The Products of the Partial Acid Hydrolysis of the Mucopeptide from Cell Walls of *Micrococcus lysodeikticus*

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Salton & Horne (1951) and McCarty (1952*a, b*) isolated insoluble material from disintegrated Gram-positive bacterial cells, which was shown by electron-microscopic examination to have retained the form of the cells from which it had been isolated, and both sets of authors concluded that they had isolated the cell walls. The procedure involved disintegrating the cells by shaking a thick suspension with fine glass beads, separating the insoluble walls from unbroken cells and soluble cell contents by differential centrifuging and then repeatedly washing the wall material with water or sodium chloride solution. Enzyme preparations which would lyse the living organisms also lysed cell walls (McCarty, 1952*a*; Salton, 1952, 1956).

Subsequently a somewhat different method was described (Cummins & Harris, 1956) for preparing wall material. This method replaced the repeated washing procedure of Salton & Horne (1951) by treatment with the enzymes trypsin, ribonuclease and pepsin. Qualitative chemical examination (Salton, 1953; Cummins & Harris, 1956) of acid hydrolysates of wall material isolated by either method showed that it had a rather simple composition. When obtained from most species it yielded four or five amino acids, some in the D-configuration (Ikawa & Snell, 1956; Snell, Radin & Ikawa, 1955), one or two hexoses and two hexosamines. It is proposed to call the material mucopeptide. The two hexosamines usually present are glucosamine and its 3-*O*- α -carboxyethyl derivative. The latter was first recognized as an unknown amino sugar universally present in mucopeptide by Cummins & Harris (1956), and was more exactly characterized by Strange & Dark (1956); it has since been synthesized by Kent (1958) (see also Strange & Kent, 1959).

Although the earlier authors thought the material we propose to call mucopeptide constituted the whole cell wall of the organism, later work has somewhat complicated this picture. For example, Baddiley, Buchanan & Carss (1958) and Armstrong, Baddiley, Buchanan & Carss (1958) have shown that a polyribitol phosphate with combined amino acids in it can be extracted from cell-wall preparations from some species. The exact inter-relationship of the mucopeptide and these other substances

is not clear. It seems likely, however, that the mucopeptide does not represent the whole cell wall in some species. Nevertheless, observations such as those of Park (1958) and Mandelstam & Rogers (1959) show that antibiotics such as penicillin and bacitracin inhibit its synthesis, and other strongly suggestive observations (Park & Johnson, 1949; Park, 1952*a, b*; Park & Strominger, 1957) have pointed to the importance of the inhibition of mucopeptide synthesis as a mechanism of action of some antibiotics.

Apart from investigations of the action of antibiotics, the mucopeptides themselves have great intrinsic interest in that they represent examples of substances containing only a limited number of both sugars and amino acids, yet, apparently, bearing considerable immunological specificity (Hundley, Ing & Krauss, 1956). Elucidation of their chemistry may help towards a further understanding of the more complicated mucoproteins.

A preliminary account of some of this work has already appeared (Perkins & Rogers, 1958).

MATERIALS AND METHODS

Growth of organisms and preparation of mucopeptide

Micrococcus lysodeikticus (National Collection of Type Cultures no. 2665) was grown on the surface of Hedley Wright (Wright, 1933) nutrient agar in Roux bottles for 48 hr. at 35°. To obtain large yields of organisms it was necessary to dry the agar thoroughly before inoculation. At the end of the period of incubation the cells were scraped from the surface of the medium with a glass rod, suspended in water (approx. 100 ml. for cells from 20 Roux bottles), washed twice with an equal volume of water and finally suspended in water to give a density of 20 mg. dry wt./ml. This suspension in 10 ml. volumes was mixed with an equal volume of glass beads and shaken on a Mickle (1948) disintegrator for 15 min. at 0–4°. The beads were removed by filtering the suspension through a Grade 1 sintered-glass filter and washed. The combined filtrate and washings were then centrifuged at 1100 g for 10 min. to sediment the unbroken cells. Some mucopeptide is also sedimented during this process, but it can be separated from the whole cells by carefully scraping the pellet with a spatula, the mucopeptide being distinguished from the whole cells by its white colour; the whole cells are bright yellow. The supernatant from this centrifuging was then

centrifuged again at 11 000 g for 10 min., during which the mucopeptide was sedimented. The deposit was washed twice with water and treated with trypsin, ribonuclease and pepsin as described by Cummins & Harris (1956). Finally the mucopeptide was washed twice with water and dried from the frozen state. Before analysis the preparation was heated to constant weight at 105°.

