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The Action of Hot Formamide on Bacterial Cell Walls

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1. The cell walls of *Corynebacterium tritici* contain much carbohydrate and their mucopeptide contains diaminobutyric acid instead of lysine or diaminopimelic acid. They are resistant to lysozyme. 2. The residue after extraction with hot formamide contains only about 10% less carbohydrate but is attacked by lysozyme. Lysozyme also slowly attacks cell walls treated with fluorodinitrobenzene and more rapidly cell walls that have been *N*-acetylated. 3. All these processes block the free γ-amino groups of diaminobutyric acid present in the untreated cell wall. Hot formamide introduces formyl groups, as shown by its ability to make formylglycine and diformyl-lysine under the same conditions. 4. *N*-Formyl groups are also introduced into the cell walls of *Micrococcus lysodeikticus* by hot formamide, but this change increases only slightly their already great sensitivity to lysozyme. *N*-Acetylation also increases sensitivity to lysozyme.

Fuller (1938) introduced the use of hot formamide for the extraction of antigens from dried bacteria. Recently the technique has been applied to isolated cell walls to remove polysaccharides from the structural mucopeptide component (McCarty, 1960; MacLennan, 1961; Heymann, Zeleznick & Manniello, 1961; Heymann, Manniello & Barkulis, 1963; Perkins, 1963). It has also been shown that treatment with hot formamide of cell walls, which were resistant to the action of lysozyme, yielded mucopeptide residues that the enzyme would attack (Krause & McCarty, 1961; Allsop & Work, 1963; Heymann, Manniello & Barkulis, 1964). The last-mentioned authors found that formamide at 170° introduced *O*-formyl groups into the cell walls of streptococci and that these groups had to be removed before lysozyme would act on the mucopeptide substrate. The connexion between sensitivity to lysozyme and the presence of *O*-acyl groups in the cell walls of some species is well known (Abrams, 1958; Brumfitt, Wardlaw & Park, 1958). The present work shows that extraction with hot formamide not only removes polysaccharides but also blocks free amino groups in the residual mucopeptide. Both of these actions may influence susceptibility to lysozyme.

METHODS

Cell walls. Bacteria were grown in shaken flask cultures and cell walls were prepared by the method of Cummins & Harris (1956). The organisms used were *Micrococcus lysodeikticus* N.C.T.C. 2665 [grown at 30° in a medium containing (per l.): NH_4Cl, 1g.; K_2HPO_4, 2g.; $MgSO_4,7H_2O$, 20mg.; L-glutamic acid, 3·6g.; DL-phenylalanine, 0·2g.; L-tyrosine 0·1g.; L-arginine, 0·65g.; sodium pyruvate, 6g.; biotin, 0·5mg.; inosine, 60mg.; this medium was brought to pH 7·6–7·8 and sterilized by autoclaving at a pressure of 10lb./in.^{2} for 15min.; $Fe(NH_4)_2(SO_4)_3$ (10ml. of a sterile solution at 2·5g./l.) was added before use] and *Corynebacterium tritici* N.C.P.P. 471 (grown at 25° in nutrient broth containing 1% of glucose added as a sterile solution). A specimen of the isolated cell walls of *Bacillus megaterium* (strain 7581) was kindly supplied by Dr E. Work.

Treatment of cell walls with formamide. The formamide used (British Drug Houses Ltd. laboratory reagent) gave a slight alkaline reaction and therefore did not contain any excess of formic acid. Formamide that had been dried over $MgSO_4$ and redistilled under reduced pressure blocked the free amino groups of the cell walls of *M. lysodeikticus* in exactly the same way.

In a typical experiment a sample of the cell walls of *C. tritici* (212mg.) was heated with 10ml. of formamide in an oil bath at 150° for 15min. The mixture was cooled and 25ml. of acid–ethanol was added (2N-HCl–ethanol; 1:19,v/v). The residue was recovered by centrifuging and washed with more acid–ethanol, with ethanol and with ether and dried *in vacuo* to give residue 1 (177mg.). The supernatant was treated with 10vol. of acetone and the resulting white precipitate was recovered by centrifuging. It was dissolved in water and dialysed repeatedly against water. The non-diffusible material was called supernatant 1.

