Catalytic Mechanisms of Enzymic Glycosyl Transfer

MICHAEL L. SINNOTT

Department of Chemistry, University of Illinois at Chicago, P.O. Box 4348, Chicago, Illinois 60680

Received March 31, 1990 (Revised Manuscript Received July 12, 1990)

Contents

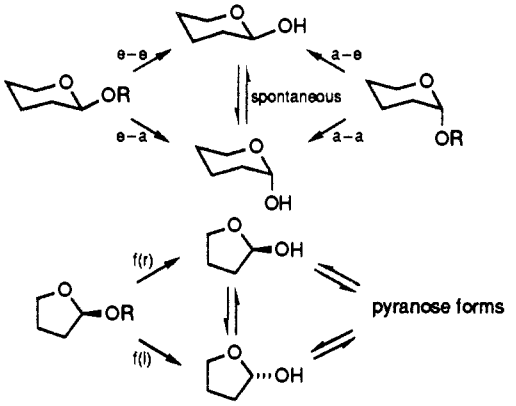
Michael Sinnott was born in Harrogate in 1944 and was raised on Merseyside. He did his undergraduate degree at the University of Oxford, spending the fourth (research) year under the supervision of Gordon Lowe, and then moved to the University of Bristol, where he worked for his Ph.D. under the supervision of Mark C. Whiting. After a postdoctoral year at Stanford, he returned to Bristol in 1969 and stayed there, with the exception of a 6-month study leave with William P. Jencks at Brandeis in 1978, unitl 1989, when he moved to the University of Illinois at Chicago, where he is now Professor of Chemistry.

I. Introduction

At least in terms of simple tonnage, glycosyl transfer must be accounted one of the most important biochemical reactions, since around two-thirds of the carbon in the biosphere exists as carbohydrate (largely cellulose and hemicellulose).[1] The reaction is formally a nucleophilic substitution at the saturated carbon of the anomeric center and can take place with either retention or inversion of the anomeric configuration. We therefore have two basic types of glycosyl-transferring enzymes—"retaining" and "inverting". They can usefully be further subdivided according to whether the sugar is in a five-membered (furanose) or a six-membered (pyranose) ring. In the case of pyranosyl-transferring enzymes it is also useful to distinguish whether, in the preferred conformation, the leaving group is equatorial or axial.[2] In this review the designation of pyranosyl-transferring enzymes as e→e, a→a, e→a, and a→e and of furanosyl-transferring enzymes as f(r) and f(i)[3,4] will therefore be adopted. This is illustrated diagrammatically in Scheme I for hydrolases, where the

0009-2665/90/0790-1171$09.50/0

SCHEME I



situation is complicated by mutarotation of the product.

Enzymes whose physiological function is the transfer of glycosyl residues between two oxygen nucleophiles, a nitrogen and an oxygen nucleophile, and even two nitrogen nucleophiles[5] or a nitrogen and a sulfur residue[6] are known. The natural occurrence of *C*-glycosides (e.g., pseudouridine) also suggests the existence of enzymes that transfer glycosyl residues to carbon nucleophiles.

Transfer of glycosyl groups between oxygen nucleophiles in the physiologically catabolic direction is carried out by three types of enzymes. In addition to glycoside hydrolases (glycosidases), there exist various polysaccharide and oligosaccharide glycosyltransferases which transfer a glycosyl residue from one sugar hydroxyl to another. These enzymes (of which the "levansucrases" and "dextransucrases" of plaque-forming oral bacteria, which convert sucrose into glucans and fructans, are examples) appear very similar mechanistically to the hydrolases. Many of them have weak hydrolase activity, and many retaining hydrolases have transglycosylation activity. The third type of catabolic O–O glycosyltransferase is rather different. The oligo- and polysaccharide phosphorylases catalyze the interconversion of a glycosidic and a glycosyl–phosphate linkage; thus, cellobiose phosphorylase catalyzes the reversible conversion of cellobiose and inorganic phosphate into an equimolar mixture of glucose and α-glucose 1-phosphate.[7] Fairly extensive mechanistic information is available on these three types of enzymes, but little on glycosyl transfer in the anabolic direction (e.g., from nucleotide diphospho sugars to a growing oligosaccharide chain), since the enzymes involved are generally unstable, present in the cell in very low concentrations, and membrane-bound in an ordered way.[8]

Glycoside hydrolases, even though their natural substrates are *O*-glycosides, seem to be remarkably indifferent to the atomic nature of the leaving group: the *lacZ* β-galactosidase of *Escherichia coli*, for example, will accept β-galactopyranosyl fluoride, azide, thiopicrate, and various galactosylpyridinium salts as substrates.[9] Therefore, it is not useful to consider enzymes that transfer glycosyl (for the most part ribofuranosyl or deoxyribofuranosyl) residues between nitrogen and oxygen nucleophiles, or between two nitrogen nucleophiles, separately from enzymes for which both glycosyl donor and acceptor are oxygen-linked.

This review will therefore cover the catalytic mechanism of *O*- and *N*-glycosidases and phosphorylases and related enzymes. The same area was covered in an article in a book published in 1987 which reviewed the literature received in Bristol before March, 1986.[3] The present review updates and amplifies this article and aims at comprehensive coverage of papers covering reaction mechanisms of glycosyl transfer published during the 1980s.

A. Equilibria in Glycosyl Transfer

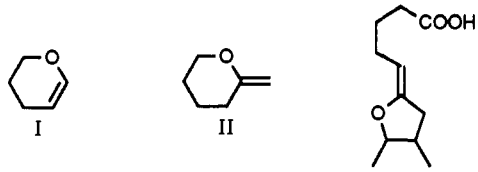
Glycoside phosphorolysis is operationally reversible (equilibrium constants, defined as [glycosyl phosphate][aglycon]/[glycoside][inorganic phosphate], being generally in the range 10^1–10^2),[10,11] whereas glycoside hydrolysis is generally considered to be irreversible. The distinction has, however, no real basis in the intrinsic properties of the molecules concerned and arises because academic studies usually refer to dilute aqueous solutions, and water, at a concentration of 55 M in itself, is a participant in the hydrolytic but not the phosphorolytic equilibrium. Under the conditions of fairly low water concentration encountered, for example, in enzyme reactors used for starch processing, glycoside hydrolysis is significantly reversible.[12]

Accurate values for equilibrium constants for disaccharide hydrolysis are remarkably difficult to find, probably because attempts to establish an equilibrium between a disaccharide and its two component monosaccharides, even using a glycoside hydrolase rather than an acid catalyst, result in the presence not only of the disaccharide of interest but also of many of its isomers. Thus, in a study of the hydrolysis of high concentrations of lactose by the *lacZ* β-galactosidase of *E. coli*, 10 chromatographic peaks corresponding to β-D-galactopyranosyl-D-glucoses and another 10 corresponding to β-D-galactosyl-D-galactoses were detected, and the authors considered it probable that all positional isomers of both disaccharides were being formed and that each transfer product could have the acceptor in the α- or β-anomeric configuration.[13] It appears, however, that these complexities were not explicitly addressed in some recent estimates of the free energy of hydrolysis of disaccharides,[14] the results of which must therefore be treated with reserve. Estimates of free energies of hydrolysis of disaccharides arrived at by combining phosphorolysis results with thermochemical measurements of the glycosyl phosphate hydrolysis are probably still reliable, though.[15] Equilibrium constants on the order of 10^2 M for disaccharides linked through pyranoside linkages, with somewhat higher figures for disaccharides linked through fructofuranoside linkages, seem realistic. The equilibrium constants for hydrolysis of *N*-glycosides are probably in the same region as those for hydrolysis of *O*-glycosides: that for hydrolysis of AMP to ribose 5-phosphate and adenine has been estimated as 170 M.[16]

B. Catalytic Flexibility and Catalytic Feebleness of Glycosidases

Despite the stereochemistry of the catalyzed reaction, with natural substrates being the most important determinant of the catalytic mechanism of glycosyltransferring enzymes, the objections of Hehre, Brewer, and their co-workers[17-23] to a simple "retaining versus inverting" classification have some force. They point out the ability of glycoside hydrolases to stereo-

specifically hydrate carbohydrate enol ethers of the general formulas I and II and the ability of some gly-



I II III

cosidases (largely those that invert the anomeric configuration of natural substances) to hydrolyze glycosyl fluorides of both anomeric configurations. They consider the stereochemistry (α or β) of the product of glycosidase action to be firmly dictated by the structure of the active site, but the protonation machinery to be flexible and, therefore, capable of transforming a range of chemical types of substrate. Thus, the anomeric configuration of the product from hydration of enol ethers always corresponds to that of the product of the action of the enzyme on a natural substrate; likewise, the final product of the action of, for example, glucoamylase on either α- or β-glucosyl fluoride is β-glucopyranose.

To the reviewer, however, it appears that although glycosidases can indeed be catalytically flexible, this occurs only at the cost of most of their efficiency: glycosyl fluorides and enol ethers are reactive compounds, and if they are bound in a microenvironment rich in catalytic functionality, it is to be expected that reactions of some description will occur. For example, simple proximity of an enol ether to a carboxyl function—as in prostacyclin-type structures (III)—can give rise to unimolecular rates of hydrolysis on the order of 1 s^{-1},[24] much the same as k_{cat} values for glycosidase-catalyzed transformations of I and II. Likewise, k_{cat} for the transformation of β-D-glucopyranosyl fluoride to α-D-glucopyranose by the normally retaining α-glucosidase of *Aspergillus niger*[23] can be estimated[25] to be around 10^4-fold bigger than the first-order rate constant for spontaneous hydrolysis of the same compound. Such a rate acceleration is small by comparison with rate enhancements brought about on substrates of the "right" anomeric configuration: yeast α-glucosidase, for example, accelerates the hydrolysis of α-glucosylpyridinium ion by a factor of 10^9, even though it can apply no proton-transfer catalysis to this reaction.[26,27]

C. Relationship between Transferase and Hydrolase Activity

With the traditional classification of glycosyltransferases by the stereochemistry of the catalyzed reaction, adopted here, it is easy to clarify the relationship between transferase activity and hydrolase activity of glycosidases and some glycosyltransferases. Many retaining glycosidases will transfer glycosyl residues to low molecular weight alcohols such as methanol, as well as to water. This activity is never found with inverting glycosidases, since were an inverting glycosidase to have nonspecific transferase activity, then it would have to be anomerically indiscriminate. Consider, for example, the hydrolysis of methyl β-glucopyranoside by an inverting glycosidase, to yield α-D-glucopyranose. If the enzyme had transferase activity to methanol, it would yield α-D-glucopyranoside from methyl β-D-glucopyranoside, and hence, by the principle of microscopic reversibility, it would have to yield methyl β-D-glucopyranoside from methyl α-D-glucopyranoside. Therefore, it would have to accept methyl α- as well as β-D-glucopyranoside as a substrate. Enzymic glucosyl transfer therefore takes place with inversion of the anomeric configuration only when the donor and acceptor are sufficiently different in structure that microscopic reversibility cannot be extended to the system.

Considerations along these lines reveal that an inverting glycoside hydrolase can only synthesize a new glycoside by reversal of the hydrolytic reaction under conditions where it is thermodynamically favored—as with the synthesis of $\alpha(1\rightarrow6)$ linkages by fungal glucoamylase in starch-processing reactors.[12] Retaining glycoside hydrolases, by contrast, work via covalent glycosyl-enzyme intermediates (vide infra): in Cleland's kinetic terminology they are ordered uni-bi reactions. At substrate and acceptor concentrations above the operational K_m values, the transfer reactions of these glycosyl-enzyme intermediates are kinetically controlled, and therefore, all possible transfer products are in principle possible from the action of a retaining glycosidase on saccharides: there may well be the buildup and decay of many transfer products before thermodynamic equilibrium is eventually achieved. At substrate and acceptor concentrations below operational K_m values, the Haldane relationships ensure that rates of forward and reverse fluxes are in the simple ratio suggested by the equilibrium constant: for example, for the β-galactosidase-catalyzed hydrolysis of lactose, if V_f is the maximal velocity in the hydrolytic direction, V_r the maximal velocity in the resynthesis direction, and $K_{i(gal)}$ the inhibition constant for galactose, a Haldane relationship is

$$K_{eq} = V_f K_{i(gal)} K_{m(glc)} / V_r K_{m(lac)}$$

and so new transfer products will not build up to concentrations in excess of their equilibrium concentrations if all species are present at concentrations well below the appropriate K values.[28]

At one time it was thought that inverting glycosidases had an exo action on a polysaccharide chain and retaining glycosidases had an endo action.[29] (Endo enzymes attack the polysaccharide chain in the middle, and exo enzymes, from the nonreducing end.) Empirically, this idea is still a useful guide,[30,31] but retaining exo enzymes[32] have since come to light, as has an inverting disaccharidase (trehalase).[33] In the case of the cellulase complex of *Cellulomonas fimi*, the exo glucanase acted with retention and the endo with inversion.[34]

D. Of Glycosyl Cations and of Stereoelectronics

The nonenzymic chemistry of the glycosidic linkage, especially in water, is dominated by electron release from the lone pairs of the oxygen atom, resulting in departure of the anomeric substituent,[35] and generation of a glycosyl cation. In solvents less polar than water glycosyl cations are too unstable to exist,[36] but in aqueous solution they are just on the border line of a real existence. By analogy with the reaction in free solution, and as a consequence of many probes of enzymic transition-state structure which indicate consid-

erable glycosyl cation character, glycosyl cation intermediates are often drawn for enzymic processes. These intermediates are often considered not to accumulate—they are high-energy intermediates in the traditional physical organic sense. However, it is not meaningful to talk of high-energy glycosyl cation intermediates, which do not accumulate, in enzyme active sites, for the following reason. In the context of the physical organic chemistry of reactions in solution, it is now realized[37] that, once a high-energy intermediate has become so unstable as to have a lifetime shorter than that of an encounter complex, the other components of the encounter complex, and the solvent shell, are necessarily involved in the reaction mechanism. In aqueous solution, the glucosyl cation is so unstable as to be on the border line of a real existence, estimates of its lifetime varying from 10^{-10} s[38] to 10^{-12} s[39]. Any glycosyl cations generated in enzyme active sites would be generated in the vicinity of the catalytic groups, and therefore any reactions supposedly involving them would necessarily be preassociation reactions: the only way a nonaccumulating, high-energy intermediate could be said to exist is if it came off the enzyme surface and became solvent equilibrated, but glucosyl cations are too unstable for that to happen.

The consequences of the direction of the lone pairs on the glycosidic oxygen in space have received much attention, and the idea has received currency that for the aglycon–glycon bond to break, the sugar ring must be a conformation such that this bond is antiperiplanar to an sp^3 lone pair on the ring oxygen atom.[40,41] Indeed, it has been firmly stated that "α-glycosides must hydrolyze via their ground state conformation, whereas β-glycosides must first assume a boat conformation in order to fulfill the stereoelectronic requirement".[40] In fact, in the case of the acid-catalyzed hydrolysis of methyl α- and β-glycosides, precisely the reverse happens,[38] and the antiperiplanar lone pair hypothesis (ALPH) requires quite implausible contortions of the pyranose ring when applied to retaining glycosidases going through covalent intermediates of opposite anomeric configuration to the substrate, since the oxocarbonium ion like transition states must, according to the dictates of ALPH, be generated in either direction from covalent intermediates with an antiperiplanar lone pair.[42] The case has been made that the "theory of stereoelectronic control" in fact represents an overinterpretation of small and elusive least motion effects,[43] and even one of its protagonists now considers that "it is not necessary to take literally the earliest formulation of stereoelectronic theory".[44]

It must be emphasized, however, that the stereoelectronic requirement for planarity of an oxocarbonium ion (and hence presumably of an oxocarbonium-like transition state) is unambiguous. Thus, in the case of furanosyl cations, the conformation of the ring is probably an envelope, with C-1, C-2, C-4, and O-4 coplanar, whereas in the case of pyranosyl cations, the analogy with aldono-δ-lactones (which can adopt half-chair or classical boat conformations in a way that varies with solvent and substitution pattern)[45] suggests that half-chair and classical boat conformations which both enable C-1, C-2, O-5, and C-5 to be coplanar must be considered. The conformations of a ribofuranosyl cation, ^3E (IV) and E_3 (V), and of a glucopyranosyl

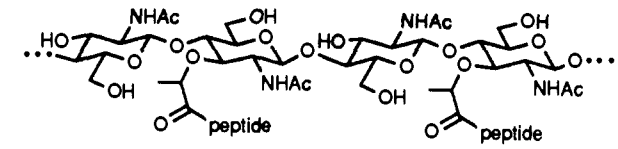
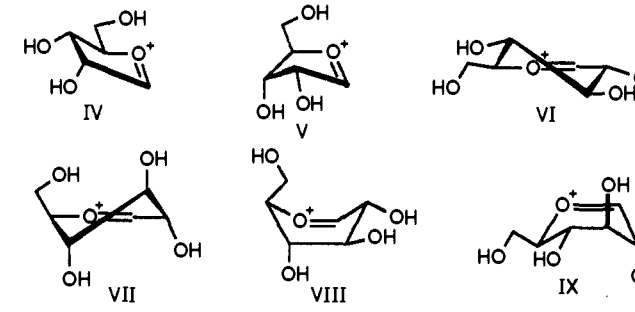


Figure 1. Natural substrate for lysozyme.

cation, $^4H_3$ (VI), $^3H_4$ (VII), $^{2,5}B$ (VIII), and $B_{2,5}$ (IX), permitted by these requirements are illustrated.



II. Enzymic Retention of the Anomeric Configuration

Koshland[46] first pointed out that it was likely that glycoside hydrolases which worked with retention of the anomeric configuration worked via a double-displacement mechanism involving an enzyme nucleophile, whereas inverting glycosidases probably worked by a single chemical step, that of displacement of the aglycon by a nucleophilic water molecule. Thirty-seven years of biochemical investigation have produced no evidence whatsoever against these mechanisms *for hydrolases*; however, in the case of glycogen phosphorylase, determination of the tertiary structure of the rabbit enzyme by X-ray crystallography has failed to reveal any plausible candidate for the enzymic nucleophile required by the double-displacement mechanism, and an "internal return" (S_Ni) mechanism has been proposed (vide infra).[47]

A. Pyranoside Hydrolases with Equatorial Leaving Groups

All available experimental evidence on this class of enzyme is consistent with Koshland's double-displacement mechanism,[46] in which the enzyme nucleophile can now be identified as the side-chain carboxylate group of an aspartate or glutamate residue, which attacks from the opposite (axial) side of the pyranose ring to the leaving group, to give a covalent glycosyl ester intermediate. It appears that most of the rate acceleration comes from various noncovalent interactions, since the enzymes hydrolyze glycosylpyridinium salts with high efficiency, with k_{cat} values 10^9–10^{12} higher than first-order rate constants for their spontaneous hydrolysis in water, in a process characterized by a high degree of oxocarbonium ion character.[3]

Other features of the catalytic mechanism of e→e glycosidases vary from enzyme to enzyme: in particular, the requirement or otherwise for some sort of electrophilic "pull" on the leaving group seems highly variable.

1. Lysozyme, the Paradigmatic e→e Glycosidase

Lysozymes hydrolyze the peptidoglycan component of the cell walls of Gram-positive bacteria, cleaving the MurNAc→GlcNAc bonds (see Figure 1). They will also

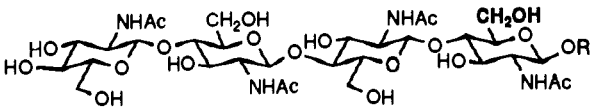


Figure 2. Alkyl chitotetraoside, a good substrate for lysozyme. The hydroxymethyl group whose interaction with the enzyme is important at the transition state is indicated in boldface.

hydrolyze chitin, a simple polymer of $\beta(1\rightarrow4)$-linked *N*-acetylglucosamine residues.

Hens' egg white lysozyme was the first enzyme for which an X-ray crystal structure became available and has become the paradigm for this class of enzyme.[3,44,48] It is small (129 amino acids, about 45 × 30 × 30 Å^3) and in the crystal roughly egg-shaped, with a well-defined cleft on one side, identified as the active site cleft because it bound chitin oligosaccharides. The structure of the lysozyme–[GlcNAc-$\beta(1\rightarrow4)$]$_2$GlcNAc complex, and some model building, suggested that the enzyme had six monosaccharide binding sites, A–F. Between sites D and E were the side chains of Asp 52 and Glu 35, in a position which suggested that Glu 35 would act as a general acid and Asp 52 as a counterion to a glycosyl cation. The X-ray crystal structures of bacteriophage T4 lysozyme[49] and goose egg white lysozyme[50] have recently also become available, and preliminary data have been reported on a fungal lysozyme.[51] Although the sequence homologies of the T4, hen egg white, and goose egg white lysozymes are weak (surprisingly, in the case of the two bird lysozymes), the tertiary structures of T4 and goose egg white lysozyme closely resemble that of the hen egg white enzyme, with Asp 20 and Asp 86, respectively, corresponding to Asp 52 of the hen egg white enzyme, and Glu 73 and Glu 11 corresponding to Glu 35.

The glycosyl-enzyme intermediate in lysozyme action was originally considered to be an ion pair of the glycosyl cation and Asp 52, which lived long enough to allow the saccharide residues in subsites D and E to diffuse away. However, there are no lysozyme substrates known for which hydrolysis of the glycosyl-enzyme intermediate is rate-determining, and so there is no information on the nature of this particular intermediate one way or another, although precedent from similar enzymes suggests it should be covalent.

Steric clashes of the C-5 hydroxymethyl group of the saccharide residue bound in subsite D with the protein were originally thought to distort this residue, but it is now clear that there is no distortion in the ground-state ES complex,[3,52] even though interactions of the hydroxymethyl group with the protein *at the transition state* are an important contributor to catalysis (evidence summarized in ref 3; see also Figure 2).

The long availability of the lysozyme tertiary structure and the smallness of the protein have made it an attractive vehicle for theoretical investigations. This is in a sense unfortunate since the synthetic organic chemical labor involved in testing these conclusions is so great that the comparison with experiment rarely takes place. Thus, Post and Karplus,[53] on the basis of a molecular dynamics simulation and an acceptance as dogma of the antiperiplanar lone pair hypothesis, were led to propose that an acyclic oxocarbonium ion intermediate, stabilized electrostatically by Asp 52, was produced by proton donation from Glu 35 to the ring oxygen atom (Figure 3). Subsequently, it was suggested that the different responses of hen and goose egg white lysozymes to the epoxyalkyl glycosides of chitin oligosaccharides may have their origin in the adoption of two mechanisms by this enzyme.[54] To the reviewer it seems rather that, with such exo affinity labels, the different response of two enzymes which are functionally similar but different in amino acid sequence is exactly what is to be expected.

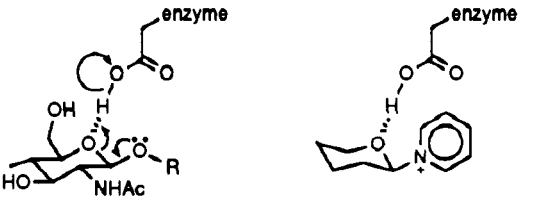


Figure 3. The ring-opening mechanism proposed for lysozyme by Post and Karplus,[53] and its inapplicability to enzymes known to hydrolyze glycosylpyridinium ions.

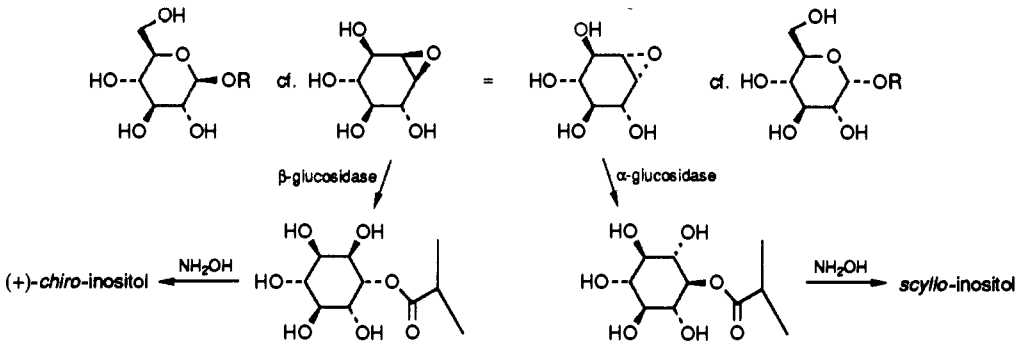
Fleet[55] made a similar proposal in respect of "β-glycosidases" in general. The efficient hydrolysis of glycosyl derivatives in which the leaving group cannot support a positive charge (such as glycosyl fluorides or pyridinium salts)[3] by many β-glycosidases, however, makes the ring opening untenable as a paradigmatic mechanism for these enzymes. Even in the case of lysozyme itself, the ring-opening mechanism is compatible with a *direct* leaving group ^{18}O kinetic isotope effect[56] and a *negative* β_{lg} value[57] only if the decomposition of some hemiacetal, rather than the initial ring opening, is rate-limiting for the truncated, disaccharide substrates on which the effects were measured.

Warshel and others[58–63] examined the effects of the electrostatic fields present in the active site of lysozyme on the energy of heterolysis of the fissile C–O bond and came to the conclusion that the enzyme active site was a supersolvent in which the dipolar transition state of the catalyzed reaction was specifically stabilized by electrostatic fields of the protein. Recently, the (essentially common) electrostatic field at the active site of a series of lysozymes has been calculated classically, and the conclusion[64] reached that the contribution to this field from Asp 52 (or its equivalent) was small and that therefore replacement of this residue by an uncharged asparagine should not destroy enzyme catalytic activity. The site-directed mutagenesis experiments of Kirsch et al.[65] were cited as experimental support of this conclusion.

These authors changed Glu 35 to a glutamine residue and could detect no activity on any substrate: changing Asp 52 to asparagine produced a protein with no activity on defined substrates (such as 4-methylumbelliferyl chitotrioside) but 5% residual activity on suspensions of *Micrococcus luteus* cells, which on prolonged incubation fell to 0.5%. Careful controls were performed to ensure that the mutant protein was not contaminated with wild-type protein arising from spontaneous deamidation. In an equivalent experiment, in which Glu 35 and Asp 52 were individually converted to their amides by chemical means, no enzyme activity could be detected in the modified proteins.[66] Similar chemical modification experiments revealed that Asp 101 (in subsite A) did not contribute significantly to binding or catalysis.[67]

Site-directed mutagenesis experiments have also been performed on human lysozyme. In initial experiments[68]

SCHEME II



it was found that mutation of Tyr 63, which had been thought to contribute to saccharide binding by hydrophobic and hydrogen-bonding interactions, had little effect on activity but that mutation of the catalytic Asp 53 (equivalent to Asp 52 in the hen egg white enzyme) to Glu yielded a protein with no action on chitin but some action on cell walls. Later experiments[69] examined the F subsite: mutation of Arg 115 to lysine had little effect but mutation to glutamine or glutamic acid altered the cleavage pattern. Mutation of Arg 115 to His gave a protein that behaved like the Lys mutant at acid pH values where the imidazole ring was protonated, but like the Glu and Gln mutants where it was deprotonated. These effects are in line with the proposed pattern of hydrogen bonding between the arginine residue and O-1 and O-5 of the sugar ring in subsite F.

Bakthavachalam and Czarnik[70] proposed a novel function for Asp 52 of lysozyme and by extension the carboxylates at the active sites of other glycosyltransferring enzymes. On the basis of a Gaussian 80 ab initio calculation using a 3-21-G basis set, they proposed that the negative charge of the carboxylate raised the energy of one of the oxygen lone pairs and hence the importance of n–σ^* interactions between this lone pair and the fissile C–O bond. This proposal requires that an oxygen atom of Asp 52 in the ES complex with substrate be directly against O-5, rather than C-1, of the saccharide residue being cleaved. As a paradigm, this proposal is directly at variance with elegant work on the stereochemistry of inactivation of α- and β-glucosidases by the D isomer of conduritol B epoxide (see Scheme II).

