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Mode of Action of a Cellulase Component from *Cellvibrio gilvus**

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The mode of action of one of four electrophoretically distinguishable β (1 → 4) glucan hydrolases from *Cellvibrio gilvus* has been investigated. Analysis of the intermediates and products during hydrolysis of cellulose-oligosaccharides and chemically reduced cellulose-oligosaccharides indicated preferential attack at the second and third glucosyl bonds from the nonreducing end of the polysaccharide chain. Kinetic studies of the release of isotope from C^{14}-cellulose-oligosaccharides labeled exclusively in the nonreducing terminal glucosyl moiety confirmed this conclusion. No glycotransferase activity could be detected, and α-cellobiose was established as the primary initial reaction product during hydrolysis of reduced cellotetraose.

Kinetic data describing simultaneous changes in the degree of polymerization (D.P.) of carboxymethylcellulose and the production of soluble reducing sugars demonstrated conspicuous differences between the mode of action of purified β-1,4-glucan hydrolases from *Cellvibrio gilvus* and the random action of either phosphoric acid or a random-cleaving hydrolase from *Myrothecium verrucaria* (Storvick and King, 1960). An indication that attack by the enzymes from *C. gilvus* might remove successive cellobiosyl moieties was obtained by qualitative examination of reaction products on paper chromatograms.

Since then greatly improved procedures for isolation of gram-lots of the lower members of the cellulose-oligosaccharide series in high purity and for quantitative microanalysis of mixtures of the cellulose-oligosaccharides have been described. With these procedures the mode of action of one of the β-1,4-glucan hydrolases of *C. gilvus* has been examined in greater detail with end-labeled substrates prepared by chemical reduction of the reducing glucose group and by addition of the C^{14}-glucose moiety to the nonreducing end of β-1,4-glucans of known structure.

With these substrates it has been possible to determine the relative rates of attack on the lower members of the cellulose-oligosaccharide series, the susceptibility of each glucosyl bond to enzymic attack, and the configuration of the reducing group as it is released from the enzyme after hydrolysis.

Methods and Results

Isolation and Characterization of Cellulose Oligosaccharides.—Cellotriose, cellotetraose, cellopentaose, and cellohexaose were isolated from fuming HCl hydrolysates of cellulose by the procedures of Miller *et al.* (1960). Heavily loaded preparative scale columns yielded sugars contaminated with the next lower member of the polymer series. The odd and even numbered members of the polymer series were therefore pooled and subjected to a second column chromatographic separation, yielding cellotriose, cellotetraose, cellopentaose, and cellohexaose samples which showed no mutual contamination when tested on paper chromatograms and which gave within 1.5% of the theoretical D.P. values when analyzed as described by Timell (1960). Complete acid hydrolysis of 10 μmoles of the pentasaccharide yielded 50.9 μmoles of glucose as determined by notatin (Saifer and Gerstenfeld, 1958). Infrared spectra, however, revealed heavy contamination with stearic acid, which was a component of the column packing. After repeated reprecipitation from water with ethanol the infrared spectra were indistinguishable from those of Higgins *et al.* (1961) for authentic compounds, and there was no indication of either carboxyl or aliphatic groups. The specific optical rotations were within 1–5% of those reported by Wolfrom and Dacons (1952) with the exception of the tetrasaccharide, which deviated by 9%. Since the solubilities decrease so rapidly as D.P. increases, deviations of this magnitude are to be anticipated, a problem to which Wolfrom and Dacons also refer.

In the solvent system ethylacetate-pyridine-water (5:2:5 v/v/v) the isolated sugars through cellopentaose migrated so that a plot of log $1/(1 - R_F)$ against D.P. was linear. The R_F of cellohexaose was too low to permit estimation.

