Enzymes Metabolizing Polysaccharides and Their Application to the Analysis of Structure and Function of Glycans

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I. General Areas of Study

A. Introduction

Enzymes metabolizing polysaccharides were used in such processes as baking and brewing for countless centuries before the relationship between the chemical structure of the polysaccharides and their modification by enzymes was known. In the past two centuries, studies of the structures and functions of polysaccharides involved in the storage of chemical energy, in the structural parts of tissues, and as information carriers, as well as the enzymes that metabolize them, have been carried out. Since the 1950s chro-

matographic and electrophoretic techniques have allowed the preparation of single proteins with a specific catalytic function, free of interfering activities.

One of the uses of enzymes is in the determination of structures of polysaccharides. The very high specificity for the anomeric linkage of a sugar allows a ready determination of this aspect. With some polysaccharides, the enzyme can be applied to the polymer; in others, a prior partial hydrolysis with a separate enzyme or with acid produces oligosaccharides, in which the nature of the glycosidic linkage can be established. Enzymes can show whether a polysaccharide preparation that produces more than one monosaccharide on complete hydrolysis is a heteropolysaccharide or a mixture of polymers. After partial depolymerization, the oligosaccharides produced are purified to single molecular species and, if this is a heteropolymer, shown to contain more than one sugar. An advantage of enzymatic hydrolysis over acidic hydrolysis is that much higher yields can be expected. If heterogeneity is due to a mixture of polymers, on incubation with requisite hydrolytic enzymes, only part of the mixture will be depolymerized.

A knowledge of the complement of enzymes involved in the biosynthesis of a polysaccharide, in conjunction with their action patterns, gives information about structural features of the polysaccharide. The synthesis or partial synthesis of a polymer with properties similar to those of the extracted polysaccharide, using the known endogenous substrates and requisite enzymes, corroborates the structure derived from degradative evidence.

The structure–function relationships of polysaccharides can be investigated by enzymatic modification and observing any change in function or properties. Monosaccharides have been removed with a glycosidase or added via a glycosyltransferase.

Examples of the uses of enzymes described in this introduction are given n Sections III–VII.

3. Estimation of Polysaccharide Content

The main advantages of using enzymes in the estimation of polysaccharide content are that one can choose enzymes that are selective for hydrolysis of a particular polysaccharide in a mixture and that hydrolysis occurs without the use of high temperatures or acidic or alkaline conditions, which modify low molecular weight products. With some enzymes, depolymerization proceeds to the monosaccharide level, and specific enzymatic methods an also be applied to the estimation of these. With others, an oligosaccharide is produced. Gel chromatography separates any unattacked polysaccharides from oligosaccharide products.

Sometimes unfractionated enzyme extracts are effective in conjunction with a specific analytical method or after removal of interfering substrates.

Examples are the incubation of chitin with snail gut extracts, followed by colorimetric estimation of 2-acetamido-2-deoxy-D-glucose (GlcNAc) with the Elson–Morgan reagent, and the estimation of cellulose, in samples free of pectin and hemicellulose, by a cell-free preparation of *Trichoderma*.

Selectivity in glycan hydrolysis is illustrated by the estimation of the content of connective tissue polysaccharides (Chapter 5): whereas both testicular and bacterial enzymes hydrolyze hyaluronic acid, only the former hydrolyzes chondroitin sulfates A and C *(1)*. Hyaluronic acid content can be selectively estimated after lysis by a hyaluronate lyase (EC 4.2.2.1) from *Staphylococcus aureus (2)*. Micro methods have been described for the estimation of the content of chondroitin sulfates A and C and dermatan sulfate (chondroitin B) in admixture with other glycosaminoglycans and these depend on the ability of chondroitin ABC lyase to depolymerize chondroitins A, B, and C to sulfated, unsaturated disaccharides but of the inability or very limited ability of chondroitin AC lyase to lyse dermatan sulfate (B). The lysis products of chondroitins A and C are then differentiated by two specific sulfatases, or the sulfated disaccharides are separated chromatographically *(3)*.

Additional enzymatic activities may be needed for complete quantitative analyses. The galactomannan content of legume seeds has been measured by hydrolysis with a mixture of α-D-galactosidase (EC 3.2.1.22) and endo-1,4-β-D-mannanase (EC 3.2.1.78), followed by estimation of the amount of galactose released with galactose dehydrogenase (EC 1.1.1.48). The presence of β-D-mannanase eliminates the precipitation of mannan chains after the partial release of galactose *(4)*. Due to the resistance of the uronosyl linkage to acidic hydrolysis and the instability of uronic acids in hot acid, prior treatment of samples with a fungal preparation increased the accuracy of the quantitative colorimetric estimation of pectin content. Other properties of polysaccharides can be utilized in estimation. Heparin can be assayed by its inhibition of pancreatic ribonuclease (EC 3.1.27.5) *(2)*.

Glycogen and starch are converted by exo-1,4-α-D-glucanase (amyloglucosidase, EC 3.2.1.3) to glucose, which is estimated with a hexokinase (EC 2.7.1.1)–D-glucose-6-phosphate dehydrogenase (EC 1.1.1.49) system *(2)* or with glucose oxidase (EC 1.1.3.4). The latter gives results that are comparable to those of acidic hydrolysis. Purified preparations must be supplemented with α-amylase (EC 3.2.1.1) for complete hydrolysis *(5)*. Whole-tissue homogenates can be used for glycogen determination. In the estimation of starch content, the granules must first be brought into solution by refluxing in water or treating with dimethyl sulfoxide or with alkali. Another approach is to use α-amylases that are stable at high enough temperatures to allow gelatinization of the granules. Exo-1,4-α-D-glucanase can also be used

in a difference procedure to measure water-soluble (1→3)(1→4)-β-D-glucans in cereal grains. The glucose content of aqueous extracts after treatment with the enzyme and after acidic hydrolysis is compared.

C. Isolation and Purification of Polysaccharides

A variety of enzymes have been used in the isolation and purification of polysaccharides. Some depolymerize and allow the removal of noncarbohydrate polymers such as protein or nucleic acid. Proteinases (EC 3.4-.-) with a low substrate specificity for any particular peptide bond efficiently depolymerize protein. Different proteinases give different results. The viscosity of submaxillary mucin decreased rapidly on proteolysis but, whereas pepsin (EC 3.4.23.1), chymotrypsin (EC 3.4.21.1), papain (EC 3.4.22.2), and trypsin (EC 3.4.21.4) produced little or no dialyzable material, Pronase almost completely degraded it to dialyzable fragments *(6)*. Ribonuclease and Pronase (EC 3.4.24.4) have been used in the preparation of bacterial and fungal polysaccharides, and lipopolysaccharide has been isolated from gram-negative bacteria by incubation with egg white lysozyme (EC 3.2.1.17) and nucleases before phenol extraction *(7)*. Other noncovalently bound polysaccharides can also be removed. Exo-1,4-α-D-glucanase hydrolyzes starch before the isolation of cell wall polysaccharides (e.g., in cereal grains).

Hydrolytic enzymes can also be applied to insoluble materials to release soluble glycopeptides or fractions from selected parts (e.g., the cell surface). Apple fruit cell walls incubated with Pronase released glycopeptides containing hydroxyproline residues *(8)*. Treatment of human gastric mucosa with Pronase *(9)* solubilized glycopeptide and, on gel chromatography, a soluble fraction with a high molecular weight was obtained that contained most of the original carbohydrate. A glycopeptide released from human erythrocytes by trypsin *(10)* had lentil phytohemagglutinin haptene inhibitory activity. Proteinases have been useful both in the isolation of glycosaminoglycans and in the determination of aspects of their structure. Bovine nasal cartilage proteoglycan, incubated with chrondroitin AC lyase *(11)*, produced protein–keratan sulfate, which was polydisperse and had a mean molecular weight of 450,000. Further incubation with papain, followed by DEAE-cellulose chromatography, gave a keratan sulfate with a molecular weight of ~10,000. Trypsin released fragments from connective tissue that consisted mainly of two polysaccharide chains joined at the reducing end by short polypeptide bridges *(12)*. Papain gave a single polysaccharide chain joined to peptide. This led to a proposed structure of a polypeptide chain substituted through serine hydroxyl groups with polysaccharide and containing unsubstituted amino acid sections of variable length.

D. Characterization of Carbohydrate–Amino Acid Linkages and Removal of Noncarbohydrate Substituents

Enzymes can be used in identifying the linkage between polysaccharide and protein. Glycopeptide, prepared from hen's ovalbumin using proteinase, had aspartic acid as the only amino acid *(13,14);* the soluble glomerular–basement membrane antigens from kidney were treated with collagenase and Pronase, and the products gave, after alkaline hydrolysis, Glc-Gal-Hyl *(15).* The proteoglycan from bovine nasal septa was incubated with testicular hyaluronidase (EC 3.2.1.35) *(16),* and the fraction excluded by gel chromatography on Sephadex G-75 was then hydrolyzed with papain. The products were chromatographed on Sephadex G-25, when keratan sulfate, amino acids, and glycopeptide were separated. More amino acids were removed by treatment with other proteinases, and the final product was identified as having serine linked to xylose, and glucuronic acid to galactose.

Several endo enzymes that split at, or near, the sugar–amino acid linkage have been prepared. Endo-β-D-*N*-acetylglucosaminidase (EC 3.2.1.96) *(17)* hydrolyzes between two GlcNAc units joined to protein in glycoproteins containing mannose, releasing the carbohydrate chain. If glycoprotein pretreated with proteinase is hydrolyzed, one of the products is GlcNAc-Asn, which can be characterized with β-aspartylacetylglucosaminidase (EC 3.2.2.11).

Noncarbohydrate groups substituting polysaccharides can be selectively removed. Chondroitin sulfatase (EC 3.1.6.4) has been found in the viscera of *Patella vulgata,* bovine aorta, and squid, and the *N*-sulfate groups in heparan sulfate were released with a sulfamidase (EC 3.10.-.-) from lymphoid tissue extracts. Deacetylase (EC 3.5.1.-) removes glycoconjugate blood group A specificity by its action on *N*-acetyl-D-galactosamine, and phosphatase (EC 3.1.3.-) partially hydrolyzes the phosphoric ester groups from amylopectin.

E. Detection and Characterization of Polysaccharides

Polysaccharides can be detected and characterized by their behavior with enzymes. The presence of chitin in the husks of germinating barley was recognized by hydrolysis with chitinase (EC 3.2.1.14), showing contamination with microorganisms that characteristically contain chitin *(18).* The composition of β-D-glucan fractions has been examined with endo-1,4-β-D-glucanase (cellulase, EC 3.2.1.4), endo-1,3(4)-β-D-glucanase (EC 3.2.1.6), lichenase (EC 3.2.1.73), and exo-1,3-β-D-glucanase (EC 3.2.1.58); oligosaccharide products have been chromatographed and the β-D-glucan characterized, or, if it is a mixture, the proportion of different β-D-glucans has been estimated. This procedure has been applied to the β-D-glucan fraction

formed from UDP-glucose by a mung bean particulate fraction and suspension-cultured ryegrass endosperm cells *(19)*.

Starch, glycogen, and phytoglycogen can be detected by their hydrolysis to glucose with exo-1,4-α-D-glucanase and to maltosaccharides by α-amylase. They are further characterized by their behavior with the debranching enzymes isoamylase (EC 3.2.1.68) and pullulanase (EC 3.2.1.41). Storage polysaccharides from the protozoan *Gregarina blaberae (20)*, the plant *Cecropia peltata (21)*, and the blue-green alga *Anacystis nidulans (22)* have been found to have phytoglycogen-like structures, from the distribution of chain lengths of the (1→4)-α-D-glucosaccharide products of debranching with isoamylase, as indicated by gel chromatography, and the small amount of debranching by pullulanase. A glucan isolated from *Cyttaria harioti* was identified as pullulan from its behavior with exo-1,4-α-D-glucanase, α-amylase, and pullulanase *(23)*.

A cell wall fraction isolated from *Lemna minor* was characterized as an apiogalacturonan, because after dilute acidic hydrolysis that released apiose it could be depolymerized to galacturonic acid. Endo-1,4-β-D-mannanase, alone and in conjunction with α-D-galactosidase, was used to detect a galactoglucomannan in *Cercis siliquastrum (24)*.

The very high specificity of some biosynthetic enzymes can be utilized for characterization. The occurrence and distribution of glycoconjugate H substances were studied using α-D-*N*-acetylgalactosaminyltransferase (A-enzyme, EC 2.4.1.40) with a labeled substrate *(25)*. Erythrocytes of blood group O, of both secretors and nonsecretors, contained H determinants that were substrates for the transferase.

Enzymatic methods offer a rapid and convenient way of comparing polysaccharides from different sources, of following any changes that occur in an endogenous polysaccharide during development and utilization, and of comparing fractions. The extent of conversion to glucose of four dextrans by a 1,6-α-D-glucosidase from *Streptococcus mitis (26)* was correlated with the proportion of (1→6)-α-D linkages. The variable galactose contents of galactomannans from different species can be measured by incubation with α-D-galactosidase and endo-1,4-β-D-mannanase *(4)*. A number of developmental, varietal, and fractionation comparisons of starches and starch fractions have been made. The percent conversion to maltose by β-amylase of the fractions precipitated by thymol and *n*-butanol (the amylose fraction) from both smooth-seeded (normal-amylose) and wrinkle-seeded (high-amylose) pea seeds decreased with increasing age of seed. The percentage of β-amylolysis is a function of the number of uninterrupted exterior (1→4)-α-D-glucan chains. The fractions not precipitated (amylopectin) did not change. Similar observations were made on normal- and high-amylose barley starches *(27)*, and tobacco leaf starches were examined during the develop-

ment and senescence of leaves and for diurnal–nocturnal changes *(28)*. The conversion to maltose of the amylose fractions from the starches of a number of plant species varied and, for the material obtained by successive leaching of starch granules, decreased as the extraction conditions became more vigorous *(29)*. The percent β-amylolysis limits of glycogens from the livers of anencephalic infants were slightly lower than those of adults and much lower (10%) than the glycogen of muscle *(30)*. Debranching enzymes have been applied to glycogens and amylopectins from different sources to determine the A/B chain ratios and chain length distribution *(31)* (see Section IV). The mixed-linkage $(1 \rightarrow 3)(1 \rightarrow 4)$-$\beta$-D-glucans from oat and barley grains, when treated with cellulase *(32)*, gave trisaccharide (**1**)* and tetrasaccharides **2** and **3** in similar yields, indicating the similarity of structures.

Glcβ1-3Glcβ1-4*Glc* Glcβ1-3Glcβ1-4Glcβ1-4*Glc*
1 **2**

Glcβ1-4Glcβ1-3Glcβ1-4*Glc*
3

Genetic differences, manifested as the absence of an enzyme or as enzymes with modified catalytic properties, can produce polysaccharides with different structures or a change in the amount of a polysaccharide.

The character of a number of glycogen storage diseases in humans can be correlated with the enzymatic status. In four types there is a lack of a degradative enzyme required for utilization (type II, lysosomal α-D-glucosidase; type III, amylo-1,6-D-glucosidase; types V and VI, phosphorylase). This leads to the accumulation of glycogen in type II and of glycogen with a shorter average unit chain length in III. In another type, IV, a synthetic enzyme, the $(1 \rightarrow 4)$-α-D-glucan branching enzyme, is deficient, and this glycogen has a longer average unit chain length *(33)*. Enzymatic deficiencies have also been detected in bacteria. There are mutants of *Escherichia coli* in which glycogen is absent due to a deficiency of glycogen synthase and is slightly decreased in amount and amylose-like when deficient in branching enzyme. In other strains the difference is due to a differing sensitivity of an enzyme to effectors. Two strains produce increased amounts of glycogen; in one the ADP-glucose pyrophosphorylase requires a lower concentration of the activator, D-fructose 1,6-bisphosphate, than that in the wild type, and in the other it needs less activator, because it is less inhibited by AMP. Lack of particular

* To simplify the presentation of this and subsequent formulas, the enantiomeric configurations and ring sizes, if pyranose, have been omitted. The enantiomeric configuration of sugars is D, except for L-fucose, L-arabinose, and L-guluronic and L-iduronic acids; for 3,6-anhydrogalactose the context of the discussion indicates which enantiomer is described. The ring size is pyranose except when indicated by the symbol *f* for furanose. The reducing end sugars in the formulas are italicized.

lysosomal hydrolases can cause malfunction of animal metabolism *(34)* [e.g., α-D-mannosidase, β-D-galactosidase, α-L-fucosidase, and an α-L-iduronidase (EC 3.2.1.76) that hydrolyzes dermatan sulfate]. In mutants of maize, the presence and amounts of (1→4)(1→6)-α-D-glucans can be related to the endogenous biosynthetic enzymes (Section IV,B). Blood group specificity has an enzymatic basis related to the biosynthetic activity for synthesis of the carbohydrate portion of the glycoconjugates *(35)*. The presence of specific glycosyltransferases determines the structure of the oligosaccharide chain and the blood group type.

F. Biosynthetic Studies

Degradative enzymes have been used in biosynthetic studies. The pattern of incorporation of ADP-glucose into glycogen produced by *Aerobacter aerogenes* glycogen synthase was determined *(36)* by supplying labeled substrate and, after isolating the glycogen, degrading it with β-amylase to maltose and the β-limit dextrin and then separating these two fractions. The label in maltose represented that in the outer part of the molecule and the label in the limit dextrin the inner portion.

The pattern of biosynthesis of starch in maize plants has also been examined *(37)*. The kernels of intact plants were allowed to take up $^{14}CO_2$, and the starch was isolated at various times. Amylose and amylopectin were separated and treated with β-amylase. The amylopectin β-limit dextrin was debranched by pullulanase, and the maltosaccharides and maltodextrins were fractionated. At each stage in this procedure, the amount of incorporated label was counted. This made it possible to measure the distribution of radioactivity between the amylose and amylopectin, as well as within these molecules.

G. Industrial Uses

A number of enzymatic processes that involve modification or synthesis of polysaccharides are used in industry, particularly in food processing *(38)* but also in industrial ethanol production and dextran synthesis and modification *(39)*.

Amylases are exploited in baking and brewing. They also remove starch hazes from solutions or starch sizes from textiles and solubilize starch for syrup formation; they are, in addition, used in the production of starch dextrins. Amylases remove starch from apple pomace in pectin production. Cereal grains have very low levels of α-amylase and moderate levels of β-amylase before germination. The α-amylase content increases markedly on germination, and the initial attack on starch granules is dependent on the

production of this α-amylase. In bread making, reasonable levels of α-amylase should be present in the dough to provide sufficient low molecular weight sugars for yeast fermentation to proceed with a suitable rate of carbon dioxide release. Residual sugars also affect the quality of the loaf. In some flours there is insufficient endogenous α-amylase, and fungal or plant preparations are added to a reproducible level of activity. In the making of cakes and sweet biscuits, the high-temperature stability of bacterial α-amylase gives a product with a softer crumb structure, because amylolytic action is more rapid at higher temperatures and inactivation takes longer during baking.

Immobilized enzymes offer advantages for some processes. The enzyme is absorbed strongly or covalently bonded to an inert, insoluble matrix. It can then be recovered after reaction or used in a continuous flow system.

Enzymes depolymerizing pectin are used in the pressing of juices from fruits, when the breakdown of cell wall material improves filtration rates, but the major use is in the clarification of fruit juices. Fungal pectinesterase, free of enzymes hydrolyzing galacturonan, can be used for producing pectin with low esterification. β-D-Glucanase removes haze from beer.

Lysozyme, when added to cow's milk, makes a product more closely resembling human milk and reduces the bacterial count. This enzyme prevents the growth of gas-forming bacteria in semihard cheeses. In the candying of fruits, the addition of preparations containing enzymes that hydrolyze pectin and cellulose assists processing. Sometimes, however, the inhibition of enzymatic activity has been useful. Some leaf extracts containing inhibitors have been used to prevent softening during pickling.

Dextran, which is used clinically as a plasma extender, can be prepared with isolated dextransucrase (EC 2.4.1.5), and this leads to a more uniform product than when whole-cell systems are used. Because the molecular size is critical, controlled hydrolysis with a dextranase (EC 3.2.1.11) gives products with the desired molecular weight range. Dextrans are also used in the production of gel chromatographic supports. Dextranases may remove dextran deposited as dental plaque, and they are used to thin sugarcane juices that contain dextran produced by contaminating *Leuconostoc* spp. Hyaluronidase has been used to facilitate the diffusion of injections.

Enzymes integral to cell structure are involved in the production of starch, pectin, gums, and cellulose for food and fiber, including paper production. Xanthan gum is of microbial origin. The breakdown of polysaccharides such as cellulose and chitin, which are produced in large quantities, is a vital part of natural processes, where it is accomplished largely by microbial systems. The possibility of harnessing selected enzymes from these processes for the preparation of glucose from cellulose waste offers a source of this compound.

An example of potential industrial use of an enzyme is the modification of

galactomannans with high galactose substitution by α-D-galactosidase to produce a polysaccharide with more desirable interaction properties in gel formation (Chapter 5, Volume 1).

II. Enzymes: Properties and Methods

A. General Aspects of Biosynthesis and Degradation of Polysaccharides

The substrates and enzymes used in *in vivo* anabolic pathways of polysaccharide biosynthesis are different from the products and enzymes of catabolism. However, starch and glycogen phosphorylases can also synthesize (1→4)-α-D-glucan linkages *in vitro,* because these enzymes catalyze an equilibrium reaction, and many hydrolases show some degree of transglycosylase activity. Polysaccharide syntheses involve nucleoside diphospho sugars or sucrose and glycosyltransferases (EC 2.4.-.-). A few synthetic enzymes transfer oligosaccharide chains to other oligosaccharide chains (e.g., branching enzyme) or cyclize oligosaccharide chains [e.g., cyclomaltodextrin glucanotransferase (EC 2.4.1.19)], and some add noncarbohydrate substituents [e.g., heparitin sulfotransferase (EC 2.8.2.12)]. The enzymes of polysaccharide catabolism are the glycosidases and glycanases (EC 3.2.-.-) and the lyases acting on polysaccharides (EC 4.2.2.-) plus a few activities such as chondroitinsulfatase (EC 3.1.6.4). Enzymatic depolymerization of carbohydrate polymers occurs in either an endo or an exo manner, and hydrolytic enzymes (EC 3.2.-.-) transfer to water. Transelimination is catalyzed by lyases (EC 4.2.-.-), which cleave C—O bonds in a polysaccharide by elimination, leaving an olefinic bond at the nonreducing end of the oligosaccharide. Polysaccharide endohydrolases and endotranseliminases catalyze an essentially random depolymerization into oligosaccharide fragments, which themselves serve as substrates for further depolymerization. Hydrolysis is often accompanied by transglycosylation, particularly as the degree of polymerization (DP) of the products decreases. In this process a segment of the oligosaccharide undergoing hydrolysis is transferred to the hydroxyl of another sugar instead of water. In reversion, which can occur at high substrate levels, there is resynthesis from free oligosaccharide fragments. Endohydrolysis requires binding of a segment of a number of glycosyl units with a section of the enzyme that contains the active site. This section has a number of binding subsites and a catalytic site. Thus, effective substrates usually contain at least approximately five sugar units. Characteristics of the substrate, such as sugar residue and sequence, the anomeric configuration, the other hydroxyl involved in the glycosidic linkage, the solubility, DP, the

extent of branching, and the nature and amount of substitution by noncarbohydrate substituents, determine the susceptibility and degree of hydrolysis and also the nature of the hydrolytic products.

Enzymes acting in an exo manner on poly- and oligosaccharides can be broadly divided into glycosidases and exoglycanases on the basis of their action patterns. Both of these release sugar units from the nonreducing end of their substrates and, if this has a repeating unit structure, release is sequential. Monosaccharides are removed by glycosidases and by some exoglycanases. Some of the latter sequentially release di-, tetra-, and even hexasaccharides. They are more active on polysaccharides and oligosaccharides with a higher DP than on disaccharides and generally show high specificity for the dimer linkage. Glycosidases show a similar or slightly higher apparent activity with disaccharides and glycosides with a noncarbohydrate aglycone than with higher oligosaccharides. Transglycosylation is associated with glycosidase action, and transfer to any hydroxyl group in the acceptor molecule is possible, but the hydroxyl on C-6 of an aldohexose is usually preferred.

A small group of enzymes debranch polysaccharides, releasing the branches as intact oligosaccharide fragments.

B. Sources

Biosynthetic enzymes can best be obtained from a source that contains the relevant polysaccharide, preferably when it is being rapidly synthesized.

There are a number of sources of degradative enzymes. They often coexist in the same organ as the polysaccharide substrate and can usually be isolated in quantity when this is being degraded (e.g., α-amylase from cereal endosperms on germination). Bacteria, fungi, and bacteriophages produce enzymes that hydrolyze polysaccharides in their host organism. Because these are often produced extracellularly in culture, they can be isolated with limited contamination by other enzymes. A number of fungal, bacterial, and other preparations such as snail gut solution contain a range of hydrolytic activities. If an enzyme is not available from these sources, it can be induced by culturing a microorganism on the polysaccharide or structurally similar compound as the sole carbohydrate source. Then the actual enzymes induced may indicate structural aspects of the polymer. A suitable microorganism can sometimes be selected by enrichment processes, when a crude mixture (e.g., from soil) is cultured with the polysaccharide and with repeated transfers and plating techniques a single culture is obtained. The phages that infect bacteria have been used as a source of degradative enzymes active on both capsular polysaccharides and lipopolysaccharides.

Biosynthetic studies are frequently performed with labeled substrates when cell particulate fractions are used. The results are often difficult to

interpret, for example, the reaction of UDP-[^{14}C]glucose with plant cell particulate fractions and the formation of variable amounts of (1 → 3)- and (1 → 4)-β-D-glucan linkages with different levels of substrate. When cell-free extracts of either synthetic or degradative enzymes are used, the presence and amount of interfering enzymes, mainly degradative, must be recognized, and this still applies to partly purified enzyme solutions. It is preferable to purify to a single protein, and the introduction of preparative chromatographic procedures based on ion exchange, molecular size, and affinity, as well as analytical methods such as gel electrophoresis and isoelectric focusing, have made it possible to purify a large range of enzymes. When an enzyme is available from a number of tissues, the relative ease of obtaining the starting material and of purification, as well as the details of the action pattern and kinetic constants, will determine the best source. An enzyme with the same name from different sources may show some differences in action pattern as well as kinetic constants.

C. Properties of Enzymes

When the properties of an enzyme are well understood, optimal reaction conditions can be applied and different experiments compared. Properties include *(40)* Michaelis constant, molar catalytic activity (or, more practically, maximum velocity), pH for maximum rate of reaction, cofactor requirements, inhibition, pH and thermal stability, temperature of inactivation, storage stability, specific activity, specificity, and action pattern. Other properties, such as electrophoretic behavior, isoelectric point, and molecular weight, serve to characterize the enzyme. Some kinetic constants provide evidence of the suitability of an enzyme from a source for a particular study. The molar catalytic activity (k_{cat}, turnover number) is not readily determined experimentally, but the maximum velocity ($V_{max} = k_{cat} \times$ enzyme concentration) can be. A comparison of relative V_{max} values indicates the more active preparation. The apparent Michaelis constant (K_m) can be expressed as polysaccharide concentration or moles of constituent sugar, but in depolymerization not all glycosidic bonds are necessarily equally available for reaction. With an exo enzyme, only the nonreducing end groups of each molecule are substrates. With both endo and exo enzymes, the DP of a molecule or chain length of a segment is probably pertinent. The inverse of the K_m is an approximate estimate of the affinity of enzyme and substrate. Then k_{cat}/K_m compares the specificity, that is, the capacity of a particular enzyme to discriminate among competing substrates. With the same enzyme preparation, this can be found experimentally by comparing V_{max}/K_m. The k_{cat}/K_m value with the same substrate compares the specificity of different enzymes. If the two enzymes are single proteins, then the V_{max} per unit

weight of protein can be used, assuming similar molecular weights and numbers of catalytic sites. The temperature of inactivation is required in procedures that use sequential reactions with different enzymes. Storage stability can be expressed as the half-life at a certain pH and temperature and should be known when long incubation times are used. The stability of enzymes may be a factor in a choice among sources. Some can be stabilized by added compounds (e.g., thiol reagents, certain ions, or inert protein). If the enzyme is a metalloenzyme, the buffer should be selected to avoid sequestering of the metal. Buffer ions inhibit some enzymes.

D. Enzymatic Assay

Enzymatic assay requires following changes in the amount of substrate or products. Methods for particular enzymes are described in "Methods in Enzymology" (Academic Press), Volumes 1, 8, 28, 41, 50, 89, and 90, and in "Methods of Enzymatic Analysis," second English edition, 1974 (Academic Press), edited by H. U. Bergmeyer. With endoglycanases, reduction in viscosity or turbidity monitors substrate changes, and starch hydrolysis can also be followed by the disappearance of the starch–iodine color. Insoluble materials such as cellulose can be converted to a derivative, for example, carboxymethylcellulose (CM-cellulose), to make a water-soluble substrate. The increase in reducing power due to released oligosaccharides or the production of water-soluble, dyed oligosaccharides from a water-insoluble, dyed polysaccharide depends on product appearance. The dyed polysaccharide can also be water soluble when the degraded products are then measured in the supernatant after precipitation of polysaccharide with ethanol. Radioactively labeled substrates can be used; hyaluronic acid has been partly deacetylated and then reacetylated with tritiated acetic anhydride. Hydrolysis products left in solution after precipitation of polymer with cetylpyridinium chloride were counted *(41)*. The action of lyases can be measured by the increase in UV absorption due to the formation of unsaturated bonds.

