BIOORGANIC CHEMISTRY 20, 77–88 (1992)

REVIEW

The Mechanism of β-Glycosidases: A Reassessment of Some Seminal Papers

RICHARD W. FRANCK

Chemistry Department, Hunter College/CUNY, 695 Park Ave., New York, New York 10021

Received April 19, 1991

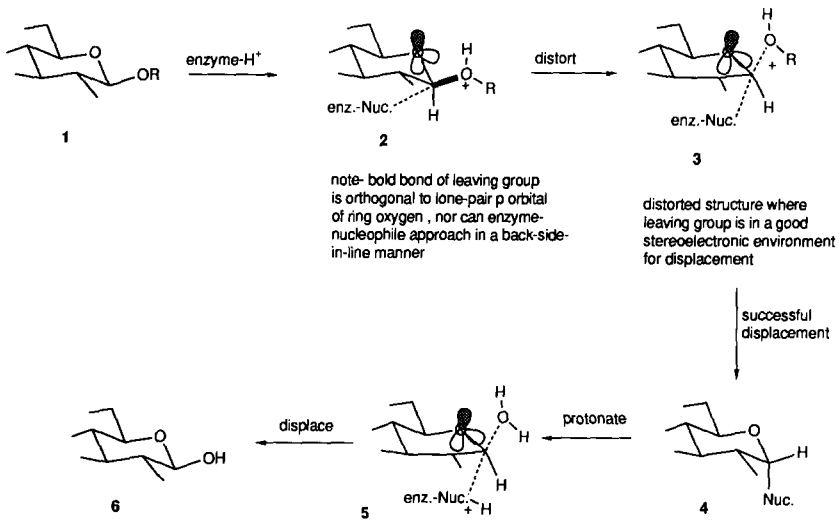
INTRODUCTION

There is a generally accepted mechanism for β-glycosidases acting upon their natural substrates, the β-glycosides (*1*), as illustrated in Scheme 1. First, the enzyme protonates the exocyclic oxygen to make it a good leaving group, but the group cannot leave directly because neither the orbital overlap of electrons of the ring oxygen nor the approach of a nucleophilic group on the enzyme is geometrically favorable. Hence, the most widely accepted postulate is that the enzyme contributes to a distortion of the protonated substrate so that both the electronic overlap and the approach of a nucleophile can be stereoelectronically correct. Displacement of the exocyclic group then takes place to form a glycosyl–enzyme adduct, which participates in a second nucleophilic step related to the first one.

As shown in the scheme, protonation of the enzymic group followed by its direct displacement leads to product. Note that the exocyclic leaving group is now axial and the stereoelectronics are suitable, but a distortion is required for the displacement step if the nucleophilic participation has S_N2 character. A nonenzymic experiment that supports the proposition that the exocyclic oxygen departure is involved in the rate-determining step of glycoside hydrolysis has been described by Sinnott (*2*). The study of the acid-catalyzed hydrolysis of a group of appropriately ^{18}O-labeled methylglucosides revealed a set of isotope effects consistent for exo C–O bond cleavage and endo C–O bond rehybridization toward sp^2. Recently, Sinnott has discounted the importance of stereoelectronic forces as control elements for these pathways (*3*). Nevertheless, it should be emphasized that, no matter what the physical driving force is for bond cleavage, it is generally accepted that a free glycosyl cation cannot have a significant lifetime and that solvent or a nucleophile must be preassociated with the anomeric carbon in some S_N2-like manner (*4*). Thus the geometry in the vicinity of the anomeric carbon must permit this association, and an equatorial exocyclic leaving group in a conventional pyranose chair conformation is not consistent with such association.

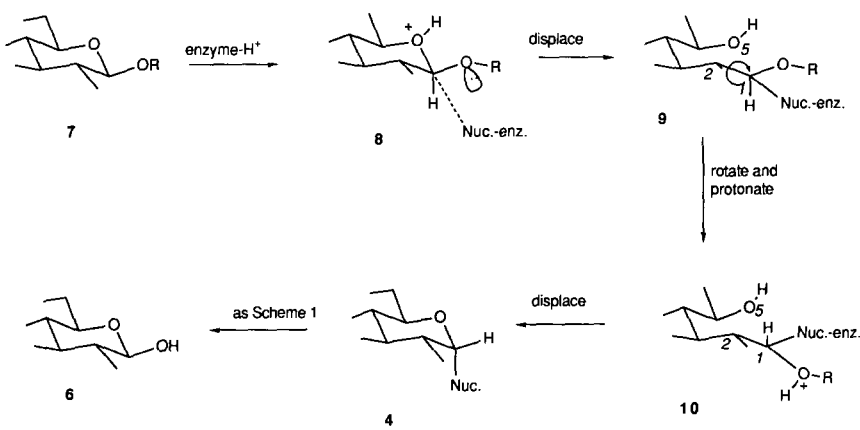
In a controversial 1986 paper (*5*), Post and Karplus suggested that their molecular

