THE CHEMICAL STRUCTURE OF THE CELL WALL OF GRAM-POSITIVE BACTERIA

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I. INTRODUCTION

In the past, the role of organic chemistry has been to establish the structure of natural products, to duplicate these products, and synthesize infinite variations of them for the amelioration of our standard of living. Recently, however, organic chemistry, together with its derived disciplines biochemistry and molecular biology, has broadened in scope and has started to give us basic knowledge about biological processes. This knowledge is valid, however, only when it is based on chemical structures, the identification of which has been made by rigorous methods and eventually supported by synthetic proofs. This knowledge is valid, in other words, only when it is based on the same foundation as the chemistry of natural products has been based in the past.

Most developments in the field of molecular biology have concerned proteins and nucleic acids. There are, however, biological phenomena (for example, the immunological protection of the cell, the duplication of the bacterial cell, and its penetration by viruses) in which carbohydrate structures play a major role. When we come to know the chemical structure of the rigid and insoluble component that gives form and protection to bacteria, we will better understand some important biological phenomena at the molecular level. To take two particularly interesting examples, we will be able to explain in chemical terms the penetration of viruses into the bacterial cell and also the action of antibiotics, such as penicillin, which block the formation of the cell wall. Only recently, moreover, has the spatial structure of egg-white lysozyme, one of the enzymes that cause the lysis of bacteria, been elucidated[1]. When we know completely the chemical structure of the lysozyme substrate, we will be in a position to explain the mechanism of enzyme action.

In recent years, methods have been developed for the preparation of cell wall material devoid of cytoplasmic or membrane material, so that chemical methods may be applied to the purified material. Cell walls of gram-positive bacteria were found to have, in general, a simpler composition than do cell walls of gram-negative bacteria, which have complex protein–lipopolysaccharidic antigens. We have to thank Salton, Work, Cummings, and Westphal, among others, for the pioneering work which determined the composition of bacterial cell walls.

One of the first cell-wall structures to be studied was that of *Micrococcus lysodeikticus,* which is the substrate traditionally used for the study of egg-white lysozyme. Taking advantage of the solubilization of the cell wall by the

enzyme, Salton and Ghuysen[2] and, independently, Perkins[3] have isolated fragments of low molecular weight, for which chemical structures have been proposed. On the basis of this work, a general structure was suggested. Polysaccharide chains composed of alternating units of 2-acetamido-2-deoxy-D-glucose (*N*-acetylglucosamine) and 2-acetamido-3-*O*-(D-1-carboxyethyl)-2-deoxy-D-glucose (*N*-acetylmuramic acid) are linked to a peptide network composed of D- and L-alanine, D-glutamic acid and L-lysine residues. Subsequently, when a polysaccharide composed of D-glucose and 2-amino-2-deoxymannuronic acid was isolated from the same cell wall[4], it was clear that even one of the relatively simple cell walls has a complex chemistry.

In the following pages, I will present the results of studies made in collaboration with Drs. Sharon, Flowers, Osawa, Nasir-ud-Din, Hoshino, Gross, Zehavi, Miss Walker, and Mrs. Jeanloz. These studies were carried out in order to solve some of the problems of carbohydrate chemistry presented by the structure of the cell wall of *M. lysodeikticus*. Specifically, I will discuss our attempt to determine the structure of the *N*-acetylglucosamine-*N*-acetylmuramic acid polysaccharide and its relation with the D-glucose and 2-amino-2-deoxymannuronic acid components; and then I will discuss the chemistry of muramic acid and the chemistry of synthetic substrates of lysozyme.

II. ISOLATION, DEGRADATION, AND FRACTIONATION OF *M. LYSODEIKTICUS* CELL WALLS

The preliminary study[2,3] of the chemical structure of the cell wall of *M. lysodeikticus* had been based on degradation by lysozyme, followed by dialysis and isolation of the components by paper chromatography; structures were proposed for these components on the basis of colour reactions and periodate oxidation. In the present study the dialyzable and nondialyzable materials were obtained under conditions similar to those previously described. They were fractionated by adsorption on columns of acidic and basic resins, on DEAE–cellulose, or by precipitation with cetylpyridinium chloride (*Figure 1*).