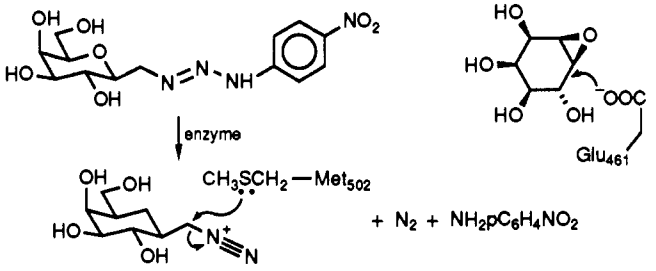
Conduritol B epoxide can be viewed so that it resembles an α- or a β-glucoside and is a mechanism-based inactivator of both types of glucosidase, reacting with the nucleophilic carboxylate group of the enzyme active site equivalent to Asp 52 of hen egg white lysozyme. Removal of the label with hydroxylamine from an inactivated β-glucosidase yields (+)-*chiro*-inositol,[71] whereas removal of the label from an inactivated α-glucosidase yields *scyllo*-inositol,[72] as shown in Scheme II. This demonstrates that the active site carboxylates are poised to attack the epoxide carbon which is equivalent to the C-1, not O-5, of the glycoside substrate. Had the active site carboxylate been poised opposite the substrate oxygen atom, it would have attacked the other epoxy carbon of the inactivator, with the eventual production of *scyllo*-inositol from α-glucosidase and (+)-*chiro*-inositol from β-glucosidase. The experiment with the α-glucosidase is particularly telling, since the enzyme carboxylate attacks the epoxide carbon corresponding to C-1 in a normally disfavored trans-diequatorial epoxide ring opening, whereas if it attacked the epoxide carbon corresponding to O-5, it would accomplish a chemically more favorable trans-diaxial ring opening.

2. *E. coli (lacZ)* β-Galactosidase

The very large amount of basic structural and mechanistic information available on this tetrameric enzyme includes the amino acid sequence of the protein monomer,[73] the base sequence of the *lacZ* gene which codes for it,[74] and identification of an active site methionine (502) by affinity labeling with (β-D-galactopyranosylmethyl)(*p*-nitrophenyl)triazene (below)[9] and of an active site glutamate (461) by affinity labeling with the conduritol C epoxide,[75] also shown below. Despite its early crystallization (reported in 1961),[76] to date its tertiary structure has defied X-ray crystallography (the enzyme has even been sent into orbit: Littke and John[77] reported crystals suitable for X-ray examination grown under conditions of microgravity in 1984). Studies of the secondary structure of the enzyme by infrared spectroscopy indicate that it is 40% β sheet, 35% α helix, 12% random coil, and 13% β turns.[78] The enzyme requires Mg^{2+} for full activity against *O*-glycosides.[9]

The covalent α-D-galactosyl ester of Glu 461[3,9] is undoubtedly an intermediate in the normal catalytic action of the enzyme, as was confirmed by site-directed mutagenesis experiments in which Glu 461 was changed to glutamine.[79] Some residual activity (around 10^{-2} of that of the wild type, the precise figure depending on the substrate used) is nonetheless observed. Careful control experiments, which indicated different K_m values and thermal stabilities for the mutant enzyme activities, were carried out. Such experiments rule out the residual activity arising from homotetrameric wild-type enzyme but leave open the possibility that the activity residues in heterotetramers of formulas such as $(\alpha_{Gln461})_3(\alpha_{Glu461})$. Nonetheless, in combination with results in which the nucleophilic aspartate in various lysozymes was mutated to an asparagine, the case that the nucleophilic carboxylate in the active site of an e→e glycosidase can be mutated to a carboxamide with retention of around 10^{-2} of the activity is compelling. To the reviewer, these results seem fatal to the idea that the nucleophilic carboxylate acts electrostatically but are what would be expected if it were a preassociated nucleophile. Bimolecular nucleophilic displacements on acetal centers (such as the methoxymethyl center)[80] are characterized by β_{nuc} values of 0.1–0.2. The pK of the nucleophilic carboxylate will be unlikely to be

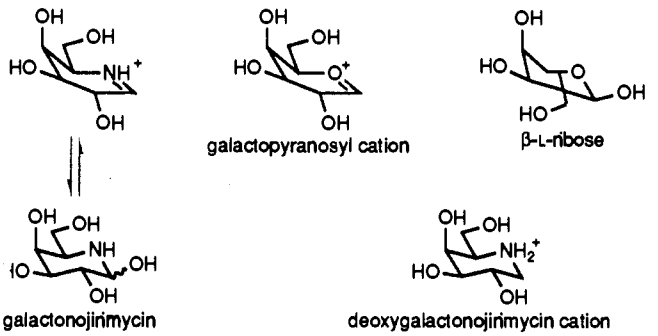
SCHEME III



greatly different from that in lysozyme[81] (~4), and the pK of an amide[82] will be ~−2. The reduction of activity on the basis of decreased nucleophilicity is thus predicted to be around an order of magnitude, as compared with the essentially complete loss of activity predicted by the electrostatic model. The recent results of Cupples et al.[470] provide convincing evidence that Glu 461 is acting as a preassociation nucleophile and that small amounts of activity can still be maintained when a completely different residue acts as such. Glu 461 was replaced with Asp, Gly, Gln, His, and Lys by the techniques of site-directed mutagenesis. In all cases except His very low residual activity was observed (10^{-2}–10^{-3} of wild type), but this was associated with very slow rates of the second chemical step, hydrolysis of the glycosyl-enzyme, and consequently low K_m values (in the micromolar range). The replacement with His reduced the degalactosylation rate by only an order of magnitude, but at alkaline pH values inactivation by substrate occurred. No simple, chemically precedented rationalization of these results was advanced by Cupples et al., and the reviewer now advances one: in the absence of a residue at position 461 that is geometrically and electronically able to act as a preassociation nucleophile, Met 502 so acts, giving a glycosyl sulfonium salt, which chemical precedent suggests should be an unstable species and slowly turn over. The basis for this speculation is that Met 502 is alkylated exclusively by (galactosylmethyl)(*p*-nitrophenyl)triazene; however, in the case of the closely homologous *ebg* enzymes discussed below, the triazene alkylates both the homologue to Glu 461 and the homologue to Met 502, the proportions depending on amino acid substitutions remote from the enzyme active site. The electrophilic site in the (galactosylmethyl)diazonium ion is 1.5 Å from that of the galactosyl cation, and at least in the *ebg* enzymes, it can alkylate both the nucleophilic glutamate and the active site methionine. The supposition that, in the absence of the carboxylate of Glu 461, Met 502 can be glycosylated is then not immediately untenable and provides a simple chemical basis for the behavior of the Glu 461 → His mutant. At low pH the His is protonated and therefore nonnucleophilic, and Met 502 is galactosylated by substrate, whereas at high pH it is now protonated, and the imidazole ring of the histidine is galactosylated. Since glycosylimidazoles are very stable compounds,[338] the galactosylhistidine does not turn over and the enzyme is inactivated by substrate (see Scheme III).

There are three main pieces of evidence that this covalent glycosyl-enzyme intermediate is produced, and goes on to product, via glycosyl cation like transition states. The first is that, where bond breaking can be shown by other criteria to be rate-determining, α-deuterium kinetic isotope effects >1.05 are observed.[9] The effect for hydrolysis of the galactosyl-enzyme is large and direct (k_H/k_D = 1.2–1.25).[9] Direct effects of this magnitude show that the glucosyl-enzyme intermediate is covalent, since there is a decrease in coordination number on going from the intermediate to the transition state for its hydrolysis: were the intermediate ionic there would be an increase in coordination number at the transition state and therefore the α-deuterium kinetic isotope effects would be inverse. The second is the active site directed irreversible inhibition by 2-deoxy-2-fluoro-β-D-galactosyl fluoride.[83] This compound is a representative of a general type of suicide substrate for retaining glycosidases developed by Withers' group: the inductive effect of the 2-fluoro group destabilizes the glycosyl cation like transition states leading to and from the covalent glycosyl-enzyme intermediates, but the good fluoride leaving group makes the 2-fluoro-2-deoxyglycosyl enzymes kinetically accessible, even though, once formed, they turn over very slowly.

CHART I



The third piece of evidence for glycosyl cation like transition states comes from the potent inhibition of *lacZ* β-galactosidase by *galacto*nojirimycin (5-amino-5-deoxy-D-galactose) and deoxy*galacto*nojirimycin[84,85] (see Chart I).

These compounds in aqueous solution can give rise to species that resemble the galactosyl cation like transition state in charge or even in charge and geometry and are therefore probably transition-state analogues. The inhibition of glycosidases by iminoalditol derivatives such as deoxy*galacto*nojirimycin is general, and the mechanism and characteristics of this inhibition are discussed below in connection with a retaining furanosidase (α-L-arabinofuranosidase) whose inhibition is best characterized. In addition to being powerfully inhibited by 1,5-dideoxy-1,5-iminoalditol derivatives, pyranosidases can also be powerfully inhibited by 1,4-dideoxy-1,4-iminoalditols, i.e. apparent analogues of furanose sugars.[86] The obvious rationalization—that the hydroxyl groups in a furanose sugar in fact mimic the hydrogen-bonded contacts of a half-chair glycosyl cation like transition state better than the hydroxyl groups of a full chair pyranose—receives support from the discovery that neutral furanoses can be quite powerful inhibitors of β-galactosidase. L-Ribose, shown above in the β-furanose form, has, for example, a K_i value of 0.21 mM.

Whereas the nucleophilic machinery used by this enzyme is unambiguous, the electrophilic machinery is not. The efficient hydrolysis of galactosylpyridinium ions[9] indicates that electrophilic or protic catalysis is

not of crucial importance. Despite their efficient hydrolysis, C–N cleavage governs both k_{cat} and k_{cat}/K_m for pyridinium salts, as is shown by linear, rather than random, Brønsted plots and significant α-deuterium kinetic isotope effects [$^{\alpha D}(V) = 1.1–1.2$]. Removal of Mg^{2+} from the enzyme has little effect on catalytic parameters for these substrates: by contrast, it slows down the rate of hydrolysis of *O*-glycosides and makes C–O bond fission rate determining;[9] some other process (called a "conformation change" in 1973,[88] when it was first identified) probably governs both first- and second-order rate constants for the hydrolysis of aryl galactosides under conditions of Mg^{2+} saturation. (A complication is that hydrolysis of the glycosyl-enzyme can become rate determining with dinitrophenyl galactoside substrates for both types of enzyme.) At the time of writing, the model that leaves fewest features unexplained is the simple one, that the Mg^{2+} ion is acting as an electrophilic catalyst.[89] With the supplementary hypothesis that the process identified as a "conformation change" in 1973 is in fact the loss of a phenolate leaving group from its first-formed complex with the enzyme-bound Mg^{2+} ion, the proposal rationalizes all the available kinetic evidence, including recent solvent kinetic isotope effect studies. The kinetic mechanism of the enzyme in the absence of Mg^{2+}, or even in its presence with substrates to the aglycon of which the Mg^{2+} cannot coordinate, is that of a simple two-step hydrolase:

$$E + \beta GalX \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} E{\cdot}\beta GalX \xrightarrow{k_{+2}} \alpha Gal{-}E + X \xrightarrow{k_{+3}} E + \beta GalOH$$

whereas the kinetic mechanism of the Mg^{2+}–enzyme with *O*-glycosides is more complex:

$$E{-}Mg + \beta GalOAr \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} E{-}Mg{\cdot}\beta GalOAr \xrightarrow{k_{+2}} \alpha Gal{-}E{-}Mg{-}OAr \xrightarrow{k_{+3}}$$

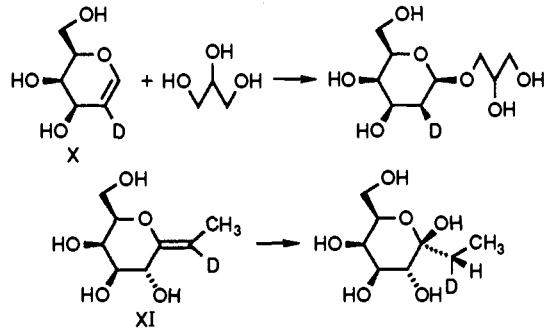
$$\alpha Gal{-}E{-}Mg + ArOH \xrightarrow[(R\ can\ =\ H)]{k_{+4}\ ROH} \alpha Gal{-}Mg{-}OR \xrightarrow{k_{+5}} \beta GalOR + E{-}Mg$$

The mechanism advanced previously[9] identified the k_{+2} step as a conformation change, but subsequent measurements of the leaving group ^{18}O kinetic isotope effects for *p*-nitrophenyl galactoside hydrolyzed by the Mg^{2+}–enzyme[90] [$^{18}(V) = 1.022$ and $^{18}(V/K) = 1.014$] cast doubt on this. Only by the assumption of a virtual transition state for this substrate, comprising the conformation change and the loss of *p*-nitrophenolate without acidic assistance, could the identification be maintained. A proton inventory on V however shows that the solvent isotope effect [$^{D_2O}(V) = 1.8$] arises from the transfer of one proton;[89] the virtual transition state, on which any solvent isotope effect should arise only from a many-proton effect on the conformational component, is thereby excluded. In the mechanism above k_{+3} would be the major contributor to V and k_{+2} to V/K; cleavage of a Mg^{2+}–phenolate bond could account for the ^{18}O kinetic isotope effect since even though the Mg–O bond is weaker than a C–O bond, the proportional change in the reduced mass on ^{18}O substitution is greater, since magnesium is heavier than carbon. The absence of a solvent isotope effect on degalactosylation, which in this model is the attack of a magnesium-bound hydroxide ion on the galactosyl-enzyme, is also accounted for. There are, however, experiments which argue that this enzyme applies Brønsted, rather than Lewis, acid catalysis to its substrates. The finding by Legler and Herrchen[91] that β-D-galactopyranosylamines bound around 10^3 times tighter than otherwise strictly analogous, but nonbasic, compounds, but only in the presence of Mg^{2+}, is compatible with an electrophilic role for this ion, since Mg^{2+} does exhibit some preference for coordination of nitrogen, rather than the isoelectronic oxygen, ligands. The NMR relaxation measurements,[92] which indicated that Mn^{2+} bound too far away from the methyl groups of methyl thiogalactoside and galactosyltrimethylammonium bromide when bound to the enzyme, have, however, to be discounted on the grounds that these inhibitors binding to Mn^{2+}–enzyme are an inaccurate model for substrates binding to Mg^{2+}–enzyme.

The most telling of the experiments indicating protic catalysis are the consequences of replacing Tyr 503 by other residues. Replacement of all the tyrosines in the molecule by fluorotyrosine results in an increase in the k_{cat} value for nitrophenyl galactosides and a shift of the alkaline ionization of the pH–rate profile to lower pH by 1.5 units, in accord with the idea that the alkaline ionization is that of a tyrosine.[93] Specific replacement of Tyr 503 by Phe by the techniques of site-directed mutagenesis[94] results in an enzyme with k_{cat} values for nitrophenyl galactosides around 10^{-3} that of wild-type enzyme and different K_m values and thermal stability. The possibility exists, as with the Glu 461 → Gln mutant, that in fact fully mutated homotetramer is wholly inactive and that existing activity is due to heterotetramers containing one or two wild-type subunits. Even if the fully mutated tetramer has activity, the size of the kinetic effect of eliminating catalysis by proton transfer from a simple phenol ($pK_a \sim 10$) to a nitrophenolate leaving group ($pK_a \sim 7$) is unprecedentedly large.[89] Although this site-directed mutagenesis experiment establishes that Tyr 503 is important for catalysis, it is difficult to see this as a consequence of the elimination of protic catalysis. A clue that the change may not be a consequence of the simple removal of the proton-donating machinery comes from other mutants, in which either Tyr 503 or Glu 461 has been replaced by a range of other amino-acids.[95] Whereas the enzyme isolated from the Tyr 503 and Glu 461 mutants had comparable activities against nitrophenyl galactosides, cells producing the Glu 461 mutants were completely inactive against X-Gal plates, whereas colonies of those producing the Tyr 503 mutants turned blue. The aglycon of X-Gal (5-bromo-4-chloro-3-hydroxyindole) is less acidic than a nitrophenol and therefore its glycosides should be more sensitive to protic catalysis of their fission. Accordingly, were the effect of mutation of Tyr 503 simply the removal of a proton donor, the effect should be seen more on X-Gal than on nitrophenyl galactosides, whereas the reverse is observed.

Experiments with carbohydrate enol ethers show that these reactive compounds can be protonated in the enzyme active site; however, the elegant stereochemical experiments of Lehmann's group show that whereas the endocyclic enol ether (X) is protonated from the α face,[96] the exocyclic enol ether (XI) is protonated from the β face.[97] Galactal (X) is probably hydrated by the mechanism general for the hydration of glycals by re-

taining glycosidases.[3] In the EI complex with this compound, the normally nucleophilic carboxylate group (Glu 461) is in contact with the hydrophobic, nonpolar double bond and its pK_a is raised, enabling it to be protonated at the operating pH of the enzyme. It transfers a proton to the α face of the galactal, generating an ion pair of the 2-deoxyglucosyl cation and the carboxylate of Glu 461, which then collapses to the 2-deoxy-α-D-glucopyranosyl ester of Glu 461, which turns over in the normal way, but more slowly.



The kinetics of the interaction of D-galactal with the enzyme have been studied thoroughly.[98] In the presence of Mg^{2+}, the compound is bound slowly and released slowly. Probably the binding is of a two-step type, like that of other glycals to other e→e glycosidases,[3] the slow step being the hydrolysis of the 2-deoxy-α-D-galactosyl-Glu 461 intermediate, but in this particular case the initial loose EI complex is not detectable at accessible concentrations of galactal. The 2-deoxygalactosyl enzyme then turns over in the normal way. In the absence of Mg^{2+}, the apparent binding is about 10^3 times weaker because the initial addition of a proton is now slow: hysteresis is also observed since the form of the Mg^{2+}-free enzyme first produced by loss of 2-deoxygalactose is apparently much more catalytically active than the normal Mg^{2+}-free form. It is very difficult to see how the correct placing of Tyr 503 on the β face of the molecule could increase the efficiency of proton donation to the α face, whereas if the Mg^{2+} were an electrophile which in the Mg^{2+} enzyme were correctly placed to coordinate to O-1 of the glycoside, a coordinated water molecule could either hydrogen bond to Glu 461 or donate a proton to the exocyclic enol ether from the β face. In the absence of Mg^{2+}, therefore, Glu 461 might be less effective at donating a proton to the galactal, since it is no longer hydrogen-bonded.

3. *E. coli (ebg)* β-Galactosidases

E. coli produces two β-galactosidases: the *ebg* enzyme is coded for by a gene at 66 min on the *E. coli* chromosome, whereas the *lacZ* enzyme maps at 8 min.[99] The *ebg* β-galactosidase contains two subunits, the genes coding for which (*ebgA* and *ebgC*) have been sequenced.[100,101] These genes, together with an *ebgB* gene of unknown function, are part of the *ebg* operon which is under negative control, the repressor protein being coded for by the *ebgR* gene.[102,103] The *ebg* system has been used extensively as a model for the study of acquisitive evolution.[104] *lacZ*-Deleted mutants cannot grow on lactose because the enzymic product of the wild-type β-galactosidase, ebg^o, is too catalytically feeble[105] and the wild-type repressor is too insensitive to β-galactosides.[106] However, if cells with *lacZ* deletions are selected for growth on lactose, certain colonies begin to grow. They do this as a consequence of spontaneous mutations in both the *ebgR* genes and the structural genes coding for the *ebg* β-galactosidase.[107] It was pointed out[104] that these double mutations occurred some 10^8 times more frequently than would be expected if the individual point mutations were to occur independently. The threat presented to the central dogma of evolutionary genetics by these data remained substantially unrecognized until another system, a phospho-β-glucosidase, was discovered which presented a similar conundrum.[108] It has since been suggested[109] that mutations are more likely to occur when the DNA is being unwound as it is transcribed; hence mutations give the appearance of occurring when they are most needed.

Hall[110] demonstrated incisively that in this system the fitness of the organism was directly related to the catalytic competence of a single enzyme, when he showed that a linear relationship existed between k_{cat}/K_m for hydrolysis of lactose by isolated enzyme and the growth rate on lactose of the bacterial strain from which the enzyme was isolated. Measurement of the Michaelis–Menten parameters for 10 substrates for each of 17 isolated *ebg* enzymes enabled the enzymes to be placed in five classes.[111] Single mutational events selected by growth on lactose give rise, 90% of the time, to a class I mutant, of which a typical representative is ebg^a, whereas single mutational events selected by growth on lactulose gave rise to a class II mutant, of which a typical representative is ebg^b; class II mutants are also produced 10% of the time by selection on lactose. Class IV mutants are the consequence of a mutational event of each type; a typical representative is ebg^{ab}. The $ebg^a \rightarrow ebg^a$ change is a consequence of a change of Asp 92 to Asn in the *ebgA* peptide, while the $ebg^a \rightarrow ebg^b$ change is a consequence of a mutation of Trp 988 to Cys in this peptide.[101]

The basic chemistry of the action of the *ebg* enzymes is similar to that of the *lacZ* enzyme,[112] as is reasonable from the 50% nucleotide identity between the *lacZ* and *ebgA* genes;[100] the enzyme appears to require Mg^{2+} for maximal activity on *O*-glycosides.[112] The nucleophilic carboxylate has been identified as that of Glu 408 (homologous with Glu 461 in the *lacZ* enzyme) by affinity labeling with (galactosylmethyl)(*p*-nitrophenyl)-triazene.[113] Improvement in efficiency of the enzyme by spontaneous mutation, as reflected in k_{cat}/K_m for lactose hydrolysis by the purified enzyme, is accompanied by a dramatic decrease (>10-fold) in the rate of hydrolysis of the galactosyl-enzyme intermediate, which becomes kinetically accessible in the case of the ebg^a, ebg^b, and ebg^{ab} enzymes.[114,115] α- and β-secondary deuterium kinetic isotope effects of around 1.1 and 1.0, respectively, have been obtained for hydrolysis of the galactosyl-enzyme intermediate.[115,116] The direct α-deuterium effect, as with the *lacZ* enzyme, establishes that the galactosyl-enzyme intermediate is covalent and that the transition state leading from it has some oxocarbonium ion character. The absence of a β-deuterium effect is not really compatible with the α-D-galactopyranosyl-Glu 408 intermediate reacting through a conformation in which the sugar ring is in the $^4C_1$ conformation (despite this being the conformation in which an oxygen sp^3 lone pair is antiperiplanar to the

breaking C–O bond),[43] since in the $^4C_1$ conformation this bond is also antiperiplanar to the C–D bond, and in any transition state with oxocarbonium ion character, such a geometrical arrangement should give rise to a significant hyperconjugative β-deuterium kinetic isotope effect.

The free energy profile of the catalyzed reaction was determined for *ebg*^{a}, *ebg*^{a}, and *ebg*^{b}.[117] No definite pattern of changes could be detected, in contrast to the suggestion of Albery and Knowles[118] that the easiest evolutionary change to bring about would be a uniform increase in binding of all internal states (intermediates and transition states).

β_{lg} values for aryl galactosides and galactosylpyridinium ions were measured for the wild-type enzyme and the evolvants and provided some indication that C–N cleavage is less advanced in the *ebg*^{a} enzyme than the wild type and that proton donation to the leaving group is more effective in the *ebg*^{b} enzyme. Given the nature of the change in the protein, this can only be through better placement of the acid (or electrophilic Mg^{2+}).[116]

4. Other β-Galactosidases

There has been extensive investigation of mammalian β-galactosidases, since lack of β-galactosidase activity causes a number of well-defined inherited disorders of metabolism. There are four reasonably well-defined activities: two lysosomal or acid enzymes (since the operating pH of the lysosome is acidic, the enzymes contained within it have acid pH optima), a neutral β-galactosidase, and an intestinal, membrane-bound enzyme referred to as "lactase–phlorizin hydrolase". Hereditary deficiency of one lysosomal enzyme results in GM_1 gangliosidosis; that of the other, Krabbe's disease.[119] The two acid β-galactosidases are genetically distinct.[120] (Galactosylmethyl)(*p*-nitrophenyl)triazene specifically inactivates the lysosomal GM_1 β-galactosidase in cultured fibroblasts,[121] and this property has been used to develop a model of GM_1 gangliosidosis.[122] The affinity label can also be used as an active site titrant and hence used to estimate the activity per protein molecule in a mild form of GM_1 gangliosidosis. The lysosomal GM_1 β-galactosidase exists as a complex of β-galactosidase, neuraminidase, and a protein that protects the complex against hydrolysis by lysosomal proteinases.[123-127] One form of combined neuraminidase and β-galactosidase deficiency was traced to a defect in the protector protein.[128]

The "lactase–phlorizin hydrolase" of mammalian intestine is responsible for the initial splitting of lactose in the diet into glucose and galactose in adults; its deficiency in humans gives rise to lactose intolerance.[129] The gene coding for it is located on the human chromosome 2.[130] The cDNA coding for the human and the rabbit enzyme has been cloned and sequenced.[131] The enzyme protein has a membrane anchor and two independent active sites, one of which splits lactose and another of which splits a wide range of β-glucosides (including the natural glucoside phlorizin). Surprisingly, both active sites are apparently labeled with conduritol B epoxide. It has been suggested that the enzyme arose by gene duplication,[131] implying that β-glucosidase and β-galactosidase activities can evolve from a common precursor.

5. β-Glucosidases

From the point of view of the bioorganic chemist, β-glucosidase is the simplest of the e→e glycosidases, yet well-defined enzymes are not readily come by. The material from sweet almonds, which is the commonest commercial source, is in fact a mixture of at least two isoenzymic forms with different kinetic properties.[132] (The occurrence of multiple molecular forms of glycosidases, the pattern of which varies with age and stage of development of the plant, is a common feature of plant biochemistry[133] which makes plant enzymes unsuitable in general for investigations of mechanism.) In fact, despite physical–organic studies of the action of sweet almond β-glucosidase going back to 1954,[134] the most incisive experiments have been done with well-characterized bacterial and fungal enzymes.

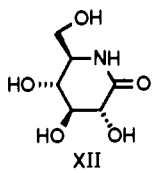
Thus, the definitive experiments showing that conduritol B epoxide labeled the same aspartate residue[135] as was alkylated by D-glucal[136] and that the 2-deoxyglucosyl enzyme generated from D-glucal was identical with the 2-deoxyglucosyl enzyme generated by the hydrolysis of *p*-nitrophenyl 2-deoxy-β-D-glucopyranoside[137] were carried out on the crystalline[138] β-glucosidase A_3 of *Aspergillis wentii*. This same enzyme was also subjected to a physical–organic study which indicated that the catalytic proton was almost completely transferred at the rate-determining transition state for aryl glucosides [$\beta_{lg}(V) \approx -0.05$ with $^{\alpha D}(V) \approx 1.1$] and that glucosyl cation like transition states were involved.[139] This enzyme binds nojirimycin and 1-deoxynojirimycin tightly ($K_i \sim 10^{-6}$ M).[140]

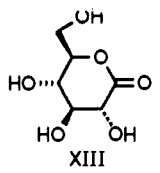
Direct evidence for the existence of a covalent, α-glucosyl-enzyme intermediate was obtained by Withers' group[141] with the enzyme from the bacterium *Alcaligenes faecalis*,[142] for which enzyme the gene had been cloned and expressed in *E. coli*.[143] Incubation with 2-deoxy-2-fluoro-β-D-glucosyl fluoride liberated 0.93 mol of fluoride ion and inactivated the enzyme. The ^{19}F NMR spectrum of the reaction mixture revealed a protein-bound fluoride resonance (δ 197.3) attributable to the 2-fluoro group of the glycosyl-enzyme intermediate (cf. the 2-fluoro group of the inactivator itself at δ 203.4). The chemical shift of the fluoro group of 2-deoxy-2-fluoroglucosyl derivatives is apparently not sensitive to the anomeric configuration at the adjacent carbon, but that of 2-deoxy-2-fluoromannosides is, and this particular β-glucosidase will (unusually) accept β-mannosides. With 2-fluoro-2-deoxy-β-mannopyranosyl fluoride as inactivator, the chemical shift of the bound 2-deoxy-2-fluoromannosyl residue (δ 201.0) is shifted only slightly upfield on denaturation of the labeled enzyme (to δ 202.6): the chemical shift of the ^{19}F nucleus of 2-deoxy-2-fluoromannose is δ 206.2 in the α-anomer and δ 224.5 in the β-anomer. Direct evidence of the catalytic competence of the 2-fluoro-2-deoxyglucosyl enzyme has recently been obtained:[485] in the presence of the acceptor β-D-glucopyranosylbenzene, the enzyme is relatively rapidly reactivated, with production of a (2-fluoro-2-deoxy-β-D-glucopyranosyl)-β-D-glucopyranosylbenzene, most likely the 4-isomer.

