Lysozyme-catalyzed Hydrolysis and Transglycosylation Reactions of Bacterial Cell Wall Oligosaccharides

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SUMMARY

Tritium-labeled tetrasaccharide, GlcNAc-β(1 → 4)-MurNAc-β(1 → 4)-GlcNAc-β(1 → 4)-MurNAc, and disaccharide, GlcNAc-β(1 → 4)-MurNAc (where MurNAc represents N-acetylmuramyl), were prepared by tritiation of the corresponding unlabeled compounds. The products, obtained in good yield, were found to be homogeneous and radiochemically pure by several chromatographic and electrophoretic criteria and indistinguishable from the starting materials.

Chemical and enzymatic studies indicated that they were uniformly labeled.

The action of lysozyme on the tetrasaccharide was studied by a combination of paper electrophoresis and paper chromatography. The major product of digestion was the disaccharide. Higher oligosaccharides were formed in the initial stages of the reaction and disappeared after prolonged incubation when only disaccharide together with considerable amounts of tetrasaccharide was present. The rate of disappearance of the tetrasaccharide changed markedly during the course of the reactions; the maximal rate increased with the increase in substrate concentration.

Four higher oligosaccharides (hexa-, octa-, deca-, and dodecasaccharides) were isolated from lysozyme digests of the tetrasaccharide-3H. Two of these were found to be identical with the hexa- and octasaccharides isolated from lysozyme digests of Micrococcus lysodeikticus cell walls. The higher oligosaccharides were readily digested by lysozyme to yield the corresponding di- and tetrasaccharides. The hexa- and octasaccharides were digested at a much faster rate than the tetrasaccharide. Addition of small amounts of octasaccharide to the tetrasaccharide increased significantly the rate of digestion of the latter.

Upon incubation of unlabeled tetrasaccharide with disaccharide-3H in the presence of lysozyme, the labeled tetrasaccharide formed did not contain significant amounts of radioactivity in the nonreducing half of the molecule. Furthermore, when this newly formed radioactive tetrasaccharide was incubated with GlcNAc-14C and lysozyme, the GlcNAc-β(1 → 4)-MurNAc-β(1 → 4)-GlcNAc contained insignificant amounts of 3H. These results indicate that in the reactions studied over 90% of the new products must be formed by transglycosylation, and not by reversal of hydrolysis.

The results obtained show that hydrolysis of the tetrasaccharide to disaccharide proceeds chiefly via transglycosylation, leading to the formation of high oligosaccharides. This mechanism is consistent with the binding data for the association of various saccharides with lysozyme and can be readily explained on the basis of the three-dimensional model of lysozyme presented by Phillips and co-workers.

In preliminary publications from this laboratory, we described studies of the action of hen's egg white lysozyme on the tetrasaccharide, GlcNAc-MurNAc-GlcNAc-MurNAc, isolated from cell walls of Micrococcus lysodeikticus (1–3). It was observed that in addition to the disaccharide GlcNAc-MurNAc, which is the major product of digestion of the tetrasaccharide by lysozyme, a number of products which moved slowly on paper chromatograms also appeared. These products were further digested by lysozyme to yield mainly the di- and tetrasaccharides. Although the slow moving compounds were not isolated and studied in detail, their mobility on paper chromatography suggested that they were the tri-, tetra-, and pentaoligomers of the cell wall disaccharide. Measurement of the rate of digestion by lysozyme of the tetrasaccharide, either by colorimetry, with a modified Morgan-Elson assay, or by polarimetry, showed that the reaction has an induction period during which the rate of release of disaccharide GlcNAc-MurNAc increases. From our observations we concluded that lysozyme can catalyze transglycosylation in addition to hydrolysis. We also suggested that, during the induction period, the tetrasaccharide reacts

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\[\text{MurNAc} = N\text{-acetylmuramic acid}\]

\[\text{GlcNAc} = \text{N-acetylglucosamine}\]
slowly and that the higher oligosaccharides formed by trans-glycosylation serve as catalysts for the hydrolysis of the tetrasaccharide. More recently, we have shown that lysozyme can transfer GlcNAc-MurNAc residues from the cell wall tetrasaccharide to a variety of mono- and disaccharide acceptors (4, 5).

Similar observations and conclusions have been reported by workers who have studied the action of lysozyme on β(1 → 4) oligomers of N-acetylglucosamine (chitin oligosaccharides) (6–10).

In this publication we describe the preparation of tritium-labeled di- and tetrasaccharides, and their use in the study of hydrolysis and transfer reactions catalyzed by lysozyme. We have further characterized the "higher oligosaccharides" formed in the incubation mixtures and have followed their synthesis and breakdown by the enzyme, as well as their effect on the hydrolysis of tetrasaccharide. These findings support our preliminary data and provide further information on the enzymatic properties of lysozyme.

**Materials and Methods**

Hen's egg white lysozyme (twice recrystallized, salt-free, Lot LYSF 638) was a product of Worthington, boar epididymal exo-β-N-acetylglucosaminidase (11) was a gift of Dr. James Conchie. N-Acetylglucosamine (Pfizer) was recrystallized from ethanol-acetone to a melting point of 202–203°. N-Acetylglucosamine-1-14C was purchased from New England Nuclear. All other materials used were of the highest purity available.

**Paper Chromatography and Paper Electrophoresis**—Whatman No. 1 paper was used for analytical experiments, and No. 3 paper for preparative work. The following solvent systems were used for descending paper chromatography: Solvent I, 1-butanol-acetic acid-water (25:6:25, upper phase); Solvent II, 1-butanol-acetic acid-water (3:1:1); Solvent III, 1-butanol-pyridine-water (2:2:1); Solvent IV, isobutyric acid-0.25 M NH3 (2:1). Paper electrophoresis was carried out under a constant voltage of 50 volts/cm with the following buffer systems: acetic acid-1 M pyridine, pH 3.5; and acetic acid-1.2 M pyridine, pH 6.5. N-Acetylaminosugars were detected as fluorescent spots by treatment of the papers with alkali reagent according to the method of Sharon and Seifter (1).

