Studies on the Cell Walls of *Micrococcus lysodeikticus*. Fractionation of the Nondialyzable Components from a Lysozyme Digest of Cell Walls†

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ABSTRACT: The nondialyzable material from a lysozyme digest of *Micrococcus lysodeikticus* cell walls was fractionated by DEAE-cellulose column chromatography. The main fraction which was rich in glucose was further divided by gel filtration into several subfractions differing in peptide:glucose ratios. Trichloroacetic acid treatment of the glucose-rich fraction yielded the glucose-*N*-acetylaminomannuronic acid polymer of Perkins linked to short fragments of murein polysaccharide backbone. The trichloroacetic acid treatment had removed most of the murein amino acids. The most highly purified glucose-*N*-acetylaminomannuronic acid polymer still contained phosphate, muramic acid, and glucosamine but insignificant amounts of amino acids. This suggests that the glucose-*N*-acetylaminomannuronic acid polymer linkage is to the backbone polysaccharide of murein and may involve muramic acid 6-phosphate.

Studies on the structure of bacterial cell walls have made great progress in the last decade. The chemical structure of the basal layer of the walls, *i.e.*, murein, has been determined in detail for a number of bacteria (Rogers and Perkins, 1968; Ghuysen, 1968). This progress resulted from the characterization of fragments of murein obtained by degradation of the insoluble wall with specific lytic enzymes and of other fragments of murein found attached to uridine diphosphate and known to be precursors of murein. Another area of rapid progress has been the isolation and partial characterization of a wide variety of species specific polysaccharides such as the teichoic acids and the group specific carbohydrates of the streptococci. These polymers are, in general, tightly associated with the cell wall through covalent linkage to the basal layer (Knox and Holmwood, 1968; Liu and Gotschlich, 1967; Hall and Knox, 1965; Hay *et al.*, 1965; Hughes, 1965; Kanetsuna, 1968; Misaki *et al.*, 1966; Ghuysen *et al.*, 1962, 1965; Button *et al.*, 1966).

An area in which progress has been slow is elucidation of the exact linkage between these various polymers and murein. There is evidence in the case of certain teichoic acids that the link may be a phosphodiester joining the terminal phosphate of the teichoic acid to C-6 of the muramic acid of the murein (Ghuysen *et al.*, 1965; Button *et al.*, 1966). In other polysaccharides the linkage to murein remains obscure.

In *Micrococcus lysodeikticus*, Perkins (1963) has shown that the wall contains a polymer composed of glucose and *N*-acetylaminomannuronic acid in roughly equal molar amounts. One of the objectives of the present work was to isolate this polymer, and specifically that portion of the polymer still attached to a fragment of murein in order to determine the nature of the linkage between polymers and to be able to elucidate the structure of the glucose-containing polymer.

Lysozyme digests of *M. lysodeikticus* cell walls have been of great value in elucidating the structure of murein. The unit disaccharide (Salton and Ghuysen, 1959, 1960; Perkins, 1960; Sharon *et al.*, 1966; Jeanloz *et al.*, 1963), the tetrasaccharide (Salton and Ghuysen, 1959, 1960), and their peptide-containing derivatives (Ghuysen, 1961; Mirelman and Sharon, 1966, 1967) have been characterized after isolation from the dialyzable portion of lysozyme digests. The nondialyzable portion of the lysozyme digest has not been studied in detail. This paper reports on the fractionation and characterization of the components of the nondialyzable fraction and in particular the isolation of the glucose-containing polymer.

Experimental Section

Materials

Micrococcus lysodeikticus Cell Walls. Dried cells of *M. lysodeikticus*, strain ATCC 4698 (obtained from Miles Chemical Co., Elkhart, Indiana), were shaken with Ballotini beads for 5 min in a Braun disintegrator with cooling. The cell walls were isolated and washed repeatedly by differential centrifugation.

Methods

Peptide determination was as done by Lowry *et al.* (1951).

Phosphate determination was as done by Chen *et al.* (1956). The samples were first ashed with 0.06 ml of 10% $Mg(NO_3)_2$ in ethanol until brown fumes appeared followed by heating at 100° in 0.3 ml of 1 N HCl for 15 min to convert pyrophosphate to inorganic phosphate.

Neutral Sugar Determination. ORCINOL REAGENT. Orcinol (1.37 g; recrystallized from benzene) was dissolved in 65% w/w H_2SO_4 (215 ml of concentrated H_2SO_4 mixed cautiously with 204 ml of H_2O).

