A SYMPOSIUM ON METABOLIC ACTIVITY AND BACTERIAL STRUCTURE

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Cell-Wall Structure and Biosynthesis

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The external structures responsible for the rigidity and integrity of bacterial cells are generally referred to as the 'cell walls' and apart from their obvious mechanical functions very little direct work has been done on the biochemical activities associated with these structures. It is generally agreed that isolated cell-wall preparations of Gram-positive bacteria are comparatively devoid of enzyme systems localized in other structures and fractions derived from disintegrated cells. With the cell walls of Gram-negative bacteria the situation is less certain as there have been several reports of enzymic activities intimately associated with 'envelope' preparations of these organisms (Marr, 1960; Hunt, Rodgers & Hughes, 1959; Salton, 1961*a*). Although the enzymic functions localized in cell walls have not been investigated extensively, walls have attracted interest in a broader biochemical sense. This report will thus be confined to some aspects of the chemistry and biosynthesis of these structures and it is not intended to be a complete review of this rapidly expanding topic (for recent reviews see Work (1961) and Salton (1961*b*)).

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Structure of cell walls of Gram-positive bacteria

The general chemical composition of the cell walls of Gram-positive bacteria has been the subject of numerous investigations and the broad features of amino acid, amino sugar and monosaccharide constitution are now well known (Cummins & Harris, 1956, 1958; Work, 1957, 1961; Salton, 1958, 1961*b*). However, the arrangement of the simple constituents identified by paper chromatography of the hydrolysed walls has remained uncertain until recent years. Thus the variety of polymers in the walls of Gram-positive bacteria can only now be defined a little more clearly.

The following classes of polymeric substances have been isolated from walls and partially or fully characterized: (1) mucopeptides or peptidopolysaccharides; (2) oligosaccharides, polysaccharides; (3) teichoic acids; (4) teichuronic acid.

It is apparent that the walls of some Gram-positive bacteria may be made up entirely of mucopeptides (1), while others contain in addition one or more of the other three classes of polymers. Mucopeptides composed of three or four principal amino acids, together with glucosamine and muramic acid, have been identified in all bacterial cell walls. Additional cell-wall components such as the teichoic acids (Armstrong *et al.* 1959) and polysaccharides are less widely distributed and the teichuronic acid polymer has so far only been reported in walls of *Bacillus subtilis* (Janczura, Perkins & Rogers, 1961). Of all the four classes of substances isolated from walls, only the chemical structure of the teichoic acid has been fully established by the beautiful work of Baddiley and his colleagues (Armstrong, Baddiley & Buchanan, 1961).

The term 'mucopeptide' was originally proposed by Mandelstam & Rogers (1959) to describe the amino sugar-amino acid-containing complexes of the walls now recognized as the structural 'backbone' common to all cell walls of Gram-positive bacteria. Whether the 'mucopeptide' of a given wall is made up of one type of 'polymer' is certainly not known at present and as shown by Salton (1956) a complex mixture of 'fragments' is obtained when a mucopeptide wall is digested by the enzyme, lysozyme. There is, however, little doubt now that the mucopeptide forms the rigid backbone component composed of covalently bonded amino acids and amino sugars. That the other cell-wall compounds are attached to the mucopeptide by weaker linkages now seems likely from the extractibility of the teichoic acids (Archibald, Armstrong, Baddiley & Hay, 1961) and the teichuronic acid of *Bacillus subtilis* (Janczura *et al.* 1961) with trichloroacetic acid (TCA) in the cold and the removal of oligosaccharide and polysaccharide residues with both picric acid (Holdsworth, 1952) and formamide (Krause & McCarty, 1961). In all cases the other wall 'polymers' have been obtained in solution, leaving behind insoluble mucopeptide residues, still possessing the structural rigidity and appearance of the native cell wall as seen in the electron microscope (Archibald, Armstrong, Baddiley & Hay, 1961; Krause & McCarty, 1961).

With the isolation and characterization of muramic acid (Strange & Dark, 1956; Strange & Kent, 1959) and its identification in the Park nucleotides (Park & Strominger, 1957) the key role of this amino sugar in the structure of the wall and spore mucopeptides became apparent. Thus the broad outline of mucopeptide structure has been established from the studies of products isolated from walls and mucopeptides digested with lysozyme and streptomyces amidase (Salton, 1956,

1957; Brumfitt, Wardlaw & Park, 1958; Ghuysen & Salton, 1960; Ghuysen, 1960; Salton & Ghuysen, 1960; Ghuysen, 1961; Primosigh, Pelzer, Maass & Weidel, 1961).