Preparation of Reduced Substrates.—Cellotetraose, cellopentaose, and cellohexaose were converted to the corresponding nonreducing sugars by reduction with $NaBH_4$. Approximately 1 μmole of borohydride and 1 μmole of sugar in 10 ml of water were heated at 96° for 30 minutes. Then an additional 0.25 μmole of borohydride was added and heating was continued for another 30 minutes. After the mixture had cooled to

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room temperature excess borohydride was destroyed by dropwise addition of glacial acetic acid. Sodium acetate and borate were then removed by passing the solution through a mixed-bed ion-exchange resin (Smith, 1960). The completeness of reduction was checked routinely by spotting 10 mg of sugar onto paper and spraying with aniline–diphenylamine–phosphoric acid reagent for reducing sugars as described by Buchan and Savage (1952). In no instance was evidence of residual reducing sugars seen. In addition, each of the reduced compounds was eluted as a single peak from the analytical column of Miller (1960) at the anticipated position.

Preparation of C^{14}-Cellulose Oligosaccharide.—UDP-glucose-C^{14} was prepared by incubating glucose-U-C^{14}, 44.2 μmoles (0.1 mc of C^{14}); ATP, 80 μmoles; UTP, 60 μmoles; $MgCl_2$, 0.25 μmole; EDTA, 25 μmoles; crystalline inorganic pyrophosphatase, 0.4 mg; crystalline hexokinase, 4 mg; and crude extract (Leloir and Trucco, 1955) of *Saccharomyces fragilis* ATCC 8608, 2.0 ml; in a final volume of 25 ml. All reagents except the crude extract were dissolved in 0.05 M Tris HCl buffer at *p*H 7.0. After incubation at 25° for 150 minutes, 12.6 μmoles of UDP-glucose-C^{14} was isolated by strip chromatography (Trucco, 1954) and found to have a specific activity of 1.2×10^6 cpm per μmole.

The UDP-glucose-C^{14} was then used in preparing cellulose oligosaccharide-C^{14}. *Acetobacter xylinum* ATCC 10245 was grown with forced-aeration in 6-liter batches and freed of cellulose according to the procedures of Hestrin and Schramm (1954). The pellet obtained from high-speed centrifugation of the sonicate of these cells was prepared as described by Glaser (1958). The reaction mixture consisted of cellohexaose, 60 mg; UDP-glucose-C^{14}, 5.0 μmoles in 5.0 ml of water; UDP-glucose, 10 μmoles in 0.25 ml of water; and 5.0 ml of crude enzyme in 0.05 M Tris buffer at *p*H 8.2 with 0.01 M $MgCl_2$ and 0.001 M EDTA. After incubation for 120 minutes at 25°, the reaction was stopped by heating at 98° for 7 minutes. The precipitated protein was removed by centrifugation, and the cellulose oligosaccharides in the supernatant were precipitated by adding 2 volumes of cold acetone. The precipitated C^{14}-sugars were then washed four times with 2:1 (v/v) acetone in water. The washed C^{14}-oligosaccharide preparation was subjected to strip chromatography on Whatman 3 MM paper with a 6:3:1 (v/v) isopropyl alcohol–water–acetic acid solvent to complete removal of oligosaccharides of D.P. below 6. Hot-water extraction of the origin yielded 30 mg of sugar having a specific activity of 96 cpm per mg. From the specific activities of the UDP-glucose-C^{14} and of the dextrin, and assuming addition of one glucose-C^{14} moiety to each cellohexaose, the mole ratio of cellohexaose and cellohexaose-C^{14} in the final oligosaccharide preparation was calculated to be 16,500:1.

Borohydride reduction of the dextrin (Timell, 1960) was followed by hydrolysis to glucose and sorbitol using 6 N HCl at 98° for 15 minutes. The products of hydrolysis were then separated by paper electrophoresis in H_2SO_4–Na_2MoO_4 buffer (Bourne *et al.*, 1959). The positions of glucose and sorbitol were determined using test strips with an alkaline silver nitrate spray (Trevelyan *et al.*, 1950). Hot-water extracts of the glucose and sorbitol areas of the electropherograms showed the C^{14}-label to be exclusively in the glucose; this demonstrates that the glucose-C^{14} was added to the nonreducing end of the cellohexaose.

The purification to which the C^{14}-oligosaccharide was subjected removes all $\beta(1 \rightarrow 4)$ glucans of 2,3,4,5, and >9 glucose units as well as any UDP-glucose or glucose. Paper chromatography of the starting glucose-U-C^{14} showed only one reducing sugar, and this reacted with glucose oxidase-peroxidase-*o*-dianisidine. The reducing sugar spot coincided with the position of C^{14} as shown by strip scanning. The biosynthetic UDP-glucose-C^{14} had an ultraviolet absorption spectrum identical with that of authentic UDP-glucose.