The assay of glycosidases can be based on the release of aglycone or sugar. Methods with aglycone typically employ a nitrophenyl- or methylumbelliferyl derivative, the release of which is measured spectrophotometrically. With some, the released sugar can be measured (e.g., glucose with glucose oxidase). Because some glycosidases are specific for the linkage to a second sugar, in the assay of these a suitable oligosaccharide must be used. The estimation of exoglycanase activity involves the measurement of reducing sugar products, and oligosaccharides with the terminal sugar reduced to an alditol are suitable as substrates.

The biosynthetic enzymes dextransucrase and levansucrase (EC 2.4.1.10) can be conveniently assayed by measurement of the fructose or glucose

released. Measurements with the glycosyltransferases that have nucleoside diphospho sugar substrates are performed mainly with a nucleoside diphospho sugar labeled in the sugar moiety, and the label incorporated into polysaccharide is measured. Sometimes an oligosaccharide serves as an acceptor.

E. Methods of Purification

For some enzyme experiments, such as the isolation of oligosaccharides in the identification of linkages in complex polysaccharides, it may not be necessary to establish definitely all the relevant activities in a preparation *(42)*. However, it can be generally stated that the purer the preparation the more significant the results. Enzymes are most commonly purified on a preparative scale by chromatography *(42a)*. Such materials as cellulose and cross-linked dextrans have been substituted to give ion exchangers that separate on a charge basis *(43)* on elution with salts. Another technique that has been useful with some enzymes is substrate elution *(44)*, when the enzyme is released from the ion exchanger by a low concentration of substrate or substrate analog. This process may have been operating in some published procedures in which the initial adsorption was primarily by ion exchange and not affinity absorption or hydrophobic bonding. The elution of a β-D-galactosidase and an α-L-arabinofuranosidase (EC 3.2.1.55) of lupin cotyledons from Con-A Sepharose with *O*-nitrophenyl β-D-galactoside and methyl α-L-arabinofuranoside, respectively, may be an example *(45)*.

Gel chromatography, which separates on the basis of molecular size, has employed supports of beaded cross-linked dextran, polyacrylamide, agarose–polyacrylamide, hydrophilic vinyl polymers, cellulose, or porous glass *(43)*. Some enzymes that react with polysaccharides have shown anomalous behavior on polysaccharide column materials *(46)*. A modification is to chromatograph the enzyme on a gel, in which it is included, and then chromatograph again on the same support but in the presence of an excess of polysaccharide substrate. The activity, bound to the polysaccharide, is then excluded and separated from the other protein that cochromatographed without polysaccharide. Yeast glycogen synthase (EC 2.4.1.11) has been chromatographed with glycogen, and starch phosphorylase with amylopectin *(47)*.

Hydrophobic chromatography *(43)*, which utilizes the association between an immobilized hydrophobic side arm and the hydrophobic surface regions on enzymes, has been applied, for example, with β-amylase *(48)*, α-galactosyltransferase (EC 2.4.1.22) *(49)*, maltodextrin *(50)* and glycogen phosphorylases, glycogen synthase *(51)*, hyaluronidase *(52)*, and β-D-galactosidase *(51)*. The introduction of charged groups, for example, via reaction between a matrix activated by cyanogen bromide and amino groups in the

side arm, at the same time as the hydrophobic chain, allows the possibility of ionic adsorption effects at low salt concentrations.

Affinity chromatography *(43,51)* offers a very convenient and rapid procedure for purifying enzymes. The column material (matrix) has a ligand attached that has a high affinity for the enzyme being purified. The K_m for the enzyme and ligand should generally be less than about 0.1 m*M*. When the ligand is a polysaccharide that can be depolymerized, if the k_{cat} is too high, little retardation may occur. β-Amylase is not held on a starch affinity column. The use of an inhibitor as ligand eliminates this problem. A spacer arm is believed to obviate steric hindrance by the matrix in the approach of the enzyme to the ligand, but polymeric ligands are often linked directly to the support. Matrix materials include agarose, cross-linked dextran, cellulose, polyacrylamide, and controlled porous-glass beads. In some reported examples, the purification may have been primarily by ion exchange. Although any chromatographic procedure is useful in enzyme purification, much higher purification can be expected with affinity chromatography than with ion exchange. Another approach for some enzymes is to use an insoluble polysaccharide or modify the polysaccharide, so that it becomes a suitable material for chromatography. This can be achieved by cross-linking polysaccharide chains with a bifunctional reagent and so dispensing with the matrix or by embedding the polysaccharide in a matrix. Pectin *(53)*, dextran *(54)*, and glycogen *(55)* have been treated this way. Table I *(56–64)* shows some selected examples of enzymes purified by affinity chromatography. The matrices were beaded agarose. Hexosylamines are inhibitors of the respective glycosidases and generally serve as ligands for these enzymes. A thio-β-D-galactoside ligand is a β-D-galactosidase inhibitor. 4-Chloromercuribenzoate combines with reactive thiol groups. Another inhibitor is the α-amylase inhibitor protein from wheat. Concanavalin A affinity is due to the glycoprotein nature of some enzymes. Apart from the antibody ligand, the remainder are substrates or their analog. Other types of ligands and eluants include enzyme effectors (e.g., AMP with glycogen phosphorylase) *(65)*. With isoamylase, the enzyme was bound to glycogen, which in turn was bound to concanavalin A. Debranching by the enzyme caused elution. In other examples of elution, cysteine competed for the mercury and released the thiol enzyme. D-Galactosyltransferase has an Mn^{2+} requirement and hence was eluted by EDTA. A change in pH modifies the binding.

F. Advantages and Disadvantages of Enzymatic Techniques

Many enzymatic procedures are rapid and can be adapted to a micro scale, an advantage when multiple measurements are to be made (e.g., comparison of a number of polysaccharide samples). Their specificity in the catalysis of

TABLE I

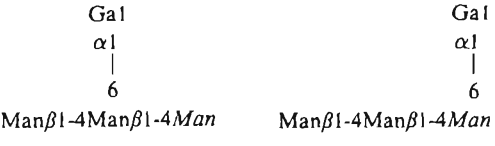
Affinity Chromatography of Carbohydrate-Metabolizing Enzymes

Enzyme (source)	Ligand	Eluant	Ref.
2-Acetamido-2-deoxy-β-D-hexosidase (*Trichomonas foetus*) (EC 3.2.1.52)	2-Acetamido-2-deoxy-β-D-glucosylamine	2-Acetamido-2-deoxy-D-glucose	*56*
β-D-Galactosidase (human liver)	1-Thio-β-D-galactoside	D-Galactonolactone	*57*
(hog small intestine)	4-Chloro-mercuribenzoate	Cysteine	*58*
(human liver)	Concanavalin A	Methyl α-D-mannoside	*59*
α-D-Mannosidase (beans) (EC 3.2.1.24)	anti-(*Phaseolus vulgaris* α-D-mannosidase) antibody	pH change	*60*
UDP-Glucose–collagen glucosyltransferase (chick embryo) (EC 2.4.1.66)	UDP-GlcA	Peptides from collagenase digestion	*61*
Starch phosphorylase (pea seeds)	Starch	Soluble starch	*47*
Isoamylase (*Cytophaga* or *Flavobacterium*)	Concanavalin A with glycogen adsorbed	Enzyme-debranched glycogen ligand and eluted	*62*
α-Amylase (yellow mealworm, human saliva)	Inhibitor proteins from wheat	Maltose	*63*
1,4-β-D-Mannanase (lucerne seed and *Basidiomycetes* spp.)	β-D-Mannan or glucomannan	β-D-Mannan	*64*

actions allows polysaccharides to be studied in mixtures. This selectivity so means that complex polysaccharides can be modified sequentially. In me cases their specificity provides the only method available, for example, e debranching of glycogen by the hydrolysis of only (1→6)-α bonds. :cause the optimal conditions for reaction are at pH values near neutral d temperatures near room temperature, this ensures that only selected ›nds react and that sugars and any conjugate structures are not altered by tremes of pH and high temperature. The need to add only catalytic ıounts of enzyme and the capacity of reactions to occur in an aqueous :dium means that water-soluble polymers can be studied without chemi-

cal modification and that reaction products can be readily separated and purified.

A disadvantage is possible contamination with other enzymes. Crystallinity is no guarantee of single function. A crystalline bacterial α-amylase contained β-D-glucanase *(66);* crystalline sweet potato β-amylase contained α-D-glucosidase *(67).* One can minimize transglycosylation and reversion by choosing suitable reaction conditions, but these possibilities should always be considered in the interpretation of results. Product inhibition can lead to incomplete reaction. This can be reduced by working at a suitable dilution and, if one product is still polymeric (e.g., a β-limit dextrin), by dialyzing or ultrafiltering during reaction. The specificity and action pattern should be investigated as thoroughly as possible; incomplete knowledge of these can give misleading results. The structure of a tetrasaccharide of one galactose and three mannose residues was described as 6^2-α-D-galactosyl-(1→4)-β-mannotriose (**4**) because on reaction with a preparation that hydrolyzed nitrophenyl-β-D-mannoside, only one mannose was released *(24).* However, the enzyme has since been shown to be an exoglycanase *(68)* that cannot hydrolyze a nonreducing terminal mannose joined to a mannose residue substituted with galactose. The correct structure of the oligosaccharide, which could be hydrolyzed by a β-D-mannosidase from snail gut, was confirmed by other methods as 6^1-α-D-galactosyl-(1→4)-β-mannotriose (**5**) *(69).*



4 **5**

In experiments that require long incubation periods, microbial and fungal contamination should be stopped by the addition of suitable agents. The low stability of some enzymes should be recognized. Denaturation by heat, pH extremes, and heavy-metal ions, as well as microbial infection of stored enzyme preparations, should be avoided. Any activators or coenzyme requirements should be known.

III. Endo Enzymes

A. Introduction

Many endo enzymes, which depolymerize polysaccharides by a random-type splitting of interior glycosidic bonds, need a region of unbranched or lightly branched glycan chain in the substrate, because they require a number of binding subsites on different glycosyl units for reaction, producing

oligosaccharides. The proportion and DP of these vary with the substrate and the enzyme and may vary for the same enzymatic activity from different sources. Oligosaccharides of lower DP engage in transglycosylation reactions, and the oligosaccharide products of final hydrolysis are often competitive inhibitors of the enzymes. Reaction is generally accompanied by retention of configuration of the anomeric carbon that is cleaved. Transglycosylation appears to occur most readily with (1→4)-β-linked D-glucans, D-mannans, and D-xylans. Disaccharides and simple glycosides are not hydrolyzed. At high disaccharide and enzyme concentrations, some reversion may also occur.

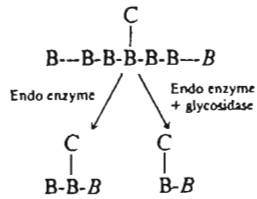
The substrates can be

1. Unbranched homopolymers with one type of repeating glycosidic linkage, such as cellulose, chitin, (1→4)-β-D-mannan, (1→3)-β-D-glucan, and amylose
2. Polymers with some degree of substitution of these homopolymers in category (1) by single sugars or short oligosaccharide chains. Substitution is at the same position on the main-chain sugars [e.g., 6-α-D-xylo-(1→4)-β-D-glucan and 6-α-D-galacto-(1→4)-β-D-mannan]
3. Unbranched homopolymers with mixed glycosidic linkages [e.g., (1→3)(1→4)-β-D-glucan]
4. Unbranched polymers in which some of the sugar units of an unbranched homopolymeric chain (category 1) are irregularly replaced by a single sugar, keeping a similar linkage [e.g., (1→4)-β-D-glucomannan and alginic acid]
5. Polysaccharides that combine the features of categories 2 and 4 such as 6-α-D-galacto-(1→4)-β-D-glucomannans
6. Branched homoglycans such as glycogen and dextran
7. Polymers in which some of the sugar units of an unbranched homopolymeric chain are irregularly replaced by a different sugar and linkage [e.g., (1→2)-L-rhamno-(1→4)-α-D-galacturonan]
8. Heteropolymers with a regular repeating heterooligosaccharide unit (e.g., chrondroitin sulfate, hyaluronic acid, agarose, and bacterial polysaccharides)
9. Heteropolymers in which a proportion of one of the sugars in a repeating heterooligosaccharide unit is replaced by another sugar (e.g., heparin)
10. Highly branched, multiple-linkage heteropolysaccharides that have regions susceptible to an endo enzyme (e.g., arabinogalactogalacturonorhamnans and plant gums)

There may be substitution by noncarbohydrate groups, for example, acetyl, lactyl, sulfate, or phosphoric ester on hydroxyl, pyruvate as an acetal, methyl ester on carboxyl, and methyl ether, which may or may not interfere

to various degrees with hydrolysis. In some cases (e.g., the sulfate on chondroitin sulfate) substitution may be necessary for hydrolysis by a particular enzyme. Substitution with sugar residues of category 1 polysaccharides gives category 2 (e.g., galactomannan), which alters the rate and degree of hydrolysis but does not necessarily stop reaction, unless the degree of substitution is high. In the endo enzyme–substrate interaction, the conformations of both are important. A number of the homopolymeric substrates that are (1→4)-β-D-linked have both bonds in the glycosidic linkage equatorial to the pyranose rings [e.g., chitin, (1→4)-β-D-mannan, cellulose and (1→4)-β-D-xylan], and this leads to a characteristic ribbonlike preferred conformation (see Chapter 5, Volume 1). These polysaccharides are insoluble, with sections of highly aligned chains in their native structure. Even oligosaccharides with a DP of 8 are insoluble, because this conformation allows the molecules to form ordered arrays in which there is strong intermolecular hydrogen bonding. With native cellulose, crystalline fibrillar micelles form. The linkage in amylose, which is (1→4) equatorial–axial, and (1→3)-β-D-glucan, which is equatorial–equatorial, leads to a helical preferred conformation, which increases water solubility and gives a random-coil structure in solution. (1→6) Linkages lead to a more flexible structure, because three bonds are involved in the linkages between pyranose rings; pustulan is reasonably water soluble. Irregular replacement (e.g., of mannose by glucose in glucomannan), which reduces the order in the chain, may also give a somewhat more soluble polymer. Branching further increases solubility *(70)*. There are a few polysaccharides that have glycofuranosyl residues (e.g., galactocarolose, fructans, and arabinans). Some helical conformations allow association between the chains of molecules in solution, which could influence enzyme–substrate interaction. In others, in which irregular interruptions of the sequence occur, some sections of a chain can associate with parts of other chains, leading to junction zones. This would allow differential interaction with enzyme within a single polymer molecule.

When the action patterns of these enzymes are being studied, it is essential that any relevant glycosidase activity be removed. If an endo enzyme contains a glycosidase or exoglycanase, then products different from those with the pure enzyme could be obtained, as in **6**.



6

The interpretation of observations on the products of depolymerization by endoglycanases is complicated by the alternative further reaction pathways that newly formed products can follow. A product can be released, enter into a transglycosylation reaction with oligosaccharides, or serve as substrate for further reaction. The end products of hydrolysis of a native substrate may be inhibitors, unlike those of a modified substrate such as CM-cellulose. The products of insoluble substrates can also be released as insoluble material or associate with insoluble substrate. Soluble derivatives of insoluble substrates (e.g., carboxymethylated) may contain substituents that affect hydrolysis. Reaction between an unbranched polymer and an enzyme can proceed in one of two extreme ways or, more likely in practice, by a mixture of these. An enzyme molecule, after scission of one polymer chain, may then bind to a fragment of that chain and continue hydrolyzing fragments of this molecule until the limiting size of oligosaccharides is reached (single-chain mechanism). Alternatively, an enzyme molecule may split one polymer chain once and then move to a different polymer chain (multichain mechanism). In branched polymers composed of interlinked chains such as amylopectin, there is a variation in that an enzyme molecule can hydrolyze a chain in one molecule and then continue hydrolyzing a fragment of that chain, move to another chain in the same molecule or to another molecule. The degree of randomness of hydrolyses varies. The multiple binding requirements of endo enzymes mean that hydrolysis occurs away from the extreme ends of the molecule. Hydrolysis of glycosidic bonds increases the reducing activity of a polymer solution proportionally to the number of bonds broken, but the decrease in viscosity depends on the posi-

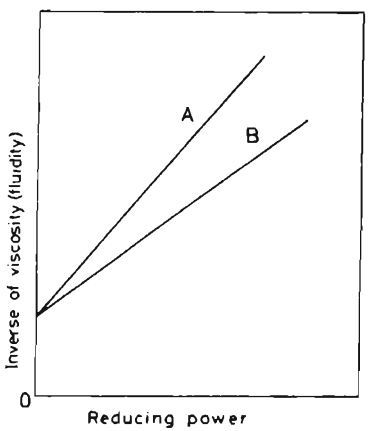


Fig. 1. Viscosity versus reducing power of endo enzymes with differing degrees of endo tracter.

tion of hydrolysis, and a plot of this decrease against reducing sugar production provides a way of comparing modes of enzymatic attack *(71,72)*. In Fig. 1 the increase in reducing power due to hydrolyzed glycosidic bonds is plotted against the increase in the inverse of the specific viscosity (specific fluidity), resulting from the lowering of the average DP of the polymer. In A, the viscosity has dropped more rapidly relative to the increase in reducing power than in B, indicating a more random endo hydrolysis. An exo mechanism produces a very low slope. The magnitude of the slope has been interpreted as a measure of the degree of randomness. The hydrolytic pattern may also be characterized from a chromatographic comparison of the products at various times *(71–73)*. Enzymes named endoglycosidases hydrolyze internal glycosidic linkages in some heterooligomeric sequences (e.g., glycoproteins).

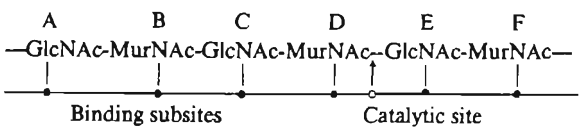
B. Endo Enzymes Hydrolyzing (1→4)-Homoglycans with Diequatorial Linkages

1. Lysozyme (EC 3.2.1.17)

Much of our knowledge of the molecular mechanism of action of endo-1,4-glycanases hydrolyzing diequatorially linked glycose residues has come from studies on hen egg white lysozyme. The catalytic mechanism has been elucidated through a combination of structural studies involving protein sequencing and X-ray crystallography, binding studies, and kinetic experiments *(74,75)*. Closely related lysozymes have been isolated from a variety of sources ranging from avian eggs to phages. Chitin and bacterial cell wall polysaccharide, composed of alternating residues of GlcNAc and 2-acetamido-3-*O*-lactyl-2-deoxy-D-glucose (MurNAc), are hydrolyzed but there is no reaction with deacetylated chitin (chitosan). The hexa- and pentasaccharides of (1→4)-β-D-linked GlcNAc and the partial hydrolysis product of cell wall peptidoglycan, $(GlcNAc\text{-}MurNAc)_3$, are substrates.

Lysozyme from hen's egg is readily purified *(75)* by crystallization. Affinity chromatography on cellulose coated with chitin, dispersed chitin, and carboxymethylchitin has also been used.

The conformation of the polypeptide chain produces an ovoid molecule with a crevice across the waist, in which the active site is located *(76)*. Chitotriose is an inhibitor, and each GlcNAc unit is bound in the active-site cleft at one of three subsites (A, B, and C). Three more sugar residues of the hexasaccharide substrate are accommodated in the cleft, as in **7**. (GlcNAc-



MurNAc)$_3$ is bound, with the bulky lactyl group extending into the solvent and the C-3 hydroxyl groups of GlcNAc fitting in the bottom of the crevice. The lactyl groups prevent the opposite face of the hexasaccharide from fitting into the crevice; that is, MurNAc cannot be held at binding subsite A, C, or E. Because cleavage occurs only at the reducing end of MurNAc, the catalytic site is between sugars B and C or D and E and, because the trisaccharide occupying binding subsites A, B, and C is not hydrolyzed, cleavage occurs between D and E. For a correct fit, pyranose ring D should be distorted from the chair to the half-chair conformation. The carboxonium ion that is an intermediate in the acidic hydrolysis of glycosides has the half-chair conformation. The model is supported by the potent inhibition by the δ-lactone formed on oxidation of tetra-*N*-acetylchitotetraose, which causes 50% inhibition at 0.7 μM *(77)*. This concentration is less than 1% of that required for 50% inhibition by the tetrasaccharide with GlcNAc at the reducing end. X-ray diffraction showed that tetra-*N*-acetyl chitotetraono-1,5-lactone in crystals of lysozyme was bound to sites A to D, with the GlcNAc residues at sites A, B, and C in a position similar to that occupied by chitotriose. The lactone ring appeared to adopt a boat conformation in site D with the hydroxymethyl group oriented axially *(76)*.

In solution studies of the binding of GlcNAc oligomers from DP 1 to 6, binding was found to increase with increasing chain length up to the trimer. A further increase in DP of 1 did not increase the binding constant; GlcNAc-MurNAc-GlcNAc-*MurNAc* is bound less firmly than GlcNAc-MurNAc-GlcNAc. This results from the distortion of the pyranose ring that is required for binding at subsite D, increasing the free energy of the bound state. It is not merely a noninteracting site but leads to unfavorable interactions. Binding at D occurs only when the substrate chain is long enough to allow binding at E, or E and F, adding a compensating negative value to the free energy of binding; (GlcNAc-MurNAc)$_3$ is bound more strongly than (GlcNAc-MurNAc)$_2$. Thus, the enzyme has three contiguous subsites, at A, B, and C on the model, in which GlcNAc moieties can bind readily, but the subsites on either side of this region do not interact favorably *(74)*. Subsites B, D, and F can accommodate either GlcNAc or MurNAc. The strongly binding subsites for a dimer are B and C. These conclusions accord with the crystallographic data.

Lysozyme catalyzes glycosyl transfer to saccharides and other alcohols as well as water, and transglycosylation also occurs with the cell wall saccharides. The reaction of (GlcNAc)$_2$ and (GlcNAc)$_3$ requires an induction period, which can be eliminated by the addition of catalytic amounts of (GlcNAc)$_4$, and the splitting of the tetrasaccharide takes place mainly by transglycosylation, in which higher oligosaccharides are formed transiently and then act as substrates for a hydrolytic reaction. Transfer of various sugars to the GlcNAc-MurNAc–lysozyme intermediate that was formed during

hydrolysis of cell wall tetrasaccharide indicated that a disaccharide is a better acceptor than a monosaccharide and that *N*-acetyl sugars interact more strongly than glucose in the acceptor site *(78)*. These experiments also provided evidence for the role of subsites E and F. Hydrolysis of chitotriose in ^{18}O-enriched water has shown that enzymatic reaction proceeds by scission of the anomeric carbon–oxygen bond. Transfer reactions to alcohols were used to study configurational changes during reaction. The cleavage of chitobiose in the presence of methanol gave only methyl β-D-GlcNAc, resulting from retention of configuration *(79)*. The similar acceptor reactivities for a variety of small alcohols with a range of p*K* values, steric effects, and nucleophilicity provided support for a reaction mechanism that is general acid-catalyzed with an intermediate carboxonium ion. Formation of the carboxonium ion with the half-chair conformation is assisted by the enzyme binding ring D in this conformation. After splitting, the sugars at E and F diffuse away, and ring D reacts with water or another acceptor.

2. Chitinase (EC 3.2.1.14) and Chitosanase (EC 3.2.1.-)

Chitinase *(80)* hydrolyzes polymers of (1→4)-β-D-linked GlcNAc residues to chitobiose and chitotriose. The trisaccharide can be further hydrolyzed but at a very much reduced rate. A small degree of hydrolysis is accompanied by a large reduction in viscosity, consistent with an endo mechanism. Chitosan *(81)* and the cell wall peptidoglycan of *Micrococcus lysodeikticus* are not hydrolyzed. The enzyme is produced by a wide range of bacteria, fungi, insects, fish, and some other animals. Preparations have been obtained from culture filtrates of *Streptomyces, Aspergillus niger,* and insects *(80,82)*. The effect of acetyl modification on hydrolysis has been studied *(81)*. The relative rates of hydrolysis of chitin derivatives, in which substituents in the *N*-acyl group had been changed, were $CH_3 > CH_3CH_2 > H > CH_3CH_2CH_2 > (CH_3)_2CH > NH_2CH_2 > ClCH_2$. Neither chitosan nor *N*-methylene-, *N*-benzylidene-, *N*-benzoyl-, *N*-nicotinyl-, or *N*-acyl chitosans (C_{15}–C_{18}) were hydrolyzed. *N*-Acetylchitosan gels were hydrolyzed 8–13 times faster than crab shell chitin, indicating that the physical form of the substrate effects the rate of hydrolysis.

Another endo enzyme that hydrolyzes the glycosidic linkages of GlcNAc has been purified to electrophoretic homogeneity from culture filtrates of *Staphylococcus aureus*. It degraded the cell walls of *M. lysodeikticus* but, unlike lysozyme, which gives products with MurNAc reducing groups, it gave oligosaccharides that had GlcNAc at the reducing end: MurNAc-*GlcNAc* was isolated from cell wall digests *(83)*.

Enzymes active on chitosan but without action on chitin are called chitosanases. The culture filtrates of *Streptomyces* contain a mixture of enzymes that partially lysed cell walls from *Mucor rouxii,* releasing hexosamine but

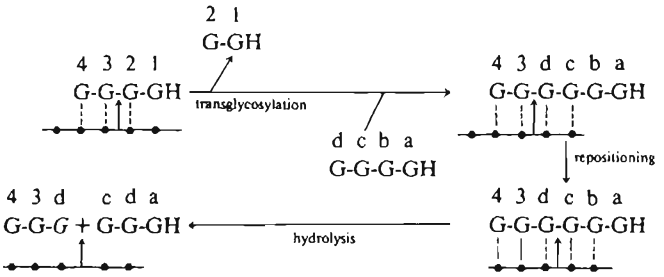
no *N*-acetylhexosamine, indicating that chitosan but not chitin was hydrolyzed. Chitosanase purified from this culture filtrate *(84)* did not hydrolyze chitin or CM-cellulose. The major products from chitosan were the di- and trisaccharides. Chitosanase from *Bacillus* R-4 also hydrolyzed in an endo manner. The purified enzyme from *Myxobacter* AL-1 showed 1,4-β-D-glucanase activity. It was electrophoretically homogeneous, with one terminal amino acid, and in denaturation studies both activities decreased at the same rate. The K_m values for chitosan and CM-cellulose were 0.3 and 1.7 mg/ml, respectively, and the V_{max} values were similar. It is an endo enzyme specific for polymers of D-glucose and D-glucosamine, and neither chitin nor amorphous acid-swollen chitin was attacked *(85)*.

Chitosanases have been used to prepare protoplasts from *Mucor* cells *(84,86)*. In surveys of microorganisms producing chitinase or chitosanase, those producing chitosanase were found to lyse live *Rhizopus* hyphae, whereas those producing chitinase could not. However, exceptions have been found. Chitinase and chitosanase may be useful in the classification of such organisms.

3. Cellulase (Endo-1,4-β-D-glucanase) (EC 3.2.1.4) with Cellulose, 6-α-D-Xylo-(1→4)-β-D-glucans, and Xanthan Gum

Cellulase has been purified from culture media of microorganisms, the gut solution of snails, and plant tissue *(71–73,87–92)*. The major hydrolysis products are cellobiose and cellotriose, and the recovery of these in high yield is consistent with cellulose being composed entirely of (1→4)-β-D-linked glucose units. Multiple activities have been reported in some culture filtrates; in some cases these may have resulted from proteolytic modification. These multiple forms can vary in their capacity to hydrolyze (1→4)-β-D-glucans and cellosaccharides *(71,88–92)*. Reduction of the terminal reducing sugar residue of a cellosaccharide to the alditol decreases the rate of hydrolysis to lower than that of the oligosaccharide with a DP of 1 less; cellotetraose is hydrolyzed more rapidly than cellopentaitol *(88)*. The susceptibility of the various linkages in cellosaccharides to hydrolysis indicates that both terminal glucosidic linkages are very much less easily split than internal linkages and that reduction of the terminal reducing glucose residue causes decreased susceptibility to hydrolysis of the penultimate bond from the glucitol end. High-pressure liquid chromatography of the products of reaction of cellooligosaccharides with *Trichoderma viride* enzymes has shown the production of intermediate fractions of higher DP than the original substrates. Cellobiitol without an equivalent amount of cellobiose was released on hydrolysis of cellotetraitol *(71)*, consistent with transglycosylation *(89)* between enzyme-bound cellobiose and cellotetraitol, forming cellohexaitol, as in Eq. (1). The

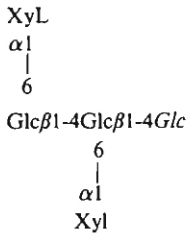
G indicates a (1→4)-linked β-D-glucose unit and GH a D-glucitol joined at the 4 position.