Residue 1 was shaken vigorously with 10ml. of water, and the mixture centrifuged. The residue was again extracted with water and centrifuged, yielding supernatant 2 (77mg. after drying) and residue 2 (87mg. after drying).

Acetylation. Aqueous suspensions of cell walls were acetylated by shaking with acetic anhydride in the presence of $NaHCO_3$ as described by Heymann *et al.* (1964). *O*-Acyl groups were removed from these and other samples either

by standing at room temperature overnight with 0·01 N-NaOH, or by incubation at pH 10 for 1 hr. at 37°. The cell walls were recovered by centrifuging and washed successively with water, ethanol and ether before drying. When the acetylated cell walls of *C. tritici*, *M. lysodeikticus* and *B. megaterium* were treated with fluorodinitrobenzene, no yellow colour developed, showing that the free amino groups present in the original cell walls had been blocked. The carbohydrate content of the preparations was not changed by the acetylation procedure.

Treatment of amino acids with formamide. Samples of glycine or L-lysine monohydrochloride (1–4 g.) were heated with 10 vol. of formamide in an oil bath at 150° for 20 min. with occasional stirring. The amino acid soon dissolved to give a pale-yellow solution, becoming mid-brown. The mixture was cooled, diluted with 3 vol. of water and passed through a column (38 cm. × 2·1 cm.) of Dowex 1 anion-exchange resin (X8; analytical grade; Cl^- form; 200–400 mesh). The column was washed with 200 ml. of water to remove formamide and unchanged amino acid, and then with 500 ml. of 2 N-acetic acid, which eluted the product.

The acetic acid solution was evaporated to dryness *in vacuo* at a bath temperature not greater than 40°. The solid residue was redissolved in a minimum volume of hot water, decolorized with charcoal and allowed to crystallize. Assuming that the products were formylglycine and diformyllysine, the yield of crystals from glycine represented 47% and from lysine 27% of the theoretical value. The crude product was twice recrystallized from hot water.

Formylglycine. Authentic formylglycine was prepared by the reaction of glycine with formic acid and acetic anhydride at 5–15° (Sheehan & Yang, 1958).

Chromatography and electrophoresis. The *N*-formyl amino acids were separated from the parent amino acids by paper chromatography in the following solvents: *A*, butan-1-ol–acetic acid–water (63:10:23, by vol.); *B*, methanol–water–pyridine–conc. HCl (32:7:4:1, by vol.); *C*, methanol–water–pyridine (20:5:1, by vol.); *D*, propan-2-ol–acetic acid–water (67:10:23, by vol.); or by paper electrophoresis at pH 7·6 in 25 mM-phosphate buffer with applied potential 9 v/cm. Amino acids were detected with ninhydrin and their *N*-formyl derivatives by the method of Rydon & Smith (1952).

The ω-DNP derivatives of lysine and its homologues were separated by paper electrophoresis in 25 mM-Na_2CO_3. For quantitative purposes the spots were eluted with 0·5 N-HCl and the extinctions measured at 353 mμ.

Estimation of formyl groups. Formic acid was estimated by the following modification of the method of Grant (1948). Samples for estimation containing *N*-formyl groups were first hydrolysed to liberate formic acid. Preliminary experiments showed that hydrolysis in N-HCl in a bath at 100° for 30 min. was adequate. Then a suitable sample was diluted to 0·9 ml. with water and treated in the same way as the blank and standards containing 0·1–0·5 μmole of formic acid (freshly diluted from a concentrated standardized solution). A 15 cm. coil of Mg ribbon (i.e. about 150 mg.) was added to each sample in a 6 in. × ⅝ in. test tube, which was then placed in an ice bath. Nine 0·1 ml. portions of conc. HCl were added at not less than 1 min. intervals, and 1 min. after the last addition the tube was removed from the ice bath. The sample was transferred with a Pasteur pipette from the excess of Mg to a clean test tube, and the first tube was washed out with 0·2 ml. of water. Then 2 ml. of the acetylacetone reagent (0·2 ml. of redistilled acetylacetone, 15·0 g. of ammonium acetate and 0·3 ml. of acetic acid, made up to 100 ml. with water; Nash, 1953) was added and the mixture was heated in a water bath at 60° for 10 min. and then cooled. The extinctions at 412 mμ were read in 1 cm. cuvettes. Under these conditions the observed extinctions bore a linear relationship to the amount of formic acid present up to 1 μmole ($\epsilon = 2460$).