Analytical methods

Paper chromatography. Descending chromatography on Whatman no. 3 paper was used throughout the work. The solvent systems found most useful were butanol-acetic acid-water (6:3:1.0:2.7, by vol.) and butanol-pyridine-water (6:4:3, by vol.; Jeanes, Wise & Dimler, 1951); for the latter it is essential that the pyridine should be freshly redistilled. The papers were subsequently sprayed with 0.1% ninhydrin in butanol for the qualitative examination of amino acids. For sugars, either the silver nitrate method of Trevelyan, Procter & Harrison (1950) was used, or the aniline phthalate spray of Partridge (1949). The amino sugar spots were made visible with the acetyl acetone and *p*-dimethylaminobenzaldehyde reagents of Partridge (1948).

Purification of paper. When substances were to be eluted from paper for subsequent analysis it was essential to use paper which had been washed. Whatman no. 3 paper initially containing low levels of peptide material was washed in *N*-HCl and then extensively with water until free of Cl⁻ ions. By this procedure the amount of elutable peptide in most batches of paper was reduced to insignificant quantities. Some batches required more vigorous treatment, such as that described by Hanes, Hird & Isherwood (1952).

Paper electrophoresis. The apparatus described by Kunkel (1954) was used with a potential difference of 700 v, usually applied for 3–4 hr. The following volatile buffers were found most useful: (1) *N*-acetic acid, pH 2.37; (2) triethylamine carbonate, pH 8.0 (Porath, 1956); (3) collidine-acetic acid (Newton & Abraham, 1954).

Estimation of amino acids. The method of Kay, Harris & Entenman (1956) was as modified by Mandelstam & Rogers (1959).

Estimation of hexosamines. In preliminary investigations the hexosamines liberated from the cell walls were estimated together as glucosamine by the method of Elson & Morgan (1933). *N*-Acetyl amino sugars were estimated by the method of Reissig, Strominger & Leloir (1955). Estimations of glucosamine and muramic acid separately were made as described below.

Separation of hexosamines. After hydrolysis of the samples in 4*N*-HCl for 4 hr. at 105° the glucosamine and muramic acid were separated by the method of J. T. Park (1957, personal communication). A column (5 mm. in diameter) of a mixture of Norit A (0.25 g.) and Celite (Johns-Manville Co. Ltd., London) (coarse, acid-washed, 0.25 g.) was washed with 2*N*-HCl. The hydrolysate was diluted to 2*N* with respect to HCl and a sample equivalent to about 4 μmoles of total hexosamine (measured as glucosamine) was added to the column. Elution with 10 ml. of water yielded the glucosamine and a further 10 ml. of 5% (v/v) ethanol in water eluted the muramic acid. That separation of the two amino sugars was complete was proved in the first experiments by paper-chromatographic examination of the eluates. The hexosamines were estimated in samples of suitable volume by the method of Rondle & Morgan (1955), recrystallized glucosamine

hydrochloride being used as a standard. The concentration of muramic acid was calculated on the assumption that, under the above-mentioned conditions, 1 mg. of muramic acid produced 27% of the colour at 530 mμ given by 1 mg. of the free base, glucosamine (Crumpton, 1958).

Terminal amino groups in peptide. Peptides dissolved in dilute NaHCO₃ solutions were treated with an ethanolic solution of fluorodinitrobenzene (Sanger, 1945) for 2 hr. at room temperature. After removal of the excess of reagent by extraction with benzene the product was hydrolysed in 4*N*-HCl for 4 hr., at which time the destruction of dinitrophenyl amino acids, even dinitrophenyl glycine, is slight (Porter, 1957). The dinitrophenyl amino acids were separated by two-dimensional paper chromatography with (a) the buffered *tert*-amyl alcohol (2-methylbutan-2-ol) solvent of Blackburn & Lowther (1951) and (b) the phosphate buffer of Levy (1954). All five dinitrophenyl amino acids and the two amino sugar derivatives could be separated by this means.

