Various catalytic reaction models have been proposed as the reaction mechanisms of glycosidases, but a reasonable and unitary model capable of interpreting both “inverting” and “retaining” glycosidase reactions remains to be established. As for the models proposed to date, the nucleophilic displacement mechanism and the oxocarbenium ion intermediate mechanism are widely known, but recently the former is widely accepted, and so the general tendency of world opinion appears to favor it. This reaction model, however, is considered to comprise some inconsistencies that cannot be neglected from the viewpoint of reactivity in organic chemistry. While the nucleophilic displacement mechanism is often applied to reactions of glycosidases, it appears unlikely that such reactions actually occur. This review argues that the oxocarbenium ion intermediate reaction mechanism is more rational than the nucleophilic displacement reaction mechanism, as the action mode of glycosidases and related enzymes.

Key words: glycosidase; \( \alpha \)-secondary isotope effects; nucleophilic displacement; oxocarbenium ion intermediate; \( \alpha \)-1,4-glucan lyase

Generally speaking, the main reaction catalyzed by glycosidases involves the hydrolysis (I), transglycosylation (II), and condensation reaction (III) as reversible reaction. In these reactions, the anomeric configuration of the substrate is retained (\( \alpha \) to \( \alpha \), or \( \beta \) to \( \beta \)) in the product by anomer-“retaining” glycosidases, but is inverted (\( \alpha \) to \( \beta \), or \( \beta \) to \( \alpha \)) by anomer-“inverting” glycosidases. Reactions I and II are the forward reaction, but reaction III corresponds to the reverse reaction in hydrolysis, which is accompanied by the synthesis of oligosaccharide. Though the retaining glycosidase catalyzes transglycosylation and also condensation to some extent, inverting glycosidase does not catalyze transglycosylation. If an inverting enzyme is capable of catalyzing a transfer reaction as well as retaining one, the product would become an anomer-inverted product.\(^{1-3}\) This entails, however, an inconsistency: the enzyme must cleave the product having different anomeric configuration by the reverse reaction, according to “the principle of microscopic reversibility.”\(^{4-7}\) Hence it appears unlikely that such a transfer reaction takes place.

Figure 1 shows simplified reaction modes of \( \alpha \)-glucosidase and glucoamylase as a retaining and an inverting glycosidases, respectively. The modes of synthesis reaction of glucobiose by transglucosylation (II) and condensation reaction (III) resemble each other except for the inversion of anomeric configuration, but in the case of \( \alpha \)-glucosidase the synthesis reaction takes place with transglycosylation and condensation, and in the case of glucoamylase the synthesis reaction occurs only by the reverse reaction, meaning condensation (III). Thus there is no transglycosylation in the reaction of the inverting enzyme as it is devoid of reaction II. The synthesis reaction catalyzed by the inverting enzyme is observed only in the reverse reaction, one of which has been designated condensation. The condensation reaction (III) must be distinguished from transglycosylation (II) as the forward reaction of the retaining enzyme, which causes no inversion in the anomeric configuration.

Various catalytic reaction models have been proposed for the reaction mechanisms of glycosidases, but a reasonable and unitary model capable of explaining both inverting and retaining enzyme reactions remains to be established. As for the catalytic reaction mechanisms proposed to date, there are two significant models, the nucleophilic displacement reaction mechanism,\(^{8}\) based on a single or double displacement reaction, and the oxocarbenium ion intermediate reaction mechanism,\(^{2,3}\) although this is based on a reaction mode that should be called the carbonium ion intermediate mechanism without oxonium ion formation.\(^{9-13}\) The nucleophilic displacement model is accepted by many investigators. However, even if the majority of researchers hold to the displacement model, the nucleophilic single and double displacement mechanisms appear to comprise inconsistencies that cannot be neglected from the viewpoint of reactivity in organic chemistry.\(^{14}\) It is difficult to explain the reaction processes, including the transition state of inverting and retaining enzymes by these mechanisms. While nucleophilic displacement models are often adopted with respect to the hydrolytic reaction of inverting and retaining glycosidases,\(^{15,16}\) it is unlikely that such reaction occur organic-chemically, as explained below. Is it possible that the catalytic reaction for the two different types of glycosidases is unified by a common mechanism?

The conceptions of the reaction mechanisms of glycosidases and related enzymes described here are based on the oxocarbenium ion intermediate reaction, and on a view of \( \alpha \)-secondary kinetic isotope effects. This description is extended to the reaction for glyco-
I. Hydrolytic Reaction from Acetal to Hemiacetal

In order to reasonably interpret the reaction processes catalyzed by glycosidases, Chiba et al. have proposed the oxocarbenium ion intermediate mechanism to be favored over the nucleophilic displacement mechanisms.2,3,14) The natural carbohydrate as the substrate have proposed -1,4-glucan lyase,17,18) the initial attack on an oxygen of acetal by hydrogen ion (H+) is essential for hydrolysis at the initial stage a, as depicted in Fig. 2A. Figure 2B shows the process that compares the mode of hydrolysis of a glucoside as acetal to that of acetal. The hydrolysis mode of glucoside is compatible with that of acetal. This implies that the reaction mechanism can be accounted for by the reaction mode of hydrolysis of acetal, that is, the oxocarbenium ion intermediate mechanism.