(1)

The crystalline, highly insoluble nature of cellulose makes it very resistant to enzymatic hydrolysis, and there is a striking difference between the digestive capacity of microorganisms and the slowness of their cell-free extracts in solubilizing native, undegraded cellulose such as cotton linters *(38)*. The system degrading cellulose is multienzyme, with at least three types of enzymes *(93,94)*. These have been called C_x, which is an endo-1,4-β-D-glucanase, C_1, and β-D-glucosidase (cellobiase, EC 3.2.1.21). The C_1 component from the culture filtrate of *T. koningii* was purified to homogeneity and shown to be an exo-1,4-β-D-glucan cellobiohydrolase (EC 3.2.1.91), *(95)*, an enzyme previously detected in culture filtrates of *Cellvibrio gilvus*. One function of the β-D-glucosidase appears to be hydrolysis of cellobiose, removing it as an inhibitor of the other enzymes. Proposals have been made for the mode of interaction of these enzymes in the hydrolysis of crystalline cellulose *(91,93,94)*.

Limited substitution of a (1→4)-β-D-glucan chain by other sugars still allows hydrolysis. Endo-1,4-β-D-glucanase hydrolyzes the (1→4)-β-D-glucan chain in 6-α-D-xylo-(1→4)-β-D-glucans from seeds *(96)*, wood, cambial tissue, and the walls of suspension-cultured dicotyledonous cells *(42,97)* and also the extracellular polysaccharide of *Xanthomonas campestris (98)*. In plant xyloglucans, xylose residues are attached to at least half of the glucose residues *(96)*. Other sugars such as β-D-galactosyl, α-L-arabinofuranosyl, and L-Fucα1-2Galβ1 may be attached to C-2 of xylose to give short side chains. Hydrolysis with endo-1,4-β-D-glucanases has released oligosaccharides that have been useful in attributing structural features *(98a)*. Exhaustive digestion of soybean xyloglucan with *T. viride* cellulase *(99)* gave **8**–**11** plus **12** and **13**, and **8** and **10** were identified from hydrolysis of a xyloglucan synthesized by a particulate fraction from suspension-cultured cells *(97)*. A hemicellulose from rice endosperm cell walls *(100)* behaved as a homogeneous polymer on sedimentation analysis by ultracentrifugation and on electrophoresis on glass paper. However, analysis of the products of



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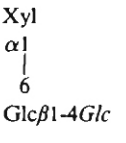

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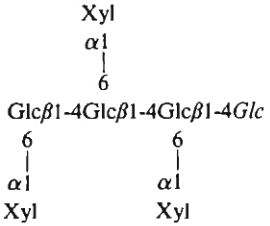

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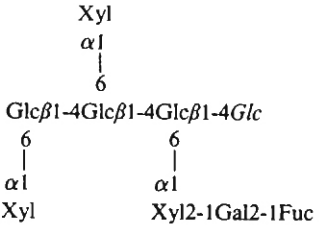

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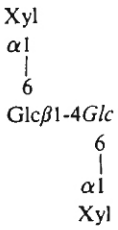

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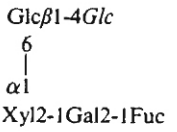

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hydrolysis with endo-1,4-β-D-glucanase indicated that it was a mixture of three polysaccharides, a xyloglucan, a (1→3)(1→4)-D-glucan and an arabinoxylan, which were firmly associated. The products of cellulase hydrolysis included glucose, cellobiose, oligosaccharide **8**, and a fraction with a structure that differed from **11** in that the oligosaccharide side chain was attached to the xylose at the nonreducing end of the glucosaccharide chain.

Xanthan gum, which has a backbone of (1→4)-β-D-glucan substituted regularly by a three-unit oligosaccharide, showed differing susceptibility to 1,4-β-D-glucanase hydrolysis under different solution conditions, and it was suggested that the degree of hydrolysis is dependent on the solution conformation of the polymer *(98)*.

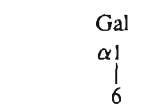
4. Endo-1,4-β-D-mannanase (EC 3.2.1.78) with β-D-Mannans and Galactomannans

β-D-Mannanase has been obtained from a range of microbial and plant sources *(64,101–106,106a)* and depolymerizes (1→4)-β-D-mannans,

(1→4)-β-D-glucomannans, and the main chains of 6-α-D-galactosyl-(1→4)-β-D-mannans and -glucomannans. The isolation, in high yield, from (1→4)-β-D-mannan of oligomers composed mainly of disaccharide and unbranched trisaccharide is in agreement with the established formula for this polysaccharide. Hydrolysis of mannotetraitol produces oligomers with a DP of more than 4 as intermediate products and gives mannobiose and mannotriose but only traces of mannobiitol as final products *(107)*. A scheme has been proposed to explain these results in which the very slow hydrolysis of a small amount of mannotetraitol leads to mannobiose bound to enzyme, which than takes part in cyclic reaction sequences involving transglycosylation with mannotetraitol, repositioning, and further transglycosylations to generate the observed products, with no need for more than very limited direct hydrolysis of mannotetraitol.

The extent of hydrolysis of a galactomannan is dependent on its galactose content, and highly substituted galactomannans undergo very limited hydrolysis *(24,101,102)*. The products have provided information about the action pattern of the enzyme and indicated aspects of polysaccharide structure, such as the distribution of galactose substituents along the mannan chain in galactomannans *(69,107–109)*. Galactomannans are more soluble than β-D-mannans and, as the galactose content decreases, V_{max} increases and K_m decreases. These changes and the lower amount of glycosidic bonds broken as the galactose content increases indicate that substitution of the main-chain mannose units by galactosyl residues interferes with the interaction between β-D-mannanase and the mannan chain. The partial removal of galactose from highly substituted galactomannans increased the rate and amount of hydrolysis. However, the isolation of significant amounts of oligosaccharides containing both mannose and galactose with a DP as low as 3 shows that substituting galactosyl groups do not interfere completely.

From identification of oligosaccharides with a DP of up to 9, released on hydrolysis of carob galactomannan, an action pattern for this enzyme from a number of sources has been proposed *(107,109)*. The only heterotri- and hetereotetrasaccharides isolated were **5** and **14**. The $1/K_m$ and V_{max} values for



(15)

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the corresponding mannosaccharide alditols increased from DP 4 to 6, indicating that at least five mannose residues bind in the region of the active site. The ribbonlike nature of the favored conformation of the (1→4)-β-D-man-

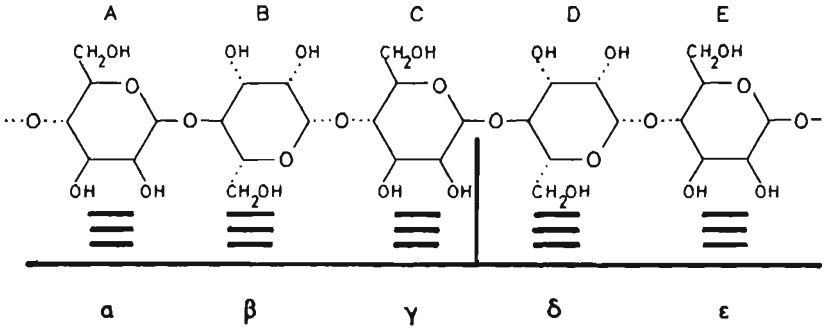
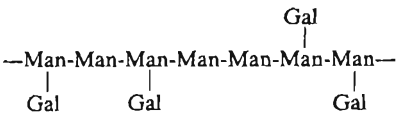


Fig. 2. Binding of 1,4-β-D-mannanase to a (1→4)-β-D-mannan chain.

nan chain, combined with an approximate twofold screw axis, means that $-CH_2OH$ groups on neighboring mannose residues lie on opposite sides of :he chain. Galactosyl substituents separated by zero or an even number of nannose residues are opposite, whereas those separated by an odd number)f mannosyl units lie on the same side of the chain as in **15** *(24)*. Then the



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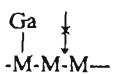
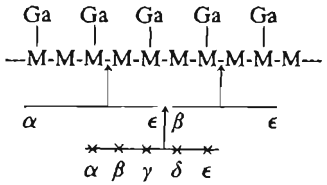
rientation of the galactosyl substituents, as well as their separation, is a ıctor in enzyme–substrate interaction. The oligomeric products of hydro-/sis can then be explained if the enzyme binds to one edge of the ribbon)nformation as in Fig. 2, with rings B and D bound by the hydroxymethyl lge of the pyranose ring and rings A, C, and E by the dihydroxyl edge. The ıtalytic site was shown to be between C and D. Substitution by a galactosyl nit of rings B or D blocks binding, but substitution of the three other rings)es not interfere with binding and hydrolysis by the *Aspergillus niger* en-'me. The isolation of mannobiose and Glcβ1-*4Man* but not cellobiose or lanβ1-*4Glc* from hydrolysis of (1→4)-β-D-glucomannan supported the 'oposal that the configuration of the 2-OH is important in ring C but ithout effect in ring B.

Thus, there is no hydrolysis of a glycosidic bond one mannose residue /ay from a substituted mannose and toward the reducing end of the poly-)r, as in **16**. Here, M and Ga represent mannose and galactose units,



16

respectively. However, the structural requirements for hydrolysis of galactomannans by β-D-mannanase cannot be stated simply in terms of the number of unsubstituted mannose residues between those that are galactosyl-substituted. Hydrolysis of the glycosidic linkage of a mannose substituted by galactose can occur if at least one unsubstituted mannose residue occurs on both sides (as in **17**), but when there are just two adjacent, unsubstituted



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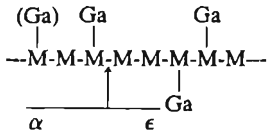
mannose residues on the reducing side of a substituted mannose residue and at least one unsubstituted residue on the nonreducing side, then the only susceptible bond is that of the mannosyl substituted by galactose (**18**). The



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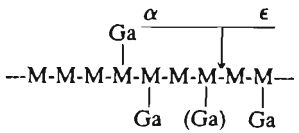
parentheses indicate an optional substitution by galactose. If two or more neighboring mannose residues are substituted, then toward the reducing end of the molecule a further sequence of only two unsubstituted mannose residues cannot be split. Three unsubstituted or a sequence of unsubstituted–substituted–unsubstituted can be hydrolyzed (**19**). Equa-



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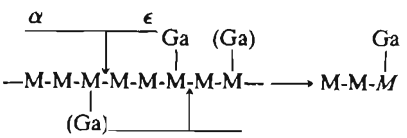
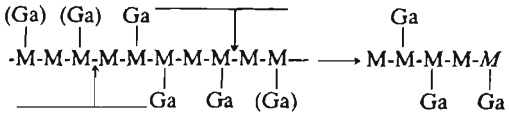
tions (2) and (3) show how two of the oligosaccharides isolated are produced.



(2)



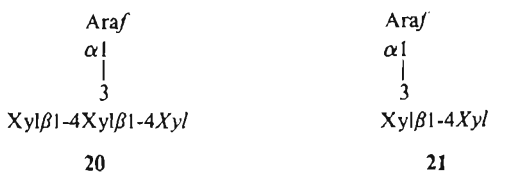
(3)

The small amount of fraction of DP > 9 in the hydrolysate and the structures of the isolated oligosaccharides of DP < 10 were not consistent with any significant amount of segments of β-D-mannan with block substitution. The products were more consistent with nonregular substitution in carob galactomannan. The fraction of DP > 9 need not be fully substituted to be resistant, and the galactose percentage indicated that it had unsubstituted mannose residues. *Aspergillus niger* β-D-mannanase hydrolyzed *Leucaena leucocephala* galactomannan, which is highly substituted, at an initial rate similar to that of carob, which has a low galactose content. In the former molecule there appears to be relatively large sections consisting of the repeating unit -Man(Gal)-Man- with few regions of contiguous unsubstituted mannosyl units *(103)*. The recovery of mannotetraose and heteropenta- and heterohexasaccharides when lucerne β-D-mannanase was used showed that this enzyme had a similar pattern of binding but that there was a difference between the hydrolytic capacities of it and *A. niger* β-D-mannanase.

5. Endo-1,4-β-D-Xylanase (EC 3.2.1.8) with Arabino- and 4-MeO-Glucuronoxylans

1,4-β-D-Xylanase acts on the (1→4)-β-D chains of xylans, arabinoxylans, glucuronoxylans, and related polymers *(106)*. The V_{max}/K_m values for xylooligosaccharides indicate a binding site of 4 to 7 xylose residues *(110,111)*.

Incubation of arabinoxylans has produced arabinoxylosaccharides, but reaction with enzymes from different sources has yielded quite different products. Wheat flour arabinoxylan with a β-D-xylanase from a *Streptomyces* species *(112)* gave large oligomeric fragments, several oligosaccharides, and traces of arabinose and xylose. The smallest heterosaccharide was 2-α-L-arabinofuranosyl-(1→4)-β-D-xylotriose (**20**). The minimum spatial

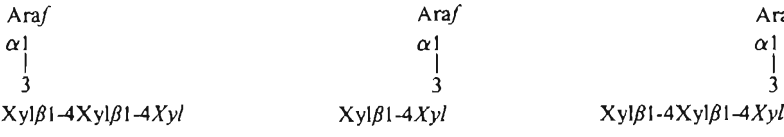


20 **21**

requirement between branches for hydrolysis appeared to be two adjacent unsubstituted units. A nonregular distribution of substituents was proposed from an examination of the hydrolysis products.

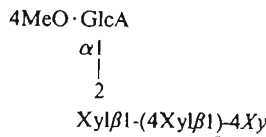
An enzyme from *Myrothecium verrucaria* released **21** from wheat straw

xylan, and it was also produced from rye flour and cocksfoot grass arabinoxylans in the presence of L-arabinonolactone, which was added to inhibit α-L-arabinofuranosidase *(113)*. Spear grass hemicellulose with *Ceratocystis paradoxa* β-D-xylanase *(114)* gave **21** and **22**. *Aspergillus niger* β-D-xylanase with rice straw arabinoxylan yielded, in addition to xylosaccharides, two heterosaccharides, the structures of which were determined enzymatically as **23** and **24**.



22 **23** **24**

4-*O*-Methylglucuronoxylans have been hydrolyzed to xylosaccharides and acidic xylosaccharides containing 4-*O*-methyl-D-glucuronic acid *(106)* having the general structure **25**, where $n = 1-5$. A partially reduced 4-*O*-



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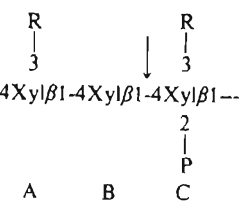
methylglucuronoxylan from aspen, resulting from borohydride treatment, released a range of oligosaccharides, including two tetrasaccharides, one acidic (**25**, $n = 1$), plus the same sequence with the uronic acid reduced. The presence of the latter was taken to indicate that some of the uronic acid residues are linked to lignin through an ester bond. Hydrolysis of a branched arabinoglucuronoxylan from redwood by a β-D-xylanase from *Sporotrichum dimorphosporum* left a nondialyzable residue (20%) plus a series of dialyzable oligosaccharides *(115)*. From the structures of these, it was concluded that the glycosidic bond on the nonreducing side of a substituted xylose residue was preferentially cleaved and that hydrolysis may have involved a region of the xylan backbone having three xylosyl residues in which O-2 of residue A and O-2 plus O-3 of residue B were unsubstituted. Residue



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C may be unbranched or branched (**26**) when R is either α-L-Ara*f* and P a hydrogen or P is 4-*O*-Me-α-GlcA and R a hydrogen. From the products of hydrolysis of 4-*O*-methylglucuronoxylan *(111)* it was concluded that the 4-*O*-methyl-D-glucuronic acid terminal unit hinders enzymatic cleavage of two (1 → 4)-β-D-xylosidic linkages in the vicinity of the branch point.

C. Endo Enzymes Hydrolyzing Homoglycans Linked by Bonds Other Than (1 → 4) Diequatorial

Homoglycans of this type, such as (1 → 3)-β-D-glucan and amylose, other than those that are linked (1 → 6), have a helical preferred conformation and the helix dimensions vary according to linkage (Chapter 5, Volume 1). Reaction commonly produces di- and trisaccharides, although in some cases the smallest product has a higher or lower DP. When the repeating unit is uronic acid, as in galacturonan, scission can involve either hydrolysis or β-elimination.

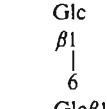
1. Endo-1,3-β-D-glucanase (EC 3.2.1.39)

1,3-β-D-Glucanases have been defined as specific or nonspecific, accord- ng to the substrates attacked and the linkages cleaved. The specific 1,3-β-D- lucanases (EC 3.2.1.39) *(116–118)* act only on (1 → 3)-β-D linkages and not n (1 → 3)(1 → 4)-β-D-glucans, such as lichenan and cereal β-D-glucans. The nonspecific 1,3(4)-β-D-glucanases (EC 3.2.1.6) *(116,119–121)* apparently ind to (1 → 3)-β-D linkages but may split either (1 → 3)- or (1 → 4)-β-D onds (see Section III, D,1). Assays with substrates having both mixed or nly (1 → 3)-β-D linkages distinguish specific and nonspecific 1,3-β-D-glu- anases and lichenases (EC 3.2.1.73), which hydrolyze a (1 → 4)-β-D link ext to a (1 → 3)-β-D bond.

The role of (1 → 3)-β-D-glucans as major structural components of yeast ell walls has generated interest in their modification during cell expansion, udding, conjugation, and the lysis of ascus walls in some species *(122)*. -Glucanase solutions have been applied to cell suspensions to prepare otoplasts. β-D-Glucanases from microorganisms that can lyse yeast cell alls can be grouped into endo enzymes, which produce oligosaccharides of ther low DP or high DP *(119,120,123–128)*, and exo-1,3-β-D-glucanases ection V,B). 1,3-β-D-Glucanases that do not lyse cell walls release oligosac- arides of low DP on hydrolysis of laminaran or pachyman and character- ically produce disaccharide, trisaccharide, and monosaccharide.

Cultures of *Cytophaga johnsonii (128)* contain enzymes that lyse thiol- ated yeast cell walls. Both (1 → 3)- and (1 → 6)-β-D-glucanases are present, d the former was separated into a component with a limited action on ast cell walls that released glucose, laminarabiose, and laminaratriose from minaran and another that lysed cell walls and hydrolyzed only long

(1→3)-β-D-glucan chains; oligosaccharides of DP 5 or more were derived from insoluble laminaran or curdlan. Other similar β-D-glucanases from *Arthrobacter (126)* did not hydrolyze soluble but could hydrolyze insoluble laminaran, suggesting that long, uninterrupted segments of (1→3)-β-D-linked glucose units were required for binding. When a (1→3)-β-D-glucan with a DP range of 11 to 14, synthesized using *Euglena* 1,3-β-D-oligoglucan phosphorylase (EC 2.4.1.30), was incubated, laminarapentaose was the major product. Yeast glucans gave mainly laminarapentaose and traces of laminarasaccharides of up to DP 10, plus some material that was immobile on paper chromatography. Pentaose formation was considered to result from initial attack rather than from accumulation at a later stage; it was excised as such from the interior section. The immobile material, when hydrolyzed by another 1,3-β-D-glucanase from the same source, yielded unbranched oligosaccharides of lower DP plus branched oligosaccharides, such as 6^2-β-D-glucosyllaminarabiose (**27**). An activity from a species of



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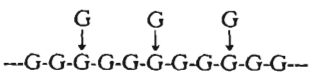
Fungi Imperfecti *(125)*, on incubation with yeast glucan solution, caused a rapid reduction in turbity combined with a comparatively slow and limited release of reducing sugar equivalents. A γ-immunoglobulin was produced against the pure enzyme, and the antigen–antibody complex retained activity against glucan but lost activity against living yeast cells. It was proposed that the enzyme could diffuse through the mannan layer to the glucan mesh of the cell wall, but the addition of antibody formed a complex of larger size, unable to diffuse.

The cell walls of *Saccharomyces cerevisiae* contain several types of glucan *(129)*. The alkali-insoluble glucan is responsible for the rigidity and tensile strength of the wall. Yeast glucan treated with 1,6-β-D-glucanase or dilute acetic acid lost a (1→6)-β-D-glucan component. Incubation of the residue, which was the major component, with 1,3-β-D-glucanase gave laminarasaccharides of DP 2–4, glucose, and gentiobiose but no higher gentiosaccharides. The structure was described as a (1→3)-β-D-glucan with 3% of (1→6)-β linkages.

A combination of enzymatic and chemical methods was used to characterize a glucan from a *Sclerotium* species *(130)*. *Rhizopus arrhizus* endo-1,3-β-D-glucanase gave glucose, gentiobiose, and laminarabiose but no laminaratriose, and there was at least 90% conversion to glucose and gentiobiose, in a

ratio of 2:1, on reaction with basidiomycete exo-1,3-β-D-glucanase. Smith degradation gave an insoluble product, which yielded glucose, laminarabiose, and laminaratriose with endo-1,3-β-D-glucanase and only glucose with exo-1,3-β-D-glucanase. When combined with the results of methylation analysis, a structure was proposed of a (1→3)-β-D-glucan backbone with single (1→6)-β-D-linked glucosyl units distributed uniformly along the chain on every third glucose residue [**28**, where the vertical arrows represent a (1→6)-β-D linkage and the hyphens a (1→3)-β-D linkage].



28

Despite their quite different gelation properties, hydrolysis of the insoluble (1→3)-β-D-glucans curdlan and pachyman by *Rhizopus arrhizus* endo-1,3-β-D-glucanase produced similar amounts of glucose, laminarabiose, and laminaratriose.

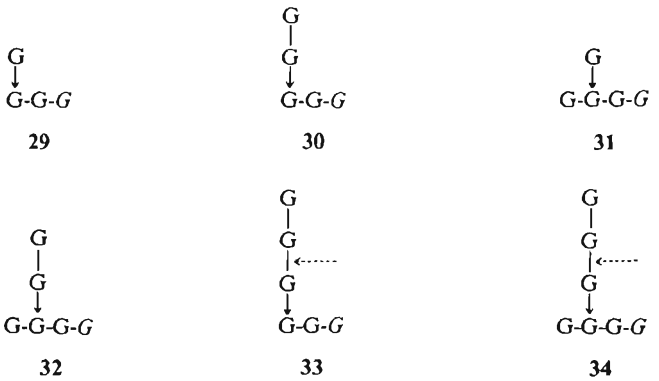
2. α-Amylase (EC 3.2.1.1) with (1→4)- and (1→4)(1→6)-α-D-Glucans, Pullulan, and Elsinan

There are a number of reviews of this enzyme (e.g., *116, 131–134*), and it is discussed in Chapter 3. It hydrolyzes amylose by an endo pattern, and the anomeric configuration of the cleaved bond is retained. The ultimate products are maltobiose, maltotriose, and some glucose. The depolymerization of amylose is not completely random *(131,133,135)*, and with most α-amylases an initial random cleavage is followed by preferential hydrolysis of one of the fragments. The initial encounter of porcine α-amylase with amylose occurred randomly and, after hydrolysis *(135)*, the fragment containing the reducing end was released. The remnant repositioned, reoccupying the entire binding site, and was further hydrolyzed. The enzyme then moved along the chain toward the nonreducing end, successively removing oligosaccharides. Values of 8 to 10 multiple attacks have been found, but with human salivary and *Aspergillus oryzae* α-amylases the values, 1.7–3.0, were lower. The degree of repetitiveness is affected by pH and other factors.

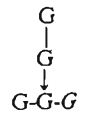
From the hydrolysis products of maltosaccharide alditols, the frequencies of splitting of particular bonds were determined *(136,137)*. This made it possible to estimate the free-energy changes on binding at the subsites. Amylase from *Bacillus subtilis* var. *amyloliquefaciens* was determined to have 10 subsites, from *Aspergillus oryzae* 8, and from takaamylase A 9. Binding at the glucose residue, the glycosidic bond of which was hydrolyzed, required a high positive free-energy change; that is, an energy input was

required. This is similar to lysozyme (Section III,B,1), where the equivalent sugar residue changes to the half-chair conformation on reaction.

With amylopectin and glycogen, the hydrolysis of the (1 → 4) chains is modified by the degree of branching, because the branch points are resistant to hydrolysis and also confer resistance on neighboring (1 → 4)-α-D linkages *(116,133)*. Hydrolysis of amylose by salivary α-amylase gave an α-limit dextrin and an oligosaccharide product with a maltose/maltotriose ratio of 2.4 : 1, whereas with amylopectin the ratio was 1.5 : 1. Six singly branched maltosaccharides were separated after incubation of salivary α-amylase with waxy maize starch [**29** – **34**, where the vertical arrows represent a (1 → 6)-α-D bond and the hyphens a (1 → 4)-α-D linkage] *(138)*. Oligosaccharides **33** and



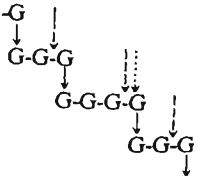
34 were susceptible to further hydrolysis at the dashed arrows. The presence of two unsubstituted glucose residues toward the reducing end from a branch point was a constant feature of the hydrolysis products. Toward the nonreducing end, one or no unsubstituted glucose units were present. The smallest branched fragment produced by pig pancreatic, barley, and *Aspergillus oryzae* α-amylases was also **29** *(139)* but that by *Bacillus subtilis* α-amylase was **35** *(140)*.



35

Pullulan is hydrolyzed by salivary α-amylase wherever there is a maltotetraosyl unit (at ·····► in **36**). An α-amylase from a culture filtrate of *Thermoactinomyces vulgaris* hydrolyzed starch to mainly maltose (74%) and glucose (12%), releasing the products in an α configuration. It also hydro-

lyzed pullulan (33%), and the products were panose (96.5%), maltose (1.5%), glucose (0.7%), isomaltose (0.3%), and higher oligosaccharides (0.4%). The cleavage points were considered to be at ——→ in **36**. It has since been



36

reported *(141)* that this enzyme attacks some (1→6)-α-D linkages in partial hydrolysates of pullulan.

Elsinan, elaborated by *Elsinoe leucospila,* is depolymerized by salivary α-amylase to 4-nigerosyl-α-D-glucose (**37**) in high yield with some 3^2-α-maltosylmaltose (**38**) *(142),* indicating a structure for the polysaccharide of maltotriosyl and maltotetraosyl residues joined by (1→3)-α-D bonds. α-Amylase from *Aspergillus oryzae* gave a tetrasaccharide (**39**) and a heptasac-

Glc
α1
|
3
Glcα1-4*Glc*

37

Glcα1-4Glc
α1
|
3
Glcα1-4*Glc*

38

Glc
α1
|
3
Glcα1-4Glcα1-4*Glc*

39

charide containing two (1→3) and four (1→4) linkages plus a fraction of high DP consisting of (1→3)-α-linked maltotriose units, showing a difference in specificity of the two α-amylases and indicating aspects of the fine structure of elsinan.

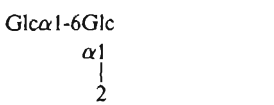
3. Endo-1,6-α-D-glucanase (Dextranase) (EC 3.2.1.11)

The limit of hydrolysis of dextran and the size of the isomaltosaccharide products are dependent on both the source of the dextranase and its concentration. With fungal dextranases, the smallest substrate that is rapidly hydrolyzed is isomaltotetraose. Bacterial dextranases do not hydrolyze isomaltotriose, and many are unable to attack the tetraose. The DP of the smallest oligosaccharide readily hydrolyzed by a particular enzyme varies from 5 to 9 *(143).* Quantitative determination of the relative amount of cleavage of different bonds in isomaltosaccharides was performed with oligosaccharides C-labeled at the nonreducing end *(144).* Analysis of the data for *Penicillium lilacinum* dextranase *(136,143,145)* gave an active site of six binding subsites. The catalytic site was at the bond between the fourth and fifth

binding subsites, numbering from the nonreducing end. Subsite 4 showed a high positive free-energy change for binding (an antibinding site). Different action patterns were found for two bacterial enzymes, a commercial dextranase and *Streptococcus mutans* dextranase.