Radioactive chromatograms or electrophoretograms were routinely analyzed with a Vanguard gas flow strip scanner. For quantitative measurements, the strips were divided into sections of 3 to 20 mm, depending on the proximity of the radioactive peaks. The paper sections were then placed in counting vials, and 0.6 ml of H2O was added to each vial. After the strips had been allowed to stand overnight, Bray's dioxane scintillation solution (12) was added, and the samples were counted in a Packard Tri-Carb scintillation counter for 5 min each.

**Anion Exchange Chromatography**—Anion exchange chromatography of the oligosaccharides (13) was carried out on Dowex 1 (acetate form) (AG1-X8, 200 to 400 mesh, Bio-Rad). Samples were added to the column (50 × 1.5 cm) in 2 ml of water and eluted with a linear gradient of acetic acid (mixing chamber, 1 liter of water; reservoir, 1 liter of 1 M acetic acid). Fractions of 10 ml were collected at a flow rate of 50 ml per hour. In some experiments a smaller column (25 × 1.1 cm) was used, and the gradient and fractions were scaled down accordingly. The acid gradients were determined by titration of samples with 0.1 N NaOH to the phenolphthalein end point. Aliquots of fractions were dried over KOH and analyzed for reducing sugars by the ferricyanide method of Park and Johnson (14). Radioactivity was assayed by counting in Bray's scintillation solution.

**Gel Filtration on Sephadex**—A column (150 × 0.9 cm) of Sephadex G-25 (fine) (Pharmacia) was used. The sample, in 0.5 ml of H2O, was placed on the column and eluted with H2O. Fractions (2 ml) were collected at a flow rate of 0.3 ml per min and analyzed for reducing sugars and radioactivity by the procedures described above.

Specific activity of radioactive saccharide preparations were in all cases determined by taking identical aliquots for scintillation counting in Bray's solution and for the determination of reducing sugars (14) with reference to standards of the same compound.

**Preparation of Cell Wall Oligosaccharides**—The cell wall tetrasaccharide GlcNAc-MurNAc-GlcNAc-MurNAc and the disaccharide GlcNAc-MurNAc were isolated from M. lysodeikticus cell walls according to the method of Sharon et al. (2, 13). They were purified by preparative paper chromatography in Solvent I.

The cell wall hexa- and octasaccharides were obtained from M. lysodeikticus cell walls in the following manner. The dialyzable fraction of lysozyme digests was prepared as previously described (2, 13), except that incubation with the enzyme was reduced to a period of 12 hours. This fraction was chromatographed on a Dowex 1-X8 (acetate) column (2.5 × 90 cm) with a gradient of acetic acid (mixing chamber, 2 liters of water; reservoir, 2 liters of 1 M acetic acid). Two fractions emerging after the tetrasaccharase were collected between 0.55 and 0.65 and 0.65 and 0.80 M acetic acid. The fractions were pooled and lyophilized. Paper chromatography of the first fraction in Solvent I showed that it consisted mainly of tetrasaccharide and a slower moving component with an RF of 0.056 (hexasaccharide), as well as smaller amounts of a component with RF 0.050 (octasaccharide). The second fraction contained no tetrasaccharide and very little hexasaccharide, and consisted mainly of octasaccharide and compounds with very small RF values, 0.024 and 0.013. Each of these two fractions was purified by preparative paper chromatography in Solvent I, and the hexa-and octasaccharides were isolated. The octasaccharide was also found to have the same RF in Solvent II as the octasaccharide isolated by Sephadex G-25 chromatography by Leyh-Bouille et al. (15).

**Tritiation of Cell Wall Disaccharide and Tetrasaccharide**—One typical preparation of tritiated tetrasaccharide is described. Tetrasaccharide (100 mg) was exposed to 5 C of carrier-free tritium (16) at a pressure of slightly less than 1 atm for 14 days, after which it was dissolved in water and lyophilized. Dissolution and lyophilization were repeated five times to remove labile tritium. The crude tetrasaccharide-3H was fractionated on the large Dowex 1-X8 column, and the fractions corresponding to the major reducing sugar peak were pooled and lyophilized. The material obtained was then passed through a column of Sephadex G-25, and the peak fractions containing reducing sugar again were pooled. Preparative paper chromatography of this saccharide in Solvent I yielded 32 mg of material; this was once again chromatographed on a large Dowex 1-X8 column to yield the purified labeled tetrasaccharide. The labeled disaccharide was prepared in a similar manner.

**Isolation of Saccharides from Partial Digestion of Tetrasaccharide-3H by Lysozyme**—The reaction mixture was prepared in...
an ice bath by mixing 0.50 ml of tetrasaccharide solution (15.0 mg per ml, 2.15 × 10^6 cpm per mg), 0.30 ml of lysozyme solution (0.45 mg per ml), and 0.20 ml of ammonium acetate-acetic acid buffer (0.5 M in acetate) to give a final pH of 5.25. The solution was incubated at 37.0° for 30 min, again chilled to 0°, and chromatographed on the small Dowex 1-X8 column.

**Time Course of Digestion of Tetrasaccharide-3H by Lysozyme**—Reaction mixtures 0.1 to 0.3 ml in volume were made up at 0° by mixing solutions of tetrasaccharide, lysozyme, and buffer, as above. An aliquot was taken at zero time, and the tube was then placed in a 37.0° bath. At various time intervals, aliquots (15 to 25 μl) were taken with a microsyringe and spotted on Whatman No. 1 paper along with appropriate references. The spotting process took up to 2.5 min for each aliquot. The paper was subjected to electrophoresis at pH 6.5 for 75 min, dried in air (without heating) until no pyridine odor remained, and then subjected to descending paper chromatography with Solvent I and on the small Dowex 1-X8 column. Whatman No. 1 paper along with appropriate references. The spotting process took up to 2.5 min for each aliquot. The paper was subjected to electrophoresis at pH 6.5 for 75 min, dried in air (without heating) until no pyridine odor remained, and then subjected to descending paper chromatography with Solvent I and on the small Dowex 1-X8 column.