PROCEDURE. Sample (0.2 ml) was mixed with 2.0 ml of orcinol reagent, heated at 100° for 15 min, and cooled. The color densities of the unknowns were compared with the densities obtained with glucose standards.

Determination of Component Amino Acids and Amino Sugars. Samples (*ca.* 3 mg) were hydrolyzed 2 hr in sealed tubes with

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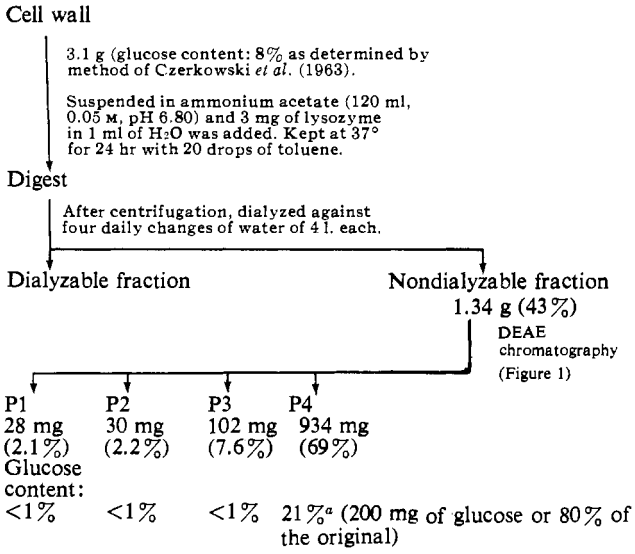
SCHEME I: Digestion of *Micrococcus lysodeikticus* Cell Walls and Fractionation of the Digest.



[a] In this paper, per cent values were not corrected for water of hydrolysis.

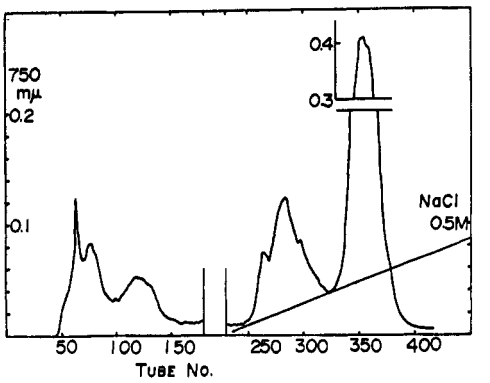


FIGURE 1: DEAE chromatography of the nondialyzable fraction. Column: 3 × 55 cm. Charge: 1.34 g of the nondialyzable fraction (NDF) of the lysozyme digest of *M. lysodeikticus* cell walls. Fraction flow rate: 6 ml/15 min. Optical densities: Lowry reaction (750 mμ) for 0.02 ml aliquots. Fractions pooled: P1 (tubes 40–94); P2 (tubes 95–160); P3 (tubes 250–315); P4 (tubes 330–390).

0.6 ml of 6 N HCl at 121° and the hydrolysates were evaporated to dryness over H_2SO_4 and moist NaOH pellets. The residues were dissolved in water and held at pH 8.0 for 2 hr at room temperature, then the pH was lowered to about 3.0. After clarification by centrifugation, aliquots were assayed by a Technicon Autoanalyzer with a known amount of norleucine as the internal standard. The procedure in the Technicon manual was modified by adjusting the pH of the first buffer to 3.05. This caused glucosamine to be eluted after alanine rather than overlapping it, so that each component, including the norleucine internal standard, was well separated from the others. In every series of experiments a known mixture of the component amino sugars and amino acids (with and without glucose) was hydrolyzed and analyzed in like manner to get the standard values for each component. It is essential both to hydrolyze the known mixtures and to treat all samples at pH 8.0 after removal of HCl in order to obtain true values for muramic acid and glucosamine.

Paper Chromatography of Neutral Sugars. Materials were hydrolyzed 2 hr in 2 N H_2SO_4 at 100° in sealed tubes. The hydrolysates were diluted with H_2O and neutralized with $Ba(OH)_2$ solution. After centrifugation, the supernatants were passed through a Dowex 50 (H^+) column. The solutions obtained were taken to dryness under reduced pressure. The residues were analyzed by paper chromatography using 1-butanol–acetic acid–pyridine–H_2O (60:3:40:30) as a moving phase and aniline hydrogen phthalate as a developer (Partridge, 1949).

