

## A Polymer Containing Glucose and Aminohexuronic Acid Isolated from the Cell Walls of *Micrococcus lysodeikticus*

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The surveys of Cummins & Harris (1956, 1958) established that the cell walls of Gram-positive bacteria all contain the amino sugars glucosamine and muramic acid (3-*O*- $\alpha$ -carboxyethylglucosamine) and the amino acids alanine, glutamic acid and either lysine or  $\alpha\epsilon$ -diaminopimelic acid. In some species either glycine or aspartic acid was also present, whereas the other amino acids found in proteins were usually absent. The component of cell-wall structure containing these compounds has been found in all bacteria so far examined and has been given the name mucopeptide (Perkins & Rogers, 1959). In addition, various non-nitrogenous sugars have been found in the cell walls of some strains, but the place of these components in the structure of the wall is not always understood. Often the complex polyol phosphate polymers known as teichoic acids (Baddiley, 1959) are present in the cell walls, and these compounds may include sugar residues glycosidically linked to the polyol. For instance, glucose occurs in the ribitol teichoic acid isolated from the cell walls of *Bacillus subtilis* (Armstrong, Baddiley & Buchanan, 1960). Sometimes, however, sugars are found that are not part of the teichoic acid but which are present in polymers independent of both teichoic acid and mucopeptide. Examples are the rhamnose-glucosamine polymer found in *Streptococcus faecalis* (Ikawa, 1961) and group A haemolytic streptococci (McCarty, 1960), and the teichuronic acid, a polymer of *N*-acetylgalactosamine and glucuronic acid, found in the cell walls of *B. subtilis* (Janeczura, Perkins & Rogers, 1961).

From previous work it was known that the cell walls of *Micrococcus lysodeikticus* contain no phosphorus (and hence no teichoic acid) and consist mainly of glucosamine, muramic acid, alanine, glutamic acid, lysine, glycine and glucose. A critical analytical study has shown that these constituents together account for 93% of the dry weight of the walls, after allowance has been made for the uptake of water during hydrolysis (Czerkawski, Perkins & Rogers, 1963). Nevertheless, certain discrepancies were brought to light, parti-

cularly: (a) the presence of a number of titratable carboxyl groups in excess of what might have been predicted after allowance had been made for the observed blocking of lysine  $\epsilon$ -amino groups; (b) the rapid liberation during acid hydrolysis of a quantity of ammonia that could not be readily accounted for by the breakdown of glucosamine, muramic acid or the amino acids; (c) the observation that more acetyl groups were present than could be derived from the *N*-acetyl derivatives of the glucosamine and muramic acid, and that these additional groups did not occur as acetic esters.

The present work describes the isolation from the cell walls of *M. lysodeikticus* of a polymer consisting of glucose and 2-acetamido-2-deoxymannuronic acid. The latter component exhibits a lability in acid solution which probably accounts for the observations of Czerkawski *et al.* (1963). A preliminary report has been published (Perkins, 1962).

### METHODS

*Cell walls.* *Micrococcus lysodeikticus* N.C.T.C. 2665 was grown, and the cell walls were prepared, by the method of Cummins & Harris (1956) as described by Perkins & Rogers (1959).

*Extraction procedures.* (1) In some experiments the cell walls were extracted with hot formamide, as described by Fuller (1938). About 10 mg. of freeze-dried cell wall was heated with 1 ml. of formamide in an oil bath at 150° for 15 min. The mixture was cooled and 2.5 ml. of acid-ethanol was added (2*N*-HCl-ethanol; 5:95, v/v). The mixture was centrifuged and the residue washed with more acid-ethanol, and then dried first with acetone and then at 105°. Excess of acetone (25 ml.) was added to the combined supernatant and washings, and a white precipitate was obtained, collected by centrifuging and washed with acetone. The formamide residue and precipitated supernatant fractions were used for chemical analysis. (2) In early experiments the cell walls were extracted by heating with 5% (w/v) trichloroacetic acid at 90° for 15 min. The extract was freed from trichloroacetic acid by repeated extraction with ether, and from ether by aeration. In later experiments the glucose polymer was extracted as follows. Freeze-dried cell-wall material was shaken successively with portions of about 50 ml. of 5% (w/v) trichloroacetic acid/g. at 35° for various periods totalling 4 or 5 days. After each extraction the acid was removed by centrifuging and replaced by a fresh portion for further extraction.

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Each extract was freed from trichloroacetic acid as described above and used for chemical analysis and other fractionation procedures. The glucose content of these extracts was not reduced by prolonged dialysis in a cellophan tube against water at 2–4°.

*Fractionation of trichloroacetic acid extracts.* (1) Ethanol precipitation. Material was precipitated from the extracts by addition of a 0.5% solution of potassium acetate in ethanol. Little precipitation occurred in the absence of potassium acetate. In a typical experiment 0.5 g. of cell wall was shaken with trichloroacetic acid at 35° for 16 hr. and for a further 24 hr. The combined extracts freed from trichloroacetic acid (40 ml.) were mixed with ethanolic potassium acetate until a thick precipitate was obtained (105 ml.). The precipitate was washed with 80 ml. of ethanol and dried with ether (74 mg.). Addition of another 105 ml. of ethanolic potassium acetate to the original supernatant gave only a small further precipitate.