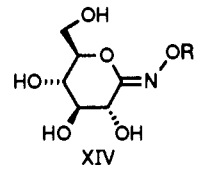
Despite its somewhat ill-defined nature, useful mechanistic data have been obtained for almond β-glucosidase. The stereochemistry of hydration of D-glucal to 2-deoxy-β-D-glucopyranose has been defined as an overall trans addition of H–OH.[144] The same

group showed that the sweet almond β-glucosidase catalyzed hydration of 2,6-anhydro-1-deoxy-D-*gluco*-hept-1-enitol (i.e., the gluco version of structure II) yielded 1-*C*-methyl-β-D-glucopyranose.[145] The enzyme is covalently inactivated by conduritol B epoxide, the group labeled in the case of the bitter almond enzyme being an aspartate.[3,4] Dale et al.[146] concluded that the sweet almond enzyme apparently operates without the application of acid catalysis to the departure of phenolate leaving groups: the hydrolysis of glucosides of phenols with $pK_a > \sim 8$ gave a value of $\beta_{lg}(V/K)$ of −1.0 and a solvent deuterium kinetic isotope effect of 1.0, whereas the rate-determining step in the hydrolysis of glucosides of phenols of $pK_a < \sim 8$ was the diffusion-controlled encounter of enyzyme and substrate. This conclusion accords with a value of $^{18}(V/K)$ of 1.036 for the hydrolysis of *p*-nitrophenyl glucoside 1-^{18}O,[56] which is too large for much O–H bond formation at the transition state. Unfortunately, as the data stand, they refer to different enzymes, the isotope effect applying to the purified A isoenzyme and the β_{lg} value to material supplied by Sigma, identified as the B isoenzyme on the basis of the chromatographic and electrophoretic properties of its major component. *Purified* sweet almond β-glucosidase B however gives a value of $\beta_{lg}(V/K)$ of −0.73 ± 0.09,[115,147] and the ratio of activities for nitrophenyl galactosides for this enzyme does not correspond to that reported by Dale et al.[146] It therefore seems not impossible that the enzyme used by Dale et al. may be the A, not the B, isoenzyme: indeed, the Sigma enzyme is identified as A in a previous paper from the same group.[148] This paper describes the inhibition by 1,5-dideoxy-1,5-imino-D-glucitol and D-glucopiperidinolactam (XII) as a function of pH; the affinity ($1/K_i$) for the lactam is sigmoid (decreasing at high pH according to an enzyme ionization of pK 6.8), whereas the affinity for the imine shows a bell-shaped dependence, with both acid and basic ionizations being governed by a pK of 6.8, the pK_a of the inhibitor as well as of the enzyme. The lactam is presumably a transition-state analogue by virtue of its half-chair conformation, whereas the imine will be a transition-state analogue by virtue of its charge when any acid catalytic group is deprotonated. As with *lacZ* β-galactosidase, an exact mimicry of the configuration of the substrate does not seem to be necessary for tight binding of an iminoalditol inhibitor: 1,5-dideoxy-1,5-imino-D-mannitol[140] and 1,4-dideoxyimino-D-glucitol[149] bind reasonably tightly ($K_i \sim 10^{-5}$–10^{-4} M at optimum pH). The lactone (XIII) behaves like a typical transition-state analogue, in that an initially formed loose complex slowly isomerizes into a tighter one.[150] There is a report that 5-amino-5-deoxy-L-glucose [(−)-nojirimycin] inhibits sweet almond β-glucosidase more powerfully than the correct isomer.[151] Another puzzle presented by this enzyme is that, whereas it rapidly hydrolyzes both diastereoisomeric glucosides of the general formula β-D-Glc*p*-OCH(OMe)$(CH_2)_n$X when X = N_3, the compounds with X = NH_2 are almost completely inert to the enzyme.[152]

Interestingly, gluconolactone oximes are tight-binding inhibitors to "emulsin",[153] probably because the conjugation of the ring oxygen lone pairs with the C=N bond makes the pyranose ring adopt the half-chair conformation, while at the same time the oxime nitrogen atom


XII


XIII


XIV

is able to hydrogen bond to any catalytic proton donor.

The β-glucosidase from the fungus *Stachybotrys atra* is reasonably well characterized and is important as one of the few retaining glycosidases for which the glycosyl-enzyme intermediate is kinetically accessible.[154,155] α-Deuterium kinetic isotope effects have been measured for the hydrolysis of the glucosyl-enzyme and found to be direct[156] [$^{\alpha D}(V) = 1.11 \pm 0.01_5$ for four substrates], in accord with this glucosyl-enzyme intermediate being covalent. Alkyl thioglycosides (and alkyl xylosides) are mixed inhibitors, the uncompetitive component of the inhibition arising from binding of the hydrophobic aglycon to the hyrophobic aglycon-binding site of the glucosyl-enzyme intermediate.[181]

Similar data exist for the β-glucosidase of *Botryodiplodia theobromae* Pat, where hydrolysis of the glucosyl-enzyme is rate-determining for *p*-nitrophenyl glucoside; for this substrate being hydrolyzed values of $^{\alpha D}(V)$ of 1.09 are observed, but when glycerol is the acceptor, this value increases to 1.21.[157] The increase in the value of $^{\alpha D}(V)$ when glycerol is the acceptor can be accounted for by a scheme involving reaction through intimate and solvent-separated ion-pair intermediates in the enzyme active site.

Although mammals are not a rich source of β-glucosidase, the medical interest in them (Gaucher's disease is an inherited deficiency of lysosomal β-glucosidase which results in accumulation of glucosylceramide in the tissues)[119] has led to a very considerable body of data on mammalian enzymes, some of which is of mechanistic significance. There are two fairly well-defined enzyme activities, lysosomal, or acid, and cytosolic, or neutral.

The human lysosomal enzyme is fairly nonspecific with respect to the leaving group and is inactivated by conduritol B epoxide;[158] this property has been used to measure turnover of the enzyme in cell culture (rat peritoneal macrophages and human fibroblasts) by following the recovery of enzyme activity after inactivation by the expoxide.[159] The lysosomal β-glucosidase of cultured human fibroblasts is inactivated in vivo by (β-D-glucopyranosylmethyl)(*p*-nitrophenyl)triazene.[121] Conduritol B epoxide has been used as an active site titrant, which can give the number of active sites in a cell and hence characterize the defective enzyme in a form of Gaucher's disease.[160] A cDNA clone for the enzyme has been isolated.[161] The biosynthesis of the enzyme involves glycosylation of a 52-kDa precursor protein.[162] The rat liver enzyme is activated by gangliosides,[163] in accord with its natural substrate being amphipathic. In vivo it acts in association with an activator protein, the primary structure of which has been determined.[164]

The nonspecific enzyme from human spleen, probably the cytosolic enzyme, like sweet almond β-glucosidase, is indifferent to the glucose substituent at C-4 and C-5, and so will hydrolyze β-galactosides, β-xylosides, β-D-fucosides, and α-L-arabinopyranosides;[165] the pig kidney enzyme is similar.[166,167] The guinea pig cytosolic

enzyme with 4-methylumbelliferyl glucoside as a substrate is activated by low concentrations of alcohols but inhibited by higher concentrations.[168] To the reviewer this appears as behavior typical of a glycosidase hydrolyzing a good substrate, for which the second chemical step, hydrolysis of the glycosyl enzyme, is rate-determining. A comparison of the human cytosolic and lysosomal β-glucosidases revealed that the cytosolic enzyme bound long-chain alkyl glucosides about 100-fold more tightly than the lysosomal enzyme.[169] The calf liver cytosolic enzyme[170] is inhibited powerfully by compounds of the deoxynojirimycin type (including bridged examples such as castanospermine); its aglycon-binding site is considered to be a narrow hydrophobic cleft. It is inactivated by bromoconduritol B epoxide and bromoconduritol F.[171]

6. Other e→e Glycosidases

Observations of mechanistic significance have been made on other enzymes of this type, but generally somewhat incidentally to other concerns. Mammalian enzymes, particularly hexosaminidase, have been studied for medical reasons. The ubiquity of the β-gluco and β-xylo linkage in structural materials of the plant kingdom has led to enzymes cleaving these linkages receiving attention from the viewpoint of plant pathology and that of biotechnological biomass conversion.[172] The enzymes which attack crystalline cellulose will be covered in a separate section (IV), since before any question of catalytic mechanism can be addressed, it is necessary to identify which enzyme from which organism is doing what, and this is rarely straightforward.

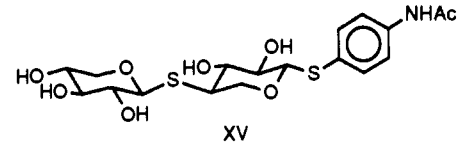
Lysosomal hexosaminidase is composed of two subunits, α and β, the genes for which are located on chromosomes 5 and 15 in humans.[173] There are two physiological forms of the enzyme, both of which are dimers: A (αβ) and B (ββ); the A enzyme is the more specific. The biological function of the enzyme is to hydrolyze terminal *N*-acetylglucosamine and *N*-acetylgalactosamine residues. A defect in the α gene results in loss of hexosaminidase A activity (Tay-Sachs disease), whereas a defect in the β gene results in loss of both hexosaminidase A and hexosaminidase B. It has been suggested that the α subunit hydrolyzes the glycoside 6-sulfates, whereas the β-subunit hydrolyzes the neutral sugars.[174] The adult-onset of Tay-Sachs disease in Ashkenazic Jewish sufferers has recently been identified as a consequence of a Gly → Ser change in the α subunit.[175]

The primary structure of another hexosaminidase, the *Streptomyces* enzyme, *endo*-β-*N*-acetylglucosaminidase H, has been determined.[176]

A retaining β-xylosidase has been purified from culture filtrates of *Penicillium wortmanni* QM7322;[177] the V_{max} values for most aryl xylosides were approximately the same,[178] and the expectation that this is because the hydrolysis of the xylosyl-enzyme intermediate is rate-determining with these substrates has been confirmed.[179] The enzyme is rapidly inactivated by (β-D-xylopyranosylmethyl)aryltriazenes, the rate of inactivation exhibiting a negative β_{lg} value when correlated with the pK_a of the aniline.[180] More recently, four isoenzymes of different kinetic[182] and immunological[183] properties have been isolated from the culture filtrates of a different isolate of this fungus (IFO 7237); the relationship between these enzymes and the enzyme on which the mechanistic studies were made is not clear.

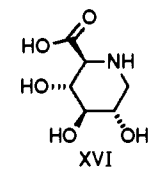
Michaelis–Menten parameters for a series of substituted phenyl β-D-xylopyranosides hydrolyzed by the β-xylosidases of *Trichoderma viride*[184] (now *Trichoderma reesei*) and *Emericella nidulans*[185] give $\beta_{lg}(V)$ values of around −0.1 and −0.2, respectively.

The sequence of the gene coding for a retaining extracellular, retaining[186] β-xylosidase (xylanase) from the soil bacterium *Bacillus pumilus* IPO has been determined,[187] and preliminary X-ray data have been reported.[188] Unfortunately, no bioorganic investigations have been carried out with this well-characterized β-xylosidase, nor with the xylanase from *Trichoderma harzianum*, on which initial X-ray crystallographic measurements have been made.[189] The nucleotide sequence of the gene coding for a xylanase (*xynZ*) in *Clostridium thermocellum* is also known.[190] The enzyme from the fungus *Sporotrichum dimorphosporum* has however been shown to be *activated* by the noncleavable thioglycoside (XV).[191]

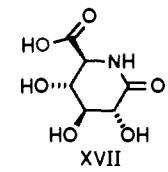


XV

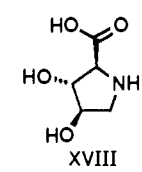
β-D-Glucuronopyranosylpyridinium ion has been shown to be a reasonably good substrate for the β-glucuronidases of *E. coli* and the snail *Helix pomatia*,[192] indicating that acid catalysis is unlikely to be of crucial importance with this enzyme. The cDNA of the *E. coli*, human placental, and rat liver enzymes has been cloned and shown to exhibit strong homology (the rat enzyme shows 77% homology with the human enzyme and 47% with the bacterial).[193] The pattern of inhibition by glucuronic acid derivatives with nitrogen in the ring is informative: The simple six-membered ring analogue (XVI) is in fact a more potent inhibitor of human liver lysosomal α-L-iduronidase than of human lysosomal β-glucuronidase[194] (K_i = 80 μM) and a less potent inhibitor than the lactam (XVII).[195] As was seen with *lacZ* β-galactosidase, in terms of these compounds being transition-state analogues, the five-membered ring compounds appear to resemble the putative half-chair conformation of the transition state rather better than fully saturated six-membered ring compounds, so that (3*R*,4*R*)-dihydroxyproline (XVIII) has a K_i of around 9 μM (against the bovine lysosomal enzyme).[196]



XVI



XVII

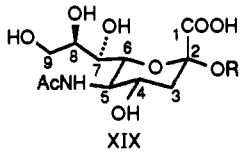


XVIII

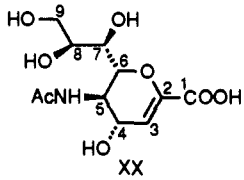
B. Sialidases and Related Enzymes

Retaining glycosidases invariably have a carboxylate group which can function as a nucleophile in the enzyme active site. In the case of the hydrolysis of sialic acids, e.g., glycosides of *N*-acetylneuraminic acid (XIX), the possibility arises that the carboxylate group which is present in the substrate, rather than an aspartate or

glutamate residue on the enzyme, may function as the nucleophile, and so these enzymes are best considered separately from other e→e enzymes. Since the acetal center in a neuraminide does not possess an anomeric hydrogen atom, determination of the initial products of action of sialidases by the usual ^1H NMR methods is not possible and is made the more difficult by the lability of most neuraminides to spontaneous decomposition. Nonetheless, by using the azide as a substrate, it has been possible to show that the *Clostridium perfringens* and *Arthrobacter ureafaciens* enzymes work with retention of configuration.[197] The gene coding for the sialidase of *C. perfringens* has been cloned and sequenced.[198]



XIX



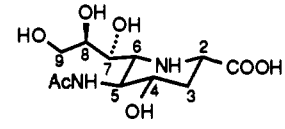
XX

It is unfortunate that workers in different disciplines have concentrated on different aspects of neuraminidase chemistry and used enzymes from different sources, with the result that a coherent picture of the action of a single protein is not available, even though there is some evidence that the action patterns of various microbial sialidases are similar.[199]

The molecular biologists have worked on the enzyme from influenza virus: their primary interest seems to have been in the immunological lability of the virus, which makes immunization against influenza so short-lived. An X-ray crystal structure of the enzyme from a type A virus at 2.9-Å resolution was published in preliminary form in the early 1980s.[200-202] The enzyme is a tetramer of 60-kDa subunit molecular weight: it is an integral membrane protein, with an N-terminal stalk on each monomer serving to anchor the enzyme in the membrane. The tetrameric structure has circular 4-fold symmetry, probably stabilized by metal ions on the symmetry axes (the enzyme is activated by Ca^{2+} ions). Each monomer has a box-shaped head (100 × 100 × 60 Å) and has six topologically identical β sheets in a propeller formation. The active site is located distally from the hydrophobic tail and is rich in charged functionality. These charged residues are confirmed in the many immunological variants of the enzyme isolated from this source. The enzyme from a type B influenza strain has recently been crystallized and examined by X-ray crystallography.[203]

A catalytic mechanism for influenza neuraminidase has been proposed on the basis of site-directed mutagenesis experiments and the X-ray crystal structure,[204] although in the opinion of the reviewer it has a number of features that lack chemical precedent. On binding of substrate, a proton is considered to be transferred from His 274 to Glu 276, which then acts as a general-acid catalyst, in the same way as Glu 35 in lysozyme. A glycosyl cation, stabilized by an enzyme carboxylate tentatively identified as Glu 277, is then formed and reacts with water in the microscopic reversal of the previous steps. As the mechanism was drawn, Glu 277 was shown as being on the same side of the sugar ring as the leaving group, therefore presumably leaving open the possibility of the substrate carboxylate acting as a nucleophile.

The bioorganic studies have for the most part used bacterial enzymes, since the neuraminidase activity of a pathogen can correlate with its virulence,[205] and therefore there is a pharmacological interest in designing neuraminidase inhibitors. The commercially available enzyme from the cholera agent, *Vibrio cholerae*, has been most studied in this connection. Thus, it was shown 20 years ago that conversion of the carboxylate group to a primary alcohol or even the isoelectronic amide resulted in compounds which were not cleaved by the enzyme;[206] neither were compounds in which the carboxylic acid group had been amidated with glycine, glutamic acid, or phenylalanine.[207] The 2,3-dehydro compound (XX) is a tight-binding inhibitor with a K_i on the order of 1 μM,[208] as is its 8-epimer,[209] whereas the 4-epimer[208] and the 7-epimer[209] are bound with K_i values on the order of 1 mM. Compound XX is a glycal, and by analogy with the interaction of other retaining glycosidases with the corresponding glycals, its hydration to *N*-acetylneuraminic acid is expected. The situation with the *V. cholerae* sialidase is not clear, but the enzyme from *Arthrobacter sialophilus* hydrates compound XX to *N*-acetylneuraminic acid at about 10^{-5} the rate at which it hydrolyzes glycosidic bonds and also hydrates the methyl ester and 4-epimer of glycal XX.[458] In accord with the importance of the stereochemistry at C-4 for the *V. cholerae* enzyme, the benzyl glycoside of 4-epi-NeuNAc is cleaved only slowly by this enzyme, even though it is cleaved at about the same rate as the neuraminide proper by the sialidases of *C. perfringens*, *A. ureafaciens*, fowl pest virus, and bovine testis.[210] An extended study of the *V. cholerae* enzyme with 4-methylumbelliferyl glycosides revealed that the 7-epi, 8-epi, 7,8-bis-epi, 7-deoxy, 8-deoxy, and 9-deoxy constitution of the glycon had no dramatic effect on Michaelis–Menten parameters (range of k_{cat}/K_m values about an order of magnitude) but that the 4,7-dideoxy glycoside was a very poor substrate.[211] In contrast to the tight binding of the enol ether type inhibitors, iminoalditol inhibitors (e.g., XXI) give only moderate inhibition.[212]

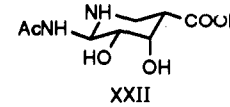


$K = 0.12$ mM

XXI

The neuraminidase from the oral bacterium *Streptococcus sanguis* can act on 4-, 7-, 8-, or 9-O-acetylated sialyl residues, unlike the *V. cholerae* enzyme.[213]

A *Streptomyces* culture was found to yield siastatin B (XXII), which is in fact a better inhibitor of sialidase than the compound above by about an order of magnitude.[214] Oddly, the enantiomer of the structure shown proved to be quite a powerful inhibitor of glucuronidase.



XXII

In addition to sialic acids, other ketosidic linkages α-substituted with a carboxylic acid are known (e.g., KDO), and enzymes cleaving these linkages have been characterized,[215] but no mechanistic work has yet been reported.

C. Pyranoside Hydrolases and Transferases with Axial Leaving Groups

All available mechanistic evidence for this class of enzyme is consistent with a general mechanism involving a covalent glycosyl-enzyme intermediate whose chemical nature is the β-glycosyl ester of an aspartate or glutamate side chain of the enzyme protein. The mechanistic picture is similar to that for e→e enzymes, and as with these enzymes, protein tertiary structures are available only for catenases (α-amylases from a fungal and a mammalian source), which have several monosaccharide binding sites. One therefore has the same problem in integrating mechanistic data into an overall picture, in that dynamic information is available for the interaction of small ligands with large, monosaccharide hydrolases, whereas protein structural information is available for small enzymes interacting with large substrates. There is evidence that a "super-secondary" structure is common to several α-(1→4) glucan hydrolases.[216]

1. α-Amylases

The nomenclature of the enzymes that hydrolyze starch can be confusing to the nonspecialist. The term "α-amylase" arises from the substrate (amylose, a linear, unbranched α(1→4) polymer of glucopyranose units) and the anomeric configuration of the products (α). The term "β-amylase" is applied analogously to enzymes that hydrolyze amylose with inversion of the anomeric configuration. "Glucoamylase" or "amyloglucosidase" is an inverting enzyme with a strictly exo action; i.e., it cleaves a single monosaccharide unit off the nonreducing end of a linear α(1→4) glucopyranose polymer. "Limit dextrinase" is an enzyme that cleaves the α(1→6) linkages in amylopectin; it is also called "pullulanase" since it hydrolyzes pullulan [a polysaccharide with the structure $[[Glcp\alpha(1\rightarrow4)]_2Glcp\alpha(1\rightarrow6)]_n$].[217] The structural elements for substrate cleavage required by a number of starch-possessing enzymes have been examined by synthesis of substrates in which one hydroxyl group at a time has been formally removed.[218]

A major practical problem in studying the action of α-amylase is that this endo-enzyme will not cleave aryl glycoside linkages efficiently, so that it is difficult to get a signal that can be continuously monitored. A fluorophoric substrate—a *p*-nitrophenyl α-maltopentaoside in which the 6-OH of the nonreducing terminal glucose residue has been replaced by the 2-pyridylamino function—solves this problem at the cost of considerable synthetic organic chemical labor.[219] The fluorescence of the aminopyridyl function is intramolecularly quenched until one of the internal glycosidic bonds is cleaved, to yield *p*-nitrophenyl glucoside or maltoside. The extended substrate is necessary to obtain readily interpretable kinetic data; experiments with maltose, maltotriose, and *p*-nitrophenyl maltoside indicate that two molecules of these substrates bind to the active site of the porcine pancreatic enzyme.[220] In accord with this idea, it has been found that chemical modification, or inhibition with a protein α-amylase inhibitor, of the porcine pancreatic enzyme can alter the relative amylase and maltosidase activity of the enzyme.[221]

The tertiary structure of an α-amylase produced by the fungus *A. wentii* (taka-amylase), and of its maltose complex, was determined by Matsuura et al.[222] Careful treatment of data produced from the hydrolysis and transglycosylation reactions of maltotriose[223] suggested that the enzyme had six and possibly seven individual monosaccharide binding sites, and that an amylose chain was cleaved between subsites 4 and 5. Model building of a maltose chain into the active site cleft appeared to confirm this. Between sugar residues 4 and 5 lay the side chains of Asp 297 and Glu 233. These were suggested to be equivalent to Asp 52 and Glu 35 of lysozyme, viz., nucleophilic carboxylate and general-acid catalyst, respectively, but in the discussion of this enzyme, both these supposedly catalytic groups were drawn on the α face of the substrate, whereas a residue not mentioned in the text, Asp 206, was drawn on the β face of the sugar residue 4, apparently in the correct position to act as a nucleophile.

A somewhat higher resolution structure of the porcine pancreatic α-amylase has since been published by a French group.[224] The protein molecule consists of three domains: A, an $(\alpha\beta)_8$ barrel (so-called TIM barrel, first recognized in triosephosphate isomerase), B, a less defined structure, and C, an eight-stranded antiparallel β barrel. This structure has been predicted to be common to several α-glucan cleaving enzymes.[216] The necessary Ca^{2+} ion has two ligands on each of the A and B domains, such that when the Ca^{2+} is removed, the two domains may be able to move with respect to each other. The catalytic residues in this enzyme were identified as Asp 197 and Asp 300. The Asp 300 corresponded to the Asp 297 in taka-amylase, but the Glu 233 residue in the pig enzyme (corresponding to Glu 230 in taka-amylase) was rejected as a catalytic group on the grounds that in a number of α-amylase sequences it was only poorly conserved. (The nucleotide sequences of the genes coding for α-amylases from a large number of sources are now becoming available: see refs 225 and 226 and references therein). These workers' candidate for an acid-catalytic group was Asp 197. This identification rationalized the loss of activity on removing calcium, since this group, on a mobile domain, would move away from the active site.

In an important paper, a covalent β-glycosyl-enzyme intermediate was identified by cryoenzymology of porcine pancreatic α-amylase and ^{13}C NMR (chemical shift data).[227] Noncovalent interactions with the C-6 hydroxy group do not appear to be important at any of the monosaccharide subsites in this enzyme, since its replacement with both hydrogen and fluorine is tolerated.[228]

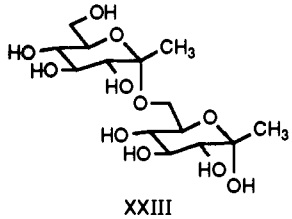
A new technique of random mutagenesis has been used to delineate the catalytically and structurally important regions of the α-amylase of *Bacillus stearothermophilus*.[484] A library of single and multiple base mutations was generated, and mutant strains were characterized by their ability to hydrolyze starch. A three-dimensional model of the *B. stearothermophilus* enzyme was constructed by sequence alignment with taka-amylase A, whose structure was known. With this procedure, it was found that those mutations which affected catalytic activity were particularly clustered around the active site and also at the interface between the central α/β barrel and the C-terminal domain.

A study has been made[229,230] of the modes of action of human salivary α-amylase, either native or expressed

in yeast,[231] using phenyl maltotetraoside and pentaoside substrates in which the hydroxymethyl group of the nonreducing terminal glucose moiety was replaced with COOH, CH_2NH_2, or CH_2I. A model of subsite structure was advanced in which there were six subsites, designated S_3, S_2, S_1, $S_{1'}$, $S_{2'}$, and $S_{3'}$: S_3 was hydrophobic, and cleavage occurred between S_1 and $S_{1'}$. The report that the human salivary enzyme had an action pattern similar to the pancreatic enzyme[232] is not readily reconcilable with the mechanism for that enzyme espoused by the X-ray crystallographers.

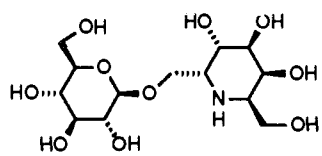
2. α-Glucosidases

The purified enzymes from rice and *Candida tropicalis* enzyme, convert the gluco exocyclic enol ether of type II to 1-*C*-methyl-α-D-glucopyranose,[145] as well as to transfer products such as compound XXIII.[234] The *C. tropicalis* enzyme hydrates D-glucal to 2-deoxy-2-deuterio-α-D-mannopyranose in D_2O.[144]

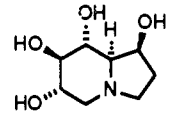


XXIII

The processing of the α-glucosyl residues of asparagine-linked carbohydrate moieties of glycoproteins is carried out by two enzymes of the endoplasmic reticulum, glucosidase I and glucosidase II, which hydrolyze the α(1→2)- and α(1→3)-linked glucosyl residues, respectively, of the initially formed oligosaccharyl-asparagine.[235] (These enzymes are different from the lysosomal α-glucosidase.) An interesting observation has been made of the relative sensitivity of glucosidases I and II to two inhibitors of the iminoalditol type: the monosaccharide analogue castanospermine (XXV), a natural product, is more potent against glucosidase I, but the disaccharide analogue XXIV, in which the methylene spacer is designed to mimic the breaking bond,[236] is more potent against glucosidase II.[237] Glucosidase I is powerfully inhibited by deoxynojirimycin and its *N*-methyl analogue.[247]

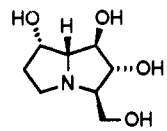


XXIV



XXV

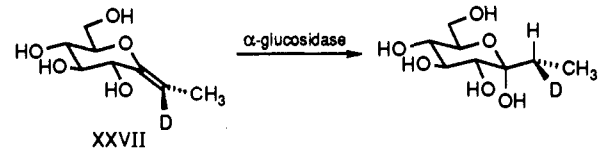
More pronounced differences in inhibition were obtained with the five-membered ring analogue australine (XXVI), which powerfully inhibited glucosidase I but had little or no activity against glucosidase II.[238]



XXVI

The lysosomal, but not the neutral, human liver α-glucosidase is powerfully inhibited by nojirimycin.[239] The sequence of the lysosomal enzyme has been determined:[240] in accord with the similarity of the catalyzed reaction, it shows significant homology with the sequence of the intestinal sucrase–isomaltase complex. It is therefore unsurprising that the enzyme is competitively inhibited by acarbose.[241]

The inactivation of simple α-glucosidases by conduritol B expoxide is slow, probably reflecting the disfavored trans-diequatorial epoxide ring opening that the process has to achieve.[242,243] In a thorough investigation, α-glucosidases from a wide range of sources (*Aspergillus niger*, pig serum, rice, buckwheat, and sugar beet seeds) were found to hydrate D-glucal in D_2O to give 2-deoxy-2-deuterio-α-D-mannopyranose by an overall trans addition.[21] In contrast to the situation with *lacZ* β-galactosidase, the proton for the hydration of the exocyclic enol ether (XXVII) by the rice and *A. niger* enzymes comes from the same side as that for hydration of the glycal, as shown.[22]



XXVII

The same direction of approach of the proton to these exocyclic enol ethers in the case of an e→e and an a→a enzyme, despite the requirements for proton donation to glycosides being opposite to each other, supports the contention that the transformation of these compounds by glycosidases is a consequence of their intrinsically high reactivity and the essentially accidental encounter with proton donors in the enzyme active site.

