Carbohydrate Research, 250 (1993) vii–xx
Elsevier Science Publishers B.V., Amsterdam

Foreword

Mechanism of enzymic glycoside hydrolysis and of glycosyl transfer by glycosidases and glycosyltransferases

Günter Legler

Institut für Biochemie, Universität Köln, Zülpicher Str. 47, D-50674 Köln (Germany)

More than 150 years ago, Wöhler and Liebig published a paper[1] which marked the beginning of glycosidase research. It described the action of an extract from sweet almonds (called "emulsin") on a substance from bitter almonds, amygdalin, which later became ill-famed as "laetrile" for its alleged effect in the treatment of cancer. Wöhler and Liebig demonstrated that almond emulsin transformed amygdalin into sugar (identified by its sweet taste and its fermentation to carbon dioxide and alcohol by added yeast!), oil of bitter almonds (benzaldehyde), and hydrogen cyanide. They also showed that boiling the almond extract abolished its ability to cleave amygdalin, indicating that the active principle might be a protein. Commenting on the results which were mutually confirmed by both authors, Liebig wrote in a letter to Wöhler (December 31, 1836): "Die Analyse des Emulsins muß Aufschluß über seine Wirkung geben. Hier Katalyse anzunehmen halte ich für Unsinn"; (The analysis of emulsin should give information on its action. I think it is nonsense to assume catalysis here).

Liebig was right, considering the prevailing ideas of his time. He was opposed to Berzelius's definition of a catalyst as an agent which could cause a chemical reaction by its mere presence. On the other hand, reactions proceeding via unstable intermediates which regenerated a catalytic species were not considered examples of true catalysis but rather special kinds of metathetical decompositions. The composition and properties of the enzymes present in almond emulsin are now known but we still have only an approximate idea of the mechanisms which cause rate accelerations of up to 10^{14}-fold. It was studies with almond emulsin and invertase which led Emil Fischer to formulate his pioneering "lock and key" hypothesis on enzyme specificity[2], later to be modified by Koshland with the idea of an "induced fit". Today β-glucosidase from almonds is still a popular enzyme, especially for studies with noncovalent and covalent inhibitors. Unfortunately, many investigators do not realize that crude preparations contain at least two different isoenzymes with widely differing molecular and kinetic properties[3], and suppliers of pure enzymes do not state which isoenzyme they sell. In order to make

SSDI 0008-6215(93)E0334-W

a meaningful comparison with published data, measurements with some standard inhibitors (α/β-D-glucose, D-glucono-1,5-lactone) should be included.

Early this century, Bourquelot demonstrated that almond emulsin catalyzed a reversible reaction and that the approach to the equilibrium starting with concentrated aqueous solutions of D-glucose and a lower alcohol was useful for the preparation of β-D-glucosides[4]. The practical usefulness of this thermodynamic approach is limited by the position of the equilibrium (it is too far on the cleavage side for aryl glycosides), by the solubility of the alcohol, and by denaturation of the glycosidase at high alcohol concentrations. It follows from the reversibility of glycosidase action that these enzymes will also catalyze transglycosylation, i.e., transfer of the glycon moiety of their substrates to hydroxyl containing acceptors other than water (aliphatic alcohols, carbohydrates). This kinetic approach to the preparation of glycosides will be favoured when the glycoside donor is cleaved more rapidly than the acceptor product, and when the activity of competing water can be reduced by a nondenaturing cosolvent, e.g., dimethyl sulfoxide. The method is still used today (see ref 5 for reviews) even though the yields rarely exceed 25% with carbohydrates as acceptors. It has the advantage over the purely chemical synthesis of oligosaccharides that regioselectivity can often be achieved without complicated protection–deprotection strategies. The contribution by Usui et al. to this issue, however, demonstrates that structural features of the acceptor molecule remote from the site of transfer have pronounced effects on the regioselectivity.

However, even in 1925, scientists were apparently not sure if reactions catalyzed by enzymes reached the same equilibrium as with inorganic catalysts. Using the binding constants for methyl β-D-glucoside and D-glucose with almond emulsin v. Euler[6] concluded that the enzymic equilibrium constant would be identical to the "natural" (acid catalyzed) one if K_m(methyl β-D-glucoside) = K_m(D-glucose). It was five years later when Haldane[7] showed that $K_{eq} = [V_{max}/K_m(\text{forward})]/[V_{max}/K_m(\text{reverse})]$. Studies with another glycosidase, invertase (β-fructofuranosidase), from yeast by Henri[8] and Michaelis and Menten[9] had laid the foundations for a mathematical description of enzyme catalyzed reactions and, more importantly, put the idea of a reversibly formed enzyme–substrate complex on a firm basis. Models for such a complex which included distinct binding sites for the glycon and aglycon moieties of the substrate were proposed by v. Euler[10] for invertase and by Pigman[11] for almond β-glucosidase.

While glycosidase research during the 1920s and 1930s centered mainly on enzyme specificity and correlations between reaction rate and substrate structure[11], mechanistic problems were tackled with some success only in the late 1950s and the following decades. Earlier studies had indicated that glycosidase action might be related to acid catalysis, thus permitting an explanation for the pH-dependence of invertase activity from pH 3.5 to 7 by the dissociation of a weakly acidic group[12] (pK_a 6.6). That glycosidases act by at least two different mechanisms followed from the discovery[13] that some amylases released their product maltose with the same anomeric configuration (α) as in the substrate, whereas others catalyzed the

hydrolysis of starch with inversion of the anomeric configuration, forming β-maltose. Later studies have shown that only a small number of glycosidases act with inversion, whereas the majority release the product aldose with retention of the anomeric configuration, presumably by a two-step mechanism involving two consecutive inversions. Referring to the experiments of Swain and Brown[14] on the bifunctional acid–base catalysis of the mutarotation of 2,3,4,6-tetra-*O*-acetyl-D-glucose, Fischer and Stein[15] proposed that glycosidases might function by a concerted action of an electrophilic (acidic) and a nucleophilic (basic) group. Specifically, such a mechanism was proposed by Wallenfels and Malhotra[16] who assumed the participation of a histidine and cysteine in catalysis by β-galactosidase of *E. coli*. A model for the participation of a carboxyl group in glycoside hydrolysis was first described by Capon[17] in 1963, where the carboxyl group of salicylic acid β-D-glucoside provided up to a 10^4-fold acceleration over the analogue *p*-hydroxybenzoic acid β-D-glucoside. Other models with catalytic groups built into the substrate were to follow; their significance for glycoside hydrolysis has been discussed by Sinnott[18].