Reactions with fluorodinitrobenzene. Samples of cell walls and derived fractions were treated in the dark at room temperature with an excess of 1-fluoro-2,4-dinitrobenzene in a mixture of 1 vol. of aq. 1% (w/v) $NaHCO_3$ and 2 vol. of ethanol. After about 2–4 hr. the mixture was centrifuged and the residue washed successively with ethanol–water (2:1, v/v), ethanol and ether and dried.

A crystalline sample of 4-DNP-2,4-diaminobutyric acid hydrochloride (m.p. 234°, decomp.) was prepared from L-2,4-diaminobutyric acid dihydrochloride (Mann Research Laboratories, New York, N.Y., U.S.A.) as described for the preparation of ε-DNP-lysine by Porter & Sanger (1948).

Digestion with lysozyme. Samples of cell wall (about 8 mg.) were suspended in 7 ml. of 0·1 M-ammonium acetate buffer, pH 6, with lysozyme at a final concentration of 100 μg./ml. (*C. tritici*) or 2 μg./ml. (*M. lysodeikticus*), and incubated at 37°. The extinctions were read in an EEL colorimeter with a blue filter, or for DNP derivatives in a Unicam SP. 500 colorimeter at 750 mμ.

Analytical methods. Amino acids were estimated on paper chromatograms by a ninhydrin method (Mandelstam & Rogers, 1959). Amino sugars were estimated by the method of Rondle & Morgan (1955). Muramic acid was separated from glucosamine on a charcoal column, as described by Perkins & Rogers (1959) except that Norit A was replaced by Ultra-Sorb ZX (British Carbo-Norit Union, West Thurrock, Grays, Essex). This charcoal was washed thoroughly with 2 N-HCl, then repeatedly with water, the fine particles being discarded each time. Each column contained a mixture of 0·5 g. of charcoal and 0·5 g. of acid-washed Celite no. 545; 12 ml. of water eluted the glucosamine, and 10 ml. of aq. 15% (v/v) ethanol eluted with muramic acid. A measure of the carbohydrate content of cell-wall samples was obtained by using the phenol–H_2SO_4 method of Dubois, Gilles, Hamilton, Rebers & Smith (1956), with glucose as a reference standard.

Elementary analyses. These were performed by Dr F. Pascher, Bonn, Germany.

RESULTS

The cell walls used in the present investigation were chiefly those of the Gram-positive bacteria *M. lysodeikticus* and *C. tritici*. Previous studies on *M. lysodeikticus* have shown that its cell walls consist of mucopeptide containing the *N*-acetyl derivatives of glucosamine and muramic acid (3-*O*-carboxyethylglucosamine) and the amino acids lysine, glycine, glutamic acid and alanine, together with a polysaccharide composed of glucose and 2-acetamidomannuronic acid (Czerkawski, Perkins & Rogers, 1963; Perkins, 1963). About 60–70% of the lysine residues have free ε-amino groups (Ingram & Salton, 1957; Perkins, 1963).

The cell walls of *C. tritici* contain a large amount

of carbohydrate and a mucopeptide consisting of the *N*-acetyl derivatives of glucosamine and muramic acid and the amino acids alanine, glycine, glutamic acid and 2,4-diaminobutyric acid (Perkins & Cummins, 1964). The latter diamino acid replaces the $\alpha\epsilon$-diaminopimelic acid or lysine that occur in the mucopeptides of most bacterial cell walls. The isolated cell walls are attacked by lysozyme hardly at all (Fig. 1).

Preparations of cell walls from *C. tritici* were extracted with hot formamide and acid–alcohol was added. Extraction of the residue with water yielded a soluble fraction (supernatant 2) consisting of carbohydrate (mainly glucose, mannose and glucuronic acid) with only traces of amino compounds. The residue (residue 2) still contained much carbohydrate measured as glucose by the phenol–sulphuric acid reaction (86% by weight as anhydroglucose, compared with 97% for the unextracted cell walls). Nevertheless, this residue, unlike the unextracted cell walls, was attacked by lysozyme (Fig. 1). After the enzyme (100 μg./ml.) had acted overnight, 51% by weight of the original sample remained insoluble (residue 3), and virtually all the dissolved material proved to be non-diffusible through cellophan (supernatant 3). Carbohydrate was found in both fractions (residue 3, 95% as anhydroglucose; supernatant 3, 57%).