DEAE-cellulose Column Chromatography. Materials were charged on a DEAE-cellulose column equilibrated with 0.005 M phosphate buffer (pH 7.0) containing 0.02 M NaCl and then eluted with the same buffer system followed by a linear gradient of NaCl in 0.005 M phosphate buffer.

Results

Fractionation of Cell Wall Lysozyme Digests. The cell wall material was digested by egg-white lysozyme and the soluble, nondialyzable part was then fractionated on DEAE-cellulose as shown in Scheme I and Figure 1. After elution of the second broad peak (P2), the other two peaks (P3 and P4) were eluted by a linear gradient of NaCl. The main fraction, P4, contained 69% of the nondialyzable fraction. This was the only fraction with glucose and it contained about 80% of the glucose in the original cell wall material. In a preliminary small scale experiment the elution pattern of material containing glucose closely paralleled P4.

Table I shows the analytical data of each peak. The hydrolysates of each contained equimolar amounts of muramic acid and glucosamine. Each peak also contained the amino acids glutamic acid, glycine, alanine, and lysine in the ratio 1:1:2:1 which is characteristic of *M. lysodeikticus* murein. However, the ratio of peptide to muramic acid varied. P1 and P3 were peptide rich whereas P2 and P4 had about equal amounts of peptide and muramic acid as did the original cell wall material. P4 differed from P2 in containing the glucose-*N*-acetylaminomannuronic acid polymer. This was indicated by the fact that P4 was the only fraction to contain glucose and to form a large amount of humin-like substance and ammonia on acid hydrolysis. Thus P4 seemed to correspond to a glucose-*N*-acetylaminomannuronic acid polymer (Perkins, 1963) attached to solubilized murein.

Gel Filtration. The main fraction obtained in DEAE chromatography (P4) was further fractionated by gel filtration. It eluted from a Bio-Gel P-60 column as a single peak with a trailing edge (Figure 2), but, as indicated by the elution pattern from a Bio-Gel P-150 column (Figure 3), it seemed to be composed of at least two main glucose-containing parts. The larger molecule contained a considerable amount of peptide and the other was relatively poor in peptide content.

When water was used as the eluting solvent instead of the NaCl–phosphate buffer system a much finer fractionation resulted even on a Bio-Gel P-60 column (Figure 4A). Each of the three main components from this column were rerun through the same column (Figure 4B–D). As can be seen, each main component retained its characteristic elution properties and was further purified by this procedure. Thus three glucose-containing fractions were obtained which differed in their peptide content. This indicates that P4, as obtained by DEAE chromatography, was a mixture of several polymers which differed in their murein:glucose-*N*-acetylaminomannuronic acid polymer ratios.

TABLE I: Analysis of Each Fraction from DEAE Chromatography.

Fraction	μmole/mg (Molar Ratios to Mur)								
	Mur[a]	GlcN[a]	Glu[a]	Glycine	Alanine	Lysine	Glucose	NH_3[b]	P[a,c]
P1	0.69	0.71	1.25	1.29	2.53	1.23	<0.06		0.013
	(1.0)	(1.0)	(1.8)	(1.9)	(3.7)	(1.8)	<(0.09)		
P2	0.94	0.99	1.01	1.05	2.10	1.01	<0.06		
	(1.0)	(1.1)	(1.1)	(1.1)	(2.2)	(1.1)	<(0.06)		
P3	0.76	0.77	1.08	1.16	2.30	1.01	<0.06		0.014
	(1.0)	(1.0)	(1.4)	(1.5)	(3.0)	(1.3)	<(0.08)		
P4	0.42	0.45	0.46	0.42	0.82	0.39	1.17	0.91	0.063
	(1.0)	(1.1)	(1.1)	(1.0)	(2.0)	(0.9)	(2.7)	(2.1)	

[a] Mur, muramic acid; GlcN, glucosamine; Glu, glutamic acid; P, organic phosphate. [b] From *N*-acetylaminomannuronic acid. [c] From samples obtained in a separate DEAE chromatography experiment.