A large step forward in our understanding of glycosidase mechanisms came in 1966/1967 when Phillips and co-workers published their crystal structure analysis of lysozyme with the competive inhibitor chitotriose[19]. The mechanism derived from the X-ray data has now become an important component of all textbooks on enzymology. Its essential features are two carboxyl groups in close proximity to the bond to be cleaved, one (Glu-35) donating a proton to the glycosidic oxygen atom, the other (Asp-52) stabilizing the glycopyranosyl cation which remains after aglycon departure with its negative charge. Formation of the cation is supposed to be aided by a deformation of the chair conformation of the GlcNAc residue bound at the cleavage site into a conformation flattened at the anomeric carbon atom. A generalisation of this, as yet hypothetical mechanism, to other glycosidases was inferred from the special inhibitory properties of glyconolactones discovered[20] in 1952, of basic sugar analogues like nojirimycin[21] and glycosylamines[22], and labeling studies with covalent active site directed inhibitors like conduritol B epoxide[23].

It was another 18 years before the next crystal structure of a glycosidase–inhibitor complex [Taka amylase A with maltose (maltotriose)] was published[24]. The results resembled those for lysozyme, i.e., the active site appeared as an extended cleft to accomodate six residues of the α-(1 → 4)-glucan chain and had a pair of carboxyl groups (Asp-292 or possibly Asp-206, and Glu-320) at the putative cleavage site. Up to 1992 ten more glycosidases had been studied by X-ray structure analysis. Interestingly, all but one (neuraminidase from influenza virus[25]) were catenases like lysozyme, i.e., their natural substrates are linear polysaccharides. In the meantime, numerous studies with inhibitors of the glyconolactone/-lactam type and with sugar analogues having an endo- or exo-cyclic basic nitrogen function at the anomeric carbon atom[26,27] provided evidence that most configuration retaining enzymes might act, in principle, by the Phillips mechanism. Points of discussion are the stereospecific S_N1 reaction of the glycosyl oxocarbenium ion

stabilized by a carboxylate with water[19,28] or a covalent glycosyl enzyme intermediate formed and decomposed by two consecutive S_N2 reactions[29,30].

The information provided by the crystal structure data, and/or the identification of essential amino acids labeled by covalent active-site directed inhibitors, made it possible to study glycosidase function by a new approach, site-directed mutagenesis. The techniques of molecular biology and genetic engineering permit the replacement of specific amino acids via cDNA constructs in which the codon for the amino acid, the function of which is to be studied, is replaced by the codon for another one. Expression of this "mutated" cDNA in suitable bacteria often yields sufficient enzyme protein to permit kinetic studies. A review by Svensson and Søgaard[31] lists 33 glycosidases, with a total of more than 200 amino acid replacements, which have been studied by this approach. In most cases, the replacement of the amino acid, thought to act as a general acid catalyst, resulted in a > 1000-fold reduction of activity. On the other hand, mutant enzymes in which the basic (anionic) amino acid had been replaced, often had a small residual activity.

While the current state of glycosidase research appears to give a well founded picture of the essential features of the mechanism, we are still far from being able to give a quantitative account of the catalytic efficiency. According to Pauling[32], enzymes have evolved towards a maximal stabilisation of the transition state, thus lowering the free energy of activation and he argued that inhibitors would bind all the more tightly the more they resembled the transition state. Wolfenden and Frick[33] have derived a quantitative relation between the rate constants of the uncatalyzed and the enzyme-catalyzed reactions, k_u and k_{cat}, and the dissociation constants of the enzyme substrate and enzyme transition state complex, K_S (or K_m) and K_T, i.e., $k_{cat}/k_u = K_S/K_T$. It is, of course, not possible to determine K_T but one can design and synthesize analogues of the putative transition state and compare their inhibition constant K_i with K_S (or K_m) and the enzymic rate acceleration factor k_{cat}/k_u. The rate constants k_u could be determined[34] only for glycosides having good leaving group aglycons, e.g., phenols with $pK_a < 8$; other glycosides, including aliphatic ones, were too stable. On this basis the lower limit for k_{cat}/k_u can be estimated to be $> 10^{14}$ for the majority of glycosidases.

As most values for K_s (K_m) are in the range of 0.1 to 10 mM, a perfect transition state analogue should have a $K_i < 10^{-16}$ M. The most potent noncovalent inhibitors known to the writer have K_i $5.5 \cdot 10^{-10}$ M (castanospermine with intestinal sucrase[35]) and $1.5 \cdot 10^{-10}$ M (*N*-dodecyl-β-D-glucosylamine with lysosomal β-glucosidase[36]). It should be noted that both systems showed a slow approach to the equilibrium state of the inhibition, with rate constants several orders below the diffusion controlled limit. Slow binding and dissociation may be an indication of the inhibitor's resemblance to the transition state, or a short lived reaction intermediate[37], but there are other explanations for this phenomenon[38]. Another inhibitor which could be considered a good analogue of the glycon moiety of the transition state is D-gluconamidine which, in its protonated form (pK_a 10.6),

has a positive charge and a planar, trigonal geometry around C-1, thus having a great similarity to the D-glucopyranosyl oxocarbenium ion. With β-glucosidase from almonds it has K_i 10 μM[39], i.e., it binds only ~10^4-fold better than D-glucose.

