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# The Action of Dilute Alkali on the Glycyl Residues of Staphylococcal Peptidoglycan

By A. R. Archibald, J. Baddiley and J. Goundry
*Microbiological Chemistry Research Laboratory, School of Chemistry, University of Newcastle upon Tyne, NE1 7RU, U.K.*

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Recent studies (Hughes & Tanner, 1968; Archibald, Coapes & Stafford, 1969) have shown that teichoic acids are readily extracted from the walls of several Gram-positive bacteria by treatment with dilute sodium hydroxide at temperatures at or below 37°C. Teichoic acids vary considerably in their lability towards alkali, but in some cases extraction may be effected without significant depolymerization and without dissolution of significant amounts of peptidoglycan; in such cases alkali provides a valuable alternative to the other procedures available for the extraction of materials from cell walls. It was observed, however, that the peptidoglycan of walls of various staphylococci dissolves fairly readily in dilute alkali, whereas that of several bacilli and lactobacilli is much more stable (Archibald *et al.* 1969). The present work shows that the ready dissolution of staphylococcal peptidoglycan in alkali is due to the lability of the glycyl peptides that form the cross-linkages in such material.

On treatment with 0.5M-sodium hydroxide at 22°C, walls of *Staphylococcus aureus* H and *Staphylococcus lactis* N.C.T.C. 2102 dissolve completely within 42h, whereas in this time less than 16% of the component amino compounds of walls of *Micrococcus lysodeikticus* are solubilized. The susceptibility towards degradation by alkali of the peptidoglycan of these walls is thus in sharp contrast with their degradation by dilute aqueous dimethylhydrazine (Anderson, Archibald, Baddiley, Curtis & Davey, 1969), which rapidly dissolves walls of *M. lysodeikticus* but which has little effect on peptidoglycan in the walls of staphylococci. This resistance of the peptidoglycan from staphylococci was ascribed to the rather more extensive cross-linking present in such material, so that its greater susceptibility to degradation by alkali was unexpected. Dissolution in alkali is not accompanied by any significant destruction of the amino sugars of the peptidoglycan, and consequently degradation from the reducing end of polysaccharide chains through saccharinic acid formation is unlikely to be responsible for the depolymerization. This conclusion is reinforced by the observation (Archibald *et al.* 1969) that walls of staphylococci dissolve in alkali even after prior treatment with sodium borohydride, and also by the consideration that the same amino sugars are present in peptidoglycans that do not dissolve in alkali. We have therefore examined the action of dilute alkali on the peptide linkages in walls of *S. aureus* H, *S. lactis* N.C.T.C. 2102 and *M. lysodeikticus* N.C.T.C. 2665.

All three wall samples were analysed on a Technicon amino acid analyser after hydrolysis in 6M-hydrochloric acid at 100°C for 16h. Samples of each wall were also dinitrophenylated and then hydrolysed in 6M-hydrochloric acid, and the resulting DNP-amino acids identified and determined by t.l.c. (Archibald, Baddiley & Shaukat, 1968). *C*-Terminal amino acids were determined by hydrazinolysis (Ghuysen, Tipper & Strominger, 1966). Results are given in Table 1.

Walls (200mg) from each organism were suspended in water (20ml) and mixed with M-sodium hydroxide (20ml). The mixtures were stirred at 22°C, and samples (10ml) were removed after 42h and 100h and immediately neutralized with M-hydrochloric acid (5ml). The staphylococcal cell walls dissolved completely within 42h, whereas the turbidity of the suspension of walls of *M. lysodeikticus* decreased only slightly. The extent of dissolution of the walls of *M. lysodeikticus* was determined quantitatively by amino acid analysis of samples of suspensions and of the supernatant solutions obtained by removal of cell walls (centrifugation at 25000*g* for 20min). These samples were heated in 6M-hydrochloric acid at 100°C for 16h in sealed tubes and then analysed on a Technicon amino acid analyser. After 42h and 100h the supernatant solutions contained 15.4% and 35.5% respectively of the total amino compounds originally present in the wall. In agreement with this, analysis of the residues of the wall from the micrococcus after 42h and 100h showed them to contain 82.6% and 63.5% respectively by weight of the amino compounds originally present.

The neutralized suspensions or solutions obtained after alkali treatment of walls of *M. lysodeikticus*, *S. aureus* and *S. lactis* were analysed as above for *N*- and *C*-terminal amino acids, and in addition samples were analysed directly for free amino acids formed during treatment with alkali. The results (Table 1) show that after 42h approx. 38% of the glycyl linkages in the peptidoglycan from *S. aureus*

Table 1. *Release of* N*-terminal,* C*-terminal and free amino acids during treatment of walls with* 0.5M*-sodium hydroxide at* 22°C

Results are expressed as μmol/g of wall. Free amino groups were analysed by dinitrophenylation and values for *N*-terminal lysine are for ε-DNP-lysine. Only trace amounts of di-DNP-lysine were detected. *C*-Terminal groups were determined by hydrazinolysis. Values are uncorrected.

