due to the great variation in the nature of the cross-link among various bacteria. In fact, the known specific hydrolytic enzymes of this group are growing in number, and they could become tools of taxonomic importance. The specific nature of the peptide bonds they cleave within peptidoglycans substantiates the existence of various types of peptide cross-links in walls of *Staphylococcus aureus* (Fig. 4), *Micrococcus roseus* R27 (Fig. 5), *M. lysodeikticus* (Fig. 6), and *Escherichia coli* and *Corynebacterium diphtheriae* (Fig. 7).

The lytic spectrum of these three *Streptomyces* endopeptidases have been studied with the walls from about 20 Gram-positive bacterial species being used as substrates (*12*). These endopeptidases do not act on DAP-containing walls (*Corynebacterium, Bacillus, Propionibacterium*). The *Streptomyces albus* GSA endopeptidase acts upon *Staphylococcus aureus, S. albus, Streptococcus pyogenes, Micrococcus radiodurans, Gaffkya tetragena*, and *Micrococcus roseus*. However, the MR endopeptidase acts only on *Micrococcus roseus*, whereas the ML endopeptidase acts on *Micrococcus lysodeikticus, M. citreus, M. flavus*, and *Sarcina lutea*. The L_{11} enzyme from *Flavobacterium* and the lytic peptidase in lysostaphin also have limited activity and hydrolyze within the pentaglycine bridge of *S. aureus* (*18*, *28*, *29*). The L_3 enzyme from *Streptomyces* (*29*, *30*) and the endopeptidase of the autolytic system of *Escherichia coli* (*31*) are specific for the direct D-Ala-meso-DAP-linkage. The L_3 enzyme acts upon *Corynebacterium diphtheriae* and *Bacillus megaterium*, but its lytic spectrum has not been fully explored. The action of endopeptidase of *Escherichia coli* has been determined only for *E. coli*; its activity on other

organisms is unknown. The activity of these two enzymes parallels that of the ML endopeptidase from *S. albus* G which acts on similar linkages involving lysine rather than DAP. These enzymes are so far the only bridge-splitting enzymes known which solubilize walls containing DAP.

Biological Role of Bacteriolytic Enzymes

It seems likely that the bacteriolytic enzymes in plants and animals have a protective role, although it is exceedingly difficult to prove that they function in this way. For example, the lysozyme in egg white presumably serves as a means of aiding in the prevention of infection of the egg albumin and embryo during the period of incubation. Similarly, the enzymes present in animal cells may aid in the prevention and dissolution of infections. A protein similar to egg-white lysozyme has been isolated from many animal tissues (*32*). The study of the structure of these substances is an exceedingly interesting area of comparative biochemistry. The isolation of these lysozymes was based on the characteristic chemical properties of these enzymes. Probably animal tissues also contain a variety of other bacteriolytic enzymes. It is known that different genera of animals and even strains within a genus vary greatly in their susceptibilities to various bacterial infections. Whether the specificity of tissue bacteriolytic enzymes plays any role in this variation in susceptibility to infection is an open question. Very few of these enzymes have been purified, and until now the methods available for their study would not have permitted adequate characterization. Perhaps improvements in techniques for fractionating proteins and the description of methods for characterizing bacteriolytic enzymes will accelerate new developments in this important area.

A second role of bacteriolytic enzymes is their function in maintenance of the biological equilibrium. These enzymes undoubtedly play an important part in bringing about the decomposition of dead bacteria in soil and sewage. Conceivably, they could also have some survival value for organisms which produced them, but it is exceedingly difficult to know whether or not the production of antibiotics or bacteriolytic enzymes in soil, for example, provides any selective advantage.

The role of the autolytic enzymes in bacteria has never been defined. They certainly can bring about the "suicide" of bacterial cultures under conditions in which synthetic processes are retarded—for example, during storage of cultures at 0°C and in culture after exhaustion of nutrients. With regard to any physiological role they might have, at least two possibilities may be suggested. The growth and duplication of bacterial cells requires the enlargement of the cell wall, a rigid structure. Conceivably the enlargement of such a structure requires the opening of "cracks" in the structure, which could be filled by new biosynthetic pieces, with a resulting enlargement of the wall. Certainly the opening of the polysaccharide chains by autolytic acetylmuramidases could permit the addition of new acetylglucosamine and acetylmuramyl-peptide fragments to the chain, with consequent wall growth. However, it is difficult to imagine such a role for the autolytic acetylmuramyl-L-alanine amidases. The linkage between acetylmuramic acid and L-alanine is synthesized in the soluble fraction of the cell, prior to its incorporation into the wall, and the opening of this linkage would not be expected to provide an initiation point for wall enlargement.