0045-2068/92 $3.00

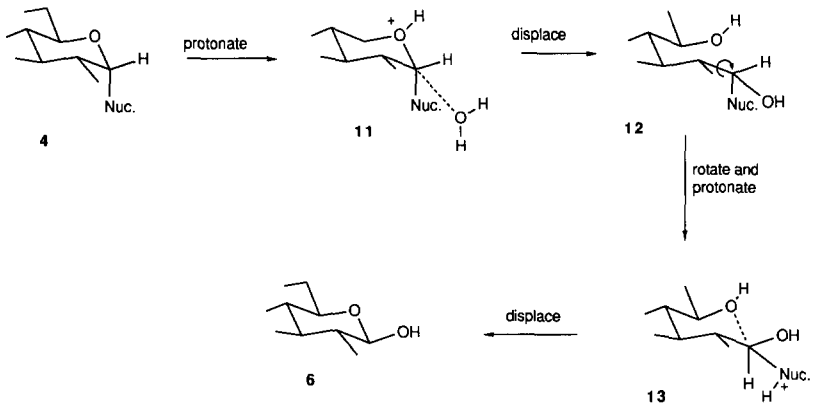


SCHEME 1

dynamic calculations of lysozyme action were more in agreement with protonation of the endocyclic oxygen whereupon nucleophilic displacement of the activated endo C–O bond would not require substrate distortion (Scheme 2). About a year earlier, Fleet proposed a similar endocyclic pathway based on an interpretation of the inhibition of glycosidase activity by hydroxylated cyclic amines (*6*). In this alternate pathway, a series of protonation–displacement–protonation–displacement processes arrives at intermediate **4** from Scheme 1 without a distortion step. Interestingly, the physical motion now required is a rotation (*7*) of the C_1–C_2 bond so that O5 can become back-side-in-line to the glycoside oxygen for the conversion of **9** to **10** in Scheme 2. Note that the enzyme-bound intermediate **4**, consistent with either mechanism, has been observed by Withers (*8*). A pathway



SCHEME 2



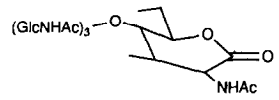
SCHEME 3

for completing the conversion of **4** to **6**, internally consistent with the rest of Scheme 2, is shown in Scheme 3. Thus, intermediate **4** is again protonated on the endocyclic oxygen, displaced by the external nucleophile, reprotonated on the enzymic nucleophilic group, and redisplaced to afford **6**. Again, a physical motion, the rotation of the C_1–C_2 bond in **12**, is required.

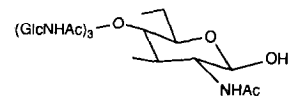
Schemes 2 and 3, implied by the Post, Karplus calculations and the Fleet hypothesis, have received little acceptance, although there are some recent data in the organic chemistry literature which are consistent with the idea of endocyclic oxygen cleavage in pyranoses (*9*). Also, in pyranose thioglycoside systems, endocyclic cleavage pathways have been demonstrated to be significant (*10*). It is the intent of this paper to reinterpret studies on β-glycosidases that have been previously invoked to support the "conventional" mechanism. It will be seen that the published data are equally consistent with the Fleet, Post, Karplus hypothesis.

THE TETRASACCHARIDE LACTONE INHIBITOR OF LYSOZYME

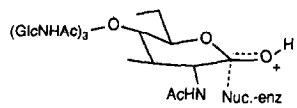
An influential paper on the mechanism of lysozyme, a very well-studied β-glycosidase, appeared nearly 20 years ago (*11*). Lienhard and coworkers reported a study of TACL, the tetrasaccharide lactone **14**, and its interaction with lysozyme (Scheme 4). They proposed that the lactone behaved as a transition-state analog. The lactone is bound more tightly to the enzyme than the unmodified tetrasaccharide **15**. The lactone ring clearly will have a geometry similar to that of flattened chair intermediate **3** in Scheme 1. Thus, the observation that the lactone binds well and that it presumably inhibits the enzyme via a structure such as **16** is consistent with **3** being a reasonable intermediate. These observations by Lienhard were used to buttress earlier arguments based on X-ray and modeling results. One fact to note is that the binding of lactone **14** is not inconsistent with an enzyme transition state that has ring oxygen protonation followed by exo oxygen participation and concomitant exo C–O bond shortening, as in **17** (Scheme 5). Furthermore, a feature of the Lienhard paper that has been overlooked is that TACA, the free acid **18**,



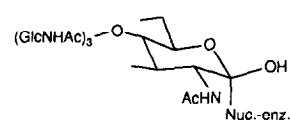
14 (TACL)