As mentioned above, the cell walls of *C. tritici* contain diaminobutyric acid, of which the γ-amino groups might be involved in cross-linking. Samples of cell walls were treated with fluorodinitrobenzene and the product was hydrolysed at 105° in 4N-hydrochloric acid for 4hr., conditions shown to give the best yield of DNP-amino acid. There were virtually no ether-extractable DNP-amino acids, and only γ-DNP-diaminobutyric acid was found in the aqueous layer. Measurement of the amount of unchanged diaminobutyric acid and its γ-DNP derivative in hydrolysates showed that about half had not reacted with fluorodinitrobenzene and was therefore presumably involved in cross-linking (moles of diaminobutyric acid/10^4g. in untreated cell wall, 2·5; in cell walls after dinitrophenylation, diaminobutyric acid, 1·3, γ-DNP-diaminobutyric acid, 1·2).

The samples of cell wall that had been treated with fluorodinitrobenzene were slowly attacked by lysozyme (Fig. 2). Control samples that had been treated in the same way with sodium hydrogen carbonate solution followed by ethanol and ether remained unattacked. Hence it appeared that substitution of the free γ-amino groups of diamino-

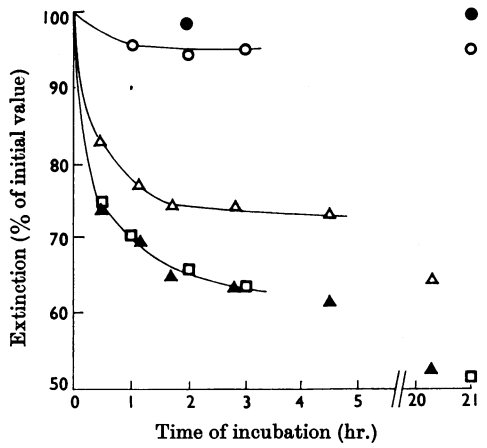

Fig. 1. A sample of the cell walls of *C. tritici* was heated with formamide at 150° for 15min. and acid–alcohol was added. The residue was extracted twice with water and then dried. Some of this residue was extracted with alkali to remove *O*-acyl groups. Another sample was treated with an excess of fluorodinitrobenzene in aq. ethanol made alkaline with $NaHCO_3$, then washed and dried. A further sample was acetylated with acetic anhydride in aq. $NaHCO_3$, and *O*-acyl groups were removed by incubation at pH10. A control sample of cell walls was extracted with alkali without other treatment. The various preparations were suspended in 0·1M-ammonium acetate buffer, pH6, and incubated at 37° with lysozyme (100 μg./ml.). Turbidities were measured in an EEL spectrophotometer. ○, Untreated cell walls; △, formamide residue; ▲, formamide residue, alkali-extracted; □, acetylated and alkali-extracted; ●, alkali-extracted.

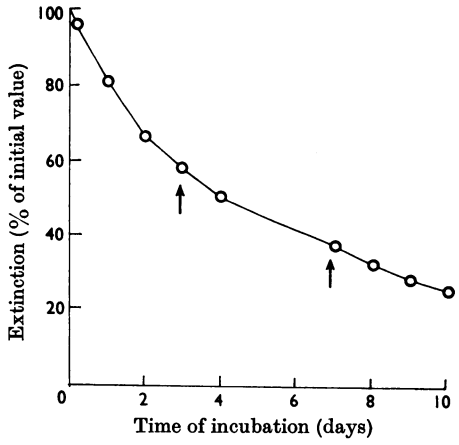

Fig. 2. Cell walls of *C. tritici* were treated with fluorodinitrobenzene. The product was suspended in 0·1M-ammonium acetate buffer, pH6, and incubated at 37° in the presence of lysozyme (100 μg./ml.). The extinction of the suspension was read in a Unicam SP. 500 spectrophotometer at 750 mμ. At the points marked by arrows the residue was centrifuged down and resuspended with fresh buffer and enzyme. Chloroform was present throughout to inhibit bacterial contamination.

butyric acid in the cell walls had induced some susceptibility to lysozyme.