A portion (57 mg.) of the first precipitate was dissolved by shaking in 4 ml. of water. Undissolved solid (0.9 mg.) was removed by centrifuging and the clear solution was examined in the polarimeter ( $[\alpha]_D^{24.5} + 24.1^\circ$ ). Ethanolic potassium acetate was then added until precipitation occurred: 6 ml., fraction 1, 11.5 mg.; 10 ml., fraction 2, 16 mg. Further additions did not lead to any more precipitation, so that 48% by wt. of the original precipitate was recovered in these two fractions, which were used for chemical studies.

(2) Curtain electrophoresis. In later experiments the glucose polymer was prepared directly from the trichloroacetic acid extracts by electrophoresis on paper in a continuous-flow apparatus (Beckman Instruments Inc., Spinco division, model CP). A portion (56.5 ml.) of the combined first and second extracts from 583 mg. of cell walls (see above) was concentrated to 30 ml. *in vacuo* and then applied to the paper curtain nearer the cathode side. The buffer was pyridine 30 ml., acetic acid 30 ml., water 10 l., pH 4.6. A constant current of 40 ma was passed, the applied potential being approximately 400 v. Fractions of about 3.5 ml. were collected, the tubes being changed automatically every 4 hr. A sample of each was analysed for glucose, and those giving a positive result were pooled to yield fractions A (tubes 19 and 20) and B (tubes 20–24), the latter material having moved further towards the anode. The pooled fractions were concentrated by freeze-drying. The glucose present in these two fractions represented 79% of that applied to the curtain.

Some of fraction B, which proved to be richer in glucose than fraction A, was again subjected to electrophoresis on the same apparatus, except that the buffer was 2*N*-acetic acid (constant current 80 ma, approx. 770 v). Two fractions were collected, from tube 11 (B<sub>1</sub>) and tubes 12–14 (B<sub>2</sub>). The glucose present represented a recovery of 81% on this second fractionation.

*Reduction of carboxyl groups in the polymer.* The method employing diborane, used by Smith & Stephen (1960), proved satisfactory. A sample of fraction B<sub>2</sub> known to contain essentially only glucose and the suspected amino-hexuronic acid was acetylated as described by Masamune, Sinohara & Okuyama (1958). A portion (7 mg.) equivalent to 13  $\mu$ moles of glucose was dried *in vacuo*; 0.1 ml. of formamide was added, followed by 0.2 ml. of dry pyridine. The mixture was cooled to 0°, and 0.18 ml. of acetic anhydride was added over a period of 20 min. The tube was

stopped and left at room temperature for 1 hr. and then at 35° for 3 days. The volatile reagents were removed *in vacuo*, and addition of about 5 ml. of 0.1 *N*-HCl produced a white precipitate. The precipitate was removed by centrifuging and washed with 0.01 *N*-HCl.

The dried precipitate (3.7 mg.) was reduced in solution in diglyme (bis-2-methoxyethyl ether). Diglyme was dried over calcium hydride and redistilled before use. Boron trifluoride etherate was also dried over CaH<sub>2</sub> and redistilled at 40 mm. Hg to yield a colourless liquid. Sodium borohydride was dissolved in diglyme (20 mg./ml.) and so was the boron trifluoride etherate (100 mg./ml.). The acetylated polymer was dissolved in 0.2 ml. of diglyme to give a clear solution, and 0.285 ml. of sodium borohydride solution was added (a precipitate formed almost immediately) followed by 0.284 ml. of boron trifluoride solution. The mixture was left at room temperature for 3.5 hr. and overnight at 2–4°. Addition of 10 ml. of ice-cold 0.05 *N*-HCl produced no precipitate and so the solution was dialysed in a cellophan tube against water (5 l.) for 48 hr. at 2–4°. The bag contents were dried *in vacuo* and dissolved in water (0.6 ml.). The resulting solution of reduced polymer was examined chemically.

*Titration.* Samples of various fractions were titrated against 0.05 *N*-NaOH solutions on a Titrator type TTT 1c (Radiometer, Copenhagen, Denmark). The solution was stirred magnetically and the alkali was measured from a micrometer syringe (Agla).

*N-Acetylation of amino sugars.* Small samples of amino sugars were converted into their *N*-acetyl derivatives by treatment of an aqueous solution with about 5 mol.prop. of acetic anhydride and enough trimethylamine solution to bring the pH to 9. After 2 hr. at room temperature the mixture was acidified with acetic acid and dried *in vacuo*, and the resulting preparation was used for chromatography.

*Conversion of hexosamines into pentoses.* The reduced polymer described above was hydrolysed in *N*-HCl for 1 hr. at 100°. The hydrolysate was dried *in vacuo* over NaOH and run on a chromatogram in pentan-1-ol-pyridine-water. The hexosamine spot was eluted and heated for 30 min. at 100° in a capillary tube with an equal volume of 2% (w/v) ninhydrin in a 4% (v/v) solution of pyridine in water (Stoffyn & Jeanloz, 1954). The final mixture was run on a chromatogram in butan-2-one-acetic acid-boric acid and the pentose produced was compared with marker spots.

