Specificity. Two parameters of the hydrolytic reactions were studied: the initial rate and the final extent of hydrolysis. Under identical conditions of assay, initial rates (10-minute incubations) of hydrolysis are similar for bovine sialyllactose, the α-1 acid glycoprotein of human serum, hemagglutination inhibitor, *Collocalia* sialomucoid, ovine submaxillary mucin, and calf brain ganglioside. Lower initial rates are observed with human sialyllactose (sialyl-(2 → 6)-lactose), bovine and porcine submaxillary mucins, and colominic acid. Prolonged incubation (2–24 hours) leads to essentially complete hydrolysis of all substrates tested except the following (the values give final extent of hydrolysis compared with theory): bovine submaxillary mucin, 50%; porcine submaxillary mucin, 84%; and calf brain ganglioside, 43%. Ovomucin and Tamm-Horsfall urinary mucoprotein are also cleaved, but comparisons with other substrates cannot be drawn because solubilities of 1 micromole per 0.25 ml were not attained.

pH Optimum. With potassium acetate buffer, the optimum is between 4.0 and 4.8, while with citrate-phosphate or Tris-maleate buffers, the optimum is approximately 5.6. No activity is observed below pH 3.0 nor above pH 8.0.

Kinetic Properties and Metal Requirements. The purified enzyme obtained from *Clostridium perfringens* shows no metal requirements and is fully active in the presence of EDTA.[11,12] In this respect, it differs sharply from the enzyme obtained from *Vibrio cholerae,* which requires calcium ion.[22] In almost all other respects, the enzymes show similar properties. *N*-Acetylneuraminic acid does not inhibit the *Clostridium* enzyme, and the K_m for bovine sialyllactose is 2.4×10^{-3} *M*.

[22] G. L. Ada, E. L. French, and P. E. Lind, *J. Gen. Microbiol.* **24**, 409 (1961).

[118] Enzymes That Degrade Bacterial Cell Walls

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Bacterial Cell Walls and Bacteriolytic Enzymes

Structure of the Substrate Hydrolyzed by Bacteriolytic Enzymes

The cell walls of virtually all bacteria are composed of two or more polymers. One of them, ubiquituous in the bacterial world, is responsible for the shape and strength of the walls and allows the cells to live in environmental conditions which are hypotonic with respect to their high intracellular osmotic pressure. This polymer is a glycopeptide built up of polyacetylhexosamine and peptide chains, and the action of all

bacteriolytic enzymes so far studied results in the fragmentation of this glycopeptide.

A schematic representation of different types of glycopeptide sheet is represented in Fig. 1. In this diagram, the *N*-acetylhexosamine residues

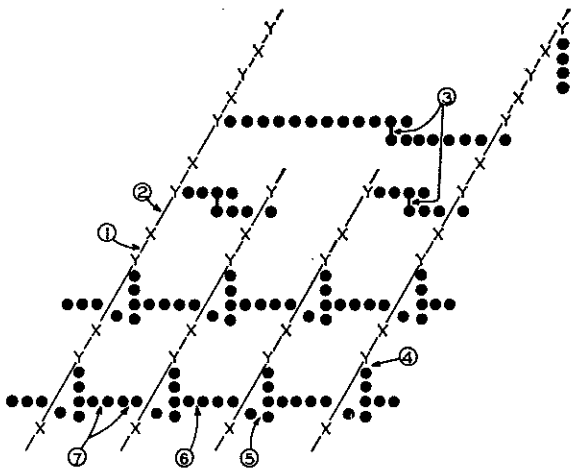

FIG. 1. A schematic representation of different types of glycopeptide sheet. The polysaccharide backbone consists of alternating residues of *N*-acetylglucosamine (X) and *N*-acetylmuramic acid (Y). Amino acids are represented by the black dots. In the lower part of the diagram, four dots dependent from a Y represent the basic tetrapeptide subunit (see text). These are cross-linked by the horizontal rows of dots. The vertical bars in the upper part of the diagram represent direct cross-linking of tetrapeptide subunits. The numbers refer to sites of enzymatic attack as listed in Table I.

form linear backbones of alternating *N*-acetylglucosamine (X) and *N*-acetylmuramic acid (Y) residues. Some of the acetylmuramic acid residues (in the walls of *Micrococcus lysodeikticus*) or all of them (in the walls of *Staphylococcus aureus*) are substituted on their carboxyl groups by peptide subunits, of which the constitutents are invariably L- and D-alanine, a diacidic amino acid (D-glutamic acid) and a dibasic amino acid (most commonly L-lysine or one of the isomers of α,α'-diaminopimelic acid). In all cases studied, the terminal amino group of L-alanine is involved in the linkage to acetylmuramic acid.

