MECHANISM AND STEREOELECTRONIC EFFECTS IN THE LYSOZYME REACTION

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I. INTRODUCTION

Lysozyme occupies a special place in the history of enzymology because it was the first enzyme to yield its three-dimensional structure to the X-ray crystallographer.[1a,b] The structural studies focused interest on a somewhat unexplored area of mechanistic chemistry: the enzyme is a β-glycosidase, and only specific acid catalysis had hitherto been identified in the hydrolysis of glycosides and of acetals in general. The early mechanistic work stimulated by the crystallographers' uniquely detailed descriptions of active site geometry and suggestions about substrate binding has been reviewed,[2,3] therefore only the salient features will be described here. In any case, the productive interaction of structural, biochemical, and mechanistic investigations has made the lysozyme story a familiar textbook example[4] and has had a major influence on our developing understanding of enzyme mechanism.[5]

This review is concerned with recent ideas on stereoelectronic effects, which suggest that reactivity at the anomeric centers of glycosides may depend on conformation in a way that has important implications for the mechanism of action of glycosidases. The lysozyme reaction is the most useful context for a discussion because of the detail in which the mechanism is defined.

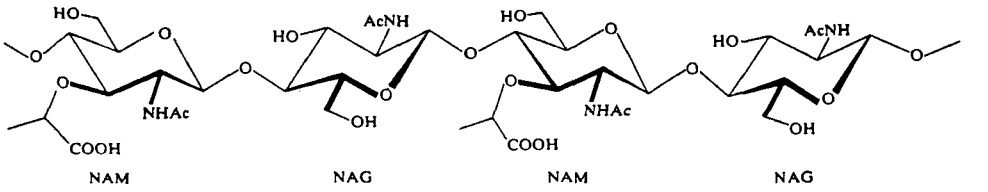
II. THE ENZYME REACTION

A. The Enzyme

The enzyme used for the structural studies, and most mechanistic work, is obtained from hen egg white. It has been set in the context of the family of vertebrate lysozymes in a major review in *The Enzymes*.[6] The enzyme is small (129 amino acids, about 45 × 30 × 30 Å) and roughly egg-shaped in the crystal, with a well-defined cleft on one side. This cleft was convincingly identified as the substrate-binding site because it is occupied by the truncated substrate NAG_3, which forms a complex with the enzyme stable enough for X-ray examination.[7]

B. The Substrate

The natural substrate of the enzyme is thought to be the alternating $(–NAG–NAM–)_x$ polysaccharide component (**1**) of bacterial cell walls.



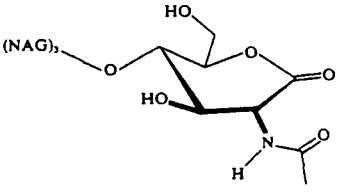
This is cross-linked through amide bonds to the lactic acid side chains of the muramic acid residues by polypeptide bridges. The enzyme cleaves the glycosidic linkage of a NAM residue, but this specificity must derive primarily from binding interactions with adjacent NAG units since NAG-oligomers derived from chitin are good substrates. In fact, only the 5-CH_2OH substituent of this residue is essential since the enzyme will also catalyze the cleavage of bonds to the anomeric center of glucose and 2-deoxyglucose, but not xylose[8] derivatives.

C. The Enzyme-Substrate Complex

The extended binding site within the cleft of lysozyme can accommodate a linear (1-4)-linked hexasaccharide in six subsites A-F. Small oligosaccharides may bind in more than one set of subsites, and even a hexasaccharide may bind partially, in more than one way. The pattern of binding at the first three (from the nonreducing end of the substrate) subsites A to C is known in detail from the X-ray structure of the enzyme-NAG_3 complex. A key question concerns the geometry of the sugar residue in subsite D because the reaction takes place at the anomeric center of this sugar.

Phillips and co-workers[6] constructed a model (Figure 1) of an enzyme-NAG_6 complex by extrapolation from the structure of the NAG_3 complex. To achieve satisfactory binding of the two terminal sugars[5,6] in subsites E and F without incurring serious nonbonded interactions with the CH_2OH group of sugar 4, it was found necessary to adjust the conformation of this sugar. In the model, the sugar in subsite D has a conformation close to the half-chair (Figure 1). This is not the ground state conformation of a pyranose, and sugar residues on both sides must be bound before it is favored.