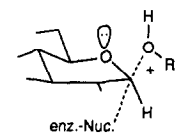
15 unmodified tetrasaccharide



16 transition-state



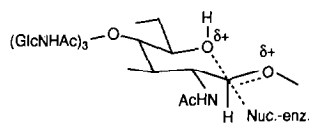
16 intermediate



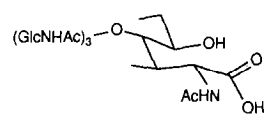
3

SCHEME 4

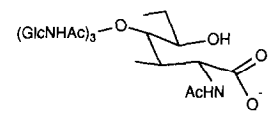
particularly when the buffer is below pH 4.5, binds to the enzyme with an affinity comparable to that of the lactone. Naturally, its ability to inhibit lysozyme at higher pHs drops off because the acid exists largely as its salt **18a**, which would not be a good binder because of electrostatic repulsive interactions with carboxylates in the enzyme active site. For example, at pH 6.2, where the assays of TACL were carried out, an initial TACA **18** concentration of 700 mM would have less than 0.7 mM of free COOH, and (*12*) *even under these conditions,* TACA **18** is an inhibitor of lysozyme. Furthermore, TACL is a substrate for lysozyme, being cleaved to TACA, *an example of endocyclic cleavage catalyzed by a β-glycosidase.* From the published data, we can observe that the rate enhancement over spontaneous ring-opening of TACL (from $t_{1/2}$ = 105 min to $t_{1/2}$ = 8 min) is not



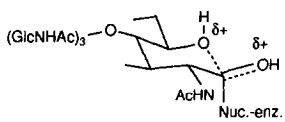
17 endocyclic cleavage transition-state that is consistent with TACL as an inhibitor



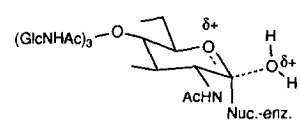
18



18a



19 observed enzyme cleavage of TACL



20 hypothetical exocyclic cleavage of TACL

SCHEME 5

spectacular; nevertheless, an enzymic endocyclic cleavage of the lactone, as in **19**, is actually taking place. But we can't rule out a rapid, but nonproductive exocyclic cleavage such as in **20**. There is another interesting consequence that should follow from the TACL to TACA conversion via endocyclic cleavage. Lysozyme should catalyze the esterification of either inhibitor to esters of TACA if the process were to be carried out in organic media in the presence of alcohols.

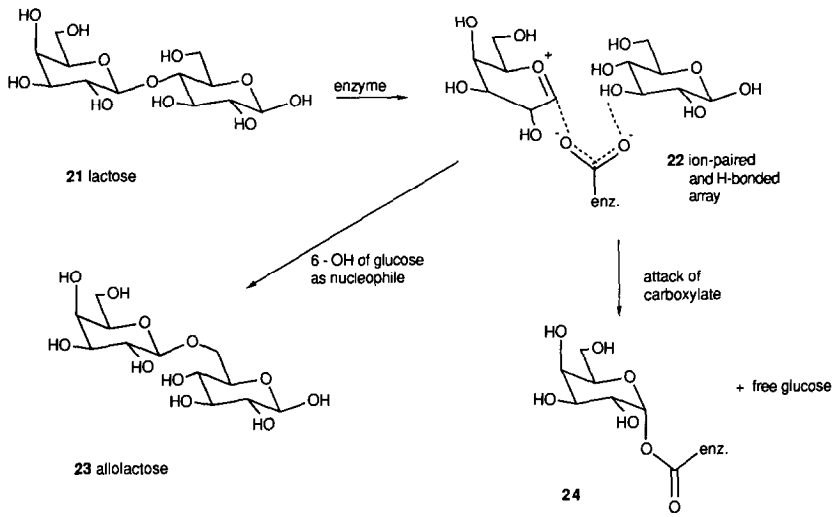
Thus, it appears that the classic Lienhard paper on TACL is not completely convincing as requiring exocyclic oxygen protonation followed by distortion, even though this pathway has become generally accepted as correct.

Sinnott's key argument against the endocyclic cleavage process in lysozyme is that there is an ^{18}O isotope effect for the exocyclic leaving group (*1d*). He argues that this effect is more consistent with a step such as illustrated for **3** to **4** in Scheme 1 as compared to the requirement of **10** converting to **4** as the rate-limiting step in Scheme 2. Since both processes are nucleophilic displacements of the same protonated C–O bond, it is not clear why the observed isotope effect is better evidence for one process than the other.

Kirby's argument against the endocyclic process is that transglycosidation with sugars catalyzed by lysozyme is observed to be faster than hydrolysis by water (*1c*). Thus, a mechanism where water were to be the nucleophile for endocyclic cleavage, affording **6** in either Schemes 2 or 3, followed by reglycosidation would be inconsistent with experimental observation. However, if transglycosidation were to occur by intermediate **11** in Scheme 3 with a glycose replacing water as the nucleophile, then the observed outcome would be reasonable for an endocyclic cleavage pathway. It seems to this reviewer that the traditional arguments for the lysozyme pathway being exocyclic are not overwhelming.