α -Amino nitrogen. This was estimated by the method of Yemm & Cocking (1955).

Glucose. This was estimated by first passing the neutralized hydrolysates (equivalent to 5–10 mg. of cell wall), obtained by heating for 2 hr. with 2*N*-H₂SO₄ at 100° (these conditions gave maximum liberation of reducing sugar), through a column (8.5 cm. × 1.1 cm.) of Zeo-Karb 225 resin (H⁺ form). The column was then washed with water (20 ml.). During this process the amino sugars and amino acids, which otherwise also react in reducing-sugar tests, are retained on the column. Examination of the effluent and washings from a column, to which cell-wall hydrolysate had been applied, by paper chromatography showed that only glucose was present. Reducing sugar in the effluent and washings was estimated by the method of King (1947). *N*-Acetylglucosamine was present in certain fractions and this substance is also not retained by the resin; its amount was estimated independently and its reducing equivalent subtracted from the total value determined.

Total phosphorus. This was estimated by the modification of the Briggs method described by Allen (1940).

Incubation with lysozyme. Samples were incubated with a final concentration of 50 μg. of crystalline lysozyme/ml. (Armour Laboratories Ltd., Eastbourne, Sussex) in 0.1*M*-ammonium acetate buffer, pH 6.1, at 37° for periods up to 20 hr.

Incubation with carboxypeptidase. Samples of some of the fragments obtained by partial hydrolysis of mucopeptide were incubated with carboxypeptidase (Armour Laboratories Ltd.) essentially as described by Fraenkel-Conrat, Harris & Levy (1955), and the digest was examined quantitatively for amino acids by paper chromatography. The number of moles of enzyme used was either one-fiftieth or one-tenth of the number of moles of glycine present in the sample studied, and incubation was carried out for various periods either at room temperature or at 37°.

RESULTS

Quantitative analysis of the cell wall

Comparatively little quantitative knowledge about the mucopeptide of Gram-positive cell walls exists and, in view of our eventual aim of understanding the structure, it was necessary that more

Table 1. *Quantitative analysis of the cell walls of Micrococcus lysodeikticus*

Two separate batches (C.W. 18 and C.W. 20) of cell wall were analysed. All results are expressed as the basis of wall dried to constant weight at 105°. Optical rotations of the amino acids were not determined.

	C.W. 18		C.W. 20	
	%	Molar ratio*	%	Molar ratio*
Alanine	20.2	2.5	16.3	1.9
Glycine	10.8	1.6	8.5	1.2
Lysine	13.1	1.0	13.3	0.95
Glutamic acid	13.0	1.0	14.1	1.0
Glucosamine	10.1	0.64	12.8	0.75
(As <i>N</i> -acetylglucosamine)	12.4	—	15.8	—
Muramic acid	19.8	0.89	22.0	0.92
(As <i>N</i> -acetylmuramic acid)	23.1	—	25.7	—
Glucose	4.1	0.31	4.0	0.28

* Expressed in relation to glutamic acid.

precise information should be obtained. Table 1 shows two sets of results for mucopeptide samples. In both instances about 97 % of the dry matter can be accounted for in terms of the five amino acids, two *N*-acetylated amino sugars and glucose. In other batches analysed the total accounted for was never less than 94 %. This calculation assumes that all the glucosamine and muramic acid present are in the *N*-acetylated form: it does not, however, take account of the *O*-acetyl which is present (Abrams, 1958; Brumfitt, Wardlaw & Park, 1958). The difference between this total result and 100 % is within the experimental error and suggests that if other substances, not estimated, are present their concentration must be low. Despite the relative agreement between the values for the total weight accounted for, discrepancies between the values for the individual components fall outside the experimental error. The sets of results shown in Table 1 were selected from a number of analyses to show the greatest extent of this variation. For example, the results for alanine differ by 25 % and those for glycine by 20–30 %. These differences are similar to those obtained during the examination of batches of mucopeptide from *Staphylococcus aureus* (Mandelstam & Rogers, 1959). Such results may suggest the possible heterogeneity of the mucopeptides.

Measurement of the total phosphorus content of the wall mucopeptide gave a result of 0.1 %, which would correspond to a polyribitol phosphate content of about 0.7 %.