*Analytical methods.* Amino sugars were estimated by the method of Rondle & Morgan (1955), often scaled down to a final volume of 3 ml. 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 ZF (British Carbo-Norit Union, W. Thurrock, Grays, Essex). Amino acids were estimated on paper chromatograms by a ninhydrin method (Mandelstam & Rogers, 1959). Total nitrogen was determined by the ninhydrin method of Jacobs (1962). For acetyl groups the method of Ludowieg & Dorfman (1960) was employed. Ammonia produced during acid hydrolysis was absorbed into 0.01 *N*-HCl in a Conway micro-diffusion unit, as described by Czerkewski *et al.* (1963), and finally estimated by the Jacobs (1960) method. Glucose was measured by an anthrone method (Mokrasch, 1954), with a heating period of 30 min. at 80°. Sialic acid was sought by the direct

Ehrlich reaction (Werner & Odin, 1952), and the thio-barbituric acid method of Warren (1959) applied to material hydrolysed in 0.1 N-H<sub>2</sub>SO<sub>4</sub> at 80° for 1 hr. The reaction used for the detection of unsubstituted hexuronic acids was that of Dische (1947). Reducing sugar was measured as glucose by the Nelson method (King, 1947).

**Paper chromatography.** The following solvent systems were employed. Whatman no. 3 paper was used except where indicated. (1) Butan-1-ol-acetic acid-water (63:10:27, by vol.; upper phase). (2) Butan-1-ol-pyridine-water (6:4:3, by vol.). (3) Phenol-water (4:1, v/v), used in an ammoniacal atmosphere. (4) Pentoses were identified on ascending chromatograms run on Whatman no. 4 paper in butan-2-one-acetic acid-saturated aq. boric acid (9:1:1, by vol.; Rees & Reynolds, 1958). The observed  $R_{\text{ribose}}$  values were: arabinose, 0.27; xylose, 0.39; lyxose, 0.44. (5) The most convenient solvent for the amino-hexuronic acids was found to be pentan-1-ol-pyridine-water (7:7:6, by vol.) as used by Heyns, Kiessling, Lindenberg, Paulsen & Webster (1959). (6) Dinitrophenyl-amino acids were separated on Whatman no. 4 paper soaked in 0.05 M-phthalate buffer (pH 6) in 2-methylbutan-2-ol saturated with the same buffer (Blackburn & Lowther, 1951). The spots were eluted with 1% (w/v) NaHCO<sub>3</sub> and read on a spectrophotometer at 350 m $\mu$ .  $\epsilon$ -Dinitrophenyllysine was run in the butanol-acetic acid-water system and eluted with N-HCl.

Amino acids and amino sugars were detected by dipping the dried chromatograms in 0.1% (w/v) ninhydrin in acetone, followed by heating at 100° for 10 min. Under these conditions the amino acids and hexosamines gave the usual purple spots, but the amino-hexuronic acids gave a light-brown colour which only became purple after standing for some hours at room temperature. The preliminary brown colour was not observed on chromatograms that had been run in phenol-NH<sub>3</sub>.

Hexoses and pentoses were observed by spraying with aniline phthalate (Partridge, 1949). Acetamido sugars were detected as described by Partridge (1948), except that the final heating was omitted.

**Paper electrophoresis.** Preliminary examination of various fractions and hydrolysis products was carried out by electrophoresis on paper with the buffer systems described above. The apparatus was based on that of Kunkel (1954) and the applied potential was about 15 v/cm.

## RESULTS

### *Extraction from the cell walls of a fraction rich in glucose*

Preparations of cell walls from *M. lysodeikticus* were treated with either hot trichloroacetic acid or hot formamide, and the extracts and residues were analysed as shown in Table 1. Although the recoveries of total material were by no means complete, it was at once apparent that all of the glucose had been extracted by either procedure, together with a lower proportion of all the other substances known to be present in the wall. Thus a means was available for obtaining an enrichment of that part of the cell-wall structure which contained glucose, and more of this material was prepared.

It was thought that both of the rather drastic extraction procedures mentioned might lead to chemical breakdown of any glucose-rich structure originally present in the cell walls. Therefore material was extracted from larger batches of frozen-dried cell walls by shaking with 5% trichloroacetic acid at 35°. Preliminary experiments showed that repeated extractions for periods up to a total of 4 days removed 70–80% of the glucose originally present, by which time about 35% of the total solid had passed into solution. Since in the successive extracts the ratio of glucose to the other components was found to decline after the 48 hr. period, combined extracts from the first and second days were used in the subsequent work.

**Bonds ruptured during extraction with trichloroacetic acid.** Since extraction of the glucose-rich polymer with 5% trichloroacetic acid at 35° proved to be so slow and, even after 4 days, incomplete, it seemed possible that some covalent bonds might be broken in the process. Cell-wall preparations were shaken at 35° with 5% (w/v) trichloroacetic acid and samples were taken each day for measurement of reducing sugar. After removal of trichloro-

Table 1. *Analysis of a cell-wall preparation after extraction with hot formamide or hot trichloroacetic acid*

Figures represent moles/10<sup>4</sup> g. of original cell wall.