In order to obtain sufficient amounts of material to be studied, a large-scale method of preparation was devised[5]. This method was based on the principle that the bacterial cell of *M. lysodeikticus* is disrupted by homogenization in the presence of glass beads. The resulting homogenate was purified by differential centrifugation, and the cell walls were further treated with trypsin. The material thus obtained is insoluble; its purity was controlled by electron microscopy (*Figure 2*) and, after degradation with lysozyme, by ultraviolet adsorption to show the absence of nucleic acids, which would indicate cytoplasmic material. Since the proportion of amino acid and carbohydrate components varies with the conditions of bacterial growth (and may also depend on the method by which the cell wall is prepared) various results have been published. A typical preparation shows the following relative proportions for each molecule of glutamic acid: 1 molecule of lysine, 1·5 molecules of glycine, 2·5 molecules of alanine, 1 molecule of 2-acetamido-2-deoxyglucose, slightly less than 1 molecule of 2-acetamido-3-*O*-(D-1-carboxyethyl)-2-deoxyglucose, 3 molecules of glucose, and an

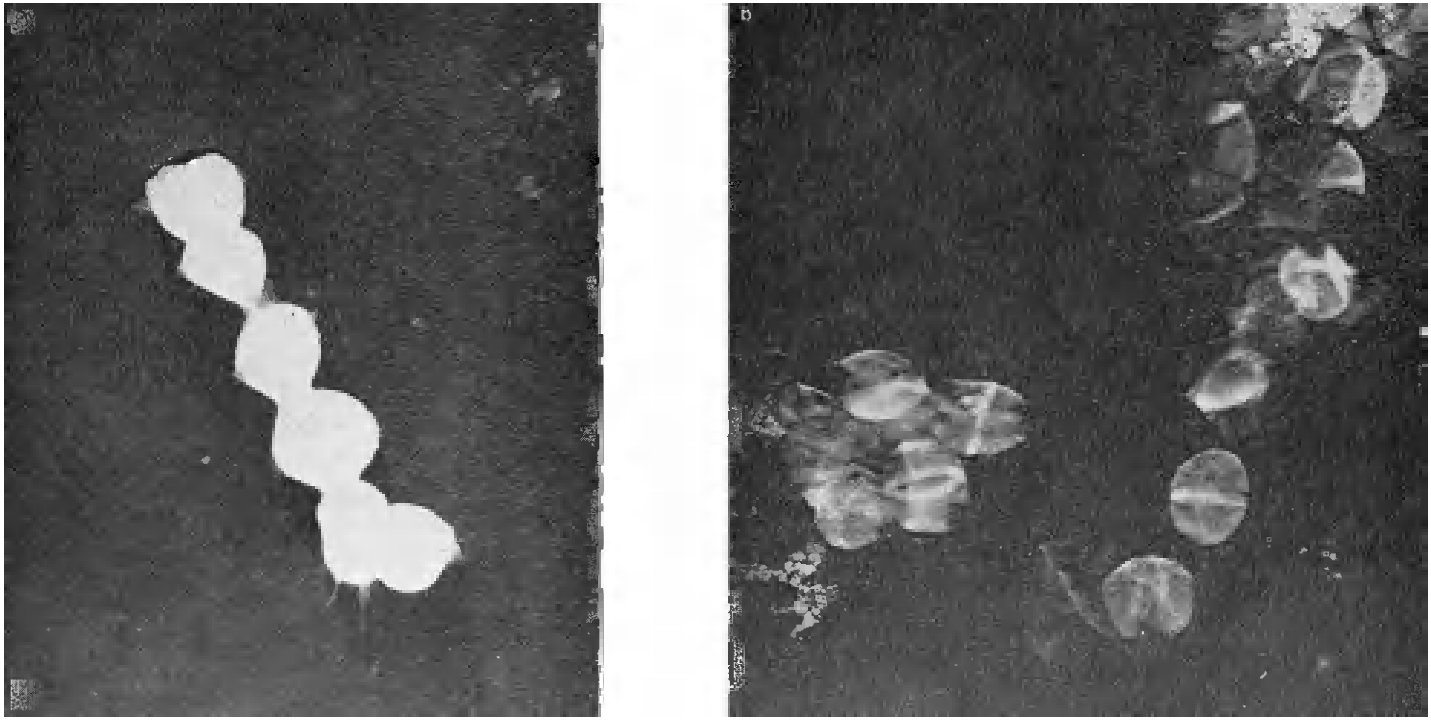

Figure 2. Electron micrographs (shadowed with chromium) of: (left) *M. lysodeikticus* cells; and (right) of isolated *M. lysodeikticus* cell walls (*Courtesy of Dr. J. Gross*)

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unknown proportion (but not more than 1 molecule) of 2-amino-2-deoxy-mannuronic acid[6,8].

