THE CHEMICAL STRUCTURE OF A DISACCHARIDE ISOLATED FROM Micrococcus lysodeikticus CELL WALL*

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The degradation by lysozyme of the cell wall of *Micrococcus lysodeikticus* yields dialyzable fragments, which include a disaccharide composed of 2-amino-2-deoxy-D-glucose (D-glucosamine) and 2-amino-2-deoxy-3-O-(D-1-carboxyethyl)-D-glucose (muramic acid). On the basis of borohydride reduction, iodine oxidation, periodate oxidation, enzymic degradation, and Morgan-Elson reaction, the structure of an O-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→6)-2-acetamido-3-O-(D-1-carboxyethyl)-2-deoxy-D-glucose, or N-acetyl-β-D-glucosaminyl-(1→6)-N-acetylmuramic acid, was proposed for this compound (Salton and Ghuysen, 1960; Perkins, 1960). From the lysozyme digest of a "muco-

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complex" extracted from the whole cell of M. lysodeikticus, Hoshino (1960) isolated a disaccharide which he considered identical to the one just described. He proposed the structure of O-2-acetamido-3-O-(D-1-carboxyethyl)-2-deoxy-D-glucopyranosyl-(1→4)-2-acetamido-2-deoxy-D-glucose, or N-acetylmuramyl-(1→4)-N-acetylglucosamine, on the basis of borohydride reduction, hypoiodite and periodate oxidation, and Morgan-Elson and Elson-Morgan tests.

Since the results of the periodate oxidation and of the Morgan-Elson reaction can be interpreted differently than has been done by these investigators (Jeanloz, 1944; Bell, 1948; Brown et al., 1950), the elucidation of the structure of the disaccharide was attempted by chemical synthesis.

In order to compare the synthetic with the natural product, a sufficient amount of the latter was necessary and a large-scale preparation of M. lysodeikticus cell walls was devised. After lysozyme digestion of the wall, the dialyzate was fractionated on a column of charcoal-Celite, using a gradient of water and ethanol as eluent, then on a column of Dowex-1 in the acetate form, using a gradient of acetic acid. The pure disaccharide, obtained in a yield of 4%, was amorphous and had a mutarotation from $[\alpha]_D^{25}$ + 10° to + 4.5° (in water, at equilibrium). Prior to analysis, the product was dried for 2-3 hr. at 50-60° in high vacuum. Anal. Calcd. for $C_{19}H_{32}N_2O_{13}$: C, 45.94; H, 6.50; N, 5.64. Found: C, 45.79; H, 6.68; N, 5.58.

This product gave, after hydrolysis, glucosamine and muramic acid, and behaved, on paper chromatograms, identically with the product described by Salton (1959). It also showed on paper the same speed of migration in two different solvents as a sample of the disaccharide kindly provided by Dr. O. Hoshino.

For the preparation of O-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→6)-2-acetamido-3-O-(D-1-carboxyethyl)-2-deoxy-D-glucose, benzyl 6-O-(2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosyl)-2-acetamido-4-O-acetyl-2-deoxy-3-O-[D-1-(methyl carboxylate)ethyl]-α-D-glucopyranoside (Flowers and Jeanloz, 1963), was saponified with sodium methoxide, then hydrogenated in the presence of Pd on charcoal. The resulting product was purified by adsorption on a column of Dowex-1 in the acetate form, followed by elution with a gradient of acetic acid, giving 67% of an amorphous product, which had a mutarotation from $[\alpha]_D^{25}$ + 16° to + 14° (in water, at equilibrium). Prior to analysis, the product was dried for 2-3 hr. at 60-70° in high vacuum. Anal. Calcd. for $C_{19}H_{32}N_2O_{13}.H_2O$: C, 44.35; H, 6.86; N, 5.44. Found: C, 44.22; H, 6.86; N, 5.00.

Since it has not been possible so far to obtain a well crystallized derivative of the natural disaccharide with a sharp m.p., comparison was made between the two amorphous products. They are very similar, as shown by almost identical infrared spectra and identical speeds of migration on paper chromatograms in three solvent systems (Table I). It is clear, however, that the two compounds are not identical, showing small, but definite differences in optical rotations and in migration on paper chromatograms in one solvent system.