Substance	Whole cell wall* (batch 29)	Treatment with 5% trichloroacetic acid (90° for 15 min.)		Treatment with formamide (150° for 15 min.)	
		Residue	Extract	Residue	Acetone-dried precipitate from extract
Glucose	4.3	0	4.9	0	4.1
Glucosamine	9.4	3.1	3.3	4.6	2.4
Muramic acid	7.6	3.1	2.0	5.3	1.0
Alanine	19.2	11.3	2.0	9.7	0.81
Glutamic acid	9.7	4.7	0.7	6.9	0.43
Lysine	10.1	4.9	1.1	7.5	0.56
Glycine	10.7	5.7	1.3	6.8	0.64

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

acetic acid with ether and neutralization, the whole sample was heated with the alkaline copper reagent used in the reducing-sugar method. Any solid material was removed by centrifuging before the final colours were read in the spectrophotometer, so that the values reported should correspond to all reducing groups present, whether in the extracted material or remaining in the residue. The proportion of amino groups set free was examined as follows. After 4 days' extraction the residue and the total material were treated with fluorodinitro-

benzene made alkaline with trimethylamine. The dinitrophenylated products were hydrolysed, and the resulting dinitrophenylamino acids were estimated at 350  $m\mu$  after extraction from chromatograms run in buffered 2-methylbutan-2-ol-water. The reducing and amino groups set free during treatment with trichloroacetic acid are shown in Tables 2 and 3. The results show that only about 0.5% of the glycosidic or 5% of the peptide linkages present in the structure were broken during the period when as much as 80% of the glucose present appeared in a soluble product.

Table 2. *Reducing groups liberated from the cell walls of Micrococcus lysodeikticus during treatment with 5% (w/v) trichloroacetic acid at 35°*

Total quantities of sugars in this sample of cell walls, expressed as mole/10<sup>4</sup> g., were: glucose, 3.4; muramic acid, 5.1; glucosamine, 8.8.

Time in trichloroacetic acid (days)	Reducing sugar (as glucose) (mole/10 <sup>4</sup> g.)
0	0.005
1	0.018
2	0.037
3	0.030
5	0.111

*Fractionation of material extracted by trichloroacetic acid*

The soluble material, rich in glucose, was precipitated with ethanolic potassium acetate, dissolved in water and fractionally precipitated as described in the Methods section. Analytical results for these fractions are presented in Table 4. Although ethanol precipitation produced a solid (fraction 1) with a glucose content about four times that of the original cell-wall preparation, it did not lead to much segregation of the various components of the original extract, except that the muramic acid content of fraction 1 was much reduced.

Table 3. *Effect of trichloroacetic acid on the number of free amino groups in the cell walls of Micrococcus lysodeikticus*

Samples of cell wall were treated with 5% (w/v) trichloroacetic acid at 35° for 4 days.

Amino acid	Percentage of total amino acid in fraction recovered as dinitrophenyl derivative		
	Untreated walls	Residue	Whole treated sample
Glycine	0	0	1.0
Glutamic acid	1.3	2.0	1.2
Alanine	1.1	2.0	3.3
Lysine ( $\alpha,\epsilon$ -bisdinitrophenyl derivative)	0.7	1.7	2.3
Lysine ( $\epsilon$ -dinitrophenyl derivative)	67	75	58

Table 4. *Analysis of fractions obtained from a cell-wall preparation by extraction with 5% trichloroacetic acid at 35° and precipitation with ethanolic potassium acetate*

The combined extract from the first 2 days was freed from trichloroacetic acid and a precipitate was obtained by adding 2.6 vol. of ethanolic potassium acetate (0.5%, w/v). The precipitate was dissolved in water and again precipitated to yield fraction 1 (1.5 vol.) and fraction 2 (2.5 vol.). The figures represent moles/10<sup>4</sup> g. of dry solid. For the combined extract the values are calculated on the basis of the mass of original cell wall used.

Sample	Glucose	Glucosamine	Muramic acid	Alanine	Glutamic acid	Glycine	Lysine
Cell walls (batch 30, freeze-dried)	3.4	8.8	5.1	—	—	—	—
Residue after extraction for 4 days	0.97	7.8	5.8	—	—	—	—
Combined extract from extraction for first 2 days	2.1	1.4	0.52	1.8	0.86	1.0	0.91
Ethanol precipitate (original)	8.3	7.5	2.9	—	—	—	—
Fraction 1 (precipitated at 60% ethanol)	13.5	6.3	0.9	8.0	4.0	4.0	4.2
Fraction 2 (precipitated at 71% ethanol)	9.5	8.5	4.0	5.1	2.5	2.5	2.5

A sample of fraction 1 was subjected to paper electrophoresis at pH 4.7 at 15 v/cm. for 1.5 hr. Strips of paper 1.5 cm. wide were cut out and eluted with water, and samples of the eluates were analysed for glucose. It was found that apart from about 13% of the added material remaining near the origin, most of the glucose (75%) occurred in the band which had moved 7.5 cm. towards the anode. Further samples from this region were hydrolysed and examined for the presence of glucosamine, muramic acid and amino acids. The results showed that the anionic material contained, for each mole of glucose, 0.26 mole of glucosamine, no muramic acid and 0.016 mole of glutamic acid. It appeared therefore that the glucose was present in a polymer that contained almost none of the known components of the cell wall having acidic groups, but which behaved as an anion at pH 4.7. As the compound rich in glucose was now known to contain amino sugar (presumably *N*-acetylated) as well, it became possible to detect it on paper by using the chlorination-potassium iodide-starch method of Rydon & Smith (1952). In this way it was shown that on paper electrophoresis at pH 2.4 the polymer still moved as an anion. Thus it was almost certain that the only ionizable groups available were carboxyl groups, and that the amino groups were blocked.