A theoretical approach by Warshel[40] could likewise only partially account for the catalytic efficiency of glycosidases. Calculations of the energetics of the reaction pathway of lysozyme by the empirical valence bond (EVB) method which included the solvation energy of the substrate in water and the electrostatic dipole and charge fields of the amino acids and peptide bonds surrounding the substrate in the active site gave a stabilization of ionic resonance forms of the transition state relative to the reaction in water. Preoriented dipoles and charge are complementary to the requirements of the their transition state and provide a kind of "supersolvent" which, in the case of lysozyme, lowers the free energy of activation by > 10 kcal/mol, corresponding to an acceleration factor of ~10^7.

Besides the natural curiosity of scientists and their striving to explain the phenomena surrounding us by a few basic principles, what are the reasons for a continuing and even rising interest in glycosidase research? Over the last 15 years more and more details have become known about glycoprotein structures and their biosynthetic pathways[41]. Elucidation of these pathways led to the unexpected finding that the biological function of glycosidases is not limited to degradative processes in the digestive tract and in lysosomes but that some of them play a crucial role in the biosynthesis of *N*-glycoproteins, i.e., that class which have their oligosaccharide chains linked to asparagine residues. In their biosynthetic pathway, common to organisms from yeasts to mammals, a precursor oligosaccharide ($Glc_3Man_9GlcNAc_2$) is transferred from a lipid carrier to specific asparagine side chains, a process located in the rough endoplasmatic reticulum (rough ER). This precursor is then "trimmed" down by two α-glucosidases and several α-mannosidases of the ER and the Golgi apparatus. In the latter organelle the various structures are completed by the transfer of additional monosaccharides (2-acetamido-D-glucose, D-galactose, D-mannose, and D-mannose-6-phosphate, sialic acid, and L-fucose) to the core oligosaccharide.

Glycosidase inhibitors became important tools for cell biologists when it was found that modifications of the core glycan structure, resulting from the inhibition of the α-glucosidases and α-mannosidases involved in the "trimming" process, had profound effects on the intracellular fate of many glycoproteins, such as, targeting to lysosomes, secretion via exocytosis, insertion into the cellular membrane, cell–cell recognition, and endocytosis (see ref 42 for reviews). Distinct effects, caused by modified glycan structures synthesized in the presence of inhibitors of "trimming" glycosidases, were first seen with hybridoma cells producing immonoglobulins D and M. Whereas IgM was secreted normally, IgD was retained in cells treated with the glucosidase inhibitor deoxynojirimycin (dNM)[43]. Differential effects of glycosidase inhibitors on enzymes, with only minor differences in substrate specificity, stimulated the interest in modified and new inhibitors. Thus, "trimming" glucosi-

dases I and II are inhibited by dNM with K_i 3 and 20 μM, respectively; *N*,*N*-dimethylation of dNM improved K_i for glucosidase I to 0.4 μM, but increased K_i for glucosidase II to 500 μM[44]. An additional stimulus of great impact on the use of glycosidase inhibitors came from recent studies with the human immunodeficiency virus (HIV) which has a heavily glycosylated protein coat. Its infectivity was greatly reduced when host cells were grown in the presence of noncytotoxic concentrations of *N*-butyl-dNM[45] or 6-*O*-butanoyl-castanospermine[46].

Modifications of glycan structure by glycosidase inhibitors are limited to *N*-glycoproteins, where they interfere with the normal processing of the $Glc_3Man_9GlcNAc_2$ oligosaccharide. *O*-Glycoproteines which have their glycan chains linked to serine or threonine can be modified to some extent by the action of neuraminidase, galactosidase, fucosidase, and others. This approach has been applied to studies on the role of carbohydrates in the receptor (lectin) mediated endocytosis of cells or circulating proteins[47]. However, in order to obtain more information on the structure–function relationships of *N*- and *O*-glycoproteins, it would be desirable to introduce other alterations by interfering with the glycosyltransferases involved in their biosynthesis. Mechanistic information on the transfer of activated glycosyl units from nucleosidediphosphate sugars to hydroxyl groups of serine/threonine, or of protein bound oligosaccharides, are scarce because kinetic studies are much more complicated than with glycosidases, usually requiring radioactive donor substrates and specific oligosaccharides acceptors. Purification of individual transferases is handicapped by their low abundance and by stability problems caused by their association with biological membranes.

Both α- and β-glycosides have the same type of precursor, i.e., the transfer must occur with retention of the anomeric configuration for α-glycosides and with inversion for β-glycosides. The latter was shown to involve a transition state with substantial sp^2 character in the reaction of galactosyltransferase with UDP-galactose and α-lactalbumin[48]. For lack of more detailed information, the design and synthesis of glycosyltransferase inhibitors appears to be limited to structural alterations of the NDP-sugar which provide noncleavable analogues which usually have less affinity for the transferase than the parent compound. Examples are α-glycopyranosylphosphonate[49] and methylenediphosphonate analogues[50].

Glycosyltransferases are specific both for the glycosyl donor and the acceptor[51]. The reaction with mono- and oligo-saccharides is highly selective for a specific hydroxyl group and often has strict requirements for the oligosaccharide structure of the acceptor, which points to enzyme acceptor interactions far removed from the catalytic site. This selectivity led to the proposal of the "one enzyme–one linkage" concept[52]. Studies with synthetic oligosaccharides, however, have shown that the structural requirements are not absolute, as some of the hydroxyl groups which do not directly participate in the glycosylation reaction can be replaced by other groups without impairment of the acceptor properties[5] (see the contribution to this issue by Nikrad et al.). Oligosaccharide analogues, where the hydroxyl group undergoing glycosylation has been replaced by hydrogen, have been used as

competitive inhibitors for various glycosyltransferases[53]. In four out of eight examples, they inhibited with K_i values in the range of the K_m of the acceptors; for the other cases, the authors assumed a catalytic role of the hydroxyl group in its own glycosylation.

The contributions to this issue related to glycosidases address most of the aspects discussed here; they can roughly be grouped into the categories: reversible inhibitors, covalent inhibitors and active-site mutated enzymes, and kinetic studies with modified substrates.

