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A Study of the Composition and Structure of the Cell-Wall Mucopeptide of *Micrococcus lysodeikticus*

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Previous work (Salton, 1953; Perkins & Rogers, 1959) has shown that acid hydrolysates of wall preparations from *Micrococcus lysodeikticus* that have been made by the method of Cummins & Harris (1956) contain glutamic acid, lysine, alanine, glycine, glucosamine, muramic acid (3-*O*-carboxyethylglucosamine) and glucose. These substances make up some 80% of the dry weight of the preparations, or 90% if it is assumed that the amino

sugars are *N*-acetylated. It has been claimed in other work that the amino sugars in wall preparations are relatively easily broken down during acid hydrolysis with the liberation of ammonia. Thus uncertainty exists about the true amounts of amino sugars present and about the origin of the ammonia found in hydrolysates of wall preparations from both staphylococci (Strominger, Park & Thompson, 1959) and lactobacilli (Ikawa & Snell, 1960). This question obviously has an important bearing on ideas about the structure of mucopeptides, more particularly in relation to the liberation of ammonia from other amino sugar-peptide sub-

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stances such as the fragment isolated from ovalbumin by Johansen, Marshall & Neuberger (1961) and the ideas that have been put forward to account for it.

Part of this work was therefore undertaken to ascertain as closely as possible (a) the composition of the wall material, and in particular the true concentration and stability of the amino sugars, (b) the nature of the acyl groups and their quantity in relation to the amount of total amino sugar. A small amount of ammonia is rapidly released during treatment of wall preparations with acid, which does not have its origin in destruction of known amino sugars. The amount of identified acetic acid liberated during acid hydrolysis was in excess of that required for the *N*-acetyl groups of the amino sugars by an amount equivalent in molar terms to the amount of ammonia that appeared rapidly.

The second part of the work depended on the first, and was concerned with the charged groups on the wall mucopeptide. Measurements were made both by direct titration and by dye-binding methods. Salton (1961) has shown that few or no α -amino groups of the amino acids in mucopeptides are free to react with fluorodinitrobenzene and that not all the ϵ -amino groups of the lysine in wall preparations from *M. lysodeikticus* are free. The suggested structure for the fragments isolated by Ghuysen (1961) from lysozyme-digested wall provides a possible reason for the unavailability of the ϵ -amino groups of lysine. Salton (1961) has also determined the number and kind of carboxyl group-terminal amino acids in mucopeptides that can be liberated by hydrazinolysis. The purpose of this part of the present work was to examine the total number of carboxyl and amino groups that could be titrated, both to ensure that steric or other factors had not led to misleading results with fluorodinitrobenzene and hydrazine, and also to see how far the observed results tallied with those expected from the various structures for the wall mucopeptide that have been suggested or implied.

A brief summary of this work has already appeared (Czerkawski, Perkins & Rogers, 1961).

MATERIALS AND METHODS

Cell walls. *M. lysodeikticus* N.C.T.C. 1665 was grown on nutrient agar contained in Roux bottles incubated at 35° for 48 hr., and the cell walls were prepared by the method of Cummins & Harris (1956). The preparations were dried from the frozen state, heated at 105° to constant weight and kept in a desiccator at room temperature over anhydrous CaCl_2 .

Reference proteins. β -Lactoglobulin was a purified sample kindly given by Dr B. A. Askonas. Crystallized bovine plasma albumin was from Armour Laboratories.

Amino acids. These were estimated as described by Mandelstam & Rogers (1959).

Amino sugars. Three different methods were used: (a) glucosamine and muramic acid were separately determined, as described by Perkins & Rogers (1959); (b) glucosamine was determined as by Cessi & Piliego (1960); (c) the total hexosamine was determined by the method of Tracey (1952).

Glucose. This was freed from the amino sugars after acid hydrolysis of the mucopeptide by the method of Perkins & Rogers (1959) and determined by both the reducing-sugar method of King (1947) and the anthrone method of Mokrasch (1954). Paper chromatography showed that the sugar estimated was glucose. The hydrolysis conditions were varied and are given in the Results section.

Acetate. This was estimated by gas chromatography, with the original apparatus of James & Martin (1952), or by the hydroxamate method of Ludowieg & Dorfman (1960). Acetate was also identified by paper chromatography (Brown, 1950).