| Amino acid content of walls | | *N*-Terminal amino acids after treatment in alkali for | | | *C*-Terminal amino acids after treatment in alkali for | | | Free amino acids present in solution after treatment in alkali for | |
|---|---|---|---|---|---|---|---|---|---|
| | | 0h | 42h | 100h | 0h | 42h | 100h | 42h | 100h |
| *Staphylococcus aureus* | | | | | | | | | |
| Muramic acid | 480 | | | | | | | | |
| Glutamic acid | 520 | 0 | 0 | 0 | | | | 0 | 0 |
| Glycine | 2240 | 0 | 642 | 1159 | 59 | 792 | 1404 | 680 | 1243 |
| Alanine | 1280 | 155 | 140 | 188 | 144 | 190 | 237 | 160 | 195 |
| Glucosamine | 1040 | | | | | | | | |
| Lysine | 480 | 52 | 213 | 292 | Trace | Trace | 10 | 0 | 0 |
| *Staphylococcus lactis* | | | | | | | | | |
| Serine | 450 | 0 | 146 | 261 | 18 | 130 | 253 | 150 | 285 |
| Muramic acid | 338 | | | | | | | | |
| Glutamic acid | 450 | 0 | 0 | 0 | | | | 0 | |
| Glycine | 1422 | 0 | 858 | 1042 | 24 | 821 | 1131 | 825 | 1020 |
| Alanine | 927 | 0 | 28 | 93 | 18 | 43 | 102 | 23 | 105 |
| Glucosamine | 828 | | | | | | | | |
| Lysine | 472 | 10 | 389 | 441 | 11 | 12 | 10 | 0 | 0 |
| *Micrococcus lysodeikticus* | | | | | | | | | |
| Muramic acid | 543 | | | | | | | | |
| Glutamic acid | 642 | 0 | 0 | 0 | | | | 0 | 0 |
| Glycine | 691 | 0 | 86 | 214 | 380 | 416 | 397 | 98 | 225 |
| Alanine | 1284 | 0 | 2 | 12 | 42 | 49 | 40 | 2 | 12 |
| Glucosamine | 593 | | | | | | | | |
| Lysine | 543 | 390 | 417 | 419 | 7 | 10 | 10 | 0 | 0 |

are hydrolysed, 44% of the ε-amino groups of lysine are liberated and 30% of the glycine is converted into the free amino acid. After 100h more than 50% of the glycine is free; no di-DNP-lysine was detected and only small increases in free *N*- and free *C*-alanine were found between 42h and 100h (most of the free alanine comes from teichoic acid rather than peptidoglycan), showing that the glycyl peptide linkages are extensively hydrolysed but that the other peptide linkages are largely unaffected. Similarly with *S. lactis* most of the glycyl linkages are hydrolysed, as are the serine residues that are present in the peptidoglycan of this organism.

Thus it appears that the glycyl bonds in the cross-linkages of staphylococcal peptidoglycan (Tipper & Berman, 1969) are readily hydrolysed in dilute alkali, whereas other peptide linkages are much more stable. This explains the susceptibility towards alkali of staphylococcal peptidoglycan and the resistance of walls of bacilli and lactobacilli, the peptidoglycan of which is cross-linked other than through glycyl peptides. The results obtained with *M. lysodeikticus* show that here also glycine, present probably as a side chain attached to the α-carboxyl group of glutamic acid (Schleifer & Kandler, 1967), is liberated under the conditions used, but that few or no other peptide linkages are attacked and so the wall does not dissolve. In order to establish the reasons for the lability of the glycyl linkages in cell walls, the action of alkali on the synthetic peptides glycylglycylglycine and L-alanyl-L-alanyl-L-alanine was examined. Samples (0.25mmol) were dissolved in 2.5ml of water, and portions (1.25ml) were removed and mixed with water (1.25ml) as controls. To the remainder of each solution was added an equal volume of M-sodium hydroxide and the solutions were incubated at 25±0.5°C. Portions (0.25ml) of each mixture were removed at intervals, neutralized and analysed on the amino acid analyser. The control solutions of the tripeptides in water showed no detectable production of alanine or glycine. Triglycine was, however, fairly extensively hydrolysed in the dilute alkali, 1.4mol of glycine being produced from 1.0mol of the tripeptide in 60h. Only 0.14mol of alanine was formed in this time from 1.0mol of trialanine, showing that the triglycine is considerably more labile towards alkali. This lability is presumably due to steric and

possibly inductive effects, and appears to account adequately for the action of dilute alkali on staphylococcal cell walls.

The analytical results suggest that probably every cross-linkage is broken after dissolution of walls in alkali, but the nature of the dissolved peptidoglycan has not yet been examined in detail. We are not aware of relevant studies of the action of dilute alkali on model peptides, nor, with one exception (Sine & Mass, 1969), of relevant studies on the action of dilute alkali on the primary structure of proteins. It appears, however, from our present findings that glycyl linkages are particularly susceptible to hydrolysis in alkali, so that it might be expected that such linkages in proteins would be hydrolysed more rapidly than most of the other linkages.

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