The basic structure of the peptide subunits is probably similar to that found in UDP-acetylmuramyl-peptide cell wall precursors. In one case, that of *S. aureus*, the presence of the sequence acetylmuramyl-L-alanyl-D-isoglutaminyl-L-lysyl-D-alanine in the cell wall has been confirmed, and as a working hypothesis, it is postulated that this or a similar DAP-con-

taining acetylmuramyl-peptide is the basic component of the peptide moiety of all bacterial wall glycopeptides. Minor structural variations are, however, encountered in the peptide subunits of certain bacterial walls. These tetrapeptides may somehow be polymerized to form longer peptide chains, as is probably the case for part of the peptide moiety in the walls of *M. lysodeikticus*.

Some or all of the peptide chains linked to adjacent polysaccharide backbones are in turn linked to each other, resulting in a network of at least two dimensions. The terminal carboxyl group of D-alanine and the terminal ϵ-amino group of L-lysine or the α'-amino group of DAP are involved in these peptide cross-linkages, either by a direct linkage (in walls of *M. lysodeikticus* and *Escherichia coli*) or by the means of peptide bridges. Tetra- or pentaglycine bridges connect the tetrapeptide subunits of the walls of *S. aureus*. Tri(L-alanyl)-L-threonyl bridges occur in the walls of *Micrococcus roseus*.

Mechanism of Action of Bacteriolytic Enzymes

On the basis of this proposed structure, the dislocation into fragments of the glycopeptide network, causing the solubilization of cell walls or the lysis of bacterial cells, can be brought about by three classes of enzymes: glycosidases which split the polysaccharide chains, acetylmuramyl-L-alanine amidases which cleave the junction between polysaccharides and peptides, and endopeptidases which split within the polypeptide chains. Most of the examples of this third class so far studied cleave the bond or the cross-linking peptide bridge between the acetylmuramyl-tetrapeptide units. It may be necessary to hydrolyze only a small number of bonds in order to induce the solubilization of the glycopeptide. This number depends on the degree of tightness of the net, that is, on the frequency with which polysaccharides are substituted by peptide subunits and with which these peptides are in turn interlinked.

That polysaccharide backbones are essential for maintaining the mechanical strength and insolubility of the glycopeptide has been known since 1952 when Salton demonstrated that Fleming's lysozyme is a glycosidase capable of degrading isolated bacterial walls. Within the past decade, numerous other bacteriolytic enzymes have been discovered. All those which were obtained in sufficiently purified form were found to be glycosidases. Very recently, however, bacteriolytic peptidases and amidases have been isolated, free of any detectable glycosidase activity. Therefore, the polypeptide moieties are also essential for the insolubility of the cell-wall glycopeptide, and in this respect, polyacetylhexosamine and peptide chains complement each other.

Walls of bacteria are never pure glycopeptide and the affinity of a

bacteriolytic enzyme for the sensitive linkages present in the glycopeptide may be partially or completely abolished by the nonglycopeptide components of the walls. Stripped glycopeptides obtained after removal of these components by treating the walls in an appropriate way, may be found sensitive to the enzyme. Much simpler accessories attached to the glycopeptide can also interfere with the activity of a lytic enzyme. Acetyl groups ester-linked to the cell-wall glycopeptide in certain strains of *M. lysodeikticus* have been shown to prevent the action of lysozyme. Some lytic enzymes probably have strict substrate requirements with respect to the size of the polymer, the extents of cross-linking, the net electrical charge, etc. Much remains to be investigated in this field.

Characterization of the Bacteriolytic Enzymes

The digestion of different bacterial walls by various bacteriolytic enzymes gives rise to fragments of highly diverse nature and molecular complexity. However, analysis of the nature of the free amino groups and reducing groups and the extent to which they have been liberated yields valuable information on the structure of the glycopeptide which has been submitted to the degradation process and on the specificity of the enzyme which has been used.

A lytic glycosidase induces the appearance in the cell-wall digest of reducing groups belonging either to *N*-acetylglucosamine (if the enzyme is an *N*-acetylglucosaminidase) or to *N*-acetylmuramic acid (if the enzyme is an *N*-acetylmuramidase). The nature of the newly formed reducing groups can be determined by borohydride reduction of the digest. The number of reducing groups is directly related to the extent of cleavage of the polysaccharide backbones. A positive Morgan-Elson reaction is indicative that this cleavage has yielded disaccharide fragments (X-Y or Y-X) either free or still attached to peptides.

A lytic intrapeptide hydrolase induces the appearance of new terminal amino and carboxyl groups. An acetylmuramyl-L-alanine amidase liberates equivalent amount of terminal amino groups of L-alanine and carboxyl groups of acetylmuramic acid residues. No routine estimation of the newly formed carboxyl groups of acetylmuramic acid is available. However, the appearance of N-terminal L-alanine without an equivalent amount of C-terminal amino acid is indicative of this type of enzyme. The absolute characterization of an enzyme as an acetylmuramyl-L-alanine amidase requires the isolation of the polysaccharide chains, if the enzyme acts directly on the walls, or of oligosaccharide fragments, if the enzyme acts in association with or subsequently to a glycosidase.