Since ethanolic precipitation appeared to be an inefficient method for concentrating this material, whereas electrophoresis enabled it to be separated from contaminating amino acids and muramic acid, a further batch was prepared directly from the trichloroacetic acid extracts on a continuous-flow paper-electrophoresis apparatus as described in the Methods section. The chief anionic fraction at pH 4.7 (fraction B) contained only small amounts of muramic acid and amino acids, and glucose made up 34% of its dry weight. Its optical rotation was  $[\alpha]_D^{23.5} + 33.5^\circ$  (*c* 1% in water). Further fractionation of this material by electrophoresis at pH 2.4 yielded a product (*B*<sub>2</sub>) that still moved somewhat towards the anode, and which contained virtually no amino acids.

*Chemical composition of the glucose-amino sugar polymer*

The polymer was now known to contain a high proportion of glucose, some amino sugar (which was only slightly adsorbed on a charcoal column and gave a colour like that of glucosamine in the Elson-Morgan reaction) and carboxyl groups which were not attributable to any of the known components of the cell walls of *M. lysodeikticus*. It seemed likely that these carboxyl groups were present in a compound that was readily broken down under the conditions of acid hydrolysis and had therefore escaped attention. The results of

Czerkawski *et al.* (1963) strongly suggest that the wall as a whole contains such a compound. Likely compounds for this role were hexuronic acids and neuraminic acid, but tests for these gave negative results.

Another type of carboxylic compound that would also give an Elson-Morgan reaction is a 2-amino-2-deoxyhexuronic acid. A sample of the glucose polymer purified by electrophoresis at pH 4.7 (see above) was hydrolysed in a sealed ampoule with *n*-hydrochloric acid at 100° for 1 hr. The hydrolysate was freed from hydrochloric acid and subjected to paper electrophoresis at pH 4.7, together with marker spots of glucosamine and 2-amino-2-deoxyglucuronic acid. Treatment with ninhydrin showed a spot near to the origin in the same position as the marker aminoglucuronic acid, having the same characteristic brown colour which turned to purple on standing. Only a trace of glucosamine was present. Samples of similar hydrolysates run on paper chromatograms also showed brown spots with ninhydrin with these *R* values relative to aminoglucuronic acid: phenol-ammonia, 0.91; pentan-1-ol-pyridine-water, 1.10. In the latter solvent the value for 2-amino-2-deoxygalacturonic acid was 0.68. Thus the unknown compound was clearly not 2-amino-2-deoxygalacturonic acid and appeared to differ also from 2-amino-2-deoxyglucuronic acid.

Samples eluted from the appropriate region of chromatograms run in pentan-1-ol-pyridine-water were converted into the *N*-acetyl derivative, as also were samples of 2-amino-2-deoxyglucuronic acid and 2-amino-2-deoxygalacturonic acid (see Methods section). The products were run on paper chromatograms in butanol-acetic acid-water, and the papers were sprayed as described by Partridge (1948). All gave the purple spots characteristic of acetamido sugars with these *R* values relative to *N*-acetylglucosamine: 2-acetamido-2-deoxyglucuronic acid, 0.48; 2-acetamido-2-deoxygalacturonic acid, 0.51; *N*-acetyl derivative of unknown compound, 0.54. Once more the unknown compound differed from both the two authentic aminohexuronic acids available.

*Breakdown of aminohexuronic acids during acid hydrolysis.* It was found that the aminohexuronic acids gave a colour in the Elson-Morgan reaction (Rondle & Morgan, 1955), with an absorption curve almost identical with that given by glucosamine, each having a maximum at about 530–535 m $\mu$ . The unknown compound derived from hydrolysates of the glucose-amino sugar polymer behaved similarly (Fig. 1), and the Elson-Morgan reaction was used to follow the disappearance of these compounds during acid hydrolysis. Fig. 2 shows the rapidity with which these compounds break down in 0.1 *N*-hydrochloric acid at 100°. The

unknown compound disappeared at a rate intermediate between that of 2-amino-2-deoxyglucuronic acid and 2-amino-2-deoxygalacturonic acid. It would obviously be difficult therefore to obtain quantitative yields of such components by acid hydrolysis of complex polymers, and this aspect of the problem is discussed below. Attempts to obtain the compound as its *N*-acetyl derivative by hydrolysis of the polymer in acetic acid or polystyrene sulphonic acid (Painter & Morgan, 1961) were unsuccessful, although a small amount of material giving the Morgan–Eelson reaction for acetamido sugars appeared in the hydrolysates.