Course of acid hydrolysis of the mucopeptide

In the first experiments mucopeptide (7 mg.) was mixed with hot (100°, 2 ml.) 2*N*-H₂SO₄ and heated in a boiling-water bath. At the times indicated in Figs. 1 and 2 samples were withdrawn, cooled rapidly to 0–4° and neutralized with solid BaCO₃. The precipitated BaSO₄ and any excess of BaCO₃ were removed by centrifuging, washed with an

equal volume of water and the combined washings and supernatant fluid analysed. It can be seen from Fig. 1 that glucose was rapidly liberated, about 70 % appearing after 5 min. and the remainder after a further 40–50 min. Material in the hydrolysate which reacted to give colour in the *N*-acetylhexosamine test rose to a maximum in 20 min. and then rapidly declined. Not all this material was free *N*-acetylhexosamine, however, since when the hydrolysate was passed through a Zeo-Karb 225 resin (H⁺ form) column, about 20 % of it was retained. This material could be eluted with 0.3*N*-HCl (Fig. 1). As the hydrolysis proceeded the amount of hexosamine-reacting substance, as distinct from the *N*-acetylated sugar, increased rapidly for the first hour and then remained approximately constant during the second hour of treatment. When the amount of hexosamine which was retained by the column but eluted by 0.3*N*-HCl was measured it rose steadily throughout the hydrolysis period of 2 hr. Some, however, which behaved very differently in its rate of elution by 0.3*N*-HCl, was clearly not free amino sugar. This material is studied in detail below. Fig. 2 shows the rate of liberation of free amino nitrogen.

Since the purpose was to study compounds containing both amino acids and amino sugars, it seemed reasonable to choose a time of hydrolysis which would leave the maximum amount of hexosamine or *N*-acetylhexosamine in some form different from the free amino sugars. The difference in behaviour on ion-exchange columns seemed a reasonable criterion and at 20 min. the concentration of such compounds was at a maximum. At this time about 80 % of the glucose was present as free hexose but only about 15–20 % of the total amino nitrogen had been liberated.

Attempts to replace 2*N*-H₂SO₄ as a hydrolytic agent by other reagents were for various reasons unsuccessful. Among those tried were 2*N*-acetic acid, 0.1*N*-HCl, 0.1*N*-H₂SO₄ and *N*-NaOH, all at

100°, and 10N-HCl at 37°. Attention was therefore directed towards the 20 min. hydrolysate in which 2N-H₂SO₄ had been used.

Fractionation of the hydrolysate

The neutralized (BaCO₃) hydrolysates were dried from the frozen state and then treated as follows.

Dialysis. The dry material was dissolved in a small volume of water and dialysed three times with agitation against 10 vol. of water, each time

overnight at 0–4°. Of the dry weight in the hydrolysate 95% was diffusible under these conditions and this diffusate was dried from the frozen state. The non-diffusible part was shown to contain all the components of the cell wall but was not examined further.

Column chromatography. A solution in water containing diffusible material from 500 mg. of mucopeptide was applied to a column (25 cm. × 1.3 cm.) of Dowex-50 (H⁺ form, 8% cross-linked). Water was passed through the column and 2.3 ml. fractions were collected. Fractions 3–9 contained reducing material and were concentrated *in vacuo* before examination by paper chromatography. After about 70 ml. of water had passed through the column further material was eluted with 0.3N-HCl and the fractions were examined for *N*-acetylhexosamine and for hexosamine. A single peak was present in the fractions 16–30. The yield of dry material was 17 mg. The next peak eluted by 0.3N-HCl contained free glucosamine and muramic acid.

Paper chromatography in butanol-acetic acid-water. (a) Material not retained by Dowex-50. A solution of the material was applied as a broad band to the washed paper, which was irrigated with the solvent for 12 hr. When sprayed for amino sugars, *N*-acetylglucosamine and *N*-acetylmuramic acid were shown to be present, together with an intermediate band ($R_{\text{glucose}} 2.4$), which also stained purple and which on acid hydrolysis yielded both amino sugars. This material was further purified by a second chromatographic run with the same solvent system when further small quantities of the free *N*-acetylated amino sugars were removed from it.