The *A. niger* enzyme catalyzes the hydrolysis of β-D-glucopyranosyl fluoride to form α-glucopyranose.[23] This transformation is remarkable, even though the rate acceleration of 10^4 is fairly modest (vide supra); careful controls were performed to show that the observed catalysis was not due to a contaminating β-glucosidase or glucoamylase activity.

Most mechanistic data are available for the yeast enzyme. It is powerfully inhibited by iminoalditol inhibitors of the five-membered ring type: thus K_i for 1,4-dideoxy-1,4-imino-D-arabinitol at optimum pH is 0.18 μM[86] whereas that for deoxynojirimycin is 33 μM.[27] Interestingly, it is not inhibited by castanospermine.[244] A kinetic study with aryl α-glucopyranosides and α-D-glucopyranosylpyridinium ions (which in their ground state are in a skew-boat conformation, probably $^1S_3$), indicated that bond breaking limited only V for the pyridinium ions; V/K for both sets of substrates, and V for aryl glucosides, was limited by a noncovalent process. The chemical transition state for the pyridinium ions involves a high degree of oxocarbonium ion character, as shown by high α- and β-deuterium kinetic isotope effects, the β effects also indicating that the C-2–D bond was approximately antiperiplanar to the cleaving C–N bond.[27] A model was advanced for the action of the enzyme in which the reactive conformation of the pyranose ring when bound to this enzyme was $^{2,5}B$. In addition to the kinetic evidence, this model can explain the lack of inhibition by castanospermine and the reluctance of this particular enzyme to hydrate glucal,[4] since neither of these ligands can readily adopt such a classical boat conformation. Incorporation of an

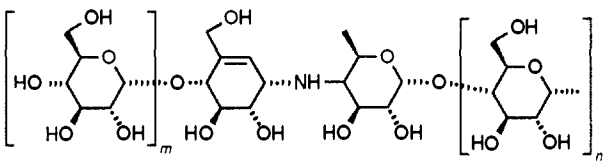
amino function into the leaving group of 1-alkoxyalkyl α-D-glucopyranosides, in such compounds as αGlcp-$OCH(OMe)CH_2NH_2$, renders them inert to α-glucosidase-catalyzed hydrolysis.[245] An approach to enzyme-activated irreversible inhibition, whose success is rather surprising, was used with this enzyme as a vehicle. 2-Chloro-1,1,2-trifluoroethyl α-D-glucopyranoside is an active site directed irreversible inhibitor;[246] presumably the enzyme hydrolyzes it to 2-chloro-1,1,2-trifluoroethanol, which then decomposes to chlorofluoroacetyl fluoride and acylates the enzyme active site at a rate competitive with loss of the acyl fluoride from the active site and random acylation of the protein.

3. Sucrase–Isomaltase

In the mammalian gut, there exist a number of membrane-associated glycoside hydrolases associated with the absorption of dietary carbohydrate. The best characterized of these is the sucrase–isomaltase of rabbit small intestine.[129,248] The enzyme consists of three domains: an anchor peptide, the isomaltase subunit, and then the sucrase subunit. The enzyme complex is excreted as a single polypeptide chain, and then the protein is cleaved proteolytically between the sucrase and isomaltase subunits. There is one active site per subunit, as was shown by affinity labeling with conduritol B epoxide. This compound alkylates an aspartate residue in the sequence -Asp-Gly-Leu-Trp-Ile-Asp-Met-Asn-Glu in both subunits.[249] But for the cleavage of the peptide link between the two active sites in sucrase–isomaltase, the domain structure is very similar in both sucrase–isomaltase and lactase–phlorizin hydrolase.[129]

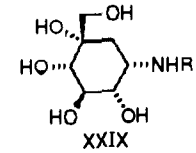
Values of $^{\alpha D}(V)$ for *p*-chlorophenyl glucoside hydrolyzed by the sucrase of 1.14, and by the isomaltase of 1.21, suggest an oxocarbonium ion like transition state.[250] A near-zero value of β_{lg} for para-substituted phenyl glucosides suggests essentially complete protonation of the leaving group at the transition state.[129]

Both enzymes are inhibited by compounds considered to be analogues of a glycosyl cation like transition state. Nojirimycin, deoxynojirimycin, and acarbose (XXVIII, $m = 0$, $n = 2$) are slow, tight-binding inhibitors of both activities.[251] Gluconolactam and gluconolactone also



XXVIII

inhibit more powerfully than substrate (K_i values around 10^{-6}–10^{-5} M). (Acarbose is one of a family of inhibitors that were intensively investigated for their α-glucosidase inhibitory power, in the hope of regulating the intake of dietary carbohydrate.[252] In the event the K_i values, in the light of the size of the inhibitor and the consequent opportunity for the enzyme to interact with many saccharide residues simultaneously, are fairly modest, e.g., 0.47 μM for acarbose at pH 6.8.) Reduction of the double bond of the pseudosaccharide unit gives a material with a K_i of 1 μM against the rat intestinal sucrase.[253] The inhibition by castanospermine[254] and substituted valiolamine derivatives (XXIX)[255]



XXIX

is in fact much more powerful, that by castanospermine being functionally irreversible (about 30% recovery of sucrase, but no recovery of isomaltase, being seen on 24-h dialysis). Inhibition by homonojirimycin (i.e., compound XXIV without the β-D-glucopyranosyl residue) is quite powerful.[256]

4. Dextransucrases

Dental caries is associated with bacterial plaque formation. A key step in plaque formation is the formation of insoluble carbohydrate polymers on the teeth by oral bacteria.[257] Because of the high free energy of hydrolysis of sucrose compared to that for hydrolysis of other glycosidic linkages, the conversion of sucrose into other carbohydrate polymers is thermodynamically favored. The enzymes catalyzing the conversion of sucrose into polymers are conventionally divided into dextransucrases, which are α-D-glucosyltransferases, and levansucrases, which are β-D-fructosyltransferases. The enzymes have weak hydrolase activity and so are conveniently discussed together with a→a hydrolases. They can also accept *p*-nitrophenyl α-D-glucopyranoside and α-D-glucopyranosyl fluoride as glucosyl donors.[258]

In accord with the mechanism being essentially that of a retaining glycosidase, modified to include acceptor binding, a D-glucosylated intermediate has been isolated for the dextransucrase of *S. sanguis* and shown to undergo partial reactions.[259,260] A hydrolytic activity of this enzyme has been detected, as is reasonable, but the evidence for the proposed *two* glucosyl-enzyme intermediates is somewhat weak to support such a radical suggestion.[261]

The glucosyl-enzyme intermediate from the enzyme from *Leuconostoc mesenteroides* has been isolated,[262] and the products of action of various acceptors and donors have been quantified.[263] This enzyme has been shown to have two binding sites by a careful study of inhibition kinetics: inhibitors can be either noncompetitive or competitive, the competitive inhibitors binding to the glucosyl donor site and the noncompetitive inhibitors binding to the acceptor site. Therefore, competitive inhibitors do not compete with noncompetitive inhibitors, but noncompetitive inhbitors compete with each other.[332]

The most studied dextransucrases are from *Streptococcus mutans* (this organism sometimes goes under the name *Streptococcus sobrinus*). They are reported to produce α(1→6), α(1→3), and some α(1→4) glucans;[264] at least the α(1→3) and α(1→6) transferase activities have been demonstrated to arise from different enzymes, as is reasonable.[265] Other workers, using a different bacterial serotype, isolated and characterized three enzymes, which synthesized respectively a highly and a sparsely α(1→3)-branched soluble α-(1→6) polymer and an insoluble α(1→3)-linked polymer.[266] The kinetics of these enzymes are complex but are compatible with a glucosyl-enzyme intermediate.[267] The inhibition patterns by the glucose disaccharides maltose [α(1→4)], isomaltose [α(1→6)], and nigerose [α(1→3)] are as expected from the enzyme specifici-

ties.[268] At least the enzyme which makes α(1→3) linkages requires a primer;[269] an enzyme producing α(1→6)-linked polymer apparently does not.[270] A β-glucosyl enzyme intermediate has been isolated in the case of the enzyme which gives a highly α(1→3)-branched α(1→6) polymer;[271] if the native glucosyl-enzyme is allowed to turn over with water as an acceptor, α-D-glucopyranose is the product, whereas if the enzyme is denatured and subjected to alkaline hydrolysis, β-D-glucopyranose is the product. This is telling evidence that the glucosyl-enzyme intermediate is a β-glucosyl ester of an aspartate or glutamate side chain.

Both *Leuconostoc* and *Streptococcus* dextransucrases require the substrate to have a hydroxyl group on positions 3, 4, and 6.[272]

5. Glycogen Debranching Enzyme

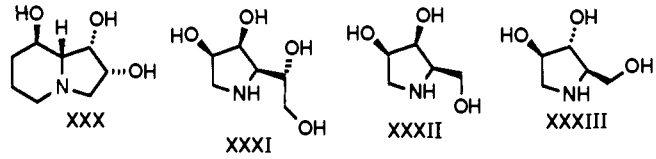
The exhaustive action of glycogen phosphorylase on glycogen results in a symmetrical limit dextran, in which the glucose unit at the branch point is substituted with a maltotetraosyl residue at both the 4- and the 6-positions. A catalytically intriguing enzyme, glycogen debranching enzyme converts the branch to a linear maltooctaose domain, susceptible to further attack by glycogen phosphorylase, by means of two activities. The first transfers a maltotriosyl residue from the maltotetraosyl residue at the 6-position of the branch to the nonreducing end of the maltotetraosyl residue at the 4-position of the branch, the product being a branch with a maltoheptaosyl residue at the 4-position and a single α-D-glucopyranosyl residue at the 6-position. The second activity is an α-glucosidase activity which hydrolyzes the single α(1→6) linkage at the branch point, to yield a linear α(1→4)-linked glucan polymer.[471-475] The rabbit muscle enzyme possesses both catalytic activities in a single polypeptide chain of M_r 165 000,[471,476,477] and crystals suitable for X-ray diffraction have been grown.[478] The transferase and glucosidase activities can function independently of each other,[471,479] but the enzyme does not bind two polysaccharide substrate molecules simultaneously; rather, a model in which a central polymer binding site is flanked on the one side by the transferase catalytic site and the other by the glucosidase catalytic site has been proposed.[471,480] Electron diffraction of this monomeric and necessarily highly flexible enzyme reveals it to be crescent- or shrimp-shaped.[481] The enzyme is rich in cysteine residues, but the kinetics and thermodynamics of the thiol/disulfide redox exchange reaction with glutathione are such that it is unlikely that this reaction, as had been suggested, plays a regulatory role.[482] The availability of titratable thiols, however, has enabled the thermodynamics of binding of maltooligosaccharides to the enzyme to be studied, since enzyme–saccharide complexes react more slowly with DTNB. Dissociation constants decrease with degree of polymerization up to maltopentaose,[483] but the effects are small (e.g., at pH 7, the value for glucose is 35 mM, for maltotriose 11.7 mM, and for maltopentaose 4.3 mM).

The molecular mechanisms of the transfer and hydrolysis reactions await detailed bioorganic study.

6. Other a→a Hydrolases

α-Mannosidase plays a key role in the processing of asparagine-linked oligosaccharide chains.[235,469] Three α-mannosidases are involved in the trimming of oligosaccharide chains, one ("ER mannosidase") in the endoplasmic reticulum and two ("Golgi mannosidases I and II") in the Golgi apparatus. There is in addition a lysosomal enzyme. The discovery that the natural product swainsonine (XXX) was a powerful inhibitor of the Golgi mannosidase II and the lysosomal enzyme,[273,274] and the perception that inhibition of glycoprotein processing was a promising strategy for the development of antiviral agents,[275-277] led to the testing of a whole range of analogues of swaisonine against mammalian enzymes; inhibition was generally unspectacular (K_i values around 10 μM).[278-281]

The inhibition of mannosidase by swainsonine (XXX) appeared to be another example of five-membered iminoalditols inhibiting the pyranosidase somewhat more powerfully than six-membered iminoalditols. However, while 1,5-dideoxy-1,5-iminomannitol was found to have a K_i value around 2 μM against Golgi mannosidase I,[282] the 1,4-iminoalditols XXXI–XXXIII have K_i values against the jack bean enzyme of around 1–10 μM.[86]



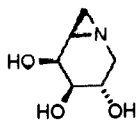
Depending on the source, inhibition of α-mannosidases by swainsonine may be freely reversible or effectively irreversible.[283] N-Alkylation of the monocyclic compounds above can alter inhibitory specificities.[284]

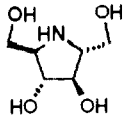
Inactivation of α-mannosidases by (α-D-mannopyranosylmethyl)(*p*-nitrophenyl)triazene, a mechanism-based reagent of a type that works generally on retaining glycosidases,[3] has been observed in a number of systems.[285] It inactivated the Golgi mannosidase I and jack bean α-mannosidase, whereas the endoplasmic reticulum enzyme was apparently resistant.[286] In another study it was found to inactivate the soluble, Golgi mannosidase I and lysosomal α-mannosidase of rat liver, but to have no action on the Golgi II enzyme.[287] At least toward reagents of this type, the behavior of an a→a enzyme is very similar to that of the e→e enzymes β-galactosidase and β-glucosidase.

A range of α-L-fucosidase inhibitors based on the 1,5,6-trideoxy-1,5-imino-L-galactitol structure have been made: 1,5,6-trideoxy-1,5-imino-L-galactitol is itself the most powerful inhibitor of the compounds studied.[288] Human α-L-fucosidase shows transglycosylation activity, and evidence for the importance of proton transfer is indicated by a value of $^{D_2O}(V/K)$ of about 1.9.[289]

An active site directed irreversible inactivator of the α-galactosidase of green coffee beans was designed[290] by using a principle similar to that involved in the inactivation of yeast α-glucosidase by 2-chloro-1,1,2-trifluoroethyl glucoside.[246] The compounds αGal-*p*-O-CH(OMe)CH_2X, where X = I or Br, are cleaved by the enzyme and the hemiacetals derived from the aglycon then decompose to bromo- or iodoacetaldehyde, which, being α-halocarbonyl compounds, alkylate the enzyme. The aglycon-binding site of this enzyme must be remarkably hydrophobic in order for the residence time of the haloacetaldehyde to be long enough to alkylate the active site. The aziridine XXXIV, considered to be a representative of a "potent new class of glycosidase

inactivators",[291] is probably a simple exo-acting affinity label for green coffee bean α-galactosidase.


XXXIV


XXXV

D. Furanoside Hydrolases and Transferases

1. Invertase

Given that the Michaelis-Menten rate law for enzyme catalysis was first demonstrated for yeast invertase (fructofuranosidase),[292] it is remarkable that the nature of the initial product of enzyme action (β-D-fructofuranose) has only recently been directly demonstrated,[293] although retention has long been assumed on the basis of the production of transfer products from alcohols.[294] Two forms of the yeast enzyme, external and internal, have a common genetic origin and differ only in that the external form is heavily glycosylated and has a signal sequence at the N-terminus.[295,296] A third form, cytosolic invertase, which requires phosphorylation for activity, has recently been detected.[297] An active site carboxylate has been identified by affinity labeling with conduritol B epoxide, but the L isomer is the active form (not the D as with α- and β-glucosidases); the label is released as *chiro*-inositol at pH 9.[298] The enzyme therefore probably works via an α-fructofuranosylated carboxylate intermediate. Compound XXXV has a K_i value of 1.5 μM, which gives some indication that glycosyl cation like transition states are involved.[299]

2. Levansucrase

The existence of fructofuranosylated carboxylate as a glucosyl-enzyme intermediate has been firmly established for the levansucrase of *Bacillus subtilis*. The enzyme transfers β-fructofuranosyl residues from sucrose to a growing levan polymer with β(2→6) and β-(2→1) linkages but has some hydrolase activity which is suppressed completely in 60% acetonitrile.[300] There is a report that the enzyme forms only α(2→6) linkages.[301] The suppression of hydrolase activity under these conditions, coupled with a 5-fold increase in k_{cat}, is further reason for thinking that the catalytic mechanism of these transferases is essentially identical with that of the corresponding hydrolase.

The fructosyl-enzyme intermediate implied by ping-pong kinetics[302] has been shown to be stable at low pH and to involve a fructosylated aspartate residue.[303] An analysis of kinetic and equilibrium relationships enabled the complete free energy profile of the catalyzed reaction to be obtained;[304] the free energy of hydrolysis of sucrose was preserved in the fructosyl-enzyme intermediate. An X-ray crystal structure at low (3.8-Å) resolution has been published,[305] but the full structure appears to await the sequence determination of the enzyme.

3. NAD$^+$ Glycohydrolases

A variety of enzymes cleave the C–N bond between ribose or deoxyribose and a pyridine or a nucleoside base. The DNA glycosylases excise modified nucleotide bases from DNA and are reasonably well characterized structurally and genetically,[306,307] but no work of significance to the catalytic mechanism has been done on them. Any form of acidic assistance to a quaternary nicotinamide leaving group is of course structurally impossible. A very remarkable report[308] that thyroidal NAD glycohydrolase hydrolyzes β-NADH and α-NAD$^+$ may well have its origin in the ability of NADH, like all glycosylamines, to spontaneously mutarotate, and nonenzymic redox reactions that yield α-NADH and β-NAD.

Hydrolysis of the glycosidic link of NAD$^+$ proceeds with retention of configuration, as is shown by the ability of the enzymes from calf spleen[309] and the venom of the banded krait[310] to catalyze base exchange and of the krait venom enzyme to transfer an ADP-ribosyl residue to methanol.[311] Subsequently, the chemical step of the calf spleen enzyme has become kinetically accessible by the use of NAD analogues lacking a carbonyl substituent at the C-3 of the pyridine, and indeed a value of $\beta_{lg}(V)$ of −0.9 has been obtained.[312]

The selectivity of the krait venom ADP-ribosyl intermediate between pyridines of various pK_a values gives a β_{nuc} value of 0.43 for pyridinolysis of this intermediate. It was suggested,[3] in the light of an estimate of β_{eq} of −1.47, that β_{lg} for the first chemical step should be around −1.0. With reasonably good substrates, the chemistry is kinetically masked also in the case of the pig brain and *Neurospora crassa* enzymes. The good substrate NAD$^+$ gives $^{\alpha D}(V/K)$ values of 1.00, but the poor substrate NMN gives values of 1.13–1.10,[313] in accord with a transition state with glycosyl cation character.

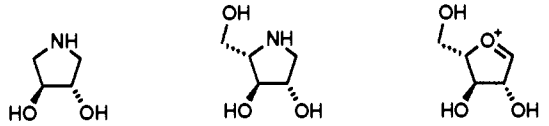
4. α-L-Arabinofuranosidase

The fungus *Monilinia* (formerly *Sclerotinia*) *fructigena* is a plant pathogen which is responsible for the brown rot of apples; it excretes a cocktail of glycoside hydrolases that attack the plant cell wall.[314] Among these are two α-L-arabinofuranosidases, AFI and AFIII (AFII being intracellular), which attack the arabinogalactan component.[315] Both AFI and AFIII work with retention of the anomeric configuration,[316] and AFIII has been purified:[317] it is a monomer of M_r 40 000. It hydrolyzes α-L-arabinofuranosylpyridinium ions with high efficiency (the ratio of k_{cat} to the spontaneous hydrolysis rate for the 4-bromoisoquinolinium ion being 2.5×10^9). Breakage of the exocyclic C–O bond limits k_{cat} for the hydrolysis of the *p*-nitrophenyl arabinofuranoside, as is shown by a 3% ^{18}O kinetic isotope effect. This isotope effect makes it likely that the absence of a detectable dependence of k_{cat} for a series of aryl arabinofuranosides on the acidity of the aglycon represents a true β_{lg} of zero, rather than the kinetic dominance of non-bond-breaking steps. The initial bond-breaking step of any ring-opening mechanism would be expected to exhibit an inverse ^{18}O leaving group kinetic isotope effect and a strongly negative β_{lg} value.

The near-zero β_{lg} value obtained for the hydrolysis of aryl arabinofuranosides by this enzyme suggests that proton donation to the leaving group is relatively far advanced in the first chemical transition state. This particular enzyme–substrate system was therefore considered a promising vehicle on which to try to detect general-acid catalysis of aglycon departure using solvent

isotope effect probes. The pH–rate profiles for V and V/K are sigmoid, with the pK governing V/K being 5.9. There is a modest (1.45) solvent isotope effect on k_{cat} but no significant one on k_{cat}/K_m, in the plateau region of the pH–rate profile, which a linear proton inventory shows to be due to one proton.[318] The pK of 5.9 is therefore in all probability the pK of the acid-catalytic group in the free enzyme.

This identification of the pK of the acid-catalytic group enabled the pattern of inhibition of this enzyme by iminoalditol inhibitors XXXVI and XXXVII to be analyzed, in particular, the question of whether they were reasonably tight binders by virtue of the resemblance of their conjugate acids to the arabinofuranosyl cation XXXVIII.



XXXVI XXXVII XXXVIII

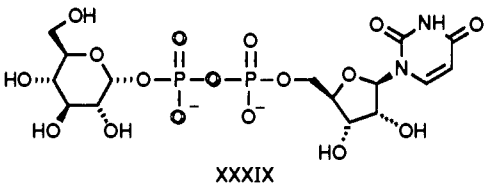
This explanation has recently fallen into disfavor because of the commonly observed increase in inhibitory potency with pH. A more detailed analysis of the whole catalytic apparatus, however, suggests that this pattern of behavior is what would be expected if these compounds were, as originally suggested, transition-state analogues. At the transition state, not only does the ring oxygen of the substrate carry a positive charge, but the acid-catalytic group of the enzyme will be partially deprotonated. The transition state will be mimicked, therefore, by the protonated inhibitor binding to deprotonated enzyme. This situation is indistinguishable by simple binding experiments from deprotonated inhibitor binding to protonated enzyme. Since by definition enzymes with an acid-catalytic group deprotonated are well away from their pH optimum, studies of the pH variation of inhibitory power of iminoalditol inhibitors lend themselves to interpretation in terms of the binding of deprotonated inhibitor to protonated enzyme.

In the case of AFIII, the studies outlined above had indicated a transition state in which the catalytic proton was largely transferred to the leaving group and had also given some indication of the pK of this acid catalytic group. Therefore, if at the transition state for this enzyme the arabinofuranose ring resembles an arabinofuranosyl cation, we would expect the conjugate acids of amines XXXVI and XXXVII to bind tightly to deprotonated enzyme. Indeed, the K_i values for both inhibitors show a bell-shaped dependence on pH, with the acid limb of the bell being governed by the pK of 5.9 obtained from the catalysis studies, and the alkaline limb being governed by the pK of the inhibitors, as measured by titration. The minimal K_i for XXXVII is 1 μM, well below the K_m values for substrates; the minimal K_i for XXXVI is 1.2 mM, likewise well below the values for *trans*-1,2-dihydroxycyclopentane derivatives.[316] The tighter binding of XXXVI and XXXVII is therefore plausibly attributed to their resemblance to the arabinofuranosyl cation XXXVIII. It is also likely as a general phenomenon that tight binding of glycosidase inhibitors of the iminoalditol type at the pH optimum of the enzyme will be observed when the pK of the inhibitor lies below that of the acid-catalytic group on the enzyme.

E. Glycosyl Transfer with Retention to and from Phosphate and Pyrophosphate

Many of the enzymes of poly- and oligosaccharide biosynthesis, which transfer glycosyl residues from nucleotide diphospho sugars, fall under this heading, but despite their importance, these enzymes are so experimentally intractable that little mechanistic work has been done on them.[8]

Mechanistic information is available on the glycogen synthetase of rabbit muscle and *E. coli* and on sucrose synthetase from wheat germ. This last enzyme has been the subject of elegant experiments involving positional isotope exchange from Raushel's group.[320] They synthesized UDP-glucose with the ^{18}O-labeling pattern shown shadowed in compound XXXIX and looked for ^{18}O exchange into the sugar C-1–phosphorus position, which would be expected if the glucosyl–oxygen bond had broken for any reason, since then the β-phosphate of the UDP product would rotate and become torsionally symmetrical. They found none except in the presence of the glucosyl acceptor fructose: even 2,5-anhydro-D-mannitol, an exact analogue of β-D-fructofuranose, did not provoke glycosyl–oxygen cleavage.



XXXIX

The *E. coli* glycogen synthetase, solubilized from the membrane by protease cleavage of the protein stalk,[321] has been available in pure form for a number of years; its amino acid sequence is now known from the sequence of the *glgA* gene.[322] It transfers glucosyl residues from ADP-glucose to maltose but not to glucose and is moderately well inhibited by gluconolactone.[323] Affinity labeling of the rabbit muscle enzyme indicated that the NH_2 terminus and Lys 38 were close to the active site.[324] The positional isotope exchange experiment analogous to the one carried out with the sucrose synthetase showed up no such exchange.[325] Glucono-δ-lactone and deoxynojirimycin were moderately powerful inhibitors, and values of $^{\alpha D}(V)$ of 1.23 and $^{\alpha D}(V/K)$ of 1.09 confirmed the expectation that oxocarbonium-like transition states were involved. The mammalian (rabbit muscle) glycogen synthetase, however, requires a primer protein ("glycogenin") which is first O-glycosylated on a tyrosine residue.[326,327]

Of the a→a phosphorylases, extensive mechanistic data are available on sucrose phosphorylase and glycogen phosphorylase.

The work on the sucrose phosphorylase of *Pseudomonas saccharophila*, though 20 years old, is quite unambiguous and is an important contribution to the overall mechanistic picture of glycosyl transfer.[328] The reaction goes through a β-glucopyranosyl-enzyme intermediate which is stable at low pH; it can be shown to be generated from the glucosyl, rather than the fructosyl, part of the sucrose molecule by ^{14}C labeling. In the direction of sucrose synthesis, in which the enzyme can use α-D-glucopyranosyl fluoride as glucosyl donor, as well as α-D-glucopyranosyl phosphate, the enzyme follows ping-pong kinetics if these are modified to allow for competitive inhibition of the donor by the

acceptor at high acceptor concentrations. Generation of the glucosyl-enzyme intermediate in the absence of acceptor will slowly generate α-D-glucopyranose (alcohols give α-glucosides). However, reagents that attack the acyl carbon of glycosyl esters (such as hydroxylamine) give β-D-glucopyranose. It is clear that a double-displacement mechanism, similar to that for a hydrolase, is operating.