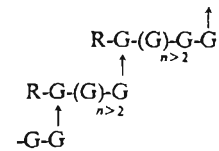
The branching in dextrans affects reaction and, if the amount is high, hydrolysis is very limited. Variations are found among enzymes from different sources. A dextranase from *Lactobacillus bifidus* gave less than half the hydrolysis of the dextran from *Leuconostoc mesenteroides* B-1415 [with (1→4) branching] than that by enzymes from *Penicillium lilacinum* and *P. funiculosum (146).* The oligosaccharides produced contained one glucose residue, joined (1→4) to an isomaltosaccharide main chain with a minimum DP of 3. With the *Penicillium* enzymes, there were two or three glucose units on the reducing side of the branch and none to three on the nonreducing side. One fraction with an isomaltohexaose main chain and two branches was separated. (1→3)-Branched oligosaccharides have been obtained on hydrolysis of B-512 (F) and *Streptococcus bovis* NCDO 1253 dextrans with preparations from *P. funiculosum* and *P. lilacinum (147).* The structures were established, using an exo-1,6-α-D-glucosidase and methylation analysis, as 3^3-α-D-glucosylisomaltosaccharides, as well as some fractions that contained isomaltosyl side chains. Hydrolysis by *P. purpurogenum* dextranase of dextrans containing (1→2)-α-D linkages *(148)* gave a mixture of tetrasaccharides and pentasaccharides, both of which contained mixed linkages, plus minor amounts of unbranched oligosaccharides. Partial acid hydrolysis of *L. mesenteroides* B-1299 dextran *(149)* removed many of the (1→2) and all of the (1→3) branch linkages, and the product was then hydrolyzed by *P. lilacinum* dextranase. The mixed-linkage pentasaccharides were identified as **40** and **41**. Because analogous oligosaccharides were not

40



41

found in hydrolysates of the original dextran, it was concluded that these were fragments from debranched segments in the acid-modified dextran and, because all the (1→2) linkages were branch linkages, that **42** repre-



42

sented a partial structure in which R is α-D-glucosyl or an isomaltosaccharide residue and the hyphens are (1→6) and the arrows (1→2) linkages.

The extent of hydrolysis by 1,6-α-D-glucanase, considered in conjunction with the percentage of (1→6) linkages as determined by methylation analysis, provides information on the amount of sequential (1→6)-α linkages in a dextran.

Exo enzymes have also contributed to the determination of structural aspects of dextrans, and these are discussed in Section V.

4. Endo-β-D-galactanases (EC 3.2.1.89 and 3.2.1.90)

The arabinogalactan from coffee beans consists mainly of a backbone of (1→3)-β-D-galactosyl residues with (1→3)-α-L-arabinofuranosyl and (1→6)-β-D-galactosyl units as side chains. Less frequently, L-arabinosyl branch units and interposed (1→6)-β-D-galactosyl units are found *(106)*. Multiple 1,3-β-D-galactanase activities were prepared from culture filtrates of *Rhizopus niveus*, and these gave 14.0% degradation of coffee bean arabinogalactan. One fraction released galactose, (1→6)-β-D-galactobiose, L-arabinose, and some heterosaccharides *(150)*.

Endo-1,4-β-D-galactanase has been obtained from culture filtrates of *Bacillus subtilis (151)* and *Phytophthora infestans*. These enzymes could not hydrolyze coffee bean arabinogalactan, in which the main linkage type is (1→3)-β. Exhaustive incubation of soybean arabinogalactan with *B. subtilis* K-50 galactanase yielded (1→4)-β-D-galactobiose as the major product, with small amounts of galactose and heterosaccharides. The three galactanases from *B. subtilis* var. *amylosacchariticus* gave galactose, (1→4)-β-D-galactobiose, and galactotriose as the major products, with trace amounts of heterosaccharides. Both enzymes caused about 30% hydrolysis. In view of the high L-arabinose content (about 30%) *(106)*, the low amount of heterosaccharides was unexpected. A possible explanation can be seen in the results of incubation with *B. subtilis* (wild type) enzyme *(151)*, when (1→4)-β-D-galactotetraose was produced with lesser amounts of galactotriose and galactobiose. However, another fraction that was obtained was excluded on chromatography on BioGel P2 and contained 87% arabinose, 6% galactose, 4% rhamnose, and 3% glucose, whereas the included oligosaccharide fractions contained less than 5% arabinose. The results suggested that the arabinose in soybean arabinogalactan occurs as arabinan subunits with a DP of at least), rather than as monosaccharides or small oligosaccharide side chains on a lactan backbone.

5. Endo-α-L-arabinofurananase (EC 3.2.1.99)

Endo-1,5-α-L-arabinofurananase occurs in culture filtrates of a range of croorganisms *(106,152)*. It preferentially cleaves 5-linked arabinofurano- residues. Beet arabinan was only about 3% hydrolyzed, presumably due

to the highly branched nature of this polysaccharide. This arabinan consists of a 5-linked backbone with 2- and 3-linked arabinofuranosyl side chains, which would limit the accessibility of the enzyme to the backbone. Arabinan, with branches partly removed by an α-L-arabinofuranosidase, was hydrolyzed by endo-1,5-L-arabinofurananase at 16 times the initial rate of the original arabinan.

6. Endo-α-D-galacturonanase (Polygalacturonase) (EC 3.2.1.15)

This enzyme is produced by a wide range of fungi, including most plant pathogens, some bacteria, and yeasts, and it occurs in plant organs and the digestive tracts of some insects *(153)*. It catalyzes random hydrolytic cleavage of glycosidic (1→4)-α-D bonds of nonesterified galacturonan and oligosaccharides, as well as those with a low amount of esterification. The preferred substrates are D-galacturonans of high DP. Activities from different sources and multiple forms from the same source may show differences in their action patterns and, in the later stages of hydrolysis, differences occur in the types and amounts of oligosaccharides. Oligomers labeled at the reducing end with tritium and at the nonreducing end as unsaturated acid, prepared by cleavage with lyase, have been used to determine action patterns *(154)*.

Endogenous endogalacturonanases are believed to play a role in plant cell wall modification (softening) during the ripening of fruits, and exogenous enzymes in bacteria and fungi are thought to have a similar role in the infection process. Ripening is generally accompanied by degradation of pectic polysaccharides and an increase in galacturonanase activity. Galacturonanase from peaches released water-soluble fragments of high DP from washed cell walls, and a similar action was proposed for the pear enzyme *(155)*. Endo-α-D-galacturonanase is one of the few isolated enzymes that causes appreciable degradation of intact plant cell walls. A purified enzyme from *Colletotrichum lindemuthianum (97)* with a purified endo-1,4-β-D-galactanase *(151)*, endo-1,5-α-L-arabinofurananase, and α-L-arabinofuranosidase *(152)* from *Bacillus subtilis* have been used in studies of the chemical structure of the cell walls of suspension-cultured sycamore cells, and a tentative structure has been proposed *(97)*. Similar studies have been performed on cell wall material from apple fruit cortical tissue.

7. Endo-α-D-galacturonan Lyase (Endopectate Lyase, Endopectin Lyase) (EC 4.2.2.10)

This enzyme catalyzes the random scission of pectate or pectin by β-elimination *(153)*. The products are reducing oligosaccharides terminated at the nonreducing end by 4-deoxy-β-L-*threo*-hex-4-enopyranosyluronic acid (ΔUA) (Fig. 3). Enzymatic elimination of both esterified and unesterified galacturonans has been reported, but nonenzymatic β-elimination is re-

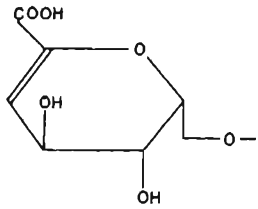


Fig. 3. 4-Deoxy-β-L-*threo*-hex-4-ene-uronic acid.

stricted to esterified galacturonans, because a free carboxyl cannot sufficiently activate the hydrogen atom at C-5. Evidence for the lack of participation of hydroxyl groups on C-2 and C-3 in catalysis came from studies of lysis of V_i-antigen with lyases from *Bacillus sphaericus* and *B. polymyxa*. V_i-Antigen is a bacterial surface polysaccharide consisting of (1→4)-linked residues of 2-acetamido-3-*O*-acetyl-2-deoxy-α-D-galacturonic acid. It was lysed similarly to deacetylated V_i-antigen and galacturonan *(156)*.

8. Endo-1,3-α-D-glucanase (EC 3.2.1.59)

1,3-α-D-Glucanase hydrolyzes polymers containing regions of consecutive (1→3)-linked α-glucosyl residues *(116,143)* and has been detected in culture filtrates of *Trichoderma, Penicillium, Streptomyces, Spicaria, Chloridium, Cladosporium,* and *Flavobacterium,* and in some cases purified and characterized. It is assayed with pseudonigeran. The products of hydrolysis by purified enzymes from different sources have varied *(157,158)*. Enzymes rom *Trichoderma viride* and *Streptomyces* and *Cladosporium resinae* produced mainly glucose with lesser amounts of nigerose, and those from *Flavobacterium* EK-14 gave a series of nigerosaccharides. 1,3-α-D-Glucanase rom *T. viride* hydrolyzed nigeran, isolichenan, and *Polyporus tumulosus* olysaccharide, as well as (1→3)-α-D-tri- to (1→3)-α-D-pentasaccharides. he hydrolysis of nigeran indicated that the linkage type in the vicinity of the leaved (1→3) linkage was not critical, and the degree of hydrolysis by the zyme from *Streptomyces* KI-8 of the (1→3)(1→4)-α-D-glucan from *entinus* may show a similar substrate specificity. In contrast, 1,3-α-D-glunase from *Cladosporium resinae (158)* was specific for (1→3)-α bonds in uninterrupted sequence; mycodextran (nigeran), isolichenan with (1→3) d (1→4), and *Leuconostoc* B-1355 soluble dextran with alternating →6) and (1→3) linkages were not substrates. Nor could the enzyme from *avobacterium* EK-14 hydrolyze this dextran, although it partly hydrolyzed insoluble dextran of *Streptococcus mutans* strains OMZ 176 and OMZ with sequences of (1→3) and (1→6) linkages. 1,3-α-D-Glucanases are ful in detecting sequences of (1→3)-α-D linkages in dextrans and offer ential for the study of the (1→3)-α-D-glucans of fungal cell walls.

9. Endo-1,6-β-D-glucanase (EC 3.2.1.75)

1,6-β-D-Glucanases are produced by a range of fungi and some bacteria *(116,159–162)*. Different enzymes vary markedly in their substrate specificity. Some are highly specific for (1→6) bonds in β-D-glucans *(160)*, whereas others also hydrolyze the glycosidic bonds of 6-O-substituted β-D-glucopyranose residues where that bond may be linked (1→6) or (1→3) to the adjacent β-D-glucopyranose residue *(124,160,161)*. A third type is represented by the enzyme from *Flavobacterium* M64, which, unlike others, hydrolyzes the octasaccharide repeating unit released from succinoglycan by succinoglycan depolymerase *(159)*. The enzyme from *Gibberella fujikuroi (161)* had no activity on the (1→3)-β-D-glucans pachyman and paramylan but readily hydrolyzed both (1→6)-β-D-glucans and poly- and oligosaccharides containing both (1→6)-β-D and (1→3)-β-D linkages. Hydrolysis of *Eisenia bicyclis* β-D-glucan, which contains both (1→6) and (1→3) linkages, produced glucose, laminarabiose, gentiobiose, as well as a series of 6-β-laminaraglucosaccharides, as illustrated in **43**. The nature of the oligo-

$$(Glc\beta1)_n\text{-}3Glc$$
$$\beta1$$
$$|$$
$$6$$
$$Glc$$

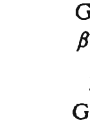
43 $(n = 1-3)$

saccharide products indicated that the enzyme can hydrolyze both (1→6)- and (1→3)-β-D-glucosidic linkages of 6-substituted glucosyl residues. 3^2-β-Gentiobiosylgentiobiose (**44**) was hydrolyzed with the release of only gentiobiose.

$$Glc\beta1\text{-}6Glc$$
$$\beta1$$
$$|$$
$$3 \qquad (47)$$
$$Glc\beta1\text{-}6Glc$$

44

One of two 1,6-β-D-glucanases from *Bacillus circulans* WL-12 *(160)*, which was unable to lyse yeast cell walls, hydrolyzed pustulan [(1→6)-β-D-glucan] by an endo mechanism to glucose and gentiobiose. The highly branched (1→6)-β-D-glucan from bakers' yeast cell walls was hydrolyzed, and one product was **45**. Branched (1→3)-β-D-glucans containing (1→6) interchain linkages were not hydrolyzed. The other enzyme, which lysed yeast cell walls, released glucose, gentiobiose, and gentiotriose from pustulan, and had some activity on (1→3)-β-D-glucans with (1→6) interchain



45

linkages. The degree of laminaran hydrolysis increased with the extent of branching. Products were gentiobiose and glucose with traces of **45**, a mixed-linkage tetrasaccharide, and gentiotriose. The capacity to cleave some (1→3) linkages near a (1→6) branch point was indicated. Oligosaccharide **45** has also been produced on hydrolysis of the acetic acid-soluble fraction from *Saccharomyces cerevisiae* cell wall glucan on incubation with a 1,6-β-D-glucanase from *Pencillium brefeldianum*, when glucose and gentiosaccharides (DP 2–5) were also released *(129)*. An activity from *Acinetobacter* hydrolyzed pustulan and lutean [(1→6)-β] but not shizophyllan [(1→3)(1→6)-β] or pachyman; laminaran was attacked slightly. Yeast cell wall glucan fractions were partly hydrolyzed *(162)*.

An enzyme partially purified from cells of *Flavobacterium* M64 *(159)* is unique in its capacity to hydrolyze the octasaccharide repeating unit (succinoglycan D) of succinoglycan, an extracellular acidic polysaccharide produced by *Alcaligenes faecalis* var. *myxogenes* 10C3. Both succinoglycan D and desuccinylated succinoglycan D are hydrolyzed to two tetrasaccharides, and these and the octasaccharide repeating unit have been characterized. The β-D-glucanase, in conjunction with succinoglycan depolymerase, that hydrolyzes a -3Galβ1-4Glcβ1-linkage in the original polysaccharide has illustrated *(163)* that the polysaccharides elaborated by *Rhizobium meliloti*, *Alcaligenes faecalis* var. *myxogenes*, and *Agrobacterium radiobacter* are identical, except for the mode of acylation. Hydrolysis of the three polysaccharides with succinoglycan depolymerase, followed by the 1,6-β-D-glucanase, gave indistinguishable paper chromatograms with two spots, each of which was due to tetrasaccharide.

10. Levanase (EC 3.2.1.65) and Inulanase (EC 3.2.1.7)

Levanase has been isolated from *Arthrobacter* and purified by adsorption to levan *(164)*. In oligofructosides of DP 3 or more, (2→6)-β-D linkages were hydrolyzed. Levanbiose and the (2→1)-β-D linkages in inulin and sucrose were not cleaved. The ultimate products of hydrolysis of unbranched oligofructosides were levanbiose and fructose in various proportions, according to the DP of the substrate. The end products of hydrolysis of a levan consisted of a mixture with an average DP of 2.5, composed of the following: fructose (10%), levanbiose (39%), (2→1)-β branched triose (7%), levantriose (7%), branched tetraose (5%), branched oligofructosides of DP 5–9 (13%)

and DP > 10 (8%). An activity from *Penicillium* that could degrade (2→6)- and (2→1)-β-D-fructan and an inulanase from *Aspergillus niger* that could degrade only (2→1)-β-D-fructan have been used to establish the structure of a fructan produced by *Aeromonas hydrophila* as a levan *(165)*.

D. Endo Enzymes Hydrolyzing Mixed-Linkage Homoglycans and Glycans with Irregular Replacement of the Single Sugar in the Hydrolyzable Homoglycan Chain

Structures included in this group are the (1→3)(1→4)-β-D-glucans, alginic acid, the (1→4)-β-D-glucomannans, and pullulan.

1. Endo Enzymes Hydrolyzing (1→3)(1→4)-β-D-Glucans

Three types of endoglucanases show activity with lichenan and the cereal gums: cellulase, 1,3(4)-β-D-glucanase, and lichenase *(116)*.

Cellulase hydrolyzes only (1→4) links next to another (1→4) link *(32,166)*, so it has no effect on *pneumococcal* RS III polysaccharide (carboxyl-reduced *Streptococcus pneumoniae* type 3-specific polysaccharide) *(167)*, consisting of repeating (1→3)- and (1→4)-β-D-glucosyl units. The oligosaccharide products from hydrolysis of lichenan and cereal β-D-glucans *(168)* included 3^2-β-D-glucosylcellobiose (**46**), 3^3-β-D-glucosylcellotriose (**47**), and 3^2-β-cellobiosylcellobiose (**48**). The ratios of trimer to tetramer released were 3.9 for lichenan and 2.2 for oat glucan.

Glcβ1-3Glcβ1-4*Glc* **46**

Glcβ1-3Glcβ1-4Glcβ1-4*Glc* **47**

Glcβ1-4Glcβ1-3Glcβ1-4*Glc* **48**

The second enzyme that cleaves (1→3)(1→4)-β-D-glucans has also been termed nonspecific 1,3-β-D-glucanase, because it can hydrolyze both the (1→3) bonds in laminaran and a (1→4) bond adjacent to a (1→3) linkage in cereal glucans. Laminaran produces mainly laminarabiose and laminaratriose, whereas lichenan and β-D-glucan give 3-β-cellobiosylglucose (**49**), and 3-β-cellotriosylglucose (**50**). The ratios of trimer to tetramer released from

Glcβ1-4Glcβ1-3*Glc* **49**

Glcβ1-4Glcβ1-4Glcβ1-3*Glc* **50**

lichenan and oat glucan were 3.6 and 1.5 *(168)*. The presence of (1→3) bonds and their position in the products suggested that the enzyme was specific for the linkage adjacent to a glucosyl residue substituted in the 3 position, rather than for cleavage of (1→3) linkages. Incubated long enough with this glucanase, the glucan from pneumococcal RS III produced only laminarabiose *(167)*, consistent with hydrolysis of (1→4) bonds adjacent to (1→3)-β-D-glucosyl linkages. It was proposed that 1,3(4)-β-D-glucanase has binding sites that attach to a laminarabiosyl residue and that additional subsites can accept either a (1→3)- or a (1→4)-linked glucosyl residue.

The third enzyme, lichenase *(66,167)*, hydrolyzes lichenan, cereal β-D-glucans, and pneumococcal RS III polysaccharide but has no action on (1→4)-β-D- (CM-cellulose) or (1→3)-β-D-glucans (laminaran). Hydrolysis of barley β-D-glucan by barley lichenase *(169,170)* or *Bacillus pumilus* lichenase *(171)* gave 3-β-cellobiosyl-D-glucose (**49**) and 3-β-cellotriosyl-D-glucose (**50**) in a molar ratio of about 2 : 1, and these together made up 80–90% of the sugars produced. The remainder included soluble oligomers of higher DP with (1→4) linkages and a 3-linked glucose at the reducing end, as well as an insoluble fraction. Hydrolysis of lichenan *(167,171)* gave **49** and lesser amounts of **50**; the enzyme cleaves (1→4) linkages adjacent to (1→3) bonds. Barley β-D-glucan and lichenan were depolymerized at the same initial rate, but the amount of degradation varied, being 28% for barley β-D-glucan and ranging from 19 to 24% for various lichenan preparations.

The partial binding and hydrolytic sites for the three enzymes are

(G represents a β-linked glucose residue)

cellulase: G1-3(4)G1-4G1↓4G1-3(4)G1-

1,3(4)-β-D-glucanase: G1-4(3)G1-3G1↓4(3)G1-

lichenase: G1-4G1-3G1↓4G1-

reducing end →

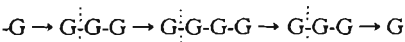
The mixed-linkage (1→3)(1→4)-β-D-glucans were shown by chemical methods to be unbranched polymers, consisting essentially of cellotriose with some cellotetraose units joined by (1→3)-β-D linkages. This structure has been confirmed enzymatically. From hydrolysis of oat and barley glucans by *Streptomyces* cellulase *(32)*, the mixed-linkage trisaccharide **46** was isolated in 60% yield with 30% of the tetrasaccharides **47** and **48**. Oligosaccharides with adjacent (1→3) linkages were not detected. A structure was suggested, as were the hydrolytic sites that would lead to the oligosaccharides isolated [**51**, where the arrows represent a (1→3)-β and the hyphens a

G → G-G-G → G-G-G (or) G-G → G-G-G → G-G-G →

51

→4)-β linkage]. Hydrolysis of cereal glucans by *Aspergillus niger* cellulase *7,166)* gave products that included cellobiose (12%), **46** (45%), and a trasaccharide fraction (16%) that was a substituted cellobiose *(166)*.

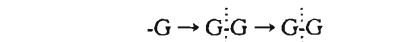
Incubation of oat seed β-D-glucan with *Rhizopus arrhizus* 1,3(4)-β-D-glunase *(32)* released as the major products trisaccharide **49** (52%) and tetraccharide **50** (30%). The hydrolysis of cereal β-D-glucan by 1,3(4)-β-D-glunase then proceeds as in **52**. Lichenase reacts similarly *(172)*. When the D-glucans from five cereals were reacted with *Bacillus subtilis* lichenase or



52

Rhizopus 1,3(4)-β-D-glucanase and the released oligosaccharides (ratio of tri to tetra, 2.5 – 3.3 : 1) were fractionated by gel chromatography, the products and their quantities indicated that all of the polysaccharides contained 30 – 31% of (1 → 3)-β linkages and that at most only a very small percentage of consecutive (1 → 3) linkages could be present *(172)*.

Structural differences between lichenan and cereal β-D-glucans are evident from a comparison of the relative amounts of oligosaccharide products from enzymatic hydrolyses, which indicate the presence of more (1 → 3) linkages and fewer cellotetraosyl units in lichenan. The production of very small but significant amounts of laminarabiose by 1,3(4)-β-D-glucanase and lichenase from lichenan indicated that an occasional single (1 → 4)-β bond may occur between two (1 → 3)-β-D-glucosidic linkages. Laminarabiose would result from hydrolysis at the dotted lines in **53**.



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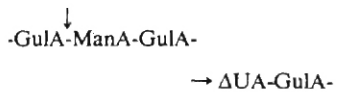
2. Endolyases Depolymerizing Alginic Acid

Enzymes that depolymerize alginate are lyases forming 4-deoxy-L-*erythro*-hex-4-enopyranosyluronic acid at the non-reducing end of the oligosaccharide released *(173)* (see Chapter 4, Volume 2).

In general, bacterial enzymes show a preference for cleaving an α-L-gulosiduronic acid linkage, whereas a β-D-mannosiduronate linkage is more susceptible to splitting by enzymes from algae and mollusks; there are separate L-guluronan and D-mannuronan lyases. An alginate lyase from *Klebsiella aerogenes (174)* with specificity for L-gulosiduronic acid linkages showed high activity with short L-guluronan segments and alginate rich in L-guluronate and showed low activity with D-mannuronate-rich alginate and short chains of D-mannuronan. β-D-Mannosiduronic acid linkages were not hydrolyzed, indicating that the low, but significant degradation of D-mannuronan was due to the few α-L-gulosiduronic acid linkages present (ManA/GulA was 5.3 : 1). Initial reaction with the enzyme gave a series of oligosaccharides, and then prolonged action gave three products, ΔUA-*GulA*, ΔUA-GulA-*GulA*, and ΔUA-GulA-GulA-*GulA*. With an L-guluronan lyase purified from a marine pseudomonad *(175)*, evidence was obtained from kinetic studies with algal and bacterial alginates with different D-mannuronate/L-guluronate ratios and from the structures of the products that the enzyme had specificity toward α-L-gulosiduronic acid linkages. The absence of D-mannuronate in the smaller oligosaccharide products showed that the enzyme cleaves at one or both of the sequences in **54** but, because



54

both ManA and GulA give the same 4-deoxy-L-*erythro*-hex-4-enopyranosyluronic acid (ΔUA) on transelimination, these cannot be differentiated.

The enzymatic activity from *Azotobacter vinelandii* phage gave ΔUA-*ManA* as a major product. From the amount of ManA in the substrate, this could have resulted from lysis of -ManA-GulA-ManA- or of -ManA-ManA-ManA-, but the mannuronosaccharides of DP > 2 could have resulted only from a sequence of ManA. Alginate lyase from the hepatopancreas of a *Littorina* species *(176)* preferentially lysed alginic acid segments rich in D-mannuronate. With alginate the V_{max} was about two-thirds the value with mannuronan fragments, but the K_m values were similar (1.9×10^{-4} *M*). Only D-mannuronic acid was detected at the reducing end of hydrolysis fragments. Alginate lyases *(177)* from the midgut gland of *Turbo cornutus* preferentially split regions rich in D-mannuronate, and a rapid decrease in viscosity of the substrate was accompanied by a gradual increase in reducing power, but there was only partial hydrolysis, with the production of limited amounts of oligosaccharides of low DP. Alginate lyases would appear to be potentially useful in the analysis of fine structure and how this reflects the gel-forming capacity of these polysaccharides.

An L-guluronan lyase from a marine pseudomonad has been used to determine the location of acetyl groups in bacterial alginates. Incubation with *Azotobacter vinelandii* alginate yielded a high molecular weight fraction, with which the acetyl groups were associated, as well as a low molecular weight fraction, free of acetyl groups. Acid hydrolysis of the high molecular weight material gave D-mannuronic acid only. In contrast, if the alginate was treated with a phage D-mannuronan lyase, the products were almost all of low molecular weight and many of the oligosaccharides were acetylated, establishing that the acetyl groups occur on the D-mannuronate residues *178)*.

L-Guluronan lyase has been used to assay D-mannuronan C-5 epimerase activity, because the newly formed L-guluronic acid residues become susceptible to lysis *(179)*.

3. Endo Enzymes Hydrolyzing Glucomannan (β-D-Mannanase and Cellulase)

The endo hydrolysis of (1→4)-β-D-glucomannan with β-D-mannanase leases a range of glucomannosaccharides at rates similar to those of hydrosis of β-D-mannans or galactomannans with a low galactose content

(24,101–107,180–182). Oligosaccharides released *(24,107,180,181)* included mannobiose Glcβ1-4*Man*, mannotriose, Glcβ1-4Manβ1-4*Man*, Manβ1-4Glcβ1-4*Man*, mannotetraose, mannopentaose, and tetra- and pentasaccharides with glucose at the nonreducing end. Thus, β-D-mannanase cannot hydrolyze β-D-glucosidic linkages but can bind to glucose residues at some subsites. A pattern of binding explaining the production of the oligosaccharides has been proposed *(107)* in which subsites β and δ (Fig. 2) can bind to a glucose residue, because at those subsites the hydroxymethyl side of the pyranose ring is bound and the stereochemistry of that side is similar in both the gluco and manno configurations.

The products of hydrolysis of lily glucomannan *(182)* by a commercial cellulase have provided information on sugar distribution in the polymer but, because commercial preparations commonly contain interfering enzymatic activities and transglycosylation alters the products, the significance of the results was limited. Konjac glucomannan was hydrolyzed with a commercial preparation that was electrophoretically homogeneous. However, the unusual nature of the products, which included a high proportion of mannosaccharides and heterosaccharides with mannose residues at the reducing end, led to the suggestion that other hydrolytic activities may have been present.

4. Endo Enzymes Hydrolyzing (1→3)(1→4)β-D-Xylans

Hydrolysis of the mixed-linkage (1→3)(1→4)-β-D-xylan from *Rhodymenia palmata* either with a crude microbial suspension from sheep rumen *(183)* or with cellulase, which was able to hydrolyze (1→4)-β-D-xylans *(184,185)*, gave (1→4)-β and mixed-linkage oligosaccharides. *Myrothecium verrucaria* cellulase *(185)* released D-xylose, (1→4)-β-D-xylobiose, -triose, and -tetraose, (1→3)-β-D-xylobiose, and the mixed-linkage trisaccharide 3^2-β-D-xylosylxylobiose (**55**). The isolation of **55** provided evidence for the coexistence of both (1→3)- and (1→4)-β-D links in the same polymer. Further information was obtained with a purified cellulase from *Stereum sangvinolentum (184)* that hydrolyzed cellodextrins and (1→4)-β-D-xylosaccharides to di- and trisaccharides, which were resistant to further hydrolysis. Transglycosylation occurred with (1→4)-β-D-xylotetraose and -pentaose but was negligible at a concentration of substrate of 0.01%, at which experiments were performed. Products included (1→3)(1→4)-β mixed-linkage oligosaccharides (**56** and **57**) plus oligosaccharides of higher DP incorporating (1→4)-β-xylotriose units linked (1→3)-β. The structures were established chemically and with a purified β-D-xylosidase from guar. It was concluded that the enzyme preferentially attacked (1→4)-β-D bonds that had (1→4)-β-D linkages on both sides but that this was not a rigorous requirement. The higher yield of hexasaccharide composed of two (1→3)-

β-D-linked (1→4)-β-D-xylotriose units compared with pentasaccharide and tetrasaccharide **57** led to the conclusion that a (1→3) linkage not only

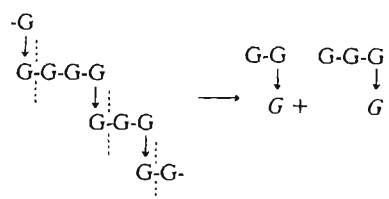
Xylβ1-3Xylβ1-4*Xyl* Xylβ1-3Xylβ1-4Xylβ1-4*Xyl* Xylβ1-4Xylβ1-3Xylβ1-4*Xyl*
55 **56** **57**

inhibited cleavage of an adjacent (1→4) bond but also reduced the rate of hydrolysis of a bond two positions removed; assuming a random distribution of (1→3) and (1→4) linkages, the occurrence of structural elements giving rise to the hexasaccharide could be expected to occur less frequently than those of penta- and tetrasaccharide in the native polysaccharide. The products were consistent with a random arrangement of (1→3) and (1→4) linkages in the ratio 1 : 2.