Enzymes also exist which, although they are not lytic themselves,

further degrade the products resulting from the action of lytic enzymes. Various classes of such enzymes are known: exoenzymes removing terminal amino acid or acetylhexosamine residues and endoenzymes splitting internal linkages present in soluble glycopeptide fragments.

Bacteriolytic Enzymes of Known Specificity

Table I lists the enzymes that degrade the glycopeptide of bacterial cell walls and of which the specificity is at least partially known.

Analytical Procedures

In applying these procedures, generally it is first established whether the enzyme is a glycosidase (appearance of reducing groups) or a peptidase or amidase (appearance of free amino groups). Then detailed procedures to determine which of the glycosidic or peptide linkages has been opened can be applied.

Appearance of Reducing Power in Soluble Fragments during Lysis of Bacterial Cell Walls

The procedure is a micromodification of the ferricyanide method of Park and Johnson as described in Vol. III of this series.[1] The reagents are unchanged except for an increase in the sulfuric acid concentration of the ferric iron reagent to 0.15 *N*. This decreases the tendency of the blue complex to precipitate when the reducing power of high oligosaccharide derivatives is being measured. The procedure is not suitable for obtaining accurate kinetics for the early stages of lysis as much reducing power remains in residual unsolubilized cell wall material. This material should be precipitated by centrifugation prior to the removal of aliquots for reducing power determination, as it tends to precipitate the blue complex.

The neutralized sample, containing reducing power equivalent to 1–5 millimicromoles of *N*-acetylglucosamine, is diluted to 100 μl in a 1-ml tube. To this 100 μl of a fresh 1:1 mixture of ferricyanide and carbonate-cyanide reagents is added. After the compounds have been mixed, heated for 15 minutes in a boiling water bath, and cooled, 250 μl of the ferric iron reagent is added. After the preparation has been mixed and has stood for 15 minutes, the blue color is read at 690 mμ. *N*-Acetylglucosamine has a molar extinction coefficient of 10^5, and the color yields from some compounds relative to an equimolar amount of *N*-acetylglucosamine are listed in Table II.

[1] G. Ashwell, Vol. III [12].

TABLE I

ENZYMES THAT DEGRADE GLYCOPEPTIDES OF BACTERIAL CELL WALLS[a]

Enzyme	Type of linkage split (see Fig. 1)
Endoacetylmuramidases	
Plant and animal lysozymes (e.g., egg white)[b]	
32 enzyme from *Streptomyces albus* G[c]	*N*-Acetylmuramyl-*N*-acetylglucosamine (1)
F_1 enzyme from *S. albus* G[d]	
B enzyme from *Chalaropsis*[e]	
T2 phage lysozyme[f]	
Endoacetylglucosamidases	
Streptococcal muralysin[g]	
Glycosidase in lysostaphin[h]	*N*-Acetylglucosaminyl-*N*-acetylmuramic acid (2)
Exoacetylglucosaminidases	
Enzyme from pig epididymis[i]	
Enzyme from *E. coli* autolytic complex[i]	
Acetylmuramyl-L-alanine amidases	
Autolysin from *Bacillus subtilis*[k]	
Enzyme from *S. albus* G[l]	
Enzyme from *E. coli* autolytic complex[i]	Acetylmuramyl-L-alanine (4)
Enzyme L_{11}, lysostaphin, and enzyme from *Myxobacterium* (see Intrapeptide hydrolases)	
Intrapeptide hydrolases	
SA endopeptidase from *S. albus* G[m,p]	D-Alanyl-glycine and D-alanyl-L-alanine (5)
ML endopeptidase from *S. albus* G[m]	D-Alanyl-L-lysine (3)
MR endopeptidase from *S. albus* G[m]	L-Alanyl-L-threonine (6)
Endopeptidase from *E. coli* autolytic complex[j,n]	D-Alanyl-*meso*-DAP (3)
L_3 enzyme from *Streptomyces*[m,o]	D-Alanyl-*meso*-DAP (3)
Lysostaphin[h]	Glycyl-glycine (7)
Nonlytic endopeptidase from *S. albus* G[p]	
L_{11} enzyme from *Flavobacterium*[m,q]	
Enzyme from *Myxobacterium*[r]	Glycyl-glycine (7) and D-alanyl-glycine (5)
Exopeptidases	
L-Alanine and glycine aminopeptidases and D-alanine and glycine carboxypeptidases from *S. albus* G[m,p]	
D-Alanine carboxypeptidase from *S. albus* G[p,m] and from *E. coli* autolytic complex[s]	

[a] The endoenzymes listed here are lytic with the exception of the acetylmuramyl-L-alanine amidase from *Streptomyces albus* G, and the nonlytic endopeptidase from *S. albus* G, which require glycopeptides solubilized by prior treatment of the walls

Appearance of *N*-Acetylamino Sugars in Soluble Fragments during Lysis of Bacterial Cell Walls

Morgan-Elson Reagent. *p*-Dimethylaminobenzaldehyde (16 g) is dissolved in acetic acid to give a total volume of 95 ml and concentrated HCl (5 ml) is added. This stock reagent is diluted 1:8 with acetic acid to give the color reagent.

