The structure of *di-* and *tetra*-saccharides released from cell walls by lysozyme and *Streptomyces* F_1 enzyme and the β (1 → 4) N-acetylhexosaminidase activity of these enzymes

Enzymic degradation of the walls of *Micrococcus lysodeikticus* with lysozyme or *Streptomyces* F_1 enzyme[1] releases *N*-acetylamino sugar compounds and N-acetylamino sugar-peptide complexes[2]. Two compounds of N-acetylmuramic acid and N-acetylglucosamine believed to be a *di*-saccharide and a *tetra*-saccharide, have been isolated from the dialysable fractions of walls digested with lysozyme and the F_1 enzyme[2,3].

To identify the nature of the N-acetylamino sugar whose reducing group is freed from glycosidic bonding by lysozyme and F_1 enzyme, the non-dialysable fractions as well as the purified *di-* and *tetra*-saccharides (from *M. lysodeikticus* walls) have been reacted with $NaBH_4$ at 0° for periods varying from 2–12 h, either in aqueous solution or in borate buffers at pH 9.2 or pH 8.0. Reduction was accompanied by a loss of N-acetylamino sugar reaction.

The $NaBH_4$-treated compounds were hydrolysed with 6*N* HCl for 16 h at 105°, and the products examined by two-dimensxional paper chromatography with pyridine–water (4 : 1, v/v) and *n*-butanol–acetic acid–water (3 : 1 : 1, v/v/v). In all cases a new compound appeared in the hydrolysates. It reacted more weakly with ninhydrin than either glucosamine or muramic acid and was detectable with $AgNO_3$–NH_3 and the SCHIFF spray reagents[4]. On electrophoresis at pH 6.4 it behaved as a neutral substance. The properties of the reduction compound are, therefore, consistent with those of the amino sugar hexitol of muramic acid (subsequently referred to as "muramicitol"). No compounds corresponding to the reduction product of glucosamine (glucosaminitol) were detected. These results provide direct experimental evidence for the suggestion[5] that the action of lysozyme and of F_1 enzyme involves the liberation of the carbonyl group of N-acetylmuramic acid. Thus, both enzymes break the glycosidic bond between C_1 of N-acetylmuramic acid and either the C_3, C_4 or C_6 atoms of N-acetylglucosamine (probably C_4 as shown by evidence below).

The ratio of glucosamine to muramicitol in the reduced *di*-saccharide was determined by conversion to the DNP-compounds with fluorodinitrobenzene after hydrolysis, separation on paper chromatograms[6] and measuring the extinctions at 360 mμ. The ratio of DNP-glucosamine to DNP-muramicitol was 1 : 0.8. [^{14}C]*tetra*-saccharide, isolated from walls of *M. lysodeikticus* grown on a casamino acid–yeast extract medium containing [^{14}C]fructose, was reduced with $NaBH_4$, hydrolysed (6*N* HCl, 16 h, 105°) and the glucosamine, muramic acid and muramicitol separated on Whatman No. 1 paper with *n*-butanol–acetic acid–water (3 : 1 : 1, v/v/v). Radioactivity of the compounds gave ratios of approximately 2 glucosamine : 1 muramic acid : 1 muramicitol. The results provide confirmatory evidence that the compounds are indeed *di-* and *tetra*-saccharides.

The nature of the linkages between N-acetylglucosamine and N-acetylmuramic acid have been investigated by the action of β-glucosidase and $NaIO_4$ oxidation. Both the *di-* and *tetra*-saccharides were hydrolyzed by β-glucosidase, yielding N-acetylmuramic acid and N-acetylglucosamine. Although hydrolysis of the compounds by β-glucosidase was not complete (the residual *di-* and *tetra*-saccharides were detectable on paper chromatograms), the free N-acetylamino sugars liberated from the *di-*

Abbreviation: DNP-, dinitrophenyl-.

saccharide and separated on paper chromatograms gave a 1:1 ratio of N-acetylglucosamine to N-acetylmuramic acid.

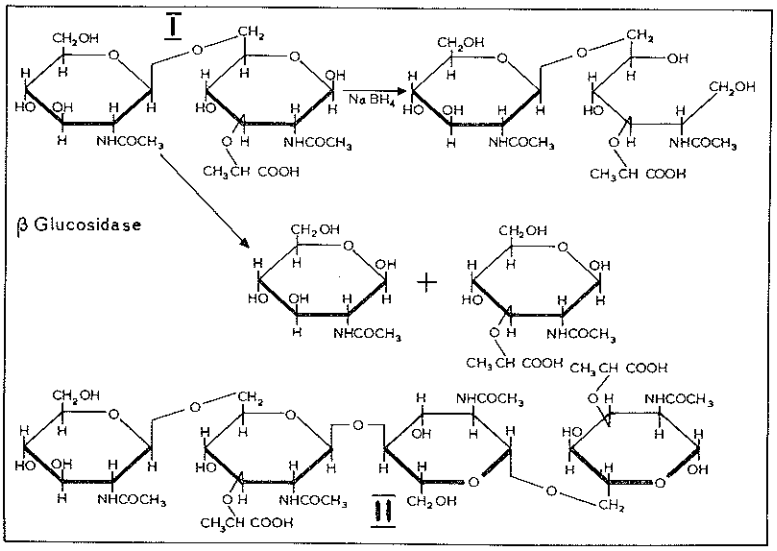

Fig. 1.