The mucopeptide is envisaged as possessing an acetyl amino sugar 'backbone' containing alternating *N*-acetylglucosamine and *N*-acetylmuramic acid residues with peptides linked through the COOH group of muramic acid. However, it is obvious that not all of the *N*-acetylmuramic acid residues carry peptide substituents since *di*- and *tetra*-saccharides have been isolated in lysozyme digests of walls (Perkins, 1960; Salton & Ghuysen, 1960). These observations suggest that *N*-acetylglucosamine and *N*-acetylmuramic acid are in the form of amino sugar repeating units. An examination of the products of partial acid hydrolysis of the walls of *Micrococcus lysodeikticus* with concentrated H_3PO_4 for 12 hr. at 37° also

Table 1. *Products identified in lysozyme and H_3PO_4 digests of Micrococcus lysodeikticus walls*

Lysozyme	H_3PO_4 , 12 hr.; 37°
Disaccharide*	Free AG† and AMA† Disaccharide*
Tetrasaccharide*	Trisaccharide?*
	Tetrasaccharide*
	Oligosaccharides*
Mucopeptides	Mucopeptides

* Compounds composed of both AG and AMA. † AG, *N*-acetylglucosamine; AMA, *N*-acetylmuramic acid.

revealed the presence of acetyl amino sugar compounds composed of *N*-acetylglucosamine and *N*-acetylmuramic acid and the nature of some of these products is compared with those obtained by lysozyme digestion in Table 1.

Confirmation that some of the muramic acid residues in the wall mucopeptide possess free carboxyl groups has been obtained by esterifying the walls of *Micrococcus lysodeikticus* with methanol-HCl and reducing the esterified walls with $LiBH_4$ in tetrahydrofuran. Upon acid hydrolysis (4*N*-HCl, 4 hr. 100°) of the walls subjected to this procedure much of the cell-wall muramic acid has been converted to a neutral amino sugar compound and the sequence of the reactions is shown in Fig. 1 (Salton, 1962).

The precise nature of the repeating unit in mucopeptides is not known. Several low-molecular-weight mucopeptides have been isolated from lysozyme-digested walls of *Micrococcus lysodeikticus* (Ghuysen & Salton, 1960) and from *Escherichia coli* mucopeptide (Primosigh, Pelzer, Maass & Weidel, 1961). Whether these simple mucopeptides form the repeating units of 10,000–20,000-molecular-weight compounds obtained from wall digests (Salton, 1956) cannot be said at present.

The problem of determining the subunit size of wall mucopeptide by conventional *N*-terminal and *C*-terminal amino acid analysis (Salton, 1961*c*) presents some difficulties, especially when the wall structure contains teichoic acids possessing ester-linked alanine. Free amino groups and *C*-terminal residues can be readily identified by reacting walls with fluoro-dinitrobenzene and anhydrous hydrazine respectively (Salton, 1961*c*). Surprisingly few free amino and *C*-terminal groups are found in some bacterial cell walls, suggesting either a high degree of cross-linking or protection of the reactive groups with substituents.

Where a variety of 'N-terminal' and 'C-terminal' groups have been detected in the wall of a given species, it is difficult to interpret the information in terms of subunit size. This has frequently been found to be the case with a wide variety of bacterial walls (Salton, 1961*c*). However, the data for walls of *Micrococcus lysodeikticus* and some of the lysozyme digest products appear to be consistent and have given a subunit size of around 2000, as shown in Table 2. A tentative model of a mucopeptide structure compatible with the data available at present is illustrated in Fig. 2.

Fig. 1. The sequence of reactions for detecting muramic acid residues (in walls) possessing free carboxyl groups involves esterification with methanol-HCl followed by reduction with lithium borohydride giving the modified 'muramic acid' compound on acid hydrolysis.