II. Nucleophilic Displacement Reaction Mechanisms

The process of hydrolysis of α-glycoside by a typical inverting glycosidase such as glucoamylase, based on the nucleophilic single displacement mechanism, is shown in Fig. 3A. This reaction scheme is generally received as the explanation of the reaction mechanism of the inverting enzymes. According to the principle of microscopic reversibility,4–7) the reverse of a reaction at equilibrium must be exact opposite of the pathway for the forward reaction. The reverse reaction of the process c to a (Fig. 3A) appears to be incorrect. However, if the configuration situation of the catalytic groups (base and acid/base) is not restored to the original situation a, the reverse reaction is inconceivable. This means that the forward reaction is perhaps wrong due to inconsistency with the principle of microscopic reversibility. The reaction of nucleophilic attack by water on the C1-carbon of the substrate glycone and electrophilic attack by the hydrogen ion (H+) of carboxyl group (acid/base) on the oxygen of the C1-O bond, and simultaneous extraction of the hydrogen ion (H+) from water by the carboxylate group (base) is unlikely to occur continuously, because the hydrogen ion (H+), as the initiation-driving force essential to the subsequent hydrolytic reaction, is missing from the catalytic group (acid/base) at the final stage c. The topological situation of the catalytic groups (base and acid/base) at the final stage c should revert to the initial situation of the stage a in the active enzyme, the reaction of which can continue without inactivation. If this is not so, the reaction demonstrated in Fig. 3A probably become dead-end reaction. That is to say, the reverse reaction (III) is impossible.

Fig. 1. Simplified Reaction Modes of α-Glucosidase (Retaining) and Glucoamylase (Inverting).

H-OR is glucose, and H-OR’, glucose as acceptor.

Fig. 2. Hydrolytic Reaction of Acetal (A) and Glucoside as Acetal (B) to Hemiacetal.

In the case of the hydrolysis of a glucoside, C of acetal molecule corresponds to C1 of glycone, R, to glycone, and R’, to C5 forming an O-ring.
Points similar to that concerning the nucleophilic single displacement can be made as to the nucleophilic double displacement mechanism (Fig. 3B). This reaction mechanism has been widely taken to explain the action mode of the retaining glycosidase. As shown in Fig. 3B, in process via the first transition state, a C1-O covalent bond is newly formed between the substrate glycone and the carboxylate group (base) of the enzyme to give an intermediate, which is also an acetal molecule. The newly formed covalent bond is usually considered to be split by nucleophilic attack of water on the C1-carbon through process to, including the second transition state. However, cleavage of the C1-O covalent bond of the glycosyl-enzyme intermediate as an acetal probably does not arise by the nucleophilic attack on the C1-carbon, because the attack of the hydrogen ion (H+) on the oxygen is essential also for the splitting of the C1-O bond between glycone and enzyme. The process from the initial stage to the stage also is a dead-end complex formation reaction of substrate and enzyme. The transition states depicted in Fig. 3A and B are similar to those of the S2-like reaction, but such transition states are impossible due to improbability of direct attack on the C1-carbon by water at the initial stage of the hydrolysis of acetal molecule. Consequently, it is inferred that there are actually no reaction depend on both nucleophilic single and double displacement mechanisms.

As for the reaction mechanism, McMurry has interpreted the catalytic reaction of inverting and retaining glycosidases by nucleophilic displacement mechanisms in his text book. The mechanisms are as follows: as Fig. 4 shows, the initial reaction (stage in Fig. 4A) of hydrolysis in both mechanisms takes place via a short-lived oxonium ion (stage in Fig. 4A) of the glycone residue of the substrate and an attack of the hydrogen ion (H+) of the carboxyl group on the oxygen of the C1-O bond (Fig. 4A). In the inverting enzyme reaction, the anomer of the product is inverted by nucleophilic attack of water on the C1-carbon of the oxonium ion (Fig. 4B). In the retaining enzyme reaction, the C1-carbon of oxonium ion is attacked by a dissociated carboxylate ion to form an intermediate between glycone and enzyme (Fig. 4C). The C1-O covalent bond of the glycosyl-enzyme is cleaved through regeneration of the oxonium ion, and the product anomer is retained by nucleophilic attack of water on the C1-carbon of the oxonium ion. It is implausible, however, that cleavage of the C1-O covalent bond of the intermediate occurs only by regeneration of the oxonium ion (stage ) in Fig. 4C. The glycosyl-enzyme intermediate is an acetal molecule, as mentioned above, and hence the splitting of the C1-O bond of the newly formed acetal is impossible without the attack of the hydrogen ion (H+) on the bond oxygen.

The process of hydrolysis from acetal to hemiacetal has been described as a generalized concept in many reports and textbooks, but the fission of the C1-O covalent bond at the stage in Fig. 4C is in conflict with the initial reaction of hydrolysis of the acetal due to the absence of hydrogen ion (H+) for the attack on the oxygen of the C1-O covalent bond. The process of the retaining glycosidase (Fig. 4C) is a dead-end reaction for the enzyme-substrate intermediate formation that terminates at the stage . Therefore, the nucleophilic double displacement reaction is improbable mechanism, as well as the nucleophilic single displacement one.
In order to solve the problem of the reaction mechanism of glycosidase, other approaches should be attempted. The oxocarbenium ion intermediate reaction appears to be plausible as a unified mechanism that is applicable to both inverting and retaining glycosidases. The mechanism must be well grounded as to chemical reactivity in organic chemistry.

III. Oxocarbenium Ion Intermediate Reaction Mechanism

Figure 5 shows the oxocarbenium ion intermediate mechanism that is plausible and adaptable to the inverting and retaining glycosidases. The resulting oxonium ion molecule at the initial stage a to c resonates with carbocation. The pyranoid ring at the transition state (stage b) exist in the first half-chair (H) conformer of the highest free energy level in the interconversions from the chair conformer (1C4) to another chair conformer (4C1) via the skew (S) and boat (B) conformers. The proposed oxocarbenium ion intermediate is applicable to both inverting and retaining glycosidases. As shown at the stages f and f' in Fig. 5, when the hydration is performed from above and below on the anomeric C1-carbon of the oxonium ion plane, in which the C1-, C2-, and C5-carbons and oxygen lie in a plane, β-anomer and α-anomer appear to be produced, respectively. The hydrolysis (I), transglycosylation (II), and condensation (III) can be reasonably interpreted without conflict with the principle of microscopic reversibility by this reaction mechanism, which is probable unitary reaction mechanism for glycosidase.