*Reduction of the polymer and identification of hexosamine.* The evidence accumulated so far suggested that the polymer consisted of glucose and an unidentified 2-amino-2-deoxyhexuronic acid, probably present as its *N*-acetyl derivative. The possibility that the unknown compound was, for instance, 2-amino-2-deoxyglucuronic acid with some substituent at C<sub>(3)</sub>, was precluded by the fact that such compounds give a colour in the Elson–Morgan reaction with a peak at 510 m $\mu$  rather than the value of 530 m $\mu$  observed (Cifonelli & Dorfman, 1958). The presence of a substituent at C<sub>(4)</sub> was

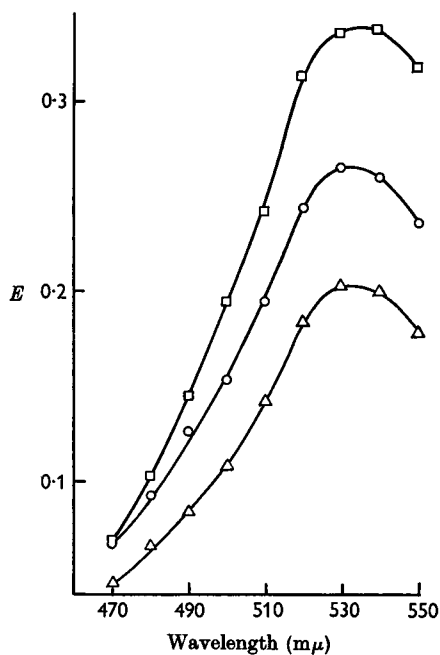


Fig. 1. Absorption curves of the final solutions produced in the Rondle & Morgan (1955) reaction. The final volume in each tube was 3 ml. Extinctions were read in 1 cm. cuvettes.  $\square$ , 2-Amino-2-deoxyglucuronic acid (0.074  $\mu$ -mole);  $\circ$ , glucosamine (0.056  $\mu$ -mole);  $\triangle$ , unknown compound from *M. lysodeikticus* (arbitrary amount eluted from a chromatogram).

also unlikely, since the *N*-acetylated compound gave the Morgan–Eelson reaction, and substitution at C<sub>(4)</sub> is known to prevent this (Kuhn, Gauhe & Baer, 1954). An unknown aminohexuronic acid could be identified by the following reactions. If its carboxyl group were reduced to a primary alcohol group the product would be a hexosamine. Treatment of this hexosamine with ninhydrin would yield a pentose in which the configuration at C<sub>(2)</sub>, C<sub>(3)</sub> and C<sub>(4)</sub> should correspond to the configuration at C<sub>(3)</sub>, C<sub>(4)</sub> and C<sub>(5)</sub> in the original aminohexuronic acid (Stoffyn & Jeanloz, 1954). Thus 2-amino-2-deoxyglucuronic acid or 2-amino-2-deoxymannuronic acid should yield arabinose, and so on.

An attempt to use sodium borohydride to reduce the methyl ester of the polymer as described by Stoffyn & Jeanloz (1960) was unsuccessful. Hydrolysis of the product yielded only unchanged aminohexuronic acid. A sample of the polymer was therefore acetylated and then reduced with diborane as described by Smith & Stephen (1960). A portion of the final product was hydrolysed in 2*N*-hydrochloric acid at 100° for 1 hr. and the hydrolysate was run on a paper chromatogram in pentan-1-ol–pyridine–water together with marker spots of 2-amino-2-deoxyglucuronic acid and glucosamine. The unknown compound suspected of being an aminohexuronic acid, which had been observed in hydrolysates of untreated polymer, was absent. A new ninhydrin-positive spot had appeared, however ( $R_{\text{glucosamine}}$  1.18). More of this new material prepared in the same way was eluted from an unsprayed chromatogram and heated with ninhydrin in aqueous pyridine. The resulting mixture was placed on a paper chromatogram and run in butan-2-one–acetic acid–boric acid with pentose markers. Spraying with aniline phthalate revealed a spot giving the characteristic colour of a pentose in the position corresponding to arabinose. This result suggested that the new compound present in hydrolysates of the reduced polymer was either glucosamine or mannosamine. The discrepancy between  $R_{\text{F}}$  values of the unknown hexosamine and glucosamine mentioned above made it likely that the unknown compound was mannosamine.

It was found that the unknown hexosamine ran in exactly the same position as an authentic sample of mannosamine in pentan-1-ol–pyridine–water and did not correspond to the other hexosamines tested ( $R_{\text{glucosamine}}$ : unknown and mannosamine, 1.18; 1,6-anhydrosamamine, 1.53; gulosamine, 1.06; allosamine, 0.96; talosamine, 1.05). The identity of the unknown hexosamine was confirmed by converting it into its *N*-acetyl derivative. This was compared with *N*-acetylglucosamine and *N*-acetylmannosamine by chromatography on borate-soaked paper in butanol–pyridine–water (Comb &

Roseman, 1958). The unknown ran identically with *N*-acetylmannosamine (*R* relative to *N*-acetylglucosamine 0.38). Since the hexosamine present in the reduced polymer was clearly mannosamine, it follows that the amino sugar present in the original polymer was almost certainly 2-amino-2-deoxymannuronic acid. At present an authentic sample of this compound is not available for comparison.