Ammonia. This was estimated by distillation at 5° into 0.01 *N*-HCl in Conway (1947) micro-diffusion units. The material in the outer ring of the units was made alkaline by the addition of a solution saturated with both Na_3PO_4 and $\text{Na}_2\text{B}_4\text{O}_7$. In the presence of amino sugars the unambiguous estimation of free NH_3 necessarily presents difficulties, since the alkaline conditions for suppressing the ionization of NH_4^+ also lead to a slow destruction of the amino sugar even at 5°, with the production of NH_3 . This problem was partly solved by measuring the rates of liberation of NH_3 under these conditions; there was an initial rapid liberation during the first 2–3 hr., followed by a continued increase equivalent to 1.5% of the total amino-sugar *N*/hr. It was assumed that only this slow liberation corresponded to destruction of amino sugar, and a time of 4 hr. at 5° was chosen for the distillation, the result being corrected for NH_3 coming from the destruction of amino sugars. This method was confirmed by studying the liberation of NH_3 from an artificial mixture of amino sugars, glucose and amino acids made up according to analyses of hydrolysates of cell walls. The amount of NH_4Cl in the centre well was finally estimated by the method of Jacobs (1960).

Pyruvic acid. This was measured as by Cavallini & Frontali (1954).

Methods for acidic and basic groups

Dye absorption. The method described by Fraenkel-Conrat & Cooper (1944) was used. A suspension of the wall preparation in buffer was gently shaken with glass beads for 24 hr. at room temperature in the presence of either Orange G or Safranin. At the end of the period of shaking the suspensions were centrifuged and the colour remaining in the supernatant solutions was measured colorimetrically at wavelengths of 477 μ for Orange G and 502 μ for Safranin. Adsorption of Safranin was carried out at pH 11.5 in the presence of buffer (250 ml. of 0.2 *M*- Na_2HPO_4 and 200 ml. of 0.1 *N*- NaOH , water to 1 l.); that of Orange G took place at pH 2.2, also with buffer (980 ml. of 0.1 *M*-citric acid and 20 ml. of 0.2 *M*- Na_2HPO_4).

Titration. The wall preparation (100 mg.) was suspended in 10 ml. of 0.2 *M*- NaCl solution in a small glass vessel containing a glass electrode connected to a pH meter (Electronic Instruments Ltd., Richmond, Surrey) previously

Table 1. *Analysis of cell-wall preparation from Micrococcus lysodeikticus*

Results refer to dry material and are from duplicate analyses on a single batch of wall. Methods used are set out in the Materials and Methods section. In calculating molar ratios the value for glutamic acid is taken as 1.00. Total recovery of material = 97% when corrected for water of hydrolysis.

Substance	%	Moles/10 ⁴ g.	Molar ratio
Alanine	16.9	19.0 ± 0.1	1.94
Glycine	8.1	10.8 ± 0.1	1.09
Glutamic acid	14.5	9.9 ± 0.2	1.00
Lysine	14.7	10.1 ± 0.2	1.02
Glucosamine	17.1	9.6 ± 0.2	0.97
Muramic acid	18.6	7.5 ± 0.1	0.76
Glucose	8.1	4.5 ± 0.2	0.46
Acetic acid (hydroxamate)	12.8	21.4	2.16
Acetic acid (gas chromatography)	12.4	20.7	2.09
Rapidly liberated NH ₃ (extrapolation)	6.8	4.0-4.5	0.41-0.46
Rapidly liberated NH ₃ (difference)*	5.4	3.3	0.34

* The difference between the total NH₃ liberated from acid hydrolysates in the Tracey (1952) method and the amount of NH₃ to be expected from the content of muramic acid and glucosamine in the preparation.

standardized with potassium phthalate buffer, pH 4.00. Stirring was with a stream of argon. Small measured amounts of either 0.01 N-HCl or 0.01 N-NaOH were then added, and after each addition the pH was measured until a constant value was obtained. The results were plotted on a graph and the number of acidic and basic groups was then calculated from the inflexion points (Bendall, 1955).