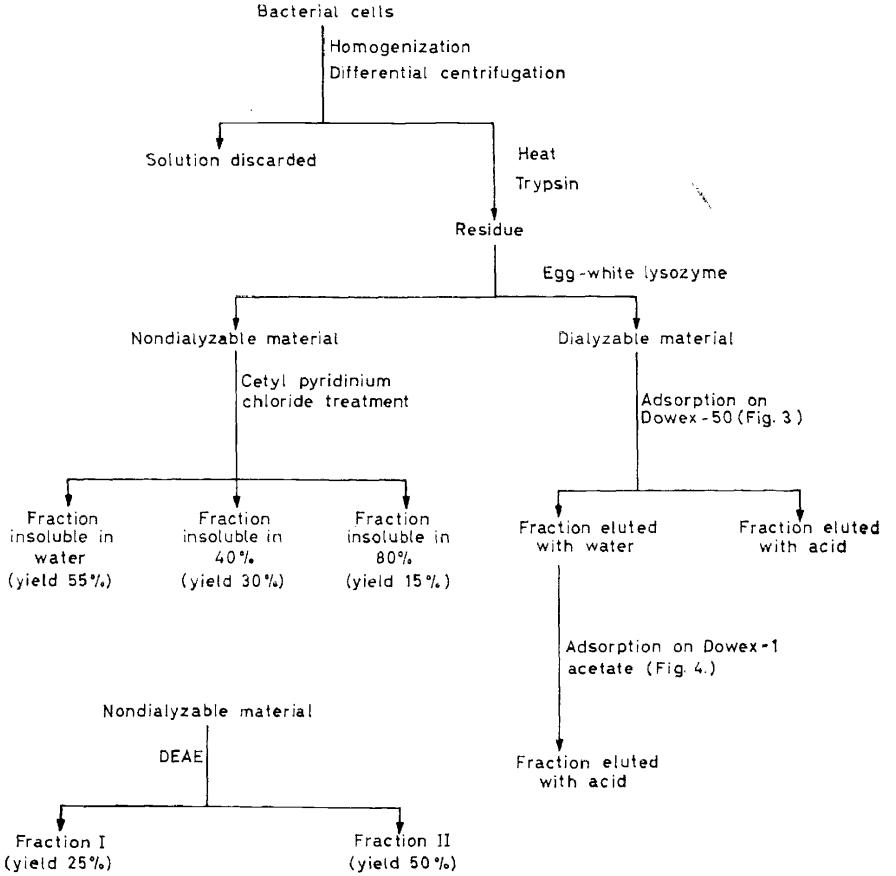


Figure 1. Scheme for the preparation of *M. lysodeikticus* cell walls, enzymatic degradation and fractionation

In previous studies of the chemical structure of the *M. lysodeikticus* cell wall degradation by lysozyme[2,3], by acid hydrolysis[9], and by methanolysis had been used[10]. Because of its selectivity, the first procedure is the most promising, although acid hydrolysis did confirm some of the results previously obtained. Methanolysis presented evidence for a covalent link between the glucose component and the glucosamine–muramic acid polysaccharide, as well as evidence for the linkage of glycine to D-glutamic acid.

Fractionation of large amounts of the dialyzable material which results from the action of lysozyme on the cell wall had been first performed by adsorption on charcoal and on Dowex–1 acetate columns; both processes were used successfully to isolate a disaccharide and a tetrasaccharide, composed of equimolecular amounts of glucosamine and muramic acid, which had been isolated earlier on a microscale. Recently, this technique was further refined[11]; as a preliminary step, the dialyzable portion of the

lysozyme hydrolyzate was adsorbed on Dowex–50. Elution was accomplished first with water and then by a gradient of hydrochloric acid (*Figure 3*). The peaks were determined by the Park–Johnson test[12] (reducing sugars),

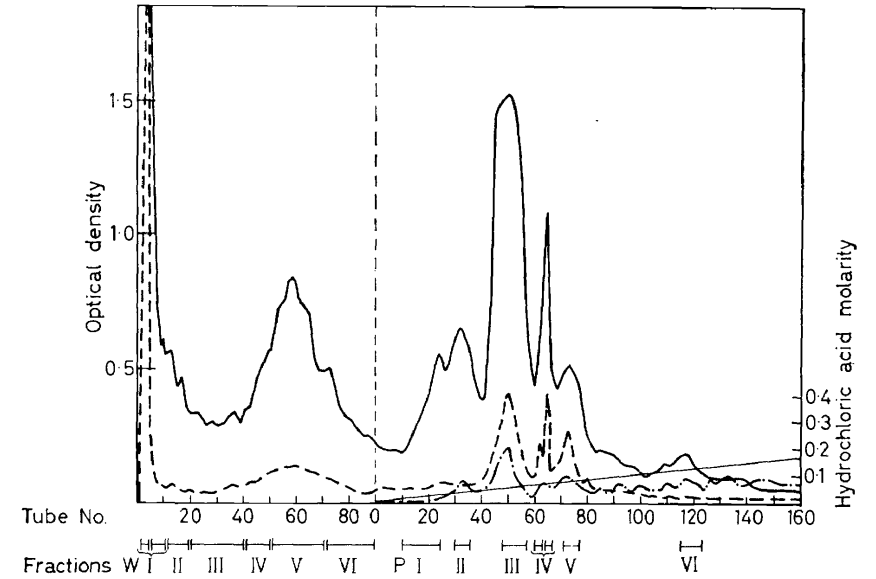


Figure 3. Fractionation on Dowex–50 of the dialyzable fraction of *M. lysodeikticus* cell walls after lysozyme degradation. Fractions W–I to VI were obtained by elution with water, fractions P I to VI by elution with a gradient of hydrochloric acid. (——) Park–Johnson test; (– – –) Morgan–Elson test; (– · – · –) ninhydrin test