**Lysozyme-catalyzed Incorporation of Disaccharide-3H into Tetrasaccharide**—A reaction mixture consisting of 15.5 mg of unlabeled tetrasaccharide, 0.20 ml of disaccharide-3H solution (7.95 mg per ml, 7.7 × 10^6 cpm per mg), 0.60 ml of lysozyme (0.45 mg per ml), and 0.20 ml of ammonium acetate-acetic acid buffer (0.5 M in acetate), to give a final pH of 5.25, was incubated at 37.0°. Aliquots of the reaction mixture (10 μl) were taken at various times during the incubation and analyzed by paper electrophoresis at pH 6.5. After 330 min, the remaining incubation mixture was chromatographed on the small Dowex 1-X8 column. The central portions of the peaks from column chromatography corresponding to disaccharide and tetrasaccharide were pooled and lyophilized, yielding products with specific activities of 7.3 × 10^6 and 1.8 × 10^6 cpm per mg, respectively, with about one-third of the radioactivity found in the tetrasaccharide. Both saccharides were found to be essentially radiochemically pure on paper chromatography in Solvent I and on the small Dowex 1-X8 column.

**Position of Label in Tetrasaccharide Labeled by Incorporation of Disaccharide-3H**—A reaction mixture consisting of 0.025 ml of bovine serum albumin solution (2 mg per ml), 0.125 ml of 0.05 M citrate buffer (pH 4.2), and 0.100 ml of boar epididymal oxo N-acetylgalactosaminidase solution (3 × 10^6 units per ml per hour, in 0.05 M citrate buffer and 0.1 M NaCl) was prepared (11). An aliquot of this mixture (50 μl) was added to each of two tetrasaccharide samples: Sample A, 150 μg of tetrasaccharide generally labeled with 3H by the Wilzbach (16) technique (2.15 × 10^6 cpm per mg); Sample B, 197 μg of tetrasaccharide labeled by lysozyme-catalyzed incorporation of disaccharide-3H, as above (1.8 × 10^6 cpm per mg). After 22 hours of incubation at 37.0°, both reaction mixtures were spotted on paper, together with appropriate reference compounds, and the paper was subjected to electrophoresis at pH 6.5. The strip corresponding to each reaction mixture was sewn onto another sheet of paper and chromatographed with Solvent I at right angles to the direction of electrophoresis. The spots were made visible with NaOH (1), and the areas corresponding to MurNAc-GlcNAc-MurNAc (electrophoretic mobility, about 1.05 times that of tetrasaccharide; Rf 0.25 in Solvent I) and to GlcNAc were cut into strips 2 × 3 cm and counted by scintillation counting.

**Transfer to N-Acetylgalactosamine-1-14C**—A solution containing GlcNAc-1-14C (50 μl, 6.6 mg per ml), lysozyme (0.40 ml, 0.566 mg per ml), water (0.35 ml), and ammonium acetate-acetic acid buffer (0.20 ml, 0.5 M), at a final pH of 5.25, was prepared. An aliquot (0.10 ml) of this solution was added to 1.2 mg of tetrasaccharide labeled by incorporation of disaccharide-3H. The GlcNAc-1-14C and tetrasaccharide-3H had specific activities of 3.1 × 10^6 and 6.0 × 10^6 cpm per μmole, respectively, when counted with the scintillation counter windows set for 3H-14C double channel counting (17). Immediately after mixing, an aliquot (40 μl) of the reaction mixture was spotted on paper and the mixture was incubated for 13 hours at 37°. Following incubation, a second aliquot was spotted, and the paper was subjected to electrophoresis at pH 6.5. The strips corresponding to each aliquot were cut up and counted by double channel scintillation. The 3H and 14C activities in each section were calculated by using the appropriate corrections for blanks and for crossover between channels.

**Acid Hydrolysis of Saccharides**—Samples of radioactive saccharides (20 μg of disaccharide-3H, 7.7 × 10^6 cpm per mg; 40 μg of tetrasaccharide-3H, 2.8 × 10^6 cpm per mg; and 100 μg of tetrasaccharide-1-14C labeled by enzymatic incorporation of disaccharide-1-14C, 1.8 × 10^6 cpm per mg) and of unlabeled disaccharide were hydrolyzed by heating at 100° for 2 hours in 0.4 ml of 2 N HCl. The hydrolysates were evaporated in a vacuum over KOH, dissolved in minimal volumes of water, and spotted quantitatively on paper. Upon paper electrophoresis (pH 3.5) and scanning, each radioactive sample showed the same pattern: two major peaks of approximately equal area, corresponding to muramic acid and glucosamine, and two small peaks, one corresponding to GlcNAc and the other to a faint spot slightly slower than glucosamine, also seen in the hydrolysate of unlabeled disaccharide.

**Amino Sugar Analyses**—Amino sugar analyses of the hexa- and octasaccharide were carried out on the long column of the Beckman/Spincaco amino acid analyzer after acid hydrolysis as above. The integration constants for muramic acid and glucosamine were taken from the work of Mirelman and Sharon (18).

**Molecular Weight Determinations**—For these procedures, the short column equilibrium sedimentation technique of Yphantis (19) was used. One per cent solutions of the saccharides in 0.2 M NaCl were equilibrated for 2 hours in the Beckman/Spincaco ultracentrifuge at 35,000 rpm at 20°. The partial specific volume, δ, for the oligosaccharides was assumed to be 0.09 (18).