As the isolated *di*- and *tetra*-saccharides were not 100 % pure, ambiguous results with periodate oxidation were anticipated. To avoid the difficulties of determining the origin of any formaldehyde formed, [^{14}C]*di*- and *tetra*-saccharides were used. Oxidation with $NaIO_4$ was performed at pH 7.5, 2°, for 30 h under the conditions giving maximum H·CHO liberation from N-acetylglucosamine[7] and blood-group substances[8]. At the end of oxidation, carrier H·CHO was added, followed by an excess of 5,5-dimethyl-1,3-cyclohexandione (dimedon) and the ^{14}C contents of the insoluble H·CHO-dimedon compounds were determined. Control periodate oxidation with [^{14}C]glucose gave 75 % of the theoretical yield of [^{14}C]formaldehyde, which on correction for the adsorption due to the carrier non-radioactive H·CHO-dimedon compound, corresponded to a 97 % yield of [^{14}C]formaldehyde. Under these conditions, the ^{14}C contents of the H·CHO-dimedon precipitated from $NaIO_4$-oxidized *di*- and *tetra*-saccharides did not differ from a control preparation of [^{14}C]*di*-saccharide treated under identical conditions but in the absence of $NaIO_4$. If it is assumed that there is nothing anomalous about the periodate oxidation of N-acetylmuramic acid under these conditions and that a normal labelling of carbon atom 6 of the hexosamine residue of muramic acid has occurred, then the results indicate 1 → 6 linkages between N-acetylglucosamine and N-acetylmuramic acid. The results of GHUYSEN[3] indicate that the *tetra*-saccharides are further degraded by lysozyme and F_1 enzyme to the *di*-saccharide. These results, together with the data from periodate oxidation, indicate that the *tetra*-saccharide can be provisionally assigned the structure of a dimer of a β (1 → 6) N-acetylglucosamine-N-acetylmuramic acid *di*-saccharide, joined by β (1 → 4) linkages.

That both lysozyme and F_1 enzyme act on β (1 → 4) N-acetylglucosaminides has been confirmed by their action on *di*- and *tetra*-chitobiose (kindly provided by

Dr. S. ROSEMAN). *Tetra*-chitobiose was broken down incompletely to *di*-chitobiose and a compound separating on paper close to free N-acetylglucosamine (very probably chitobiose and/or N-acetylglucosamine). The *di*-chitobiose yielded the compound or compounds corresponding to chitobiose and/or N-acetylglucosamine.

The proposed structure for the *di*-saccharide (I) and its degradations with $NaBH_4$ and β-glucosidase together with the structure of the *tetra*-saccharide (II) are summarized below.

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Received September 21st, 1959

* Associé du Fonds National de la Recherche Scientifique, Belgium. This research was performed during an appointment (J.M.G.) supported by the International Cooperation Administration under the Visiting Research Scientists Program administered by the National Academy of Sciences of the United States of America.

Mechanism of enzymic formation of threonine from O-phosphohomoserine

The final step in threonine biosynthesis in yeast[1] has been shown by WATANABE *et al.*[2] to require 2 enzyme fractions, Hser kinase (Reaction (1)), and a second fraction catalyzing an elimination of P_i coupled with isomerization to form L-Thr from PHser (Reaction (2)).

$$\text{L-Hser} + \text{ATP} \longrightarrow \text{PHser} + \text{ADP} \qquad (1)$$

$$\text{PHser} + H_2O \longrightarrow \text{L-Thr} + P_i \qquad (2)$$

$$\text{L-Hser} + \text{ATP} + H_2O \longrightarrow \text{L-Thr} + \text{ADP} + P_i \qquad (3)$$

The enzyme catalyzing Reaction (2) has been purified 500-fold from *Neurospora*, and appears to be a single protein, which we shall call threonine synthetase[3]. The reaction requires pyridoxal phosphate, and proceeds to completion, forming equimolar amounts of Thr and P_i. It has not so far been possible to show the occurrence of Reaction (1) in extracts of *Neurospora*. PHser was prepared with yeast Hser kinase.

The Thr synthetase reaction has been carried out in water labeled with ^3H, ^2H or ^{18}O. As shown in Table I, Expt. 1, incubation of PHser and enzyme in $H_2{}^{18}O$ led to the incorporation of about 1 atom of solvent O into Thr, and none into P_i. P_i is therefore eliminated from PHser by a C-O cleavage. Mechanisms involving the inter-

Abbreviations: PHser, O-phosphohomoserine; Hser, homoserine; Thr, threonine; ATP, ADP, adenosine tri- and -diphoxphate; P_i, orthophosphate.