by the Morgan–Elson test[13] (acetamidodeoxy sugars), and by the ninhydrin test (amino acids). Each peak was investigated by paper chromatography in two solvent systems and by paper electrophoresis. The first peak, eluted with water, was further purified by adsorption on Dowex–1 (CH_3COO^-), and then was eluted with a gradient of acetic acid (*Figure 4*); the resulting peaks were determined in the manner described above. The results of the

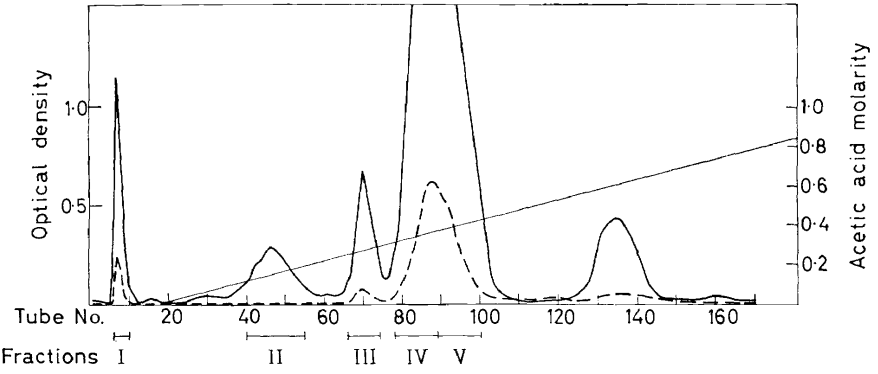


Figure 4. Fractionation on Dowex–1 acetate of the fraction W–I eluted from the Dowex–50 column. Fraction I was obtained by elution with water, and Fractions II to V by a gradient of acetic acid. (——) Park–Johnson test; (– – – –) Morgan–Elson test

paper chromatography and of the electrophoresis were reported on a two-dimensional map (*Figure 5*): at least six substances reacted with the aniline phosphate reagent and at least three reacted with both the aniline phosphate and ninhydrin reagents.

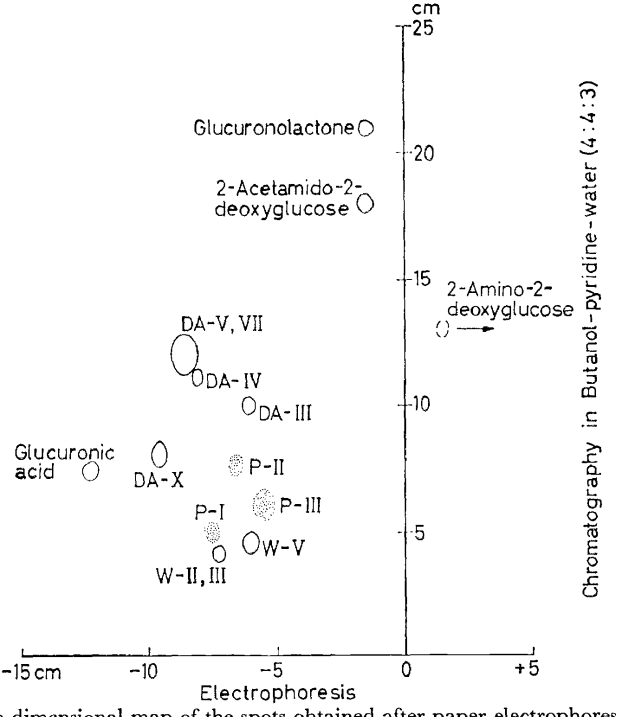


Figure 5. Two-dimensional map of the spots obtained after paper electrophoresis and paper chromatography. All spots reacted with the aniline phosphate reagents. Spots corresponding to P–I, P–II, and P–III reacted, in addition, with the ninhydrin reagent