Analytical and Counting Procedures.—Radioactivity was measured after direct plating using a thin-window Geiger-Mueller tube or by scintillation counting in a Packard Tri-Carb scintillation counter. A minimum of 2500 counts over background was measured in all samples.

UDP-glucose was determined spectrophotometrically at 262 mμ using a reference molar absorbancy index of 10^4.

Reducing sugars were determined colorimetrically, using reference curves for the corresponding member of the cellulose-oligosaccharide series (Nelson, 1944). Nonreducing sugars were determined using the phenol–sulfuric acid reagent of Timell (1960) with glucose as standard.

The viscosimetric assay of King (1956) was used to standardize all enzyme solutions. Component I derived from culture filtrates of *C. gilvus* by zone electrophoresis as described by Storvick and King (1960) was used throughout.

Polarimetric determinations were conducted in a Rudolph High Precision Polarimeter in a darkroom using a sodium lamp as light source. Readings were made to 0.001° arc in a 1-dm tube having a total volume of 0.75 ml.

The microanalytical column of Miller (1960) was found to be satisfactory for resolution of all members of the cellulose polymer series from glucose through cellohexaose. For the present purposes it was also necessary to resolve the corresponding reduced polymer series (sorbitol through pentaglucosylsorbitol) simultaneously. The only modification of the original procedure that was required was a 50% increase in the amount of column packing. With this change all members of the two-polymer series were resolved completely except glucose and sorbitol, the reduced oligosaccharides being eluted immediately prior to the corresponding members of the reducing sugar polymer series. The accuracy of the analysis was improved by drying the eluted fractions in a forced-draft oven at 90–100° and analyzing the dried residues as described by Timell (1960). Adjustment for variations in the alcohol content of succeeding fractions was then unnecessary.

The composition of partial enzymic hydrolysates was determined by incubating 0.8–2.0 μmoles of substrate and 55–90 units of enzyme in 0.3–0.4 ml of 0.05 M potassium phosphate at *p*H 7.0 for 10–120 minutes. The reactions were stopped by boiling for 15 minutes, and aliquots were applied to the analytical column. Representative results of hydrolysis of reduced cellopentaose, reduced cellohexaose, and cellohexaose appear in Table I.

The relative rates of hydrolysis of the cellulose-oligosaccharide series between D.P. 2 and 6 was determined in reaction systems consisting of 11.5 units of enzyme and 3.0 μmoles of substrate in 2.0 ml of 0.05 M potassium phosphate at *p*H 7.0 and at 40°. Immediately after mixture of the prewarmed reactants and at 10-minute intervals thereafter aliquots of 100 μl were transferred to 2.0 ml of the alkaline copper reagent used for analysis of reducing sugars. Over at least the first 20 minutes all systems followed zero-order kinetics. Only data representing 15% hydrolysis of the substrate or less were used in calculating the rates of hydrolysis in order to avoid errors introduced by the

TABLE I
COMPOSITION OF PARTIAL HYDROLYSATES OF PENTA- AND HEXASACCHARIDES

Product	Substrate: Reduced Cello-pentaose (μmole)	Reduced Cello-hexaose (μmole)	Cello-hexaose (μmole)
Glucose	0	0.083	0.022
Glucosylsorbitol	0.044	0	0
Cellobiose	0.590	0.281	0.493
Cellobiosylsorbitol	0.606	0.325	0
Cellotriose	0.042	0.360	0.369
Cellotriosylsorbitol	0	0.330	0
Cellotetraose	0	0.030	0.040
Cellotetraosylsorbitol	0.024	0	0
Cellopentaose	0	0	0
Cellopentaosylsorbitol	0	1.475	0
Cellohexaose	0	0	0.018

fact that for the penta- and hexasaccharides the products of the initial cleavage are themselves substrates. Zero-order reaction velocities were calculated to be as follows in μmoles ml^{-1} min^{-1}: cellobiose, 0; cellotriose, 0.158; cellotetraose, 1.68; cellopentaose, 2.38; and cellohexaose, 3.11.