5. Endo Enzymes Hydrolyzing Pullulan [Pullulanase (EC 3.2.1.41) and Isopullulanase (EC 3.2.1.57)]

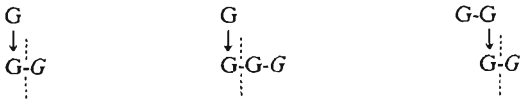
Since the initial discovery of pullulanase in *Enterobacter aerogenes,* it has been detected in a number of microorganisms (Chapter 3). With pullulan, it released oligosaccharides that are multiples of maltotriose linked (1→6)-α by random scission of (1→6)-α bonds *(116).* Exhaustive hydrolysis also gave 6.6% of maltotetraose *(186),* which occurs as segments in the interior of the molecule, and these are susceptible to hydrolysis by α-amylase. *Enterobacter aerogenes* pullulanase is very heat stable, and some activity reappears on cooling after heating at 100°C for several minutes. The substrate specificity of pullulanase *(187)* and of the pullulanase-type enzymes of plant origin, which are called limit dextrinases and whose physiological substrate is endogenous starch limit dextrin, is described in Chapter 3 and Section IV.

Isopullulanase (pullulan 4-glucanohydrolase) has been prepared from cell extracts and from culture filtrates of *Aspergillus niger,* grown on either rice or wheat bran koji medium, and a similar activity has been detected in culture filtrates of *Arthrobacter globiformis (188).* Both enzymes depolymerized pullulan in a random endo manner to the tri- and tetrasaccharides, 6-α-maltosyl-D-glucose (isopanose) (**58**), and 6-α-maltotriosyl-D-glucose (**59**). The latter formula was presumed from the known structure of pullulan. It was concluded that isopullulanase splits (1→4) linkages of α-D-glucose residues linked through the 6 position as in **60** [where the hyphens represent (1→4)-α



60 **58** **59**

and the arrows (1 → 6)-α linkages]. Panose (**61**) was hydrolyzed to isomaltose and D-glucose, 6^3-α-D-glucosylmaltotriose (**62**) to isomaltose and maltose and 6^2-α-maltosylmaltose (**63**) to isopanose and D-glucose, with rates,



61 **62** **63**

relative to pullulan at 100, of 71, 110, and 25, respectively. 6^2-α-Maltosylmaltotriose, isopanose, isomaltotriose, isomaltose, maltotriose, and maltose were unaffected.

E. Endo Enzymes Hydrolyzing Glycans with Oligosaccharide Repeating Units or Masked Repeating Units

The structures of a number of polysaccharides involve an oligosaccharide repeating unit. This may be a disaccharide, as in the glycosaminoglycans, or an oligosaccharide of higher DP, as in the (capsular) exopolysaccharides of bacteria. The repeating unit may be masked by modification after polymerization, as when some of the D-glucuronic acid residues in heparin are isomerized to those of L-iduronic acid. A number of enzymes depolymerize these molecules, such that a repeating unit or multiple of this unit is released. Those catabolizing the glycosaminoglycans can be endo or exo and hydrolases or lyases.

1. Enzymes Depolymerizing Hyaluronate, Chondroitin 4-Sulfate, Dermatan Sulfate, and Chondroitin 6-Sulfate

These enzymes include hyaluronoglucosaminidase (EC 3.2.1.35), hyaluronoglucuronidase (EC 3.2.1.36), hyaluronate lyase (EC 4.2.2.1), chondroitin ABC lyase (EC 4.2.2.4), chondroitin AC lyase (EC 4.2.2.5), chondroitin B lyase (EC 4.2.2.-), and chondroitin C lyase (EC 4.2.2.-) *(189–191)*. The specificity and application to the quantitative estimation of the chondroitin sulfates and hyaluronate and the structural analysis of glycosaminoglycans are discussed in Chapter 5.

Gel chromatography of the reaction products of chondroitin ABC lyase from *Proteus vulgaris* indicated an exo action *(191)*, but an examination of the lysis pattern of oligosaccharides and the corresponding oligosaccharide alditols was interpreted as showing an endo mechanism for the human placental enzyme *(192)*. Chondroitin AC lyase from the same source was considered to act in both an endo and an exo manner. From studies with labeled oligosaccharides, testicular hyaluronoglucosaminidase was found to

bind five neighboring -GlcA-GlcNAc- units, with the catalytic site between units 2 and 3 from the nonreducing end *(193)*.

Some of these enzymes have been used in comparison studies. Changes in young, articular cartilage from long bones, growth cartilage, adult surface cartilage from the arthrosic process, and normal adult articular cartilage *(194)* as well as those occurring on fetal development have been compared using chondroitin lyase *(195)*. Depolymerization of aggregating chains of dermatan sulfate with testicular hyaluronidase gave larger amounts of hexa-, octa-, and decasaccharides than of nonaggregating chains. These oligosaccharides were further degraded with chondroitin AC lyase to tetrasaccharides with L-iduronic acid positioned internally in the sequence. Thus, alternating sequences of D-glucuronic and L-iduronic acids were present in aggregating chains but were rare in nonaggregating chains *(196)*.

2. Enzymes Depolymerizing Heparin and Heparan Sulfate

These enzymes include heparin lyase (heparinase, EC 4.2.2.7), heparitinase (EC 4.2.2.8), and endo-β-D-glucuronidase and are discussed in Chapter 5. Comparative aspects of the structure of a heparan sulfate from lobsters were probed with heparinase and heparitinase *(197)*. It was degraded much less extensively than beef lung heparin, with negligible depolymerization by heparitinase, indicating a structure intermediate between heparin and heparan sulfate. Whale heparin *(198)* was partly digested with heparinase to an octasaccharide with a high affinity for antithrombin III. The sugar sequence was determined by ^{13}C-NMR spectroscopy in conjunction with further lysis to smaller oligosaccharides with heparinase and heparitinase, and the structure was related to the binding capacity.

An endo-β-D-glucuronidase purified from human placenta by affinity chromatography on heparan sulfate–Sepharose 4B released fragments from heparan sulfate with GlcNAc adjacent to the GlcA residue at the reducing end. Carboxyl-reduced heparan sulfate inhibited enzyme action, low molecular weight heparan sulfate (MW 3000) was not attacked, and heparin and N-desulfated heparan sulfate were poor substrates *(199)*.

3. Endo-β-D-galactosidase (Endo-β-D-galactanase) Hydrolyzing Keratan Sulfate)

Keratan sulfate is hydrolyzed by an endo-β-D-galactosidase that has been found in a *Pseudomonas* species, a *Coccobacillus* species, and *Escherichia freundii (200)*. Keratan sulfate was hydrolyzed to a mixture of oligosaccharides of which the smallest was GlcNAc · $6SO_4\beta$1-3*Gal*. The oligosaccharide profile varied according to the source of keratan sulfate, indicating a use for the enzyme in detailed structural studies. Other glycosaminoglycans were not hydrolyzed, but oligosaccharide chains of glycolipids and glycoproteins

with a —Galβ1-4(3)GlcNAcβ1-3Galβ1-4 Glc (or GlcNAc) sequence were. Two proteokeratan sulfates were separated from rabbit corneal stroma, and after papain digestion both could be digested with *E. freundii* endo-β-D-galactosidase *(201)*.

4. Agarase (EC 3.2.1.81)

β-Agarase cleaves agarose and related polysaccharides at the (1→4)-β-D linkage, between D-galactose and 3,6-anhydro-L-galactose (An.Gal). The main oligosaccharide products are neoagarobiose (**64**), tetrasaccharide (**65**), and hexasaccharide, with a predominance of tetrasaccharide. Usually, sulfated oligosaccharides of higher DP are also present. The α linkage between An.Gal and galactose is susceptible to acidic hydrolysis, yielding agarobiose (**66**). Hydrolysis of agar by a preparation from *Pseudomonas kyotoensis* and

-An.Galα1-3Galβ1-4An.Galα1-3Galβ1-

64 (Neoagarobiose)

65 [(neoagarobiose)$_2$]

66 (Agarobiose)

isolation of neoagarobiose and (neoagarobiose)$_2$, together with the separation of agarobiose from an acidic partial hydrolysis, allowed the formulation of the structure of agarose as alternating repeating residues of (1→3)-β-D-linked galactose and (1→4)-α-L-linked An.Gal (Chapter 4, Volume 2) *(173)*. The estimation of agarose content in agar and in admixture with carrageenan, which may occur in the same algae, can be performed enzymatically, because only agarose is susceptible to hydrolysis by *Pseudomonas atlantica* β-agarase.

The extent of hydrolysis of agarose and related galactans such as porphyran by β-agarase decreases according to their difference in structure from the idealized agarose formula. The enzyme is relatively specific for An.Gal linked to galactose. Hydrolysis of desulfated porphyran gave a tetrasaccharide fraction of 6^3-*O*-methyl(neoagarobiose)$_2$ (**67**) and 6^1-*O*-methyl(neoagarobiose)$_2$ (**68**) in the ratio 5 : 1, indicating that the glycosidic linkage of

An.Galα1-3(6Me)Galβ1-4An.Galα1-3*Gal*

67

An.Galα1-3Galβ1-4An.Galα1-3(6*Me*)*Gal*

68

methylated galactose is hydrolyzed at about one-fifth the rate of nonmethylated galactose. The distribution of 6-*O*-methylgalactose in all the fractions from the hydrolysate was consistent with a random distribution of methyl groups. Sulfated oligosaccharides were very resistant to hydrolysis, and it was suggested that a sequence of at least two contiguous nonsulfated disaccharide units was needed for hydrolysis. Reaction of sulfated agarose followed by gel chromatography of the products gave a sulfated fraction of high DP with a large amount of sulfation, indicating the presence in the polymer of blocks of regions substituted with sulfate, which were resistant to hydrolysis *(202)*.

Structural differences in the agars from several *Gracilaria* species have been probed using purified extracellular β-agarase *(203)* and a cell wall β-(neoagarobiose)$_2$ hydrolase (EC 3.2.1.-) *(204)* from *Pseudomonas atlantica* with the aim of providing an index of gelling properties (agarose content). The latter enzyme is an exohydrolase that removes neoagarobiose units sequentially from the nonreducing terminus of neoagarosaccharides. On hydrolysis of the agars with β-agarase and separation of neutral and charged oligosaccharides by ion-exchange chromatography, the same pattern of neutral oligosaccharides was obtained from all preparations, but the amounts of each oligosaccharide, **64**, **65**, **67**, and 6^1-O-methylneoagarobiose (**69**), var-

An.Galα1-3(6*Me*)*Gal*
69

ied. Incubation of the agars with the two enzymes gave as neutral products **64** and **69**, and the ratio varied according to the amount of 6-*O*-methylgalactose n the polysaccharide. Tetrasaccharide **70** was produced from two species

An.Galα1-3Galβ1-4An.Galα1-3*Gal*
46
| |
pyr
| |
70 (pyr = pyruvate ketal)

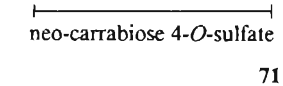
hat contained pyruvate. Higher molecular weight, sulfated oligomers were resent in all hydrolysates and were more resistant to hydrolysis by (neogarobiose)$_2$ hydrolase. The enzymatic technique distinguished agars by ıeir oligosaccharide fragments but did not establish a relationship to gelling ıpacity, probably because it did not indicate the arrangement of charged ·oups.

The enzymatic degradation of gels has been studied with a highly purified ;arase from a *Pseudomonas*-like bacterium *(205)*. Incubation with Sephare 6B caused swelling and increased the transparency of the gel, and this was lieved to result from a decrease in the size of double-helical aggregates hapter 5, Volume 1). The change in molecular sieving properties indicated

that the polysaccharide chains around large voids in the gel were more rapidly degraded than those around small voids, but this may have been a consequence of the high molecular weight (210,000) of the enzyme. If incubation was combined with mechanical disruption, the solubilization was increased from 65 to 95%. Gel chromatography of the products of hydrolysis of melted beads of Sepharose 4B showed a single peak, whereas the products of hydrolysis of unmelted beads always gave two peaks, independently of the degree of degradation. One peak was of oligosaccharides, but the other was of higher molecular weight material (~25,000). This was related to the structure of the gel and the resistance of the double-helical regions to enzymatic attack.

5. κ-Carrageenanase (EC 3.2.1.83)

Enzymes hydrolyzing carrageenan have been found in marine mollusks and culture filtrates of a marine bacterium *(173,206)*. Activities specific for the κ and λ fractions of carrageenan were obtained from the cell-free culture medium of *Pseudomonas carrageenovora.* The rate and amount of hydrolysis of κ-carrageenans from different marine algae varied. The polymer from *Hypnea musciformis* was hydrolyzed at a higher initial rate and to a much greater extent than that from *Gigartina acicularis.* Those from agarophytes were not hydrolyzed. Hydrolysis occurred specifically at the (1→4)-β-D linkages of κ-carrageenan (**71**) in a random endo manner (An.Gal is 3,6-an-



71

hydro-D-galactose). Products included a resistant fraction and a series of sulfated oligosaccharides, neocarrabiose-4-*O*-sulfate (**71**), the tetrasaccharide sulfate, hexasaccharide sulfate, and octasaccharide sulfate. The di- and tetrasaccharides made up more than 95% of the oligosaccharide fraction and the resistant fraction contained more galactose and sulfate than did κ-carrageenan. Treatment of this fraction with alkali released 19% sulfate with equivalent anhydrogalactose formation. It was then degraded 75% by κ-carrageenanase, so enzyme action was dependent on the presence of the carrabiose 4-sulfate unit. The enzyme could not hydrolyze (1→4)-β-D linkages adjacent to disaccharides that contained D-galactose 6-sulfate, disulfate, or 3,6-anhydro-D-galactose 2-sulfate. The results of enzymatic studies showed that 80% of the carrageenan molecule consists of (1→4)-β-linked neocarrabiose 4-*O*-sulfate units and, in the remainder, the anhydrogalactose units are replaced by galactose sulfate.

6. Mycodextranase (1,3-1,4-α-D-Glucan 4-Glycanohydrolase) (EC 3.2.1.61)

Mycodextran (nigeran), an unbranched polymer of regularly alternating (1→3)- and (1→4)-α-D-glucopyranose residues, is hydrolyzed by mycodextranase. Activity was prepared from culture filtrates of *Penicillium melinii (207)*, which produced nigerose and 4^2-α-nigerosylnigerose (**72**). The

Glcα1-3Glcα1-4Glcα1-3*Glc*
72

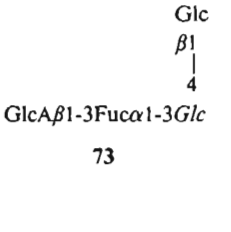
tetrasaccharide was a poor substrate and, with a large amount of enzyme, nigerose but no D-glucose was released. On hydrolysis of nigeran, nigerose and the tetrasaccharide were released simultaneously, and the molar ratio of tetra- and disaccharide was constant at about 1.5 during digestion. Inhibition studies showed that lactose did not bind, but maltose and nigerose did, with similar K_i values ($6-7 \times 10^{-2}$ *M*), suggesting that the enzyme has sites binding to both (1→3)- and (1→4)-linked α-D-glucose residues. The nigerosyl tri- and tetrasaccharides were more effective inhibitors, with K_i values of 3×10^{-2} and 4.8×10^{-3} *M*, respectively. A model for an active site of eight glucose units and a catalytic site between residues 4 and 5 was proposed. The substrate requirement is rigid. Nigeran was hydrolyzed quantitatively, but amylose, with all (1→4)-α-D linkages, and pseudonigeran, with all (1→3)-α-D linkages, were not affected. Hydrolysis of nigeran released mainly nigerose and the tetrasaccharide $(nigerose)_2$, confirming the structure of repeating 1→4)- and (1→3)-α linkages. Examination of the 3–5% of trisaccharide eleased indicated that about one-half of the reducing ends and one-quarter of the nonreducing ends of the polymer chains had (1→4)-α linkages. Isolichenan was hydrolyzed slightly, but no tetrasaccharide was released, indiating that it contains no segments of three consecutive nigerose units linked 1→4) but has a majority of segments with one or two consecutive (1→3) onds surrounded by (1→4) linkages, in agreement with the previously oposed structure. The high specificity has been useful in confirming the ructure of nigeran, for studying the location and amount of nigeran in ngal cell walls *(208)*, and for preparing nigerose *(209)*.

7. Endoglycanases That Depolymerize Bacterial Polysaccharides

A number of bacteriophage glycanases selectively endohydrolyze one kage type in exopolysaccharides of bacteria to produce oligosaccharide its plus multiples of these, offering an alternative method of fragmentan to chemical procedures *(210)*, the advantage being that *O*-ester and ketal ups are not removed. The bacteriophages can be purified by polyethylene col precipitation or by isopycnic centrifugation *(211)*. Bacteriophage ysaccharide hydrolase, incubated with the exopolysaccharide of *Kleb-*

siella aerogenes serotype 54, released acylated tetrasaccharides based on structure **73** *(210,212)*. The enzyme hydrolyzed a Glcβ1-4GlcA bond. An octasaccharide was also obtained, which could be further hydrolyzed to tetrasaccharides. Reduction of D-glucuronic acid to D-glucose residues prevented enzyme action.

A glycanase associated with *Klebsiella* bacteriophage 11 *(211)* catalyzed the depolymerization of alkali-treated serotype 11 polysaccharide, giving oligosaccharides of one or two repeating units. It cleaved the polysaccharide chain at → 3Glcβ1 ↓ 3GlcAβ1- by hydrolysis, mainly to **74**. Hydrolysis also



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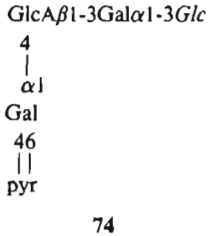

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occurred after Smith degradation of the polymer, when the repeating unit became **75** but, after esterification and borohydride reduction, which converted glucuronic acid to glucose residues, there was no hydrolysis.

-3GlcAβ1-3Galα1-3Glcβ1-
75

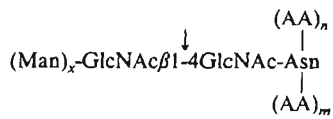
A model of the stereochemical arrangement of bonds and groups in the two sugars on either side of the glycosidic bond hydrolyzed by K13 phage *(213)* has been devised. It was suggested, that for reaction, a carboxyl group may have to be located at a certain distance from this linkage. When 74 polysaccharides were reacted with 55 bacteriophages *(214)*, in most cases in which the one polysaccharide was hydrolyzed by several phages (9 of 11), the same bond was split. In most cases (33 of 36) the polymer chains were hydrolyzed on either side of negative charges, but uronic acid was not produced at the reducing end of the oligosaccharide. In 33 of 36, the reducing end formed was substituted at position 3 and, in 32 of 36, β-D-glycosidic linkages were broken.

A comparison *(215)* of the NMR spectra of *Klebsiella* 18 exopolysaccharide and the hexasaccharide derived by depolymerization with bacteriophage 18 indicated that the solution conformations were similar despite the large difference in DP. The structures of *Klebsiella* K21 and K32 exopolysaccharides were determined after initial phage depolymerization, without loss of pyruvate ketal groups *(216)*, and the position of the D-galactosyl unit in the trisaccharide released by phage depolymerization of *Klebsiella* K63

polysaccharide was found to be at the nonreducing end, because it was released by α-D-galactosidase, leaving an aldobiouronic acid *(217)*.

F. Endo Enzymes Hydrolyzing Complex Polysaccharides

Most endo enzymes do not readily attack more complex polysaccharides, because these may lack uninterrupted sequences of similar sugars and linkages that are subject to endoglycanase attack, and they are often highly branched, resulting in more interference with endo action. A number of endohydrolases that selectively cleave the oligosaccharide chains from glycoproteins have been useful in structure determination *(17,218)*. Several preparations have been described that hydrolyze at the chitobiose section linked to asparagine in chains containing mannose (**76**), releasing the oligo-



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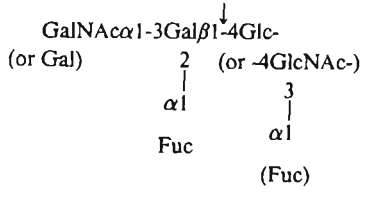
saccharide chain less one GlcNAc as a unit. The specificity of these enzymes from different sources varies according to the nature of the $(Man)_x$ section and any further substitution of the chain by other sugars. They are called endo-*N*-acetyl-β-D-glucosaminidases and have been assayed with the tritiated dansyl derivative of the asparagine glycopeptide and also by measuring the binding of glycoprotein to ^{125}I-iodinated concanavalin A, because the removal of oligosaccharide chains terminated by mannose residues destroys binding activity *(219)*.

As well as the release of oligosaccharide chains for the determination of heir structures, there have been some other uses for endoglycosidases. *Saccharomyces* invertase treated with the enzyme from *Streptomyces plicatus* ost almost all oligosaccharide chains, and this allowed the determination of ie molecular weight and subunit molecular weight of the protein portion *20)*. Phospholipases have been hydrolyzed to study the influence of the igar chains on the glycoprotein *(221)*, and the growth-dependent alterions of glycopeptides from human diploid fibroblasts have been examined. hese were incubated with Pronase followed by endo-*N*-acetyl-β-D-glucosainidases and then successively degraded with α- and β-D-mannosidases *22)*. The structures of the oligosaccharide chains of ovalbumin were reed following the application of two endo-*N*-acetyl-β-D-glucosaminidases the hydrolysis of glycopeptide fractions *(223)*. Incomplete hydrolysis by e but complete hydrolysis by the other of one of the fractions indicated a xture.

Another enzyme, which releases oligosaccharide chains by hydrolyzing between GlcNAc and asparagine, has been extracted from almond and jack bean seeds *(224)*. The released oligosaccharide contains two GlcNAc residues. It is relatively nonspecific for the oligosaccharide, and both complex and high-mannose chains are released (DP 3–11). It has been applied to bromelain glycopeptide and ovalbumin. Digestion of desialylated fibrinogen removed 40% of neutral sugars with no change in clotting capacity.

A third enzyme is endo-β-D-galactosidase, which has been isolated from *Streptococcus pneumoniae (224a)* and purified by chromatography. It hydrolyses the carbohydrate chains of glycoproteins with structure **77** at the



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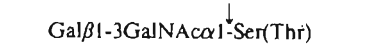
position indicated by the arrow and releases oligosaccharides from blood group A and B mucins. Another endo-β-D-galactosidase has been isolated from *Escherichia freundii* (Section III,E,8) and has been used in the elucidation of the structures of oligosaccharides of blood group glycolipids *(225)*.

A fourth enzyme, endo-*N*-acetyl-α-D-galactosaminidase, was separated from *S. pneumoniae (226,227)*. Hydrolysis occurred at the position shown by the arrow in **78** to produce a disaccharide. A similar activity was detected



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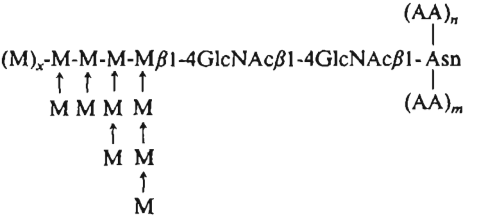
in *Clostridium perfringens*. Disaccharide has been released from asialoglycopeptide fractions of mouse and human melanoma, fetuin and human bronchial glycoprotein, as well as antifreeze glycoprotein and pig submaxillary mucin.

The plant gums, which are highly branched acidic heteropolymers, are resistant to microbial attack but gum tragacanth, partly degraded by dilute acid, on incubation with an enzyme preparation gave (1→4)-α-D-galacturonobiose and -triose and Xylβ1-3*GalA (228)*. Polysaccharide from soybean was partly degraded with acid and then, after incubation, Xylβ1-3*GalA* and the trisaccharide Xylβ1-3GalAα1-4*GalA* released *(229,230)*. A D-arabino-D-galactan from the cell wall of *Mycobacterium phlei* was treated with an enzyme preparation from a soil bacterium, and a series of (1→5)-α-D-ara-

binofuranosaccharides, up to the tetraose, were isolated. Because a high molecular weight fraction containing most of the D-galactose remained, the D-arabinose was considered to come from side chains *(231)*.

An endo-1,6-α-D-mannanase (EC 3.2.1.-) was purified from a soil organism obtained by enrichment culture on the (1→6)-α-D-mannan backbone of the highly branched mannoprotein from *Saccharomyces cerevisiae (232)*; the mannan was prepared by removal of (1→2)- and (1→3)-α-D side chains from yeast mannan with an exo-1,2-1,3-α-D-mannanase (EC 3.2.1.77). Endo-1,6-α-D-mannanase was used to determine the structure near the mannan–protein linkage region. A mutant of *S. cerevisiae* that produced a yeast mannan with a predominantly unbranched mannan chain was treated first with endo-1,6-α-D-mannanase and then with endo-*N*-acetyl-β-D-glucosaminidase to give an oligosaccharide with the formula $(Man)_x$-*GlcNAc*, where x was about 12. On acetolysis a series of (1→2)-α- and (1→3)α-D-mannose oligosaccharides plus oligosaccharides with mannose linked (1→4)-β-D to GlcNAc indicated a general structure for the original mannoprotein of 79 [where the arrows represent (1→2) or (1→3) linkages and the hyphens (1→6)-α-D linkages].



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G. Conclusions about Endoglycanases

The properties of the various endoglycanases show some similarities, with a few clear exceptions in particular areas. The pH optima for most are in the acidic region, generally around 5–6, but those for some are as low as 3. Exceptions are the lyases, which are most active near 8. The molecular weights of many are below 50,000, and these are single polypeptide chains that are stable to storage and to temperatures above ambient (up to 50°C). Their solutions can often be frozen without denaturation. Pullulanase, the lyases, and some of the enzymes hydrolyzing glycoprotein chains and bacterial polysaccharides have higher molecular weights. The K_m values with their native substrates are generally in the range 0.1–1 mg/ml. The major prod-

ucts of hydrolysis of homoglycan chains are mainly a mixture of di- and trisaccharides, but a number of exceptions are found. When there is an oligosaccharide repeating unit, oligomers of two and three repeating units are formed. The pattern of hydrolysis of chitin by lysozyme provides a model for the action pattern of a number of these enzymes. Hydrolysis involves binding of the polysaccharide at multiple subsites on either side of the active site, with interaction to neighboring individual sugars. Six sugars are involved with lysozyme. More have been implicated with other enzymes. The free-energy changes on binding have been calculated from the frequency of bond hydrolysis for some enzymes *(136,137)*, indicating subsite affinities. There is evidence with several endoglycanases that binding to the sugar lying next to and on the nonreducing side of the sugar whose glycosidic bond is being hydrolyzed is of prime significance. The free-energy change on binding at this subsite is negative and very high in α-amylase from *Aspergillus oryzae (136)*, lysozyme *(134)*, and *Pseudomonas lilacinum* dextranase *(143)*. Binding to the sugar whose glycosidic bond is broken involves a positive free-energy change, and with lysozyme the half-chair conformation of the carboxonium ion intermediate affords a better fit. Other positions show lower free-energy changes, some negative, others positive. All of these enzymes have a characteristically endo pattern of hydrolysis. In the α-amylase from *Bacillus amyloliquefaciens (136)*, which is also an endo enzyme but with considerable nonrandom hydrolytic character, the subsites with negative free-energy change are more uniform in amount and spread more evenly between the two sides of the catalytic site. The amount and sign of the free-energy change on binding at particular sites and the distribution of sites probably influence the degree of endo character. After scission, the balance in free-energy change between the sites would affect whether a fragment is released or repositioned on the active site. Substitution of a homoglycan chain affects the degree and pattern of hydrolysis, and there is considerable variation in the effect of this on different enzymes. There are also differences in the action patterns between the same enzyme from different sources.

Endoglycanases appear to be absolutely specific for the anomeric linkage, but there are examples in which specificity for the intersugar linkage is not so strict, for example, in the hydrolysis of $(1\rightarrow3)(1\rightarrow4)$-$\beta$-D-xylans and reaction with 1,3(4)(nonspecific)-β-D-glucanase. In most cases specificity for the sugar is high, but minor structural modifications may still allow reaction. β-D-Mannanase can hydrolyze glucomannans, giving oligosaccharides containing glucose; a chitosanase that hydrolyzed cellulose has been described; and some cellulases hydrolyze β-D-xylans in which the linkage is the same as in cellulose and the component sugar has the same configuration as glucose, except for lacking a hydroxymethyl group. Transglycosylation with endoglycanases, particularly those like lysozyme and cellulase, is believed to lead

to products with the same linkage as the substrate, because they contain stereospecific binding sites on either side of the catalytic site. However a 1,4-β-D-xylanase from *Cryptococcus albidus (233)* formed (1→3)-β-D linkages. The absence of a hydroxymethyl substituent may allow less specificity in binding.

The major use for the endoglycanases in structural studies is the production of oligosaccharide fragments for further examination, particularly from heteroglycans.