Reversible inhibitors.—Three papers in this group are on glycosylmethylamines. These are, in general, less potent inhibitors than those basic glycon analogues which have their basic centre directly linked to the anomeric carbon atom, i.e., glycosylamines, 1,5-deoxy-1,5-iminohexitols (deoxynojirimycins), and 5-amino-5-deoxypyranoses (nojirimycins). An increase in the distance between C-1 and the positive charge of the protonated inhibitor by only one C–C bond length appears to result in greatly diminished ionic and hydrogen bond interactions with the active site carboxylates. Glycosylmethylamines have, however, the advantage of stability (over glycosylamines and nojirimycins) and an orientation of possible nitrogen substituents which is much better suited for interactions with the aglycon binding site than *N*-substituents of deoxynojirimycins.

BeMiller et al. (p 93) have studied the inhibition of β-galactosidase from *E. coli* by *N*-substituted β-D-galactopyranosylmethylamines of different basicity. Strong inhibition (K_i 0.2 to 7.8 μM, based on the concentration of free base) was found only with inhibitors having $pK_a \geqslant 8$. The corresponding nonbasic galactosyl formamides (β-D-Gal-CO-NH-R) had 1 200–12 000-fold larger K_i values. It should be noted that *N*-substituted β-galactosylamines of comparable hydrophobicity inhibit 20- to 300-fold better than the corresponding methylamine derivatives[54], in spite of their lower basicity (pK_a 5.3 to 6.6).

β-Glucosidase from almonds has a rather low glycon specificity: β-D-glucosides, -galactosides, and -fucosides are hydrolysed with comparable efficiency. Lai and Martin (p 185) have prepared β-glycosylmethylamines having the D-*gluco*-, D-*galacto*-, and D-*fuco*-configuration. Compared with published data for the corresponding glycosylamines[55], the methylamine derivatives are 2.2-, 10-, and 220-fold less potent inhibitors, respectively. This demonstrates that the nitrogen atom of basic glycon related inhibitors should be directly linked to the anomeric carbon atom to cause strong inhibition.

Dietrich and Schmidt (p 161) have synthesized several *N*-substituted α-D-glucopyranosylmethylamines by two novel routes and studied their inhibition with α-glucosidase from yeast. In contrast with the findings by BeMiller et al. with *E. coli* β-galactosidase, the best inhibitor was the weakly basic aniline derivative *N*-phenyl β-D-glucopyranosylmethylamine (K_i 11 μM). The two enantiomers of the more basic *C*-phenyl isomers, with a benzylic amino group, had K_i 38 and 1 100 μM, respectively. The nonbasic analogue α-D-glucopyranosyl benzene had K_i 1 300 μM.

Many, but not all, glycopyranosidases are inhibited by basic glycon analogues having an NH group in a hydroxylated five-membered ring with similar or even higher strength than by their six-membered counterparts, in spite of their better structural resemblence to the glycon part of the substrate. Liessem et al. (p 19) have extended the studies on the correlation of ring size with inhibitory potency with a new hydroxylated pyrrolidine related to 2-acetamido-2-deoxy-D-galactose. 2-Acetamido-1,4-imino-1,2,4-trideoxy-D-galactitol inhibited the β-subunit of Hexoaminidase A with K_i 18 μM, i.e., with the same strength as the nonbasic analogue 2-acetamido-2-deoxy-D-gluconohydroximino-1,4-lactone (K_i 20 μM). The corresponding six-membered *gluco*-configured analogues, 2-acetamido-1,2-dideoxynojirimycin[35] and 2-acetamido-2-deoxy-D-gluconohydroximino-1,5-lactone[56] inhibit 20- and 45-fold better. The inhibitory potency of the five-membered iminogalactitol is, nevertheless, surprisingly large as the two five-membered inhibitors have distinctly different conformations (crystal structure). Interestingly, the α-subunit of hexosaminidase A (measured with 4-methylumbelliferyl 2-acetamido-2-deoxy-D-glucopyranoside-6-sulfate) was inhibited by the two five-membered inhibitors with K_i 220 and 248 μM, respectively.

Two other five-membered basic sugar analogues, 2,5-dideoxy-2,5-imino-D-mannitol (DIM) and -D-glucitol synthesized by a novel route developed by Stütz were tested by Legler et al. (p 67) with α-glucosidase from yeast, β-glucosidases from almonds, *Asp. wentii*, and bovine kidney, and β-fructofuranosidase (invertase) from yeast. DIM was a much better inhibitor than the D-*gluco* isomer with all enzymes tested and was also better than 1-deoxynojirimycin (dNM) except for the *Aspergillus* enzyme and invertase, the latter being not inhibited at all by dNM [i.e., K_i (dNM) > 5000 μM vs. 6.8 to 1.1 μM for DIM]. The lack of inhibition of invertase by dNM, a basic analogue of D-glucopyranose, required a re-evaluation of its inactivation by the L-enantiomer of conduritol B epoxide (1,2-anhydro-*myo*-inositol) which had been postulated to interact with the glucose binding site of invertase[57].

'Nojiritetrazoles' can be regarded as derivatives of hexono-1,5-lactams in which a tetrazole ring is annelated to the hexose skeleton, providing a rigid structure with planar geometry at C-1. Ermert et al. (p 113) have synthesized the D-*gluco* and D-*manno* isomers and tested them with five α- and β-glucosidases and -mannosidases. A good linear correlation was seen between log V_{max}/K_m with log $1/K_i$ when glucosidase and mannosidase activity of each enzyme was compared with the *gluco*- and *manno*-configured inhibitors, thus demonstrating the configurational selectivity of the inhibitors. Where a comparison with published data can be made, the tetrazols are seen to have a similar inhibitory potency as the corresponding hexono-1,5-lactones. This shows that the tetrazole ring does not impair enzyme–inhibitor interactions. If these resemble enzyme–substrate interactions the endocyclic bond cleavage mechanism of substrate hydrolysis[58] appears unlikely.