To determine whether the newly created reducing groups were released from the enzyme in the α- or the β-configuration 85 μmoles of triglucosylsorbitol produced by reduction of cellotetraose were incubated with 2100 units of enzyme at 24° in 2.0 ml of 0.005 M potassium phosphate at *p*H 7.0. A 0.75-ml portion was immediately placed in the polarimeter tube and readings of optical rotation were taken at 1-minute intervals for 36 minutes. Then 0.1 ml of concd NH_4OH was mixed with 1.0 ml of the remaining reaction mixture to catalyze mutarotation, and several more readings of optical rotation were made. The data are presented in Figure 1.

Evidence that transferase activity resulting in reversion of substrate to products of higher D.P. did not occur was obtained by incubating 10 μmoles of cellotetraose and 104 units of enzyme at 40° in 2.0 ml of 0.05 M potassium phosphate at *p*H 7.0 for 20 minutes. Analysis of the reaction mixture after inactivation of the enzyme by boiling revealed only cellobiose and residual cellotetraose. Hydrolysis was 20% complete at this point. Either cellopentaose or cellohexaose would have been observed under these conditions had they accounted for as much as 0.01% of the substrate.

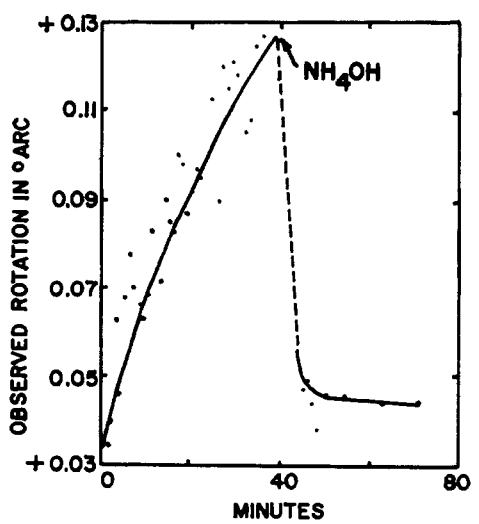

FIG. 1.—Changes in optical rotation during enzymic hydrolysis of triglucosyl-sorbitol and subsequent mutarotation Experimental details are given in the text.

Acid hydrolysis of the C^{14}-oligosaccharide was studied in a system consisting of 5.0 mg of labeled dextrin in 3.0 ml of 1.0 N HCl at 98°. At the times indicated in Figure 2, 0.5-ml samples were withdrawn and neutralized with 0.083 ml of 6 N NaOH. The glucose was then isolated by column chromatography (Miller, 1960) and dried *in vacuo*. The glucose sample was dissolved in 4.0 ml of water and duplicate 0.5-ml portions were used for sugar determinations. The C^{14} content of 3.0 ml of the remaining glucose was determined in a scintillation counter. The results are summarized in Figure 2.

Enzymic hydrolysis was studied at 37° in a system consisting of 5.0 mg of C^{14}-oligosaccharide in 2.50 ml of water, 105 units of cellulase in 0.30 ml of water, and 0.2 ml of 0.05 M potassium phosphate at *p*H 7.0.

Samples of 0.5 ml were withdrawn at the times shown in Figure 3 and inactivated by heating at 98° for 3 minutes. These samples were then processed and analyzed as described in the preceding paragraph except that the cellobiose fraction was used. From the enzymic hydrolysates neither C^{14} nor chemically detectable amounts of reducing sugar were found in the fractions where glucose would occur.

Figures 2 and 3 also indicate the theoretical relationship of total C^{14} in the final hydrolytic product to the per cent hydrolysis. The theoretical curve in Figure 2 was derived assuming a system in which (*a*) a single hydrolytic event was associated with each formation of an enzyme-substrate complex, (*b*) all glucosyl bonds were equally susceptible to hydrolysis, and (*c*) a substrate consisting of celloheptaose was labeled exclusively in the terminal nonreducing glucose moiety. Assumptions (*a*) and (*c*) also apply to the theoretical curve in Figure 3, but here it was further assumed that cellobiosyl units were cleaved from the nonreducing end of the substrate.