Table 2. Subunit size of walls and soluble products from *Micrococcus lysodeikticus*

	Wall	Lysozyme NDF	Mucopeptides
Molecular weight	—	10,000–20,000	1200 2400
Subunit from			
ε-NH ₂ lysine	2500	2500	—
C-terminal glycine	2000	2000	—

Many structural problems remain to be solved and it is not known at present how subunits of the type proposed in Fig. 2 would be joined together to give the higher-molecular-weight (10,000–20,000) components possessing peptide to disaccharide (*N*-acetylmuramic acid-*N*-acetylglucosamine) ratios greater than 1.

Structure of walls of Gram-negative bacteria

Isolated walls of Gram-negative bacteria are both physically and chemically more complex than those of most Gram-positive organisms (Salton, 1961*b*). The protein, lipid, polysaccharide complexes form part of the wall and in addition a

rigid mucopeptide layer has been clearly demonstrated in the elegant studies of Weidel, Frank & Martin (1960). Thus there is now abundant evidence that walls of Gram-negative bacteria possess a mucopeptide of similar composition to that found in Gram-positive bacteria and it is the mucopeptide component which represents the 'basal structure' containing muramic acid and diaminopimelic acid and D-glutamic acid as the most characteristic substances (Work, 1957).

Fig. 2. A tentative model of a mucopeptide subunit based on data obtained from *Micrococcus lysodeikticus* walls.

Biosynthesis of cell walls

Interest in the problem of cell-wall biosynthesis was stimulated by the discovery that penicillin action on bacteria involved an inhibition of mucopeptide formation and consequent accumulation of nucleotide precursors (Park & Strominger, 1957; Park, 1952; Mandelstam & Rogers, 1959). Various aspects of the biosynthesis of walls have been dealt with in reviews by Strominger (1960), Work (1961) and Salton (1961*b*) and the present discussion briefly covers only certain facets of the problem.

Amino acids

The simplest level at which wall synthesis has been studied is at the level of biosynthesis of some of the most characteristic building blocks (amino acids and amino sugars) of the wall mucopeptide. Thus the origins of the D-isomers of the amino acids alanine, aspartic acid and glutamic acid as well as the formation of α,ϵ -diaminopimelic acid (DAP) in its various isomeric forms (LL, DD and *meso*) are relevant to the problem of the biosynthesis of walls. Pathways for the biosynthesis of the latter amino acid (DAP) have been discussed in detail elsewhere (Rhuland, 1960; Work, 1961).

Alanine racemase has been known for some time in bacteria (Wood & Gunsalus, 1951) and the presence of such an enzyme could fulfil the function of providing

D-alanine for cell-wall precursors. The origin of D-glutamic acid in bacteria has been investigated in relation to the formation of the γ -D-glutamyl capsular polypeptides (Thorne, 1956). No glutamic racemase could be detected in the organisms capable of producing D-glutamyl peptides, but they did, however, contain enzymes effecting an indirect conversion to D-glutamic acid by a transamination reaction between D-alanine and α -ketoglutaric acid (Thorne, 1956). Whether cell-wall D-glutamic acid arises by a similar pathway cannot be said at present. That the D-glutamate may also originate from a specific racemase in some organisms has now been demonstrated with an enzyme purified from extracts of *Lactobacillus fermenti* by Tanaka, Kato & Kinoshita (1961). The *meso* isomer of diaminopimelic acid occurs more widely in cell walls than does the LL isomer (Salton, 1961*b*; Work, 1961) and a racemase which interconverts the LL or *meso* isomer to an equilibrium mixture of the two has been described by Antia, Hoare & Work (1957). The origin of the D-isomer of aspartic acid in bacterial cell walls has not, so far as the author is aware, been investigated.

Another enzymic reaction which may have a bearing on the biosynthesis of cell-wall compounds includes the amino acid-activating system for D-alanine detected by Baddiley & Neuhaus (1959) in *Lactobacillus arabinosus*.

Amino sugars

The two amino sugars *N*-acetylglucosamine and *N*-acetylmuramic acid occur in all of the bacterial cell walls so far studied. The biosynthesis of amino sugars has been discussed in some detail in the excellent review by Roseman (1959). The pathways for the formation of *N*-acetylglucosamine and *N*-acetylgalactosamine have been studied with bacterial enzymes by Roseman and his colleagues (Roseman, 1959). It is thus likely that cell-wall acetylglucosamine originates from some of these described enzymic reactions.