THE LACTOSE-ALLOLACTOSE ISOMERIZATION

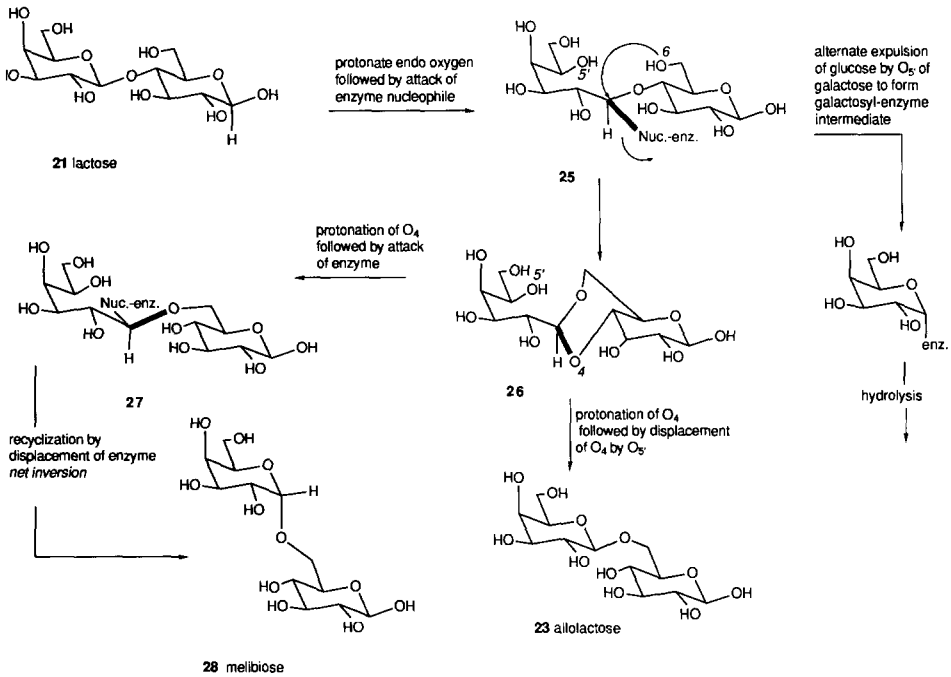
A second well-studied system is the β-galactosidase of *Escherichia coli*. It catalyzes two processes, the cleavage of lactose to galactose plus glucose and the isomerization of lactose to allolactose where now glucose is β-linked via its 6-oxygen to galactose. Careful experimentation has shown that the glucose portion of the disaccharide does not become free of the enzyme during the isomerization process (*13*). Thus, free, labeled glucose is not incorporated into allolactose during the lactose–allolactose isomerization. In order to be consistent with the accepted view that β-glycosides cleave via their exocyclic linkage, Sinnott proposed a novel mechanism to account for the retention of glucose with its galactose partner during the lactose isomerization (*14*). The novelty is that a carboxylate ion acts to keep the glucose associated with its galactose partner via a kind of ion-pair, even though the glucose has no charge (Scheme 6). Capture of the ion-pair by glucose O_6 yields allolactose whereas capture by carboxylate affords enzyme-bound galactose, which is then hydrolyzed in later steps. In later papers on the subject of glycosidase mechanism, Sinnott himself discounts really free oxonium ions as intermediates in these processes (*1*). Even without ion-pairing, it is always possible to postulate some sort of special binding site for the liberated glucose which prevents its



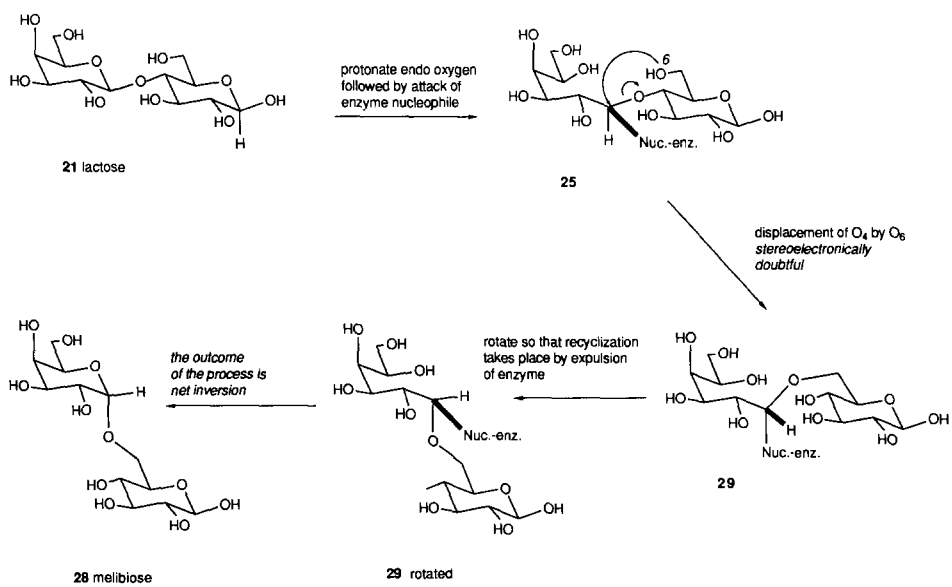
SCHEME 6

diffusion into free solution. This sort of special binding has been discussed for lysozyme by Chipman (*15*).