The supernatant 1 and residue 2 from treatment with hot formamide of the cell walls of *C. tritici* were allowed to react with fluorodinitrobenzene. Very little yellow coloration occurred and acid hydrolysis and isolation of the products showed that no γ-DNP-diaminobutyric acid had been formed. Since there were no amino acids in the water-soluble material (supernatant 2) it was evident that the treatment with formamide had blocked the free amino groups found in the intact cell walls. Cell walls of *M. lysodeikticus* and *B. megaterium*, which are known to contain free ϵ-amino groups respectively of lysine and diaminopimelic acid (Salton, 1961), were treated in the same way. Again, the treatment with hot formamide caused a complete 'masking' of the free amino groups.

Free amino acids were also heated with formamide under similar conditions. Glycine passed into solution to give a product that was examined by paper electrophoresis at pH 7·6. This compound did not react with ninhydrin but gave the Rydon & Smith (1952) reaction for amide linkages and moved towards the anode. A large sample was prepared and recrystallized twice from hot water. When observed on the hot-stage microscope (Kofler) the crystals underwent a change in crystal habit to form long needles at 125–135° and finally melted at 148–149°. Authentic formylglycine showed exactly the same change in crystal habit and melting point, contrasting with the literature value of 153–154° (decomp.) (Fischer & Warburg, 1905). Repeated recrystallization and careful drying did not raise the melting point of either sample, and the mixed melting point was undepressed (Found: compound from reaction of formamide and glycine, C, 34·9; H, 5·1; N, 13·7%; neutral equiv., 102·8; authentic formylglycine, C, 35·2; H, 5·0; N, 13·8%; neutral equiv., 102·6. Calc. for formylglycine: C, 35·0; H, 4·9; N, 13·6%; neutral equiv., 103·1). The unknown and authentic compounds were found to be identical by paper chromatography in several solvent systems (solvent *A*, R_F 0·51; solvent *B*, R_F 0·73; solvent *D*, R_F 0·78). When the compound from the reaction of formamide and glycine was hydrolysed in N-hydrochloric acid at 100° for 30 min. free glycine reappeared. The liberated formic acid was estimated, the yield being 102% of the theoretical value. It is concluded that the compound was formylglycine.

L-Lysine monohydrochloride was also heated with formamide and the acidic product isolated in the same way. It melted at 128–130° with no sign of the change in crystal form seen with formylglycine (Found: C, 47·2; H, 6·9; N, 13·7%; neutral equiv., 202·5. Calc. for diformyl-lysine: C, 47·5; H, 7·0; N, 13·9%; neutral equiv., 202·2). The yield of formic acid on hydrolysis was 104% of the theoretical value. It therefore seems likely that both amino groups of lysine were substituted by heating with formamide to yield 2,6-diformyl-lysine.

In view of these results, the free amino groups of the cell-wall samples were probably also substituted with formyl groups. Samples of the residues obtained from cell walls after heating with formamide, with and without extraction with alkali to remove *O*-formyl groups, were hydrolysed and the liberated formic acid was estimated. Table 1 shows that in the treated cell walls of *M. lysodeikticus* the *N*-formyl content corresponded to 57% of the total lysine, which is similar to the value found for substitution with DNP groups (Perkins, 1963). The *N*-formyl

Table 1. *Analysis of cell-wall fractions*

Cell-wall samples were heated with formamide at 150° for 15 min. The residue obtained after the addition of acid–alcohol was extracted with water.

	Composition (moles/10^4 g. of dried sample)			
	M. lysodeikticus		*C. tritici*	
Substance	Untreated* wall	Formamide residue (prep. 101)	Untreated wall	Formamide residue
Glucosamine	9·6	7·7	1·6	0·9
Muramic acid	7·5	5·7	1·0	0·3
Alanine	19·0	20·2	1·5	1·4
Glutamic acid	9·9	10·9	2·2	2·4
Glycine	10·8	9·3	3·0	3·1
Lysine	10·1	9·7	—	—
Diaminobutyric acid	—	—	2·8	4·5
Formyl (total)	—	6·0	—	4·0
N-Formyl	—	5·5	—	2·2

* Results from Czerkawski *et al.* (1963).

content of the treated cell walls of *C. tritici* was equivalent to 49% of the total diaminobutyric acid, corresponding closely to the proportion found as DNP-diaminobutyric acid (48%). The values for amounts of amino acids in the residue from the cell walls of *M. lysodeikticus* are higher than reported by Perkins (1963). It seems probable that in the present work the water-washing introduced after the addition of acid–alcohol gave a purer preparation, since the former analysis give a recovery of only 55% compared with 80% in the present work.