The results of many structural and binding studies are consistent with this picture.[6] Tetrasaccharides with various modified sugars at the nonreducing end bind more strongly if the modified sugar lacks the 5-CH_2OH group[8-9b] or naturally adopts the half-chair conformation.[10,11] A crystal structure[12] of the complex with the NAG_3-lactone (2) confirms that it occupies subsites A to D, with the lactone ring adopting a sofa (or boat) conformation. And NMR studies in solution indicate that *N*-acetylglucosaminides bind in subsites C and E but not in subsite D.[13]



More recently, it has become possible to calculate preferred conformations for enxyme-oligosaccharide complexes based on the coordinates established for the enzyme-NAG_3 complex. There is general agreement among the authors concerned[14-17] that a stable complex can be formed between lysozyme and NAG_6 without distorting the $^4C_1$ conformation of the sugar residue occupying subsite D. These calculations are valuable and are rapidly increasing

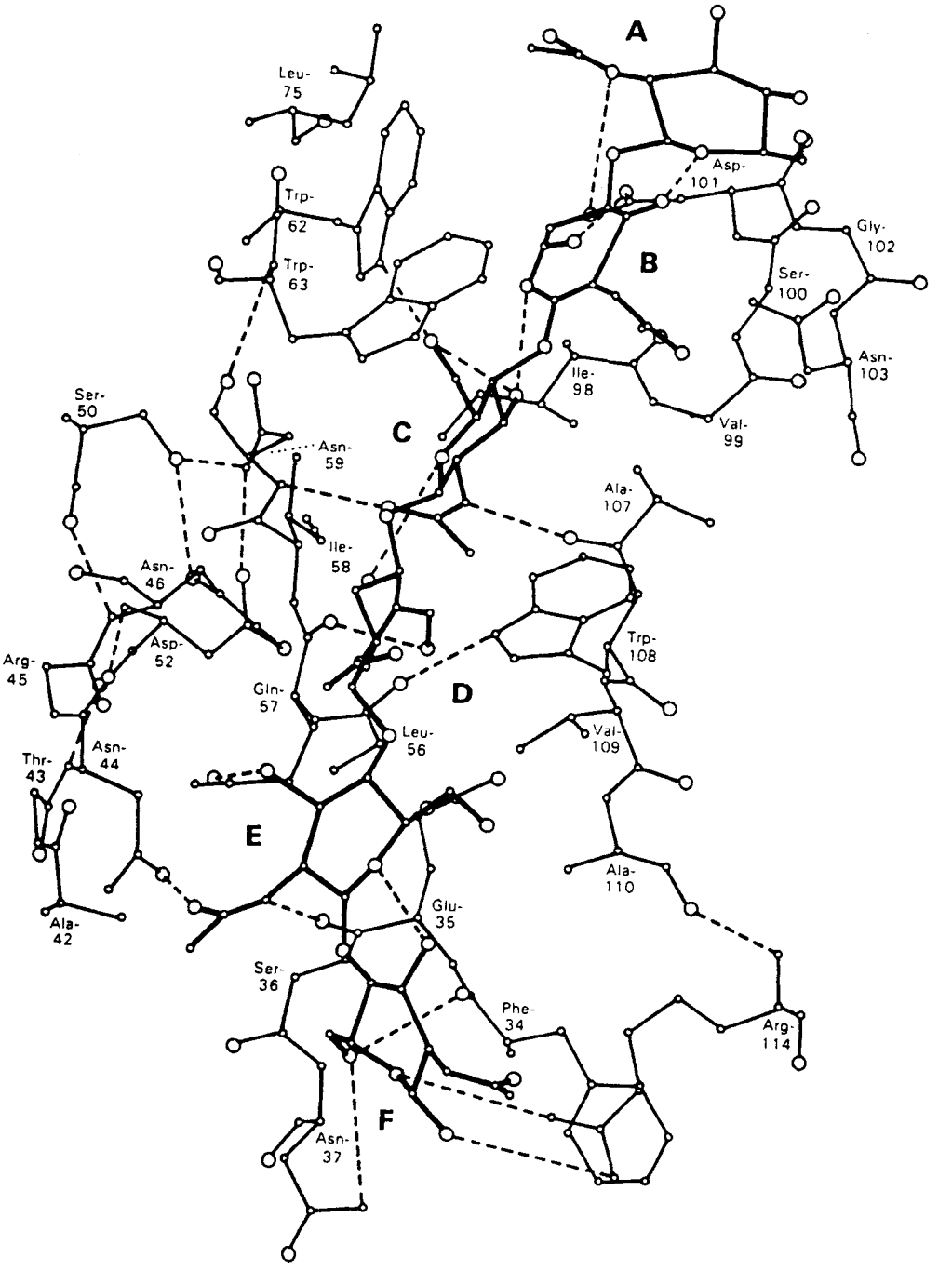


FIGURE 1. The model proposed by Phillips and co-workers for hexasaccharide binding to lysozyme. The figure depicts the active-site region of the enzyme; the substrate bonds are drawn with bolder lines. (From Perkins, S. J., Johnson, L. N., Phillips, D. C., and Dwek, R. A., *Biochem. J.*, 193, 554, 1981. With permission.)