**RESULTS**

**Tritiated Cell Wall Saccharides**—After a number of purification steps, which are summarized in Table I, a preparation of tritium-labeled cell wall tetrasaccharide with a specific activity of 2.8 × 10^6 cpm per mg on scintillation counting in Bray's dioxane solution (or about 3 mC per mmole) was obtained. The tetrasaccharide-3H preparation was found to be radiochemically pure by a number of criteria. When the purified tritiated tetrasaccharide was diluted with unlabeled tetrasaccharide and the
had a specific activity of $7.7 \times 10^6$ cpm per mg. This material gave single, symmetrical peaks on paper chromatography with Solvents I, II, and III and on paper electrophoresis at pH 6.5. After dilution with nonradioactive saccharide, it chromatographed on Dowex 1-X8 as a single peak with constant specific activity.

**Products of Action of Lysozyme on Cell Wall Tetrascarhide—** The results of fractionation of a partial lysozyme digest of tetrasaccharide-$3H$ on a Dowex 1-X8 column are given in Fig. 1. The peaks corresponding to the disaccharide and tetrascarhide, as well as the fractions eluted after the tetrascarhide, were pooled and lyophilized. Upon paper chromatography in Solvent I, the di- and tetrascarhide fractions migrated as single spots, whereas the latter fractions were not homogeneous. They were further purified by preparative paper chromatography in Solvent I, yielding labeled hexa-, octa-, deca-, and dodecascarhides. The $R_f$ values, electrophoretic mobilities, and behavior on anion exchange resin chromatography of the products listed in Table II suggest that these six compounds are members of a series of oligomers of the formula (GlcNAc-MurNAc)$_n$. The relation mobilities on paper of the oligosaccharides in Solvent I obey the well known relationship (20) between $R_f$ and degree of polymerization, $n$, as shown in Fig. 2.

The hexasaccharide-$3H$ and octasaccharide-$3H$ obtained from the partial lysozyme digest of tetrasaccharide-$3H$ are also identical in their mobility upon paper electrophoresis at pH 6.5, and on paper chromatography (Solvent I) with those of the hexasaccharide and octasaccharide isolated from bacterial cell walls. The latter were shown by acid hydrolysis to contain equal amounts of the two amino sugars glucosamine and muramic acid, and their molecular weights were determined by equilibrium sedimentation experiments to be 1377 and 1800, respectively (calculated values, 1452 and 1930). Furthermore, both the hexa- and octasaccharides were readily digested by lysozyme to give mainly the corresponding di- and tetrascarhides.

**Course of Reaction—** The complex reaction mixtures obtained upon incubation of the cell wall oligosaccharides with lysozyme could also be separated into their components by a combination of paper electrophoresis and paper chromatography. Following this separation, the relative amounts of radioactivity due to each component in the mixture at any given time could be determined. Fig. 3 shows the results of a typical experiment in which lysozyme was incubated with tetrasaccharide-$3H$ and an aliquot was analyzed by a combination of paper electrophoresis and paper chromatography.

**TABLE II**

<table>
<thead>
<tr>
<th>Step</th>
<th>Sample weight $^a$</th>
<th>Activity $^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lyophilization</td>
<td>100</td>
<td>$1.7 \times 10^8$</td>
</tr>
<tr>
<td>Dowex 1 chromatography</td>
<td>68</td>
<td>$2.2 \times 10^7$</td>
</tr>
<tr>
<td>Sephadex G-25 chromatography</td>
<td>51</td>
<td>$1.65 \times 10^7$</td>
</tr>
<tr>
<td>Paper chromatography, Solvent I</td>
<td>32</td>
<td>$3.5 \times 10^4$</td>
</tr>
<tr>
<td>Dowex 1 chromatography</td>
<td>19</td>
<td>$2.8 \times 10^4$</td>
</tr>
</tbody>
</table>

$^a$ The weight of radioactive material was determined by comparison of the reducing value of the product with that of an authentic sample of unlabeled tetrasaccharide.

$^b$ Radioactivity was determined by scintillation counting in Bray's solution.

**Fig. 1.** Ion exchange chromatography of the products from a lysozyme digest of tetrasaccharide-$3H$. The tetrasaccharide (0.50 ml of a $1.5 \times 10^{-3}$ M solution, $2.15 \times 10^5$ cpm per $\mu$ mole) was incubated at $37^\circ$ with hen's egg white lysozyme (0.50 ml, 0.45 mg per ml) in 0.5 M ammonium acetate-acetic acid buffer, pH 5.25 (0.20 ml). After 30 min, the digest was placed on a Dowex 1-X8 acetate column (1.1 X 25 cm). Linear gradient elution was carried out with acetic acid (reservoir, 1 liter of 1.5 M acetic acid; mixing chamber, 1 liter of H$_2$O), and fractions of 8.5 ml were collected at a flow rate of about 0.5 ml per min. Every 10th tube was titrated with 0.1 M NaOH for the gradient (— — —, 0.2-ml aliquots). Analysis of every fourth tube for determination of reducing sugars was done by the method of Park and Johnson (14) (— — —, 0.1-ml aliquots), and every second tube was counted for 5 min in Bray's scintillation solution (— — —, 0.1-ml aliquots).

The disaccharide, tritiated and purified by a similar procedure, mixture was rechromatographed on Dowex 1-X8, the saccharide emerged as a single peak with a constant specific activity across the peak. Upon paper chromatography with Solvents I, III, and IV, and upon paper electrophoresis at pH 3.5 and 6.5, the purified tetrascarhide gave only single, symmetrical radiactive peaks at positions equivalent to those of references of authentic unlabeled tetrascarhide.