DISCUSSION

The frequency of attack on the various glucosyl bonds of cellulose-oligosaccharide can be estimated from the data in Table I. Numbering the glucosyl bonds of cellohexaose as No. 1–No. 5 starting at the nonreducing end of the molecule has been adopted for convenience in the discussion which follows.

With the reduced pentasaccharide as substrate the absence of glucose, cellotetraose, and reduced cellotetraose indicates that bonds No. 1 and No. 4 were not appreciably hydrolyzed. Cleavage at bond No. 2 yields cellobiose and reduced cellotriose whereas cleavage at bond No. 3 yields reduced cellobiose and cellotriose. Observation of the predicted mole ratios of each of these pairs of products confirms the capacity of the analytical column to resolve the reaction products adequately. Calculation of the moles of cellotriose produced expressed as a per cent of the total moles of trisaccharide products indicates that 6.5% of the cleavages occurred at bond No. 3. The analogous calculation using the data from cellobiose and reduced cellobiose estimates that 6.9% of the cleavages occurred at bond No. 3.

Hydrolysis of reduced cellohexaose confirms the failure of the enzyme to attack bond No. 4 since no reduced cellobiose was found. Both hexasaccharide substrates indicate that bond No. 5 was also not attacked and that cleavages at bond No. 1 occurred with a frequency of less than 2%. The trisaccharide data obtained from both hexasaccharides indicate 48% of the cleavages to occur at bond No. 3 and 50% to occur at bond No. 2.

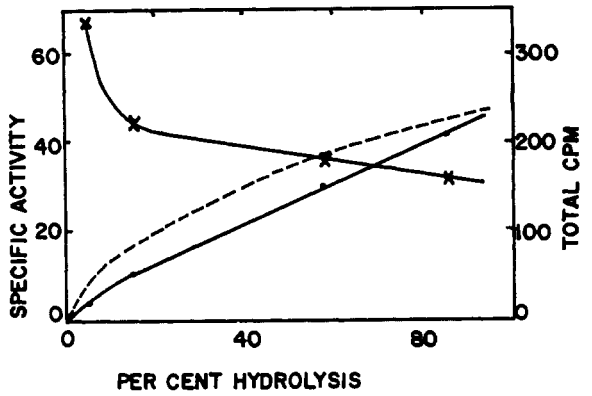

FIG. 2.—Hydrolysis of cellulodextrin-C^{14} by hydrochloric acid. Samples were taken after incubation for 0.05, 7, 30, and 120 minutes. Total counts observed in glucose (●—●), theoretical total counts in glucose (.....), specific activity of glucose (cpm per mg, ×—×). Experimental details appear in text.

It would appear then that as the D.P. of substrate increases the frequency of attack at bond No. 3 rises from 0% in the tetrasaccharide, which yields only cellobiose, to 6–7% in the pentasaccharide, to 48% in the hexasaccharide. The only other bond susceptible to hydrolysis may be No. 2, the glucose observed in the products of hexasaccharide probably arising from hydrolysis of bond No. 2 of the trisaccharide produced by the initial hydrolytic event.

Reduction of the reducing group of the hexasaccharide appears to have no appreciable effect on the site of bond attack since the frequencies of attack at specific bonds was not altered. In the case of the pentasaccharide reduction may have suppressed attack at bond No. 3, but the question cannot be answered (as with the hexasaccharides) by examining the products of hydrolysis of the nonreduced pentasaccharide, because cleavages at both bond No. 2 and bond No. 3 yield identical products.

Both the release of α-cellobiose and the absence of transferase activity suggest single displacement or a related reaction mechanism.