In fact, endocyclic cleavage supplies a more satisfying rationale for the intramolecularity of the lactose–allolactose isomerization and its independence from the hydrolysis mechanism. The first step is the enzymatic protonation of the ring oxygen followed by endocyclic C–O cleavage to form **25**. Then, the initial intermediate **25** is subjected to a second displacement when O_6 of the glucose expels the enzyme to form **26**. If, alternately, glucose is expelled by O_5 of galactose, then a simple galactosyl enzyme is formed which will lead to hydrolysis product. To continue with the transglycosidation–rearrangement pathway, the enzyme simply catalyzes the displacement of O_4 by $O_{5'}$ to form allolactose with the required β-configuration. An alternate pathway to form **27** via the enzyme nucleophile displacing O_4 followed by expulsion of the enzyme nucleophile by the galactose $O_{5'}$ would form melibiose with the inverted α-stereochemistry. This general mechanism leads to the interesting prediction that acetal **26** should be a substrate for **E. coli** β-galactosidase and should afford allolactose. The diastereomeric acetal related to **26** (not shown) would also be an interesting substrate. It would probably be part of a product mixture if a chemical synthesis of **26** were attempted. Scheme 7 using one endocyclic cleavage requires a three-step process; each step is essentially identical in mechanism, namely protonation of a leaving group followed by nucleophilic displacement with inversion. It accounts for the observed intramolecularity of the lactose–allolactose conversion. A second concise mechanism, shown in Scheme 8, where the glucose O_6 acts as a nucleophile toward the anomeric carbon with the O_4 of glucose as the leaving group (the oxygen which had previously been exocyclic) to give **29** suffers from two problems. A consequence of intermediate **29** is that subsequent recyclization of the opened galactose via expulsion of the enzymic nucleophile would afford **28**, a disaccharide with the α-configuration,



SCHEME 7



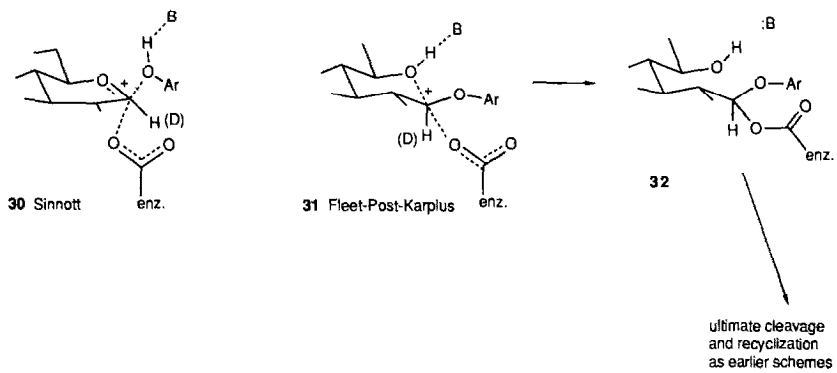
SCHEME 8

i.e., melibiose, which is not the product observed. Another difficulty with this mechanism is that the geometry of the internal displacement of O_4 by O_6 is not ideal.

An analysis of the lactose–allolactose system is complicated by the fact that, superimposed on its isomerization process, the enzyme also catalyzes a transglycosidation when external saccharide donors are added to the system. Huber has shown that the hydrolysis and the transglycosidation (as opposed to the isomerization) have different sensitivities to pH, in the range from pH 7.0 to 10.0 (*16*). It is interesting that Huber concludes from his studies with lactose as well as *p*- and *o*-nitrophenyl galactosides as substrates that there must be two complexes to account for his observations. Thus, there must be a galactosyl–enzyme complex to rationalize the hydrolysis while there must be a galactosyl–glucosyl–enzyme complex to explain the transglycosidation. Although Huber presents no structures, this reviewer submits that covalent intermediate **25** in Scheme 7 could very well be what Huber has detected in his studies. Inter alia, a version of Scheme 7 can account for the observed intermolecular transferase properties of many glycosidases, again with the prediction that there must be ring-opened acetal intermediates analogous to **26** that would be substrates.