Since Perkins[4] had described the isolation, from *M. lysodeikticus* cell wall, of a polysaccharide composed of D-glucose and 2-amino-2-deoxymannuronic acid, attempts to fractionate the nondialyzable part of the *M. lysodeikticus* digest were based on adsorption on diethylaminoethylcellulose and on fractional precipitation with cetylpyridinium chloride[8]. Both methods have been used extensively for the purification of polysaccharides which contain uronic acid. In addition to the usual amino acids, the nondialyzable fraction contained 32 per cent glucose, 10 per cent glucosamine, 7 per cent muramic acid and an unknown (but no more than 15 per cent) percentage of 2-amino-2-deoxymannuronic acid; this last component, which is degraded in large part by acid hydrolysis, has never been determined quantitatively, since no method of measurement in presence of amino acids and muramic acid has been devised as yet. Fractionation with cetylpyridinium chloride gave three main fractions, one insoluble in water, the other two insoluble in ethanol solutions of 40–50 per cent and 80–90 per cent, respectively (*Figure 1*). Adsorption on diethylaminoethylcellulose, followed by elution with a phosphate buffer gradient at pH 6, gave two main fractions in yields of 25 per cent and 50 per cent.

Table 1. Properties of the fractions obtained from the nondialyzable fraction of *M. lysodeikticus* cell walls after lysozyme degradation

Fractions	$[\alpha]_D$ *in water* (*degrees*)	*N* (%)	*P* (%)	*Acetyl* (%)	*Hexoses* (%)	*Hexosamine* (%)	*Muramic Acid* (%)	*Amino Acids* (%)			
								Glu	*Ala*	*Gly*	*Lys*
CPC–Insoluble in water	+31	7·2	1·1	20·1	32	10	7	6	7	10	8
CPC–Insoluble in 40% ethanol	+18	11·5	0·5	17·0	8	10	4	3	10	4	8
CPC–Insoluble in 80% ethanol	+40	4·9	2·7	17·9	45	8	5	3	4	2	2
DEAE–I	+22				23	10	7	+	+	+	+
DEAE–II	+34				34	7	7	+	+	+	+

No clear-cut difference was found among the various fractions obtained by cetylpyridinium chloride precipitation or diethylaminoethylcellulose adsorption. Glass-fibre electrophoresis in phosphate buffer at pH 6·2 showed the presence of two components, but sedimentation analysis showed only one homogeneous peak. Optical rotation and quantitative determination of the components showed the two main fractions, obtained from each procedure of separation, to be quite similar (*Table 1*); consequently the fraction giving a cetylpyridinium complex insoluble in water was investigated further.

III. CHEMICAL STRUCTURE OF ISOLATED FRAGMENTS

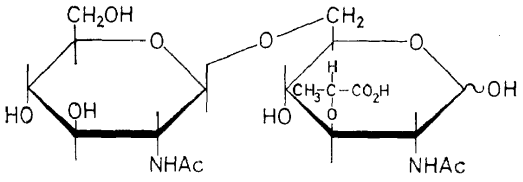
A. Dialyzable component

The water eluate of the Dowex-50 column could be separated into four main fractions on Dowex-1 (CH_3COO^-). Two of these fractions correspond to the di- and tetrasaccharide previously isolated (DA-V-VII and DA-X respectively). The two other fractions correspond to a disaccharide (DA-IV) and a tetrasaccharide (DA-III), as was determined from the reducing properties and from the speed of migration on paper electrophoresis. The four components gave, after hydrolysis, 2-amino-2-deoxyglucose. In addition, upon paper electrophoresis, the first two components showed a spot corresponding to 2-amino-3-*O*-(D-1-carboxyethyl)-2-deoxyglucose (muramic acid). However, when the last two components were tested, the spot did not move quite as fast as the one produced by muramic acid. Furthermore, the spot produced by the acidic component of the two last-mentioned substances gave a much weaker reaction with the alkaline silver nitrate reagent, a characteristic of sugars having the *manno-* or *talo-* configuration. The colour reaction shown by this acidic component, after treatment with ninhydrin, did not correspond to that given by 2-amino-2-deoxymannuronic acid. Further investigations to elucidate the structure of this component are in progress.

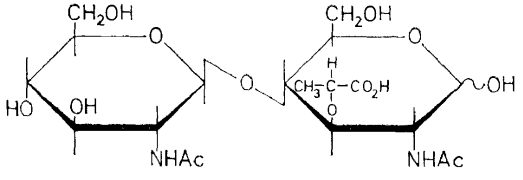
1. *Structure of disaccharide DA-V-VII*

Structure (I) was proposed for the disaccharide DA-V-VII by earlier investigators[2,3], who relied mainly on periodate oxidation and colour reactions. Later, the synthesis of this disaccharide was achieved[12-14]. Since a comparison of the crystalline, fully-acetylated methyl ester of the natural product with that of the synthetic product has shown that the two are not identical, the structure of *O*-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1 → 4)-2-acetamido-3-*O*-(D-1-carboxyethyl)-2-deoxy-D-glucose (II) has been proposed for the natural disaccharide[7,15].