Polyhydroxylated piperidines and pyrrolidines of appropriate configuration are highly efficient glycosidase inhibitors with K_i in the micro- to submicro-molar

range, i.e., they inhibit several hundred- to 10^5-fold better than their cyclic oxygen analogues. In order to explore the minimum structural requirements Jiricek et al. (p 31) have synthesized two 1,3-diamino-1,3-dideoxytetrols configurationally related to D-glucose and D-galactose and tested their inhibitory potency with β-glucosidase from almonds and β-galactosidase from *E. coli*. The inhibition constants K_i ranged from 3 to 10 mM and showed a moderate discrimination between the two enzymes. Conversion of the diamino inhibitors into their cyclic urea and thiourea derivatives resulted in an about 10-fold reduction of inhibitory potency in spite of freezing two degrees of freedom of internal rotation and a conformation resembling the structures of the presumed glycosyl cation intermediate. This demonstrates the importance of a cyclic structure combined with a basic group for good inhibition.

Acarviosin is a pseudo-disaccharide consisting of valienamine linked by an NH-bridge to C-4 of D-quinovose; its methyl α-glycoside inhibits α-glucosidase from yeast with $K_i \sim 0.4$ μM. Ogawa and Aso (p 177) have transformed the quinovose moiety of acarviosin into the less polar 2-deoxy- and 2,3-dideoxy-1,6-anhydro derivatives which inhibited about 10-fold better. From a comparison with the polar 2-acetamido-2-deoxy- and the hydrophobic 2-deoxy-2-thiotolyl-derivatives the authors conclude that hydrophobic interactions with the aglycon site of α-glucosidase plays an important role.

Covalent inhibitors and active-site mutated enzymes.—Covalent inhibitors, designed for a specific reaction with a functional group of the active site, should be of the suicide or mechanism-based type, i.e., they should have, in addition to their affinity for the glycon site, a moderately reactive group in a position equivalent to the anomeric centre which can be transformed into a highly reactive species by the catalytic capabilities of the enzyme. Inhibitors which fulfil these requirements are glycon related epoxides and epimines[27] and the glycosylmethyltriazenes introduced by Sinnott[59]. However, the position of the glycosylmethylcarbenium ion produced from the latter appears to be just out of reach of the catalytic groups, as shown by the labeling of Met-502 in β-galactosidase from *E. coli* which is adjacent[60] to the catalytic acid Tyr-503 (see also the discussion about the cationic centre of protonated glycosylmethylamines). Glycosylmethylcarbenium ions are also formed from *C*-glycosyl diazomethanes, as shown by Lehmann[61] with β-galactosidase from *E. coli*. In this case, however, about 50% of the bound inhibitor had reacted with a carboxylic group of the active site.

Inhibitors for labeling the aglycon site should have, in addition to a group of high intrisic reactivity, a high affinity for the enzyme in order to avoid reactions outside the active site. Examples of this type are *N*-bromoacetyl glycosylamines[62], glycon analogues and pseudosaccharides with the vinyl oxirane group[63], and the photolabile diazirine compounds, first applied to label a nonenzymic carbohydrate binding protein[64] and, in addition to reactions with amylases, to create a bifunctional inhibitor for β-galactosidase in combination with the vinyloxirane group[65].

Bar-Guilloux et al. (p 1) describe experiments designed to label the active site of

trehalase with two epimeric 1-thio-α-D-glucopyranosides having the highly reactive vinyloxirane group in a carbocyclic aglycon. The compounds showed neither competitive nor irreversible inhibition, thus demonstrating the high aglycon specificity of trehalase.

The inhibitors synthesized by BeMiller et al. (p 101) resemble the glycosylmethyltriazenes[59] and *C*-glycosyl diazomethanes[61] in that they form a carbenium ion on being protonated by an acidic group of the active site. Their reactive diazomethylcarbonyl group is, however, much more stable in aqueous solution than the other two, permitting studies down to pH 4 where the others decompose rapidly, making kinetic measurements difficult or impossible. The results with β-galactosidase from *A. oryzae* (reversible inhibition with K_i 30 mM, inactivation with k_i (max) 0.56 min^{-1}) and protection experiments with phenyl 1-thio-β-D-galactopyranoside, showed that the inactivation was caused by a covalent reaction at the active site. No inhibition was observed with β-galactosidase from *E. coli*; the authors ascribe this to the low acidity of the proton donating group (pK_a 8.5, presumably Tyr-503). Another explanation could be that the larger distance between the anomeric carbon and the reactive group of the inhibitor (two C–C bond lengths instead of one) puts it out of reach of the acid or a suitable nucleophile of the *E. coli* enzyme.

Black et al. (p 195) have inactivated a β-glucosidase from *Agrobacterium faecalis* and an exoglucanase from *Cellulomonas fimi* with *N*-bromoacetyl-β-D-glucosylamine and -β-cellobiosylamine respectively. Reaction of the inhibitors at the active site followed from the kinetics of activity loss and from protection experiments with competitive inhibitors. The stoichiometry of the reaction determined, by ion spray mass spectrometry, was found to be 1 mol inhibitor per mol protein for the *C. fimi* enzyme whereas it was 2 to 4 for the enzyme from *A. faecalis*, in spite of clearcut kinetics for both enzymes. Apparently, nonspecific reactions are of similar rate with respect to the active site and do not effect the specificity of the reaction.

The study presented by Huber and Chivers (p 9) is on a mutated β-galactosidase from *E. coli* in which Glu-461, thought to be essential for catalysis[66], had been replaced by glycine. The almost inactive mutated enzyme could be reactivated to some extent by various nucleophiles (e.g., azide, imidazole, carboxylates, but not by acids with $pK_a < 4$ or amines with $pK_a > 9$) when enzyme and nucleophile were incubated together with substrate. In many cases, a β-galactosyl adduct with the nucleophile could be identified in the activation process. A possible explanation is that the mutated enzyme forms an α-galactosyl intermediate with Glu-537 which is hydrolyzed very slowly (Glu-537 had been labeled with concomitant inactivation with 2,4-dinitrophenyl 2-deoxy-2-fluoro-β-D-galactopyranoside[67]). Activation could then occur with nucleophiles which react faster than water with this alternate galactosyl enzyme.