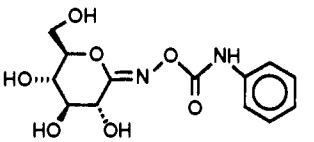
1. Glycogen Phosphorylase

The mechanistic picture for glycogen phosphorylase, which catalyzes the reaction of inorganic phosphate with an α(1→4)-linked glucan that removes the nonreducing terminal glucose unit as α-D-glucopyranosyl phosphate, is complicated by the many binding sites and its conformational nonrigidity.[329,330] In the dimeric enzyme from rabbit muscle it is possible to discern an oligosaccharide primer binding site, an AMP binding site, and a purine binding site, which are all remote from the catalytic site. In addition to heterotropic interactions within a subunit, there are homotropic and heterotropic interactions between subunits. The enzyme is also phosphorylated by ATP and glycogen phosphorylase kinase, which converts largely inactive phosphorylase *b* to active phosphorylase *a*; there is a specific phosphatase which dephosphorylates the enzyme. To a first approximation, conformational changes can be discussed in terms of a change from T (inactive form) to R (active form), mixed TR dimers not being formed, although the model is inadequate in detail.[331,333,334]

Because of the conformational flexibility of the enzyme, mechanistic deductions from the X-ray crystal structure of either phosphorylase *a*[335] or phosphorylase *b*[336] have not been easy,[487] and at the time of writing it is not entirely clear whether the enzyme proceeds by a double-displacement mechanism like sucrose phosphorylase, or whether by a novel S_Ni reaction. X-ray crystallographic measurements can be actively misleading: the proximity of His 376 to the anomeric center of a reacting glucose residue led to the suggestion that there was a β-glucosylated imidazole as an intermediate,[337] a proposal at variance with the known hydrolytic stability of such structures.[338] α-D-Glucopyranose, 1,2-cyclic phosphate binds to the catalytically active (R state) of phosphorylase *b*, presumably at the active site.[339] If it binds only 1 Å away from the true site of a reacting glucose residue, then Glu 671 is correctly placed for nucleophilic attack on the β face of the glucosyl ring that is transferred.[340] Modification of the enzyme with a water-soluble carbodiimide, however, reveals Glu 664 and Asp 671 to be essential residues.[341]

The interaction of enol ethers, such as glucal, with glycogen phosphorylase is different from their interaction with a normal retaining glycosidase. Glucal reacts in the presence of phosphate (or arsenate) to add a 2-deoxy-α-D-glucopyranosyl residue to the nonreducing end of the primer: if the reaction is run in D_2O, a 2-deoxy-2-deuterioglucose residue is added, not, as one would expect in the case of an a→a glycosidase, a 2-deoxy-2-deuteriomannose residue.[342] Although different from what is observed with a→a glycosidases, this stereochemistry is what would be expected if the initial reaction with glucal were protonation by the enzymic acid catalyst coupled with trans attack by the enzyme nucleophile, possible Glu 671. The enzyme utilizes D-glucoheptenitol (i.e., the gluco version of compound II) in the absence of primer, to give 1-*C*-methyl-α-D-glucopyranosyl phosphate.[47] If the reaction is run in D_2O, one deuteron is incorporated into the methyl group. The reaction has been followed in the crystal by use of synchroton radiation; no intermediate could be detected.[343] Very recently, the X-ray structure of the AMP–oligosaccharide–heptulose 2-phosphate complex has been solved:[488] there is a short hydrogen bond between the phosphate groups bonded to the heptulose and to the pyridoxal cofactor, and the dihedral angle about the C-2–O-2 bond of the heptulose phosphate is that disfavored by the exo-anomeric effect.

Compound XL, in which C-1 is trigonal, bound neatly at the active site of phosphorylase *b* when examined by X-ray crystallography[344] and was cleanly competitive with glucose 1-phosphate in free solution.[345] α-D-Glucopyranosyl fluoride is only a very poor glucosyl donor, in stark contrast to the situation with sucrose phosphorylase.[346]



XL

These data from the European groups are interpreted in terms of an S_Ni mechanism for the enzyme.[3,47] In the phosphorolysis direction, proton transfer from the phosphate to the glycosidic oxygen of the glycogen substrate results in C–O fission and the production of an ion pair in which the positive charge of the glycosyl cation is stabilized, perhaps electrostatically, from the same side of the sugar ring as the departing leaving group. The ion pair then collapses to α-glucopyranosyl phosphate without the intervention of a covalent β-glucosyl intermediate at all. There is the chemical precedent for this mechanism in the results from solvolysis of α-D-glucosyl fluoride in mixtures of ethanol, trifluoroethanol, and phenol, which produces large quantities of retained product derived from the most acidic component of the solvent.[36] In this system internal return from an intramolecularly hydrogen-bonded leaving group ROH···F^- is taking place. However, the evidence in favor of the internal return mechanism for glycogen phosphorylase is essentially negative—there is no enzyme group on the β face of the substrate which could plausibly act as a nucleophile—or based on the reactions of intrinsically highly reactive enol ethers, whose reaction patterns may in reality represent only modest rate enhancements. Arguing against the mechanism is the (apparently redundant) presence of pyridoxal phosphate in the enzyme active site and a whole series of experiments addressing the problem of its role, which have recently been reviewed.[486] There is even a report from Indian workers that the starch phosphorylase from tapioca leaves contains no pyridoxal phosphate.[347] Moreover, the S_Ni mechanism must envisage a large glucose leaving group and a large phosphate acid/nucleophile on the same (hindered) face of the sugar ring.

The aldehyde group of the pyridoxal phosphate forms a Schiff base with a lysine residue of the protein in the active site. There is undoubtedly interaction between

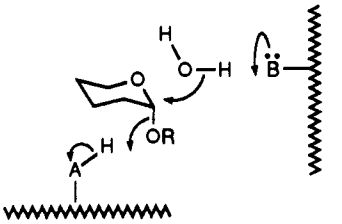
the phosphate of the cofactor and the inorganic phosphate substrate: if the covalent diphosphate is made, it slowly transfers a glucosyl residue to the acceptor.[341,348] The pH dependencies of the pyridoxal enzyme using HPO_3H^- and FPO_3H^- as phosphorylysing agents instead of inorganic phosphate are unaltered, despite a difference of pK_a values of 1.8 pK units, arguing against an acid-catalytic role for the inorganic phosphate.[349] Likewise, fluorophosphate did not alter its ionization state during catalysis, and V_{max} values for a series of phosphates and phosphites showed little variation, despite large changes in pK. Reconstitution of the enzyme with 6-fluoropyridoxal phosphate rather than pyridoxal phosphate itself did not alter the pH–rate profile.[334] Even addition of an extra phosphate group seems to have no very great effect: pyridoxal 5′-triphosphoglucose transfers a glucosyl residue to glycogen at about $^1/_4$ the rate of the diphospho compound.[350] The binding of fluorodeoxy glucose and mannose derivatives has been interpreted in terms of defined hydrogen-bonding interactions with the crystallographically identified glucose binding site;[351] binding of glucose stabilizes the inactive T state. The success of this approach provides some assurance that this site has been correctly identified: acarbose, prime candidate for a transition-state analogue, in fact binds in the glycogen storage site.[352]

The question of the nature of the transition state(s) has been addressed in two ways. No significant α-deuterium kinetic isotope effect was found on the reaction,[353] but this could easily be because an isotopically silent step was rate-determining. The enzymic reactions of a series of deoxy and fluorodeoxy glucosyl phosphates were examined;[354] they are poor substrates (10^3–10^4 times slower than glucose 1-phosphate itself), so the chemistry is very likely rate-limiting. Their rates of acid-catalyzed hydrolysis (i.e., rate of unimolecular departure of $H_2PO_4^-$ from the anomeric center) had previously been measured and found to conform to the pattern of reactivity expected on the basis of the inductive effect of the substituent.[355] V_{max} values for the enzyme reaction correlated with the spontaneous rate with a correlation coefficient of 0.9 and a gradient of 0.8. Since the nonenzymic reactions proceed, if not through discrete carbonium ions, then at least through highly oxocarbonium ion like transition states, the enzymic reaction must proceed through a similar species.

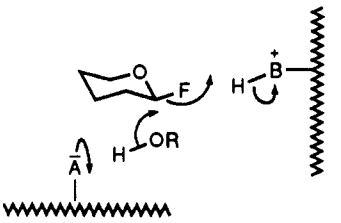
III. Enzymic Inversion of the Anomeric Configuration

Inversion of the anomeric configuration in the course of phosphorylase action is easy to establish experimentally, and perhaps for this reason inversion seems as common a stereochemical outcome of phosphorolysis as retention. The impression obtained from the literature is that retention is the commoner course of glycosidase action, but this may merely be a consequence of the experimental difficulties encountered in demonstrating inversion. Most retaining glycosidases have some transferase activity to alcohols, and therefore, a stereochemically stable product can be obtained with catalytic amounts of enzyme and examined at leisure. Establishment of inversion, however, requires quantities of enzyme sufficient to make the total flux to product comparable to that from accumulated product to the mutarotational equilibrium mixture, and that the analytical system (NMR or optical rotation, neither of them particularly sensitive) be capable of responding on a time scale short compared to the mutarotational constants. With many mammalian enzymes these conditions cannot be met.

The mechanism of those glycosidases acting with inversion of the anomeric configuration has for many years been discussed in terms of the single displacement by a nucleophilic water molecule first suggested by Koshland.[46] The departure of the aglycon is envisaged as being assisted by a general-acid-catalytic group on the enzyme and the nucleophilicity of the water molecule as being enhanced by an enzymic general base.[356]



Quite the best evidence for the operation of this mechanism with at least some inverting enzymes comes from Hehre's discovery that inverting glycosidases hydrolyze the appropriate glycosyl fluorides of both anomeric configurations.[357] The hydrolysis of the fluoride of the same anomeric configuration as the *O*-glycoside substrate exhibits normal Michaelis–Menten kinetics, whereas the hydrolysis of the "wrong" fluoride shows a greater than linear increase in rate of fluoride ion release with substrate concentration at low substrate concentration, and fluoride ion release is stimulated by the addition of glycosyl derivatives which would ordinarily be considered competitive inhibitors. In these cases, and also in some of the cases where the enzyme acted upon the wrong fluoride alone, it was possible to isolate transfer products. Thus, in the case of the glucoamylase of *Rhizopus niveus* and the glucodextranase of *Arthrobacter globiformis*, addition of methyl α-D-glucopyranoside to a solution of enzyme and β-glucopyranosyl fluoride led to the production of methyl α-maltoside.[358]



These data are elegantly rationalized by the suggestion that the wrong fluorides are hydrolyzed by a resynthesis–hydrolysis mechanism as shown above, in the first step of which the active form of the enzyme is the tautomer of the form which is active in the normal hydrolytic reaction.

A. Pyranoside Hydrolases with Equatorial Leaving Groups

Although there are reports of $\beta(1\rightarrow3)$ glucan hydrolases,[29,359,360] a β-mannosidase,[361] and an endoxylanase[362] acting with inversion of configuration, mechanistic detail is confined for the most part to two enzymes, the β-xylosidase of *B. pumilus*[363] and the cellobioside hy-

drolase II of *T. reesei*.[32] A histidine has been implicated in the active site of an inverting exo-β(1→3)-glucanase from a fungus ("Basidomycete QM 806") by modification experiments; more significantly, a fairly large solvent deuterium kinetic isotope effect has been detected [$^{D_2O}(V) = 2.5$].[359] Very recently it has been discovered that β(1→3) glucanases (whether inverting or retaining is not yet known) form part of plant defense systems.[364-366]

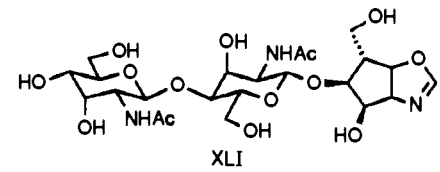
1. β-Xylosidase of B. pumilus

The considerable body of work on this enzyme has been carried out for the most part by two groups, that of De Bruyne in Ghent and that of Okada in Osaka, the Belgian group working on the kinetics and mechanism and the Japanese group on the molecular biology. At the time of writing, however, it is difficult to get other than circumstantial evidence that the results from the two groups apply to essentially the same protein. The strain used by the Belgian workers (designated *B. pumilus* 12) was one originally chosen for its ability to clear "squeegee" in flour extracts.[367] The "Belgian" enzyme is a dimer of 60-kDa subunits and is intracellular (it indeed is a thiol enzyme).[368]

The "Japanese" enzyme is part of the xylan-degrading system of a strain of the bacterium (designated IPO) isolated from a Thai paddy field. The system was in earlier papers described as consisting of an extracellular β-xylanase (the product of the *xynA* gene, whose sequence had been determined)[187] and *two* intracellular β-xylosidases, I and II, of which the first showed similarities with the "Belgian" enzyme in subunit molecular weight, structure, and K_m values.[369] In a later paper, recording the sequence of the *xynB* gene,[370] reference is made only to two enzymes that cleave β-xylosyl bonds, the extracellular xylanase (or *xynA* gene product) and the intracellular β-xylosidase (or *xynB* gene product), which is a dimer of 65–70-kDa subunit molecular weight.

The "Belgian" enzyme hydrolyzes xylo oligosaccharides, most effectively xylobiose[371] and aryl xylosides but not alkyl xylosides.[372] On the basis of the ability of the enzyme not only to bind and react with xylosyl derivatives held in the $^4C_1$ conformation but also to bind glycosyl derivatives held in the $^1C_4$ conformation and arabinofuranosides, it was suggested that the natural substrate was a hemicellulose fragment, produced by an extracellular xylosidase (now probably identified as the *xynA* gene product), containing the β-D-Xyl*p*-(1→4)(3-O-α-L-Ara*f*)-Xyl*p* structure.[373] A value of β_{lg} (V) of between −0.2 and −0.3[374] and $^{\alpha D}(V)$ values between 1.03 and 1.09[156] for aryl xyloside hydrolysis are consistent with some oxocarbonium ion character in the transition state.

The enzyme will hydrolyze α- as well as β-xylopyranosyl fluoride, using the resynthesis–hydrolysis mechanism, as is shown by the detection of transfer products.[20] All mechanistic data for this enzyme are consistent with the Koshland single-displacement mechanism. In accord with this, the powerful inhibition of this enzyme by ribopyranose derivatives[375] may be a consequence of their being transition-state analogues by virtue of the axial C-3 OH group of the β-D-ribopyranose unit mimicking the incoming water molecule. If this is so, then the powerful inhibition of insect chitinase by "allosamidin" (XLI) may be circumstantial evidence that this insect chitinase is in fact an "inverting" enzyme.[376]



XLI

2. The Inverting Cellobiohydrolase of T. reesei

The tertiary structure of the enzymically active core of the cellobiohydrolase II (CBHII) of *T. reesei* has been determined.[377,378] The native enzyme possesses an N-terminal region which is responsible for binding to crystalline cellulose, linked to the core, catalytically active protein by a polypeptide rich in serine and threonine that are O-glycosylated. Proteolytic cleavage can release this core which has full activity on small synthetic substrates but does not bind to cellulose. This protein is a large, single-domain α/β protein with a central β barrel made up of seven parellel strands. The first six strands are connected by α-helices while the connection between the sixth and seventh strands is irregular. The fold is therefore similar to but different from the $(\alpha/\beta)_8$ structure observed, for example, in α-amylase.

The active site was located by determining the crystal structure in the presence of the inhibitor *o*-iodobenzyl 1-thiocellobioside; this is an inhibitor since the enzyme requires at least three contiguous glucosyl residues for activity.[379] The active site is a tunnel at the C-terminal end of the β-sheet structure of the barrel (rather than the cleft often observed with endo-acting enzymes). It has four subsites, cleavage occurring between subsites B and C, where the catalytic groups, Asp 175 and Asp 221, are located. Both these catalytic groups are on the β face of the bound substrate; on the α face there is a narrow tunnel, leading to the surface of the protein, filled with water molecules. There is apparently *no* plausible candidate for the general base B in the Koshland single-displacement mechanism. At the time of writing it is not clear whether CBHII can be taken as a paradigm for all inverting glycosidases, but the reviewer considers the Hehre work on the hydrolysis of the wrong fluorides, which requires the presence of B, to be so compelling that the likelihood is that CBHII is unusual. Indeed, even though CBHII hydrolyzes α- as well as β-cellobiosyl fluoride, attempts to establish a resynthesis–hydrolysis mechanism for the α-anomer have so far failed.[380]

There seems to be some cooperativity between glucose binding sites, since glucose increases the affinity for cellobiose and cellotriose by factors of 10–20.[420]

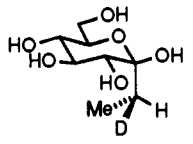
B. Pyranoside Hydrolases with Axial Leaving Groups

1. Trehalase

Trehalose is a nonreducing sugar (α-D-glucopyranosyl-α-D-glucopyranose), and all known enzymes hydrolyzing it do so with inversion of the anomeric configuration,[33,381] to yield a molecule each of α- and β-glucopyranose. A report that the pig enzyme retains the anomeric configuration is in error.[382] Yeast has an

acid and a neutral form of the enzyme.[383]

The rabbit renal cortex and the *C. tropicalis* enzymes both hydrolyze β- as well as α-glucopyranosyl fluoride. In the presence of glucose a small amount of resynthesized trehalose can be detected.[384] The *T. reesei* enzyme behaves essentially similarly, but in the case of this enzyme substantial amounts of the transfer product α-D-glucopyranosyl-α-D-xylopyranose can be obtained by the use of xylose as an acceptor.[18] Enzyme from this source hydrates enol ether XXVII to XLII,[222] indicating a proton donor on the β face of the bound substrate.



XLII

α-Mannopyranosyl-α-D-glucopyranose[385] and its sulfur analogue[386] are powerful competitive inhibitors of the cockchafer enzyme (being about 2 orders of magnitude more tightly bound than the corresponding gluco compound), whereas α-D-mannopyranosyl-α-D-mannopyranose does not bind at all. The axial OH group of the mono manno compound may be well enough placed to make similar hydrogen-bonded contacts to the nucleophilic water molecule, and thus the compound may be in some sense a transition-state analogue.

2. Glucoamylase

Glucoamylases (also sometimes called amyloglucosidases or glucodextrinases) hydrolyze the nonreducing terminal glucose residue off α(1→4)-linked amyloses or dextrins. The minimum requirement for a good *O*-glycoside substrate is usually the maltosyl unit, although glucoamylases will hydrolyze nitrophenyl glucoside very slowly and α-D-glucopyranosyl fluoride, which has an excellent leaving group with low steric demands, as rapidly as maltooligosaccharides.[358] It would be helpful if all enzymes given the name glucoamylase had the common mechanistic features of exo α-glucosidase action, β-glucopyranose as first product, and maximal activity on substrates with specific, carbohydrate leaving groups. The enzyme in the mammalian gut described as "maltase–glucoamylase" apparently retains the configuration of the substrate.[129]

The enzymes from *A. globiformis* and *Paecilomyces varioli* hydrate D-glucal, the product in D_2O being 2-deoxy-2-deuterio-β-D-glucopyranose; i.e., the enzyme catalyzes a trans-diequatorial hydration in which the proton is added to the substrate from the β face of the molecule. This suggests the presence of a specific acid–base catalytic group on this side of the substrate, viz., the general base that partly deprotonates the incoming nucleophilic water molecule.[21]

The enzymes from *Aspergillus saitoi* and *Rhizopus sp.* are quite powerfully inhibited by amino alcohols with no stereochemistry (e.g., aminoethanol, 2-amino-2-methylpropane-1,3-diol), with K_i values in the millimolar range;[387] as with the inhibition of intestinal sucrase–isomaltase by tris(hydroxymethyl)aminomethane,[388] these data supply a perspective to mechanistic inferences based largely on the inhibitory capacities of hydroxylated amines.

Careful fluorescence measurements of the binding of D-glucono-δ-lactone, glucose, and methyl α- and β-glucosides to the *R. niveus* glucoamylase have unraveled a pattern of behavior which supports the idea that the lactone resembles the transition state more than glucose or glucosides.[389] The binding of maltose to the *R. niveus* enzyme has been shown to take place even at pH values where both catalytic groups are ionized.[390]

α-Tritium kinetic isotope effects (necessarily V/K competition effects) for hydrolysis of α-D-glucopyranosyl fluoride by the glucoamylases of *R. niveus*, *A. niger*, *P. varioli*, and the *Ar. globiformis* glucodextranase have been measured: they are all high (1.26, 1.19, 1.19, and 1.17, respectively, corresponding to deuterium effects of 1.17–1.12) and indicate a high degree of oxocarbonium ion character.[233]

The enzyme from *A. niger* is produced on the ton scale for use in industrial starch processing. In fact, two enzymes (amyloglucosidases I and II) are produced from the same gene, by differential processing of a 169 bp intron.[391] Despite the single-displacement mechanism as apparently requiring the simultaneous operation of a general acid and a general base, the solvent isotope effect in the glucoamylase-catalyzed hydrolysis of *p*-nitrophenyl glucoside arises from the transfer of one proton.[380]

The glucoamylase from *Aspergillus awamori*, which is apparently identical with that from *A. niger*, has been subjected to a study by site-directed mutagenesis.[489] Asp 176, Glu 179, and Glu 180, suggested by differential labeling experiments to be in the active site, were changed to their corresponding amides, Asn or Gln. No activity against maltose or isomaltose and only very feeble activity against maltoheptaose (k_{cat}/K_m reduced by a factor of 10^3) were found for the Glu 179 → Gln mutant, whereas the kinetic changes consequent upon the Glu 180 → Gln change were smaller, the biggest being a 200-fold reduction in k_{cat}/K_m for maltose. The changes consequent upon the Asp 176 → Asn change were smaller still, the maximum change being a 70-fold reduction in k_{cat}/K_m for maltose.

3. β-Amylase

It is to be expected that the catalytic machinery of α- and β-amylases will be very different: the precedent of the bifunctional glycosidases of the mammalian gut (sucrase–isomaltase, lactase–phlorizin hydrolase, and maltase–glucoamylase) suggests that such bifunctional single polypeptides have arisen from the duplication of an ancestral gene. Therefore, the recent report of a single precursor protein containing α- and β-amylase activity in *Bacillus polymyxa* is very remarkable.[392]

The sweet potato enzyme was the first inverting glycosidase shown to hydrolyze the wrong fluoride, by the resynthesis–hydrolysis mechanism.[357] It also catalyzes the hydration of maltal.[17] The reaction is so slow that it was not possible to determine the anomeric configuration of the product, but by running the reaction in D_2O, it was possible to show that the proton was added to the double bond from the β face. The reaction is subject to a very large solvent deuterium isotope effect [$^{D_2O}(V) = 8$], which a linear proton inventory showed to be due to the transfer of one proton, presumably the one transferred to carbon.

The soybean enzyme possesses a thiol group close to, but not in, the active site; modification with methyl 2,4-dinitrophenyl disulfide yields a product with 9%

residual activity, but bulkier thiol reagents give lower activities.[393] It has been affinity labeled by 2,3-epoxypropyl α-glucopyranoside,[394] which alkylates Glu 186[395] in a reaction exhibiting a bell-shaped pH–rate profile.

C. Transfer of Furanosyl Residues with Inversion

ADP ribosylation of proteins is associated with various pathogenic infections;[396] the acceptor is a protein arginine residue in the case of diphtheria toxin and a cysteine residue in the case of the pertussis (whooping cough) toxin.[6] The inversion of configuration observed in the action of ADP-ribosyltransferases suggests that the reactions proceed through a direct displacement mechanism; where examined, the kinetics are such as to implicate a ternary complex of enzyme, NAD^+, and acceptor, in accord with this idea.[397] The sequence of the gene coding for diphtheria toxin has been determined,[398] and the techniques of site-directed mutagenesis have been used to change Glu 148 to aspartate.[399] The resulting mutant enzyme had only 0.6% of the activity of the wild type, suggesting that Glu 148 played a catalytic role.

The electronic effect of the nucleophilicity of the acceptor guanidino group on the rate of cholera toxin catalyzed ADP ribosylation is significant: 13 substituted (benzylideneamino)guanidines gave a Hammett ρ value of -0.37 ± 0.08 calculated on both V and V/K.[400]

Mechanistic data are available on only one inverting furanoside hydrolase, the AMP nucleosidase of *Azotobacter vinelandii*, but these mechanistic data are very extensive and allow us to form a remarkably detailed picture of the enzyme transition state.[401] The enzyme, which hydrolyzes AMP into ribose 5-phosphate and adenine, has been crystallized and preliminary X-ray data have been reported.[402] The monomers of the *E. coli* and *A. vinelandii* enzymes are arranged as hexamers in the crystal.[403]

Four V/K kinetic isotope effects (α-deuterium and tritium, β-deuterium, anomeric ^{14}C, leaving group ^{15}N) were measured for hydrolysis of AMP in the presence and absence of an allosteric activator (MgATP).[404] As is reasonable, all the effects are lower for the allosterically activated enzyme, indicating that some isotopically silent step has become partially rate determining. The effects on the nonactivated enzyme are considered to be intrinsic, on the basis of their high values (1.030 for ^{15}N, 1.044 for ^{14}C, 1.061 for β-deuterium) and the similarity of these values to those for acid-catalyzed hydrolysis (1.030, 1.044, and 1.077, respectively). These effects all indicate a transition state with substantial oxocarbonium ion character; the α-deuterium kinetic isotope effect is therefore a puzzle, being surprisingly low (1.045). The effect is undoubtedly experimentally correct since the α-tritium effect was measured as well and the value (1.069) is close to what would be predicted by the Swain–Schaad relationship $[(k_H/k_T) = (k_H/k_D)^{1.44}]$. The transition state was examined with the aid of the isotope effect modeling program BEBOVIB-IV[405] and the conclusion reached that in the enzyme-catalyzed reaction the nucleophilic water molecule was forced onto the anomeric center, stopping full expression of the α-deuterium kinetic isotope effect. This α-deuterium kinetic isotope effect indeed appears very sensitive to the nature of the groups at the reaction center: NMN, a poor substrate but with a quaternary pyridine leaving group, gives a V/K effect of 1.16, which must be close to intrinsic.[406]

Similar ^{14}C and secondary deuterium effects had previously been measured for the *E. coli* enzyme.[407]

D. Transfer to and from Phosphate and Pyrophosphate with Inversion

Some mechanistic studies have been reported on isolated enzymes of this class, but except for the nucleoside phosphorylases, extended mechanistic investigations have not been carried out. Thus, V_{max} for transfer of a glucuronyl residue from UDP-glucuronic acid to a series of substituted phenols by a UDP-glucuronyl transferase from pig liver was shown to be correlated with σ^- of the phenol substituent, a Hammett ρ value of +2.2 being obtained. These data suggested a mechanism in which the phenol was essentially deprotonated and little bonding to the incoming glucuronyl residue had occurred.[408] The cDNA sequence of the rat liver enzyme has been determined.[409]

1. Nucleoside Phosphorylases

The rabbit muscle purine nucleoside phosphorylase has been claimed to have a Theorell–Chance, rather than a ternary complex, mechanism.[410] The mammalian enzyme is a tetramer of 32-kDa subunits, the *E. coli* enzyme is a hexamer of 23-kDa molecular mass subunits, and the latter enzyme has been crystallized and subject to preliminary X-ray investigation.[411] Some cooperativity in the binding of inorganic phosphate ($n_H = 0.5$) has been reported for the *E. coli* and *Salmonella typhimurium* enzymes.[412] The difference in the kinetic and mechanistic properties of this enzyme in mammals and in pathogens or parasites[413] makes it an attractive target for pharmaceuticals. Thus, it was found that both calf spleen and *E. coli* enzymes accepted N-7- and C-8-substituted inosine and guanosine, but only the *E. coli* enzyme accepted N-1-substituted substrates.[414] The human erythrocyte enzyme accepted 5′-deoxy and 2′,5′-dideoxyinosine and could also tolerate epimerization of the substrate at C-4′, but could not tolerate alterations to the 3′ OH group.[415]

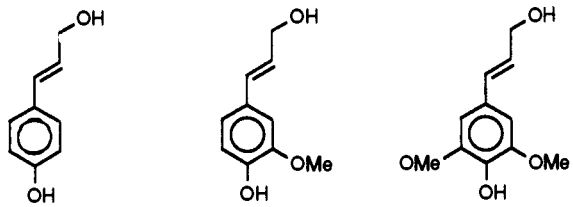
A study of α-deuterium kinetic isotope effects with the *E. coli* enzyme revealed an interesting pattern of behavior. The V/K effect was 1.00 over the whole pH range with adenosine as a substrate, but with the poorer substrate inosine, values indicative of an oxocarbonium ion mechanism were obtained at the acid (1.10) and alkaline (1.18) pH extrema, even though the effect fell to zero at the pH optimum.[416] The results were very plausibly interpreted in terms of an intrinsic effect of about 1.18, masked by isotopically silent steps near the pH optimum. An intrinsic effect of this magnitude is however very difficult to reconcile with the results with AMP nucleosidase, and with what is known about the effect of a preassociated nucleophile on α-deuterium kinetic isotope effects in model systems.[80] In these model systems, as the preassociated nucleophile gets "softer", the effect gets bigger, so that one would expect a bigger effect for AMP nucleosidase than for purine nucleoside phosphorylase, the reverse of what is observed. A reinvestigation of α-deuterium kinetic isotope effects in the purine nucleoside phosphorylase catalyzed phosphorolysis of inosine by equilibrium perturbation[11]

gave results differing only marginally from those of the previous workers. It thus appears that α-deuterium kinetic isotope effects may be very sensitive to the precise structure of the enzyme active site, in a way other kinetic isotope effects are not. This work[11] also revealed that the N-3-isomer of inosine was a substrate, implying that acidic assistance of the departure of the leaving group was no more critical with this enzyme than with many other glycosyl-transferring enzymes.