The synthesis of this disaccharide by condensation of 2-acetamido-1, 6-di-*O*-acetyl-3-*O*-[D-1-(methyl carboxylate)ethyl]-β-D-glucopyranose (III) with 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranosyl bromide, or by starting from di-*N*-acetylchitobiose (IV) and adding the lactyl side chain, was attempted, but has not been successful as yet.



(I)



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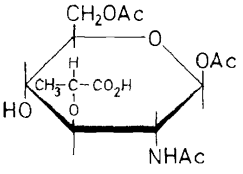

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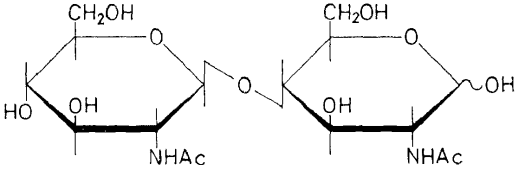
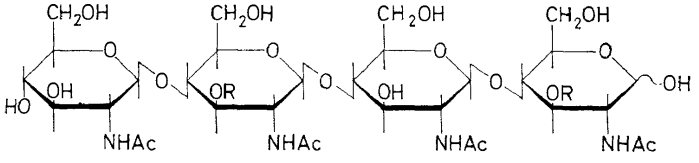


(IV)

2. *Structure of tetrasaccharide DA–X*

Structure (V) has been proposed for the tetrasaccharide DA–X. The proposal was based on the observation that when lysozyme splits the tetrasaccharide, not only do some higher molecular oligosaccharides result from transglycosylation, but also two molecules of the disaccharide described



(V) R = CH_3CHCO_2H

in the preceding paragraph are produced[16]. The linkage between C–1 of the non-reducing 2-acetamido-3-*O*-(D-1-carboxyethyl)-2-deoxy-β-D-glucopyranosyl residue and the 2-acetamido-2-deoxy-β-D-glucopyranosyl residue has been assumed to be (1 → 4), since lysozyme attacks oligosaccharide derived

from chitin. Some additional evidence for this linkage was gained from the results of the periodate oxidation of a trisaccharide obtained by enzymatic degradation of the tetrasaccharide[17]. Moreover, the hydrolyzate of the methylated nondialyzable fraction gave, in large proportion, the 3,6-dimethyl-ether of 2-amino-2-deoxy-D-glucose (*see* next paragraph), which is a further indication for a (1 → 4) linkage.

B. Nondialyzable component

The fraction precipitated by cetylpyridinium chloride in water solution presents a composition very similar to that of the main fraction eluted from the diethylaminoethylcellulose column; it was, therefore, investigated[8] further.

Attempts to separate a polysaccharide composed of glucose and 2-amino-2-deoxymannuronic acid by treatment according to the method of Perkins[4], with trichloroacetic acid for 48 hours at 35°, was not successful. The result was a peptidoglycan, isolated in a yield of 97 per cent, which still contained 31 per cent glucose, 11 per cent glucosamine, and 9 per cent muramic acid; this substance was not degraded further by treatment with lysozyme.

In order to ascertain the presence of 2-amino-2-deoxy-mannuronic acid, the fraction investigated was treated with diborane in diglyme solution; the reduced product showed, after hydrolysis, the presence of 2-amino-2-deoxymannose.

1. *Periodate oxidation*

The peptidoglycan obtained from the water-insoluble cetylpyridinium complex was oxidized with excess periodate and then reduced with sodium borohydride. It was further treated with dilute acid and dialyzed. The dialyzate showed the presence of glycerol, and the remaining nondialyzable peptidoglycan contained 2 per cent glucose, 9 per cent glucosamine, and 14 per cent muramic acid. A second periodate oxidation removed the remaining glucose units.

2. *Methylation*

The peptidoglycan was methylated with dimethyl sulphate and sodium hydroxide in an atmosphere of nitrogen at low temperature. The process was repeated until the content in methoxyl groups reached a limit at 18·1 per cent; the infrared spectrum indicated complete methylation. After acid hydrolysis and removal of the acid, the hydrolyzate was fractionated on a Dowex–50 column with a gradient of hydrochloric acid.