The disaccharide, tritiated and purified by a similar procedure,
If a number of aliquots at various time intervals were analyzed by this method, the change in concentration of each component with time could be followed. Fig. 4 shows the course of a reaction in which a solution of tetrasaccharide-^3H (7.5 × 10^{-3} M) was digested by lysozyme (300 µg per ml) in a pH 5.25 buffer at 37°C. It can be seen that the reaction followed a complex pattern, with the rate of digestion of tetrasaccharide and appearance of disaccharide varying markedly with time. The maximal rate of disappearance of the tetrasaccharide which occurred under the experimental conditions used (between 5 and 15 min of incubation) was found to be 8 × 10^{-2} M per g of enzyme per hour.

FIG. 3. Analysis of the products of a reaction mixture by high voltage paper electrophoresis and paper chromatography. Tritium-labeled tetrasaccharide (0.1 ml, 7.5 × 10^{-3} M, 2.15 × 10^6 cpm per µmole) was incubated with hen's egg white lysozyme (0.30 mg per ml) in 0.5 M ammonium acetate-acetic acid buffer (0.04 ml). Distilled water (0.060 ml) was added to give a final solution volume of 0.2 ml. After 17 min of incubation, an aliquot (0.03 ml) was removed from the reaction mixture and spotted on paper. The subsequent steps, involving first paper electrophoresis (pH 6.5), then paper chromatography (Solvent 1), and finally liquid scintillation counting of the cut up paper, were carried out as described in the text; 2, 4, 6, 8, 10, and 12 denote the di-, tetra-, hexa-, octa-, deca-, and dodecasaccharides, respectively.

TABLE III

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Substrate concentration</th>
<th>Enzyme concentration</th>
<th>Rate of digestion</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Tetrasaccharide</td>
<td>3.8 × 10^{-4} M</td>
<td>0.3</td>
<td>3.8 × 10^{-7} M/g enzyme/hr</td>
</tr>
<tr>
<td>2. Tetrasaccharide</td>
<td>2.5 × 10^{-4} M</td>
<td>0.3</td>
<td>7 × 10^{-4} M/g enzyme/hr</td>
</tr>
<tr>
<td>3. Tetrasaccharide</td>
<td>2.5 × 10^{-4} M</td>
<td>0.03</td>
<td>2.5 × 10^{-4} M/g enzyme/hr</td>
</tr>
<tr>
<td>4. Tetrasaccharide</td>
<td>2.9 × 10^{-4} M</td>
<td>0.03</td>
<td>12 × 10^{-4} M/g enzyme/hr</td>
</tr>
<tr>
<td>5. Hexasaccharide</td>
<td>2.4 × 10^{-4} M</td>
<td>0.03</td>
<td>12 × 10^{-4} M/g enzyme/hr</td>
</tr>
<tr>
<td>6. Octasaccharide</td>
<td>3.8 × 10^{-3} M</td>
<td>0.3</td>
<td>3.8 × 10^{-7} M/g enzyme/hr</td>
</tr>
<tr>
<td>7. Tetrasaccharide with unlabeled octasaccharide (1 × 10^{-4} M)</td>
<td>3.8 × 10^{-3} M</td>
<td>0.3</td>
<td>10^{-1} M/g enzyme/hr</td>
</tr>
</tbody>
</table>

It can be seen that the reaction followed a complex pattern, with the rate of digestion of tetrasaccharide and appearance of disaccharide varying markedly with time. The maximal rate of disappearance of the tetrasaccharide which occurred under the experimental conditions used (between 5 and 15 min of incubation) was found to be 8 × 10^{-2} M per g of enzyme per hour. We have also measured the rate of digestion of tetrasaccharide as affected by changes in concentration of substrate and of enzyme. The results given in Table III show that the rate of digestion...
isolated from bacterial cell walls was added to the tetrasaccharide-\(^{3}H\) \((3.8 \times 10^{-3} \text{ M})\), the digestion by lysozyme \((300 \, \mu\text{g per ml})\) of the tetrasaccharide was greatly accelerated (Fig. 7). After 20 min of incubation at 37\(^\circ\), the reaction mixture with added octasaccharide contained nearly 30\% of the radioactivity in the form of disaccharide, while in a similar reaction mixture, without added octasaccharide, only about 10\% of the radioactivity was in the form of disaccharide. The maximal rate of digestion of tetrasaccharide with added octasaccharide was 5 times faster than that of the tetrasaccharide alone, at the same concentration of substrate and enzyme (Table III, Experiments 2 and 7).

**Incorporation of Disaccharide\(^{-3}H\) into Tetrasaccharide**—When the tetrasaccharide obtained by transfer of unlabeled disaccharide from unlabeled tetrasaccharide to labeled disaccharide was cleaved at the nonreducing terminal end by the pig epididymal exo\(\beta\)N-acetylglucosaminidase, the GlcNAc released contained only 2.2\% of the radioactivity in the sample, while the MurNAc-GlcNAc-MurNAc had 97.8\% of the activity. Since both sugar moieties of the disaccharide\(^{-3}H\) incorporated are known to be labeled to a similar extent, it appears that the label was almost exclusively incorporated in the reducing end of the tetrasaccharide.

The position of the radioactive label was even more clearly manifested by the lysozyme-catalyzed transfer from this tetrasaccharide to GlcNAc-1\(^{-14}C\). The results of the electrophoretic decreased to a large extent with decrease of substrate concentration.

The labeled hexasaccharide and octasaccharide isolated from a reaction mixture of tetrasaccharide-\(^{3}H\) by lysozyme were found to be very rapidly broken down by lysozyme (Table III). The course of digestion of the hexasaccharide \((2.9 \times 10^{-4} \text{ M})\) is given in Fig. 5, and that of the octasaccharide \((2.4 \times 10^{-4} \text{ M})\) in Fig. 6. In each case, only 30 \(\mu\text{g}\) of lysozyme per ml were used.

When a small amount of the octasaccharide \((1 \times 10^{-4} \text{ M})\)
saccharide was at least 20-fold greater in the reducing end than the nonreducing end of the tetrasaccharide as from the reducing end. This also indicates that the incorporation of disaccharide-H into tetrasaccharide was at least 20-fold greater in the reducing end than in the nonreducing end.