*Quantitative composition of the glucose-amino sugar polymer.* From the qualitative results already presented it is clear that the polymer under investigation contains glucose and 2-acetamido-2-deoxymannuronic acid and no other recognizable components. No method exists for the measurement of combined aminohexuronic acid. The rapid breakdown of these compounds in hot dilute hydrochloric acid (Fig. 2) means that estimation by the Elson-Morgan reaction of the amount of amino sugar liberated during acid hydrolysis of the polymer is most unlikely to give a correct answer. The best yield of chromatographically isolated aminomannuronic acid obtained in practice (hydrolysis in *N*-hydrochloric acid, 100°, 1 hr.) represented only 0.05 mole compared with the glucose present, assuming that the colour value given in the Rondle & Morgan (1955) reaction was the same as for aminoglucuronic acid. However, since glucose only accounted for about 35% of the dry weight of the polymer, it seemed likely that the true content of aminohexuronic acid was much higher.

It is known that glycosides of amino sugars are much more stable to acid hydrolysis than their *N*-

acylated derivatives (Moggridge & Neuberger, 1938; Foster, Horton & Stacey, 1957). Anomalous results obtained when the glucose-aminohexuronic acid polymer was hydrolysed with more concentrated acid suggested that the low yields of isolated aminomannuronic acid might be attributable to this factor. A sample of polymer was hydrolysed in 4*N*-hydrochloric acid at 100° for 15 min., and amino sugar was estimated by the Elson-Morgan reaction, with glucosamine as a standard. The value obtained corresponded to 0.83 mole for each mole of glucose known to be present, suggesting that in the whole polymer there might be equimolar amounts of glucose and aminomannuronic acid. However, chromatographic examination of a similar hydrolysate showed that only a small amount of free aminomannuronic acid was present, the bulk of the material being present on the base line. A possible explanation of this result is that the strong acid hydrolysis quickly removed the *N*-acetyl groups to give an acid-resistant polyglycoside, which under the alkaline conditions of the Elson-Morgan reaction was broken down to yield the characteristic chromogen. Indirect methods had to be used therefore to establish the molar proportions of glucose and aminomannuronic acid present in the polymer.

A sample of polymer known to be free from other constituents of the whole cell wall (*B*<sub>2</sub>) was dialysed against a large volume of distilled water and used for analysis. The free carboxyl group present was titrated against standard alkali, and total nitrogen, acetyl and glucose were estimated with these results: glucose, 1.00 mole; total nitrogen, 0.98 mole; acetyl, 0.95 mole; carboxyl group, 1.05 equiv. It seems probable therefore that the polymer consists of equimolar proportions of glucose and 2-acetamido-2-deoxymannuronic acid. On this basis the amount of glucose present after hydrolysis should represent 47.5% of the dry weight of the polymer. The glucose content of the sample *B*<sub>2</sub> after dialysis and drying *in vacuo* over phosphorus pentoxide at 20° was 35.5% (the highest recorded), corresponding to 75% of the calculated value. This discrepancy between the observed and calculated values may have been due to inadequate drying of the sample, although, of course, it remains possible that yet another unrecognized component makes up the difference.

*Liberation of ammonia during acid hydrolysis.* Czerkawski *et al.* (1963) studied the rate of liberation of ammonia from a preparation of the cell walls of *M. lysodeikticus* during hydrolysis with 4*N*-hydrochloric acid. They found that ammonia was rapidly set free at first, and only subsequently was the rate reduced to a lower value corresponding to the observed disappearance of glucosamine and muramic acid. The number of moles of ammonia

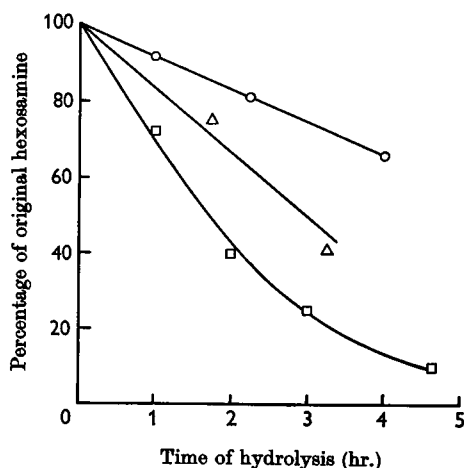


Fig. 2. Acid hydrolysis of aminohexuronic acids. Samples were hydrolysed in sealed ampoules in 0.1*N*-HCl at 100°. Amino sugar was estimated by the method of Rondle & Morgan (1955). O, 2-Amino-2-deoxygalacturonic acid; □, 2-amino-2-deoxyglucuronic acid; Δ, unknown compound isolated from *M. lysodeikticus*.