The methylated sugars were identified by a variety of methods: paper chromatography in four different solvent systems, paper electrophoresis, periodate oxidation of the sugar (or of its glycitol derivative) followed by paper chromatography, gas–liquid chromatography of the methyl glycoside and of its trimethylsilyl derivative, degradation of the 2-amino-2-deoxy sugars with ninhydrin and, finally, crystallization of the sugars (or of the

azoyl derivative of the corresponding glycitol). The results are reported in *Table 2.*

Table 2. *O*-Methyl derivatives isolated from the hydrolysate of a methylated nondialyzable fraction (CPC–Complex insoluble in water)

Starting material:	1·37 g
Methylated material recovered:	0·98 g (OCH_3: 18%)
Methylated material hydrolysed:	0.46 g
O-Methyl derivatives isolated:	3-*O*-Methyl-D-glucose (16 mg)
	2,3-Di-*O*-methyl-D-glucose (32 mg)
	2,3,4-Tri-*O*-methyl-D-glucose (50 mg)
	2-Amino-2-deoxy-3-*O*-methyl-D-glucose (14 mg)
	2-Amino-2-deoxy-3,6-di-*O*-methyl-D-glucose (29 mg)

The methylated sugars isolated in order of decreasing amounts are as follows: the 2,3,6-trimethyl, 2,3-dimethyl and 3-methyl ethers of D-glucose for the neutral sugars; and the 3,6-dimethyl and 3-methyl derivatives of 2-amino-2-deoxy-D-glucose for the amino sugars.

IV. TENTATIVE CHEMICAL STRUCTURE OF THE CARBOHYDRATE MOIETY

The carbohydrate components of the *M. lysodeikticus* cell wall identified up to the present time are 2-amino-2-deoxy-D-glucose (D-glucosamine), 2-amino-3-*O*-(D-1-carboxyethyl)-2-deoxy-D-glucose (muramic acid), D-glucose and 2-amino-2-deoxymannuronic acid. The possibility that other unidentified sugars exist in this particular cell wall cannot be excluded; one of them may be a compound very similar to muramic acid.

As a result of the work of Salton, Perkins, and their associates, it has been assumed that the first two compounds and the last two form two separate polysaccharides. In view of the work described in the preceding paragraphs, this hypothesis should be modified. While it is not as yet possible to ascertain the position of the 2-amino-2-deoxymannuronic acid component, it seems most likely that the D-glucose units are covalently linked with the peptidoglycan composed of glucosamine and muramic acid.

There is no reason to doubt the existence of a glycan chain consisting of alternating units of *N*-acetylglucosamine and *N*-acetylmuramic acid, an hypothesis suggested by earlier investigators. However, until such a chain has been isolated, devoid of peptide side-chains, until its molecular weight has been determined, and until its complete chemical structure has been elucidated, it will not be possible to ascertain the role of this glycan in the structure of the cell wall.

The 2-acetamido-2-deoxy-β-D-glucopyranosyl and 2-acetamido-3-*O*-(D-1-carboxyethyl)-2-deoxy-β-D-glucopyranosyl units are linked at C–4, as is suggested by studies of the dialyzable di- and tetrasaccharides, and by the fact that the 3,6-dimethyl ether of 2-amino-2-deoxy-D-glucose has been isolated in the largest proportion from the methylated derivative of one of the nondialyzable fragments (*Table 2*). The amount of 3-methyl ether of 2-amino-2-deoxy-D-glucose that has been isolated from the hydrolyzate of the methylated nondialyzable fraction is too large to be discounted as a result

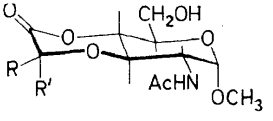
of incomplete methylation; its presence may indicate branching along the chain of the peptidoglycan.

Perkins has shown, by the isolation (in a very small yield) of a polysaccharide consisting of glucose and 2-amino-2-deoxymannuronic acid, that these two components are linked together. The results of the methylation, in agreement with those of the periodate oxidation, have established that the D-glucose units are linked at C–6 and that none of them is located at the terminal, non-reducing end of a chain. It is quite logical to assume that they are the first components of a branch chain attached to the chain of the peptidoglycan and that they are linked only to the glucosamine residues; but there is no direct evidence as yet to support this suggestion. Furthermore, it is not known whether the chains starting with the D-glucose units consist of alternating units of D-glucose and 2-amino-2-deoxymannuronic acid components; nor is it known in which position the 2-amino-2-deoxymannuronic acid units are linked. The large proportion of 3-methyl and 2,3-dimethyl ethers of D-glucose suggest extensive ramification of the side-chains. Much work remains to be done until a chemical structure of the whole bacterial cell wall can be presented.

V. PRESENT STATUS OF MURAMIC ACID CHEMISTRY

The chemistry of 2-amino-3-*O*-(D-1-carboxyethyl)-2-deoxy-D-glucose (muramic acid), a component found in all bacterial cell walls has been the subject of a few recent reviews[18–21]. Some of the problems concerning the structure of the bacterial cell wall which have been investigated recently in our laboratory include the following: the configuration of the side-chain; new methods of synthesizing muramic acid; and the preparation of derivatives which may serve as substrates for lysozyme (such as the β-D-glycosides), or which may be used to elucidate the structure of the peptidoglycan chain (such as the methyl ethers).