THE PYRIDINIUM GLYCOSIDE AND ARYL GLYCOSIDE SUBSTRATES

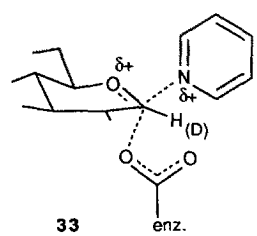
Sinnott and Legler and co-workers have carried out a series of fascinating studies with unnatural substrates of β-glucosidases (*17*). The principal conclusions, supporting the exocyclic cleavage process, are based on thorough analyses of two data sets. One method involves secondary isotope effects of an anomeric C–D bond ($k_{H/D}$ approximately 1.2). The second technique uses correlations of log k_{cat} values of the enzymic hydrolysis with pK_a of the pyridine and phenol leaving groups. What is left unsaid in the phenol studies is that not only is the correlation of log k_{cat} with pK_a very poor (goodness of fit well below 0.5), but that there is no correlation between log k_{cat} and the pK_a of other oxygen leaving groups such as simple alcohols, sugars, and carboxylic acids. For example, the k_{cat}/k_m for the DNP leaving group is $\frac{1}{5}$ of that for glucose. Except for the data point for 2,4-dinitrophenol, the correlation looks better for an inverse sensitivity to phenol acidity, with two parallel lines where one family has C=O or N=O groups in para conjugation to the phenol and the other does not. Thus, the rather unconvincing correlation for phenol leaving groups actually suggests a change in mechanism or a dual mechanism for the DNP leaving group. When a similar experiment was done in the lysozyme series, the DNP substrate had a k_{cat}/k_m value of $\frac{1}{50}$ of the natural substrate. This can be interpreted as a retardation of the protonation and leaving of the endocyclic group (*18*). It is worthy of note that similar data treatments in studies of the acid-catalyzed hydrolysis of aryl thioglycosides and aryl sulfoxyglycosides have been reported (*9b*). Here, a change of mechanism from endocyclic to exocyclic cleavage is clearly indicated by the respective negative and positive ρ values observed. Thus, the method and the results of the aryl glycoside "Hammett" study under discussion are not doubtful. It is their correspondence with the



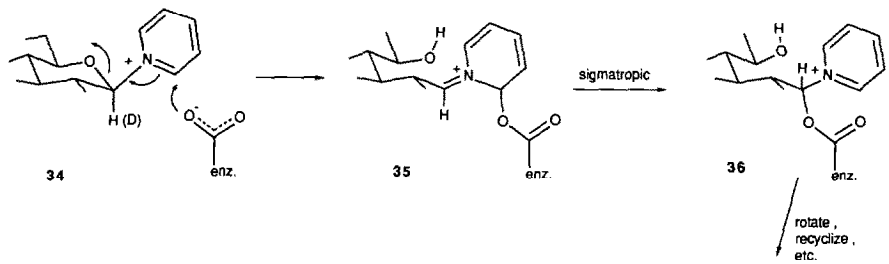
SCHEME 9

proposed exocyclic cleavage mechanism that is dubious. The isotope effect data can be interpreted to say that the anomeric carbon is becoming somewhat trigonal in the transition state. However, the C–O bond that is breaking, endo- or exocyclic, is not distinguished in the experiment reported. Thus, the mechanism could be either as drawn by Sinnott (**30**) or as suggested by Fleet–Post–Karplus (**31–32**) shown in Scheme 9 with no more of a Procrustean data treatment in one case or the other.

The pyridinium glycoside studies afford a much better correlation of k_{cat} (for pyridinium = 0.023 s^{-1} compared to 335 for natural substrate) with the pK_a of the leaving group (conjugate acid) than in the phenol case just discussed. However, an alternate mechanism of pyridine cleavage can be envisaged. Whereas Sinnott suggests a transition state (Scheme 10) quite similar to the cases of oxygen leaving groups, one could instead invoke the very well-known attack at C-2 of quaternized pyridines as a key step, as shown in Scheme 11. The appeal of the hetero-aromatic pathway of Scheme 11 is that the C-2 position is much less sterically hindered than the aromatic carbon of the sugar, and of course, the observed isotope effects ($k_H/k_D \approx 1.1$ for α-D) and pK_a sensitivity ($\log k_{cat}/pK_a \approx -1$) are also consistent. A simple prediction would follow from a quaternary ammonium glycoside derived from an amine having a pK_a similar to that of pyridine, e.g., trifluoroethylamine $pK_a = 5.6$. Since it could not undergo remote attack, it would not be a substrate