Trichloroacetic Acid Treatment of P4. Perkins had originally obtained the glucose-*N*-acetylaminomannuronic acid polymer (GM-polymer) almost free of murein components directly from the cell wall material by extraction with 5% trichloroacetic acid at 35°. Consequently, in order to obtain GM-polymer from P4, P4 was digested in 5% trichloroacetic acid solution at 37° for 10 days. During this treatment, gradual and continuous increases in ninhydrin value and reducing power were observed, suggesting nonspecific splitting of peptide, amide, and glycosidic bonds rather than hydrolysis of a specific linkage between the GM-polymer and murein. Trichloroacetic acid was extracted with ether and the aqueous layer was dialyzed against water. The small molecular weight ninhydrin- and orcinol-positive substances were removed by dialysis and the nondialyzable material was fractionated by chromatography on DEAE in the same way as in Figure 1. In this case, the bulk of the peptide appeared soon after the start of the NaCl gradient in a broad peak and the orcinol-positive material followed in a separate but overlapping peak as shown in Figure 5.

To remove the peptide-positive material more completely from the glucose-containing polymer, the fractions containing orcinol-positive material (tubes 300–380) were combined, concentrated under reduced pressure, dialyzed to remove inorganic salts, and then passed through a Dowex 50 (H^+) column twice. Dowex 50 (H^+) firmly adsorbed the peptide-containing material whereas the bulk of the glucose-containing polymer passed through the column without being adsorbed. This material was passed through a Bio-Gel P-150 column (as in Figure 3) and the orcinol-positive fraction was dialyzed and freeze-dried to yield 34 mg from 140 mg of P4.

In a separate experiment, the glucose-rich, peptide-poor part of P4 obtained without trichloroacetic acid treatment, that is, the main peak from Bio-Gel P-60 gel filtration (Figure 4D), was also treated with Dowex 50 (H^+) and Bio-Gel P-150 as above (Figure 6).

Table II shows the analysis of the various glucose-containing fractions obtained. As can be seen, of the three components isolated from P4, 1–1 had a molar ratio of pentapeptide (Glu, Gly, Lys, 2 Ala) to amino sugar (Mur) of greater than

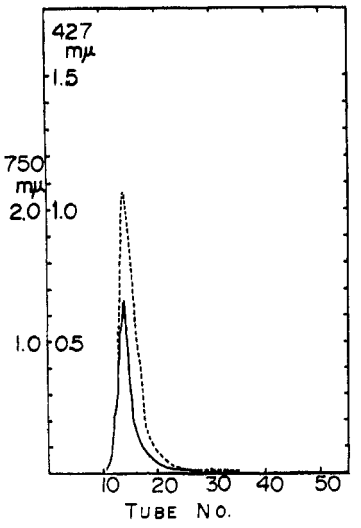


FIGURE 2: Bio-Gel P-60 gel filtration of P4. Column: 1.7 × 50 cm, equilibrated with 0.005 M phosphate buffer–0.5 M NaCl (pH 7.0). Charge: 5.5 mg/0.5 ml. Fraction flow rate: 2.6 ml/15 min. Optical densities: Lowry reaction (750 mμ) for 0.1-ml aliquots (——); orcinol reaction (427 mμ) for 0.1-ml aliquots (- - - - -).

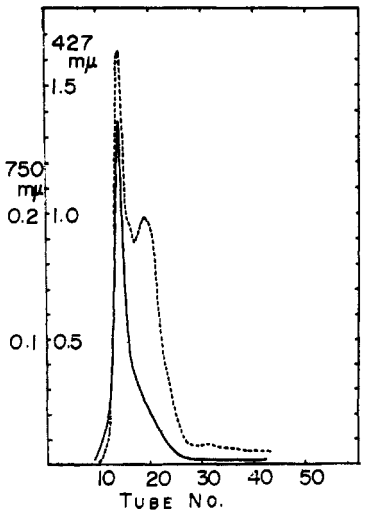


FIGURE 3: Bio-Gel P-150 gel filtration of P4. Column: 1.7 × 52 cm equilibrated with 0.005 M phosphate buffer–0.5 M NaCl (pH 7.0). Charge: 5.7 mg. Fraction flow rate: 2 ml/15 min. Optical densities: Lowry reaction (750 mμ) for 0.2-ml aliquots (—); orcinol reaction (427 mμ) for 0.2-ml aliquots (- - - -).

