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Cross-Linking of Bacterial Cell Walls with Glutaraldehyde

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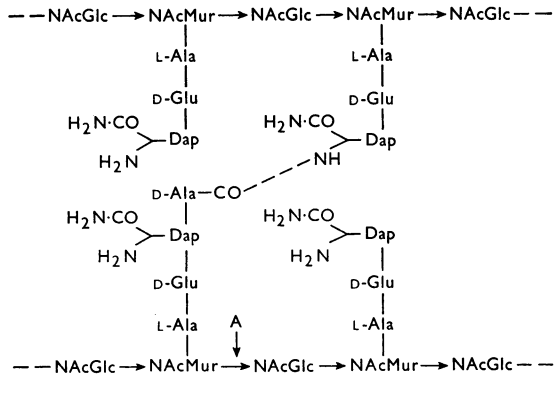
Many bacterial mucopeptides (e.g. *Staphylococcus aureus*) are highly cross-linked and have very few free amino groups (Salton, 1961). However, in *Bacillus subtilis* and *Bacillus licheniformis* mucopeptide (structure I) only about two-thirds of the peptide side chains are linked together. The linkage is through a residue of D-alanine from the α-carboxyl terminal of a diaminopimelic acid of one chain to the ε-amino group of diaminopimelic acid in a second chain (Hughes, 1968; Warth & Strominger, 1968), forming heptapeptide dimer units. A substantial number of ε-amino groups of diaminopimelic acid are free and are available to reagents such as 1-fluoro-2,4-dinitrobenzene. We have examined the reaction of these groups with a classical cross-linking reagent, glutaraldehyde. The treated material is solubilized only very slowly by lysozyme although the glycosidic linkages of the glycan chains of the modified mucopeptide are readily hydrolysed by the enzyme.

Bacillus subtilis 168 mucopeptide (40mg) was suspended in 0.1M-sodium phosphate buffer, pH 7.0 (5.0ml). Samples (0.5ml) were treated under various conditions with glutaraldehyde solutions (0.1ml). The samples were shaken occasionally at room temperature. After thorough washing with water the residues were treated with lysozyme (1mg) in 20mM-ammonium acetate, pH 6.5 (3.0ml), and the rate of solubilization was followed by turbidity measurements. The samples that had been treated for at least 5h with the more concentrated solutions (0.2–1.0%) of glutaraldehyde were dissolved more slowly than the control. The optimum conditions were chosen as 0.33% glutaraldehyde at room temperature for 5h.

Mucopeptide treated with glutaraldehyde under these conditions was incubated with lysozyme and the turbidity of the solution fell to 58% of the original value after 5h (Fig. 1). An untreated control was completely digested by lysozyme within 30min. Samples (0.2ml) were taken at intervals and assayed for substances reacting as *N*-acetylhexosamine in the modified Morgan–Elson reaction (Reissig, Strominger & Leloir, 1955), with a heating time in borate buffer of 35min. This reaction is a test for the reducing disaccharide unit, *N*-acetylglucosaminyl-β-(1→4)-*N*-acetylmuramic acid, that is released by lysozyme. Lysozyme hydrolysed about 85% of susceptible linkages after 5h treatment of the control mucopeptide (Fig. 1) and a similar value (79%) was obtained for the glutaraldehyde-treated mucopeptide. About one-half of these bonds in both cases were split very quickly, indicating that the treatment with glutaraldehyde had not affected the accessibility of a substantial proportion of the glycosidic linkages to lysozyme. In the later part of the curve the modified mucopeptide was hydrolysed more slowly than in the control, perhaps owing to restricted diffusion of the enzyme in the insoluble matrix.

The amino acid and amino sugar content of mucopeptide remained unchanged after glutaraldehyde treatment except for a variable decrease (17–27%) in diaminopimelic acid. Since the cross-links introduced by glutaraldehyde are probably stable to acid hydrolysis (Quicho & Richards, 1966; Richards & Knowles, 1968), this result indicates that about 30–50% of the available amino groups had reacted with glutaraldehyde.