**DISCUSSION**

The substrates most commonly used for the assay of lysozyme are cells or cell walls of *M. lysodeikticus*. The structure of these substrates, however, is highly complex, and studies with them give only limited information on the mechanism of action of lysozyme. The finding that lysozyme can act upon low molecular weight oligosaccharides derived from chitin or from bacterial cell walls (21) provided for the first time a useful tool for the detailed investigation of the enzymatic properties of lysozyme. Such studies became especially significant after the three-dimensional structures of lysozyme and lysozyme-saccharide complexes were elucidated by the x-ray crystallographic investigations of Phillips and co-workers (22-25).

Earlier work (1-10) on the pattern of action of lysozyme on the oligosaccharide substrates showed the reaction to be complex, involving both hydrolysis and transglycosylation. In order to follow quantitatively the fates of the oligosaccharide molecules in the course of the lysozyme-catalyzed reactions, it became desirable to prepare radioactively labeled substrates. Since no good synthetic substrates for lysozyme are yet available, we prepared the tritium-labeled tetrasaccharide GlcNAc-MurNAc-GlcNAc-MurNAc, starting with the unlabeled natural product.

The procedure described in this paper for the preparation of the radioactive tetrasaccharide tritiated by the Wilzbach technique (10) gives a product suitable for the studies of lysozyme. Following tritiation, the labeled tetrasaccharide was obtained in good over-all yield (19%) by a series of purification steps. The product was found to be homogeneous and radiochemically pure by several chromatographic and electrophoretic criteria, and to be identical with the unlabeled authentic compound. After partial digestion of the labeled tetrasaccharide with lysozyme, the disaccharide and tetrasaccharide isolated from the reaction mixture had the same specific activity (see Fig. 1), indicating that the preparation contained no radioactive impurity which is attacked by the enzyme in a mode different from that of the unlabeled saccharide. The tetrasaccharide isolated from the partial digestion reaction could be further digested by lysozyme to the same extent as the original preparation.

The Wilzbach tritium-labeling technique is known to be random, and chemically similar hydrogens on a molecule exposed to such tritiation are expected to be exchanged to a similar degree. Thus, one might expect the two GlcNAc moieties in the tetrasaccharide-H preparation to be labeled to an equal extent, and the two MurNAc moieties to be labeled to an equal extent, although the labeling in GlcNAc and MurNAc might be rather different. Initially all hydrogen positions in the molecule become labeled when exposed to tritium gas, but the hydrogens in the “labile” positions (in this case, hydrogens attached to oxygen and nitrogen atoms) lose their radioactivity upon repeated dissolution and lyophilization. It may be safely assumed, therefore, that in the final product only hydrogens linked to carbon atoms are radioactively labeled. The MurNAc residues contain more hydrogens attached to carbon than GlcNAc, and this would account for the greater extent of labeling in the MurNAc residues in comparison to the GlcNAc residues. As the assumption of uniform labeling in the two halves of the tetrasaccharide is important in interpreting the results, the distribution of H in the tetrasaccharide was investigated by various chemical and enzymatic experiments.

When acid hydrolysis of the tetrasaccharide-H and the disaccharide-H (and the tetrasaccharide labeled by enzymatic incorporation of disaccharide-H) was carried out, it was found that the glucosamine and muramic acid moieties in each saccharide were labeled to about the same extent (based on the number of carbon-linked hydrogens in each monosaccharide). Digestion of the tetrasaccharide-H with exo-β-N-acetylglucosaminidase revealed that 17% of the H in the tetrasaccharide preparation was contained in the nonreducing terminal GlcNAc moiety, as compared with a theoretical value of 21% based on completely random labeling. All these results indicate that the tetrasaccharide molecules can be considered to be uniformly labeled.
The tritium-labeled disaccharide was prepared from unlabeled disaccharide by methods similar to those used for the preparation of the labeled tetrasaccharide. This compound was shown to be homogeneous and radiochemically pure and, although not tested as rigorously as the tetrasaccharide-\( _{3}^{4}\)H, seemed also to be uniformly labeled.

In addition to the disaccharide and tetrasaccharide, at least four radioactive higher oligosaccharides have been identified in reaction mixtures after incubation of lysozyme with the cell wall tetrasaccharide-\( _{3}^{4}\)H. Our previous hypothesis (1-3) that such saccharides are higher oligomers of the formula (GlcNAc-MurNAc), has been further strengthened. The \( R_{\Phi} \) values of the six oligosaccharides in Solvent I followed the expected relationship between \( R_{\Phi} \) and degree of polymerization, \( n \), for \( n = 1 \) to 6 (Fig. 2), and the electrophoretic mobilities and behavior on anion exchange chromatography of these compounds were also as expected. Since all of these saccharides except the di- and tetrasaccharide disappear from reaction mixtures after sufficiently prolonged incubation, the saccharides of molecular weight higher than the tetrasaccharide must be susceptible to hydrolysis by lysozyme to yield the di- and tetrasaccharide. This has in fact been shown directly for the hexa- and octasaccharide (Figs. 5 and 6). The hexa- and octasaccharide-\( _{3}^{4}\)H isolated from reaction mixtures of tetrasaccharide-\( _{3}^{4}\)H incubated with lysozyme were also identical, by chromatographic and electrophoretic criteria, with two compounds isolated from the bacterial cell wall. The latter compounds were found to have molecular weights corresponding to a hexa- and an octasaccharide, respectively, and to contain equimolar amounts of glucosamine and muramic acid.