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with lytic enzymes. The endopeptidase and the acetylmuramyl-L-alanine amidase from *E. coli* cells have not been obtained free of endoacetylmuramidase and therefore it is not known whether or not they are lytic by themselves. The endoenzymes L_{II}, L_3, lysostaphin, and the enzyme from *Myxobacterium* hydrolyzed more than one type of linkage. From the data published at present, it is impossible to decide which activity is responsible for the lysis. The glycosidase in lysostaphin has been reported to be nonlytic for *S. aureus*.[h]

[b] M. R. J. Salton, *Bacteriol. Rev.* **21,** 82 (1957).
M. R. J. Salton and J. M. Ghuysen, *Biochim. Biophys. Acta* **45,** 355 (1960).

[c] J. M. Ghuysen and J. L. Strominger, *Biochemistry* **2,** 1110, 1119 (1963).

[d] E. Munoz and J. M. Ghuysen, unpublished.

[e] J. Hash, *Arch. Biochem. Biophys.* **102,** 379 (1963).
D. J. Tipper, J. L. Strominger, and J. M. Ghuysen, *Science* **146,** 781 (1964).

[f] J. Primosigh, H. Pelzer, D. Maas, and W. Weidel, *Biochim. Biophys. Acta* **46,** 68 (1961).
W. Katz, *Z. Naturforsch.* (1964).

[g] S. S. Barkulis, C. Smith, J. J. Boltralik, and H. Heymann, *J. Biol. Chem.* **239,** 4027 (1964).
S. S. Barkulis, C. Smith, J. J. Boltralik, and H. Heymann, *Bacteriol. Proc.* P32 (1964).

[h] C. A. Schindler and V. T. Schuhardt, *Proc. Natl. Acad. Sci.* **51,** 414 (1964).
H. P. Browder, W. A. Zygmut, J. R. Young, P. A. Tavormina, *Biochem. Biophys. Res. Commun.* **19,** 383 (1965).
D. J. Tipper and J. L. Strominger, *Biochem. Biophys. Res. Commun.* **22,** 48 (1966).

[i] J. Findlay, J. Levvy, and S. A. Levvy, *Biochem. J.* **77,** 170 (1960).

[j] H. Pelzer, *Z. Naturforsch.* **18b,** 950 (1963).

[k] F. E. Young, D. J. Tipper, J. L. Strominger, *J. Biol. Chem.* **239,** 3600 (1964).

[l] J. M. Ghuysen, M. Leyh-Bouille, and L. Dierickx, *Biochim. Biophys. Acta* **63,** 286 (1962). See also footnote *c*.

[m] J. M. Ghuysen, J. F. Petit, E. Munoz, and K. Kato, *Federation Proc.* **25,** 410 (1966).

[n] W. Weidel and H. Pelzer, *Advan. Enzymol.* **26,** 193 (1964).

[o] Y. Mori, K. Kato, T. Matsubara, and S. Kotani, *Biken's J.* **3,** 139 (1960).

[p] J. M. Ghuysen, D. J. Tipper, C. Birge, and J. L. Strominger, *Biochemistry* **4,** 2245 (1965).

[q] K. Kato, S. Kotani, T. Matsubara, J. Kogami, S. Hashimoto, M. Chimori, and T. Kazekowa, *Biken's J.* **3,** 155 (1962).

[r] J. C. Ensign and R. S. Wolfe, *Bacteriol. Proc.* p. 33 (1964).
D. J. Tipper, J. M. Ghuysen, and J. L. Strominger, *Federation Proc.* **23,** 379 (1964).

[s] W. Leutgeb and W. Weidel, *Z. Naturforsch.* **18b,** 1065 (1963).

TABLE II
RELATIVE COLOR YIELDS IN THE REDUCING POWER DETERMINATION

Compound	Reducing power
X	1
XY	1.5
XYXY	1.35
XYXYXYXY	0.85
YX	0.75

Procedure. This is a micromodification of that described by Reissig, Strominger, and Leloir.[2] The sample, containing the equivalent of 1–5 millimicromoles of *N*-acetylglucosamine in 20 μl of 1% $K_2B_4O_7$, is heated for 30 minutes in a boiling water bath. After cooling, fresh color reagent (90 μl) is added, the mixtures are incubated for 20 minutes at 37° and read at 585 mμ. The molar extinction coefficient of some oligosaccharides are listed in Table III. The presence of peptides amidically

TABLE III
MOLAR EXTINCTION COEFFICIENTS IN THE MORGAN-ELSON PROCEDURE

Compound	Time of heating at 100° (minutes)		
	7	30	60
X	20,000	14,000	9,500
XY	3,500	9,500	8,000
XYXY	—	1,000	—
YX	300	1,600	3,100
Y	19,000	13,500	10,000

linked to *N*-acetylmuramic acid residues does not prevent color production.