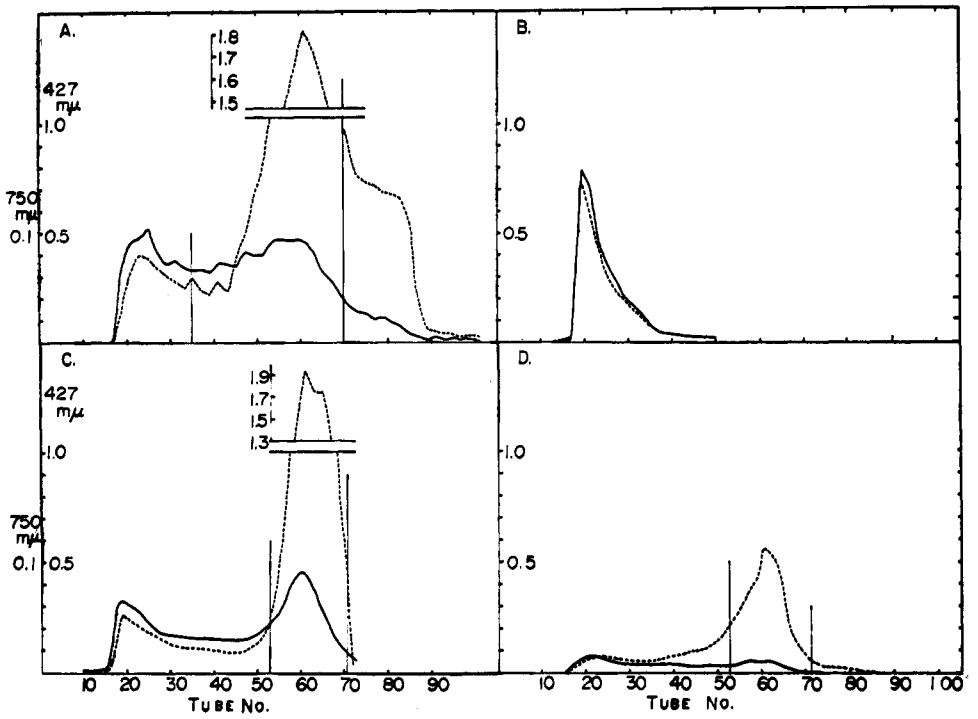


FIGURE 4: Bio-Gel P-60 gel filtration of P4 and refiltration of subfractions. Column: 3 × 62 cm, H_2O as liquid phase. Fraction flow rate: 6.9 ml/16 min. Optical densities: Lowry reaction (750 mμ) for 0.05-ml aliquots (——), except 0.1-ml aliquots were used in part B, orcinol reaction (427 mμ) for 0.05-ml aliquots (- - - - -) except 0.1-ml aliquots were used in part B. (A) Sample = 350 mg of P4/3 ml. (B) Sample = fractions from tubes 18 to 35 from 4A combined and concentrated. (C) Sample = fractions from tubes 36 to 69 from 4A combined and concentrated. (D) Sample = fractions from tubes 70 to 89 from 4A combined and concentrated.

TABLE II: Analysis of the Glucose-Containing Fractions.

Fraction	μmole/mg (Molar Ratios to Mur)						Total Amino Acids (%)	Total Amino Sugars (%)	Glucose (%)	Ratio[i] (P:G)
	Mur[h]	GlcN[h]	Glu[h]	Glycine	Alanine	Lysine				
1–1[a]	0.39	0.46	1.06	1.06	2.15	1.00				
	(1.0)	(1.2)	(2.7)	(2.7)	(5.5)	(2.5)				0.22
2–3[b]	0.32	0.41	0.24	0.25	0.48	0.22				
	(1.0)	(1.3)	(0.8)	(0.8)	(1.5)	(0.7)				0.046
3–3[c]	0.23	0.35	0.07	0.07	0.13	0.06				
	(1.0)	(1.5)	(0.3)	(0.3)	(0.6)	(0.3)	3.4	14.2		0.016
P4[d]	0.22	0.32	0.09	0.09	0.18	0.09				
	(1.0)	(1.4)	(0.4)	(0.4)	(0.8)	(0.4)			31	0.022[f]
CCl_3COOH-	0.13	0.20	0.02	0.03	0.02	0.01				
treated[e] P4[e]	(1.0)	(1.6)	(0.1)	(0.2)	(0.1)	(0.1)	0.75	8.2	41[g]	0.003[g]

[a] Main peak shown in Figure 4B. [b] Main peak shown in Figure 4C. [c] Main peak shown in Figure 4D after Dowex-50 (H^+) treatment and Bio-Gel P-150 gel filtration. [d] After Dowex 50 (H^+) treatment. [e] After Dowex 50 (H^+) treatment and Bio-Gel P-150 gel filtration. [f] Before Dowex 50 (H^+) treatment, this ratio was 0.079. [g] Before Bio-Gel P-150 gel filtration. Other analytical data were almost the same both before and after this gel filtration. [h] Abbreviations as in Table I. [i] Ratio of peptide to glucose.