IV. α-Secondary Kinetic Isotope Effects

α-Secondary deuterium (D) and tritium (T) kinetic isotope effects, α-SD(T)KIEs, have been observed in the hydrolytic reactions of α- and β-glycosidases including anomer inverting and retaining enzymes. The concept of α-SD(T)KIEs is briefly elucidated as follows: in the hydrolytic reactions of the substrate substituted H of the C1-hydrogen bond by D or T and the unsubstituted substrate, the rate (kDαT) of hydrolysis of the former becomes lower than that (kH) of hydrolysis of the latter. As indicated in the steps c and d in Fig. 5, the occurrence of stable oxonium ion intermediate, the resonance forms between oxonium ion and carbocation essentially generated by cleavage of the C1-O bond, causes a slow down in the hydrolysis rate (kDαT). The kH/kDαT value is larger than 1 (unity). This kinetic
phenomenon is called \(\alpha\)-SKIEs.\(^{11,25}\) Insets A and B in Fig. 6 display the schematic curves of the potential energy at the initial stage and the activated stage of the substrate molecule. As indicated in inset A, when \(\alpha\)-SD(T)KIEs are observed, the difference in zero-point energy levels (\(E_H\) and \(E_{D \text{ or } T}\)) between the initial state and the activated state is \(E_H < E_{D \text{ or } T}\), that is, \(k_H > k_{D \text{ or } T}\). The secondary kinetic isotope effects are explained to be fundamentally same as primary kinetic isotope effects.\(^{26–29}\) In the case of inset B, on the other hand, the enzyme zero-point energy levels in the initial state and the transition state between the normal substrate and the substituted one are equivalent, \(E_H = E_{D \text{ and } T}\), that is, \(k_H = k_{D \text{ and } T}\). Hence \(\alpha\)-SKIEs are not observed. Besides the cases of \(E_H < E_{D \text{ or } T}\) and \(E_H = E_{D \text{ or } T}\), a special case, \(E_H > E_{D \text{ or } T}\),
is theoretically conceivable, inverse kinetic isotope effects. In the case, the \( k_{H}/k_{D} \) values are less than unity, but it may be unnecessary to take this into consideration in the reaction of glycosidase.

The ratio \( k_{H}/k_{D} \) values have been reported for \( \alpha\)-SD(T)KIEs in hydrolytic reactions by many glycosidases. The values for \( \alpha\)-SDKIEs range from 1.10 to 1.16 in the hydrolysis of \([1,1'\text{-}^{2}H]\)isomalto, \( p\)-chlorophenyl \( \alpha\text{-}[1\text{-}^{2}H]\)glucoside, and \( \alpha\text{-}[1\text{-}^{2}H]\)glucosyl fluoride \( \beta\)-glucosidases (retaining), glucoamylase- \( \beta\text{-}\) (inverting), and glucodextranase (inverting). The \( k_{H}/k_{O} \) value in the hydrolysis of \( \alpha\text{-}[1\text{-}^{2}H]\)trehalose by trehalase (inverting) is 1.53. The \( k_{H}/k_{O} \) values for \( \alpha\text{-}STKIEs \) range are from 1.17 to 1.26 in the hydrolysis of \( \alpha\text{-}[1\text{-}^{3}H]\)glucosyl fluoride, \( \beta\text{-}\) and glucodextranase. Also in \( \beta\text{-}\)-glucosidase, the values for \( \alpha\text{-}SDKIEs \) range are from 1.10 to 1.12 in the hydrolysis of \( p\)-nitrophenyl \( \beta\text{-}[1\text{-}^{2}H]\)glucoside, \( \beta\text{-}\) and from 1.11 to 1.19 in the hydrolysis of phenyl or \( p\)-nitrophenyl \( \beta\text{-}[1\text{-}^{2}H]\)chitobiose by lysozyme. The value for \( \alpha\text{-}STKIEs \) is 1.19 in the hydrolysis of \( [1,1'\text{-}^{2}H]\)chitotriose by lysozyme. The \( \alpha\text{-}SD(T)KIEs \) values, as explained above, imply that hydrolytic reactions by many \( \alpha\text{-} \) and \( \beta\text{-}\)glycosidases occur through oxocarbenium ion intermediate. Recently, it was reported that relatively high values for \( \alpha\text{-}SDKIEs \) were observed in the synthesis reaction of \( \alpha\text{-}\) and \( \alpha\text{-}\)trehalose-6-phosphate by \( \alpha\text{-}\) and \( \beta\text{-}\)trehalose-6-phosphat synthase (UDP forming) as a retaining glycosyltransferase (EC 2.4.1.15): the values for \( \alpha\text{-} \) and \( \beta\text{-}SDKIEs \) are 1.20 and 1.16, respectively, and the value for \( \alpha\text{-}STKIEs \), 1.16. \( \beta\text{-}STKIEs \) this implies that, in the enzyme classified into the category of transferase (EC 2 groups) too, the splitting reaction of the \( \text{C}_{1}\text{-O} \) bond of UDPglucose occurs through resonance between oxocarbenium ion and the carboxylation intermediate.