Kinetic studies with modified substrates.—From the effects on the enzymic constants k_{cat} and K_m, caused by structural modifications of the glycon and

aglycon moieties of the substrate, valuable information can be obtained on details of enzyme–substrate interactions and the catalytic mechanism. Early studies with β-glucosidase from almonds had shown that this enzyme does not tolerate the methylation or acetylation of individual hydroxyl groups, and that hydrophobic interactions with the aglycon site improve substrate binding and catalytic efficiency except with tertiary aglycons[68]. Later investigations showed that the replacement of glycon hydroxyl groups by smaller[69] (hydrogen) or similar size[70] (fluorine) also had profound detrimental effects mainly on k_{cat}. This was most pronounced when the hydroxyl group on C-2 had been replaced. The reasons for similar effects on k_{cat} being produced by substituents with both smaller and larger electron withdrawing potential than -OH have been discussed in ref 27.

The term "pseudosubstrate" as used here refers to sugar related compounds that can undergo a chemical reaction catalyzed by a glycosidase[27]. They often form long-lived intermediates, thereby acting as slow, reversibly inhibitors. Examples are glycals and sugar derivatives having a C=C double bond at C-1 of the parent hexose (hexenitols, octenitols) and glycosyl fluorides. A transition from pseudosubstrates to irreversible inhibitors is seen with 2-deoxy-2-fluoroglycosyl fluorides and the corresponding 2,4-dinitrophenyl glycosides.

Amyloglucosidase, an "inverting" glycosidase, cleaves the terminal nonreducing glucose from α-(1 → 4)-glucan chains; (1 → 6)-α-glucosidic bonds are also hydrolyzed but 30- to 50-fold slower. Palcic et al. (p 87) have extended studies on the catalytically competent conformations of maltosides and isomaltosides bound at the active site by introducing a C-methyl group at C-6 of methyl α-isomaltoside. This had practically no effect on k_{cat}, but K_m for the *R* isomer was 25-fold smaller and for the *S* isomer 3.5-fold larger than K_m for the parent isomaltoside. This is explained by the relative ease of the compounds to reach the conformation required for an optimal interaction of aglycon hydroxyl groups with the active site.

Even though acid catalysis is not possible with the hydrolysis of β-glycopyranosyl pyridinium ions, they have been shown to be good substrates for configuration retaining glycosidases, especially when they are based on pyridines of low pK_a; rate acceleration factors of up to 10^{12} have been observed[71], Padmaperuma and Sinnott (p 79) have extended studies on the substrate properties of glycosyl pyridinium ions to three "inverting" glycosidases (β-xylosidase from *Bacillus pumilis*, 1,3-glucanase from *Sporotrichum dimorphosporum*, and amyloglucosidase II from *A. niger*). It was found that these enzymes also have the potential to hydrolyze β-xylosyl and β-(α-)glucosyl pyridinium ions with acceleration factors of up to 10^9 over the uncatalyzed rates. The dependence of log k_{cat} on pK_a of the pyridine was similar but less pronounced than with the "retaining" glycosidases, i.e., rate acceleration was higher with pyridinium ions having more basic leaving groups. As a direct interaction of an essential carboxylate group with the glycon C-1 or the pyridinium ring as discussed for the "retaining" glycosidases is unlikely, only noncovalent forces and the deprotonation of the attacking water molecule are considers to be effective for catalysis. The endocyclic cleavage mechanism[58] can thus be ruled out.

Many α- and β-glycosidases catalyze the hydrolysis of glycosyl fluorides with the "correct" anomeric configuration with similar or even higher rates than the corresponding 4-nitrophenyl glycosides. It was first shown for α-glucosidase from *A. niger* that the absolute specificity for either α- *or* β-glycosides does not hold when glycosyl fluorides are used as substrates[72]. That the hydrolysis of a substrate with the "wrong" anomeric configuration is not exceptional was shown by the study contributed by Matsui et al. (p 45). α-Glucosidase from rice and sugar beet seeds and β-glucosidase from almonds were found to act on α- and β-glucosyl fluorides in the same way as the *Aspergillus* enzyme, i.e., V_{max} with the "wrong" substrate was 0.4 to 1% of V_{max} for the "correct" one (0.07% with β-glucosidase). In all cases, the anomeric configuration of the product D-glucose corresponded to the anomeric specificity of the enzyme. The model proposed for the mechanism of catalysis with different glycosylic substrates (including D-glucal) features a flexible response to the substrate configuration in the first step (formation of a glycosyl enzyme) and a conserved second step (hydrolysis of the glycosyl enzyme) in which the product hexose (2-deoxyhexose in the case of D-glycals) is released in the anomeric configuration dictated by the specificity of the enzyme.

Usui et al. (p 57) describe the application of the transglycosylation capacity of a maltotriose forming amylase from *Streptomyces griseus* to the preparation of chromogenic substrates for the assay of amylases. The glycosyl donor was maltotetrose, 4-nitrophenyl α- and β-glucoside were used as acceptors; transglycosylation was enhanced relative to donor hydrolysis by the addition of 50% dimethyl sulfoxide to reduce the activity of competing water. Interestingly, the regiospecificity was effected by the anomeric configuration of the acceptor. While maltotriose was transferred almost exclusively to the 4-OH of the α-configurated acceptor, it was, in addition, transferred to the 3-OH of 4-nitrophenyl β-glucoside with comparable efficiency. The latter product predominated with longer reaction times, presumably at the expense of the 4-maltotetraoside formed initially.

Glycosyltransferases.—The two papers on glycosyltransferases in this issue are on the preparation of oligosaccharides (10- to 20-mg scale) by routes which exploit the regioselectivity of the transferases to link monosaccharides to specific hydroxyl groups in unprotected acceptor saccharides synthesized chemically. The contribution by Nikrad et al. (p 145) is on the synthesis of sialyl Lewis^x and Lewis^a tetrasaccharides which play an important role in the adhesion of leukocytes to endothelial cells when they have expressed complementary cell surface lectins (selectins) in the inflammatory process. The fucosyltransferases, contained in a preparation from human milk are specific for the 3- and 4-OH of GlcNAc in the acceptor saccharides D-Neu5Ac-(2 → 3)-β-D-Gal-(1 → 4)-β-D-GlcNAc-O-$(CH_2)_8CO$-OCH_3 (for Lewis^x) and α-D-Neu5Ac-(2 → 3)-β-D-Gal-(1 → 3)-β-D-GlcNAc-O-$(CH_2)_8CO$-OCH_3 (for Lewis^a). Whereas the β-Gal residue at C-4/C-3 of the acceptor GlcNAc are of prime importance for the fucosyltransferases both enzymes tolerate substitutions of the 2-acetamido group. Its replacement by -OH, $-NH_2$, $-N_3$, and the propionamido group affected the rate of fucosyl transfer by

less than ±50%, thus permitting an efficient synthesis of Lewis oligosaccharide analogues to explore the binding specificity of selectins.