2.5 while in 2–3 the ratio (peptide:Mur) was somewhat less than 1 and in 3–3 the peptide:Mur ratio was 0.3. Presumably some peptide-rich polymers were removed from fraction 3–3X by the additional treatment with Dowex 50 (H^+) and filtration through Bio-Gel P-150. As can also be seen from Table II, trichloroacetic acid treated P4 purified by Dowex 50 (H^+) treatment and Bio-Gel P-150 gel filtration yielded a product rich in the glucose-*N*-acetylaminomannuronic acid polymer (94% assuming 1:1 glucose:*N*-acetylaminomannuronic acid), but which still contained significant amounts of amino sugars and traces (less than 1%) of amino acids.

Figure 7 shows the paper electrophoretic behavior of GM-polymer from trichloroacetic acid treated and original nondialyzable fraction. From the results in the three different buffer systems, it seems clear that trichloroacetic acid treatment has hydrolyzed the nondialyzable fraction in a manner

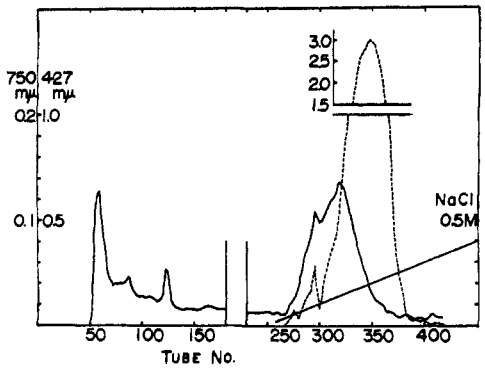


FIGURE 5: DEAE chromatography of trichloroacetic acid treated P4. Column: 1.7 × 55 cm. Charge: trichloroacetic acid treated P4 (140 mg of original P4). Fraction flow rate: 2 ml/15 min. Optical densities: Lowry reaction (750 mμ) for 0.1-ml aliquots (——); orcinol reaction (427 mμ) for 0.1-ml aliquots (- - - -).

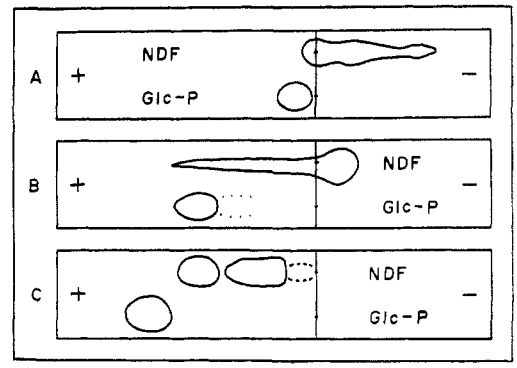


FIGURE 7: Paper electrophoresis of GM-polymer. (A) pH 2.6 in 1 N acetic acid; 10 V/cm, 1 hr. (B) pH 5.4 in pyridine–acetic acid buffer (acetic acid–pyridine–H_2O, 2:4:1000); 10 V/cm, 2.5 hr. (C) pH 9.0 in 0.025 M borate buffer; 10 V/cm, 3 hr. GM-polymer: fractions obtained by stepwise elution (NaCl concentration, 0.2–0.25 M) from DEAE column of trichloroacetic acid treated nondialyzable fractions. Papers were stained by Cl_2–KI–starch method (Rydon and Smith, 1952).

which releases the GM-polymer from the large fragments of murein with their free amino groups. As shown in Table II, trichloroacetic acid–P4 still contained a considerable amount of amino sugars (*ca.* 8%). The nature of the bonds split by trichloroacetic acid is obscure, but since GM-polymer still contains the amino sugars glucosamine and muramic acid but only traces of amino acids, the GM-polymer is clearly linked to the murein disaccharide oligomer which remains after lysozyme and trichloroacetic acid digestion.