Although most workers have referred to the formation in lysozyme-catalyzed reactions of saccharides of higher molecular weight than the starting material, or of products containing parts of two different starting materials joined together, as being due to transglycosylation reactions, there could be some question of whether this is the only interpretation possible. In particular, it is of interest to what extent such products are formed by true transglycosylation (Equation 1) and to what extent by the “reverse of hydrolysis” (Equation 2).

\[
\begin{align*}
R_{1}-O\rightarrow R_{2} + HO_{3} & \rightarrow R_{1}-O\rightarrow R_{2} + H-O\rightarrow R_{2} \quad (1) \\
R_{1}-OH + HO_{3} & \rightarrow R_{1}-O\rightarrow R_{2} + H_{2}O \quad (2)
\end{align*}
\]

In two experiments carried out by us to test this point, it was found that the new products in question were formed almost exclusively by true transglycosylation. On incubation of unlabeled tetrasaccharide with disaccharide-\( _{3}^{4}\)H in the presence of lysozyme, at most 5% of the label incorporated into the tetrasaccharide was in the nonreducing half of the molecule. If the labeled tetrasaccharide were formed by reverse of hydrolysis from two disaccharide moieties, the label would be expected to be random. Upon incubation of tetrasaccharide-\( _{3}^{4}\)H labeled only in the reducing half together with GlcNAc-\( _{3}^{4}\)C in the presence of lysozyme, the GlcNAc-MurNAc-GlcNAc formed contained a very small amount of tritium (Fig. 8), consistent only with incorporation of less than 5\% of the GlcNAc-MurNAc from the reducing end of the tetrasaccharide. If the triasaccharide were formed by reverse of hydrolysis from free disaccharide (produced by digestion of the tetrasaccharide) and GlcNAc, half the GlcNAc-MurNAc incorporated would be from the labeled, reducing end of the tetrasaccharide; on the other hand, if the triasaccharide were formed by the perhaps more likely path of reverse of hydrolysis from the tetrasaccharide and GlcNAc to form a pentasaccharide, which then would be hydrolyzed, it would contain GlcNAc-MurNAc from the reducing end only. Thus, our experimental results show that in the reactions investigated over 90\% of the new product must be formed by true transglycosylation.

Analysis of the change in composition with time of reaction mixtures of lysozyme and tetrasaccharide-\( _{3}^{4}\)H (Fig. 4) indicates that transglycosylation plays a very important role in the overall process of hydrolysis of the tetrasaccharide to disaccharide. During the course of the reaction, the fraction of the total weight of saccharide in the form of the hexa- and higher oligosaccharides may exceed 25\%. There seems to be a definite correlation between the concentration of higher oligosaccharides at a given time and the rate of formation of disaccharide. This is clearly not a coincidence, as a separate experiment showed that the addition of small amounts of octasaccharide to tetrasaccharide-\( _{3}^{4}\)H at the outset of the reaction strongly accelerated the hydrolysis of the tetrasaccharide by lysozyme (Fig. 7). The hexa- and octasaccharides are themselves very rapidly hydrolyzed by lysozyme (Figs. 5 and 6, and Table III).

The above features of the reactions of cell wall oligosaccharides with lysozyme may be understood in terms of a few simple hypotheses, which are also supported by data for association of various saccharides with lysozyme (26) and by the model of the three-dimensional structure of the lysozyme molecule proposed by Blake et al. (24, 25). These hypotheses are as follows.

1. The cell wall tetrasaccharide GlcNAc-MurNAc-GlcNAc-MurNAc is most strongly bound to lysozyme in a nonproductive manner, one which does not lead to bond cleavage. As we described in a previous paper (26), this would be in Subsites A to D of the active site of lysozyme. This mode of binding is expected to be preferred because Subsites A, B, and C are the strong binding subsites, but Subsite C is prohibited to the MurNAc moiety. Although Subsite D is “unfavorable,” the sum of the contributions of C and D is favorable (26).

2. A weaker, and therefore less probable, mode of binding of the tetrasaccharide is in Subsites C, D, E, and F. This mode, with a bond lying across the “catalytic region,” between D and E (25), may lead to bond cleavage. From the comparison of the binding constant of the hexaasaccharide (5) with that of the tetrasaccharide, Subsites E and F contribute about 1.7 kcal per mole to \(-\Delta F^{\circ}\) of association of a saccharide interacting with both of them. As A and B together would contribute 5.7 kcal per mole to \(-\Delta F^{\circ}\), the tetrasaccharide would be bound in the nonproductive mode, ABCD, about 1000 times more often than in the productive mode, CDEF.

3. The cell wall hexaasaccharide is likely to be bound almost entirely across A to F, with the second bond from the reducing end, between D and E, susceptible to cleavage. The octasaccharide and higher oligosaccharides will also be bound across A to F, with the rest of the molecule extending beyond these subsites on one or both sides.

4. After a bond lying across D and E is cleaved, the section of the saccharide lying in E and beyond may be released as the free saccharide. The section lying up to D remains as an intermediate with some finite lifetime (4), which may be attacked either by water or by a hydroxyl group of another saccharide.

5. Subsites E and F interact with an attacking acceptor saccharide, and thus lead to the specific formation of new glycosidic linkages (4, 5). This interaction also favors transglycosyla-
tion over hydrolysis. There is probably little enhancement of attack by an acceptor larger than a disaccharide, as compared with a disaccharide.