Since the free amino groups of cell walls had been shown to be blocked in samples that had been made sensitive to lysozyme, the effect of acetylating these groups was also studied. Heymann *et al.* (1961, 1964) had shown that heating streptococcal cell walls with formamide at 170° for 1hr. introduced many *O*-formyl groups, which inhibited the action of lysozyme. Therefore the residues of the cell walls of *M. lysodeikticus* and *C. tritici* after heating with formamide were treated with dilute sodium hydroxide solution to remove *O*-acyl groups before they were tested for susceptibility to the action of lysozyme. Samples of untreated cell walls, walls treated with formamide with and without subsequent extraction with alkali and walls acetylated and then extracted with alkali were exposed to the action of lysozyme. The observed decreases in turbidity are shown in Fig. 1 (*C. tritici*), Fig. 3 (*M. lysodeikticus*) and Fig. 4 (*B. megaterium*).

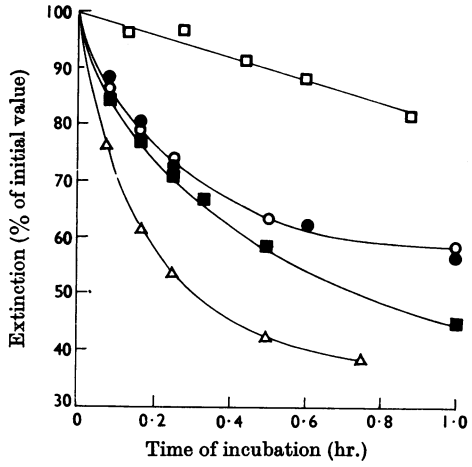

Fig. 3. Cell walls of *M. lysodeikticus* (prep. 113) were heated with formamide at 150° for 15 min. and acid–alcohol was added. The residue was extracted twice with water and then dried. Some of this residue was extracted with alkali to remove *O*-acyl groups. Further samples of the same original wall preparation were: (*a*) alkali-extracted, (*b*) dinitrophenylated and (*c*) acetylated and then alkali-extracted. Portions of all these products were suspended in 0·1M-ammonium acetate buffer, pH6, and incubated at 37° with lysozyme (2μg./ml.). Turbidities were measured in an EEL colorimeter. ○, Untreated cell walls; ■, formamide residue, alkali-extracted (the formamide residue without alkali extraction gave results so similar that the curve is omitted for the sake of clarity); ●, alkali-extracted; △, acetylated and alkali-extracted; □, dinitrophenylated.

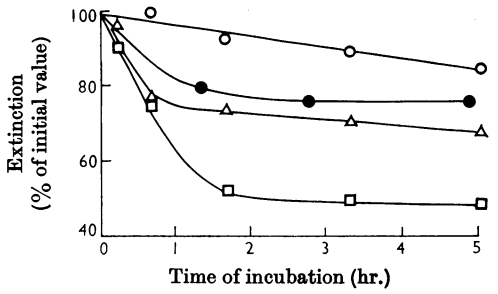

Fig. 4. Cell walls of *B. megaterium* strain 7581 were (*a*) extracted with hot formamide, (*b*) acetylated and alkali-extracted, and (*c*) alkali-extracted as described for *C. tritici* in the legend to Fig. 1. Samples were suspended in 0·1M-ammonium acetate buffer, pH6, and incubated at 37° with lysozyme (100μg./ml.). The extinctions of the suspensions were read in a Unicam spectrophotometer. ○, Untreated cell walls; △, acetylated and alkali-extracted; □, formamide residue, not alkali-extracted; ●, alkali-extracted.

Table 2. *Effect of acetylation on sensitivity of the cell walls of* M. lysodeikticus *to lysozyme*

Samples of various preparations of cell walls before and after acetylation and alkali extraction were suspended in 0·1M-ammonium acetate buffer, pH6, and incubated at 37° with lysozyme (2μg./ml.).