The clearest difference, however, is shown by the color values obtained in both Morgan-Elson tests, using the Reissig et al. (1955) modification at 3 min. and 35 min. heating time, and the Aminoff et al. (1957) modification. For comparison, crystalline 2-acetamido-3-O-(D-1-carboxyethyl)-2-deoxy-α-D-glucose (N-acetylmuramic acid) (Flowers

Table I

Paper chromatography of natural and synthetic disaccharides

Compounds	$R_{N\text{-acetylglucosamine}}$ in solvent mixtures*			
	A	B	C	D
Natural Disaccharide	1.05	1.0	0.4	0.75
Synthetic Disaccharide**	1.00	1.0	0.4	0.75

* A. Butanol-acetic acid-water 4:1:5
B. Butanol-acetic acid-water 3:1:1
C. Butanol-pyridine-water 6:4:3
D. Pyridine-ethyl acetate-acetic acid-water 5:5:1:3

** *O*-2-Acetamido-2-deoxy-β-*D*-glucopyranosyl-(1→6)-2-acetamido-3-*O*-(*D*-1-carboxyethyl)-2-deoxy-*D*-glucose

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and Jeanloz, 1963)† was also tested under the same conditions, and the results are reported in Table II. The low color yield obtained with the natural disaccharide has also been observed by previous investigators (Salton and Ghuysen, 1960; Perkins, 1960; Hoshino, 1960).

The data just mentioned show that the natural disaccharide isolated from *M. lysodeikticus* cell walls is not an *O*-(*N*-acetyl-β-*D*-glucosaminyl)-(1→6)-*N*-acetylmuramic acid disaccharide. Preliminary experiments repeating the reduction with sodium borohydride (Salton and Ghuysen, 1960) have shown the conclusion of these authors, that muramic acid is the reducing moiety, is correct. Consequently, the only structure left for the natural disaccharide would be that of *O*-(β-*N*-acetylglucosaminyl)-(1→4)-*N*-acetylmuramic acid,

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† In order to obtain crystalline *N*-acetylmuramic acid with the procedure of Carroll (1963), it is necessary to use double the amount of *N*-acetoxyphthalimide recommended (Osawa and Jeanloz, unpublished).

Table II

Color yield in the Morgan-Elson test of natural and synthetic derivatives of muramic acid*

Compounds	Reissig *et al.* modification		Aminoff *et al.* modification
	3 min.	35 min.	
Natural Disaccharide	0.20	0.67	0.55
Synthetic Disaccharide**	0.88	1.45	4.3
N-acetylmuramic Acid	0.95	1.15	2.1

* Relative to an equimolar amount of *N*-acetylglucosamine tested under identical conditions. All measurements were made in the Klett-Sumerson Colorimeter, filter No. 56.

** *O*-2-Acetamido-2-deoxy-β-D-glucopyranosyl-(1→6)-2-acetamido-3-*O*-(D-1-carboxyethyl)-2-deoxy-D-glucose

or *O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1→4)-2-acetamido-3-*O*-(D-1-carboxyethyl)-2-deoxy-D-glucose.

Such a structure is different from the one proposed by Salton and Ghuysen, and by Perkins, as well as from the one proposed by Hoshino, but is not in contradiction with the observed properties of the natural disaccharide. Whereas a negative Morgan-Elson test has been taken as indicative of a C-4 substitution in an acetylamino sugar (Kuhn *et al.*, 1954; Jeanloz and Trémège, 1956), a slight positive Morgan-Elson reaction has also been observed, despite the substitution at position 4, with *N*, *N*'-diacetylchitobiose (Salton and Ghuysen, 1960) and, after prolonged time of reaction, with 2,4-diacetamido-2,4-dideoxy-α-D-glucose (Jeanloz and Rapin, 1963).

In order to ascertain the correctness of this revised structure, as well as to explain the results of the periodate oxidation and of the Morgan-Elson reaction, methylation of the disaccharide and preparation of partially methylated derivatives of muramic acid have been undertaken.

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