The kinetics of the uridine phosphorylase of *E. coli* implicate a ternary complex;[417] it[418] and the thymidine phosphorylase from the same organism[419] have been crystallized and subjected to preliminary X-ray investigation.

IV. The Enzymic Hydrolysis of Cellulose

Of the carbon bound in the biosphere, it has been estimated[1] that around 96% exists as plant biomass, which is essentially lignocellulose. Lignocellulose itself is an intimate complex of three primary polymers—lignin, hemicellulose, and cellulose. Lignin is a random copolymer, the first step in the biosynthesis of which is the nonenzymic coupling of radicals derived from the alcohols XLIII–XLV. Hemicelluloses are soluble glycans of various structures (i.e., xylans and arabinoxylans). Cellulose is a crystalline polymer of β(1→4)-linked glucose residues which is totally insoluble in water.



Lignocellulose thus presents those biological systems which degrade it with three simultaneous problems in catalysis: the random polymer lignin, the soluble carbohydrates hemicellulose, and the insoluble polymer cellulose. The problem in catalysis is so great that comparatively few classes of organisms have solved it. Most studied among these are the filamentous fungi, particularly *Trichoderma* species, although attention has been paid to actinomycetes.[172,421]

The very many, randomly generated, asymmetric centers in lignin make it impossible for any enzyme degrading this polymer to rely much on the "Circe effect" (the transduction of intrinsic binding energy to lower the free energy of activation of the catalyzed reaction).[422] Accordingly, the catalytic strategy adopted by those enzymes that have been studied in detail—those from white rot fungi, most notably *Phanerochaete chyrsosporium*—is what has been termed "enzymatic combustion"; the fungus produces a series of high-potential peroxidase enzymes (some of which produce smaller and more diffusible one-electron oxidants such as Mn^{III} complexes) which act purely as powerful one-electron oxidants:[423] there is no noncovalent interaction between enzyme protein and substrate. The problem of hemicellulose degradation is a problem in the hydrolysis of soluble carbohydrates no different to the hydrolysis of any other glycosidic linkage, in which the Circe effect plays a major role. The problem of cellulose hyrolysis is of hyrolyzing an insoluble polymer.

A. The Cellulase Complex of *T. reesei*

This filamentous fungus (formerly called *T. viride*) is the best studied cellulolytic microorganism: it produces a complete set of extracellular enzymes required for the breakdown of crystalline cellulose.[424-426] The system has been studied for many years, but progress was very slow until recently, largely because of the multiple molecular forms of the enzymes, some of which are proteolytic cleavage products, present in culture filtrates: during purification and storage the isoenzyme composition varies.[427] For this reason mechanistic experiments carried out before the application of the methods of molecular genetics to the system will be difficult to relate to structure and sequence. Thus, Capon and Thomson[428] showed, by examining the rates of hydrolysis of 4-(cellooligosaccharyl) 3′,4′-dinitrophenyl xylosides by endoglucanases isolated from culture filtrates, that noncovalent interactions with the C-5 hydroxymethyl were not important for catalysis, whereas interactions with the C-2 hydroxyl residue were, but the enzyme (or enzyme proteolytic fragment) to which these experiments apply can probably now never be identified. Similar problems may be encountered with the affinity labeling work of Legler and Bause.[429] These authors prepared a series of ω-epoxyalkyl β-D-cellobiosides and found optimum inactivation of a cellulase with the pentyl compound. Quite reasonably, they proposed that the epoxyalkyl group bound in the site normally occupied by the fissile monosaccharide residue.

T. reesei produces at least one β-glucosidase, two endoglucanases, and two enzymes called variously exoglucanases or cellobiohydrolases, although six endoglucanases and three exoglucanases have been detected as proteins (some of these probably being proteolytic cleavage products).[430] The cellobiohydrolases (designated CBHI and CBHII) play a central role in the hydrolysis of cellulose, acting synergistically to degrade both crystalline and amorphous cellulose.[431-433] The synergy increases with decreasing enzyme concentration.[434] Although CBHI produces cellobiose when allowed to act on soluble glucans, its designation as an exo enzyme is brought into doubt by the discovery of Chanzy et al., using immunogold staining, that it bound to crystalline cellulose all the way along the cellulose chain.[435] By the use of β-cellobiosyl fluoride as a substrate, it was shown that CBHI acted with retention of configuration and CBHII with inversion.[32]

The genes for CBHI,[436] CBHII,[437] and the endoglucanases EGI[438] and EGII/EGIII[439] have been isolated and characterized (the II/III designation arising because endoglucanases isolated in different laboratories were designated endoglucanase II and endoglucanase III, but turned out to be the same enzyme). Remarkably, considering the difficulties, the structure of CBHI[440] and the partial structure of EGII[441] were determined at the protein level. The amino acid sequences of CBHI and CBHII show no overall homology, in accord with their different stereochemistries of action, but a 35 amino acid region of 70% homology occurs in all four enzymes. In CBHII and EGII/III this occurs at the N-terminus, while in CBHI and EGI it is at the C-terminus. This domain has no catalytic activity in either CBHI or CBHII but is required for full binding on cellulose and has some cellulose binding activity of itself.[442] It can

be removed from both CBHI and CBHII by limited proteolysis: the resulting catalytic domains have unaltered properties toward soluble cellulose but no longer act on crystalline cellulose.[443] The conformation of this 36-residue fragment has been determined by NMR spectroscopy, using nuclear Overhauser effects in the 2-D mode (as well as COSY and HOHAHA sequences).[444] It has been suggested that possible preferential adsorption sites for cellulases on the cellulose microfibrils might be the exposed chains at the obtuse corners.[445] A binding site deficient, catalytically active core protein of EGIII has also been isolated (from culture filtrates) and shown to be active on soluble cellulose but not on the crystalline material.[446]

An active site carboxylate group (Glu 126) has been identified in CBHI by modification with Woodward's reagent K.[447] This could well be the nucleophilic carboxylate group expected on the basis of the rententive stereochemistry of the catalyzed reaction. As an e→e enzyme it is a remarkably poor catalyst: the k_{cat} value for 4-methylumbelliferyl cellobioside is only 0.002 s^{-1}, whereas the value for the lactoside, which differs only in the configuration of the remote 4-OH group, the value is around 0.04 s^{-1}.[448] k_{cat} values for other e→e enzymes such as *lacZ* β-galactosidase can be around 10^3 s^{-1}.

The reviewer tentatively advances a model of the hydrolysis of crystalline cellulose that takes into account the stereochemistry of the catalyzed reaction, the very low activity of CBHI, and the inability of EGI and EGII/III to hydrolyze crystalline cellulose. It is a more detailed version of the model advanced by Enari and Niku-Paavola[426] and has some features in common with the ideas of C_1 and C_x components of cellulase advanced many years ago by Reese[449] and espoused by Wood.[450] The C_1 component was considered to disaggregate the cellulose chains and make them susceptible to attack by the endoglucanase, or C_x component. The Enari–Niku–Paavola model envisages that initial attack on cellulose takes place with CBHI and CBHII and that this attack produces soluble glucan chains which are further attacked by the endoglucanases. The reviewer suggests that CBHI is in fact Reese's C_1 component and that its function is to bind to cellulose and then to convert a cellulose chain at the surface of the molecule to a glycosyl-enzyme intermediate, and then to stop, or turn over very slowly. In generating a cellulose-bound glycosyl-enzyme intermediate, it will also generate a "loose", nonreducing end which can be acted upon by CBHII. Two or more molecules of CBHI bound to the same glucan chain will allow the cellooligosaccharyl-CBHI to diffuse off the crystal and then turn over.

The overall rate of hydrolysis of cellulose under biotechnological conditions tends to be limited by the rate of hydrolysis of cellobiose to glucose, since CBHI and CBHII are subject to product inhibition;[451] mutant strains, producing more β-glucosidase, are being explored.

B. *Penicillium pinophilum*

On the basis of synergism between two immunologically distinct hydrolases (probably the equivalent of CBHI and CBHII) in solubilizing cellulose, it has been suggested that the two enzymes attack the two possible different cellobiose end groups in the cellulose fibril (the unit cell of cellulose is based on cellobiose, not glucose).[452] This proposed origin of the two different cellobiohydrolases does not address their different stereochemistries of action (at least in the *T. reesei* system).

The enzyme complex from *P. pinophilum* may be more complex than that of *T. reesei*, endoglucanase, as well as CBHI and CBHII, being required to solubilize crystalline cellulose.[453] However, a comparison of the activities of CBHI and CBHII enzymes from the two sources acting on synthetic aryl glycosides reveals very close similarities indeed (the maximum difference in k_{cat} values for cleavage at any glycosidic bond in any of the substrates was a factor of 3.5).[454] Nonetheless, it was claimed that this fungus produces five major endoglucanases.

C. *C. fimi*

Like many cellulolytic bacteria, this organism possess a multienzyme complex, of glucanases and β-glucosidase. Remarkably, the endoglucanase component acts with inversion and the exoglucanase component with retention.[34]

There is a cellulose binding domain in an endoglucanase from this organism, since it can be cloned to the end of alkaline phosphatase; the alkaline phosphatase can then be immobilized on cellulose.[456] The cellulose binding domains of the endocellulose (*CenA* gene product) and the exoglucanase (*Cex* gene product) have been separately excised proteolytically and shown not to affect catalysis of soluble substrates.[457] The nucleotide sequence of the *CenA* gene is known and is homologous with that of CBHII.[34]

D. Other Organisms

The role of the ability to be adsorbed on crystalline cellulose (which can now be reasonably associated with the cellulose binding domains detected in many enzymes) has been correlated with the ability to hydrolyze it by means of a correlation involving enzymes from a large number of sources.[459] A linear correlation exists between the activity on crystalline cellulose and the product of the activity on amorphous cellulose and the independently measured adsorption constant for the enzyme in question.

The endo type cellulase from *A. niger* has been shown to hydrate cellobial to give β-2-deoxycellobiose: if the reaction is run in D_2O, the deuteron is in the axial position.[19] The exo cellulase from *Irpex lacteus*, surprisingly also a retaining enzyme, behaves similarly, but more slowly.[19]

P. chrysosporium, also called *Sporotrichum pulverulentum* and *Chrysosporium lignorum*, has been investigated from the point of view of cellulase production. Its ligninases, rather than cellulases, have been the focus of most attention, but cellulases have been isolated and characterized from culture filtrates.[460] An inverting cellobiohydrolase was also identified.[461]

Three iminoalditol inhibitors of the 4-*O*-(cellooligosaccharyl)-1,5,6-trideoxy-1,5-iminoalditol type have been made and shown to inhibit the endoglucanases of *Trichoderma fusca*, as expected. K_i values are however modest (10–100 μM).[462]

Whereas fungi excrete individual cellulase components into the external medium, bacteria appear to

operate by multienzyme complexes ("cellulosomes"). Synergy in the degradation of crystalline cellulose by two components of the cellulosome of *Clostridium thermocellum* has been observed.[463] An endoglucanase from this bacterium has been crystallized and subjected to preliminary X-ray investigation.[464]

An endoglucanase has been isolated from *Schizophyllum commune*. Its amino acid sequence has been determined and shown to be 40% homologous with that of hen egg white lysozyme.[465] By modification with diethyl pyrocarbonate the catalytic groups have been identified as Glu 33 and Asp 50.[466] The binding of substrates and inhibitors has been examined by difference spectroscopy and considered to indicate a similar pattern of behavior to lysozyme.[467] This enzyme has also been affinity labeled with ω-epoxypentyl cellobioside.[468]

V. Acknowledgments

Work in the author's laboratory has been funded by the United Kingdom Science and Engineering, and Agricultural and Food, Research Councils and by the United States National Institutes of Health, which are duly thanked.

Registry No. Glycosidase, 9032-92-2; phosphorylase, 9035-74-9.

VI. References

(1) Gruber, E. *Papier* **1976**, *30*, 533.
(2) For the common sugars glucose, galactose, xylose, and mannose, which adopt the $^4C_1$ conformation in water (Angyal, S. J. *Angew. Chem., Int. Ed. Engl.* **1969**, *8*, 157), the equatorial anomeric orientation corresponds to the β configuration and the axial orientation to the α configuration. This is not however general—for example, formal removal of merely the C-5 hydroxymethyl group of a β-D-galactopyranoside turns it into an α-L-arabinopyranoside—and so reference to, e.g., "β-glycosidases" can mislead.
(3) Sinnott, M. L. In *Enzyme Mechanisms*; Page, M. I., Williams, A., Eds.; Royal Society of Chemistry: London, 1987; p 259.
(4) Lalégerie, P.; Legler, G.; Yon, J. M. *Biochimie* **1982**, *64*, 977.
(5) For example, see: Soman, G.; Narayan, J.; Martin, B. L.; Graves, D. J. *Biochemistry* **1986**, *25*, 4113.
(6) West, R. E.; Moss, J.; Vaughan, M.; Liu, T.; Liu, T.-Y. *J. Biol. Chem.* **1985**, *260*, 14428.
(7) Schimz, K.-M.; Broll, B.; John, B. *Arch. Microbiol.* **1983**, *135*, 241.
(8) For example, see: (a) Sutherland, I. W. *Annu. Rev. Microbiol.* **1985**, *39*, 243. (b) Shibaev, V. N. *Adv. Carbohydr. Chem. Biochem.* **1986**, *44*, 277. (c) Watkins, W. M. *Carbohydr. Res.* **1986**, *149*, 1. (d) Paulson, J. C.; Colley, K. J. *J. Biol. Chem.* **1989**, *264*, 17615.
(9) Sinnott, M. L. *FEBS Lett.* **1978**, *94*, 1.
(10) Dedonder, R. A. *Annu. Rev. Biochem.* **1961**, *30*, 347.
(11) Krenitsky, T. K.; Lehikoinen, P. K.; Sinnott, M. L. *Biochem. J.* **1989**, *257*, 355.
(12) van Beynum, G. M. A.; Roels, J. A.; van Tilburg, R. *Biotechnol. Bioeng.* **1980**, *22*, 643.
(13) Huber, R. E.; Hurlburt, K. L. *Arch. Biochem. Biophys.* **1986**, *246*, 411.
(14) (a) Goldberg, R. N.; Tewari, Y. B. *J. Biol. Chem.* **1989**, *264*, 9897. (b) Tewari, Y. B.; Goldberg, R. N. *J. Biol. Chem.* **1989**, *264*, 3966.
(15) (a) Goldberg, R. N.; Tewari, Y. B.; Ahluwalia, J. C. *J. Biol. Chem.* **1989**, *264*, 9901. (b) Tewari, Y. B.; Steckler, D. K.; Goldberg, R. N.; Gitomer, W. L. *J. Biol. Chem.* **1988**, *263*. 3670.
(16) De Wolf, W. E.; Emig, F. A.; Schram, V. L. *Biochemistry* **1986**, *25*, 4132.
(17) Hehre, E. J.; Kitahata, S.; Brewer, C. F. *J. Biol. Chem.* **1986**, *261*, 2147.
(18) Kasumi, T.; Brewer, C. F.; Reese, E. T.; Hehre, E. *J. Carbohydr. Res.* **1986**, *146*, 39.
(19) Kanda, T.; Brewer, C. F.; Okada, G.; Hehre, E. J. *Biochemistry*, **1986**, *25*, 1159.
(20) Kasumi, T.; Tsumuraya, Y.; Brewer, C. F.; Kersters-Hilderson, H.; Claeyssens, M.; Hehre, E. J. *Biochemistry* **1987**, *26*, 3010.
(21) Chiba, S.; Brewer, C. F.; Okada, G.; Matsui, H.; Hehre, E. J. *Biochemistry* **1988**, *27*, 1564.
(22) Weiser, W.; Lehmann, J.; Chiba, S.; Matsui, H.; Brewer, C. F.; Hehre, E. J. *Biochemistry* **1988**, *27*, 2294.
(23) Hehre, E. J.; Matsui, H.; Brewer, C. F. *Carbohydr. Res.* **1990**, *198*, 123.
(24) Bergman, N.-Å.; Jansson, M.; Chiang, Y.; Kresge, A. J.; Yin, Y. *J. Org. Chem.* **1987**, *52*, 4449.
(25) (a) Jones, C. C.; Sinnott, M. L. *J. Chem. Soc., Chem. Commun.* **1977**, 767. (b) Cocker, D.; Sinnott, M. L. *J. Chem. Soc., Perkin Trans. 2* **1975**, 1391.
(26) Hosie, L.; Marshall, P. J.; Sinnott, M. L. *J. Chem. Soc., Perkin Trans. 2* **1984**, 1121.
(27) Hosie, L.; Sinnott, M. L. *Biochem. J.* **1985**, *226*, 437.
(28) Segal, I. H. *Enzyme Kinetics*; John Wiley: New York, Chichester, Brisbane, and Toronto, 1975; p 544.
(29) Parrish, F. W.; Reese, E. T. *Carbohydr. Res.* **1967**, *3*, 424.
(30) Chiba, S.; Kimura, A.; Matsui, H. *Agric. Biol. Chem.* **1983**, *47*, 1741.
(31) Nakakuki, T.; Azuma, K.; Kainuma, K. *Carbohydr. Res.* **1984**, *128*, 297.
(32) Knowles, J. K. C.; Lehtovaara, P.; Murray, M.; Sinnott, M. L. *J. Chem. Soc., Chem. Commun.* **1988**, 1401.
(33) Alabran, D. M.; Ball, D. H.; Reese, E. T. *Carbohydr. Res.* **1983**, *123*, 179.
(34) Withers, S. G.; Dombrowski, D.; Berven, L. A.; Kilburn, D. G.; Miller, R. C.; Warren, R. A. J.; Gilkes, N. R. *Biochem. Biophys. Res. Commun.* **1986**, *139*, 487–494.
(35) Sinnott, M. L. In *The Chemistry of Enzyme Action*; Page, M. I., Ed.; Elsevier: Amsterdam, 1984; p 389.
(36) Sinnott, M. L.; Jencks, W. P. *J. Am. Chem. Soc.* **1980**, *102*, 2026.
(37) Jencks, W. P. *Chem. Soc. Rev.* **1981**, *10*, 345.
(38) Bennet, A. J.; Sinnott, M. L. *J. Am. Chem. Soc.* **1986**, *108*, 7287.
(39) Amyes, T. L.; Jencks, W. P. *J. Am. Chem. Soc.* 1989, *111*, 7888.
(40) Deslongchamps, P. *Stereoelectronic Effects in Organic Chemistry*; Pergamon: Oxford, 1983.
(41) Kirby, A. J. *Acc. Chem. Res.* **1984**, *17*, 305.
(42) Sinnott, M. L. *Biochem. J.* **1984**, *224*, 817.
(43) Sinnott, M. L. *Adv. Phys. Org. Chem.* **1988**, *24*, 113 and references therein.
(44) Kirby, A. J. *CRC Crit. Rev. Biochem.* **1987**, *22*, 283.
(45) (a) Wałaszek, Z.; Horton, D.; Ekiel, I. *Carbohydr. Res.* **1982**, *106*, 193. (b) Nelson, C. R. *Carbohydr. Res.* **1979**, *68*, 55.
(46) Koshland, D. E. *Biol. Rev.* **1953**, *28*, 416.
(47) Klein, W.; Im, M. J.; Palm, D. *Eur. J. Biochem.* **1986**, *157*, 107.
(48) (a) Blake, C. C. F.; Mair, G. A.; North, A. C. T.; Phillips, D. C.; Sharma, V. R. *Proc. R. Soc. London, B* **1967**, *167*, 365. (b) Imoto, T.; Johnson, L. N.; North, A. C. T.; Phillips, D. C.; Rupley, J. A. In *The Enzymes*; Boyer, P. Ed.; Academic: New York, 1972; Vol. 7, p 665.
(49) Anderson, W. F.; Grütter, M. G.; Remington, S. J.; Weaver, L. H.; Matthews, B. W. *J. Mol. Biol.* **1981**, *147*, 523.
(50) Grütter, M. G.; Weaver, L. H.; Matthews, B. W. *Nature* **1983**, *303*, 828.
(51) Harada, S.; Kitadokoro, K.; Fujii, T.; Kai, Y.; Kasai, N. *J. Mol. Biol.* **1989**, *207*, 851.
(52) Pincus, M. R.; Scherage, H. A. *Acc. Chem. Res.* **1981**, *15*, 299.
(53) Post, C. B.; Karplus, M. *J. Am. Chem. Soc.* **1986**, *108*, 1317.
(54) Høj, P. B.; Rodriguez, E. B.; Stick, R. V.; Stone, B. A. *J. Biol. Chem.* **1989**, *264*, 4939.
(55) Fleet, G. W. J. *Tetrahedron Lett.* **1985**, *26*, 5073.
(56) Rosenberg, S.; Kirsch, J. F. *Biochemistry* **1981**, *20*, 3196.
(57) Lowe, G.; Sheppard, G.; Sinnott, M. L.; Williams, A. *Biochem. J.* **1967**, *104*, 893.
(58) Warshel, A.; Levitt, M. *J. Mol. Biol.* **1976**, *103*, 227.
(59) Pincus, M. R.; Scheraga, H. *Macromolecules* **1979**, *12*, 633.
(60) Warshel, A. *Acc. Chem. Res.* **1981**, *14*, 284.
(61) Warshel, A. *Proc. Natl. Acad. Sci. U.S.A.* **1984**, *81*, 444.
(62) Warshel, A.; Sussman, F.; Hwang, J.-K. *J. Mol. Biol.* **1988**, *201*, 139.
(63) Warshel, A. *Biochemistry* **1981**, *20*, 3167.
(64) Dao-Pin, S.; Liao, D.-I.; Remington, S. J. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 5361.
(65) Malcolm, B. A.; Rosenberg, S.; Corey, M. J.; Allen, J. S.; de Baetselier, A.; Kirsch, J. F. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 133.
(66) Kuroki, R.; Yamada, H.; Moriyama, T.; Imoto, T. *J. Biol. Chem.* **1986**, *261*, 13571.
(67) Fukamizo, T.; Hayashi, K.; Goto, S. *Eur. J. Biochem.* **1986**, *158*, 463.
(68) Muraki, M.; Jigami, Y.; Morikawa, M.; Tanaka, H. *Biochim. Biophys. Acta* **1987**, *911*, 376.
(69) Muraki, M.; Morikawa, M.; Jigami, Y.; Tanaka, H. *Eur. J. Biochem.* **1989**, *179*, 573.
(70) Bakthavachalam, V.; Czarnik, A. W. *Tetrahedron Lett.* **1987**, *28*, 2925.