Free *N*-acetylglucosamine or *N*-acetylmuramic acid is determined by the same procedure but with a heating time of 7 minutes.

Determination of Total Hexosamines

Color Reagent. Two volumes of the same stock reagent used in the Morgan-Elson procedure are diluted with five volumes of acetic acid.

Procedure. Samples containing 10–100 millimicromoles of total hexosamine are lyophilized and then hydrolyzed in 20 μl of 3 *N* HCl for 4 hours at 95° in a sealed tube. The hydrolyzates are neutralized with 20

[2] J. L. Reissig, J. L. Strominger, and L. F. Leloir, *J. Biol. Chem.* **217**, 1954 (1955).

μl of 3 *M* NaOH, and aliquots (30 μl) are removed and transferred to 3 ml tubes. Removal of HCl by lyophilization leads to destruction of muramic acid. The neutralization must be fairly exact, but an error of 1 μl of 3 *N* HCl or NaOH can be tolerated. Saturated $NaHCO_3$ solution (10 μl) and freshly prepared 5% (v/v) acetic anhydride solution (10 μl) are added and mixed. After the tubes have stood at room temperature for 10 minutes, during which time *N*-acetylation occurs, the excess anhydride is hydrolyzed by heating the tubes in a boiling water bath for exactly 3 minutes and then immersing them in a cold water bath. The time of heating is critical if identical color yields are to be obtained from muramic acid and glucosamine, since muramic acid yields chromogen in the presence of $NaHCO_3$.[3] Five per cent $K_2B_4O_7$ (50 μl) is added, and after the samples have been mixed, heated in a boiling water bath for 7 minutes, and cooled, 700 μl of color reagent is added. The solutions are mixed, incubated at 37° for 20 minutes, and read at 585 mμ. Glucosamine and muramic acid have a molar extinction coefficient of 11,500.

Determination of the Nature of the Liberated Reducing Groups

If the glycosidic linkages in the cell wall are the same as those found in *S. aureus* or *M. lysodeikticus*, that is all β-1,4, then a comparison of the results of reducing power and Morgan-Elson determinations will enable the nature of the reducing groups to be predicted.

Opening all the Y-X linkages results in liberation of both high reducing power and an equivalent amount of material reactive as acetylhexosamine in the modified Morgan-Elson reaction (30 minutes' heating in borate buffer). The opening of the X-Y linkage, however, results in appearance of reducing groups without corresponding appearance of acetylhexosamine-reactive material. To determine the extent of hydrolysis of the glycosidic linkages, it is necessary to follow the appearance of reducing groups to a maximum value and to compare this to the total hexosamine content of the preparation.

When fragments containing oligosaccharides of chain length of six or less can be obtained, the nature of the liberated reducing groups can be determined by a subtractive method involving reduction. The method is not affected by the substitution of peptide on the lactyl moiety of muramic acid residues.

Chromatographic Procedure. A sample containing 100 millimicromoles of total hexosamine is reduced in 30 μl of fresh, unbuffered 0.1 *M* $NaBH_4$ for 3 hours at room temperature. A control consists of an identical sample in 30 μl of 0.1 *M* $NaBH_4$ which had been previously acidified

[3] J. M. Ghuysen, and J. L. Strominger, *Biochemistry* **2**, 1119 (1963).

and reneutralized. The product and the control are acidified with concentrated HCl (15 μl) and hydrolyzed for 3 hours in a water bath at 95°. After lyophilization, the residues are chromatographed in two dimensions using freshly prepared *n*-butanol–acetic acid–water (3:1:1) and then pyridine–water (4:1). The compounds are detected with ninhydrin.

This procedure separates all the common cell-wall glycopeptide components. The action of an acetylhexosaminidase, followed by reduction and hydrolysis, will result in the disappearance from the chromatogram of the hexosamine whose reducing group has been liberated. The extent of disappearance will be proportional to the extent of hydrolysis of glycosidic bonds. Quantitatively, the conversion of amino sugar to reduced amino sugar is best followed using the Beckman-Spinco amino acid analyzer.

Enzymatic Procedure. Reduction, with the same control, is performed as for the chromatographic procedure. After acid hydrolysis and lyophilization, the boric acid in the residue is removed by dissolving it in anhydrous methanol and evaporation *in vacuo*. This is repeated twice. The glucosamine content of the residue is then determined using the specific glucosamine 6-phosphate *N*-acetylase, as described by Lüderitz *et al.*[4] If the enzyme was an endoacetylglucosaminidase, the glucosamine content of the final product will be reduced in proportion to the extent of the enzymatic hydrolysis.