IV. Debranching Enzymes

There are several enzymatic activities that hydrolyze the (1→6) linkages in (1→4)(1→6)-α-D-glucans, removing the branch chains. They are classified as hydrolases of *O*-glycosyl bonds. The discussion in this section includes enzymes that hydrolyze branch chains only at the branch point, removing branches of more than one monosaccharide unit as the complete oligosaccharide. Thus, an α-D-galactosidase hydrolyzing the single galactose side chains of galactomannans is not discussed. Amylo-1,6-D-glucosidase is briefly mentioned because of its historical significance. Debranching enzymes have found extensive use in the determination of fine structure of amylopectin, glycogen, and phytoglycogen. They have been named (microbial) pullulanase, (plant) limit dextrinase (pullulanase, EC 3.2.1.41), isoamylase (EC 3.2.1.68), and amylo-1,6-D-glucosidase:4-α-D-glucotransferase (EC 3.2.1.33:EC 2.4.1.25). Their contribution to an understanding of the structure of (1→4)(1→6)-α-D-glucans is discussed in Chapter 3 and has been reviewed *(116,133,187)*.

A. Action Patterns

Amylo-1,6-D-glucosidase:4-α-D-glucanotransferase is a bifunctional enzyme from mammalian and fungal sources, where it takes part in the degradation of glycogen. Glycogen, amylopectin, and their β-limit dextrins react slowly and incompletely *(187)*. The best substrate is a phosphorylase limit dextrin with an optimal A-chain length of four glucose units. The terminology is derived from glycogen or amylopectin structures. The A chains are joined by a single (1→6)-α-D linkage to another chain. The B chains are linked to another chain through a (1→6)-α-D linkage and have at least one other chain linked to them through a 6-hydroxyl group. The single C chain in a molecule has the only free reducing group. This is illustrated diagrammatically in Fig. 4. The enzyme catalyzes the transfer of three of the four glucose residues in an A chain of the phosphorylase limit dextrin to another A chain

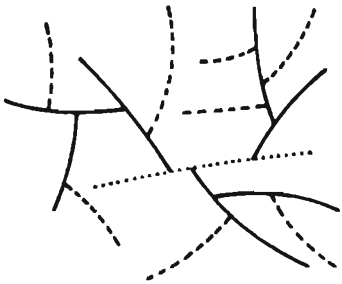


Fig. 4. Chain types in (1→4)(1→6)-α-D-glucans (—, A chains; —, B chains; · · ·, C chain).

and then hydrolyzes the remaining single glucose stub. In small oligosaccharides composed of two (1→4)-α chains linked (1→6)-α, the chain with the reducing end sugar must be at least four glucose units; 6^3-α-D-glucosylmaltotetraose is the smallest substrate.

The action pattern of limit dextrinase with oligosaccharides and polysaccharides resembles that of pullulanase, but that of isoamylase differs. The α-D configuration of the hydrolyzed bond is retained on reaction by all three *(234)*. With dextrins of low DP (up to 7) *(235)*, neither pullulanase nor isoamylase removed single glucose stubs or hydrolyzed isopanose. Pullulanase catalyzed a very low initial rate of release of maltose from 6^2-α-maltosylmaltose (**80**), but isoamylase had no action [the hyphens represent (1→4)-α and the arrows (1→6)-α bonds]. 6^3-α-Maltosylmaltotriose (**81**) was readily hydrolyzed by pullulanase and plant limit dextrinase but only very slowly by isoamylase. Pullulanase hydrolyzed 6^3-α-maltotriosylmaltotriose (**82**) as readily as pullulan and isoamylase hydrolyzed it slowly, at a lesser rate than polysaccharides. Oligosaccharide substrates for pullulanase composed of two (1→4)-α chains linked (1→6)-α, with two glucose units in the reducing (1→4)-α chain and substituted (1→6) at the nonreducing glucose residue (**80**) or with three glucose units in the reducing chain and substituted 6^2 (**83**), were less readily hydrolyzed than compounds with the reducing chain substituted 6^3 (**81**, **82**, and **84**). Isoamylase requires at least three glucose units in

G-G	G-G	G-G-G	G-G-G-G	G-G-G
↓	↓	↓	↓	↓
G-*G*	G-G-*G*	G-G-*G*	G-G-*G*	G-G-G-*G*
80	**81**	**82**	**83**	**84**

the reducing chain to show any action. Once there are three glucose residues in the reducing chain and two in the nonreducing chain, the initial rate of

hydrolysis with pullulanase and limit dextrinase is of the same order as with pullulan or with the nonasaccharide derived from pullulan. The rates with isoamylase are lower for oligosaccharides than for glycogen or amylopectin but increase in the order **81** < **82** < **84**, and **84** was hydrolyzed at about one-third the rate of amylopectin. Oligosaccharides can undergo reversion at high concentrations.

Isoamylase debranches glycogen, phytoglycogen, and amylopectin but shows very slight reaction with pullulan. Relative V_{max}/K_m values with these substrates of 875, 2180, and 1150, respectively, compared with 0.5 for pullulan, have been reported *(236)* for the enzyme from *Pseudomonas amyloderamosa*. Pullulanase and limit dextrinase react with pullulan, much less readily with amylopectin, and even less easily with phytoglycogen and glycogen. Comparative relative V_{max}/K_m values for *Enterobacter aerogenes* pullulanase *(236)* were 59 (pullulan), 0.009 (amylopectin), 0.0016 (phytoglycogen), and 0.0009 (glycogen), and for the limit dextrinase from rice seeds *(237)* the values were 16.7, 0.003, 0.00045, and 0.00046, respectively. Pullulanase attacks dextrins more readily than the parent (1→4)(1→6)-α-D-glucans. The limit dextrinase from oat seeds had relative V_{max}/K_m values of 5.9 and 1.1 for pullulan and amylopectin β-limit dextrin, respectively *(238)*. The extent of debranching can be dependent on the concentration of enzyme and substrate and the period of incubation. In a number of experiments *(239)* in which moderate levels of activity were used, pullulanase debranched about half the amylopectin molecule. Although pullulanase splits pullulan in an endo pattern, gel chromatography of a series of mixtures of partly debranched amylopectin showed that exterior chains were preferentially removed *(240,241)*, leaving a high molecular weight core. If high levels of enzymes were incubated with amylopectin, debranching was essentially complete *(240)*. The β- and α-limit dextrins of amylopectin were fully debranched more readily than the parent polysaccharide *(239,242)*. Glycogen was only very slightly debranched (< 5%) by moderate levels of pullulanase *(240,242)* but, if a much higher amount of enzyme was applied, this could be increased to 30%. Exterior chains were removed. Glycogen β-limit dextrin, with moderate levels of enzyme, was more readily but still incompletely attacked (up to half) *(242)*. The isoamylase from *P. amyloderamosa* hydrolyzed amylopectin, but high levels of enzyme relative to substrate were necessary for complete hydrolysis *(240)*. The outer chains were preferentially removed from glycogen, but both inner and outer chains of amylopectin were hydrolyzed simultaneously. Examination of the hydrolysis products of the β-limit dextrins of amylopectin and glycogen with both *P. amyloderamosa (235)* and *Cytophaga (62)* isoamylases showed that some maltose was released.

B. Debranching Enzymes and the Fine Structure of (1→4)(1→6)-α-D-Glucans

Debranching enzymes can be used in a number of ways to establish fine structural details of branched (1→4)(1→6)-α-D-glucans. After debranching, the (1→4)-α chains can be chromatographed on Sephadex G-50 or G-75, when the elution pattern gives a chain length versus frequency distribution. The first study of this type, when whole starches and β-limit dextrins were debranched with pullulanase, led to a revision of the Meyer treelike structure *(243)* to give a formula more in accord with physicochemical data from viscosity and ultracentrifugation. When isoamylase, which can completely debranch glycogen, became available, this procedure was extended *(240,243,244)* and glycogen gave a single, slightly skew, included peak and phytoglycogen a pattern intermediate between this and that of amylopectin, which is bimodal. The chain lengths of outer chains of glycogens were compared with inner chains by first treating the glycogens with very high levels of pullulanase, which preferentially hydrolyzed some outer chains, affecting about 30% debranching *(244)*. The remainder of the molecule was then debranched with isoamylase and the average chain length determined. Pullulanase was used to compare the chain length distributions of waxy, normal, and high-amylose starches *(245)*. The whole starches were first converted with β-amylase to the limit dextrins, which depolymerized and removed unbranched (1→4) chains (amylose) before debranching and chromatography. Debranching with isoamylase showed a different distribution of chain lengths in native amylopectin and the (1→4)(1→6)-α-D-glucans synthesized from D-glucose 1-phosphate and maltosaccharides by phosphorylase and branching enzyme (Q-enzyme) and also from amylose and branching enzyme *(243)*. The presence of (1→6)-α branch points in the amylose fraction of starches was detected by the increase in the percent conversion to maltose by β-amylase after debranching *(246)*.

Another aspect of the fine structure of (1→4)(1→6)-α-D-glucans is the ratio of A to B chains. The A chains can be recognized by treatment of the polysaccharide with β-amylase, which reduces these to maltose or maltotriose stubs, so that after debranching they can be clearly distinguished from the longer B chains. Probability predicts that equal amounts of maltose and maltotriose will be produced. The first estimate was made for amylopectin *(247,248)* with a limit dextrinase (R-enzyme) and separation of the oligosaccharides by charcoal–celite chromatography. The fraction of A chains is calculated from Eq. (4). An estimate for glycogen was made using phosphor-

$$\frac{\text{Average unit chain length}}{2.5} \times \frac{100 - \%\ \text{amylolysis}}{100} \times \frac{\%\ \text{maltose} + \text{maltotriose}}{100} \qquad (4)$$

ylase to reduce the A chains to stubs of four glucose units and then de-

branching with amylo-1,6-glucosidase. The significance of these results has been discussed *(116)*. The availability of pullulanase made it possible to extend the method to glycogen β-limit dextrin *(31)*. Maltose and maltotriose were separated by paper chromatography and were released in equimolar quantities. A fully enzymatic method was introduced that used the different action patterns of pullulanase and isoamylase *(249)*, in particular the relative ease of hydrolysis of maltosyl and maltotriosyl stubs by isoamylase and pullulanase. The β-limit dextrin was incubated with isoamylase, alone initially, and then the incubation mixture was divided and pullulanase was added to one-half. Reducing power was estimated for each sample with copper reagent. The A/B chain ratio was estimated directly from the spectrophotometric absorbance (*A*) readings of the Somogyi estimations; Eq. (5)

$$\text{A/B chain ratio} = \frac{2(A_{\text{isoamylase + pullulanase}} - A_{\text{isoamylase}})}{2A_{\text{isoamylase}} - A_{\text{isoamylase + pullulanase}}} \tag{5}$$

gives the A/B chain ratio. For accuracy, the method depends on isoamylase hydrolyzing all maltotriose units and no maltose units, but it has been shown that, at least at some levels of isoamylase activity, both the *Pseudomonas amyloderamosa (235)* and the *Cytophaga (62)* enzymes release some maltose from maltosaccharides and β-limit dextrins and that the rate of release of maltotriose chains is low. The amount of isoamylase is critical *(241)*. Also, the subtractions in the calculation reduce the reproducibility of results *(241,250)*. However, the method can be useful for comparative studies, due to its ease of application. The A/B chain ratio of amylopectin, from which outer chains had been removed by pullulanase, was found to be lower than in the original molecule, as is consistent with the nonsymmetric shape found by physicochemical methods.

2. Debranching of Molecules Other Than (1→4)(1→6)-α-D-Glucans

The value of debranching enzymes in the determination of fine structure f such molecules as glycogen and amylopectin demonstrates the potential sefulness of this type of enzyme in the determination of structures of other olysaccharides. An enzyme described as a dextran-1,2-α-debranching en-/me from *Flavobacterium* has been reported; by the use of this enzyme ngle glucose residues linked (1→2) could be estimated in various dextrans *51)*. The enzyme released glucose and appeared to interact with the →6)-α chain. It is not known if its action pattern would include the release oligosaccharide chains. Another group of enzymes has been described that drolyzes glycoproteins at or near the linkage point of oligosaccharide to

protein without further degradation of the released oligosaccharide chain. The specificity of these is described in Section III,F.

V. Exo Enzymes

A. Characterization of Exoglycanases and Glycosidases

Two other groups of enzymes that have been used in studies on polysaccharides are the exoglycanases and glycosidases. Both remove sugar units from the nonreducing end of a polymer. Some exoglycanases remove oligosaccharide units, and some of these act from the reducing end. If the substrate has repeating units, there is sequential removal as incubation proceeds. Because some glycosidases react at high rates with polysaccharides, the final result resembles that with exoglycanases releasing monosaccharides. The two groups differ in their substrate requirements and pattern of hydrolysis and are distinguished by several experimental criteria *(252)*, but they are most readily characterized by their relative rates of hydrolysis of oligosaccharides of increasing DP. In general, with exoglycanases, as the DP increases from 2 to 6, V_{max} increases and K_m decreases; that is, the most effective hydrolysis by exoglycanases involves the recognition and binding of about four to six units, although only the terminal unit is released in reaction. Examples of this behavior have been shown by exo-1,3-β-D-glucanase *(252,253)*, glucodextranase (exo-1,6-α-D-glucanase; EC 3.2.1.70) *(254)*, exo-1,4-α-D-glucanase *(252,255)*, and exo-1,4-β-D-mannanase (EC 3.2.1.-) *(68)*.

With glycosidases, the nonreducing terminal sugar whose glycosidic bond is being cleaved is primarily recognized by the enzyme. Many glycosidase substrates have a noncarbohydrate aglycone. The size, charge, hydrophobicity, and stereochemistry of the aglycone affect the rate of hydrolysis. In the hydrolysis of a homologous series of oligosaccharides by a glycosidase, as the DP increases, the reciprocals of the K_m as well as the V_{max} values, expressed as moles of terminal glycoside, usually either do not vary significantly or decrease. β-D-Xylosidases (EC 3.2.1.37) from *Aspergillus niger* and *Malbranchea pulchella (256)* and β-D-mannosidase from *Helix pomatia (257)* provide examples. Hydrolysis by glycosidases generally occurs with retention of configuration of the C-1 hydroxyl, whereas hydrolysis by exoglycanases proceeds with inversion of configuration. This takes place with β-amylase, exo-1,4-α-D-glucanase, α- and β-D-glucosidases, dextranglucosidase, glucodextranase, exo-1,4-β-D-mannanase, and exo-1,3-β-D-glucanase, but exceptions have been found. The capacity of D-glucosidases to control the anomeric configuration of the sugar formed was shown by reaction with D-glucal

(258). Sweet almond β-D-glucosidase converted it initially to 2-deoxy-β-D-glucopyranose, and *Candida tropicalis* α-D-glucosidase initially produced the α anomer. In deuterium oxide, α-D-glucosidase catalyzed specific axial deuteration at C-2, forming 2-deoxy-α-D-[2-$^2H_{ax}$]glycopyranose, and the β-enzyme caused equatorial deuteration. An exoglycanase, glucodextranase from *Arthrobacter globiformis*, reacted with 2,6-anhydro-1-deoxy-D-gluco-hept-1-enitol (Fig. 5), creating a new anomeric configuration specifically *(259)*. Glycosidases and exoglycanases can also be distinguished by the effect of linkage type in an oligosaccharide substrate on susceptibility to hydrolysis. Glycosidases generally have less specific requirements for hydrolysis. Most glycosidases hydrolyze dimers linked (1→2), (1→3), (1→4), and (1→6), although differences in relative rates are displayed. An α-D-mannosidase from jack bean cleaved mannobioses linked variously at different rates *(260)*. Exoglycanases are more specific for the linkage type, for example, β-amylase, which hydrolyzes only (1→4)-α-D linkages. Specific competitive inhibitors are useful in characterization. Aldonolactones are potent inhibitors of glycosidases but have a limited effect on exoglycanases. Inhibition is competitive, and the lactone must have the same ring size and configuration as the sugar residue of the substrate. In most cases the β-D-glycosidase is inhibited to a greater extent and inhibition is independent of the aglycone *(252,261,262)*. One molecule of D-glucono-(1→5)-lactone competes successfully with more than 100 molecules of substrate for β-D-glucosidase, but 1000 times as much lactone is needed to give a similar inhibition of exo-1,3-β-D-glucanase. Other compounds [e.g., aminoglycosides and nojirimycin *(262,263)*] can also be used for inhibition studies. Another distinguishing feature is that glycosidases more readily catalyze transfer reactions to other sugars. By the use of these criteria a number of activities have been differentiated. However, there is not always consistency in these properties for a particular enzyme, and in many cases the tests have not been performed. An exoglycanase can indicate the extent of an unbranched sequence of the same sugar from the nonreducing end of the chain.

The differences in the action patterns of exoglycanases and glycosidases may be due in part to the relative free-energy changes on binding of sugars at

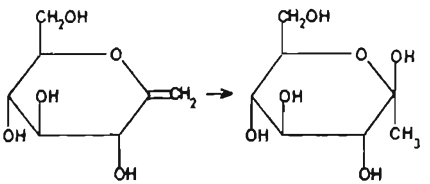
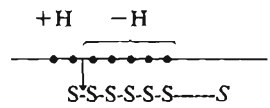


Fig. 5 Stereospecific reaction of 2,6-anhydro-1-deoxy-D-gluco-hept-1-enitol.

and near the nonreducing end of the glycan. Both hydrolases would have a very high positive change associated with an area on the enzyme near the catalytic site and just past the binding site of the nonreducing end sugar. This would ensure that they could bind only in a manner that released the nonreducing end unit. In exoglycanase hydrolysis, three to five sugar residues on the reducing side of the catalytic site would have a high aggregate affinity, resulting in a chain of up to six sugar residues being required for optimal rates (85). (The positive and negative signs refer to the change in free energy on



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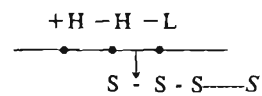
binding, H and L indicate whether the value is high or low, and S represents a monosaccharide unit.) If affinity for the terminal unit were low, rapid release after scission would occur. If this affinity were high, product inhibition and transglycosylation might occur. With a glycosidase (86), there would be high



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affinity for the nonreducing terminal sugar. In glycosidases that hydrolyze disaccharides, there may be a low affinity for binding to the sugar on the reducing side of the catalytic site. Hydrolases with intermediate character would have more affinity for one or more sugars on the reducing side of the catalytic site. There may be a range of types of activity between two extremes. Some enzymes that remove oligosaccharides from exterior chains of branched polysaccharides in an exo manner appear to also have a slower endo action on the resulting limit glycans.

B. Exoglycanases That Release Monosaccharides

1. Exo-1,3-β-D-glucanase (exo-1,3-β-D-glucosidase) (EC 3.2.1.58)

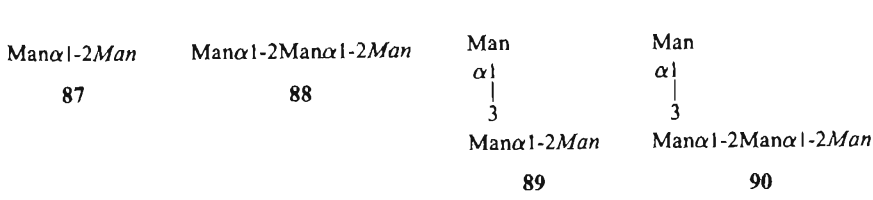
Exo-1,3-β-D-glucanases *(116)* have been purified from a wide range of microorganisms, particularly yeasts, in which they are believed to take part in the cellular processes of expansion and budding *(122)*. Enzymes from different sources vary in their capacity to cleave substrates with (1→3) and (1→6) linkages and periodate-treated laminaran. A basidiomycete exo-1,3-

β-D-glucanase and an activity from *Sclerotinia libertiana* hydrolyzed laminarasaccharides without transglycosylation *(264)*.

Exo-1,3-β-D-glucanases, in combination with other 1,3-β-D-glucan hydrolases, have been used to characterize glucans synthesized by fungi and yeasts (see Section III,C,1). When a polysaccharide from *Sclerotium glucanicum* was hydrolyzed with a basidiomycete species exo-1,3-β-D-glucanase that bypasses (1→6)-β branch points, the ratio of glucose to gentiobiose was constant (2:1) during 10–90% hydrolysis, indicating that the (1→6)-β-D-glucosyl residues were evenly distributed along the (1→3)-β chain, on every third glucose *(130)*. A similar structure was determined for an extracellular glucan from the culture fluid and from sclerotia of *Sclerotium libertiana*. Up to 73% hydrolysis of the former by basidiomycete exoglucanase gave a constant ratio (2:1) of D-glucose and gentiobiose, and only D-glucose was released from the residual polysaccharide after Smith degradation *(265)*. When the alkali-insoluble glucan from fruiting bodies of *Lentinus edodes* was incubated with basidiomycete exo-1,3-β-D-glucanase, the sequence of oligosaccharides released (glucose plus gentibiose and gentiosaccharides of DP > 2) and the occurrence of a resistant residue were interpreted as showing a structure with an outer section of (1→3)-β-D chains substituted (1→6) with some β-D-glucose residues, a middle section of (1→6)-β-D-glucan, and an inner core of highly branched glucan with (1→6)- and (1→3)-β-D linkages. It was suggested that the gentiosaccharides of DP > 2 may have been produced from a separate (1→6)-β-D-glucan by a low level of endo-1,6-β-D-glucanase contaminant *(266)*. The exo-1,3-β-D-glucanase from a basidiomycete species almost completely solubilized yeast cell walls, and it has been applied to the analysis of cell wall carbohydrates and to the formation of protoplasts. Laminaran fractions from zoospores and mycelia of *Phytophthora palmivora (267)* were hydrolyzed by an exo-1,3-β-D-glucanase from *Sporotrichum dimorphosporum* into glucose, gentiobiose, and a trace of laminarabiose. The amount of gentiobiose corresponded closely to the proportion of (1→6) branches in each sample, as found by methylation analysis.

2. Exo-α-D-mannanase (Exo-1,2-1,3-α-D-mannosidase) (EC 3.2.1.77)

Exo-α-D-mannanases differ in specificity. The enzymes from *Flavobacterium dormitator (268)* and *Acinetobacter* sp. hydrolyzed (1→2) linkages, whereas the enzymes from *Arthrobacter (269)* cleaved (1→2) and (1→3) linkages and, much more slowly, (1→6). *Arthrobacter* exo-α-D-mannanase hydrolyzed mannosaccharides **87**–**90**, obtained by acetolysis of *Saccharomyces cerevisiae mannan*, completely to mannose. Tetrasaccharide **90** was



hydrolyzed at about 30% of the rate of **87** and **88**, indicating that the (1 → 3) linkage is less readily hydrolyzed than the (1 → 2) linkage. This tetrasaccharide was fully resistant to hydrolysis by *F. dormitator* and *Acinetobacter* exo-α-D-mannanases. The difference in specificity explains the greater hydrolysis of yeast mannan by the *Arthrobacter* enzyme (40%) than by the *F. dormitator* (30%) and *Acinetobacter* (25%) enzymes. Exhaustive hydrolysis of yeast mannan with *Arthrobacter* exo-α-D-mannanase gave cleavage of all the (1 → 2) and (1 → 3) bonds, leaving a mannoprotein inner core that consisted of (1 → 6)-linked α-D-mannose units, because it could be degraded with an endo-1,6-α-D-mannanase *(232)*. The *Flavobacterium* enzyme inverted configuration on hydrolysis.

3. Exo-1,4-α-D-glucanase (Amyloglucosidase, Glucoamylase, exo-1,4-α-D-glucosidase) (EC 3.2.1.3)

With this enzyme, glucose is released sequentially from the nonreducing ends of (1 → 4)-α-D- and (1 → 4)(1 → 6)-α-D-dextrins and polysaccharides *(116,255,270–272)*, and hydrolysis proceeds with inversion of configuration of the released glucose. Amylopectin is cleaved at three to eight times the rate of maltose and up to twice the rate of maltotriose, and the K_m values in milligrams per milliliter for glycogen, starch, and amylopectin are at least an order of magnitude lower than that for maltose. The initial rates of hydrolysis of maltotetraose, maltopentaose, and maltohexaose are similar to those of amylopectin and soluble starch. Usually (1 → 4) bonds are split more readily. The comparative ease of hydrolysis of (1 → 4) and (1 → 6) linkages varies with the source of the enzyme and other linkages in the vicinity of the hydrolyzed bond. Maltose was hydrolyzed about 80 times as fast as isomaltose, but panose was split at 60 times the rate of isomaltose *(273)*. Reaction of isomaltose, panose, 6^3-α-D-glucosylmaltotriose, and 6^3-α-D-glucosyl-6^3-α-maltotriosylmaltotriose with four exo-1,4-α-D-glucanases also showed the significance of other linkages neighboring the hydrolyzed bond. One of the fractions from *Cladosporium resinae (255,270)* appeared to cleave (1 → 6) linkages as readily as (1 → 4). There was a rapid hydrolysis of pullulan relative to starch compared with other exo-1,4-α-D-glucanases, and this could be related to the much lower K_m value (0.58 mg/ml). 6^3-α-D-Glucosyl-6^3-α-maltotriosylmaltotriose also had a low K_m value.

α-D-Glucosidic linkages other than (1 → 4) and (1 → 6) are cleaved at a

greatly reduced rate. The enzyme from an *Aspergillus niger* preparation *(274)* hydrolyzed the (1→3)-α bond in nigerose at 6.6% and the α-(1-1)-α bond in trehalose at 0.07% the rate of maltose. In another preparation *(273)*, the relative rate with nigerose was 1.1% and that with the enzyme from *C. resinae*, 0.6% *(270)*. Major uses for exo-1,4-α-D-glucanase are in the estimation of starch and glycogen content and the quantitative conversion of starch to glucose. It has been applied to some dextrans, in which it slowly hydrolyzes (1→6) and (1→4)-α-D linkages *(143)*. The dextran from *Leuconostoc mesenteroides* B-1415 was degraded 10%, and subsequent incubation with dextranase liberated isomaltosaccharides without branches. A clinical dextran was degraded 33%.

4. Exo-1,4-α-D-Galacturonanase (Exopolygalacturonase) (EC 3.2.1.67)

This enzyme catalyzes the hydrolytic cleavage of (1→4)-α-D bonds of galacturonosyl residues at the nonreducing terminus of galacturonan chains, releasing D-galacturonic acid *(153,155)*. The rate of hydrolysis by plant and microbial exo-α-D-galacturonanases is either independent of or proportional to the DP. Examination of products by gel and paper chromatography *(155,275)* indicated a multichain mechanism. In general, substrates with ΔUA at the nonreducing terminus are not attacked, but enzymes that are exceptions have been isolated from extracts of an aerobic *Bacillus* species and from *Aspergillus niger* mycelium *(153)*. Modification, by reduction or oxidation of the reducing terminus, of a polymeric substrate does not affect hydrolysis but does affect the rate with oligomers *(276)*. Preparations that preferentially hydrolyze oligomers have been obtained from *A. niger* and a *Bacillus* species. Pectic acid is incompletely degraded *(275)* because substitution restricts hydrolysis. The extent of degradation of pectate from peaches, bast hemp, citrus fruits, carrot roots, orange peel, and bast ramie by an enzyme from carrots ranged from 7 to 56% *(153)*. A preparation from apple cortical tissue released 16% of the uronic acid residues of apple cell wall fractions *(277)*. Exogalacturonanases do not hydrolyze highly methyl-esterified pectin; the enzyme from ripe peaches had no action on citrus pectin that was 70% esterified.

5. Exo-1,6-α-D-glucanase (Glucodextranase, exo-1,6-α-D-glucosidase) (EC 3.2.1.70)

Enzymes releasing D-glucose in an exo manner from dextran have been purified from *Arthrobacter globiformis (278)* and *Streptococcus mitis (26)* and called glucodextranase and dextranglucosidase, respectively. The characteristics of these enzymes differ. On hydrolysis of isomaltosaccharides of DP 2–7 at the same molar concentration, there was an increase in the rate of

hydrolysis with the *A. globiformis* enzyme as the DP increased, whereas with the *S. mitis* enzyme the initial rates of hydrolysis changed only slightly. Also, the former inverted the configuration of the released glucose and the latter retained the configuration. This indicates that the *Arthrobacter* enzyme is an exoglycanase and the *Streptococcus* enzyme is a glycosidase with a high specificity for the penultimate sugar and the position of linkage to it. The latter is discussed further in Section V,D,3. The extent of hydrolysis of dextrans by *Arthrobacter* exo-1,6-α-D-glucanase is inversely related to the type and amount of α-D linkages other than (1→6), according to their location.

6. Phosphorylase (EC 2.4.1.1)

This enzyme catalyzes a reversible reaction, leading in one direction to partial depolymerization of (1→4)(1→6)-α-D-glucans, such as glycogen and amylopectin, by a phosphorolytic mechanism that transfers D-glucosyl units from (1→4) chains to phosphate. Reaction starts at the nonreducing end of a chain and stops six glucosyl units from a (1→6) branch point. Only A chains and the outer section of B chains before a (1→6) linkage can react, and the product is a phosphorylase limit dextrin *(116,131)*. Muscle glycogen phosphorylase is subject to a complex control mechanism *(279)*, but the control of starch phosphorylase in plants is much less elaborate *(280)*. Phosphorylase has been used in a number of studies of starch and glycogen, which involved the preparation of a limit dextrin *(116)*. Highly purified phosphorylases have been obtained from a number of sources, and X-ray crystallographic examination of phosphorylases *a* and *b* have established aspects of the bonding between the enzyme and substrates, the cofactor and effectors *(281)*.