The SDKIEs for the non-enzymatic reactions in acid-catalyzed hydrolysis also have been examined using methyl \( \alpha\text{-} \) and \( \beta\text{-}\)glycosides substituted deuterium at \( \text{C}_{1}\text{-} \), \( \text{C}_{2}\text{-} \), and \( \text{C}_{5}\text{-}\)hydrogen atoms of glycoses, the kinetic isotope effects of which are designated \( \alpha\text{-} \), \( \beta\text{-} \), and \( \gamma\text{-}SDKIEs \) respectively. In the non-enzymatic reaction for methyl \( \alpha\text{-} \) and \( \beta\text{-}\)glucosides (solvolysis, 2.0 \( \text{M} \) \text{HClO}_{4} at 80 °C), the \( \alpha\text{-} \) and \( \beta\text{-}SDKIEs \) for both substrates are observed to be \( k_{H}/k_{D} \) values of 1.13 and 1.11, while those for methyl \( \beta\text{-}\)glucoside are 1.09 and 1.05, respectively, but no \( \gamma\text{-}SDKIEs \) were noticed in either substrate. These results are important in understanding the reaction mechanism of glycosidase, because they suggest that the reaction in non-enzymatic hydrolysis also proceeds on the basis of the oxocarbenium ion intermediate mechanism.

Figure 6 shows the relation of free energy levels as the reaction progresses, the level of which are related to the potential energy levels of the ground and activated states (insets A and B). Figure 6A suggests a relationship between free energy and reaction progress during hydrolytic reaction in oxocarbenium ion intermediate mechanism (Fig. 5). In the hydrolytic reaction of the substrate replaced H of the \( \text{C}_{1}\text{-}\)hydrogen bond by D or T, \( \alpha\text{-}SD(T)KIEs \) have been observed in many glycosidases, implying the presence of carboxylation arising from resonating oxocarbenium ion. A momentarily stable situation in the resonance (stages c and d in Fig. 5) brings about a delay of the hydrolysis rate dependent on \( \alpha\text{-}SKIEs \). This means that the reaction of glycosidase proceeds through an oxocarbenium ion intermediate in \( S_{n}1\)-like reaction. Figure 6B and C indicate the same schematic relationship between free energy and reaction progress, as shown in Fig. 6A, but in the cases of nucleophilic single and double displacement mechanisms, no \( \alpha\text{-}SD(T)KIEs \) are observed, since there is no process of oxonium ion and carboxylation. Nevertheless, \( \alpha\text{-}SD(T)KIEs \) have been observed in the hydrolytic reaction of inverting enzymes such as glucoamylase and trehalase. The energy levels (T,) in Fig. 6A and B correspond to the transition states and the rate-limiting of the reaction processes in Fig. 3A and Fig. 5, but in the case of T2 (Fig. 6C) the energy levels of \( \Delta G_{1}\) and \( \Delta G_{2}\) at two transition states are depicted as equal as a matter of convenience. If the energy levels are \( \Delta G_{1} > \Delta G_{2} \), the rate-limiting step might be present in the first transition state (stage b in Fig. 3B). If \( \Delta G_{1} < \Delta G_{2} \), the rate-limiting step might be present in the second transition state (T2, stage e in Fig. 3B). Thus it appears to be difficult to decide the rate-limiting step in the reaction. From the hydrolysis mechanism of acetal molecule and the data of \( \alpha\text{-}SD(T)KIEs \), it can be assumed that in the hydrolysis of glycosidase the oxocarbenium ion intermediate is more likely than the \( \text{C}_{1}\text{-O} \) covalent bond of the glycosyl-enzyme intermediate, which probably is not essential in the reaction of retaining glycosidase.

V. MECHANISM-BASED INACTIVATION

The reason why the nucleophilic displacement model is widely accepted by a great many researchers is obscure and complicated, but a reason probable is attributed to numerous reports on the mechanism-based inhibitors. Available inhibitors, \( \text{2-deoxy-2-fluoro-} \alpha\text{-d-glucopyranosyl fluoride} \), \( \text{1,1-difluoroalkyl-glucoside} \), and \( \text{5-fluoro-} \alpha\text{-d-glucopyranosyl fluoride} \), regarded as substrate analogs, and conduritol B epoxide as a different type of inhibitor, have been synthesized as a novel class of enzyme-activated irreversible inhibitors, which are all mechanism-based inhibitors (suicide substrate). In mechanism-based inhibitors, usually glycine analog specifically binds to a carbonylexyl group at the active site directly involved in the activity, and then the glycosidase is irreversibly inactivated. The mechanism-based inactivation reaction for many \( \alpha\text{-} \) and \( \beta\text{-}\)glycosidases and \( \beta\text{-}\)glucan glycodextranases \( \text{3,35} \) have been examined using the inhibitors named above. Most of the substrate analogs directly modify a catalytic carbonyl of enzyme, but 1,1-difluoroalkyl-glucoside is distinct from the modification reaction of the other analogs. The compound as an inhibitor is the substrate of an \( \alpha\text{-}\)glycosidase. In the inactivation reaction, the aglycone residue released in the hydrolysis by \( \alpha\text{-}\)glycosidase modifies \( \alpha\text{-}\)glycosidase itself with an intermediate to bring about inactivation. The importance of mechanism-based inactivation with inhibitors lies in the fact that the time-dependence for inactivation indicates the curve of a definite pseudo-first-order reaction. This means that the reaction modes are dead-end, almost irreversible reactions, and glycine analogs have been confirmed to connect to the carbonylexyl group in the catalytic site. The identifica-
tion of such a covalent C₁-O bond has been regarded as a proof of the nucleophilic double displacement mechanism. However, even if the glycone analog is proved to be linked to a carboxylate group essential for the catalytic activity, the glycosyl-enzyme intermediate by the covalent-O bond is not always possible in the hydrolytic reaction of natural substrate. The reactions of substrate analogs and glycosidases examined to date are just the same as general mechanism-based modification with a suicide substrate, and accordingly it cannot be concluded to be the evidence that the usual hydrolysis of normal substrate by glycosidase occurs through the nucleophilic double displacement mechanism.