When it was required to examine the material after lysozyme hydrolysis 200 mg. of the wall preparation was first suspended in 10 ml. of 0.2 M-NaCl (pH adjusted to 6.0 with 0.01 N-HCl) and incubated at 35° with 1 mg. of crystalline lysozyme for 2 hr. At the end of this period portions of the resulting solution were diluted with an equal volume of 0.2 M-NaCl, adjusted to pH 4.0 and titrated as described above.

RESULTS

Composition of the wall substance

Amino acids. The wall preparations were hydrolysed with 6 N-hydrochloric acid at 105° for various times and the concentration of amino acids was estimated. It was found that alanine and glycine were completely liberated within 2-4 hr., but that free glutamate and lysine appeared more slowly. In 16 hr. hydrolysis was complete, and Table 1 shows the concentrations of the amino acids found. The molar relationship between them is almost exactly 2:1:1:1 for alanine, glycine, glutamic acid and lysine respectively. These values were repeatedly found during numbers of analyses of the two particular batches of cell wall examined here, and resemble batch CW. 20 described by Perkins & Rogers (1959). It is therefore probable that this is a truer picture than that of the somewhat higher proportion of alanine (2.5) reported earlier for batch CW. 18.

Amino sugars and ammonia liberation. Muramic acid, glucosamine and ammonia were estimated after various periods of treatment with 4 N-hydrochloric acid at 105°. Both cell-wall preparations and an artificial mixture of glucosamine, muramic

Fig. 1. Liberation of muramic acid, glucosamine and NH₃ from cell walls by acid hydrolysis. Samples of cell wall were hydrolysed in sealed ampoules in 4 N-HCl at 105°. Δ , Glucosamine; $\square$, muramic acid; $\circ$, ammonia.

acid, glucose and the four relevant amino acids were examined. Fig. 1 shows the results obtained. A peak value for both muramic acid and glucosamine, irrespective of the method used for estimation, was reached in about 30-40 min. The rate of destruction was then rather more rapid for the glucosamine fraction than for muramic acid for 2-3 hr. Both amino sugars eventually came to a steady rate of destruction amounting to about 1.5%/hr. The destruction of total hexosamine in an artificial mixture containing glucosamine, muramic acid, glucose and amino acids in the proportions present in the cell walls proceeds steadily at about 1%/hr.

Ammonia liberation corresponded to destruction of amino sugar except during a period of rapid evolution, which had already begun when the

first sample was taken (after 15 min.). After about 4 hr. the rate became steady and was about equal to that of the destruction of the amino sugars. The total amount of ammonia liberated between 4 and 19 hr. was 3.5 moles, compared with the destruction of 1.2 moles of muramic acid and 2.2 moles of glucosamine, giving a total of 3.4 moles of total hexosamine destroyed. Thus the latter phase of slow liberation of ammonia is probably due to destruction of glucosamine and muramic acid but its early rapid liberation is not.

The total 'hexosamine' in a 4 hr. acid hydrolysate was also estimated by the Tracey (1952) method, which involves steam-distillation of the hydrolysate under alkaline conditions. The total ammonia found amounted to 20.4 moles/ 10^4 g. of the original preparation. The total content of glucosamine and muramic acid calculated from Fig. 1 by correcting for acid destruction was $9.6 + 7.5 = 17.1$ moles/ 10^4 g. Thus the amount of ammonia in excess of that which could possibly have come from these two amino sugars is 3.3 moles/ 10^4 g. Another way of calculating this excess of ammonia is to extrapolate the line for the slow and approximately steady liberation of ammonia that occurs from about 2 to 19 hr. to cut the ordinate axis. By this method it would appear that about 4.0–4.5 moles of ammonia/ 10^4 g. are rapidly liberated by acid hydrolysis.

Liberation of acetate. If the volatile acid present in hydrolysates arose only from the hydrolysis of the *N*-acetyl groups of glucosamine and muramic acid, there should be a total concentration of acetic acid equivalent to 17.1 moles/ 10^4 g. No unequivocal evidence has hitherto existed that all the NH_2 groups of the amino sugars were substituted by acetyl groups. Table 1 shows that the number of moles of volatile acid identified as acetic acid by gas chromatography and paper chromatography exceeded the number of moles of amino-sugar by about 4 moles/ 10^4 g., an amount about equivalent to the ammonia liberated rapidly during acid hydrolysis. The intact preparation before acid hydrolysis liberated no acetic acid.