Similarly, muramic acid can be measured enzymatically by destroying glucosamine with glucosamine deaminase and then measuring the residual total amino sugar.[5] After reduction and hydrolysis, the action of an endoacetylmuramidase will manifest itself in the disappearance of residual muramic acid.

By these procedures, it can be established whether a glycosidase is an acetylglucosaminidase or an acetylmuramidase. The determination of the exact specificity of the glycosidase is more difficult and requires the elucidation of the precise chemical nature of the linkage which has been split.

Appearance of Free Amino Groups during Lysis of Bacterial Cell Walls

FDNB Reagent. Fluorodinitrobenzene, 130 μl in 10 ml 100% ethanol.

Procedure. Samples containing 5–25 millimicromoles of amino group in 20 μl of 1% $K_2B_4O_7$ are mixed with FDNB reagent (2 μl) and immediately placed in a water bath at 60°. After 30 minutes, they are

[4] O. Lüderitz, A. R. Simmons, O. Westphal, and J. L. Strominger, *Anal. Biochem.* **9**, 263 (1964).

[5] H. J. Risse and O. D. Lüderitz, *Biochem. Z.* **341**, 1 (1964).

acidified with 2 *N* HCl (80 μl) and read at 420 mμ.[6] At this wavelength and pH, dinitrophenol derived from the excess reagent has negligible absorption while the *N*-dinitrophenyl (DNP) derivatives of amino acids have a molar extinction coefficient of 5200. As in the case of reducing power, the insolubility of polymeric DNP derivatives precludes the determination of accurate kinetics in the initial phases of lysis of cell walls. This difficulty can be overcome if the DNP derivatives are first hydrolyzed, as in the determination of N-terminal groups described below. Otherwise, the kinetics of the cell wall digestion may be related to the liberation of soluble fragments with free terminal amino groups. The residual unhydrolyzed cell wall material must then be removed by centrifugation prior to applying the FDNB procedure.

A further difficulty arises in the case of cell walls having a high intrinsic content of N-terminal groups, such as those due to the ϵ-amino groups of lysine in *M. lysodeikticus*. These may mask the liberation of a smaller proportion of new amino groups, but they have been eliminated in this case by hydroxymethylation of the cell walls with ethylene oxide. The product was still sensitive to the ML endopeptidase and to lysozymes. Cell walls are suspended at a concentration of 20 mg/ml in fresh 10% aqueous ethylene oxide, adjusted at pH 8 with NaOH, and agitated gently for 48 hours. They are isolated by centrifugation; thoroughly washed with water, and lyophilized.

N-Terminal and Free Amino Acids

Samples containing 10–50 millimicromoles of each N-terminal group are mixed with 10% $K_2B_4O_7$ and water to give a total volume of 100 μl of 1% $K_2B_4O_7$. FDNB reagent identical to that used for determination of total amino groups (10 μl) is added. The solutions are mixed and incubated at 60° for 30 minutes. After acidification with concentrated HCl (50 μl), the DNP derivatives of free amino acids are extracted with ether (100 μl, three times). Most DNP peptides are not extracted. The residual ether in the aqueous phase is evaporated at 60°, then the tubes are sealed and hydrolyzed for 6 hours in a water bath at 95°. The DNP derivatives of the N-terminal amino acids are then extracted with ether (100 μl, three times) leaving mono-DNP lysine and mono-DNP diaminopimelic acid derivatives in the aqueous phase. The ether extracts are evaporated with gentle agitation in a 37° bath and then dried *in vacuo*. Controls consist of two standard mixtures of the cell wall amino acids, one of which is hydrolyzed after dinitrophenylation, and a third control of ϵ-DNP lysine or mono-DNP diaminopimelic acid in

[6] O. H. Lowry, unpublished procedure.