The D-isomery at C–2 of the side chain of muramic acid was assigned after comparison of the optical rotation of muramic acid with that of optically active ethers of D-lactic acid[22] and after consideration of the mechanism of the reaction assumed to take place in the synthesis starting with L-chloropropionic acid[23]. These hypotheses are, however, open to criticism; we are attempting to prove (or disprove) the proposed configuration by using different methods. The preparation of the lactone (VI) {m.p. 246–8°, $[\alpha]_D$ + 227° in acetone}

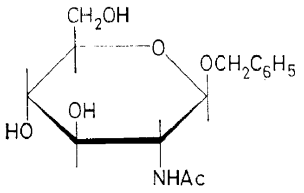


(VI) R = H, R' = CH_3
(VII) R = CH_3, R' = H

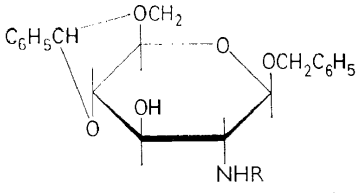
has been reported previously[24]. A similar lactone (VII), which differs solely in the isomery of the lactyl chain, has now been prepared {m.p. 254–6°, $[\alpha]_D$ + 168° in acetone}[25]. When sufficient amounts of both lactones are available, determination of their optical rotatory dispersion and

of the nuclear magnetic resonance spectra may give additional information on the conformation of the methyl group of the lactyl side chain.

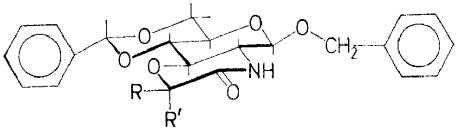
Preparation of a disaccharide composed of glucosamine and muramic acid, in which the latter component is linked at C–4, has not as yet been accomplished. The bulky lactic acid side-chain seems to present a major obstacle, since an attempt to prepare the 4-benzoate of the 6-trityl ether was not successful. In order to study less hindered intermediates, a lactam derivative has been prepared through the sequences (VIII) to (XII)[26].



(VIII)



(IX) R = Ac
(X) R = H
(XI) R = CO–C(H)(Cl)–CH3



(XII) R = CH_3, R' = H
(XIII) R = H, R' = CH_3

Study of the optical rotatory dispersion and nuclear magnetic resonance spectra of the lactam (XII) and of the isomeric lactam (XIII) formed during the synthesis may also establish the configuration of the lactyl side chain.

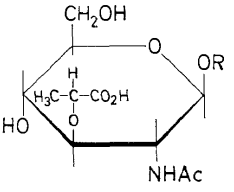
VI. LYSOZYME SUBSTRATES

When the site of action of egg-white lysozyme in the bacterial cell wall was elucidated and when the spatial structure of the enzyme was determined, there was added incentive to prepare synthetic substrates.

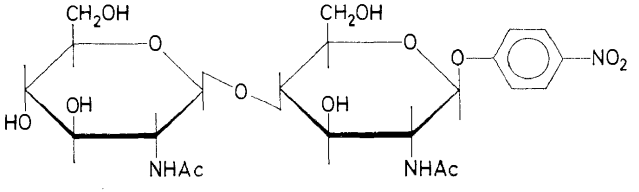
In order to study the specificity of egg-white lysozyme and its interaction with muramic acid derivatives, both the *p*-nitrophenyl (XIV) and benzyl β-D-glycosides (XV) have been synthesized[27]. Both products have been shown to be resistant to enzymatic degradation; similar results have been reported for other β-D-glycosides of muramic acid[19].

Many years ago, degradation products of chitin were known to be substrate for egg-white lysozyme; consequently interest has centred on di-*N*-acetylchitobiose. Thus, recently, the *p*-nitrophenyl β-D-glycoside of the disaccharide (XVI) has been synthesized and shown to be a reactive substrate for the enzyme[28]. However, the large proportion of enzyme necessary to obtain a rate sufficient to be measured raises some doubt on the validity of the

assumption that in a bacterial cell wall the linkage split by the enzyme is of the same type as in chitin [β-D (1 → 4)]. Work now in progress on the synthesis of various oligosaccharides of 2-acetamido-2-deoxy-D-glucose will



(XIV) R = $C_6H_5NO_2$-*p*
(XV) R = $CH_2C_6H_5$



(XVI)

help to solve this problem, and will give further information on the structure of the bacterial cell wall.

CONCLUSION

The problems I have briefly discussed show how complex a task it is to describe in chemical terms the organization of just one biological component (the cell wall) of rather simple organisms (bacteria). The results are, nevertheless, encouraging, because they show that the tools for such studies are available. But it is clear, at the same time, that many of the present procedures must be performed automatically if the desired solutions are to be found in a not too distant future.

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