**VI. Reversible Reaction of Glycosidases**

Hehre *et al.* have been ascertained that glucoamylase (an inverting enzyme) synthesizes maltose with other glucobiases from β-glucose, and β-amylyase (another inverting one), maltotetraose from β-maltose, through the reverse reaction of hydrolysis.⁵⁹ It was found that α-amylyase (a retaining enzyme) also catalyzed to form maltotetraose from α-maltose. The synthesis of maltose from β-glucose by glucoamylase proceeds through dehydration from the C₁-hydroxyl group of β-glucose and the hydrogen of C₄-hydroxyl one of aglycone (Fig. 7, scheme A), and in the case of β-amylyase the formation of maltotetraose at the active site was interpreted to proceed *via* the same mechanism as in that of glucoamylase.⁶⁰ Besides, it was demonstrated for the first time that several α-amylys produced malto-oligosaccharides from α-glucose fluoride by transglycosylation by Hehre *et al.* The retaining enzyme is capable of synthesizing oligosaccharides by both of the forward and reverse reactions (process III and II in Fig. 7B and C), but in the inverting enzyme it is noteworthy that oligosaccharides are produced only by the reverse reaction. Usually, the formation of oligosaccharides by retaining enzyme (forward reaction II in Fig. 7C) and that by the reverse reactions of inverting and retaining enzymes (Fig. 7A and B) have been called transglycosylation and condensation, respectively. If the reaction process of glucoamylase shown in Fig. 7A depends on the oxocarbenium ion intermediate mechanism, it should be depicted like that in Fig. 7D.

Further, β-amylyase was found to act on α- and β-maltosyl fluoride (-F), producing β-maltose and hydrogen fluoride (HF).⁶¹ and glucoamylase was found to act on α- and β-glucosyl fluoride, producing β-glucose and HF.⁶² In order to explain the fact that β-maltose is formed by β-amylyase action, the schemes have been proposed as follows: (A) maltotetraose + H₂O ⇌ β-maltose + maltose; (B) α-maltosyl-F + H₂O ⇌ β-maltose + HF; (C) β-maltosyl-F + β-maltosyl-F →...
\[ \beta\text{-maltotetraosyl-F} + \text{H}_2\text{O} \rightleftharpoons \beta\text{-maltose} + \beta\text{-maltosyl-F} \]. The forward (\( \rightarrow \)) and reverse (\( \leftarrow \)) reactions are the hydrolysis and synthesis (condensation), respectively, and the inverting glycosidase is capable of synthesizing oligosaccharides only by the reverse reaction. Figure 8 represents the possible mechanisms for the reaction occurring at the active site, drawn on the basis of proposed schemes\(^{59}\). Reaction I (Fig. 8A and B) is the hydrolysis and the reaction II (Fig. 8A and C) is the reverse reaction (condensation). \(\text{\beta-Glucose, apparently formed from } \alpha\text{- and }\beta\text{-glucosyl fluorides, can be explained as the hydrolysis of the substrate in scheme B and of the synthetic product in scheme C, respectively. In these reaction models (A, B, and C), a carboxylate ion (base) is assumed to have a catalytic function in addition to B:H, whose residue has been identified as the carboxyl group (acid/base).}\(^{63}\) Each functional group is viewed as having a dual role that alternates between the two types of functions. As stated above (Fig. 1), at the termination of reaction I, the topologies of the catalytic groups do not revert to the initial state, implying that the reaction is dead-end, but this infringes on the principle of microscopic reversibility. Therefore, it is reasonable that the reaction schemes A, B, and C should be altered as A', B', and C', according to oxocarbenium ion intermediate mechanism, respectively.

The reaction mechanism shown in Fig. 8, denoted in terms of a concerted mechanism\(^{61}\) is interpreted as catalytic flexibility\(^{62,64}\) or versatility\(^{65}\) at active site of glycosidase by Hehre et al. The forward reaction shown in Fig. 8A and B appear to show a single displacement mechanism, in which the cleavage of the C\(_1\)-O or -F bond by direct attack of water is probably inconceivable. The same concerted mechanism has been applied to several inverting enzymes, including glucoamylase\(^{62}\) and glucoolstexanase,\(^{62}\) trehalase\(^{64,65}\) and \(\beta\text{-xylosidase}\(^{66}\) As an example of the reaction for the concerted mechanism, the hydrolytic and synthesis reactions of \(\alpha\text{-trehalose}\) by trehalase particularly taken from among the above-mentioned inverting glycosidases are shown Fig. 9.
Trehalase is found to form β-glucose from both α- and β-\(\text{D-glucosyl fluorides}\). The reaction pathway is explained by schemes I and II: (I) \(\alpha\text{-D-glucosyl fluoride (or } \alpha,\alpha\text{-trehalose)} + \text{H}_2\text{O} \rightarrow \beta\text{-glucose} + \text{hydrogen fluoride (or } \alpha\text{-D-glucose); (II) } \beta\text{-D-glucosyl fluoride + } \alpha\text{-D-glucose} \rightarrow \alpha,\alpha\text{-trehalose + hydrogen fluoride (HF). Based on the schemes A, B, and C in Fig. 9, it is represented that trehalase utilizes \(\alpha\)- and \(\beta\)-glucosyl fluorides to afford \(\beta\)-glucose. However, it is unlikely that the conversion from carboxylate (base) to carboxyl group (acid/base) is reiterated. In this respect, there is an imperfection in the mechanism (Fig. 9A, B, and C). Thus it appears to be correct that schemes A, B, and C should be modified to \(\text{A}', \text{B}', \text{and C}'.\) There is an implicit consent in reaction \(\text{B}',\) which is denoted as the forward reaction, but practically it is the reverse reaction (condensation) for the synthesis of \(\alpha,\alpha\text{-trehalose},\) corresponding to the reversal of scheme \(\text{C}',\) and is not so-called transglycosylation. Needless to say, the inverting glycosidase is capable of catalyzing the synthesis of oligosaccharide only by condensation, as shown in Fig. 1.