Cell wall prep. no.	Glucose content (moles/10^4g.)	Extinction after 45min. (% of initial value): Untreated cell walls	Extinction after 45min. (% of initial value): *N*-Acetylated cell walls
104	4·4	60	32
105	5·8	42	32
109	4·9	49	35
113	7·0	58	38

The sensitivity of the cell walls of *C. tritici* to lysozyme was unaffected by extraction with alkali, showing that *O*-acyl groups did not cause their resistance to the enzyme. Extraction with formamide yielded a residue that was attacked by lysozyme, and this sensitivity was further increased by the removal of *O*-formyl groups. Acetylation, which also blocked the free amino groups as shown by the unreactivity of the product towards fluorodinitrobenzene, produced a similar degree of sensitivity to lysozyme.

The cell walls of *M. lysodeikticus* are, of course, very readily attacked by lysozyme. Extraction with alkali did not affect their susceptibility to the enzyme, and extraction with formamide produced only a slight increase. In the particular preparation of cell walls used in this experiment (Fig. 3), hot formamide had not introduced any *O*-formyl groups, since the observed formyl content was the same before and after treatment with alkali. Correspondingly, there was no difference in the sensitivity to lysozyme of these two samples. A different preparation of the cell walls contained after treatment with hot formamide a small proportion of *O*-formyl groups (Table 1). This residue was a little more resistant to lysozyme than the untreated cell walls and became slightly more sensitive than the original walls only after removal of the *O*-formyl groups with alkali. As shown in Fig. 3, acetylation of the free amino groups in the cell walls of *M. lysodeikticus* produced an even greater susceptibility to lysozyme, whereas dinitrophenylation decreased it. A number of different preparations of the cell walls of *M. lysodeikticus* were *N*-acetylated and tested for sensitivity to lysozyme. Acetylation always increased their sensitivity but to varying extents not related to the carbohydrate content (Table 2).

The cell walls of *B. megaterium* showed a somewhat similar response to acetylation or to the action of hot formamide (Fig. 4). Once more these reagents blocked the free amino groups and increased sensitivity to lysozyme. However, treatment with alkali alone produced some effect, so that the natural resistance of the cell walls of this strain of *B. megaterium* to lysozyme was in part due to the presence of alkali-labile (probably *O*-acyl) groups.

DISCUSSION

Hot formamide has often been used to isolate polysaccharide antigens from bacteria and to separate polysaccharides from the muco peptide of isolated bacterial cell walls. It is clear from the present work that if these walls have free amino groups, as many do (Salton, 1961), then heating with formamide may convert these groups into their formyl derivatives, thus modifying the chemical composition and charge of the residual cell-wall material. Stegemann (1960) studied the effect of heating gelatin with formamide at 135° for 24hr. and found that about one-quarter of the formamide molecules incorporated had 'formamidolysed' the chain. It seems possible that here, too, the ϵ-amino groups of lysine and hydroxylysine had been formylated, but this point was not examined.

The effect of treatment with formamide on the susceptibility of bacterial cell walls to the action of lysozyme is complex. In some species, removal of polysaccharide may allow the enzyme to attack its substrate (Krause & McCarty, 1961; Allsop & Work, 1963), although sometimes *O*-formyl groups introduced by the reagent must first be removed (Heymann *et al.* 1964). The present work suggests that the blocking of free amino groups may also facilitate lysozyme action, perhaps by favouring the approach of the very basic enzyme.

The cell walls of *M. lysodeikticus*, which are highly susceptible to the action of lysozyme, contain a large number of free amino groups, and blocking of these amino groups by treatment with formamide or acetylation enhances lysozyme action. However, with these cell walls, introduction of the bulky dinitrophenyl group partially inhibits the action of the enzyme. In contrast with these results, Allsop & Work (1963) found that extraction of the cell walls of propionibacteria with formamide produced a lysozyme soluble residue without apparently blocking the few end amino groups present. Evidently, 'masking' of free amino groups is only one factor to be considered in understanding why hot formamide makes some cell walls sensitive to lysozyme.

Krause & McCarty (1961) found that extraction of the cell walls of streptococci with formamide caused some destruction of amino sugars. The analytical values given in the present work also suggest that amino sugars may have been destroyed in the cell walls of *C. tritici*, although, of course, it remains possible that they were preferentially extracted by the formamide.

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