Minor components. During the examination of the acid hydrolysates of the wall preparations, small amounts of formaldehyde and formic acid were identified, but did not amount to more than about 0.1–0.2 mole/ 10^4 g. A somewhat larger amount of pyruvic acid, amounting to about 1.5 moles/ 10^4 g., was also found. The origin of these small amounts of material was not identified.

Acidic and basic groups

The titration curve obtained with both the solid wall preparation and with the same material after it had been made soluble with lysozyme is shown in Fig. 2. As might be expected from the supposed

action of lysozyme as a β -(1 $\rightarrow$ 4)-glycosidase, no new charged groups were liberated by enzymic hydrolysis. The small shift in the curve that occurred would be consistent with the effect of rupturing hydrogen bonds on the strengths of already pre-existing charges. The important points of inflexion (i.e. the mid-points between increasing or decreasing slope) on both curves are at about 3.5 and 10.5. The uncorrected isoelectric point of the material was at pH 4.0. Calculation of the amount of CO_2H groups titrated gave a result of 16.1 moles/ 10^4 g. for the unhydrolysed material and 16.3 moles/ 10^4 g. for the sample hydrolysed by lysozyme. For the basic groups the corresponding values were 5.8 and 5.6. When the solid mucopeptide was titrated in the presence of 2% of formaldehyde the pK of the basic groups was shifted towards the acid region and the alkali uptake between pH 7.0 and 9.0 corresponded to 4.8 moles/ 10^4 g. The pK values of the modified and unmodified basic groups show that they are in fact likely to be due to the ϵ - NH_2 groups of the lysine present.

The results of the determinations of the CO_2H group content of the unhydrolysed wall preparations, obtained by using Safranin as the dye and working at pH 11.5 in phosphate buffer, were in approximate agreement with those obtained by direct titration, giving a value of 17.7 moles/ 10^4 g. This value was obtained by adjusting the system so that not more than 50% of the dye was adsorbed by the mucopeptide or a standard protein

Fig. 2. Titration curves obtained with cell wall and with the same preparation after dissolution by lysozyme. O, Untreated cell wall; ▲, lysozyme digest of cell wall. The change in slope at the end of the alkaline titration was difficult to determine with precision.

and comparing the amounts adsorbed by known weights of material. The standard proteins used were albumin and β -lactoglobulin, the values for which were assumed to be those stated by Cohn & Edsall (1943).

The values for the basic groups determined by the binding of Orange G at pH 2.2 were in gross disagreement with those obtained by direct titration, being 1.7 as compared with 5.7 moles/ 10^4 g. by the latter method. Possible reasons for this discrepancy are discussed below.

DISCUSSION

The results of the analyses of the present batch of cell walls for amino acids and the known amino sugars (glucosamine and muramic acid) largely confirm those previously published. There seems no doubt that in these preparations the molar proportions of glutamic acid:lysine:glycine:alanine are 1:1:1:2. This result is important to the interpretation of other data. When treated with 5-fluorouracil these cells, just like staphylococci (Rogers & Perkins, 1960), accumulate a 5-fluorouridine diphosphate-linked mucopeptide having the amino acids glutamic acid, lysine and alanine in the proportions 1:1:3 (H. J. Rogers & H. R. Perkins, unpublished observations). Thus deductions about the structure of wall mucopeptide from those of apparent intermediates that accumulate in inhibited cells are not to be trusted. Rogers & Perkins (1959) and Janczura, Perkins & Rogers (1961) pointed out that acceptance of analyses of whole wall as representing that of a single simple mucopeptide may lead to confusion and error. This point is made by Mandelstam & Strominger (1961), who have confirmed the finding (Mandelstam & Rogers, 1959) that, after the removal of teichoic acid with its ester-attached alanine from staphylococcal wall preparations, the mucopeptide has only two molecules of alanine to one of glutamate, and not three, as had previously been thought (Park & Strominger, 1957; Strominger *et al.* 1959; Hancock, 1960). However, Mandelstam & Strominger (1961) consider that the ester alanine of teichoic acid represents the third alanine of the mucopeptide, i.e. that it is bound in peptide linkage by its amino group, although Archibald, Armstrong, Baddiley & Hay (1961) showed that such a linkage is most improbable. By the use of this hypothesis, it is still possible to maintain that the mucopeptide part of the Park (1952) compound is incorporated without further modification. The present work confirms that the wall of *M. lysodeikticus*, which has no detectable teichoic acid associated with it (Armstrong, Baddiley, Buchanan, Carss & Greenberg, 1958), has only two molecular proportions of alanine to one of glutamate, despite the ability of

the cells to accumulate a nucleotide-mucopeptide compound with three molecules of alanine.