(b) Bound hexosamine material eluted from Dowex-50 by 0.3N-HCl. Also by chromatography with butanol-acetic acid-water, this material was separated after 4 hr. into three bands which stained reddish purple with Elson-Morgan reagent together with a small amount of material which did not move from the origin. On hydrolysis all three bands which moved were shown to contain both amino acids and amino sugars but only the slowest band contained glucose.

Paper electrophoresis. Materials in the bands on the paper chromatograms were eluted with water and, after concentration of the solutions *in vacuo*, examined by paper electrophoresis.

Oligosaccharide fraction

This fraction (A in Fig. 3) was present along with the free *N*-acetyl amino sugars and the glucose. The behaviour on the column together with the reaction given in the Reissig *et al.* (1955) *N*-acetylglucosamine test have been taken to indicate that at least part of the amino sugars present is acetylated. Table 2 shows the analytical results obtained

Fig. 1. Course of acid hydrolysis of mucopeptide with 2N-H₂SO₄ at 100°. ○, *N*-Acetylhexosamine expressed in terms of *N*-acetylglucosamine; □, hexosamine, and ●, bound hexosamine, both expressed in terms of glucosamine; △, glucose.

Fig. 2. Course of liberation of α-amino nitrogen from mucopeptide during hydrolysis with 2N-H₂SO₄ at 100°.

Fig. 3. Fractionation of partial acid hydrolysates of mucopeptide from *M. lysodeikticus*. Compositions of the solvent used for paper chromatography and of the buffers used for paper electrophoresis are given in the Materials and Methods section.

Table 2. Composition of two oligosaccharide fractions isolated by the methods given in the text (A in Fig. 3)

Hydrolysis was for 4 hr. with 4N-HCl at 100°. Results are expressed relative to glucosamine. (a) and (b) represent two separately isolated batches.

	Amount (μmoles)	
	(a)	(b)
Glucosamine	1.0	1.0
Muramic acid	2.1	2.5
Glycine	0.17	0.20
Alanine	0.08	0.08
Glutamic acid	Trace	0.12
Apparent <i>N</i> -acetylhexosamine*	— †	1.50

* *N*-Acetylhexosamine measured on the unhydrolysed material by the Reissig, Strominger & Leloir (1955) test. Result is expressed in terms of *N*-acetylglucosamine.

† Not examined.

after acid hydrolysis of the fraction. The colour given by batch (b) in the *N*-acetylhexosamine test was equivalent to about 1.5 μmoles of *N*-acetylglucosamine compared with 1.0 μmole of glucosamine and 2.5 μmoles of muramic acid. The exact significance of this result is difficult to interpret owing to lack of knowledge of the effects of substitution on the intensity of colour given in the *N*-acetylhexosamine test by *N*-acetylmuramic acid. It does, however, suggest that a considerable proportion of one of the acetylhexosamines has a free C-1 position.

In both preparations small amounts of amino acids are also present. These, however, are very small and vary considerably between the two batches. It is not considered that these are likely to be part of the molecule but arise from small amounts of peptides occurring as contaminants.

Some of the oligosaccharide was incubated with lysozyme overnight. When the product was chromatographed in butanol-acetic acid-water and the paper sprayed with Elson-Morgan spray (Partridge, 1948) only a single spot of the original unchanged material could be detected. It appears therefore that the linkage between glucosamine and muramic acid in this material is unaffected by lysozyme.

Muramate-glucosamine-glycine mucopeptide

This material appeared at point *B* in the fractionation scheme shown in Fig. 3. Table 3 shows the composition of hydrolysates obtained by heating the material for 4 hr. with 4*N*-HCl at 100°. Again two separately isolated batches were examined and the results agreed reasonably well, suggesting an approximate molecular composition of one molecule of glucosamine, one of muramic acid and one of glycine, although one of the values for glycine appears to differ from unity by more than might reasonably be attributed to experimental error. Again small amounts of other amino acids were present. In this instance it is less possible to exclude the amino acids from being part of the molecule. Apart from the values of lysine there is close agreement between the values for the others in the two batches. The amounts, however, are small and if they are part of the same molecule as the amino sugars and glycine a molecular weight within the region of 5000–10 000 would be indicated, which is not consistent with the behaviour of the material on paper and on columns or with its rapid diffusibility through cellophan.