The optimum D.P. of substrate appears to be six or greater from the measurement of the initial rates of hydrolysis of the oligosaccharide series under conditions of zero-order kinetics. From the facts that (*a*) the rate of hydrolysis of cellotriose is only 5 to 10% of the rate of hydrolysis of the larger members of the polymer series, (*b*) the trisaccharide is not seen as an extracellular product in cultures growing on cellulose (Hulcher and King, 1958), and (*c*) cellotriose is a conspicuous product of the hydrolysis of both cellopentaose and cellohexaose, it is inferred that *Cellvibrio gilvus* elaborates an enzyme with high activity on cellotriose which has not yet been observed. A number of possibilities exist, among them a simple hydrolase yielding cellobiose plus glucose, a hydrolase with strong glucotransferase action yielding cellobiose plus glucose plus cellotetraose from two molecules of cellotriose, or an active absorption system specific for the trisaccharide. With what is known now, however, the failure of cellotriose to accumulate extracellularly remains unexplained as does the nature of the C_1 system responsible for the initial attack on native cellulose (King, 1961; Storvick and King, 1960).

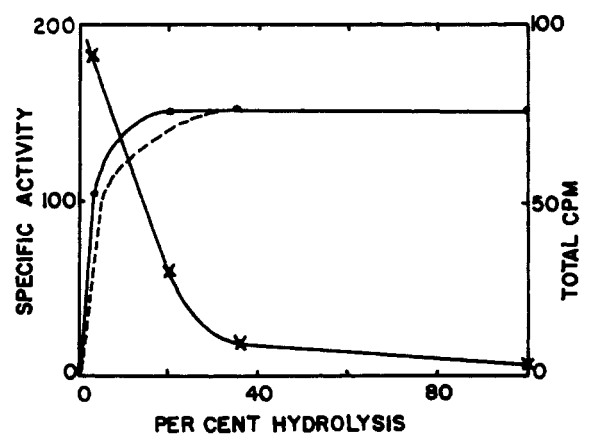

FIG. 3.—Hydrolysis of cellulodextrin-C^{14} by purified cellulase component from *Cellvibro gilvus*. Samples were taken after incubation for 1.5, 10, 40, and 120 minutes. Total counts observed in cellobiose (●—●), theoretical total counts in cellobiose (.....), specific activity of cellobiose (cpm per mg, ×—×). Experimental details appear in text.

An earlier report included the confusing observation that qualitative studies using paper chromatography as the detecting system failed to demonstrate hydrolysis of cellotetraose (Storvick and King, 1960). The improved analytical procedures used here as well as the use of more reliable procedures for isolation and characterization of the oligosaccharide substrates make it apparent that the tetrasaccharide is hydrolyzed.

The data on hydrolysis of the C^{14}-oligosaccharide serve to confirm that reduction of the reducing glucose moiety to sorbitol does not radically alter the susceptibility of the substrate to hydrolysis. The isotope-labeled substrate also confirms that the attack is distinctly nonrandom, occurring preferentially from the nonreducing end of the oligosaccharide chain.

The reason for the decrease in specific activity of the glucose produced during random hydrolysis by HCl is perhaps not obvious. For celloheptaose-C^{14} only cleavages at bonds 1 and 6 release glucose. Only cleavage at bond 1 yields glucose-C^{14}. In the initial hydrolytic event, then, half of the glucose-yielding possibilities yield glucose-C^{14}, and the specific activity (cpm per mg) of this glucose should be 7/2 that of the substrate. As hydrolysis proceeds each cleavage at bonds 2, 3, 4, or 5 creates two new terminal glucosyl units, both unlabeled. Consequently, the probability of a glucose-C^{14}-yielding hydrolysis diminishes and the specific activity of the accumulated glucose should fall.

All the data are consistent with an enzyme acting by a single-displacement mechanism resulting in hydrolysis of the second and third bonds from the nonreducing end of the substrate. It would appear that a minimum of two glucosyl moieties must lie on the nonreducing side of the bond attacked. A requirement for two glucosyls or a glucosylsorbitol on the reducing side of the bond being hydrolyzed may also exist.

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Structure of the Cell Wall of *Staphylococcus aureus*, Strain Copenhagen. I. Preparation of Fragments by Enzymatic Hydrolysis

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The cell wall of *S. aureus*, strain Copenhagen, has been solubilized through the action of the "32 enzyme" isolated from *Streptomyces albus* G. This solubilization was the consequence of hydrolysis of acetylamino sugar linkages in the cell wall, and resulted in the formation of two compounds which were separated by gel filtration and electrophoresis, the glycopeptide and a teichoic acid–glycopeptide complex. Each of these two compounds was further hydrolyzed by a second purified enzyme from *S. albus* which cleaved the linkages between acetylmuramic acid and L-alanine in the glycopeptide. An oligosaccharide fraction and a peptide fraction, obtained from this treatment of the glycopeptide, were also separated by gel filtration. In addition to these two substances, an undegraded teichoic acid was liberated from the teichoic acid–glycopeptide complex by this enzyme. The structures of these three major components of the wall will be reported in following papers.