(71) Legler, G. *Hoppe-Seyler's Z. Physiol. Chem.* (a) **1968**, *349*, 767. (b) **1970**, *351*, 25.
(72) Braun, H.; Legler, G.; Deshusses, J.; Semenza, G. *Biochim. Biophys. Acta* **1977**, 483, 135.
(73) Fowler, A.; Zabin, I. *Proc. Natl. Acad. Sci. U.S.A.* **1977**, *74*, 1507.
(74) Kalnins, A.; Otto, K.; Rüther, U.; Müller-Hill, B. *EMBO J.* **1983**, *2*, 593.
(75) Herrchen, M.; Legler, G. *Eur. J. Biochem.* **1984**, *138*, 527.
(76) Wallenfels, K.; Malhotra, O. P. *Adv. Carbohydr. Chem.* **1961**, *16*, 239.
(77) Littke, W.; John, C. *Science* **1984**, *225*, 203.
(78) Arrondo, J. L. R.; Muga, A.; Castresana, J.; Bernabeu, C.; Goñi, F. M. *FEBS Lett.* **1989**, *252*, 118.
(79) Bader, D. E.; Ring, M.; Huber, R. E. *Biochem. Biophys. Res. Commun.* **1988**, *153*, 301.
(80) Knier, B. L.; Jencks, W. P. *J. Am. Chem. Soc.* **1980**, *102*, 6789.
(81) Dahlquist, F. W.; Jao, L. Raftery, M. *Proc. Natl. Acad. Sci. U.S.A.* **1966**, *56*, 26.
(82) Arnett, E. M.; Scorrano, G. *Adv. Phys. Org. Chem.* **1976**, *13*, 83.
(83) Withers, S. G.; Rupitz, K.; Street, I. P. *J. Biol. Chem.* **1988**, *263*, 7929.
(84) Legler, G.; Pohl, S. *Carbohydr. Res.* **1986**, *155*, 119.
(85) 1,5-Dideoxy-1,5-imino-D-galactitol was first made by Paulsen's group, and its inhibition of the β-galactosidase of pig small intestinal mucosa was briefly mentioned (Paulsen, H.; Hayauchi, Y.; Sinnwell, V. *Chem. Ber.* **1980**, *113*, 2601). 5-Amino-5-deoxy-D-galactose was very recently isolated from *Streptomyces lydicus* and renamed "galactostatin" (Miyake, Y.; Ebata, M. *Agric. Biol. Chem.* **1988**, *52*, 661; *ibid.* **1988**, *52*, 1649), in apparent ignorance of this work and the work of Legler and Phol.[84]
(86) Fleet, G. W. J.; Nicholas, S. J.; Smith, P. W.; Evans, S. V.; Fellows, L. E.; Nash, R. J. *Tetrahedron Lett.* **1985**, *26*, 3127.
(87) Huber, R. E.; Brockbank, R. L. *Biochemistry* **1987**, *26*, 1526.
(88) Sinnott, M. L.; Souchard, I. J. L. *Biochem. J.* **1973**, *133*, 89.
(89) Selwood, T.; Sinnott, M. L. *Biochem. J.* **1990**, *268*, 317.
(90) Rosenberg, S.; Kirsch, J. F. *Biochemistry* **1981**, *21*, 3189.
(91) Legler, G.; Herrchen, M. *Carbohydr. Res.* **1983**, *116*, 95.
(92) Loeffler, R. S. T.; Sinnott, M. L.; Sykes, B. D.; Withers, S. G. *Biochem. J.* **1979**, *177*, 145.
(93) Ring, M.; Armitage, I. M.; Huber, R. E. *Biochem. Biophys. Res. Commun.* **1985**, *131*, 675.
(94) Ring, M.; Bader, D. E.; Huber, R. E. *Biochem. Biophys. Res. Commun.* **1988**, *152*, 1050.
(95) Cupples, C. G.; Milier, J. H. *Genetics* **1988**, *120*, 637.
(96) Lehmann, G.; Ziegler, B. *Carbohydr. Res.* **1977**, *58*, 73.
(97) Lehmann, J.; Schlesselmann, P. *Carbohydr. Res.* **1983**, *113*, 93.
(98) Viratelle, O. M.; Yon, J. *Biochemistry* **1980**, *19*, 4143.
(99) Bachmann, B. J.; Low, K. B.; Taylor, A. L. *Bacteriol. Rev.* **1976**, *40*, 116.
(100) Stokes, H. W.; Betts, P. W.; Hall, B. G. *Mol. Biol. Evol.* **1985**, *2*, 469.
(101) Hall, B. G.; Betts, P. W.; Wootton, J. C. *Genetics* **1989**, *123*, 635.
(102) Hall, B. G.; Hartl, D. *Genetics* **1975**, *81*, 427.
(103) Hall, B. G.; Zuzel, T. *J. Bacteriol.* **1980**, *144*, 1208.
(104) Reviewed by: Hall, B. G. *Evol. Biol.* **1982**, *15*, 85.
(105) Hall, B. G.; Hartl, D. *Genetics* **1974**, *76*, 391.
(106) Hall, B. G. *Genetics* **1978**, *90*, 673.
(107) Hall, B. G.; Clarke, N. D. *Genetics* **1977**, *85*, 193.
(108) Hall, B. G. *Genetics* **1988**, *120*, 887.
(109) Davis, B. D. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 5005.
(110) Hall, B. G. *J. Mol. Biol.* **1976**, *107*, 71.
(111) Hall, B. G. *Biochemistry* **1981**, *20*, 4042.
(112) Burton, J.; Sinnott, M. L. *J. Chem. Soc., Perkin Trans. 2* **1983**, 359.
(113) Fowler, A. V.; Smith, P. J. *J. Biol. Chem.* **1983**, *258*, 10204.
(114) Li, B. F. L.; Osborne, S.; Sinnott, M. L.; Yates, D. W. *J. Chem. Soc., Perkin Trans. 2* **1983**, 365.
(115) Elliott, A. C.; Li, B. F.; Morton, C. A. J.; Pownall, J. D.; Selwood, T.; Sinnott, M. L.; Souchard, I. J. L.; Stuart-Tilley, A. K. *J. Mol. Catal.* **1988**, *47*, 255.
(116) Li, B. F. L.; Holdup, D.; Morton, C. A. J.; Sinnott, M. L. *Biochem. J.* **1989**, *260*, 109.
(117) Hall, B. G.; Murray, M.; Osborne, S.; Sinnott, M. L. *J. Chem. Soc., Perkin Trans. 2* **1983**, 1595.
(118) Albery, W. J.; Knowles, J. R. *Biochemistry* **1976**, *15*, 5631.
(119) Neufeld, E. F.; Lim, T. W.; Shapiro, L. J. *Annu. Rev. Biochem.* **1975**, *44*, 357.
(120) Kobayashi, T.; Shinnoh, M.; Goto, I.; Kuroiwa, Y. *J. Biol. Chem.* **1985**, *260*, 14982.
(121) van Diggelen, O. P.; Galjaard, H.; Sinnott, M. L.; Smith, P. J. *Biochem. J.* **1980**, *188*, 337.
(122) Singer, H. S.; Tiemeyer, M.; Slesinger, P. A.; Sinnott, M. L. *Ann. Neurol.* **1987**, *21*, 497.
(123) Verheijen, F.; Brossmer, R.; Galjaard, H. *Biochem. Biophys. Res. Commun.* **1982**, *108*, 868.
(124) Hogeveen, A. T.; Verheijen, F. W.; Galjaard, H. *J. Biol. Chem.* **1983**, *258*, 12143.
(125) van der Horst, G. T. J.; Galjart, N. J.; d'Azzo, A.; Galjaard, H.; Verheijen, F. W. *J. Biol. Chem.* **1989**, *264*, 1317.
(126) Verheijen, F. W.; Palmeri, S.; Galjaard, H. *Eur. J. Biochem.* **1987**, *162*, 63.
(127) Verheijen, F. W.; Palmeri, S.; Hoogeveen, A. T.; Galjaard, H. *Eur. J. Biochem.* 1985, *149*, 315.
(128) van Diggelen, O. P.; Hogeveen, A. T.; Smith, P. J.; Reuser, A. J. J.; Galjaard, H. *Biochim. Biophys. Acta* **1982**, *703*, 69.
(129) Semenza, G. In *Mammalian Ectoenzymes*; Kenny; Turner, Eds.; Elsevier B. V.: Amsterdam, 1987; p 265.
(130) Kruse, T. A.; Bolund, L.; Grzeschik, K.-H.; Ropers, H. H.; Sjöström, H.; Norén, O.; Mantei, N.; Semenza, G. *FEBS Lett.* **1988**, *240*, 123.
(131) Mantei, N.; Villa, M. Enzler, T.; Wacker, H.; Boll, W.; James, P.; Hunziker, W.; Semenza, G. *EMBO J.* **1988**, *7*, 2705.
(132) Helferich, B.; Kleinschmidt, T.; *Hoppe Seyler's Z. Physiol. Chem.* **1965**, *340*, 31.
(133) See, e.g.: Dey, P. M. *Adv. Carbohydr. Chem. Biochem.* **1978**, *35*, 341. Akazawa, T.; Miyata, S. *Essays Biochemistry* **1982**, *18*, 40.
(134) Nath, R. L.; Rydon, H. N. *Biochem. J.* **1954**, *57*, 1.
(135) Bause, E.; Legler, G. *Hoppe-Seyler's Z. Physiol. Chem.* **1974**, *355*, 438.
(136) Legler, G.; Roeser, K.-R.; Illig, H.-K. *Eur. J. Biochem.* **1979**, *101*, 85.
(137) Roeser, K.-R.; Legler, G. *Biochem. Biophys. Acta* **1981**, *657*, 321.
(138) Legler, G. *Hoppe-Seyler's Z. Physiol. Chem.* **1967**, *348*, 1359.
(139) Legler, G.; Sinnott, M. L.; Withers, S. G. *J. Chem. Soc., Perkin Trans. 2* **1980**, 1376.
(140) Legler, G.; Jülich, E. *Carbohydr. Res.* **1984**, *128*, 61.
(141) Withers, S. G.; Street, I. P. *J. Am. Chem. Soc.* **1988**, *110*, 8551.
(142) Day, A. G.; Withers, S. G. *Can. J. Biochem. Cell. Biol.* **1986**, *64*, 914.
(143) Wakarchuck, W. W.; Kilburn, D. G.; Miller, R. C.; Warren, R. A. *Mol. Gen. Genet.* **1986**, *205*, 146.
(144) Hehre, E. J.; Genghof, D. S.; Sternlicht, H.; Brewer, C. F. *Biochemistry* **1977**, *16*, 1780.
(145) Hehre, E. J.; Brewer, C. F.; Uchiyama, T.; Schlesselmann, P.; Lehmann, J. *Biochemistry* **1980**, *19*, 3557.
(146) Dale, M. P.; Kopfler, W. P.; Chait, I.; Byers, L. D. *Biochemistry* **1986**, *25*, 2522.
(147) Pownall, J. D. *B.Sc. Thesis*, University of Bristol, Bristol, U.K., 1978.
(148) Dale, M. P.; Ensley, H. E.; Kern, K.; Sastry, K. A. R.; Byers, L. D. *Biochemistry* **1985**, *24*, 3530.
(149) Kuszmann, J.; Kiss, L. *Carbohydr. Res.* **1986**, *153*, 43.
(150) Tanaka, A.; Ito, M.; Horomi, K. *J. Biochem.* **1986**, *100*, 1379.
(151) Chida, N.; Furuno, Y.; Ogawa, S. *J. Chem. Soc., Chem. Commun.* **1989**, *1230*.
(152) Lehmann, J.; Ziser, L. *Carbohydr. Res.* **1989**, *188*, 45.
(153) Beer, D.; Vasella, A. *Helv. Chim. Acta* **1986**, *69*, 267.
(154) Aerts, G. M.; De Bruyne, C. K. *Biochim. Biophys. Acta* **1981**, *660*, 317.
(155) De Bruyne, C. K.; Aerts, M.; De Gussem, R. L. *Eur. J. Biochem.* **1979**, *102*, 257.
(156) van Doorslaer, E.; van Opstal, O.; Kersters-Hilderson, H.; De Bruyne, C. K. *Bioorg. Chem.* **1984**, *12*, 158.
(157) Umezurike, G. M. *Biochem. J.* **1988**, *254*, 73.
(158) Grabowski, A.; Gatt, S.; Kruse, J.; Desnick, R. J. *Arch. Biochem. Biophys.* **1984**, *231*, 144.
(159) Das, P. K.; Murray, G. J.; Barranger, J. A. *Eur. J. Biochem.* **1986**, *154*, 445.
(160) Grabowski, G. A.; Osiecki-Newman, K.; Dinur, T.; Fabbro, D.; Legler, G.; Gatt, S.; Desnick, R. J. *J. Biol. Chem.* **1986**, *261*, 8263.
(161) Ginns, E. I.; Choudhary, P. V.; Martin, B. M.; Winfield, S.; Stubblefield, B.; Mayor, J.; Merkle-Lehman, D.; Murray, G. J.; Bowers, L. A.; Barranger, J. A. *Biochem. Biophys. Res. Commun.* **1984**, *123*, 574.
(162) Erickson, A. H.; Ginns, E. I.; Barranger, J. A. *J. Biol. Chem.* **1985**, *260*, 14319.
(163) Basu, A.; Glew, R. H. *J. Biol. Chem.* **1985**, *260*, 13067.
(164) Sano, A.; Radin, N. S.; Johnson, L. L.; Tarr, G. E. *J. Biol. Chem.* **1988**, *263*, 19597.
(165) Maret, A.; Salvayre, R.; Negre, A.; Douste-Blazy, L. *Eur. J. Biochem.* **1983**, *133*, 283.
(166) Pöcsi, I.; Kiss, L. *Biochem. J.* **1988**, *256*, 139.
(167) Huber, R. E.; Brockbank, R. L. *Biochem. Cell. Biol.* **1988**, *66*, 830.
(168) Gopalan, V.; Glew, R. H.; Libell, D. P.; De Petro, J. J. *J. Biol. Chem.* **1989**, *264*, 15418.
(169) Gopalan, V.; Daniels, L. B.; Glew, R. H.; Clacyssens, M. *Biochem. J.* **1989**, *262*, 541.

(170) Legler, G.; Bieberich, E. *Arch. Biochem. Biophys.* **1988**, *260*, 427.
(171) Legler, G.; Bieberich, E. *Arch. Biochem. Biophys.* **1988**, *260*, 437.
(172) Eriksson, K.-E. *Pure App. Chem.* **1981**, *53*, 33.
(173) Neufeld, E. F. *J. Biol. Chem.* **1989**, *264*, 10927.
(174) Kytzia, H.-J.; Sandhoff, K. *J. Biol. Chem.* **1985**, *260*, 7568.
(175) Paw, B. H.; Kaback, M. M.; Neufeld, E. F. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 2413.
(176) Robbins, P. W.; Trimble, R. B.; Wirth, D. F.; Hering, C.; Maley, F.; Maley, G. F.; Das, R.; Gibson, B. W.; Royal, N.; Biemann, K. *J. Biol. Chem.* **1984**, *259*, 7577.
(177) Deleyn, F.; Claeyssens, M.; Van Beeumen, J.; De Bruyne, C. K. *Can. J. Biochem.* **1978**, *56*, 43.
(178) Deleyn, F.; Claeyssens, M.; De Bruyne, C. K. *Can. J. Biochem.* **1980**, *58*, 5.
(179) Deleyn, F.; Claeyssens, M.; De Bruyne, C. K.; de Bruyn, A. *Can. J. Biochem. Cell. Biol.* **1985**, *63*, 204.
(180) Sinnott, M. L.; Tzotzos, G. T.; Marshall, S. E. *J. Chem. Soc., Perkin Trans. 2* **1982**, 1665.
(181) Aerts, G. M.; Van Opstal, O.; De Bruyne, C. K. *Carbohydr. Res.* **1985**, *138*, 127.
(182) Matsuo, M.; Fujie, A.; Win, M.; Yasui, T. *Agric. Biol. Chem.* **1987**, *51*, 2367.
(183) Win, M.; Matsuo, M.; Yasui, T. *Agric. Biol. Chem.* **1987**, *51*, 3151.
(184) Matsuo, M.; Yasui, T. *Agric. Biol. Chem.* **1984**, *48*, 1845.
(185) Matsuo, M.; Yasui, T. *Agric. Biol. Chem.* **1984**, *48*, 1853.
(186) Pangbangred, W.; Shinmyo, A.; Kinoshita, S.; Okada, H. *Agric. Biol. Chem.* **1983**, *47*, 957.
(187) Fukasaki, E.; Panbangred, W.; Shinmyo, A.; Okada, H. *FEBS Lett.* **1984**, *171*, 197.
(188) Moriyama, H.; Hata, Y.; Yamaguchi, H.; Sato, M.; Shinmyo, A.; Tanaka, N.; Okada, H.; Katsube, Y. *J. Mol. Biol.* **1987**, *193*, 237.
(189) Rose, D. R.; Birnbaum, G. I.; Tan, L. U. L.; Sadler, J. M. *J. Mol. Biol.* **1987**, *194*, 755.
(190) Grêpinet, O.; Chebrou, M.-C.; Béguin, P. *J. Bacteriol.* **1988**, *170*, 4582.
(191) Comtat, J.; Defaye, J.; Driguez, H.; Ohleyer, E. *Carbohydr. Res.* **1985**, *144*, 33.
(192) Dalgaard, L. *Acta Chem. Scand.* **1983**, *B37*, 923.
(193) Powell, P. P.; Kyle, J. W.; Miller, R. D.; Pantano, J.; Grubb, J. H.; Sly, W. S. *Biochem. J.* **1988**, *250*, 547.
(194) Cenci di Bello, I.; Dorling, P.; Fellows, L.; Winchester, B. *FEBS Lett.* **1984**, *176*, 61.
(195) Niwa, T.; Tsuruoka, T.; Inouye, S.; Naito, Y.; Koeda, T.; Niida, T. *J. Biochem.* **1972**, *72*, 207.
(196) Rule, C. J.; Wurzburg, B. A.; Ganem, B. *Tetrahedron Lett.* **1985**, *26*, 5379.
(197) Friebolin, H.; Brossmer, R.; Keilich, G.; Ziegler, D.; Supp. M. *Hoppe-Seyler's Z. Physiol. Chem.* **1980**, *361*, 697.
(198) Roggentin, P.; Rothe, B.; Lottspeich, F.; Schauer, R. *FEBS Lett.* **1988**, *238*, 31.
(199) Friebolin, H.; Baumann, W.; Hauck, M.; Kurz, D.; Wajda, R.; Weisshaar, G.; Keilich, G.; Ziegler, D.; Brossmer, R.; von Nicolai, H. *Hoppe-Seyler's Z. Physiol. Chem.* **1984**, *365*, 1309.
(200) Shaw, M. W.; Lamb, R. A.; Erickson, B. W.; Briedis, J. D.; Choppin, P. W. *Proc. Natl. Acad. Sci. U.S.A.* **1982**, *79*, 6817.
(201) Varghese, J. N.; Laver, W. G.; Colman, P. M. *Nature* **1983**, *303*, 35.
(202) Colman, P. M.; Varghese, J. N.; Laver, W. G. *Nature* **1983**, *303*, 41.
(203) Bossart, P. J.; Sudhakar Babu, Y.; Cook, W. J.; Air, G. M.; Laver, W. G. *J. Biol. Chem.* **1988**, *263*, 6421.
(204) Lentz, M. R.; Webster, R. G.; Air, G. M. *Biochemistry* **1987**, *26*, 5351.
(205) Harth, G.; Haidaris, C. G.; So, M. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 8320.
(206) Brossmer, R.; Holmquist, L. *Hoppe Seyler's Physiol. Chem.* **1971**, *352*, 1715.
(207) Eschenfelder, V.; Brossmer, R.; Wachter, M. *Hoppe-Seyler's Z. Physiol. Chem.* **1983**, *364*, 1411.
(208) Kumar, V.; Kessler, J.; Scott, M. E.; Patwardhan, B. H.; Tanenbaum, S. W.; Flashner, M. *Carbohydr. Res.* **1981**, *94*, 123.
(209) Zbiral, E.; Brandstetter, H. H.; Christian, R.; Schauer, R. *Liebigs Ann. Chem.* **1987**, 781.
(210) Gross, H. J.; Kovac, A.; Rose, U.; Watzlawick, H.; Brossmer, R. *Biochemistry* **1988**, *27*, 4279.
(211) Zbiral, E.; Schreiner, E.; Salunkhe, M. M.; Schulz, G.; Kleineidam, R. G.; Schauer, R. *Liebigs Ann. Chem.* **1989**, 519.
(212) Baumberger, F.; Vasella, A.; Schauer, R.; *Helv. Chim. Acta* **1988**, *71*, 429.
(213) Varki, A.; Diaz, S. *J. Biol. Chem.* **1983**, *258*, 12465.
(214) Noshimura, Y.; Wang, W.; Kondo, S.; Aoyagi, T.; Umezawa, H. *J. Am. Chem. Soc.* **1988**, *110*, 7249.
(215) Altmann, F.; März, L.; Stirm, S.; Unger, F. M. *FEBS Lett.* **1987**, *221*, 145.
(216) MacGregor, E. A.; Svensson, B. *Biochem. J.* **1989**, *259*, 145.
(217) Manners, D. J. *Essays Biochem.* **1974**, *10*, 37.
(218) Bock, K. *Pure Appl. Chem.* **1987**, *59*, 1447.
(219) Omichi, K.; Ikenaka, T. *J. Biochem.* **1986**, *99*, 291.
(220) Seigner, C.; Prodanov, E.; Marchis-Mouren, G. *Eur. J. Biochem.* **1985**, *148*, 161.
(221) (a) Nakatai, H. *Arch. Biochem. Biophys.* **1988**, *263*, 364. (b) Ishikawa, K.; Hirata, H. *Arch. Biochem. Biophys.* **1989**, *272*, 356.
(222) Matsuura, Y.; Kusunoki, M.; Harada, W.; Kakudo, M. *J. Biochem.* **1984**, *95*, 697.
(223) Suganama, T.; Matsuno, R.; Ohnishi, M.; Hiromi, K. *J. Biochem.* **1978**, *84*, 293.
(224) Buisson, G.; Duée, E.; Haser, R.; Payan, F. *EMBO J.* **1987**, *6*, 3909.
(225) Gobius, K. S.; Pemberton, J. M. *J. Bacteriol.* **1988**, *170*, 1325.
(226) Long, C. M.; Virolle, M.-J.; Chang, S.-Y.; Chang, S.; Bibb, M. J. *J. Bacteriol.* **1987**, *169*, 5745.
(227) Tao, B. Y.; Reilly, P. J.; Robyt, J. F. *Biochim. Biophys. Acta* **1989**, *995*, 214.
(228) Braun, P. J.; French, D.; Robyt, J. F. *Carbohydr. Res.* **1985**, *143*, 107.
(229) Nagamine, Y.; Omichi, K.; Ikenaka, T. *J. Biochem.* **1988**, *104*, 409.
(230) Omichi, K.; Ikenaka, T. *J. Biochem.* **1988**, *104*, 881.
(231) Omichi, K.; Shiosaki, K.; Matsubara, K.; Ikenaka, T. *J. Biochem.* **1989**, *106*, 646.
(232) Nagamine, Y.; Omichi, K.; Ikenaka, T. *J. Biochem.* **1988**, *104*, 667.
(233) Matsui, H.; Blanchard, J. S.; Brewer, C. F.; Hehre, E. J. *J. Biol. Chem.* **1989**, *264*, 8714.
(234) Schlesselman, P.; Fritz, H.; Lehmann, J.; Uchiyama, T.; Brewer, C. F.; Hehre, E. J. *Biochemistry* **1982**, *21*, 6606.
(235) Kornfeld, R.; Kornfield, S. *Annu. Rev. Biochem.* **1985**, *54*, 631.
(236) Liu, P. S. *J. Org. Chem.* **1987**, *52*, 4717.
(237) Kaushal, G. P.; Pan, Y. T.; Tropea, J. E.; Mitchell, M.; Liu, P.; Elbein, A. D. *J. Biol. Chem.* **1988**, *263*, 17278.
(238) Tropea, J. E.; Molyneux, R. J.; Kaushal, G. P.; Pan, Y. T.; Mitchell, M.; Elbein, A. D. *Biochemistry* **1989**, *28*, 2027.
(239) Chambers, J. P.; Elbein, A. D.; Williams, J. C. *Biochem. Biophys. Res. Commun.* **1982**, *107*, 1490.
(240) Hoefsloot, L. H.; Hoogeveen-Westerveld, M.; Kroos, M. A.; van Beeumen, J.; Reuser, A. J. J.; Oostra, B. A. *EMBO J.* **1988**, *7*, 1697.
(241) Calder, P. C.; Geddes, R. *Carbohydr. Res.* **1989**, *191*, 71.
(242) Legler, G.; Lotz, W. *Hoppe Seyler's Z. Physiol. Chem.* **1973**, *354*, 243.
(243) Shou-jun, Y.; Su-guo, G.; Yu-cheng, Z.; Shu-zheng, Z. *Biochim. Biophys. Acta* **1985**, *828*, 236.
(244) Saul, R.; Chambers, J. P.; Molyneux, R. J.; Elbein, A. D. *Arch. Biochem. Biophys.* **1983**, *221*, 593.
(245) Jegge, S.; Lehman, J. *Carbohydr. Res.* **1985**, *142*, 47.
(246) Halazy, S.; Danzin, C.; Ehrhard, A.; Gerhart, F. *J. Am. Chem. Soc.* **1989**, *111*, 3484.
(247) Schweden, J.; Borgmann, C.; Legler, G.; Bause, E. *Arch. Biochem. Biophys.* **1986**, *248*, 335.
(248) Hauser, H.; Semenza, G. *CRC Crit. Rev. Biochem.* **1983**, *14*, 319.
(249) Hunziker, W.; Spiess, M.; Semenza, G.; Lodish, H. F. *Cell.* **1986**, *46*, 227.
(250) Cogoli, A.; Semenza, G. *J. Biol. Chem.* **1975**, *250*, 7802.
(251) Hanozet, G.; Pircher, H.-P.; Vanni, P.; Oesch, B.; Semenza, G. *J. Biol. Chem.* **1981**, *256*, 3703.
(252) Truscheit, E.; Frommer, W.; Junge, B.; Müller, L.; Schmidt, D. D.; Wingender, W. *Angew. Chem., Int. Ed. Engl.* **1981**, *20*, 738.
(253) Hayashida, M.; Sakairi, N.; Kuzuhara, H.; Yajima, M. *Carbohydr. Res.* **1989**, *194*, 233.
(254) Danzin, C.; Erhard, A. *Arch. Biochem. Biophys.* **1987**, *257*, 472.
(255) Horii, S.; Fukase, H.; Matsuo, T.; Kameda, Y.; Asano, N.; Matsui, K. *J. Med. Chem.* **1986**, *29*, 1038.
(256) Kite, G. C.; Fellows, L. E.; Fleet, G. W. J.; Liu, P. S.; Scofield, A. M.; Smith, N. G. *Tetrahedron Lett.* **1988**, *29*, 6483.
(257) McGhee, J. R.; Michalek, S. M. *Annu. Rev. Microbiol.* **1981**, *35*, 595.
(258) Binder, T. P.; Côté, G. L.; Robyt, J. F. *Carbohydr. Res.* **1983**, *124*, 275.
(259) Parnaik, V.; Luzio, G. A.; Grahame, D. A.; Ditson, S. L.; Mayer, R. M. *Carbohydr. Res.* **1983**, *121*, 257.
(260) Luzio, G. A.; Parnaik, V. K.; Mayer, R. M. *Carbohydr. Res.* **1983**, *121*, 269.
(261) Ditson, S. L.; Sung, S. M.; Mayer, R. M. *Arch. Biochem. Biophys.* **1986**, *249*, 53.
(262) Robyt, J. F.; Walseth, T. F. *Carbohydr. Res.* **1978**, *61*, 433.
(263) Robyt, J. F.; Eklund, S. H. *Carbohydr. Res.* **1983**, *121*, 279.
(264) Sato, S.; Koga, T.; Inoue, M. *Carbohydr. Res.* **1984**, *134*, 293.
(265) Asem, K. G.; Kenney, A. C.; Cole, J. A. *Arch. Biochem. Biophys.* **1986**, *244*, 607.
(266) Shimamura, A.; Tsumori, H.; Mukasa, H. *FEBS Lett.* **1983**, *157*, 79.