The concerted reaction mechanism is referred to as the Hehre resynthesis-hydrolysis mechanism (Fig. 10).\(^{67-73}\) William and Withers\(^{69}\) have cited it as an example of the mechanism for the hydrolysis of "wrong" anomeric fluoride, meaning mistaken recognition of anomeric configuration, in the synthesis and hydrolysis of 4-\(\beta\text{-xylobiose formed from } \alpha\text{-xylosyl fluoride}^{66}\) for inverting \(\beta\text{-xylosidase (Fig. 10A).}\) Kasumi \textit{et al.} have explained that it requires the reversal of the roles of catalytic groups to continue the reaction of transglycosylation and hydrolysis of 4-\(\beta\text{-xylobiose (or } 4\text{-} \beta\text{-xylobiosyl fluoride) by } \beta\text{-xylosidase, and that reaction A occurs beyond the requirement of the principle of microscopic reversibility.}\(^{66}\) This description intimates an important consideration that the concerted reaction mechanism may be in conflict with the principle. However, if the disposition of functional groups reverts
to common states for inverting glycosidases, as in Fig. 10A’, B’, and C’, according to the oxocarbenium ion intermediate mechanism. Hehre’s mechanism does not infringe on the principle, because the reaction is based on the mechanism relating to the inverting β-glycosidase.

On the other hand, several α-glycosidases and a β-glycosidase have been pointed out to be capable of hydrolyzing both α- and β-glycosyl fluoride.74,75 Is it conceivable that the reaction with two substrates having different anomeric configurations are directly hydrolyzed by an individual α- or β-glycosidase? Hehre et al. reported that Aspergillus niger,74 sugar beet seed,75 and rice seed75 α-glycosidases can catalyze the hydrolysis of β-glycosyl fluoride to produce α-glucose, and rice seed75 β-glycosidase hydrolyzes α-glucosyl fluoride to yield β-glucose. These abilities of the α- and β-glycosidases are described to act wrongly with inversion on a substrate having α- or β-anomeric configuration. Especially, A. niger α-glucosidase was isolated and crystallized from the enzyme source of A. niger transglucosidase Amano (a mixture of α-glucosidase and glucoamylase in the main).76 However, there is a possibility that the α-glucosidase is always contaminated by a trace amount of glucoamylase, though the removal of β-glucosidase as a contaminant is carefully done, and even if the enzyme is in crystal form. The relative ratio catalytic efficiency ($V/K_m$) hydrolysis of β-glucosyl fluoride ($β$-GF)/α-glucosyl fluoride ($α$-GF) is evaluated to be $1/6,000$. If the α-glucosidase used contains a trace of glucoamylase, β-maltosyl fluoride (or β-isomaltosyl fluoride) can be formed via the same reverse reaction C’ as shown in Fig. 8, and then the product is immediately split to α-glucose and β-glucosyl fluoride by α-glucosidase (forward reaction B, in Fig. 7).

As for plant glycosidases, however, it is of importance to check for contamination with trehalase. Trehalase is widely distributed in the living world including plants.14 Though trehalase hidden in almond β-glucosidase has been confirmed, contamination with trehalase in sugar beet and rice α-glucosidases has not been checked. The ratios of the $V/K_m$ value for the hydrolysis of β-GF/α-GF are calculated to be $1/2,600$ in sugar beet α-glucosidase and for that of β-GF/α-GF, $1/5,600$ in rice α-glucosidase, and for that of α-GF/β-GF, $1/17,000$ in almond β-glucosidase. If these plant α-glucosidases contain traces of trehalase, α,α-trehalose will be formed from β-glucosyl fluoride via reaction
process B’ (condensation) depicted in Fig. 9, trehalase splits trehalose and then 
via process C’ (hydrolysis) to give equimolecular β-glucose and α-glucose. In the case of β-glucosidase, it is assumed that trehalase hydrolyzes α-glucosyl fluoride directly to afford β-glucose by reaction scheme A’ in Fig. 9, and some of β-glucose changes into α-glucose. So long as the possibility of contamination by another enzyme is not completely excluded, it is not clear that α- and β-glucosidases can wrongly hydrolyze β- and α-glucosyl fluoride, respectively. Hence wrong cleavage of anomeric C12 would lead to the synthesis of α- and β-glycoside (or β-celllobioside and laminaribioside), and the latter D481G utilized β-glucosyl fluoride and α-glucose. In the synthesis reactions by the mutated β- and α-glycosidases, it is important that the anomeric configuration inherent in the specificity of each enzyme is maintained in the synthesized product, because the reaction processes are evidence of the reverse reaction, that is, condensation, but are not so-called transglycosylation, though depicted as the forward reaction.

Figure 11 shows the reaction schemes for glycosynthase E358A proposed by Withers and coworkers.77,78 These schemes (Fig. 11A and B) are accepted by many investigators, and are referred to in papers77,78 and reviews,81,82 but there is some confusion as to the processes. Probably, the schemes (Fig. 11A and B) correspond to the reverse reaction (Figs. 8C and 10A for α- and β-glycosidases, respectively) in the concerted mechanism,61 whose mechanism cannot be readily accepted without partial modification, as explained above. In the scheme in Fig. 11A,69,77,81 the leaving fluorine (F) is released as hydrogen fluoride (HF). Where does the initiator hydrogen (H) of HF generated come from? In the scheme in Fig. 11B,78,82 the leaving fluoride ion (F-) is released as fluoride ion (F-). Is the ion present as it is? In addition, at the termini of synthesis reactions A and B, the catalytic carbonylates convert into carboxyl groups. If the carboxyl groups do not revert to the initial carbonylates, the continuance of reactions A and B appears to be inconceivable. It can hence be assumed that the reaction mechanism is not plausible.