7. Other Exoglycanases Releasing Monosaccharides

Exo-1,3-α-D-glucanase (exo-1,3-α-D-glucosidase, EC 3.2.1.84) from *Aspergillus nidulans (282)* completely depolymerized (1→3)-α-D-glucan from the same source, gave 50% hydrolysis of *Schizophyllum commune* α-D-glucan, but had no action on nigeran or an acid-degraded dextran B-512(F). It released glucose from a water-insoluble dextran from *Streptococcus mutans*, indicating its use in structural studies of dextrans *(143)*.

An exo-1,4-β-D-mannanase from guar seeds *(68)* released mannose from mannosaccharides and the rate of hydrolysis of (1→4)-β-mannopentaose was twice that of the tetraose, which in turn was five times that of trisaccharide. 6^1-α-D-Galactosyl-(1→4)-β-mannobiose and mannotriitol, which were readily hydrolyzed by β-D-mannosidase, were resistant or hydrolyzed very slowly. β-D-Mannan, solubilized by alkali treatment, was a substrate and there was a very slow hydrolysis of insoluble native mannan.

Oligogalacturonoside lyase (EC 4.2.2.6), which cleaves oligosaccharides of α-D-galacturonic acid linked (1→4), eliminating a monosaccharide as 4-deoxy-L-*threo*-hexose-5-ulosonic acid from the reducing end of the oligomer, occurs in cell extracts of *Erwinia* and *Pseudomonas* spp. *(283)*. The optimal substrate is the tetrasaccharide, but α-D-galacturonan is also attacked.

A partially purified enzyme from *Saccharomyces fragilis (284)* hydrolyzes inulin, the D-fructofuranose moiety of raffinose and sucrose, as well as branched levans of high molecular weight. It differs from invertase in showing higher activity with polymers. The enzyme appears to hydrolyze terminal D-fructofuranosyl units from oligosaccharides and linear or branched D-fructofuranans, irrespective of the linkage to the next sugar.

Two exo enzymes, partially purified from Jerusalem artichoke tubers, hydrolyzed (2→1)-β-D-fructofuranosyl oligosaccharides at increasing rates up to a DP of 5 to 6 (enzyme A) or 6 to 8 (enzyme B) *(285)*. A D-glucosyl residue at the other end of the oligosaccharide reduced the rate of enzyme action. Both were virtually inactive against branched bacterial levans, sucrose, and raffinose.

C. Exoglycanases That Release Oligosaccharides

1. Exocellobiohydrolase (1,4-β-D-Glucan Cellobiohydrolase, C_1-Enzyme) (EC 3.2.1.91)

Much of the interest in this enzyme follows from its apparent identity with C_1-enzyme, which acts synergistically with endo-1,4-β-D-glucanase in the degradation of microcrystalline cellulose (see Section III,B,4) *(91,93, 94,285a)*. Acid-swollen cellulose and cellosaccharides are rapidly hydrolyzed, releasing cellobiose with some glucose but no higher oligosaccharides. The DP decreases slowly. Hydrolysis of CM-cellulose ceases after removal of a few unsubstituted residues from the nonreducing end, and the decrease in viscosity is insignificant. The susceptibility of microcrystalline cellulose varies. Reaction is enhanced by β-D-glucosidase and endo-1,4-β-D-glucanase. The former removes cellobiose, which is a competitive inhibitor of cellobiohydrolase. The latter increases the number of nonreducing termini suceptible to cellobiohydrolase attack. Cellobiohydrolase is specific for the (1→4)-β-D linkage. A purified enzyme from *Flavobacterium solani* did not hydrolyze (1→2)-β-, (1→3)-β- (laminaran), or (1→6)-β-D-glucans (lutean). Barley β-D-glucan reacted slightly, but this may have been due to a contaminating lichenase. The cellobiohydrolases from *Trichoderma viride* and *Irpex lacteus (286)* were exceptions to the rule that exoglycanase hydrolysis proceeds with inversion of configuration. However, an exo-1,4-β-D-

glucanase from *Sporotrichum pulverulentum*, which also released glucose, reacted with inversion *(287)*.

2. β-Amylase (EC 3.2.1.2)

β-Amylase hydrolyzes (1→4)-α-D-glucans by sequential removal of maltosyl units from the nonreducing terminus *(116,132,133)*. Unbranched chains with an even-numbered DP are converted to maltose, and those with an odd numbered are converted to maltose plus maltotriose, which is then slowly hydrolyzed to maltose and glucose. In branched (1→4)(1→6)-α-D-glucans, such as glycogen and amylopectin, hydrolysis occurs only in the outer chains and stops two or three glucose units from the (1→6) branch points. β-Amylase has been used extensively, particularly in association with other enzymes, in establishing fine structural details of starch and glycogen (see Chapters 3 and 4 and Section IV). Average chain length can be estimated by debranching, followed by treatment with β-amylase and estimation of the glucose produced from the chains that have an odd-numbered DP (half), and this compared with total glucose content. The complete hydrolysis of material leached at 70°C by water from starch granules indicated that it was unbranched (1→4)-α-D-glucan. The incomplete hydrolysis of fractions leached at higher temperatures and produced by *n*-butanol–thymol fractionation showed that these were not simple unbranched chains *(133)*. The extent of β-amylolysis to maltose and a β-limit dextrin indicates the amount of exterior chains in branched (1→4)(1→6)-α-D-glucans and, used in conjunction with debranching enzymes, initially established the treelike structure, the modification of this to include B chains that are further substituted only by B chains, and the A/B chain ratio *(116,241,247–249)*. The structure of a singly branched pentasaccharide derived from digestion of amylopectin with *Bacillus subtilis* α-amylase was shown to be **35** from its lack of reaction with β-amylase and the production of 2 mol of glucose and 1 mol of panose with exo-1,4-α-D-glucanase *(140)*.

Potato amylopectin contains a small amount of phosphoric ester groups. From the results of debranching with isoamylase and reaction of the (1→4)-α chains with β-amylase, it was proposed that the phosphoric ester groups are located mostly in B chains and distributed over the whole of these chains, except in the vicinity of the (1→6)-α branching linkages *(288)*.

3. Other Amylases Degrading Starch

An amylase from *Pseudomonas stutzeri* (EC 3.2.1.60) *(289)* produced large amounts of maltotetraose from (1→4)-α unbranched and (1→4)(1→6)-α branched D-glucans, and the fourth bond from the nonreducing end of maltohexa- and maltopentaose was hydrolyzed specifically. On incubation, amylopectin and glycogen gave maltotetraose and limit

dextrins. In another study it was found at high concentrations it could further hydrolyze by an endo mechanism. Maltotetraose was still the only initial product, but the final percentage of hydrolysis was 75%, and cross-linked starch, insoluble blue-dyed starch, and the maltotetraose segments of pullulan were cleaved. A third examination of this enzyme *(290)* indicated an exo action in which maltotetraose was released as the α anomer.

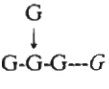
Klebsiella pneumoniae produces an exohydrolase (EC 3.2.1.98) *(291)* that releases maltohexaose from amylose and amylopectin and also acts on β-limit dextrins of amylopectin and glycogen, forming branched oligosaccharides of DP 6–8. It was suggested that the binding site, which normally requires a chain of (1→4)-linked α-D-glucose residues, may have the capacity to accept as substrate a chain in which one of the (1→4) linkages is replaced by a (1→6)-α-D bond. All the products had a (1→4) hexaose structure with a glucosyl or maltosyl substituent linked (1→6) at the third or fourth glucose residue from the reducing end. This enzyme has potential use in the examination of the fine structural detail of glycogens and amylopectin. An amylase from *Bacillus circulans* G-6 released maltohexaose in the α configuration in 30% yield from starch and was considered to be an endo enzyme, but a preparation from *B. circulans* F-2 released maltohexaose, which was believed to come from the nonreducing ends. The product was then split into maltotetraose and maltose *(292)*.

4. Isomaltodextranase (G_2-Dextranase) (EC 3.2.1.94)

Isomaltodextranase, purified from *Arthrobacter globiformis,* hydrolyzed the penultimate linkage from the nonreducing terminus of isomaltosaccharides and dextrans, releasing isomaltose. Activity increased from isomaltotriose to isomaltohexaose *(293)*, as expected for an exoglycanase. However, reaction proceeded with retention of anomeric configuration, and transglycosylation occurred. On hydrolysis of dextran 2000, which consists of all 1→6)-α-D linkages, there was gradual formation and subsequent disappearance of isomaltotetraose. The addition of glucose at the concentration of 3.3% to an isomaltodextranase digest produced isomaltotriose and, in the early stages, formation of this exceeded isomaltose release, indicating a higher capacity of the enzyme to transfer to the primary hydroxyl of glucose than to water. Transglycosylation also occurred on incubation with oligosaccharides of DP 3–6. With very high concentrations of isomaltose, tetraose and traces of isomaltohexaose were formed. Isomaltodextranase can bypass some bonds that are not (1→6), and some of these can be hydrolyzed. The pattern has been studied with oligosaccharides. 2^2-α-D-Glucosyl-, 3^2-α-D-glucosyl-, and 4^2-α-D-glucosylisomaltoses and 6^1-α-D-glucosylkojibiose were not hydrolyzed, but 2-, 3-, and 4-α-isomaltosylglucoses were split to isomaltose and glucose. 3^3-α-D-Glucosylisomaltotriose (**91**) was hydrolyzed

91

as indicated [(1→6)-α-D linkages are shown as hyphens and the other linkages as arrows. 2^2-α-D-Glucosyl-, 3^2-α-D-glucosyl-, and 4^2-α-D-glucosylisomaltose have been identified as products of reaction with dextran *(293,294)*. Even-numbered side chains were completely removed, but odd-numbered side chains left a single glucose unit. A (1→6)-α chain became resistant when the penultimate residue at the nonreducing end was substituted at C-2, C-3, or C-4 as in **92**. Hydrolysis of *Leuconostoc mesenteroides* B-1355-S



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dextran gave isomaltose and 3^2-α-D-glucosylisomaltose in a molar ratio of 5.6 : 1, leaving a limit dextran, and oligosaccharides with a DP of 4 (**93**) and 5 (**94**) appeared transiently *(295)* [the arrows represent (1→3)-α-D bonds].

93 **94**

Methylation analysis of the limit dextran indicated a highly branched structure with an average repeating unit of six α-D-glucosyl residues, consisting mainly of alternate (1→6) and (1→3) linkages. These results, in conjunction with chemical methods applied to the original dextran, indicated that the polymer contained alternating (1→6) and (1→3) bonds and that consecutive (1→3)-linked sugars were absent. In two dextrans, the presence of previously unrecognized (1→3) linkages was detected.

5. Other Exo Enzymes Releasing Oligosaccharides

Exoisomaltotriohydrolase (EC 3.2.1.95) was prepared from culture filtrates of *Brevibacterium fuscum (296)*. Isomaltotriose was released and the specific viscosity of dextran was only slowly reduced, consistent with an exo action.

Exo-D-galacturonan lyase (exopolygalacturonate lyase, EC 4.2.2.9) splits the penultimate (1→4)-α-D-galacturonosyl bond of α-D-galacturonan, starting at the reducing end of the polymer. An unsaturated disaccharide is

produced by elimination *(153,297)*. Trigalacturonate gave a high rate of lysis but lower and similar rates were found for substrates of DP 4, 5, and 6. The enzyme from *Clostridium multifermentans* has a similar reaction rate with substrates of DP 3, 4, and 12. Highly esterified pectin was not attacked. This activity has been reported in other microorganisms. An exolyase that released unsaturated trisaccharide from the reducing end of α-D-galacturonan was purified from *Streptomyces nitroporeus*. It had a higher affinity for pectin 10–30% esterified than for pectate.

Exo-1,4-β-D-Mannobiohydrolase was isolated from the culture fluid of *Aeromonas hydrophila (298)*. The K_m values for mannotri-, mannotetra-, and mannopentaose were 0.51, 0.24, and 0.13 m*M*, respectively. Mannobiose was released from (1→4)-β-D-mannans but not from galactomannans or glucomannans. Transglycosylation occurred. The extent of hydrolysis of coffee and codium β-D-mannans was low (3.6 and 13.8%, respectively), which may have been a consequence of a small amount of galactosyl substitution. An exoarabinanase that releases (1→5)-α-L-arabinofuranotriose units from (1→5)-α-L-arabinan has been isolated from *Erwinia caratovora (298a)*.

D. Glycosidases

1. General Properties and Action Patterns

Glycosidases *(218,261)* remove glycosyl groups from a nonreducing terminus and, in a homoglycan, further incubation leads to sequential removal of similar units. As well as catalyzing hydrolysis (the transfer of the glycosyl unit to water), they also transfer to another glycosyl unit, usually to any hydroxyl, but there is a preference for the primary position *(252)*. The products of transfer reactions are susceptible to further hydrolysis. All glycosidases have an absolute specificity for the anomeric configuration and one enantiomer *(262)*. The specificity for the sugar released varies for different preparations. Some react with only one configuration, but others release sugars with epimeric differences. Drawing conclusions about the specificities of glycosidases is complicated by questions of enzymatic homogeneity. Two β-D-galactosidases from lupin seeds *(45)* had a number of other activities after DEAE-cellulose and gel chromatography but lost most or all of these on concanavalin A–agarose chromatography. In contrast, a β-D-galactosidase from human liver, characterized as a single protein by isoelectric focusing and immunotitration with an antibody prepared to the purified enzyme, also hydrolyzed β-D-glucosides *(299)*. In addition, frequently, selected modifications to substituents, which involve a reduction in size still allow reaction *(51,300)*. Some α-D-galactosidases hydrolyze α-D-fucosides in which the

6-CH_2OH is replaced by $-CH_3$ and β-L-arabinosides in which it is replaced by H. Lectin-binding patterns of two liver glycosidases indicated that one had both β-D-glucosidase and β-D-xylosidase activities and the other β-D-galactosidase, β-D-fucosidase (EC 3.2.1.38), and α-L-arabinosidase *(299)*. However, not all reductions in size of substituents have a minor effect. A β-D-glucosidase from *Aspergillus wentii* hydrolyzed the 4-methylumbelliferyl β-glycoside of 2-deoxy-D-glucose at 4×10^{-6} the rate of the glucoside *(301)*.

Although some glycosidases have a high specificity for a noncarbohydrate aglycone and show little or no action on oligosaccharides *(302)*, a number hydrolyze oligosaccharides. For many of these the position of glycosidic linkage to the penultimate sugar from the nonreducing terminus is not critical *(303)*. Most glycosidases hydrolyze oligosaccharides with (1→2), (1→3), (1→4), and (1→6) linkages but at variable rates. An α-D-glucosidase from sweet corn *(304)* split (1→4)-, (1→3)-, (1→2)-, and (1→6)-α-glucodisaccharides at decreasing rates. However, some preparations are more selective. An enzyme specific for (1→2)-α-D-mannosidic linkages was obtained from *Aspergillus niger*. It hydrolyzed Manα1-2*Man* and Manα1-2Manα1-2*Man* and could not release mannose from yeast mannan, ovalbumin, or fetuin. Another enzyme from the same source hydrolyzed (1→4)-α and (1→6)-α bonds linked to D-mannose or GlcNAc residues *(305)*. Manα1-3*Man* and Manα1-2*Man* were cleaved at approximately 5% of the rate of the 4 isomer. Some have other specific requirements: a 1,2-α-D-mannosidase from rabbit liver microsomes was affected by phospholipids *(306)*. A number of glycosidases also release monosaccharide at significant rates from polysaccharides. Although most microbial α-D-galactosidases as well as plant enzymes hydrolyze melibiose and raffinose, many have little or no action on the α-D-galactosyl residues in galactomannan *(300)*; enzymes from a number of plant sources, including seeds of coffee bean, soybean, alfalfa (lucerne), and guar *(307,308)*, readily release these galactosyl residues. The enzymes from soybean, coffee bean, and fig *(309)* split terminal, nonreducing α-D-galactosyl residues from glycoproteins and glycolipids, but *Mortierella vinacea* α-D-galactosidase did not. The α-D-glucosidase from sweet corn *(304)* hydrolyzed starch and, although the K_m was high relative to reaction with maltose, the V_{max} was also high. Kinetic parameters for maltosaccharides of DP from 2 to 13 were consistent with a glycosidase function.

Some activities reacting with polysaccharides or glycoproteins hydrolyze selectively. Chicken embryos contain an enzyme that released glucose from Glcα1-2Gal-hydroxylysine and basement membrane *(310)* but had no action on maltose and *p*-nitrophenyl α-D-glucoside. Acetylation of the ε-amino group in hydroxylysine greatly reduced the rate. An enzyme activity partly purified from *Saccharomyces cerevisiae* released the terminal (1→2)-

linked α-D-glucose residue from $Glc_3 \cdot Man_9 \cdot GlcNAc_2$ and is probably involved in the processing of the oligosaccharide chains of glycoproteins *(311)*. At least two activities have been recognized: one that cleaves the terminal (1→2)- and another that splits the next two (1→3)-α-D-glucosyl linkages. Further processing to ultimately produce complex chains involves specific α-D-mannosidases *(312)*. Sometimes the variation can be sufficient to make fine distinctions among different structures. A β-D-galactosidase from *Streptococcus pneumoniae (313)* cleaved only a Galβ1-4GlcNAc linkage, but this sequence underwent scission only if the GlcNAc was not substituted with (1→3)-α-Fuc. Jack bean β-D-galactosidase, in contrast, can split all linkage types if used at sufficiently high concentrations *(314)*.

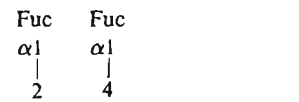
Remote substituent sugars in the primary structure can affect hydrolysis by some glycosidases. The action of jack bean α-D-mannosidase on mannosyl residues at one nonreducing terminus of the carbohydrate chains of bovine rhodopsin was prevented by the presence of a GlcNAc residue at another nonreducing end *(315)*. Removal of the GlcNAc by incubation with *N*-acetyl-β-D-hexosaminidase rendered these α-D-mannosyl residues susceptible to hydrolysis by α-D-mannosidase. The hydrolysis of two terminal, α-L-linked fucose residues from a milk octasaccharide by an α-L-fucosidase from almonds was dependent on their position in the oligomer *(316)*. Only the L-fucose residue closer to the nonreducing end of the oligosaccharide was cleaved. After treatment of the resultant heptasaccharide with a mixture of β-D-galactosidase and *N*-acetyl-β-D-hexosaminidase, the second fucose residue could be hydrolyzed by the almond α-L-fucosidase. Fucα1-2Galβ1-*p*NO_2-phenyl is a substrate for α-L-fucosidase specific for the Fucα1-2Gal linkage. To assay this enzyme, after α-L-fucosidase reaction β-D-galactosidase is added and the released *p*NO_2-phenol is measured *(317)*.

2. Some Applications of Glycosidases to the Determination of Structure and Function of Glycoproteins

Glycosidases can be used to determine the sugar sequence and anomeric nkage in the carbohydrate chains of glycoproteins after digestion with :oteinase or a suitable endohydrolase and separation of the different oligoccharides *(17,218)*. In conjunction with methylation analysis, Smith degdation, and NMR spectroscopy, complete structures can be established. In quence analysis, enzymes should be specific for the hydrolyzed glycosyl ɔup but have the capacity to split linkages to any hydroxyl group on the xt sugar. Bacterial and fungal glycosidases are generally most suitable. ycosidases specific for one sugar and its anomeric linkage but differing in :cificity for the interchain linkages, in particular α-D-mannosidase, are ɔ useful for further characterization.

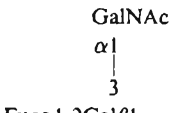
Incubations with α-D-mannosidase and *N*-acetyl-β-D-glucosaminidase established the general sequence of sugars in fractionated oligosaccharides prepared by Pronase digestion of hen ovalbumin *(14,17)*. Reaction with a purified β-D-mannosidase from hen oviduct showed that the first D-mannose residue joined to the GlcNAc-GlcNAc-Asn sequence of ribonuclease B glycopeptide had a β-D-anomeric linkage *(318)*. Glycosidase digestions have contributed to a number of determinations of glycoprotein structures [e.g., IgA immunoglobulin *(319)*] and, in a more recent example that gives details of sequential treatment *(320)*, the structures of two glycopeptide fractions from pulmonary glycoprotein were determined. ^{13}C-NMR spectroscopy has been applied to digests, to follow the kinetics of α-D-mannosidase action on oligosaccharide chains from hen ovalbumin, to facilitate stopping reaction at a particular stage of cleavage, as well as to determine the structures of the residual oligosaccharides *(260)*. The monosaccharide units that significantly contribute to binding between glycopeptide and lentil phytohemagglutinin have been determined by sequential glycosidase hydrolysis of the glycopeptides and examination of residual agglutinin activity *(10)*.

Blood group glycoconjugates, the derived oligosaccharides, and other active oligosaccharides have been modified by glycosidases, defining the relationships between the structure and function of these molecules. Treatment of a milk hexasaccharide (**95**) showing Le^b hemagglutination inhibitor activ-



95

ity with an α-L-fucosidase released the L-fucose residue joined to D-galactose, with loss of Le^b and development of Le^a hemagglutination inhibitor activity. A pentasaccharide prepared from blood group Le^b substance with structure **95** but lacking the glucose residue behaved similarly *(321)*. An *N*-acetyl-α-D-galactosaminidase activity in *Clostridium perfringens (322)* reacted with A-active porcine submaxillary mucin and derived oligosaccharides. Incubation with blood group A erythrocytes caused the loss of A activity due to **96** with simultaneous production of H activity, characteristic of the O blood group type, due to the production of **97**.



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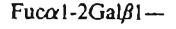

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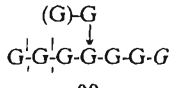
A glycoprotein from potatoes was shown by hydrolysis with α-D-galactosidase to have α-D-galactosyl units linked to serine and, by the use of α-L-arabinofuranosidase, the L-arabinofuranosyl chains with a DP of 4 were found to be terminated with an α-L-linkage, in contrast to the three inner linkages, which were β *(323)*.

3. Some Applications of Glycosidases to the Determination of Structure and Function of Polysaccharides Other Than Glycoproteins

Dextrans are susceptible to hydrolysis by a number of α-D-glucosidases and exo-α-D-glucanases *(143)*. An enzyme from *Cladosporium resinae (324)* specifically cleaved (1→3) but not (1→6) bonds. (1→3)-α-D-Linked residues that occurred as branch points were not hydrolyzed. This enzyme has been used to demonstrate that there are (1→3)-α-D-glucosyl residues at some of the nonreducing termini of soluble dextran B-1355. A dextran that contained 32% of (1→3)-α linkages, synthesized with *Streptococcus mutans* dextransucrase, underwent limited hydrolysis (< 1%) with the *C. resinae* enzyme. The limit dextran that remained could then be hydrolyzed (16%) by isomaltodextranase. Because the latter enzyme can bypass a single (1→3) linkage but could not attack the dextran before treatment with the *C. resinae* enzyme, the result indicated that the dextran contained nonreducing termini with two or more consecutively linked (1→3)-α-D-glucosyl units.

Dextranglucosidase has been purified from *Streptococcus mitis (26)* and some strains of *S. mutans*. It released glucose by hydrolyzing (1→6)-α-D linkages and could not hydrolyze (1→3)- or (1→4)-α-D linkages. Studies of hydrolysis of branched oligosaccharides showed no action on glucosyl residues linked (1→6) to a glucose residue substituted at C-2, C-3, or C-4. A 1→6)-linked glucose residue at the nonreducing end could be hydrolyzed if t were joined to a glucose unit whose linkage to the next sugar was other than 1→6), provided that no branch linkages were involved. Hydrolysis occurred as shown by dashed lines in **98** and **99** [where hyphens represent a



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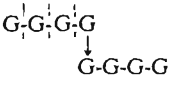


99

→6) linkage and arrows any other α-D linkage]. This pattern of hydrolysis s allowed conclusions to be made about some dextran structures. In dexn B-512, chemical studies had shown that 85% of the side chains conned only one or two glucose residues. Because *Streptococcus mitis* dexnglucosidase gave 22% hydrolysis, it was calculated that the side chains ger than 2 glucose residues would need to have an average chain length 3.

The removal of D-galactose from a number of galactomannans by α-D-galactosidase from the endosperm of lucerne seed *(308,325)* gave a (1→4)-β-D-mannan of high molecular weight, which was essentially devoid of galactose, as is consistent with the accepted formula for galactomannans of a (1→4)-β-D-mannan chain substituted with galactose residues. Examination of a series of galactomannans from which the galactose had been partially removed enzymatically showed that the high viscosity of these glycans is due to the (1→4)-β-D-mannan chain.

α-L-Arabinofuranosidase was isolated from microbial and plant sources, and it hydrolyzed glycosides and exterior arabinofuranosyl linkages in beet arabinan *(326)*, arabinoxylan *(327)*, and plant cell wall and intercellular polysaccharides *(45,328)*. Gum arabic and larch arabinogalactan were not attacked. A number of polysaccharides containing exterior L-arabinofuranosyl units were incubated with α-L-arabinofuranosidase, giving various amounts of hydrolysis. Beet arabinan was incompletely degraded by *Scopolia japonica* α-L-arabinofuranosidase, and this was ascribed to the presence of L-arabinopyranosyl, D-galactosyl, and (1→2)-linked α-L-arabinofuranosyl residues in the polysaccharide *(326)*.

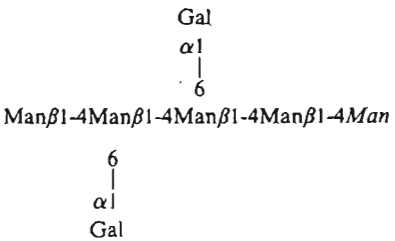
β-D-Galactosidase from lupin cotyledons gave a significant but incomplete hydrolysis of modified lupin cell wall polysaccharide and gum arabic, from both of which most of the L-arabinofuranosyl units had been previously removed by partial, dilute acid hydrolysis *(45)*.

A structure for the linkage region between the polysaccharide and protein of bovine corneal proteokeratan sulfate was established by a procedure that used endo-*N*-acetyl-β-D-glucosaminidase in conjunction with *N*-acetyl-β-D-glucosaminidase, α-L-fucosidase, and β-D-galactosidase *(329)*. Digestion of rooster comb hyaluronate with β-D-glucuronidase and β-*N*-acetylhexosaminidase combined with dialysis gave 99.6% conversion to monosaccharides and oligosaccharides that were transferase products, as is consistent with the whole molecule being unbranched and made up of D-glucuronic acid and GlcNAc, with negligible amounts of other sugars *(330)*.

4. Some Applications of Glycosidases to the Determination of Structures of Oligosaccharides Derived from Polysaccharides (Other Than Glycoproteins)

Heterooligosaccharides released on incubation of konjac glucomannan with endo-β-D-mannanase were characterized with β-D-glucosidase and β-D-mannosidase as having sequences Glc-*Man* and Glc-Man-*Man (181)*. The location of galactosyl groups on a series of galactomannosaccharides, resulting from hydrolysis of galactomannan with β-D-mannanase, were determined with *Helix pomatia* β-D-mannosidase *(69,109,256)*. Both 6^1-α-D-galactosyl-(1→4)-β-D-mannobiose (**14**) and 6^1-α-D-galactosylmannotriose

(**5**) gave 6-α-D-galactosyl-D-mannose and 1 or 2 mol of mannose on reaction. A heptasaccharide produced mannopentaose and 2 mol of D-galactose on α-D-galactosidase treatment. Incubation with β-D-mannosidase released 1 mol of D-mannose and a hexasaccharide, locating one D-galactose group on the second D-mannose from the nonreducing end. Partial hydrolysis with α-D-galactosidase gave a mixture, from which the hexasaccharide fraction was recovered by gel chromatography. Hydrolysis of this with β-D-mannosidase produced 6^3-α-D-galactosylmannotriose and 6^4-α-D-galactosylmannotetraose, identifying the heptasaccharide as 6^3, 6^4-di-α-D-galactosylmannopentaose (**100**).



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Products of hydrolysis of rice straw arabinoxylan with *Aspergillus niger* β-D-xylanase were further examined with a β-D-xylosidase from the same source *(331)*. The oligosaccharide fragments from cellulase *(Trichoderma viride)* hydrolysis of mung bean xyloglucan have been further examined with β-D-glucosidase *(99)*. The characterization of the oligosaccharides produced on hydrolysis of the mixed-linkage (1→3)(1→4)-β-D-xylan from *Rhodymenia palmata* by β-D-xylanase was assisted by the use of a guar β-D-xylosidase *(184)*, and a β-D-galactosidase aided the identification of the oligosaccharides released on hydrolysis of coffee arabinogalactan by β-D-galactanase *(150)*. Oligosaccharides resulting from nitrous acid treatment of heparan sulfate were degraded with either β-D-glucuronidase (EC 3.2.1.31) or α-L-iduronidase, followed by *N*-acetyl-α-D-glucosaminidase (EC 3.2.1.50), demonstrating that GlcNAc was preceded by a nonsulfated uronic acid, which could be either acid, but was followed by D-glucuronic acid and that very few uronic acid residues were sulfated. The GlcNAc in the major fraction was sulfated *(332)*. An octasaccharide, prepared from pig heparin by partial deaminative cleavage, with a high affinity for antithrombin, was shown to have L-iduronic acid at the nonreducing end by hydrolysis to a heptasaccharide with α-L-iduronidase *(332a)*. The structure of a tetrasaccharide isolated by gel chromatography after chemical degradation of bovine corneal keptidokeratan sulfate was proposed from treatment with β-D-galactosidase,

α-D-mannosidase, and α-L-fucosidase in combination with methylation analysis *(333)*.