known concentration and which is also hydrolyzed. The residues from the ether extracts are redissolved in 0.05 *M* NH_3 (20 μl) and aliquots are chromatographed on thin-layer plates of silica gel G.[7] Sequential development in the same dimension with a basic solvent and an acidic solvent allows the complete separation of all the DNP derivatives present. The plates are first developed with solvent A (*n*-butanol–1% ammonia (w/v), 1:1—upper phase) at room temperature. Then, after thorough drying in a stream of cold air, the plates are developed with solvent B (chloroform–methanol–acetic acid, 85:14:1—single phase) at 2°. The mono-DNP lysine and mono-DNP diaminopimelic acid remaining in the aqueous HCl layers are extracted with water-saturated *n*-butanol (100 μl, twice) and the extracts are dried *in vacuo*. The residues are then chromatographed in solvent C (benzyl alcohol–chloroform–methanol–water–conc. ammonia, 30:30:30:6:2) with only one development. After drying, the spots are transferred to 1-ml tubes, using a suction device, and eluted from the gel by vigorous mixing (twice for 10 seconds using a Vortex mixer) with 0.01 *M* ammonia—methanol, 1:1 (200 μl). After centrifugation, the optical density of the supernatants is measured at 360 mμ. Molar extinction coefficients are about 15,000 and 25,000 for unhydrolyzed mono- and bis-DNP amino acid derivatives, respectively, and these figures are reduced by 10–20% by the hydrolytic procedure. As far as possible, the whole procedure is performed in the dark or with tungsten lamp illumination to minimize photodecomposition. DNP glycine seems to be particularly acid-labile and this can result in low recovery if the hydrolysis is too vigorous. With practice, one can achieve by this chromatographic procedure a reproducibility of ±5%. The hydrolysis conditions described above were, however, found to be insufficient in one case. Lysis of the cell walls of *M. roseus* with the MR endopeptidase from *S. albus* G (see Table I) results in the liberation of N-terminal threonine. After dinitrophenylation any hydrolysis as described above, this is almost entirely liberated as the *N*-ϵ-(DNP threonyl)-lysine dipeptide derivative, which is not ether extractable, and has a mobility in solvent C similar to that of *N*-ϵ-DNP lysine. Hydrolysis for 15 hours in 6 *N* HCl is required for the liberation of DNP threonine. DNP alanyl-alanine and some other DNP-dipeptides are ether extractable. Also considerable difficulty has been found in determining the N-terminal glycine of polyglycines where the yield is found to decrease sharply with chain-length greater than 3. Thus considerable caution should be used in the interpretation of preliminary data obtained by this procedure.

[7] V. M. Brenner, A. Niederweiser, and G. Pataki, *Experientia* **17**, 145 (1961).

Carboxyl-Terminal Amino Acids

Samples containing 10–50 millimicromoles of each C-terminal amino acid are dried, sealed with redistilled[8] hydrazine (40 μl) and incubated for 16 hours at 60°.[9] After the excess reagent has been dried off *in vacuo* over H_2SO_4, the residue is dissolved in water (100 μl) and redistilled benzaldehyde (20 μl) is added. The tubes are thoroughly mixed for 10 seconds at 5-minute intervals during 1 hour. An aliquot (90 μl) of the supernatant is removed and is retreated with benzaldehyde (20 μl) in the same way. An aliquot (80 μl) of the supernatant is extracted twice with ether (100 μl) and then dried *in vacuo*. The free amino acids are determined as described above.

Where it has been determined that lysis involves the production of N-terminal or C-terminal alanine, the site of action of the enzyme can be further defined by determination of the configuration of the terminal alanine. For instance, an acetylmuramyl-L-alanine amidase will liberate N-terminal L-alanine, and a peptidase splitting intertetrapeptide cross links will liberate C-terminal D-alanine.

Enzymatic Determination of D- and L-Alanine

Total D-alanine and L-alanine are measured in aliquots of HCl hydrolyzates after lyophilization. N-terminal D- and L-alanine are determined by the decrease in D- and L-alanine which occurs when the peptide is dinitrophenylated prior to hydrolysis. C-terminal D- and L-alanine are determined in the products of hydrazinolysis. Because some of the enzymes involved in the determinations are inhibited by components of the mixtures obtained after hydrolysis of dinitrophenylated peptides and after hydrazinolysis, the alanine in these mixtures must be purified prior to the determination.

Purification of Alanine by Dowex 50 Chromatography. Dowex 50-H^+ ($\times$8, 100–200-mesh) is packed in a column 10–15 cm in length and 0.8 cm in diameter. After washing with 4 *N* HCl (20 ml), the column is equilibrated with 1.5 *N* HCl (20 ml). The sample, dissolved in about 1 ml of 1.5 *N* HCl, is applied to the column, which is then eluted with 20 ml of 1.5 *N* HCl at a rate of 0.2 ml/minute. Fractions of 1 ml are collected. If the total alanine of the sample exceeds 20 millimicromoles, the alanine can be located by drying 100 μl aliquots of the fractions and following the procedure for free amino acid determination described previously. As little as 10 millimicromoles is sufficient for the determinations of D- and L-alanine, however, and when only a small amount of alanine is

[8] N. L. Drake, *Org. Syn.* **24**, 53 (1944).
[9] M. R. J. Salton, *Biochim. Biophys. Acta* **52**, 329 (1961).

available for the determinations, it can be located in the eluate if ^{14}C-alanine (e.g., 0.2 millimicromole of material of specific activity 40 μC/micromole or higher) has been previously mixed with the applied sample. Aliquots of the fractions (20 μl) are then counted. Alanine is reproducibly eluted in a volume of 4–5 ml, centered at about 12 ml after the beginning of the elution. The alanine-containing fractions are lyophilized.