The total analysis of the wall, allowing for water of hydrolysis (which was not allowed for in Perkins & Rogers, 1959), accounts for 95–97 % of its weight. This includes about half a molecular proportion of ammonia, which is released rapidly during acid hydrolysis and is not likely to come either from destruction of glucosamine or muramic acid or from amide-substitution of carboxyl groups. A similar amount of acetic acid in excess of that necessary for acyl-substitution of the amino groups of the amino sugars is also present. The ammonia, the excess of acetic acid and the traces of formaldehyde, formic acid and pyruvic acid, may all come from a *N*-acetylated amino-sugar compound equimolar in amount with the glucose also present in the wall. This substance would have to be very much more acid-labile than the two amino sugars already known to be present. It is probable that the ammonia and excess of acetyl groups and the small amounts of the other substances arise during acid hydrolysis from *N*-acetamidomannuronic acid that is present in a separable polymer, which contains equimolar amounts of glucose and this new compound; however, the isolated material itself fails to give ammonia during acid hydrolysis. Possible reasons for this failure are discussed by Perkins (1963).

We may, then, on the basis of the present and other work (Perkins & Rogers, 1959; Salton & Ghuyssen, 1960; Ghuyssen, 1961), suppose that wall preparations from *M. lysodeikticus* contain about 85 % of one or more mucopeptides containing *N*-acetylmuramic acid, *N*-acetylglucosamine, glutamic acid, lysine, glycine, alanine in the molecular proportions 0.8:1:1:1:1:2, and about 15 % of a glucose-*N*-acetamidomannuronic acid polymer. We now consider the bearing of the present results on the structure of the major mucopeptide part of the wall.

Sufficient evidence is available from earlier work to make it clear that at least in part of the mucopeptide the two amino sugars are joined together by glycosidic linkages, as shown in Fig. 3. Peptides are joined to most of the muramic acid residues through the amino group of one of their alanine residues to the carboxyl group of the amino sugar. Some muramic acid residues, however, are not so substituted, since, after the action of lysozyme, disaccharide with no peptide attached can be isolated (Perkins, 1960; Salton & Ghuyssen, 1960). The minimum proportion of unsubstituted muramic acid would appear to be about 20 % of the total. The hypothetical structure shown in Fig. 3 differs from that originally proposed by Brumfitt, Wardlaw & Park (1958) only by the presence of one disaccharide in five without a peptide chain

SUMMARY

1. The major constituents of wall preparations from *Micrococcus lysodeikticus* are 2-acetamido-3-O-carboxyethyl-2-deoxyglucose (*N*-acetylmuramic acid), 2-acetamido-2-deoxyglucose (*N*-acetylglucosamine), glutamate, lysine, glycine and alanine in the molecular proportions 0.8:1:1:1:1:2. This mucopeptide accounts for 85 % of the wall.

2. Also present in acid hydrolysates are approximately equimolar proportions of ammonia, glucose and acetic acid. The ammonia and acetic acid do not arise from breakdown of the known *N*-acetyl groups of the amino sugars.

3. Acid-base titration of the wall preparations and of these preparations after hydrolysis by lysozyme shows the presence of about 16 moles/10⁴ g. of basic groups with pK about 10.5. The isoelectric point for the whole material was 4.0.

4. The conclusions from these results are that about half the ϵ -amino groups of lysine in the whole mucopeptide are combined, possibly with a similar proportion of the γ -carboxyl groups of the glutamic acid. The total titratable acidic and basic groups are too few to be accounted for by a simple structure consisting of a polysaccharide chain with short peptide chains attached to the muramic acid and provide evidence for rather extensive cross-linking in the whole polymer.

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