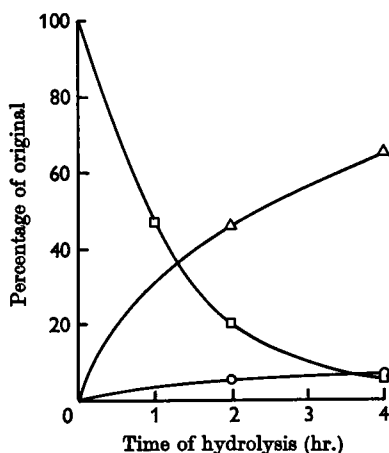


Fig. 3. Liberation of  $\text{NH}_3$  during acid hydrolysis of amino-glucuronic acid and glucose-amino sugar polymer. Samples of 2-amino-2-deoxyglucuronic acid and of the glucose-aminomannuronic acid polymer (fraction B) were hydrolysed in 4N-HCl at  $100^\circ$ . The graph shows the disappearance of 2-amino-2-deoxyglucuronic acid (as measured by the Elson-Morgan reaction) and the number of moles of  $\text{NH}_3$  liberated either from 1 mole of aminoglucuronic acid, or an amount of polymer containing 1 mole of glucose (as measured by the anthrone reaction). □, Moles of amino-glucuronic acid; △, moles of  $\text{NH}_3$  liberated from amino-glucuronic acid; ○, moles of  $\text{NH}_3$  liberated from the polymer.

set free rapidly was equal to the number of moles of glucose present. The discovery of the presence of a polymer containing equimolecular proportions of glucose and the acid-labile aminomannuronic acid suggested that the latter component might give rise to the ammonia. Samples of 2-aminoglucuronic acid and the glucose-aminomannuronic acid polymer were hydrolysed in 4N-hydrochloric acid at  $100^\circ$  in sealed ampoules, and the amount of ammonia liberated was estimated. The results are shown in Fig. 3. Contrary to expectations, although ammonia appeared during hydrolysis of aminoglucuronic acid, very little was liberated from the polymer. A possible explanation of this paradox is discussed below.

#### DISCUSSION

The analysis of the cell walls of *M. lysodeikticus* by Czerkawski *et al.* (1963) showed that the material contained an excess of acetyl groups and of ammonia liberated during acid hydrolysis beyond what would be expected from the most probable structure built up of known constituents. The number of moles of each of these excess components was approximately equal to the number of moles of

glucose present. It seemed possible therefore that an unknown molecule was present in an amount about equimolar with the glucose, which contained an acetyl residue and a carboxyl group and yielded ammonia on acid hydrolysis. The identification in the present work of a polymer derived from the same cell-wall preparation containing equimolar proportions of glucose and acetamidomannuronic acid provides a possible explanation. Acetamidomannuronic acid contains the necessary acetyl and carboxyl groups, and will yield ammonia on acid hydrolysis. However, acid hydrolysis of the isolated and purified polymer did not yield more than about 0.07 mole of ammonia compared with the glucose present. It may well be that, in the intact cell-wall structure, the order in which the various acid-labile bonds are hydrolysed is different from that in the isolated polymer. If in the whole structure the acetyl substituents of aminomannuronic acid are protected from acid hydrolysis, then the glycosidic links joining it to the next sugar molecule will be relatively acid-labile (Moggridge & Neuberger, 1938; Foster *et al.* 1957). Once acetamidomannuronic acid is set free, it will be readily broken down to yield, amongst other things, ammonia. On this hypothesis the acetyl groups in the isolated polymer, being relatively unprotected, would be removed first to give a polymer, which was only slowly hydrolysed to yield aminomannuronic acid and thence ammonia. This reasoning would account for the fact that, although small yields of the ninhydrin-positive aminomannuronic acid can be found in hydrolysates of the isolated polymer, it has never been recorded as a component of hydrolysates of the whole cell wall of this or any other organism.

Aminogalacturonic acid has been found in Vi antigen preparations from *Salmonella typhosa*, *Escherichia coli* and *Paracolobactrum ballerup* (Heyns *et al.* 1959). This is one of the more acid-stable aminohexuronic acids (see Fig. 2), and it is possible that this class of compound may be found in other bacterial products. Indeed Morse (1962) has already obtained antigenic material from a strain of *Staphylococcus aureus* that contains a component resembling the aminohexuronic acids in some of its properties. Brief acid hydrolysis of a sample of this material supplied by Dr Morse, followed by paper chromatography, has shown that it indeed contains a substance giving the brown ninhydrin colour characteristic of aminohexuronic acids. The  $R_f$  would suggest that this substance was not aminomannuronic acid or aminogalacturonic acid, but could possibly be aminoglucuronic acid.

The mode of linkage between the glucose-aminomannuronic acid polymer and the mucopolypeptide part of the structure remains unknown.



Although the carboxylic acid groups could be held by amide linkages, the results in Table 3 show that at most only a small proportion of them could have been bound in this way, at least in that part of the polymer which was extracted by trichloroacetic acid at 35°. Similarly, if the polymer were held by glycosidic bonds, then only an occasional bond at the end of a long chain could be involved (Table 2). Another possible type of covalent binding which has not been examined here is an ester link. Such a bond could conceivably be hydrolysed slowly under the conditions used for extraction. However, the results of Czerkawski *et al.* (1963) would suggest that most of the carboxyl groups were free in the complete wall structure, since they could be reversibly titrated.

#### SUMMARY

1. A glucose-rich polymer can be extracted from cell walls prepared from *Micrococcus lysodeikticus* either by hot formamide or trichloroacetic acid at 35°.

2. The glucose-rich polymer was acidic but contained no amino acids, glucosamine or muramic acid.

3. The acidic component of the polymer was found to be an acid-labile aminohexuronic acid.

4. Reduction of the carboxyl groups of the polymer to primary alcohol groups gave rise to mannosamine, suggesting that the acidic component was 2-amino-2-deoxymannuronic acid.

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