The paper by Kashem et al. (p 129) aims at the synthesis of different oligosaccharides based on β-D-GlcNAc-(1 → 3)-β-D-Gal-(1 → 4)-β-D-GlcNAc-O-$(CH_2)_8CO$-OCH_3 by the sequential application of α-(2 → 6)- and α-(2 → 3)-sialyltransferases from rat liver, α-(1 → 3/4)-fucosyltransferases from human milk, and β-(1 → 4)-galactosyltransferase from bovine milk.

REFERENCES

1 F. Wöhler and J. Liebig, *Ann. Pharmazie*, 22 (1837) 1–24.
2 E. Fischer, *Untersuchungen über Kohlenhydrate und Fermente*, Vol. I, Verlag Berlin, 1909, p. 843.
3 B. Helferich and T. Kleinschmidt, *Hoppe-Seyler's Z. Physiol. Chem.*, 348 (1967) 753–758; T. Kleinschmidt and J. Horst, *Hoppe-Seyler's Z. Physiol. Chem.*, 352 (1971) 603–614; G. Legler and F. Witassek, *Hoppe-Seyler's Z. Physiol. Chem.*, 355 (1974) 617–623.
4 E. Bourquelot, *Ann. Chim.*, 29 (1913) 145; *J. Pharm. Chim.*, 10 (1914) 361, 393; cited from ref 6, p 315.
5 E.J. Toone, D.S. Simon, M.D. Bednarski, and G.M. Whitesides, *Tetrahedron*, 45 (1989) 5365–5422; H. Waldmann, *Nachr. Chem. Tech.*, 40 (1992) 828–834.
6 H. v. Euler, *Chemie der Enzyme*, 3rd ed., Vol. I, J.F. Bergmann, München, 1925, p 306.
7 J.B.S. Haldane, *Enzymes*, Longmans, London, 1930.
8 V. Henri, *Lois Générales de l'Action des Diastases*, Herman, Paris, 1903; cited from H.L. Segal, in P.D. Boyer, H. Lardy, and K. Myrbäk (Eds.), *The Enzymes*, 2nd ed., Vol. 1, Academic Press, New York, 1959, p 147.
9 L. Michaelis and M.L. Menten, *Biochem. Z.*, 49 (1913) 333.
10 H. v. Euler, *Hoppe-Seyler's Z. Physiol. Chem.*, 143 (1925) 79.
11 W.W. Pigman, *Adv. Enzymol.*, 4 (1946) 41–73.
12 H. v. Euler, ref 6, p 62–68.
13 R. Kuhn, *Ber. Dtsch. Chem. Ges.*, 57 (1924) 1965–1969; *Liebigs Ann. Chem.*, 139 (1924) 24–29.
14 C.G. Swain and J.F. Brown, *J. Am. Chem. Soc.*, 74 (1952) 2534–2538.
15 E.H. Fischer and F.A. Stein, in *The Enzymes*, 2nd ed., Vol. 4, Academic Press, New York, 1960, p 303.
16 K. Wallenfels and O.P. Malhotra, *Adv. Carbohydr. Chem.*, 16 (1961) 239–298.
17 B. Capon, *Tetrahedron Lett.*, (1963) 911–913.
18 M.L. Sinnott, in M.I. Page (Ed.), *The Chemistry of Enzyme Action*, Elsevier, Amsterdam, 1984, pp 389–431.
19 D.C. Phillips, *Sci. Am.*, 215 (5) (1966) 78–90; C.C.F. Blake, G.A. Mair, A.T.C. North, D.C. Phillips, and V.R. Sarma, *Proc. Roy. Acad. Sci. (London), Ser. B*, 167 (1967) 378–387.
20 G.A. Levvy, *Biochem. J.*, 65 (1952) 464–472; G.A. Levvy and S.M. Snaith, *Adv. Enzymol.*, 36 (1972) 151–181.
21 T. Niwa, S. Inouye, T. Tsuruoka, Y. Koaze, and T. Niida, *Agric. Biol. Chem.*, 34 (1970) 966–972.
22 H.-Y. Lai and B. Axelrod, *Biochem. Biophys. Res. Commun.*, 54 (1973) 463–468.
23 G. Legler, *Hoppe-Seyler's Z. Physiol. Chem.*, 345 (1966) 197–214.
24 Y. Matsuura, M. Kusonoki, W. Harada, and M. Kakudo, *J. Biochem.*, 95 (1984) 697–702.
25 J.N. Varghese and P.M. Colman, *J. Mol. Biol.*, 221 (1991) 473–486.
26 M.L. Sinnott, *Chem. Rev.*, 90 (1990) 1171–1202.
27 G. Legler, *Adv. Carbohydr. Chem. Biochem.*, 48 (1990) 319–384.
28 N.C.J. Strynadka and M.N.G. James, *J. Mol. Biol.*, 220 (1991) 401–424.
29 M.L. Sinnott, *FEBS Lett.*, 94 (1978) 1–9.
30 K.-R. Roeser and G. Legler, *Biochim. Biophys. Acta*, 657 (1981) 321–333.
31 B. Svensson and M. Søgaard, *J. Biotechnol.*, 29 (1993) 1–37.
32 L. Pauling, *Chem. Eng. News*, 24 (1946) 1375–1379.