These hypotheses explain the observed behavior of lysozyme with cell wall oligosaccharides. The tetrasaccharide itself is cleaved only slowly because it is bound largely in a nonproductive mode. By reactions such as those in Equations 3 and 4, however, it may slowly form higher oligosaccharides.

\[
\text{GlcNAc-MurNAc-GlcNAc-MurNAc} + E \rightarrow \text{GlcNAc-MurNAc-GlcNAc-MurNAc} \sim E + \text{GlcNAc-MurNAc}
\]  

\[
\text{GlcNAc-MurNAc} \sim E + \text{GlcNAc-MurNAc}
\]  

A saccharide such as the hexasaccharide is quite reactive, as it is preferentially bound in a productive mode. It may act as a "chain carrier" in a cyclic series of reactions which lead to a net result of rapid hydrolysis of the tetrasaccharide to disaccharide, as outlined in Equations 5 to 8.

\[
\text{GlcNAc-MurNAc-GlcNAc-MurNAc-GlcNAc-MurNAc} + E \rightarrow \text{GlcNAc-MurNAc-GlcNAc-MurNAc} \sim E + \text{GlcNAc-MurNAc-GlcNAc-MurNAc}
\]  

\[
\text{GlcNAc-MurNAc-GlcNAc-MurNAc} \sim E + \text{GlcNAc-MurNAc-GlcNAc-MurNAc}
\]  

\[
\text{MurNAc-GlcNAc-MurNAc} \rightarrow \text{GlcNAc-MurNAc-MurNAc}
\]  

\[
\text{MurNAc-GlcNAc-MurNAc} \rightarrow \text{GlcNAc-MurNAc-MurNAc}
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\]  

\[
\text{GlcNAc-MurNAc-GlcNAc-MurNAc} \sim E + \text{GlcNAc-MurNAc-GlcNAc-MurNAc}
\]  

\[
\text{GlcNAc-MurNAc-GlcNAc-MurNAc-GlcNAc-MurNAc} \sim E + \text{GlcNAc-MurNAc-GlcNAc-MurNAc}
\]  

As large amounts of disaccharide build up, however, the overall reaction is inhibited, because the disaccharide intercepts the "active intermediates," and leads to a decrease in the amounts of higher oligosaccharides available. The reaction then slows down considerably to a stage at which there is very little net cleavage of tetrasaccharide, and much of the tetrasaccharide is not hydrolyzed.

This scheme also would explain the increase in over-all rate of conversion of tetrasaccharide to disaccharide with increasing tetrasaccharide concentration (Table III), even beyond the concentration at which the enzyme would be saturated. Since the over-all rate must depend on the amount of higher oligosaccharides available and on the efficiency of the transglycosylation process, it is dependent on the competition of saccharides with water for the active intermediates. This competition is, of course, dependent on saccharide concentration.

In work with GlcNAc-GlcNAc-GlcNAc and other chitin oligosaccharides, Rupley and Gates (7) also observed increased rates of hydrolysis by lysozyme with increasing concentrations of substrate beyond the point of saturation of the enzyme with one substrate molecule. The explanation they offered, that the active complex for cleavage must be one in which a second molecule of substrate is bound in DEF after the first is bound in ABC, cannot possibly apply in the case of the cell wall oligosaccharides. In addition, it is difficult to imagine how the enzyme site, which models indicate can accommodate a hexasaccharide in Subsites A to F, could accommodate two trisaccharides which have one more oxygen atom than a hexasaccharide in the same subsites. As the phenomenon concerned is certainly explained in the case of cell wall oligosaccharides by a complex mechanism involving transglycosylation to form reactive higher oligosaccharides, we suggest that such an explanation may be more appropriate for the reaction of chitin oligosaccharides as well.

In experiments in which hexasaccharide-3H or octasaccharide-3H at low concentration (2.9 \( \times \) 10^{-3} and 2.4 \( \times \) 10^{-3} m, respectively) is hydrolyzed by lysozyme, the reactions appear to follow comparatively simple courses. At these concentrations, the saccharides apparently do not compete effectively with water for the "reactive intermediates" formed after cleavage, and relatively little material resulting from transglycosylation is formed. The distribution of products of the hydrolysis of the octasaccharide (Fig. 6) also indicates that the two expected modes of hydrolysis (coming from formation of complexes in which all six subsites are occupied, i.e. at the second and fourth bonds from the reducing terminus of the substrate) occur in about equal amounts.

The relative simplicity of these reactions allows one to make an estimate of an important kinetic parameter of the enzymatic reaction. The concentrations of substrate used, although low enough to prevent extensive transglycosylation, are higher than the dissociation constants of the enzyme-substrate complexes formed (K, dissociation for the lysozyme-hexasaccharide complex, is about 3 \( \times \) 10^{-8} M (5)). Since the enzyme must be essentially entirely in the form of productive complexes and reactive intermediates, in these cases the rate of hydrolysis of the substrate enables one to estimate the turnover number for the enzyme. This turnover number for the digestion of the hexa- and octasaccharides is thus of the order of 30 moles of substrate per mole of enzyme per min.

It should be noted that the rate of hydrolysis of these higher oligosaccharides is comparable to the rate of digestion of whole bacterial cells by lysozyme. Since the cell wall polysaccharide comprises about 5% of the dry weight of M. lysodeikticus, a suspension of 250 \( \mu \)g of dry bacteria per ml is about 2.5 \( \times \) 10^{-3} M in susceptible MurNAc-GlcNAc linkages. With 10 \( \mu \)g of lysozyme per ml operating with a turnover of 30 moles of substrate per mole of enzyme per min, the rate of hydrolysis would be about 2 \( \times \) 10^{-3} M per min. In turbidimetric experiments with these concentrations of enzyme and M. lysodeikticus cells, the half-time for clearing of the bacterial suspension is on the order of 1 min (3). As it is unlikely that clearing involves the hydrolysis of all susceptible bonds, the hexa- and octasaccharides are probably as good substrates for lysozyme as the whole bacterial cell.

The study of the action of lysozyme on generally labeled cell wall oligosaccharides, together with studies of association constants of various saccharides with lysozyme, provides a clear picture of the interaction between lysozyme and its substrates. It is evident that each of five or six subsites in the active site of lysozyme plays a role in determining the course that the enzyme-catalyzed reactions will take. The relative rates of digestion of various saccharides, as well as the products formed in lysozyme reactions, may be predicted on the basis of the properties of...
these sites, and these properties are in full agreement with the three-dimensional model of lysozyme developed by Phillips et al. (21–25). The important problem which remains to be solved is the detailed mechanism of the bond cleavage step in lysozyme-catalyzed reactions.

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