Phosphate Content of Various Fractions. The *M. lysodeikticus* cell walls and the nondialyzable fraction of the lysozyme digest of cell walls contained 0.05 μM organic phosphate/mg. As shown in Table I, P-4, the only fraction from DEAE chromatography containing the glucose-*N*-acetylaminomannuronic acid polymer, was slightly enriched in organic phosphate whereas the fractions lacking GM polymer contained considerably less phosphate. Trichloroacetic acid treatment of P-4 followed by treatment with Dowex 50 (H^+) resulted in a further enrichment of phosphate in the GM-polymer fraction.

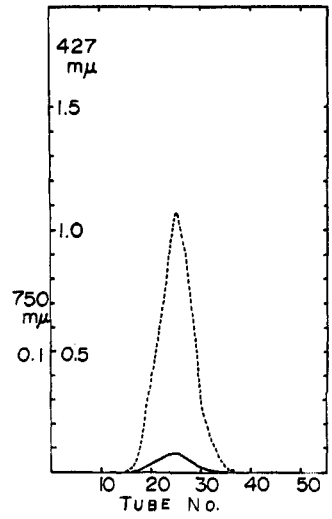


FIGURE 6: Bio-Gel P-150 gel fitration of Dowex treated 3–3. Column and fraction flow rate: same as in Figure 3. Sample = combined fractions from tubes 54 to 71 in Figure 4D after Dowex 50 (H^+) treatment and concentration. Optical densities: Lowry reaction (750 mμ) for 0.03-ml aliquots (——); orcinol reaction (427 mμ) for 0.03 ml aliquots (- - - -).

Nature of the Phosphate in P-4 GM-polymer. Liu and Gotschlich (1967) reported that 7.1% of the muramic acid in *M. lysodeikticus* cell walls was present as muramic acid 6-phosphate. Since this would suggest that over 75% of the phosphate of purified cell walls from *M. lysodeikticus* was present as muramic acid 6-phosphate, and the remainder was likely due to contaminating membrane phospholipids (Mirelman *et al.*, 1971), it came as no surprise to find that the phosphate present in P-4 was muramic acid 6-phosphate. A 127-mg sample of P-4 was hydrolyzed in 6 N HCl at 110° for 75 min. After removal of HCl and passage through an 11 × 0.8 cm column of Dowex 50 (H^+), a sample was recovered which contained 4.6 μM total phosphate. A similar hydrolysate of *M. lysodeikticus* cell walls was prepared for comparison. By paper chromatography, both samples contained a ninhydrin-positive organic phosphate component (the principal phosphate-containing compound) which moved 4.6–4.8 cm in 18 hr in 1-butanol–acetic acid–water (3:1:1). When aliquots of the samples were incubated with alkaline phosphatase overnight at room temperature in 0.2 M ammonium carbonate (pH 9.6) with 3 μl of *Escherichia coli* alkaline phosphatase (Sigma) followed by paper chromatography, the spot originally present at 4.6 cm had disappeared, and a new ninhydrin-positive spot corresponding to muramic acid (12.7 cm) appeared. No spot corresponding to glucosamine (8.7 cm) appeared. By quantitative analysis on a Beckman amino acid analyzer, over two-thirds of the phosphate in the hydrolysate of P4 was present as muramic acid 6-phosphate, assuming muramic acid 6-phosphate and glutamic acid give the same color yield with ninhydrin (peak at 56 min *vs.* 99 min for glutamic acid). Thus, it is concluded that the phosphate attached to the GM-polymer is muramic acid 6-phosphate.

Another sample of GM-polymer was obtained from nondialyzable fraction following Dowex 50 (H^+) treatment by digestion with *Flavobacterium* L_{11} muramyl-L-alanine amidase (Kato *et al.*, 1962) and then again with lysozyme followed by Dowex 50 (H^+) treatment. This sample contained 0.084 μmole/mg of phosphoric acid, 0.22 μmole/mg of muramic acid, and 0.30 μmole/mg of glucosamine. The phosphate was not released by either alkaline or acid phosphatase. Thus it appears that the fragment of murein to which GM-polymer is

attached contains phosphate, muramic acid, and glucosamine but no amino acids.

Discussion

We have shown that the nondialyzable material from a lysozyme digest of *M. lysodeikticus* cell walls can be separated into four fractions by DEAE chromatography. The three smallest fractions contained essentially equimolar amounts of glucosamine and muramic acid and a variable amount of the peptide containing glutamic acid, glycine, lysine, and alanine in the ratio 1:1:1:2. These fractions lacked the glucose-*N*-acetylaminomannuronic acid polymer. The main fraction contained this polymer in addition to muramic acid, glucosamine, glutamic acid, glycine, lysine, and alanine in the ratio 1:1:1:1:1:2.