The pKa values of carboxyl groups of the side chains in peptide linkage of protein are generally known to be about 4.3–4.7 for the β- and γ-carboxyl groups of aspartic acid and glutamic acid.83 McIntosh et al. reported that the pKa values of functional carboxylate (base, Glu78 and carboxyl group (acid/base, Glu172) of a retaining xylanase of Bacillus circulans were 4.6 (pKα1) for Glu78 and 6.7 (pKα2) for Glu172, and that

VII. Reaction Mechanism of Mutant Glycosidase

Mutant glycosidase, so-called “glycosynthase,” constructed by replacing the catalytic carboxylate (base) of glycosidase with different amino acid residues, was reported for the first time for Agrobacterium sp. retaining β-glucosidase, the catalytic carboxylate of which was Glu358. The mutant E358A substituted Glu358 with alanine was prepared.77 The action pattern was found to convert from retaining to inverting enzyme, and thus oligosaccharide derivatives were synthesized from α-glucosyl fluoride, and many β-glycosides (or β-glycobiosides) without hydrolytic activity.77,78 Mutant enzymes for β-glucanase and α-glucosidase were prepared by replacing the functional carboxylate (base, Glu134) with alanine in retaining 1,3,1,4-β-glucanase,79 and the carboxylate (base, Asp481) with glycine in retaining α-glucosidase.80 The actions of the two mutants E134A79 and D481G80 also were confirmed to convert from retaining to inverting mode, and the mutants catalyzed the synthesis of oligosaccharide derivatives, that lacked the activity of hydrolysis. The former E134A utilized α-laminaribiosyl fluoride and β-glucoside (or β-celllobioside and laminaribioside), and the latter D481G utilized β-glucosyl fluoride and α-glucose. In the synthesis reactions by the mutated β- and α-glucanses, it is important that the anomeric configuration inherent in the specificity of each enzyme is maintained in the synthesized product, because the reaction processes are evidence of the reverse reaction, that is, condensation, but are not so-called transglycosylation, though depicted as the forward reaction.

Fig. 11. Reaction Schemes (A) and (B) of a Mutant Enzyme (Glu358Ala) as Glycosynthase Constructed from Retaining β-Glucosidase.77
both of a mutant Q78 of which E78 was replaced by Gln and the xylanase bereft of the activity by modification of E78 with 2,4-dinitrophenyl 2-deoxy-2-fluoro-β-xyloside (a suicide substrate) exhibited the same pKₐ value of 4.2. 84) The conversion of functional carboxyl group (acid/base) of native xylanase into carboxylate (base) in reaction (condensation) of mutant E358A converted scheme C. Scheme C in Fig. 11 means the reverse reaction. A and B in Fig. 11 should be revised to be like correct, it appears to be plausible that reaction schemes A and B in Fig. 11 can be surmised that the carboxylate (Fig. 11A) involved in the initial reaction is the carboxyl group. As has been described by Phillips, 9,11) the pH value (4.3–4.7), and it remains in the acidic state. On the other hand, the carboxylate (Asp52, Kₐ) than ordinary pH values for glycosidases are known to be low and induces the negative charge. 86,87) The pKₐ value should be higher than normal situation. It was first reported that β-1,4-xylanase releasing from the reducing end of β-1,4-oligosaccharide have been reported. 70) These mutants produce β-1,4-xylotriose from α-xylobiosyl fluoride and β-xylose, but they are intact inverting enzymes, and hence the reaction mechanism appears to be basically ambiguous as to whether such mutants fall under the category of glycosynthase. Interesting papers 70–73,89) and reviews 81,82) on the synthesis of oligosaccharides by mutant enzymes have been published, but the reaction mechanism should be reexamined carefully.

### VIII. Hydration Reaction to Glycal and Enitol as Other Synthetic Substrates Having No O-Glycosidic Bond

Glycal and enitol are special synthetic substrates of glycosidases, except for glycosyl fluorides. In 1972, it was first reported that Escherichia coli β-galactosidase catalyzed the addition of water 80) to d-galactol to give 2-deoxy-d-galactose, maintaining β-anomeric configuration in the product. Subsequently, the hydration to d-glucal was found to be catalyzed also by α- and β-glycosidases, forming 2-deoxy-α-D-[2-(axial)-2H]glucose and 2-deoxy-β-D-[2-(equatorial)-2H]glucose, respectively.
by using $^2\text{H}_2\text{O}$ (D$_2$O).$^{91}$ The hydration to $\beta$-glucal by glucoamylase$^{92}$ and glucodextranase$^{92}$, and to maltal by $\beta$-amylase$^{93,94}$ was examined for inverting glycosidases. The anomeric configuration characteristic of each enzyme was found to be maintained in the hydration of all glycosidases, including exo- and endo-cellulases.$^{95}$

Typical reaction schemes selected from the hydration processes of glycosidases are depicted in Fig. 12, in which schemes A and B show the mechanisms expected for the hydration reaction by Hehre et al.$^{96}$ although the origin of the deuterium ion (D$^+$) in the initial reaction is obscure. According to the oxocarbenium ion intermediate model, however, the hydration processes should be revised to be like the reaction schemes in Fig. 12A and B. The oxocarbenium ion intermediate shows a striking resemblance to the hydration mechanism of $\beta$-glycal lacking the O-glycosidic bond.

An additional reaction of water to special synthetic compounds, enitols, is to be expected. $E.\ coli$ $\beta$-galactosidase was found to catalyze the conversion of $\beta$-galacto-heptenitol and $\beta$-galacto-octenitol to 1-deoxy-$\beta$-$\beta$-galact-heptulose$^{96}$ and to 1,2-dideoxy-$\beta$-$\beta$-galact-octulose, respectively, with $\beta$-anomeric conformation.