(267) Mooser, G.; Shur, D.; Lyou, M.; Watanabe, C. *J. Biol. Chem.* **1985**, *260*, 6907.
(268) McAlister, D.; Doyle, R. J.; Taylor, K. G. *Carbohydr. Res.* **1989**, *187*, 131.
(269) Hanada, N.; Takehara, T. *Carbohydr. Res.* **1987**, *168*, 120.
(270) Yamashita, Y.; Hanada, N.; Takehara, T. *Biochem. Biophys. Res. Commun.* **1988**, *150*, 687.
(271) Mooser, G.; Iwaoka, K. R. *Biochemistry* **1989**, *28*, 443.
(272) Tanriseven, A.; Robyt, J. F. *Carbohydr. Res.* **1989**, *186*, 87.
(273) Tulsiani, D. R. P.; Harris, T. M.; Touster, O. *J. Biol. Chem.* **1982**, *257*, 7936.
(274) Abraham, D. J.; Sidebotham, R.; Winchester, B. G.; Dorling, P. R.; Dell, A. *FEBS Lett.* **1983**, *163*, 110.
(275) Blough, H. A.; Pauwels, R.; de Clercq, E.; Cogniaux, J.; Sprecher-Goldberger, S.; Thiry, L. *Biochem. Biophys. Res. Commun.* **1986**, *141*, 33.
(276) Sunkara, P. S.; Bowlin, T. L.; Liu, P. S.; Sjoerdsma, A. *Biochem. Biophys. Res. Commun.* **1987**, *148*, 206.
(277) Winkler, D. A.; Holan, G. *J. Med. Chem.* **1989**, *32*, 2084.
(278) Elbein, A. D.; Szumilo, T.; Sanford, B. A.; Sharpless, K. B.; Adams, C. *Biochemistry* **1987**, *26*, 2502.
(279) Molyneux, R. J.; Roitman, J. N.; Dunnheim, G.; Szumilo, T.; Elbein, A. D. *Arch. Biochem. Biophys.* **1986**, *251*, 450.
(280) Tadano, K.; Morita, M.; Hotta, Y.; Ogawa, S.; Winchester, B.; Cenci di Bello, I. *J. Org. Chem.* **1988**, *53*, 5209.
(281) Cenci di Bello, I.; Fleet, G.; Namgoong, S. K.; Tadano, K.-I.; Winchester, B. *Biochem. J.* **1989**, *259*, 855.
(282) Bischoff, J.; Kornfeld, R. *Biochem. Biophys. Res. Commun.* **1984**, *125*, 324.
(283) Tulsiani, D. R. P.; Broquist, H. P.; Touster, O. *Arch. Biochem. Biophys.* **1985**, *236*, 427.
(284) Al Daher, S.; Fleet, G.; Namgoong, S. K.; Winchester, B. *Biochem. J.* **1989**, *258*, 613.
(285) Docherty, P. A.; Kuranda, M. J.; Aronson, N. N.; BeMiller, J. N.; Myers, R. W.; Bohn, J. A. *J. Biol. Chem.* **1986**, *261*, 3457.
(286) McDowell, W.; Tlusty (Trusty?), A.; Rott, R.; BeMiller, J. N.; Bohn, J. A.; Myers, R. W.; Schwartz, R. T. *Biochem. J.* **1988**, *255*, 991.
(287) Docherty, P. A.; Aronson, N. N. *Biochim. Biophys. Acta* **1987**, *914*, 283.
(288) Fleet, G. W. J.; Namgoong, S. K.; Barker, C.; Baines, S.; Jacob, G. S.; Winchester, B. *Tetrahedron Lett.* **1989**, *30*, 4439.
(289) White, W. J.; Schray, K. J.; Legler, G.; Alhadeff, J. A. *Biochim. Biophys. Acta* **1987**, *912*, 132.
(290) Lehmann, J.; Ziser, L. *Carbohydr. Res.* **1988**, *183*, 301.
(291) Tong, M. K.; Ganem, B. *J. Am. Chem. Soc.* **1988**, *110*, 312.
(292) Michaelis, L.; Menten, M. L. *Biochem. Z.* **1913**, *49*, 333.
(293) Shomi, N.; Onodera, S. *Agric. Biol. Chem.* **1988**, *52*, 2347.
(294) Bacon, J. S. D. *Biochem. J.* **1952**, *50*, xviii.
(295) Chu, F. K.; Takase, K.; Guarino, D.; Maley, F. *Biochemistry* **1985**, *24*, 6125.
(296) Williams, R. S.; Trumbly, R. J.; MacColl, R.; Trimble, R. B.; Maley, F. *J. Biol. Chem.* **1985**, *260*, 13334.
(297) App, H.; Holzer, H. *J. Biol. Chem.* **1989**, *264*, 17583.
(298) Braun, H. *Biochim. Biophys. Acta* **1976**, *452*, 452. *Ibid.* **1977**, *485*, 141.
(299) Card, P. J.; Hitz, W. D. *J. Org. Chem.* **1985**, *50*, 891.
(300) Chambert, R.; Petit-Glatron, M.-F. *Carbohydr. Res.* **1989**, *191*, 117.
(301) Yamamoto, S.; Iizuka, M.; Tanaka, T.; Yamamoto, T. *Agric. Biol. Chem.* **1985**, *49*, 343.
(302) Chambert, R.; Gonzy-Treboul, G.; Dedonder, R. *Eur. J. Biochem.* **1974**, *41*, 285.
(303) Chambert, R.; Gonzy-Treboul, G. *Eur. J. Biochem.* **1976**, *71*, 493.
(304) Chambert, R.; Gonzy-Treboul, G. *Eur. J. Biochem.* **1976**, *62*, 55.
(305) Lebrun, E.; Van Rapenbush, R. *J. Biol. Chem.* **1980**, *255*, 12034.
(306) Sancar, A.; Sancar, G. B. *Annu. Rev. Biochem.* **1988**, *57*, 29.
(307) Percival, K. J.; Klein, M. B.; Burger, P. M. J. *J. Biol. Chem.* **1989**, *264*, 2593.
(308) De Wolf, M. J. S.; van Dessel, G. A. F.; Lagrou, A. R.; Hilderson, H. J. J.; Dierick, W. S. H. *Biochem. J.* **1985**, *226*, 415.
(309) Schuber, F.; Travo, P.; Pascal, M. *Bioorg. Chem.* **1979**, *8*, 83.
(310) Yost, D. A.; Anderson, B. M. *J. Biol. Chem.* **1982**, *257*, 767.
(311) Yost, D. A.; Anderson, B. M. *J. Biol. Chem.* **1983**, *258*, 3075.
(312) Tarnus, C.; Schuber, F. *Bioorg. Chem.* **1987**, *15*, 31.
(313) Bull, H. G.; Ferraz, J. P.; Cordes, E. H.; Ribbi, A.; Apitz-Castro, R. *J. Biol. Chem.* **1978**, *253*, 5186.
(314) Byrde, R. J. W.; Fielding, A. H.; Archer, S. A.; Davies, E. In *Fungal Pathogenicity and the Plant's Response*; Byrde, R. J. W., Cutting, C. V., Eds.; Academic Press: London, 1973; p 39.
(315) (a) Laborda, F.; Fielding, A. H.; Byrde, R. J. W. *J. Gen. Microbiol.* **1973**, *79*, 321. (b) Laborda, F.; Archer, S. A.; Fielding, A. H.; Byrde, R. J. W. *J. Gen. Microbiol.* **1974**, *81*, 151.
(316) Fielding, A. H.; Sinnott, M. L.; Kelly, M. A.; Widdows, D. *J. Chem. Soc., Perkin Trans 1* **1981**, 1013.
(317) Kelly, M. A.; Sinnott, M. L.; Herrchen, M. *Biochem. J.* **1987**, *245*, 843.
(318) Selwood, T.; Sinnott, M. L. *Biochem. J.* **1988**, *254*, 899.
(319) Axamawaty, M. T. H.; Fleet, G. W. J.; Hannah, K. A.; Namgoong, S. K.; Sinnott, M. L. *Biochem. J.* **1990**, *266*, 245.
(320) Singh, A. N.; Hester, L. S.; Raushel, F. M. *J. Biol. Chem.* **1987**, *262*, 2554.
(321) Fox, J.; Govons, S.; Preiss, J. *Methods Enzymol.* **1972**, *28*, 539.
(322) Kumar, A.; Larsen, C. E.; Preiss, J. *J. Biol. Chem.* **1986**, *261*, 16256.
(323) Fox, J.; Kawaguchi, K.; Greenberg, E.; Preiss, J. *Biochemistry* **1976**, *15*, 849.
(324) Mahrenholz, A. M.; Wang, Y.; Roach, P. J. *J. Biol. Chem.* **1988**, *263*, 10561.
(325) Kim, S. C.; Singh, A. N.; Raushel, F. M. *J. Biol. Chem.* **1988**, *263*, 10151.
(326) Campbell, D. G. C.; Cohen, P. *Eur. J. Biochem.* **1989**, *185*, 119.
(327) Smyth, C.; Caudwell, F. B.; Ferguson, M.; Cohen, P. *EMBO J.* **1988**, *7*, 2681.
(328) Mieyal, J. J.; Abeles, R. H. In *The Enzymes, 3rd Edition*; Boyer, P. D., Ed.; Academic Press: New York, 1972; Vol. 7, p 515.
(329) Fletterick, R. J.; Sprang, S. R. *Acc. Chem. Res.* **1982**, *15*, 361.
(330) Helmreich, E. J. M.; Klein, H. W. *Angew. Chem., Int. Ed. Engl.* **1980**, *19*, 441.
(331) Withers, S. G.; Sykes, B. D.; Madsen, N. B.; Kasvinsky, P. J. *Biochemistry* **1979**, *18*, 5342.
(332) Kobayashi, M.; Yokoyama, I.; Matsuda, K. *Agric. Biol. Chem.* **1986**, *50*, 2585.
(333) Withers, S. G. *J. Biol. Chem.* **1985**, *260*, 841.
(334) Change, Y. C.; Graves, D. J. *J. Biol. Chem.* **1985**, *260*, 2709.
(335) Spring, S. R.; Goldsmith, E. J.; Fletterick, R. J.; Withers, S. G.; Madsen, N. B. *Biochemistry* **1982**, *21*, 5364.
(336) Johnson, L. N.; Jenkins, J. A.; Wilson, K. S.; Stura, E. A.; Zanotti, V. G. *J. Mol. Biol.* **1980**, *140*, 565.
(337) Tagaya, M.; Fukui, T. *J. Biol. Chem.* **1984**, *259*, 4860.
(338) Bourne, E. J.; Finch, P.; Nagpurkar, A. G. *J. Chem. Soc., Perkin Trans. 1* **1972**, 2202.
(339) Withers, S. G.; Madsen, N. B.; Sykes, B. D. *Biochemistry* **1981**, *20*, 1748.
(340) Withers, S. G.; Madsen, N. B.; Sprang, S. R.; Fletterick, R. J. *Biochemistry* **1982**, *21*, 5372.
(341) Takagi, S.; Kobayashi, M.; Matsuda, K. *J. Biochem.* **1989**, *105*, 933.
(342) Klein, H. W.; Palm, D.; Helmreich, E. J. *Biochemistry* **1982**, *21*, 6675.
(343) Hajdu, J.; Acharya, K. R.; Stuart, D. I.; McLaughlin, P. J.; Barford, D.; Oikonomakos, N. G.; Klein, H.; Johnson, L. N. *EMBO J.* **1987**, *6*, 539.
(344) Barford, D.; Schwabe, J. W. R.; Oikonomakos, N. G.; Acharya, K. R.; Hajdu, J.; Papageorgiou, A. C.; Martin, J. L.; Knott, J. C. A.; Vasella, A.; Johnson, L. M. *Biochemistry* **1988**, *27*, 6733.
(345) Papageorgiou, A. C.; Oikonomakos, N. G.; Leonidas, D. D. *Arch. Biochem. Biophys.* **1989**, *272*, 376.
(346) Palm, D.; Blumenauer, G.; Klein, H. W.; Blanc-Muesser, M. *Biochem. Biophys. Res. Commun.* **1983**. *111*, 530.
(347) Kumar, A.; Sanwal, G. G. *Arch. Biochem. Biophys.* **1982**, *217*, 341.
(348) Withers, S. G.; Madsen, N. B.; Sykes, B. D.; Takagi, M.; Shimomura, S.; Fukui, T. *J. Biol. Chem.* **1981**, *256*, 10759.
(349) Withers, S. G.; Shechosky, S.; Madsen, N. B. *Biochem. Biophys. Res. Commun.* **1982**, *108*, 322.
(350) Tagaya, M.; Horinishi, N.; Fukui, T. *J. Biol. Chem.* **1987**, *262*, 3092.
(351) Street, I. P.; Armstrong, C. R.; Withers, S. G. *Biochemistry* **1986**, *25*, 6021.
(352) Goldsmith, E. J.; Fletterick, R. J.; Withers, S. G. *J. Biol. Chem.* **1987**, *262*, 1449.
(353) Firsov, L. M.; Bogacheva, T. I.; Bresler, S. E. *Eur. J. Biochem.* **1974**, *42*, 605.
(354) Street, I. P.; Rupitz, K.; Withers, S. G. *Biochemistry* **1989**, *28*, 1581.
(355) Withers, S. G.; Percival, M. D.; Street, I. P. *Carbohydr. Res.* **1989**, *187*, 43.
(356) Thoma, J. A.; Koshland, D. E. *J. Biol. Chem.* **1960**, *235*, 2511.
(357) Hehre, E. J.; Brewer, C. F.; Genghof, D. S. *J. Biol. Chem.* **1979**, *254*, 5942.
(358) Kitahata, S.; Brewer, C. F.; Genghof, D. S.; Sawai, T.; Hehre, E. J. *J. Biol. Chem.* **1981**, *256*, 6017.
(359) Jeffcoat, R.; Kirkwood, S. *J. Biol. Chem.* **1987**, *262*, 1088.
(360) Ohno, N.; Nono, I.; Yadomae, T. *Carbohydr. Res.* **1989**, *194*, 261.
(361) McCleary, B. V. *Carbohydr. Res.* **1982**, *101*, 75.
(362) Frederick, M. M.; Frederick, J. R.; Fratzke, A. R.; Reilly, P. J. *Carbohydr. Res.* **1981**, *97*, 87.

(363) Kersters-Hilderson, H.; Claeyssens, M.; Van Doorslaer, E.; De Bruyne, C. K. *Carbohydr. Res.* **1976**, *47*, 269.
(364) Shinshi, H.; Wenzler, H.; Neuhaus, J.-M.; Felix, G.; Hofsteenge, J.; Meins, F. *Proc. Natl. Acad. Sci. US.A.* **1988**, *85*, 5541.
(365) Kombrink, E.; Schröder, M.; Hahlbrock, K. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 782.
(366) Van den Bulcke, M.; Bauw, G.; Castresana, C.; Van Montagu, M.; Vandekerckhove, J. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 2673.
(367) Simpson, F. J. *Can. J. Microbiol.* **1954**, *1*, 131.
(368) Saman, E.; Claeyssens, M.; De Bruyne, C. K. *Eur. J. Biochem.* **1978**, *85*, 301.
(369) Pangbangred, W.; Kawaguchi, O.; Tomita, T.; Shinmyo, A.; Okada, H. *Eur. J. Biochem.* **1984**, *138*, 267.
(370) Moriyama, H.; Fukusaki, E.; Crespo, J. C.; Shinmyo, A.; Okada, H. *Eur. J. Biochem.* **1987**, *166*, 539.
(371) Van Droorslaer, E.; Kersters-Hilderson, H.; De Bruyne, C. K. *Carbohydr. Res.* **1985**, *140*, 342.
(372) Kersters-Hilderson, H.; Loontiens, F.; Claeyssens, M.; De Bruyne, C. K. *Eur. J. Biochem.* **1969**, *7*, 434.
(373) Marshall, P. J.; Sinnott, M. L. *Biochem. J.* **1983**, *215*, 67.
(374) Kersters-Hilderson, H.; Van Doorslaer, E.; De Bruyne, C. K. *Carbohydr. Res.* **1978**, *65*, 219.
(375) Claeyssens, M.; De Bruyne, C. K. *Biochim. Biophys. Acta* **1978**, *533*, 98.
(376) Sakuda, S.; Isogai, A.; Matsumoto, S.; Suzuki, A.; Koseki, K. *Tetrahedron Lett.* **1986**, *27*, 2475.
(377) Bergfors, T.; Rouvinen, J.; Lehtovaara, P.; Caldentey, X.; Tomme, P.; Claeyssens, M.; Pettersson, G.; Teeri, T.; Knowles, J.; Jones, T. A. *J. Mol. Biol.* **1989**, *209*, 167.
(378) Rouvinen, J.; Bergfors, T.; Teeri, T.; Knowles, J. K. C.; Jones, T. A. *Science* **1990**, *249*, 380.
(379) van Tilbeurgh, H.; Pettersson, G.; Bhikhabi, R.; de Boeck, H.; Claeyssens, M. *Eur. J. Biochem.* **1985**, *148*, 329–334.
(380) Selwood, T.; Sinnott, M. L. In *Molecular Mechanisms of Bioorganic Processes*; Golding, B. T., Bleasedale, C., Eds.; Royal Society of Chemistry: London, 1990; in press.
(381) Defaye, J.; Driguez, H.; Henrissat, B.; Bar-Gilloux, E. *Carbohydr. Res.* **1983**, *124*, 265.
(382) Nakano, M.; Brewer, C. F.; Kasumi, T.; Hehre, E. J. *Carbohydr. Res.* **1989**, *194*, 139.
(383) Mittenbühler, K.; Holzer, H. *J. Biol. Chem.* **1988**, *263*, 8537.
(384) Hehre, E. J.; Sawai, T.; Brewer, C. F.; Nakano, M.; Kanda, T. *Biochemistry* **1982**, *21*, 3090.
(385) Defaye, J.; Driguez, H.; Henrissat, B.; Bar-Gilloux, E. *Nouv. J. Chim.* **1980**, *4*, 59.
(386) Bock, K.; Defaye, J.; Driguez, H.; Bar-Gilloux, E. *Eur. J. Biochem.* **1983**, *131*, 595.
(387) Iwama, M.; Takahashi, T.; Inokuchi, N.; Koyama, T.; Irie, M. *J. Biochem.* **1985**, *98*, 341.
(388) Semenza, G.; von Balthazar, A.-K. *Eur. J. Biochem.* **1974**, *41*, 149.
(389) Hiromi, K.; Tanaka, A.; Ohnishi, M. *Biochemistry* **1982**, *21*, 102.
(390) Ohnishi, M.; Hiromi, K. *Carbohydr. Res.* **1989**, *195*, 138.
(391) Boel, E.; Hansen, M. T.; Hjort, H.; Høegh, I.; Fiil, N. P. *EMBO J.* **1984**, *3*, 1581.
(392) Uozumi, N.; Sakurai, K.; Sasaki, T.; Takekawa, S.; Yamagata, H.; Tsukagoshi, N.; Udaka, S. *J. Bacteriol.* **1989**, *171*, 375.
(393) Nomura, K.; Mikami, B.; Nagao, Y.; Morita, Y. *J. Biochem.* **1987**, *102*, 333.
(394) Isoda, Y.; Nitta, Y. *Agric. Biol. Chem.* **1988**, *52*, 271.
(395) Nitta, Y.; Isoda, Y.; Toda, H.; Sakiyuma, F. *J. Biochem.* **1989**, *105*, 573.
(396) Ueda, K.; Hayaishi, O. *Annu. Rev. Biochem.* **1985**, *54*, 73.
(397) Osborne, J. C.; Stanley, S. J.; Moss, J. *Biochemistry* **1985**, *24*, 5235.
(398) Greenfield, L.; Bjorn, M. J.; Horn, G.; Fong, D. A.; Buck, G. A.; Collier, R. J.; Kaplan, D. A. *Proc. Natl. Acad. Sci. U.S.A.* **1983**, *80*, 6853.
(399) Tweten, R. K.; Barbieri, J. T.; Collier, R. J. *J. Biol. Chem.* **1985**, *260*, 10392.
(400) Soman, G.; Narayan, J.; Martin, B. L.; Graves, D. J. *Biochemistry* **1986**, *25*, 4113.
(401) DeWolf, W. E.; Emig, F. A.; Schramm, V. L. *Biochemistry* **1986**, *25*, 4132.
(402) Giranda, V. L.; Berman, H. M.; Schramm, V. L. *J. Biol. Chem.* **1986**, *261*, 15307.
(403) Giranda, V. L.; Berman, H. M.; Schramm, V. L. *J. Biol. Chem.* **1989**, *264*, 15674.
(404) Parkin, D. W.; Schramm, V. L. *Biochemistry* **1987**, *26*, 913.
(405) Mentch, F.; Parkin, D. W.; Schramm, V. L. *Biochemistry* **1987**, *26*, 921.
(406) Skoog, M. T. *J. Biol. Chem.* **1989**, *261*, 4451.
(407) Parkin, D. W.; Schramm, V. L. *J. Biol. Chem.* **1984**, *259*, 9418.
(408) Magdalou, J.; Hochman, Y.; Zakim, D. *J. Biol. Chem.* **1982**, *257*, 13624.
(409) Mackenzie, P. I. *J. Biol. Chem.* **1986**, *261*, 14112.
(410) Lewis, A. S. *J. Biol. Chem.* **1977**, *252*, 732.
(411) Cook, W. J.; Ealick, S. E.; Krenitsky, T. A.; Stoeckler, J. D.; Helliwell, J. R.; Bugg, C. E. *J. Biol. Chem.* **1985**, *260*, 12968.
(412) Jensen, K. F.; Nygaard, P. *Eur. J. Biochem.* **1975**, *51*, 253.
(413) el Kouni, M. H.; Naguib, F. N. M.; Niedzwicki, J. G.; Iltzsch, M. H.; Cha, S. *J. Biol. Chem.* **1988**, *263*, 6081.
(414) Bzowska, A.; Kulikowska, E.; Darzynkiewicz, E.; Shugar, D. *J. Biol. Chem.* **1988**, *263*, 9212.
(415) Stoeckler, J. D.; Cambor, C.; Parks, R. E. *Biochemistry* **1980**, *19*, 102.
(416) Stein, R. L.; Cordes, E. H. *J. Biol. Chem.* **1981**, *256*, 767.
(417) Vita, A.; Huang, C. Y.; Magni, G. *Arch. Biochem. Biophys.* **1983**, *226*, 687.
(418) Cook, W. J.; Koszalka, G. W.; Hall, W. W.; Narayana, S. V. L.; Ealick, S. E. *J. Biol. Chem.* **1987**, *262*, 2852.
(419) Cook, W. J.; Koszalka, G. W.; Hall, W. W.; Burns, C. L.; Ealick, S. E. *J. Biol. Chem.* **1987**, *262*, 3788.
(420) Van Tilbeurgh, H.; Loontiens, F. G.; Engelborgs, Y.; Claeyssens, M. *Eur. J. Biochem.* **1989**, *184*, 553.
(421) McCarthy, A. J. *FEMS Microbiol. Rev.* **1987**, *46*, 145.
(422) Jencks, W. P. *Adv. Enzymol.* **1975**, *43*, 219.
(423) Kirk, T. K.; Farrell, R. L. *Annu. Rev. Microbiol.* **1987**, *41*, 465.
(424) Mandels, M.; Reese, E. T. *Dev. Ind. Microbiol.* **1964**, *5*, 5.
(425) Montenecourt, B. S. *Trends. Biotechnol.* **1983**, *1*, 156.
(426) Enari, T.-M.; Niku-Paavola, M.-L. *CRC Crit. Rev. Biotechnol.* **1987**, *5*, 67.
(427) Niku-Paavola, M.-L.; Lappalainen, A.; Enari, T.-M.; Nummi, M. *Biochem. J.* **1985**, *231*, 75.
(428) Capon, B.; Thomson, J. W. *Bioorg. Chem.* **1979**, *8*, 147.
(429) Legler, G.; Bause, E. *Carbohydr. Res.* **1973**, *28*, 45.
(430) Beldman, G.; Searle-Van Leeuwen, M. F.; Rombouts, F. M.; Voragen, F. G. H. *Eur. J. Biochem.* **1985**, *146*, 301.
(431) Fägerstam, L.; Pettersson, L. G. *FEBS Lett.* **1980**, *119*, 97.
(432) Henrissat, B.; Driguez, H.; Viet, C.; Schülein, M. *BioTechnology* **1985**, *3*, 722.
(433) Niku-Paavola, M.-L.; Lappalainen, A.; Enari, T.-M.; Nummi, M. *Biotechnol. Appl. Biochem.* **1986**, *8*, 449.
(434) Woodward, J.; Lima, M.; Lee, N. E. *Biochem. J.* **1988**, *255*, 895.
(435) Chanzy, H.; Henrissat, B.; Vuong, R. *FEBS Lett.* **1984**, *172*, 193.
(436) (a) Teeri, T.; Salovuori, I.; Knowles, J. *BioTechnology* **1983**, *1*, 696. (b) Shoemaker, S.; Schweikart, V.; Ladner, M.; Gelfand, D.; Kwok, S.; Myambo, K.; Innis, M. A. *BioTechnology* **1983**, *1*, 691.
(437) (a) Teeri, T. T.; Lehtovaara, P.; Kauppinen, S.; Salovuori, I.; Knowles, J. K. C. *Gene* **1987**, *51*, 43. (b) Chen, C. M.; Gritzali, M.; Stafford, D. W. *BioTechnology* **1987**, *5*, 274.
(438) Penttilä, M.; Lehtovaara, P.; Nevalainen, H.; Bhikhabhai, R.; Knowles, J. *Gene* **1986**, *45*, 253.
(439) Saloheimo, M.; Lehtovaara, P.; Penttilä, M.; Teeri, T. T.; Ståhlberg, J.; Johansson, G.; Pettersson, G.; Claeyssens, M.; Tomme, P.; Knowles, J. *Gene* **1988**, *63*, 11.
(440) Fägerstam, L. G.; Pettersson, L. G.; Engström, J. Å. *FEBS Lett.* **1984**, *167*, 309.
(441) Bhikhabhai, R.; Pettersson, L. G. *FEBS Lett.* **1984**, *167*, 301.
(442) Johansson, G.; Ståhlberg, J.; Lindeberg, G.; Engstrom, Å. *FEBS Lett.* **1989**, *243*, 389.
(443) Tomme, P.; van Tilbeurg, H.; Pettersson, G.; van Damme, J.; Vandekerckhove, J.; Knowles, J.; Teeri, T.; Claeyssens, M. *Eur. J. Biochem.* **1988**, *170*, 575.
(444) Kraulis, P. J.; Clore, G. M.; Nilges, M.; Jones, T. A.; Pettersson, G.; Knowles, J.; Gronenborn, A. M. *Biochemistry* **1989**, *28*, 7241.
(445) Henrissat, B.; Vigny, B.; Buleon, A.; Perez, S. *FEBS Lett.* **1988**, *231*, 177.
(446) Ståhlberg, J.; Johansson, G.; Pettersson, G. *Eur. J. Biochem.* **1988**, *173*, 179.
(447) Tomme, P.; Claeyssens, M. *FEBS Lett.* **1989**, *243*, 239.
(448) van Tilbeurgh, H.; Claeyssens, M.; De Bruyne, C. K. *FEBS Lett.* **1982**, *149*, 152.
(449) Reese, E. T.; Siu, R. G. H.; Levinson, H. S. *J. Bacteriol.* **1950**, *59*, 485.
(450) Wood, T. M. *Ferment. Technol. Today* **1972**, 711.
(451) Kawamori, M.; Ado, Y.; Takasawa, S. *Agric. Biol. Chem.* **1986**, *50*, 2477.
(452) Wood, T. M.; McCrae, S. I. *Biochem. J.* **1987**, *234*, 93.
(453) Wood, T. M.; McCrae, S. I.; Bhat, K. M. *Biochem. J.* **1989**, *260*, 37.
(454) Claeyssens, M.; van Tilbeurg, H.; Tomme, P.; Wood, T. M.; McCrae, S. I. *Biochem. J.* **1989**, *261*, 819.
(455) Bhat, K. M.; McCrae, S. I.; Wood, T. M. *Carbohydr. Res.* **1989**, *190*, 279.
(456) Greenwood, J. M.; Gilkes, N. R.; Kilburn, D. G.; Miller, R. C.; Warren, R. A. J. *FEBS Lett.* **1989**, *244*, 127.
(457) Gilkes, N. R.; Warren, R. A. J.; Miller, R. C.; Kilburn, D. C. *J. Biol. Chem.* **1988**, *263*, 10401.
(458) Flashner, M.; Kessler, J.; Tanenbaum, S. W. *Arch. Biochem. Biophys.* **1983**, *221*, 188.

(459) Klyosov, A. A.; Mitkevich, O. V.; Sinitsyn, A. P. *Biochemistry* **1986**, *25*, 540.
(460) Eriksson, K.-E.; Pettersson, B. *Eur. J. Biochem.* **1975**, *51*, 193.
(461) Streamer, M.; Eriksson, K.-E.; Pettersson, B. *Eur. J. Biochem.* **1975**, *59*, 607.
(462) Liotta, L. J.; Bernotas, R. C.; Wilson, D. B.; Ganem, B. *J. Am. Chem. Soc.* **1989**, *111*, 783.
(463) Wu, J. H. D.; Orme-Johnson, W. H.; Demain, A. L. *Biochemistry* **1988**, *27*, 1703.
(464) Joliff, G.; Béguin, P.; Millet, J.; Aubert, J.-P.; Alzari, P.; Juy, M.; Poljak, R. J. *J. Mol. Biol.* **1986**, *189*, 249.
(465) Yaguchi, M.; Roy, C.; Rollin, C. F.; Paice, M. G.; Jurasek, L. *Biochem. Biophys. Res. Commun.* **1983**, *116*, 408.
(466) Clarke, A. J.; Yaguchi, M. *Eur. J. Biochem.* **1985**, *149*, 233.
(467) Clarke, A. J.; Yaguchi, M. *Biochim. Biophys. Acta* **1986**, *870*, 401.
(468) Clarke, A. J.; Strating, H. *Carbohydr. Res.* **1989**, *188*, 245.
(469) Elbein, A. D. *CRC Crit. Rev. Biochem.* **1984**, *16*, 21.
(470) Cupples, C. G.; Miller, J. H.; Huber, R. E. *J. Biol. Chem.* **1990**, *265*, 5512.
(471) Gillard, B. K.; Nelson, T. E. *Biochemistry* **1977**, *16*, 3978.
(472) Nelson, T. E.; Palmer, D. H.; Larner, J. *Biochim. Biophys. Acta* **1970**, *212*, 269.
(473) Van Hoof, F.; Hers, H. G. *Eur. J. Biochem.* **1967**, *2*, 265.
(474) Bates, E. J.; Heaton, G. M.; Taylor, C.; Kernohan, J. C.; Cohen, P. *FEBS Lett.* **1975**, *58*, 181.
(475) Nelson, T. E.; Watts, T. E. *Mol. Cell. Biochem.* **1974**, *5*, 153.
(476) White, R. C.; Nelson, T. E. *Biochim. Biophys. Acta* **1974**, *365*, 274.
(477) White, R. C.; Nelson, T. E. *Biochim. Biophys. Acta* **1975**, *400*, 154.
(478) Osterlund, B. R.; Hayakawa, K.; Madsen, N. B.; James, M. N. G. *J. Mol. Biol.* **1984**, *174*, 557.
(479) Gillard, B. K.; White, R. C.; Zingaro, R. A.; Nelson, T. E. *J. Biol. Chem.* **1980**, *255*, 8451.
(480) Nelson, T. E.; White, R. C.; Gillard, B. K. *ACS Symp. Ser.* **1979**, *88*, 131.
(481) Scraba, D. G.; Bradley, R. D.; Fitzgerald, P. M. D.; Madsen, N. B. *FEBS Lett.* **1988**, *240*, 133.
(482) Cappel, R. E.; Bremer, J. W.; Timmons, T. M.; Nelson, T. E.; Gilbert, H. F. *J. Biol. Chem.* **1986**, *261*, 15385.
(483) Takrama, J.; Madsen, N. B. *Biochemistry* **1988**, *27*, 3308.
(484) Holm, L.; Koivula, A. K.; Lehtovaara, P.; Hemminki, A.; Knowles, J. K. C. *Protein Eng.* **1990**, *3*, 181.
(485) Withers, S. G.; Warren, R. A. J.; Street, I. P.; Rupitz, K.; Kempton, J. B.; Aebersold, R. *J. Am. Chem. Soc.* **1990**, *112*, 5887.
(486) Palm, D.; Klein, H. W.; Schinzel, R.; Buehner, M.; Helmreich, E. J. M.; *Biochemistry* **1990**, *29*, 1099.
(487) Johnson, L. N.; Barford, D. *J. Biol. Chem.* **1990**, *265*, 2409.
(488) Johnson, L. N.; Acharya, K. R.; Jordan, M. D.; McLaughlin, P. J. *J. Mol. Biol.* **1990**, *211*, 645.
(489) Slerks, M. R.; Ford, C.; Reilly, P. J.; Svensson, B. *Protein Eng.* **1990**, *3*, 193.