Although the main fraction could not be fractionated further on the basis of size when passed through a column of Bio-Gel P-60 in the presence of NaCl and phosphate buffer, separation into three different fractions could be achieved on Bio-Gel P-60 with water as elutrient. The lower the peptide content of the polymer the longer it was retained by the Bio-Gel though the size of the polymers was mainly due to the glucose-*N*-acetylaminomannuronic acid polymer. A more highly purified sample of the glucose-*N*-acetylaminomannuronic acid polymer was obtained following treatment with trichloroacetic acid and Dowex 50 (H^+). This preparation was low in peptide content and appeared to contain about one-third less muramic acid than glucosamine. In fact, this apparent deficiency of muramic acid was seen in all preparations as the amount of murein components remaining attached to the glucose-*N*-acetylaminomannuronic acid polymer decreased.

The apparent low muramic acid content of the more highly purified GM-polymer preparations is probably caused by the increased proportion of muramic acid present as muramic acid 6-phosphate. Liu and Gotschlich (1967) first reported the presence of muramic acid 6-phosphate in *M. lysodeikticus* walls, a fact we confirm, and the organic phosphate in the preparations was found to increase with increasing GM-polymer content. It is roughly estimated that under the conditions used for acid hydrolysis (6 N HCl, 2 hr, 121°) less than 50% of the muramic acid 6-phosphate would be hydrolyzed. The unhydrolyzed material is not included in our analyses for muramic acid and hence one would expect the greatest apparent losses of muramic acid in the purified GM-polymer fractions which contain the most phosphate along with a lowest murein content. Thus the various steps involved in purification of the GM-polymer resulted in the loss of murein amino acids, the retention of a significant amount of murein amino sugars, and an enrichment in the phosphate content of the material. In order to confirm these observations on material obtained by gentler means, a sample of comparable purity was obtained without trichloroacetic acid treatment by use of *Flavobacterium* L_{11} muramyl-L-alanine amidase to remove peptide. This sample also contained almost 40% more glucosamine than muramic acid and in addition contained 1 mole of phosphate for every 2.6 moles of muramic acid.

These results suggest that the glucose-*N*-acetylaminomannuronic acid polymer linkage is to the backbone polysaccharide of murein and may involve muramic acid 6-phosphate.

A paper by Campbell *et al.* (1968) has recently appeared which also relates the presence of muramic acid 6-phosphate to the linkage between GM-polymer and murein. Their results and ours are in essential agreement that the GM-polymer as isolated, albeit after different treatments, remains attached to a fragment of murein consisting of perhaps a hexasaccharide that includes one muramic acid 6-phosphate.

The present work shows additionally that a GM-polymer–murein–glycan complex free of amino acids can be obtained after treatment with trichloroacetic acid and Dowex 50 (H^+). This indicates clearly that a peptide is not directly involved in the linkage of the two wall polymers. On the other hand, our finding that the murein fragment remains attached to the GM-polymer even after treatment with 5% trichloroacetic acid for 10 days at 37° indicates that the release of GM-polymer from *M. lysodeikticus* walls by trichloroacetic acid (Perkins, 1963) is not due to hydrolysis of the bond which directly links the GM-polymer to the murein fragment whether or not the link is through a phosphodiester bond.

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CORRECTIONS

"Ligase Joining of Oligodeoxythymidylates," by Clifford L. Harvey* and Rosemary Wright, Volume 11, Number 14, July 4, 1972, page 2667.

On page 2668 the reaction mixtures given under the heading "Assay for Joining" and the heading "Large-Scale Preparation of $p(dT)_{18}$" should include 6.6 mM $MgCl_2$.

"Kinetic Studies on Substrate–Enzyme Interaction in the Adrenal Cholesterol Side-Chain Cleavage System," by Shlomo Burstein,* Jane Dinh, Nana Co, Marcel Gut, Heinz Schleyer, David Y. Cooper, and Otto Rosenthal, Volume 11, Number 15, July 18, 1972, page 2883.

On page 2889 (after eq 3a) the expression for m_2 should contain $-4k_1'k_3$ and not $+4k_1'k_3$.