An examination of the free amino end-group of this material by the fluorodinitrobenzene technique failed to reveal any dinitrophenyl glycine, although *N*-dinitrophenyl glucosamine was present. It is therefore concluded that the amino group of the glycine is covalently linked in this compound.

Muramate-alanine-glycine-glutamate-lysine mucopeptide

This material appeared at point *C* (Fig. 3) during the fractionation. Three separate isolations were made and the analyses of acid hydrolysates are recorded in Table 4. The procedures for isolating batches (b) and (c) were considerably more rigorous than those for batch (a) and, as can be seen, glucosamine, which was present in considerable amounts in (a), was almost eliminated. Similarly, the proportions of alanine and lysine were considerably increased in batches (b) and (c) as compared with batch (a). Even, however, in the later batches the results do not correspond with any very simple ratio of the component molecules. This may suggest that the compound has a high molecular weight. So far we have not had enough of this material to examine it by physical methods.

Alternatively, of course, despite the vigorous purification procedure adopted, the final material obtained may still be a mixture of closely similar compounds. The material was examined for free amino end-groups but only lysine substituted in the ϵ -amino group could be isolated. Before hydrolysis the compound reacts to give a colour in the *N*-acetylhexosamine test, which suggests that the amino group of muramic acid is acetylated; if so, this would prevent it reacting with fluorodinitrobenzene. The absence of any other free amino group may suggest that the peptide chain is linked through its terminal amino group to the carboxyl group of muramic acid.

Incubation with carboxypeptidase produced little or no effect on this molecule. Even when 0.1 mole of enzyme was used for every mole of glycine present in the compound and incubation was continued overnight at 37° there was no appreciable liberation of amino acid from the mucopeptide. At most only 10% of the alanine was set free and no other amino acid appeared. Likewise incubation with lysozyme left the molecule unchanged.

Table 3. *Analysis of a muramate-glucosamine-glycine mucopeptide*

Isolation and analysis of this compound after hydrolysis with 4*N*-HCl for 4 hr. at 100° was as described in the text (*B* in Fig. 3). Results are expressed relative to glucosamine. (a) and (b) represent two separately isolated batches of material.

	Amount (μ moles)	
	(a)	(b)
Glucosamine	1.0	1.0
Muramic acid	1.2	1.1
Glycine	0.93	0.75
Alanine	0.15	0.16
Glutamic acid	0.11	0.14
Lysine	0.10	0.20

Table 4. *Analysis of a muramate-alanine-glycine-glutamate-lysine mucopeptide*

Isolation and analysis of this compound after hydrolysis with 4*N*-HCl for 4 hr. at 100° was as described in the text (*C* in Fig. 3). Results are expressed relative to muramic acid, which is taken as 4 μ moles. (a), (b) and (c) represent three separately isolated batches purified by slightly different methods.

	Amount (μ moles)		
	(a)	(b)	(c)
Muramic acid	4.0	4.0	4.0
Alanine	2.9	3.4	3.9
Glycine	2.5	2.2	2.4
Glutamic acid	1.6	1.7	1.7
Lysine	1.1	1.5	1.6
Glucosamine	0.29	Trace	0.19

*Other peptide material isolated but not
examined in detail*

Two other mucopeptide fractions were also isolated (*D* and *E*, Fig. 3) but not studied in great detail. One (*D*) of these contained about four molecules each of muramate and alanine and one molecule of glycine as the major constituents. The other (*E*) contained all the compounds in the original cell-wall mucopeptide except muramate. It is to be noted that this latter fraction had glucose as part of the molecule.

DISCUSSION

Analysis of the wall structure from this organism showed that about 94–97 % of its dry weight can be accounted for in terms of the seven components alanine, glutamate, glycine, lysine, glucose, *N*-acetylglucosamine and *N*-acetylmuramic acid. Thus there is considerable reason for saying that the cell wall of this organism as prepared by the method (Cummins & Harris, 1956) used here, consists only of mucopeptide. If other substances are also involved they must be very small in quantity. The low phosphorus content also indicates that very little teichoic acid can be present, an observation which confirms the work of Baddiley *et al.* (1958), who failed to find polyol phosphates in cell walls from *Micrococcus lysodeikticus*.