On the other hand, $\alpha$-glucan lyase and $\beta$-glucosidases,$^{98}$ glucodextranase,$^{99}$ and trehalase.$^{100}$ Only $\alpha$-glucosidase formed a product having an $\alpha$-anomeric configuration; glucodextranase and trehalase gave a common $\beta$-product. Reaction mechanisms of hydration have been proposed for glycosidases. The proposed reaction mechanisms$^{98,99}$ of $\alpha$-glucosidase (retaining) and glucodextranase (inverting) chosen from among such glycosidases are shown in Fig. 13A and B. From $\alpha$-gluco-hept-1-enitol, $\alpha$-glucosidase and glucodextranase produced 1-deoxy-$\alpha$-$\beta$-gluco-heptulose and 1-deoxy-$\beta$-$\beta$-gluco-heptulose via reactions A and B, respectively. Reaction schemes A and B, however, appear not to be appropriate, so that it is reasonable to modify the processes A and B in the schemes to A$'$ and B$'$.

In addition, $\alpha$-glucosidase (retaining) and trehalase (inverting) have been confirmed to catalyze the hydration of $\alpha$-gluco-oct-2-enitol to yield 1,2-dideoxy-$\alpha$-$\beta$-gluco-octulose and 1,2-dideoxy-$\beta$-$\beta$-gluco-octulose, respectively.$^{100}$ Reaction mechanisms for $\alpha$-glucosidase and trehalase are demonstrated in Fig. 14A and B. However, since the proposed reaction mechanism appears to be inappropriate as an explanation of reaction processes, the scheme should be corrected like in Fig. 14A$'$ and B$'$. In the hydration of special synthetic compounds, it is of interest that the reaction is initiated through oxonium ion formation.

**IX. Reaction Mechanism of Exo-$\alpha$-1,4-glucan Lyase**

$\alpha$-Glucans, starch and glycogen, are usually considered to be hydrolyzed by endo- and exo-hydrolases, releasing glucooligosaccharide and glucose, and to be broken down by phosphorylase, yielding glucose 1-phosphate. Besides the processes of hydrolysis and phosphorolysis, however, another $\alpha$-glucan-degrading pathway catalyzed by exo-$\alpha$-1,4-glucan lyase (EC 4.2.2.13) releasing 1,5-anhydrofructose from non-reducing terminus of $\alpha$-1,4-glucan, the so-called the...
third α-1,4-glucanolytic system of starch and glycogen \(^{101}\) has been found in fungi \(^{102}\) and red algae, \(^{103–105}\) and the lyase was first purified from red seaweed. Afterwards the enzymes have been found to be widespread in the biological world. \(^{101,106–108}\) The pathway for lyase action is outlined in Fig. 15. \(^{109–111}\) As drawn there, 1,5-anhydrofructose, produced from the non-reducing end of α-1,4-glucan by lyase, is converted into 1,5-anhydroglucitol by NADPH-dependent 1,5-anhydrofructose reductase. \(^{112}\)

The amino acid sequential alignments of α-1,4-glucan lyase from fungi and red algae were revealed to be highly homologous to a series of α-glucosidases (EC 3.2.1.20) belonging to glucoside hydrolase family 31. \(^{113}\) This suggests that the reaction mechanism of α-1,4-glucan lyase is closely correlated with that of α-glucosidase. Two reaction mechanisms \(^{113–115}\) for lyase have been proposed, as shown in Fig. 16A and B. Both reaction processes depend on the double displacement mechanism, and hence the initial reaction forms the covalent intermediate between a glycosyl residue and lyase, followed by extraction of the hydrogen ion (H\(^+\)) from C\(_2\)-H bond with the carboxylate (base). In practice, however, it is questionable whether these schemes are correct. In the two transition states of the initial reactions shown in Fig. 16A and B, the coordination numbers at anomeric C\(_1\) are indicated as six-coordinates, but five-coordinates may be suitable to C\(_1\) in this case. The formation of covalent intermediate by the C-O bond between the anomeric C\(_1\) and the carboxylate of lyase makes it too difficult to extract the hydrogen atom of C\(_2\)-H bond, though it is easy to extract the H-atom of the C\(_2\)-H bond in the oxocarbenium ion intermediate when anomeric C\(_1\) becomes carbocation (C\(^+\)). Thus the pathway depicted in Fig. 16C appears to be rational. The hydration to the oxonium ion inter-

![Fig. 14. Reaction Mechanism of Hydration to D-Gluc-oct-2-enitol by α-Glucosidase (A) and Trehalase (B). \(^{100}\)
Schemes (A) and (B) are corrected to be like (A') and (B').](image)

![Fig. 15. Outlines of Pathways for Exo-α-1,4-glucan Lyase Action.\(^{110,111}\)](image)
mediate produced in the initial reaction is ordinary the hydrolytic reaction of α-glucosidase, but this does not occur in the lyase reaction. The proton of the C2-H bond is extracted from the resonant carboxylation intermediate accompanied with oxonium ion intermediate by carboxylate(base) to give 1,5-anhydrofructose (enol form). As for the reaction splitting α-glucosidic bond by the enzyme such as α-1,4-glucan lyase, it can be surmised that the oxonium ion and its resonant carbocation is formed essentially as shown in Fig. 16C. This is considered to be a proof in support of the oxocarbenium ion intermediate model. The formation of an oxonium ion in the initial reaction and the attack on oxygen of the C1-O bond by hydrogen ion (H+) of the carboxyl group (acid/base) appears to be common to all cases of the cleavage of the glycosidic O-bond of carbohydrate.

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