VI. Anabolic Enzymes

Although polysaccharide depolymerases have made the major contributions in enzymatic studies of the structure and properties of these polymers, information from biosynthetic reactions has also contributed.

A. Action Pattern and Specificity of Glycosyltransferases

The substrates for glycosyltransferases (EC 2.4.-.-) consist of a donor molecule, which may be one of a variety of nucleoside diphospho sugars or sucrose and an acceptor, in the general reactions (6) and (7). The substrate

$$\text{Nucleoside diphospho sugar} + \text{HO-acceptor} \rightarrow \text{sugar-O-acceptor} + \text{nucleoside diphosphate} \quad (6)$$

$$\text{Sucrose} + \text{glucan (or fructan) acceptor} \rightarrow \text{dextran (or fructan, levan)} + \text{fructose (or glucose)} \quad (7)$$

for sialic acid transfer is the nucleoside monophospho sugar. The biosynthesis of polysaccharides is discussed in Chapter 2, and some specific topics receive attention in Chapter 5 and in Chapter 4, Volume 2. With a nucleoside diphospho sugar substrate, the acceptor may be a carbohydrate, certain amino acids in a protein (e.g., serine, threonine, or hydroxylysine), or a lipid. Glycosyltransferases synthesizing homoglycans utilize a saccharide acceptor in which the linkage type is the same as the homoglycan. For significant rates of reaction it must usually contain at least two monosaccharide units, and the rate of synthesis increases with increasing DP.

When the acceptor sugar is at the nonreducing terminus of an unbranched chain, repeated substitution causes elongation, generating an unbranched chain. If substitution occurs on another hydroxyl concurrently with or followed by repeated substitution of the main-chain hydroxyl, then branching occurs. Branching may also result from substitution of a sugar away from the nonreducing terminus. A particular enzyme is specific for the sugar being transferred from the donor, for the sugar being substituted, for the position of linkage on this sugar, and for the new anomeric linkage. The nature of the anomeric linkage of the sugar at the nonreducing terminus of the acceptor and the position of linkage to the penultimate sugar, as well as the identity of this sugar, are critical or affect the rate strongly. Thus, each glycosyltransferase is specific for the linkage synthesized and recognizes the terminal monosaccharide and usually some additional primary or secondary structure in

the acceptor. In particle-bound preparations of *Cryptococcus laurentii,* four separate GDP-mannosyltransferases (EC 2.4.1.48) were differentiated; these catalyzed transfer to form different α-D linkages, Manα1-2Man, (1→6), (1→3), and Man-Xyl, in the biosynthesis of the cell wall *(334).* β-D-Galactoside-2,6-α-sialyltransferase (EC 2.4.99.-) reacted only with the terminal sequence Galβ1-4GlcNAc-, and no incorporation was observed with Galβ1-3GalNAcα1-Thr/Ser or GalNAcα1-Thr/Ser. Asparagine-linked oligosaccharide chains of asialotransferrin with the sequence Galβ1-4GlcNAc- could be either sialylated on the galactose or fucosylated on the GlcNAc but not both. The addition of one sugar blocked the addition of the other *(313).* The only glycoprotein that could act as an acceptor for the galactosylhydroxylysylglucosyltransferase (EC 2.4.1.66) from chicken embryo was collagen *(335).* Separate glycosyltransferases for glycoprotein and lipopolysaccharide in cells are found *(336).*

Monosaccharide transformations nearly always occur before polymerization. Exceptions are the postpolymerization epimerization of D-mannuronic to L-guluronic acid residues in alginic acid *(337),* leading to an unbranched glycan with a nonregular pattern of sugars, and the conversion of D-glucuronic to L-iduronic acid residues in heparin *(338)* and dermatan sulfate. Heparin is an unbranched glycan with a disaccharide repeating unit in which some monomer units of one of the sugars in the disaccharide have been modified. Because oligosaccharides with a nonreducing L-iduronic acid end group were not acceptors in heparin formation, polymerization before epimerization was indicated. Another postpolymerization modification is the conversion of some D-galactose 6-sulfate residues in carrageenan to 3,6-anhydrogalactose *(339),* again giving a masked repeating unit in an unbranched polymer, as is consistent with other structural evidence. Chitosan is formed by deacetylation of chitin *(339a).*

When the acceptor is a protein, as in the addition of the first sugar in the glycan chain of a proteoglycan or glycoprotein, the best molecule is the whole endogenous protein stripped of carbohydrate, followed by peptides produced by partial hydrolysis and then exogenous smaller peptides. With the D-xylosyltransferase involved in chondroitin synthesis, glycine is always found on the C-terminus of the serine acceptor site. The dipeptide Ser-Gly is inactive, but Ser-Gly-Gly reacts with a high K_m *(340).* In the native proteoglycan, although about one-half of the serine residues are not substituted, these will not accept D-xylose, even if the chondroitin chains have been degraded with hyaluronidase. In the synthesis of the GalNAc–amino acid linkage of sheep submaxillary mucin, the only acceptor was the core protein *41).* The D-galactosyltransferase involved in collagen biosynthesis has high specificity for disaccharide-free collagen or glomerular basement protein *42).*

B. Biosynthetic Enzymes and Polysaccharide Structures

The structures of polysaccharides are a consequence of the action pattern of the enzyme or enzymes implicated in their synthesis and the presence of these at the production site.

The specificity of the glycosyltransferases that have nucleoside diphospho sugar substrates for both the anomeric linkage and one position of attachment to the acceptor sugar, combined commonly with the requirement of a primer with a DP of at least 2, means that a single enzyme plus a particular nucleoside diphospho sugar produces an unbranched homoglycan [e.g., amylose *(280)*], and expected structures are consistent with those determined by chemical procedures and enzymatic degradation.

The structures of a number of heteropolysaccharides, in which there is a repeating disaccharide unit, are also consistent with the biosynthetic evidence. The main chain of chondroitin has a repeating unit of D-glucuronic acid and GalNAc residues in an unbranched structure, which is connected to protein via a tetrasaccharide sequence joined to serine. Cell-free preparations of embryonic chicken cartilage transferred radioactively labeled D-glucuronic acid and GlcNAc from the UDP derivatives of these sugars to endogenous acceptors, with the composition and susceptibility to appropriate hydrolytic enzymes of chondroitin sulfate *(343,344)*. Desulfated oligosaccharides served as acceptors. When the nonreducing terminal sugar was D-glucuronic acid, only GalNAc could be transferred and, when the nonreducing terminal sugar was GalNAc, only D-glucuronic acid reacted, in agreement with the structure of a regularly repeating disaccharide unit in the main chain. The monosaccharide sequence in the region of the linkage to protein was shown from the strict sequence of addition of the four sugars with the appropriate glycosyltransferases. There are six glycosidic linkage types in chondroitin, and six separate enzymatic activities have been defined, one for each linkage.

The biosynthesis of an unbranched heteroglycan, $(1\rightarrow4)$-β-D-glucomannan, which has nonregular replacement of D-mannose by D-glucose, has been investigated *(345,346)*. An enzyme preparation from *Phaseolus aureus* contained D-mannosyltransferase (EC 2.4.1.32) and D-glucosyltransferase. With only GDP-mannose, a $(1\rightarrow4)$-β-D-mannan was formed and, with only GDP-glucose, a $(1\rightarrow4)$-β-D-glucan was formed. If both nucleoside diphospho sugars were present, glucomannan was formed. The D-glucosyltransferase optimally required the continual production of D-mannose-containing acceptor molecules, but the D-mannosyltransferase did not require the production of D-glucose-containing acceptors. However, it was severely inhibited by GDP-glucose, and these properties were considered to lead to the synthesis of glucomannans with nonregular replacement by D-glucose instead of two homopolymers.

Some branching occurs when a transferase is specific for a free hydroxyl in a sugar that has already been substituted, and this leads to a regular pattern of branching. The glucosyltransferases that react with sucrose, forming dextrans, may have less specificity for the hydroxyl group being substituted, leading to nonregular structures with variable linkages, or multiple enzymatic activities may be involved. The branching enzymes that form amylopectin, glycogen, and phytoglycogen make a specific (1→6) linkage from preformed (1→4) chains but, because these transfer polymerized (1→4) segments with a range of DP values, they produce a regular linkage pattern with variable chain lengths. The average chain lengths for these three molecules are significantly different *(243,347)*, reflecting different characteristics of the particular branching activity. It has been proposed that branching, which is interchain, requires two (1→4)-α-D chains forming a double-helical structure.

Branching may be affected by conformational aspects of the substrate. A UDP-GlcNAc-transferase from hen oviduct that further substitutes the branched D-mannose residue in biantennary glycoprotein chains, producing "bisecting" GlcNAc, was sensitive to substitution of the two antennas at their termini by GlcNAc residues *(348)*. Extension of branches may also be interdependent. Bovine colostrum *N*-acetyl-β-D-glucosaminide-1,4-β-D-galactosyltransferase, which incorporates D-galactosyl units into **101**, substi-

101

uted the (1→6)-linked GlcNAc first. From kinetic data it was proposed that he new D-galactosyl residue on the (1→6)-linked GlcNAc enhanced the .ceptor properties of the (1→3)-linked GlcNAc, increasing the rate of ubstitution at the latter *(349)*.

In a number of polysaccharides, a regular repeating oligosaccharide se-uence results from another pattern of biosynthesis. An understanding of ıe biosynthetic pathway of the lipopolysaccharides from the cell walls of -am-negative bacteria played a significant part in the elucidation of the ature of their structure. It led to the concept of a regular repeating structure bacterial heteropolysaccharides, in contrast to plant heteropolysaccha-ides, which have a much less regular arrangement. These lipopolysaccha-ides characteristically consist of a core section linked to lipid, and attached this core is the O side chain. In establishing the structures of both these ctions, considerable use was made of mutants lacking an enzyme for their osynthesis *(350)*. These mutants had all the glycosyltransferases required

for polysaccharide synthesis but lacked the full complement of activities for the synthesis of a nucleoside diphospho sugar that was a substrate for the inclusion of the sugar component into the polysaccharide. On addition of the appropriate monosaccharide, synthesis could proceed [e.g., a mutant lacking glucosephosphate isomerase (EC 5.3.1.9)]; if supplied with D-glucose, UDP-glucose could then be produced by this mutant and hence the core structure could be completed. Biosynthesis takes place by the sequential addition of monosaccharides. The addition of sugar requires the presence of an acceptor with the requisite sugar at the nonreducing end, the glycosyltransferase, and the nucleoside diphospho sugar derivative of the monosaccharide being added.

Synthesis of the O chains involves a different mechanism, and the nature of the final structure reflects the pattern of biosynthesis. Again, mutants deficient in an enzyme for making a particular nucleoside diphospho sugar can synthesize complete lipopolysaccharide only if the requisite sugar derivative is added. In contrast to the R core, O side chains have a repeating oligosaccharide structure; the repeating units are presynthesized on a lipid before incorporation into the polysaccharide, and this leads to a regular repeating oligosaccharide unit in the O side chain.

A number of glycoconjugates have specific sequences of sugars in the carbohydrate portion, which is due to the specificity of the glycosyltransferases, although all chains may not be the same length. Elongation of oligosaccharide chains, linked through threonine or serine, proceeds via stepwise addition without prior involvement of lipid *(351)*. In the structures linked through asparagine, synthesis requires the previous construction of an oligosaccharide–lipid, which is then transferred as oligosaccharide to the β-amide of an asparaginyl residue, which occurs in the sequence Asn-AA-Thr, where the indeterminate amino acid can be one of a number. The oligosaccharide can then be modified by the removal of some sugars with glycosidases *(311,352)* and the addition of others by sequential glycosyltransferase action. The chain is in almost all cases terminated when neuraminic acid is added.

In contrast to the regularity of structure of many polysaccharides, there is another pattern found, particularly in plant polysaccharides (e.g., plant gums, galactomannan, and plant cell wall polysaccharides). In these, although the proportion of component sugars from a particular species is relatively constant and there are certain regularities of structure [arabinoxylans, for example, have a (1 $\rightarrow$ 4)-β-D-xylan backbone] the assembly of the whole molecule is less ordered. The synthesis by a particulate fraction from suspension-cultured soybean cells of xyloglucan, which has a (1 $\rightarrow$ 4)-β-D-glucan backbone substituted (1 $\rightarrow$ 6) by α-D-xylose residues, was dependent on the presence of both nucleoside diphospho sugars, as is consistent with concurrent incorporation of both sugars into the polymer *(99)*.

The coexistence of the requisite enzyme and the polysaccharide in the same organ or organelle has been shown in many cases. The endosperms of grains of maize have starch synthase and branching activities *(280,353)*. Multiple branching enzymes are present, one producing the phytoglycogen structure and others amylopectin. A lower concentration of one or more of the latter in the amylose extender mutant than in normal maize produces the less highly branched structure found in this mutant.

Occasionally, enzymes have been used to prepare a sufficient amount of a polysaccharide for it to be isolated and its properties studied. Starch phosphorylase was incubated with α-D-glucosyl phosphate to make a series of (1→4)-α-D-glucan molecules that varied in their DP, and the series was used to establish the relationship between chain length and iodine staining *(354)*, potentiometric titration with iodine, retrogradation, and optical properties *(355)*. The higher molecular weight fractions resembled amylose. A mixture of starch synthase (EC 2.4.1.21) and branching enzyme with ADP-glucose synthesized a (1→4)(1→6)-α-D-glucan that resembled native amylopectin *(356)*.

The second type of enzyme synthesizing polysaccharides has sucrose as the donor molecule and transfers either the D-glucose or D-fructofuranose moiety to an acceptor. When these activities are isolated, the *in vitro* preparations of polysaccharide are similar to the whole-cell products. Dextransucrase (D-glucosyltransferase, EC 2.4.1.5) *(143,357)* transfers D-glucose from sucrose to an acceptor that contains α-D-glucosyl residues, forming (1→6)- and as well (1→3)-, (1→4)-, and (1→2)-α-D linkages. Amylosucrase (EC 2.4.1.4) *(358)* transfers D-glucose to form (1→4)- and possibly (1→6)-α-D linkages. Levansucrase (EC 2.4.1.10) *(359)* transfers D-fructofuranose from sucrose to the 6 and 1 hydroxyl groups of fructofuranosyl units, forming a branched (2→6)(2→1)-β-D-fructan in which the average chain length of the (2→6) chains is about 10–12 D-fructofuranose units. Inulin *(360)* is formed by the transfer of D-fructofuranose, forming a (2→1)-β-D-fructan.

Dextransucrase preparations have been obtained from *Leuconostoc*, *Streptococcus*, and *Lactobacillus* species. The degree of specificity of particular enzymes has not been established with complete certainty. Experimental difficulties arise from the tendency for aggregation between dextransucrase and dextran, the loss of activity on purification, and the possibility of proteolytic modification. One possibility is that the cellular activity is a mixture of enzymes, each specific for one of the hydroxyl groups undergoing substitution. Another is that a single enzyme may have differing affinities for different hydroxyl positions. Both factors may be operating *(361–365a)*. Activities from different sources produce polymers with various proportions of the possible linkages *(357)*, and existing substituents or DP affect the position of further substitution. Another aspect of dextransucrase action is that some radiochemical evidence indicates that, with the enzyme from

Leuconostoc, extension occurs at the reducing end of the growing polymer *(366).* This follows from the absence of released D-[^{14}C]glucose with exo-1,4-α-D-glucanase after a pulse of label and the isolation of D-[^{14}C]glucitol on reduction and hydrolysis. However, the diminished dextran synthesis with dextransucrase from *Leuconostoc mesenteroides* in the presence of exo-1,4-α-D-glucanase was ascribed to competition between the exo-D-glucanase and dextransucrase for D-glucosyl groups, and it was concluded that addition occurred at the nonreducing end of the growing dextran *(367).* Under experimental conditions differing from those employed elsewhere *(366)* the dextransucrase activity from *Streptococcus mutans* added D-glucose to the nonreducing end of isomaltosaccharides of DP ≤ 6 and formed a (1$\rightarrow$3)-α-D branching linkage if the DP was above 6 *(361).*

Levansucrase from *Bacillus subtilis* synthesized a levan, suggesting that it may be specific for the primary hydroxyls with a preference for the 6 position, but the presence of two enzymes with similar physical properties is also possible. *Actinomyces viscosus* produced soluble extracellular and cell wall-associated activities, which were identical in molecular weight, kinetic properties, and antisera reactions, and both synthesized branched levan *(368).*

Inulosucrase, from at least some plant sources, cannot use sucrose as both donor and acceptor. The primary step in inulin biosynthesis requires the enzyme sucrose 1^{F}-fructosyltransferase (EC2.4.1.99), which converts two molecules of sucrose to the trisaccharide ^{F}1-fructofuranosylsucrose and D-glucose. The trisaccharide is the smallest acceptor taking part in the reaction catalyzed by 2,1-β-D-fructan 1^{F}-fructosyltransferase (EC2.4.1.100) *(360),* forming (2$\rightarrow$1)-β-D-fructan. Inulin from some plant sources is a low molecular weight, unbranched molecule, and the requirement for the trisaccharide as the smallest acceptor suggests a more specific transfer, in accord with the unbranched structure of inulin. D-Fructan with only (2$\rightarrow$6)-β-D linkages has been isolated from a *Lolium* species. Three enzymes have been purified from asparagus roots, sucrose 1^{F}-fructosyltransferase 2,1-β-fructan 1^{F}-fructosyltransferase,and 6^{G}-fructosyltransferase *(369).*

Amylosucrase from *Neissera perflava* was freed from maltase (α-D-glucosidase) and phosphorylase, when it synthesized a polysaccharide from sucrose with the properties of glycogen *(358).* The preparation may have contained branching activity, so it is not known whether it was specific only for the formation of (1$\rightarrow$4)- or for (1$\rightarrow$6)-α-D linkages as well.

C. Some Applications of Glycosyltransferases to the Determination of Structure and Function

Glycosyltransferases have restored the function to glycoconjugates from which a terminal sugar or sugars have been removed by glycosidases and also

interconverted glycoconjugates with one activity into one with another, indicating relationships between structure and function in this type of molecule *(351)*. The β-D-galactoside-binding glycoprotein from liver was inactivated on incubation with neuraminidase. Treatment with CMP-*N*-acetylneuraminate–D-galactosyl-glycoprotein sialyltransferase (EC 2.4.99.1) and CMP-*N*-acetyl neuraminate restored the native binding capacity *(370)*. Resialylation stopped complex formation between glycoprotein molecules, allowing them to bind exogenous D-galactosides. Blood group substance H, which has the partial structure **97**, on treatment with L-fucosyl(1→2)-D-galactose-α-3-*N*-acetylgalactosaminyltransferase (EC 2.4.1.40) and UDP-GalNAc gave a product that inhibited the agglutination of type A erythrocytes by anti-A antiserum, as is consistent with the conversion of blood group substance H to A by the addition of GalNAc to the 3-hydroxyl of the penultimate sugar residue from the nonreducing end. This gave the partial oligosaccharide structure **96** *(321)* and indicates the relationship between the H and A structures. The enzyme has been purified from human blood *(371)* and porcine submaxillary glands *(372)* and was absolutely specific for the terminal sequence Fucα1-2Gal-. Sometimes glycosyltransferase reactions have given information about aspects of unknown structures. The D-galactose-binding glycoprotein from rabbit liver was found to have β-D-galactose linked (1→4) to GlcNAc and not (1→3) from reaction with CMP-*N*-acetylneuraminate–D-galactosyl-glycoprotein sialyltransferase and CMP-*N*-acetylneuraminic acid. This enzyme has a marked preference in the acceptor for the sequence Galβ1-4GlcNAc- and does not react with (1→3)- or (1→6)-linked β-D-galactose *(370)*. Human milk D-galactosyltransferase has been used to estimate the average glycan chain length of the soluble non-cross-linked peptidoglycan secreted by *Micrococcus luteus* cells incubated with penicillin. D-Galactose was transferred from UDP-[^{14}C]Galactose to C-4 of GlcNAc residues at the ends of the oligosaccharides. Chain length could then be estimated after digestion with lysozyme *(373)*.

VII. Further Uses

There are additional ways of using the enzymes already described, as well as other enzymes, in studies of the structure and function of polysaccharides.

D-Galactose oxidase (EC 1.1.3.9), which oxidizes the primary hydroxyl groups of D-galactosyl or GalNAc and reacts with nonreducing terminal D-galactosyl units in a polysaccharide, is useful in modification. The aldehyde group that is formed can be either reduced with sodium borotritiide back to a primary alcohol, thus labeling the D-galactose, or further oxidized

with a mild chemical reagent, converting it to D-galacturonic acid. The content of D-enantiomer in the mixture of D- and L-galactose in a hydrolysate of seaweed galactan has been determined by selective oxidation of the D-isomer to *meso*-D-*galacto*-hexodialdose and estimation with ^{1}H-NMR spectroscopy *(374)*. Organization of the surface glycoconjugates in human erythrocytes was analyzed by labeling the D-galactose and GalNAc residues with NaB^3H_4 after prior oxidase treatment *(375)*, and a similar procedure was applied to red blood cell membranes *(376)*. The Gal-Gal-Glc-ceramide from human kidney tissue was shown to have the terminal D-galactose unit in the α-D configuration by converting it to D-[^{3}H]galactose and then detecting labeled D-galactose after hydrolysis with α-D-galactosidase *(377)*. The role of terminal D-galactosyl residues in platelet aggregation was indicated by the removal of this property after oxidation of collagen with D-galactose oxidase *(378)*. The nature of the D-galactosyl linkage in the lipopolysaccharide of *Salmonella typhimurium* was established by oxidation of D-galactosyl to D-galacturonosyl residues with D-galactose oxidase and bromine water before hydrolysis. No D-galactosyl oligosaccharides could be detected on acid hydrolysis of the original polysaccharide due to the lability of the glycosidic linkage, but after oxidation the increased stability of the uronosyl linkage in acid gave an aldobiouronic acid with D-galacturonic acid linked to the 3 position of D-glucose *(379)*. The linkage type of terminal D-galactosyl to GlcNAc could be distinguished in asialoglycopeptides, after oxidation to D-galacturonic acid, from acidic hydrolysis and identification of the aldobiouronic acid released *(380)*.

Enzymes metabolizing carbohydrates can be combined with nonenzymatic processes to define structural aspects. The DP of glucan was determined after reduction and hydrolysis from an estimation of the D-glucitol produced by the reducing end group with D-glucitol dehydrogenase (EC 1.1.1.14), when the reaction was linked to NAD reduction. The average chain length of (1→3)- and (1→4)-linked glycans was determined from enzymatic measurement of the glycerol resulting from a Smith degradation, when it was produced by sugars at the nonreducing termini of the molecule. The glycerol was phosphorylated with glycerol kinase (EC 2.7.1.30), which was then oxidized by glycerolphosphate dehydrogenase (EC 1.1.1.8), and changes were followed spectrophotometrically by the reduction of NAD. Erythritol from a Smith degradation can be estimated by similar types of reactions.

Another use for enzymes that metabolize polysaccharides is the production of oligosaccharides. With the appropriate polysaccharides, the endo enzymes described in Section III provide both homo- and heterooligosaccharides, and some exoglycanases produce oligosaccharides. The mixtures produced by endohydrolases can be separated into individual compounds by chromatography. For example, a series of (1→4)-α-D-galacturonosyl oligo-

saccharides, up to the nonamer, have been separated by chromatography on thick paper or TLC, after incubation of pectic acid from *Chara globate* with endo-D-galacturonanase from *Saccharomyces fragilis (381)*, and also by passing D-galacturonan through a column of the same enzyme linked to agarose and then separating oligomers of DP up to 5 by gel chromatography on polyacrylamide *(382)*. Nigerose has been prepared by treating nigeran with 1,4-α-mycodextranase and separating it from mixed-linkage oligosaccharides of higher DP by charcoal chromatography *(209)*. Yeast endo-1,3-β-D-glucanase, hydrolyzing either laminaran or yeast (1 → 3)-β-D-glucan, gave laminarabiose in 100 times the yield of chemical hydrolysis *(383)*. An alternative to chromatography is the removal of unwanted products with a microorganism. Xylobiose has been prepared from β-D-xylan by incubation with a culture filtrate of *Streptomyces* that contained β-D-xylanase followed by selective removal of D-xylose by a yeast strain *(384)*. Heterooligosaccharides can also be prepared. 6-α-D-Galactosyl (1 → 4)-β-D-mannosaccharides of DP up to 9 have been separated after digestion of carob galactomannan with a β-D-mannanase from *Aspergillus niger* by gel chromatography on polyacrylamide *(109)*. Incubation of guar galactomannan with D-galactose oxidase followed by bromine oxidation made it possible to prepare the aldobiouronic acid GalAα1-6*Man* by acidic hydrolysis due to the high stability of the D-galactopyranosiduronic acid bond *(385)*. If the polymer chain has mixed linkages, high yields of a particular oligosaccharide can often be obtained. Isopanose was isolated in 90% yield after charcoal–celite chromatography of the hydrolysis products of pullulan with isopullulanase from *Aspergillus niger*, and panose was obtained in 70% yield by a similar procedure using *Thermoactinomyces vulgaris* α-amylase *(386)*. 6^3-α-D-Glucosylmaltotriose was produced in about 40% yield by incubation of pullulan with low levels of pullulanase, combined with high levels of β-amylase, when β-amylase removed maltose from the nonreducing end, preventing hydrolysis of the 6-D-glucosyl (1 → 6)-α-D bond *(387)*.

Oligosaccharides can also be synthesized by using the transglycosylation capacity of glycosidases. For example, D-glucose-pentose disaccharides *(388)* and Glcα1-6*GlcNH*$_2$ *(389)* have been prepared by incubation of phenyl-α-D-glucoside with pentoses or of maltose with D-glucosamine and an extract from *Tetrahymena pyriformis*.

Another approach is to use a biosynthetic enzyme. Dextransucrase and levansucrase have given a number of oligosaccharides. Levansucrase from *Arthrobacter levanicum*, on incubation with sucrose and galactose, synthesized Galα1-2βFru*f*, and this enzyme also transferred the D-fructofuranosyl group from sucrose to the C-1 of the free aldose of a variety of sugars to give sucrose analogs. Dextransucrase has also provided a number of oligosaccharides. Leucrose (Glcα1-5*Fru*) and isomaltulose (Glcα1-6*Fru*) as well as dex-

tran, were synthesized on incubation of sucrose with dextransucrase as the concentration of fructose increased *(390)*. Panose and Glcα1-3Glcα1-4*Glc* were formed from maltose *(391)* and Glcα1-1βGal*f* and Glcα1-1βMan from D-galactose and D-mannose *(392)*. Isomaltosaccharides labeled at the nonreducing end *(144)* were prepared with dextransucrase, [^{14}C]sucrose, and excess isomaltosaccharide. Also, isomaltotriose labeled with ^{14}C in the glucose at the reducing end was made by transferring isomaltose from dextran to D-[^{14}C]glucose with *Arthrobacter globiformis* exodextranase (EC 3.2.1.94) *(393)*. This was extended at the nonreducing end with unlabeled sucrose and dextransucrase, giving a series of isomaltosaccharides labeled at the reducing end.

Oligosaccharides related to blood group H substance have been synthesized in 20- to 50-mg amounts using stable, partly purified preparations of bovine *N*-acetyl-D-glucosamine:β-4-D-galactosyltransferase and porcine β-D-galactose:α-2-L-fucosyltransferase *(394)*.

Polymer with an increased average chain length was synthesized from amylopectin and α-D-glucosyl phosphate with potato starch phosphorylase. The reaction with glycogen indicated differences in the access of enzyme and substrate to the nonreducing ends of branches in this molecule. Whereas almost all A chains in amylopectin could be extended, only a few reacted in glycogen *(395)*.

Structural features of chemically synthesized polysaccharides have been examined enzymatically. The (1$\rightarrow$6)-D-glucan formed from 1,6-anhydro-2,3,4-tri-*O*-benzyl-β-D-glucopyranoside was treated with dextranase, which indicated 2% of linkages other than (1$\rightarrow$6)-α-D. The residual portion could be hydrolyzed with exo-1,4-α-D-glucanase and α-D-glucosidase, but no hydrolysis occurred with β-D-glucosidase *(396)*. A mannan synthesized similarly was examined with an exo-α-D-mannanase from *Arthrobacter,* and there were shown to be less than 1% of non-α-D linkages *(397)*.

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