SCHEME 10

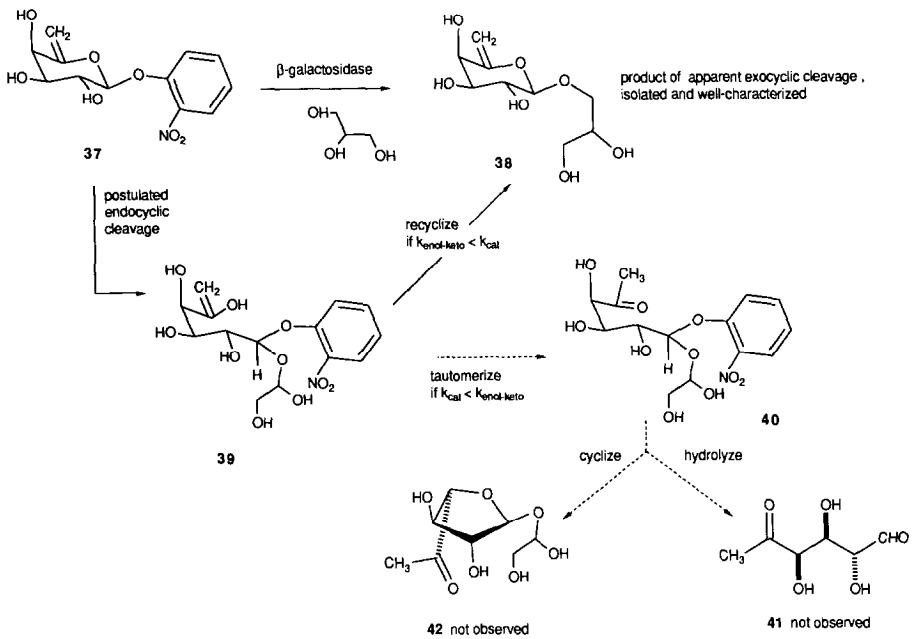


SCHEME 11

for the enzyme, nor would it undergo acid-catalyzed cleavage since protonation of the endocyclic oxygen would be retarded. There is already a suggestion of these outcomes from other work by Sinnott where the trimethylammonium derivative of glucose is a stable substance. However, it could be argued that the pK_a of trimethylamine, 9.8, would correspond to an extremely slow rate of reaction as a leaving group upon extrapolation from the Sinnott data where leaving groups in the pK_a 5 range were examined. It is worthy of note that the idea of nucleophilic attack on the pyridine ring may be useful for explaining the anomalous behavior observed by Hosie and Sinnott in a study of α-glycosidases (*19*). A concluding thought on this section is that the studies with unnatural leaving groups are interesting, but not conclusive as to the enzyme mechanism and, in fact, may actually be involved with unnatural enzymic pathways.

THE Δ-5,6-HEXENOPYRANOSIDE SUBSTRATE

As part of an elegant body of work on enzymic transformations of sugar derivatives, Lehmann has performed an experiment which was interpreted to favor the Sinnott view of exocyclic cleavage, and with which the alternate endocyclic hypothesis is, at first glance, not consistent (*20*). Thus, the *o*-nitrophenyl substrate **37**, upon incubation with glycerol and a β-galactosidase at pH 6.8 afforded a modest yield of **38** as the only characterized organic product. If endocyclic cleavage of **37** had occurred, one would have guessed that some of the known **41** or perhaps the unknown **42** should have been detected (Scheme 12). This assumption would be correct only if the rate of ketonization of **39** were fast compared to k_{cat} of the enzyme. The rate for **39** is not known, but if one uses the data for simple enols (*21*) where k (H_2O) is approximately $10^{-2}/s^{-1}$ and $k(OH^-)$ is 10^7 M^{-1}/s^{-1}, the enzyme k_{cat} of approximately 200 is significantly faster than the ketonization rate up to a pH of 8. Thus, even though the preferred conversion of **39** to **38** rather than to **40** seems counterintuitive, the preference is perfectly reasonable based on this simple comparison of known rate constants (*22*). There is a slight possibility that the *o*-nitrophenyl system is aberrant. It has been observed that in the *E. coli* β-galactosidase lactose–allolactose system described above, the behavior of the *o*-nitrophenyl galactoside is not entirely consistent with either lactose or *p*-



SCHEME 12

nitrophenyl galactoside substrates. Thus, the most telling experiment with this Δ-5,6 substrate would use glucose as the leaving group that exchanged with glycerol. Nonetheless, contrary to the original conclusions drawn from this interesting experiment, the absence of keto products experiment does not rule out the endocyclic pathway of glycoside cleavage.

In conclusion, a reanalysis of some key experiments pertaining to the mechanism of β-glycosidases has shown that the evidence is not overwhelmingly in favor of the exocyclic cleavage hypothesis. It may in fact be the case that these enzymes can catalyze both endo- and exocyclic pathways depending on the substrate, and perhaps each system must be individually analyzed without trying to impose a monolithic scheme for all systems. It is the opinion of this reviewer that the Post–Karplus–Fleet endocyclic proposal deserves further serious consideration and some further experimental testing.

ACKNOWLEDGMENTS

This review was written during a sabbatical leave with partial support by the Deutsche Akademisches Austausch Dienst. and NSF-INT 8712570. Thanks are due to Professors A. Kirby, J. Thiem, C. Post, J. Knowles, and G. Legler for their reading of the manuscript and their clarifying comments, to Professor Thiem for the hospitality provided by the Institut für Organisches Chemie, Universität Hamburg, and to Professor Legler for a preprint and some unpublished data.