One obvious difficulty in studying the undergraded bacterial cell-wall mucopeptides is their complete insolubility in all but disruptive solvents. The result of this intransigence is that the homogeneity of the starting material is unproved and it is impossible to say whether the fragments isolated here have their origin from one or more than one high-molecular-weight compound. The observed variation in the relative amounts of the amino acids and sugars from batch to batch of cell wall points to the need for caution in this respect. However, the present work has shown that all seven component compounds are covalently interlinked and has disposed of the unlikely but possible hypothesis that the original mucopeptide consists entirely of a number of polymers each built from only one or two of the seven components. The composition of the fragments examined in detail shows that in some part of the molecule glucosamine and muramate are joined together. This provides some support for the hypothesis of an amino sugar backbone to the original mucopeptide, as suggested by Brumfitt *et al.* (1958). Lysozyme does not affect this isolated oligosaccharide, which seems to make it unlikely that the major action of lysozyme is to split the bond between the two amino sugars in the absence of further substitution. This is consistent with the statement (Salton, 1956) that a disac-

charide of glucosamine and muramate is liberated from the cell-wall mucopeptide by the action of lysozyme.

The second fragment isolated contains the two amino sugars and glycine. No terminal glycine amino group can be demonstrated, which suggests that this group is already linked to some other molecule. The presence of the carboxyl group in muramate makes it tempting to suggest that this is the group with which the amino group of glycine is combined to give a type of peptide. No definite proof for this suggestion exists, however, and it remains possible that the glycine amino group is combined in some way with the hydroxy groups of the sugars: however, no such compounds have been described.

Not only were the oligosaccharide and the glycine-oligosaccharide resistant to the action of lysozyme, but so also was the third, more complicated fragment. Thus so far no fragment less than the whole polymer has been isolated which is susceptible to lysozyme action. The larger fragment was also resistant to the action of carboxypeptidase despite the failure to demonstrate a free amino group. The absence of a reactive amino group may suggest that, in this fragment also, the carboxyl group of muramate is combined with the terminal amino group. The absence of carboxypeptidase action might be explained either by the presence in the molecule of the sugar group or alternatively by the presence of amino acids in the *D*-configuration, either of which might be expected to prevent hydrolysis by the enzyme.

The total yields of the materials examined have been small. For example, a total of only about 3 % of the weight of the original wall mucopeptide was obtained as at least three different peptides and probably more. Thus it is not yet safe to hypothesize about the structure of the whole wall. It is, however, possible to say that some parts of the muramic acid and glucosamine are linked together and that some of the oligosaccharide is linked to glycine probably by way of the carboxyl group of the muramic acid and the amino group of glycine. Further work depends on finding more satisfactory methods for hydrolysing the wall mucopeptide and better methods for separating the resultant small mucopeptides.

SUMMARY

1. The quantitative composition of mucopeptide from cell walls of *Micrococcus lysodeikticus* is given. About 95 % of the dry weight of the wall preparation can be accounted for.

2. Acid hydrolysis of the mucopeptide has been studied.

3. Three fragments have been isolated from the acid hydrolysates: (*a*) an oligosaccharide containing

glucosamine and muramic acid; (b) a mucopeptide containing glycine, muramic acid and glucosamine; (c) a mucopeptide containing muramic acid, glycine, lysine, glutamate and alanine.

4. All the fragments isolated are resistant to the action of lysozyme.

5. The significance of the mucopeptide fragments for the structure of the wall mucopeptide is discussed.

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The Incorporation of Amino Acids into the Cell-Wall Mucopeptide of Staphylococci and the Effect of Antibiotics on the Process

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When washed suspensions of staphylococci in buffered glucose are incubated in the presence of certain single amino acids labelled with ^{14}C , radioactivity is incorporated into the fraction of the cells insoluble in hot trichloroacetic acid (Gale & Folkes, 1953). This process is chloramphenicol-resistant and penicillin-sensitive. Other work (Salton & Horne, 1951; McCarty, 1952a, b; Salton, 1953; Cummins & Harris, 1956) has shown that, after

disruption of Gram-positive bacterial cells, material can be obtained, either by differential centrifuging alone or by a combination of centrifuging and enzyme treatment, which appears, by electron-microscopic examination, to be made up of the cell walls of organisms. Acid hydrolysis and paper chromatography have shown that this material consists preponderantly, in most Gram-positive organisms, of a few amino acids together with