Bacterial cell walls are heteropolymeric substances of unusual complexity. They contain polysaccharide, polypeptide, and in some cases lipid components. Interest in their structures has been catalyzed in recent years by interest in a number of biological phenomena in which the structure or biosynthesis of cell walls is important. These include inhibition of bacterial growth by some antibacterial agents, lysis of bacteria by enzymes from a variety of sources, and specific toxic and immunological responses to bacterial infection. The structure and biosynthesis of the cell wall of *Staphylococcus aureus*, strain Copenhagen, the object of the present investigation, have been studied in connection with the mechanisms of action of some antibacterial substances (cf. Strominger, 1962). The cell wall is composed of two polymers, a glycopeptide and a teichoic acid, which are separated by trichloroacetic acid at elevated temperatures (or by prolonged extraction in the cold). It has been recognized in the course of studies of the structures of these polymers (Mandelstam and Strominger, 1961; Sanderson *et al.*, 1962) that their separation by acid is hydrolytic and that some damage to both polymers (i.e., random cleavage of chemical bonds) occurs. In the present work, therefore, wall structure has been studied following degradation with two specific, purified enzymes employed at about *p*H 5. These enzymes, purified from the culture filtrate of *Streptomyces albus* G, were isolated employing lysis of the cell walls of *Bacillus megaterium* as the assay (Welsch, 1947; Ghuysen, 1960; Ghuysen *et al.*, 1962a). One of these enzymes, amidase, cleaves the amidic linkage of acetylmuramic acid and L-alanine. The mechanism of action of the second enzyme employed, the "32 enzyme," believed to be an acetylhexosaminidase (Ghuysen *et al.*, 1962a), has been elucidated in the course of this work.

In the first paper the isolation of fragments of the cell wall following enzymatic hydrolysis will be described. In following papers the structures of the three components obtained, the oligosaccharides, the peptide, and the teichoic acid, will be reported.

* Supported by research grants from the U. S. Public Health Service (NIAID E-1902) and National Science Foundation (G-18742).

Materials and Methods

Cell Walls.—These walls were prepared from logarithmic phase cells of *S. aureus*, strain Copenhagen, by repeated disintegration with glass beads in a Nossal disintegrator. Glass beads were removed on a sintered glass filter, and the filtrate was centrifuged at 30,000 × *g*. Disintegration was repeated until a clear supernatant solution was obtained on centrifugation, and the cell wall pellet had a homogenous grayish-white appearance. This usually required three disintegrations of 4 minutes each. The pellet finally obtained was washed five times in the cold with water and then lyophilized. In some studies a glycopeptide obtained from the walls was employed. This material was obtained by hydrolysis of cell walls in 10% trichloroacetic acid at 60° for 12 hours. This is the minimum time under these conditions required to solubilize 95% of the organic phosphate (i.e., the teichoic acid). The insoluble glycopeptide was removed by centrifugation, washed with water, and dried.[1]

Enzymes.—Purification of the "32 enzyme" and of the amidase by zone electrophoresis in a sucrose gradient have been described (Ghuysen *et al.*, 1962a). The preparations employed contained 1.05 and 1.30 mg of protein per ml, respectively.

Paper Electrophoresis.—This was carried out on Whatman No. 3 MM filter paper in 0.2 M pyridine-acetate buffer, *p*H 3.8. The electrophoresis was carried out under "Varsol 3" (Enco Division, Humble Oil and Refining Co.) cooled to 0° in the 5000-v apparatus manufactured by Servonuclear Corp., N. Y.

Analytical Procedures.—Analyses were performed for

[1] We are very grateful to Mr. Rodney Wishnow who prepared the cell walls and glycopeptide used in this study.