An alternative possibility is that, although the cell wall may be freely permeable to small molecules, large molecules may not be able to pass through it. It may thus be necessary for gaps to be opened in the wall in order that large molecules (such as secreted proteins) pass from the cell to the medium or from the medium into the cell. There is some evidence that an autolytic acetylmuramyl-L-amidase may be associated with competency, that is, the transformation of these cells by DNA may require that gaps be opened in the wall through which the large molecules can pass (*24, 33*). Such a function would require a mechanism for the repair of gaps introduced in this way, but no such mechanism has been described. The spore lysins are a specialized group of autolytic enzymes which function during outgrowth of the vegative cell. The manner in which all of these autolytic systems are kept under control is an intriguing problem .

Conceivably bacteriolytic enzymes represent a class of potentially useful chemotherapeutic agents. However, the difficulty of purifying proteins, their instability relative to smaller organic molecules, and the potential toxicity of foreign proteins for animals has not encouraged exploration of substances of this kind, even though they have turned up in routine screening of antimicrobial substances. However, a great deal of attention has been given recently to a powerful bacteriolytic enzyme, lysostaphin, which has a narrow antibacterial spectrum but has high activity against an important pathogen, *Staphylococcus aureus* (*18, 34*). All strains of *S. aureus* obtained from patients infected in hospitals were susceptible to this agent (*35*), and animals infected experimentally with such strains have been cured as a result of treatment with this enzyme (*36*). On a weight basis, it is five to ten times as potent as penicillin G as an antibacterial agent in vitro (*37, 38*) and on a mole basis 500 to 1000 times as active (*37*). It is thus among the most potent antibacterial substances to have been isolated from nature. This and other bacteriolytic enzymes could conceivably find a place in the chemotherapy of infectious diseases. A beginning has been made in trials of this substance in persons who are nasal carriers of *S. aureus* (*39*).

Finally, it may be stated that the study and purification of bacteriolytic enzymes is an essential part of the study of the structure and biosynthesis of bacterial cell walls. These structures which are essential for the survival of bacteria in their normal environment are among the most complex and diverse structures found in nature. They are extraordinarily large macromolecules, and since they completely surround the bacterial cell, they must be larger than any molecule which lies within the cell. The study of such structures requires the use of hydrolytic agents of high specificity, and purified bacteriolytic enzymes are just such tools. It is of great interest that the cleavage of almost any chemical bond within the structure results in solubilization of the cell wall, and the diversity of hydrolyses which result in solubilization is already great. Both carbohydrases and peptidases are included among these enzymes, and studies of them have led to an important generalization regarding cell wall structure. The insolubility and rigidity of the wall are not due to the properties of any one of the polymers which it contains, but rather they are the consequence of a highly ordered cross-link-

ing system. The repeating units of acetylglucosamine and acetylmuramyl tetrapeptide in the polysaccharide are cross-linked through peptide bridges involving the additional amino groups of dibasic amino acids and the carboxyl group of D-alanine, the last amino acid in the tetrapeptide. The fundamental units of the cell wall, acetylglucosamine and acetylmuramyl tetrapeptide, appear to be linked to each other in similar ways in different genera of bacteria, while the scanty information available suggests that the nature of the cross-links may be highly specific to a given genus or species. In any event these cross-links result in a three-dimensional structure of great strength, rigidity, and insolubility which serves to protect the bacterial cell in a variety of environmental circumstances. The loosening of this structure by the selective hydrolysis of any linkage within it results in solubilization of the wall and is usually followed by lysis and death of the bacterial cell.

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10. Abbreviations used are: DAP, diaminopimelic acid; UDP, uridine diphosphate; SA, *Staphylococcus aureus*; MR, *Micrococcus roseus*; ML, *Micrococcus lysodeikticus*; Ala, alanine; Gly, glycine; Lys, lysine; Glu, glutamic acid.
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