The biosynthesis of muramic acid (the 3-0-lactyl ether of D-glucosamine) has not been fully investigated. Strominger (1960) reported the following enzymic reaction of uridine diphosphonucleotides (UDP) in extracts of *Staphylococcus aureus*:

Richmond & Perkins (1960) also investigated the origin of the lactic acid residue of muramic acid and their results were consistent with the idea that phosphoenolpyruvate was the immediate precursor.

Whether muramic acid is synthesized at the nucleotide level has not been conclusively established. Zilliken (personal communication) has suggested the biosynthesis of muramic acid at a *di*- or *tri*-saccharide level by the addition of an unknown 3-C fragment to form the O-lactyl side chain.

The possibility of a more direct route of biosynthesis has been explored in the author's laboratory using extracts of *Micrococcus lysodeikticus* (Salton, unpublished observations). The investigations of Roseman (1959) have suggested that the amino sugar phosphates are probably the most important intermediates for the formation of *N*-acetyl amino sugars. To test the possibility that muramic acid synthesis may occur via glucosamine-6-phosphate (GM-6 P) crude extracts of *M. lysodeikticus*

were incubated with GM-6 P and phosphoenolpyruvate in M/15 phosphate buffer at pH 7.0. A compound which corresponded to muramic acid-6-phosphate (prepared by incubating Boehringer crystalline yeast hexokinase, ATP, Mg^{2+} and muramic acid and separated by paper chromatography) was detected in the reaction mixture. Thus these results tentatively suggest that muramic acid may originate from the following reaction:

Glucosamine-6-phosphate + phosphoenolpyruvate $\rightarrow$ muramic acid-6-phosphate + Pi.

Confirmation of this pathway must await further purification of the enzyme system present in the crude extracts. Which of the three possible pathways suggested for biosynthesis of muramic acid will be the principal one involved in wall formation remains to be established. It is perhaps relevant to this problem that muramic acid-6-phosphate has already been isolated from bacteria by Ågren & de Verdier (1958) as well as a compound believed to be UDP-muramic acid-6-phosphate.

Cell-wall intermediates and biosynthetic pathways

The original observations of Park (1952) on the structure of the nucleotides accumulating in penicillin-treated *Staphylococcus aureus* gave the first indications of the types of compounds acting as cell-wall precursors (Park & Strominger, 1957). Compounds of identical structure have also been obtained by Rogers & Perkins (1960) when 5-fluorouracil was used as an inhibitor of mucopeptide biosynthesis. Thus the use of substances inhibiting wall formation has greatly facilitated the detection and isolation of cell-wall nucleotide precursors (Strominger, 1960).

The number of nucleotides isolated from both untreated and penicillin- or fluorouracil-inhibited bacteria has increased rapidly during the past few years, and some of the earlier data have been summarized by Strominger (1960) and Salton (1961*b*). Uridine nucleotides containing muramic acid and amino acids including diaminopimelic acid have also been isolated from *Escherichia coli* by Strominger (1960) and Comb, Chin & Roseman (1961). Until recently only uridine nucleotides containing muramic acid had been found in bacteria, but the detection in extracts from *Aerobacter cloacae* of adenylyl nucleotides containing muramic acid and peptides is of considerable interest (Cooksey, Anwar, Roy & Watson, 1961). The formation of thymidine diphosphorhamnose by extracts of *Streptococcus faecalis* which contains considerable quantities of rhamnose in the wall has now been established by Pazur & Shuey (1960) and it seems likely that this nucleotide may also be a cell-wall precursor compound.

Transfer of the amino sugar-peptide residues as well as polyol and sugar moieties of the various nucleotides to mucopeptide and/or wall has not been conclusively demonstrated. However, the nature of the nucleotides accumulating in the presence of various antibiotics makes it extremely likely that some of the suggested pathways for wall biosynthesis (Strominger, 1960; Salton, 1961*b*) may not be far from reality.

The site of cell-wall biosynthesis within the bacterial cell has not been established, but studies of the anatomy of thin sections of cells support the view that wall formation may occur in a limited area rather than over the entire surface of the protoplast. It seems inevitable that the membranes or membranous organelles are concerned in some way in wall synthesis. The hypothesis that the intermediates

are formed at intracellular levels and that the transferring and/or 'polymerizing' mechanisms are located in the membrane in intimate contact with the cell-wall acceptor seems an attractive basis for further experimental exploration.

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