The simplest interpretation of the result obtained is that glutaraldehyde joins together two contiguous side chains in the case where both tripeptides carry



Structure (I): peptide side chains in *Bacillus subtilis* mucopeptide. Two tripeptide side chains and a dimer heptapeptide formed from two tripeptides by a D-alanine residue cross-bridge (broken line), with their free amino groups, are shown. A, Linkages broken by lysozyme. Abbreviations: NAcGlc, *N*-acetylglucosamine; NAcMur, *N*-acetylmuramic acid; Dap, diaminopimelic acid.

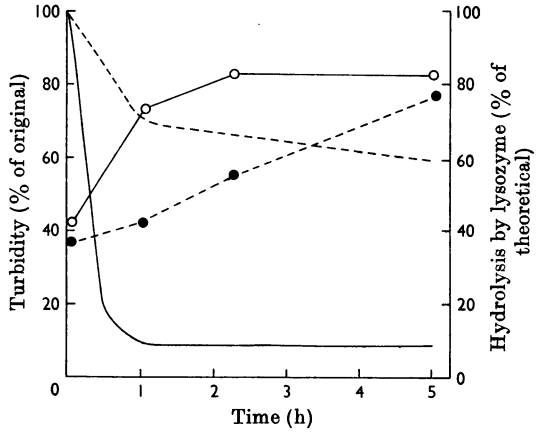


Fig. 1. Action of lysozyme on glutaraldehyde-treated mucopeptide. *Bacillus subtilis* mucopeptide (8mg) was treated with 0.33% glutaraldehyde in 0.1M-phosphate buffer, pH7 (0.6ml), at room temperature for 5h. Lysozyme digestion of the washed mucopeptide and of untreated mucopeptide (8mg) was then followed turbidimetrically (——, control; ----, glutaraldehyde-treated sample) and by analysis of Morgan–Elson-positive material (○—○, control; ●--●, glutaraldehyde-treated sample).

free ε-amino groups. The distance between contiguous peptides along a single polysaccharide backbone (structure I) would be at maximum the length of the disaccharide unit (about 10.3 Å). The mechanism proposed (Richards & Knowles, 1968) for the cross-linking reaction with glutaraldehyde would allow a cross-bridge considerably longer than 10 Å, but the most common cross-bridge, containing a five-carbon chain, would fit neatly the distance between adjacent peptide side chains, particularly since these peptides almost certainly have some lateral flexibility. A cross-link of this type would keep two adjacent tripeptides together even after cleavage at the glycosidic linkage (bond A in structure I) between them and the modified mucopeptide would be dissolved less readily by lysozyme than untreated material, as is observed. In contrast with the results obtained with glutaraldehyde, mucopeptide that had been treated with 1,5-difluoro-2,4-dinitrobenzene such that most of the free amino groups were substituted was solubilized by lysozyme as quickly as the untreated mucopeptide. Presumably this cross-linking reagent was unable to link together the peptide side chains of mucopeptide, as suggested for glutaraldehyde, and had reacted predominantly as a monofunctional reagent.

In *Staphylococcus aureus* the mucopeptide is completely solubilized by hydrolysis of the polysaccharide backbones (Ghuysen & Strominger, 1963). Presumably the large number of cross-links between peptide side chains of this mucopeptide are topologically not equivalent to the cross-bridges introduced artificially into *Bacillus subtilis* mucopeptide with glutaraldehyde. Indeed the increased resistance to lysis of the modified mucopeptide would be detrimental to proper cell growth where a certain plasticity of the wall, controlled by autolytic enzymes, may be required (Shockman, Pooley & Thompson, 1967).

Whole cell walls of *Bacillus subtilis* and *Bacillus licheniformis* are also made considerably more resistant to solubilization by lysozyme after treatment with glutaraldehyde under the standard conditions. Examination in the electron microscope of glutaraldehyde-treated walls exposed to lysozyme for 5–18h showed that wall morphology had been extensively retained. No change in wall composition of either organism was found after treatment with glutaraldehyde except for a loss (30–35%) of diaminopimelic acid. In addition, the ester-linked D-alanine residues present in the teichoic acids of the walls had reacted quantitatively with the reagent. If the ester linkages survived the incubation at pH7.0, the treatment of walls with glutaraldehyde would form both intramolecular cross-bridges in a single teichoic acid chain and join teichoic acid chains together by intermolecular bonds, provided the chains were sufficiently close together in the native wall.

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