Reagents and Equipment

Enzymes: Crystalline D-amino acid oxidase (17 mg/ml) is prepared from hog kidney.[10] Commercially available preparations of low purity are not suitable for this determination, but the purified enzyme supplied by Worthington Biochemical Corp., Freehold, New Jersey, is suitable

Catalase from beef liver, 20 mg/ml, recrystallized, Sigma Chemical Co., St. Louis

Lactate dehydrogenase (crystalline suspension from rabbit muscle, type 2, 10 mg/ml, Sigma Chemical Co., St. Louis)

L-Glutamate-pyruvate (L-alanine-α-ketoglutarate) transaminase (crystalline suspension, 10 mg/ml, C. F. Boehringer and Soehne Gmbh, Mannheim)

Stock DPNH solution, 2.5 mg/ml in 0.1 *M* Na_2CO_3

Pyrex tubes, 3 ml, chosen for uniformity in diameter, freedom from scratches and defects, and ability to fit in the cell holder of the fluorometer

Farrand model A-2 fluorometer used with Corning filter 7-60 in the primary position and filters 3-72 and 4-70 in the secondary position. (For further details on fluorometric determination of DPNH, see Lowry and Passonneau)[11]

Procedure for Determination of D-*Alanine*

$$\text{D-Alanine} \xrightarrow{\text{D-amino acid oxidase}} \text{pyruvate} + NH_3 + H_2O_2$$

$$H_2O_2 \xrightarrow{\text{catalase}} H_2O + \tfrac{1}{2}\,O_2$$

$$\text{Pyruvate} + \text{DPNH} \xrightarrow{\text{LDH}} \text{lactate} + \text{DPN}$$

Triplicate aliquots containing 0.5–1.5 millimicromoles of D-alanine are dried in 3-ml tubes. Forty microliters of 0.1 *M* pyrophosphate, pH 8.3, containing, per milliliter, 5 μl catalase and 5 μl D-amino acid oxidase, is added. The tubes are mixed and incubated at 37° for 3 hours. Controls

[10] V. Massey, G. Palmer, and R. Bennett, *Biochim. Biophys. Acta* **48**, 1 (1961).
[11] O. H. Lowry and J. V. Passonneau, Vol. VI [111].

consist of a blank, at least two levels of D-alanine, and a set of tubes containing an excess of pyruvate. One milliliter of DPNH solution (diluted just before use 1:80 with 0.05 *M* potassium phosphate, pH 6.8) is then added; after mixing, the tubes are read in the fluorometer. The sensitivity is adjusted so that the DPNH solutions give near maximum deflection of the galvonometer. Five microliters of lactate dehydrogenase (diluted 1:5 with 0.05 *M* potassium phosphate, pH 6.8) is then added; after mixing and incubation at 37° for 30 minutes, the tubes are again read in the fluorometer. The decrease in fluoroscence due to oxidation of DPNH to DPN is proportional to the amount of D-alanine present.

Procedure for Determination of L-*Alanine*

$$\text{L-Alanine} + \alpha\text{-ketoglutarate} \xrightleftharpoons{\text{transaminase}} \text{pyruvate} + \text{L-glutamate}$$

$$\text{Pyruvate} + \text{DPNH} \xrightarrow{\text{LDH}} \text{lactate} + \text{DPN}$$

The reagent mixture contains 35 ml of 0.05 *M* potassium pyrophosphate, pH 8.0, 30 μl of lactate dehydrogenase, 150 μl of 50 m*M* α-ketoglutarate, and 50 μl of the stock DPNH solution. Aliquots containing 0.5–1.5 millimicromoles of L-alanine are dried and mixed with 1 ml of this incubation mixture. After reading in the fluorometer, 6 μl of the transaminase suspension is added to each tube, which is mixed and incubated at 38° for 2½ hours. The tubes are then read again in the fluorometer.

Remarks

As noted earlier, the procedures are very susceptible to impurities and with questionable samples an internal standard should be used. This is prepared by mixing 0.5–1 millimicromole of the unknown alanine sample with a similar amount of D- or L-alanine, depending upon the determination.

It may be noted that large amounts of D-amino acid oxidase and L-alanine-α-ketoglutarate (L-glutamate–pyruvate) transaminase are employed in the procedure. This is due to the high K_m's of these two enzymes for D- and L-alanine, respectively.

Determination of Total Amino Acids

Samples containing 10 to 50 millimicromoles of each amino acid are hydrolyzed in 6 *N* HCl for 12 hours at 95°. After removal of the HCl by lyophilization, the amino acids are dissolved in 1% $K_2B_4O_7$ (100 μl) and the previously described procedure for free amino acids is followed.

ERRATUM

METHODS IN ENZYMOLOGY, VOLUME VIII

Page 699, line 3: should read "1:800"
instead of "1:80"