33 R. Wolfenden and L. Frick, in M.I. Page and A. Williams (Eds.), *Enzyme Mechanisms*, Roy. Soc. Chem., London, 1987, pp 97–122.
34 G. Legler, M.L. Sinnott, and S.G. Withers, *J. Chem. Soc., Perkin Trans. 2*, (1980) 1376–1383.
35 C. Danzin and A. Ehrhard, *Arch. Biochem. Biophys.*, 257 (1987) 472–475.
36 P. Greenberg, H.H. Merrill, D.C. Liotta, and G.A. Grabowski, *Biochim. Biophys. Acta*, 1039 (1990) 12–20.
37 J.V. Schloss, *Acc. Chem. Res.*, 21 (1988) 348–353.
38 J.F. Morrison and C.T. Walsh, *Adv. Enzymol.*, 61 (1988) 201–302.
39 B. Ganem and G. Papandreou, *J. Am. Chem. Soc.*, 113 (1991) 8984–8985.
40 A. Warshel, *Acc. Chem. Res.*, 14 (1981) 284–290.
41 R. Kornfeld and S. Kornfeld, *Annu. Rev. Biochem.*, 54 (1985) 631–664.
42 (a) U. Fuhrmann, E. Bause, and H. Ploegh, *Biochim. Biophys. Acta*, 825 (1985) 95–110; (b) A.D. Elbein, *Annu. Rev. Biochem.*, 56 (1987) 497–534; (c) T.W. Rademacher, R.B. Parekh, and R.A. Dwek, *Annu. Rev. Biochem.*, 57 (1988) 785–838; (d) B. Winchester and G.W.J. Fleet, *Glycobiology*, 2 (1992) 199–210.
43 N. Peyeras, E. Bause, G. Legler, R. Vasilov, L. Claeson, P. Peterson, and H. Ploegh, *EMBO J.*, 2 (1983) 823–832.
44 H. Hettkamp, G. Legler, and E. Bause, *Eur. J. Biochem.*, 142 (1984) 85–90.
45 R.A. Gruters, J.J. Neefjes, M. Tersmette, R.E.Y. de Goede, A. Tulp, H.G. Huisman, F. Miedema, and H.L. Ploegh, *Nature (London)*, 330 (1987) 74–77.
46 P.S. Sunkara, D.L. Taylor, M.S. Kang, T.L. Bowlin, A.S. Tyms, and A. Sjoerdsma, *Lancet*, 334 (1989) 1206.
47 G.J. Ashwell and J. Harford, *Annu. Rev. Biochem.*, 51 (1982) 531–554.
48 S.C. Kim, A.N. Singh, and F.M. Raushel, *Arch. Biochem. Biophys.*, 267 (1988) 54–59.
49 M.M. Vaghefi, R.J. Bernacki, N.K. Dalley, B.E. Wilson, and R.K. Robins, *J. Med. Chem.*, 30 (1987) 1387–1390.
50 M.M. Vaghefi, R.J. Bernacki, W.J. Hennen, and R.K. Robins, *J. Med. Chem.*, 30 (1987) 1391–1399.
51 T. Beyer, J. Sadler, J. Raerik, J. Paulson, and R. Hill, *Adv. Enzymol.*, 52 (1981) 23–175.
52 A. Hagopian and H.H. Eylar, *Arch. Biochem. Biophys.*, 128 (1968) 422–430.
53 O. Hindsgaul, K.J. Kanwal, G. Srivasta, M. Blaszyk-Thurin, S.L. Crawley, L.D. Heerze, and M. Palcic, *J. Biol. Chem.*, 266 (1991) 17858–17862.
54 E. Kappes and G. Legler, *J. Carbohydr. Chem.*, 8 (1989) 371–388.
55 D.E. Walker and B. Axelrod, *Arch. Biochem. Biophys.*, 187 (1978) 102–107.
56 M. Horsch, L. Hoesch, A. Vasella, and D.M. Rast, *Eur. J. Biochem.*, 197 (1991) 815–818.
57 H. Braun, *Biochim. Biophys. Acta*, 485 (1977) 141–146.
58 R.W. Franck, *Bioorg. Chem.*, 20 (1992) 77–88.
59 M.L. Sinnott and P.J. Smith, *Biochem. J.*, 175 (1978) 523–528.
60 M. Ring, D.E. Bader, and R.E. Huber, *Biochem. Biophys. Res. Commun.*, 152 (1988) 1050–1055.
61 M. Brockhaus and J. Lehmann, *Carbohydr. Res.*, 63 (1978) 301–306.
62 F. Naider, Z. Bohak, and J. Yariv, *Biochemistry*, 11 (1972) 3202–3207.
63 (a) G. Legler and W. Lotz, *Hoppe-Seyler's Z. Physiol. Chem.*, 354 (1973) 243–254; (b) J. Lehmann and B. Neumann, *Carbohydr. Res.*, 200 (1990) 355–361.
64 G.L. Jung, K.R. Holme, C.P.J. Glaudemans, J. Lehmann, and H. Lay, *Carbohydr. Res.*, 174 (1988) 87–98.
65 R.E. Huber, J. Lehmann, and L. Ziser, *Carbohydr. Res.*, 214 (1991) 35–41.
66 M. Herrchen and G. Legler, *Eur. J. Biochem.*, 138 (1984) 527–531.
67 J.C. Gebler, R. Aebershold, and S.G. Withers, *J. Biol. Chem.*, 267 (1992) 11126–11130.
68 S. Veibel, in J.B. Sumner and K. Myrbäck (Eds.), *The Enzymes*, Vol. 1, Academic Press, New York, 1950, pp 584–620.
69 G. Legler, *Acta Microbiol. Acad. Sci. Hung.*, 22 (1975) 403–409; K.-R. Roeser and G. Legler, *Biochim. Biophys. Acta*, 657 (1981) 321–333.
70 S.G. Withers, K. Rupitz, and I.P. Street, *J. Biol. Chem.*, 263 (1988) 7929–7932.
71 C.C. Jones, M.L. Sinnott, and I.J.L. Souchard, *J. Chem. Soc., Perkin Trans. 2*, (1977) 1191–1198.
72 E.J. Hehre, H. Matsui, and C.F. Brewer, *Carbohydr. Res.*, 198 (1990) 123–132.