REFERENCES

1. (a) SINNOTT, M. L. (1984) *in* The Chemistry of Enzyme Action, p. 359, Elsevier, New York; (b) SINNOTT, M. L. (1987) *in* Enzyme Mechanisms, p. 259, Royal Society of Chemistry, London; (c) KIRBY, A. J. (1987) *in* CRC *Crit. Rev. Biochem.* **22,** 283–315; (d) SINNOTT, M. J. (1990) *Chem. Rev.* **90,** 1171–1202; (e) LEGLER, G. (1990) *Adv. Carbohydr. Chem. Biochem.* **48,** 319–384.
2. BENNET, A. J., AND SINNOTT, M. L. (1986) *J. Am. Chem. Soc.* **108,** 7287.
3. SINNOTT, M. L. (1988) *Prog. Phys. Org. Chem.* **24,** 113–204.
4. (a) YOUNG, R. R., AND JENCKS, W. D. (1977) *J. Am. Chem. Soc.* **99,** 8238; (b) MCCLELLAND, R. A., AND AHMED, M. (1978) *J. Am. Chem. Soc.* **100,** 7031.
5. (a) POST, C. B., AND KARPLUS, M. (1986) *J. Am. Chem. Soc.* **108,** 1317; (b) POST, C. B., DOBSON, C. M., AND KARPLUS, M., Computer Modeling of Carbohydrate Molecules (French, A. D., Brady, J. W., Eds.), ACS Symposium Series No. 430, Ch. 23, Washington D.C. (a thorough discussion of the earlier communication).
6. FLEET, G. W. J. (1985) *Tetrahedron Lett.* **26,** 5073.
7. The shorthand description of the required motion as rotation about only one bond is an oversimplification. What is required in terms of geometry is that the O5 become colinear and in back of the exocyclic oxygen bond to the anomeric carbon. If rotation about the C1–C2 bond were the only motion available to the proposed ring-opened intermediate that would permit the O5 to move the position required for backside-in-line attack on the glycosidic oxygen, there would be a serious problem of the entire leaving group having to vacate its binding site. Another corollary would be a rather large motion of the enzymic nucleophile which is connected to the peptide chain. However, examination of a simple model shows that the O5 can become colinear-in-back to the glycoside oxygen by a combination of small motions so that the C1–C2 bond does not have to do all the work. Also, interestingly, there does seem to be enough flexibility in the two side-chain bonds of the aspartate nucleophile to permit enough of the required movement.
8. (a) WITHERS, S. G., AND STREET, I. P. (1989) *in* Plant Cell Wall Polymers, Ch. 43, Amer. Chem. Soc., Washington DC; (b) Withers, S. G., Warren, R. A. J., Street, I. P., Rupitz, K., Kempton, J. B., and Aebersold, R. (1990) *J. Am. Chem. Soc.* **112,** 5887.
9. (a) GUINDON, Y., BERNSTEIN, M. A., AND ANDERSON, P. C. (1987) *Tetrahedron Lett.* **28,** 2225; (b) GUINDON, Y., AND ANDERSON, P. C. (1987) *Tetrahedron Lett.* **28,** 2485; (c) GUPTA, R. B., AND FRANCK, R. W. (1987) *J. Am. Chem. Soc.* **109,** 6554.
10. (a) CLAYTON, C. J., HUGHES, N. A., AND SAEED, S. A. (1967) *J. Chem. Soc. C,* 644; (b) Wagner, G., and Wagner, M. (1964) *Arch. Pharm.* **297,** 348.
11. SECEMSKI, I. I., LEHRER, S. S., AND LIENHARD, G. E. (1972) *J. Biol. Chem.* **247,** 4740.
12. It must be noted that in the enzyme, the glutamate carboxyl at the active site is protonated at pH 6.2; thus, there could conceivably be a high concentration of TACA in its protonated form.
13. HUBER, R. E., KURZ, G., AND WALLENFELS, K. (1976) *Biochemistry* **15,** 1994.
14. SINNOTT, M. L. (1978) *FEBS Lett.* **94,** 1.
15. CHIPMAN, D. L. (1971) *Biochemistry* **10,** 1714.
16. HUBER, R. E., GAUNT, M. T., SEPT., R. L., AND BABIAK, M. J. (1983) *Canad. J. Biochem. Cell Biol.* **61,** 198.
17. LEGLER, G., SINNOTT, M. L., AND WITHERS, S. G. (1980) J.C.S. *Perkin II,* 1376 and many other papers from both the Legler and the Sinnott groups.
18. BALLARDIE, F. W., CAPON, B., CUTHBERT, M. W., AND DEARIE, W. M. (1977) *Bioorg. Chem.* **6,** 483.
19. HOSIE, L., AND SINNOTT, M. L. (1985) *Biochem. J.* **226,** 437.
20. LEHMANN, J., AND REINSHAGEN, H. (1970) *Justus Liebigs Ann. Chem.* **732,** 112.
21. CAPON, B., GUO, B-Z. KWOK, F. C., SIDDHANTA, A. K., AND ZUCCO, C. (1988) *Acc. Chem. Res.* **21,** 135.
22. For an example from the shikimate pathway where an intramolecular aldol reaction is faster than ketonization of an enol see BARTLETT, P. A., AND SATAKE, K. (1988) *J. Am. Chem. Soc.* **110,** 1628–1630.