in sophistication. The most recent represents[17] a 55-pS molecular dynamics simulation in which the conformations of the amino acid side chains of the protein are also allowed to vary. This is important since it is known from NMR studies that there is high conformational mobility in the cleft region[18] and that there are important conformational changes on binding even monosaccharides. For example, a ^{13}C NMR study[20] showed that the enzyme becomes much less conformationally flexible on binding *N*-acetylglucosamine.

What is not clear from this work is how far the enzyme-hexasaccharide structures described represent productive complexes. The expectation is that they will best represent Michaelis complexes. However, there is good evidence from presteady state[21] and cryoenzymological[22] studies that the initial enzyme-hexasaccharide complex undergoes two kinetically significant conformation changes before covalent bond breaking occurs. This is supported by structural studies using the more specific trisaccharide NAM–NAG–NAM, which is found to bind in subsites B–C–D. The NMR spectrum of the bound trisaccharide shows that the conformation of the reducing-terminal NAM residue is undistorted,[23] and this is confirmed by a crystal structure determination.[24] But detailed comparison with the structure of the enzyme-NAG_3-lactone (**2**) complex shows that the transition-state analog penetrates significantly more deeply into the cleft of the enzyme. (It is also known to bind more tightly.) Again, the conclusion is that the enzyme-NAM–NAG–NAM complex is probably best regarded as a guide to the structure of the Michaelis complex.

D. The Active Site

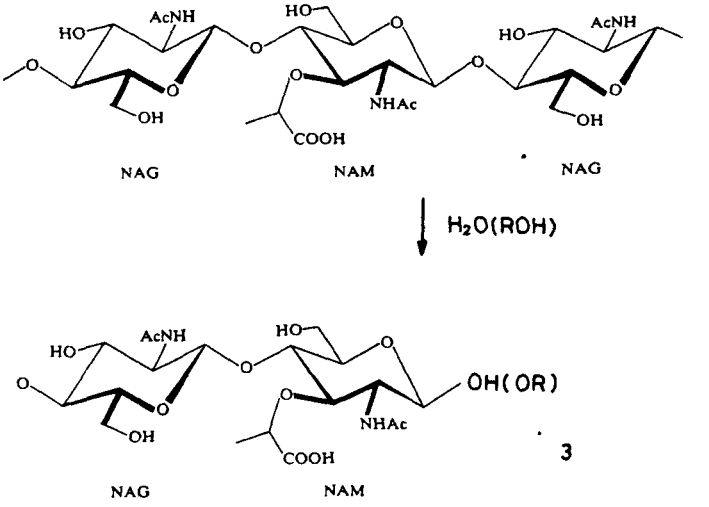
Even an approximate three-dimensional structure of the enzyme-substrate complex makes it a simple matter to identify the functional groups in the active-site region. Within striking range of the anomeric center of the sugar residue bound in subsite D, where bond-cleavage occurs, are two carboxyl groups, those of glutamic acid-35 (Glu-35) and aspartic acid-52 (Asp-52). Chemical modification of these groups destroys catalytic activity,[25-27] and there appears to be no ambiguity in identifying them as the two groups on the enzyme responsible for chemical catalysis.

Of the ten carboxyl groups of lysozyme (two glutamic acids, seven aspartic acids, and the terminal carboxyl), two have unusual pK_as, Asp-66 (2.0 tightly H-bonded) and Glu-35 with pK_a about 6. The remainder, including Asp-52, have normal (4.51) pK_as and may be assumed to be fairly freely solvated.[28-30] Microscopic constants are given by Parsons and Raftery:[28] only the pK_a of Glu-35 is significantly changed on binding chitin oligomers, jumping to over 8 when the cleft-filling glycol-chitin is bound.[31]

These results are all consistent with the picture deduced from model building, which shows Glu-35 situated in a depression in the active-site region of the cleft lined with hydrophobic groups, where it will be screened even more efficiently from solvent when the cleft is filled by bound substrate. These effects act to suppress the ionization of Glu-35, which will thus be present in the COOH form at the pH optimum around 5, where Asp-52 is predominantly ionized.

E. The Reaction

Lysozyme catalyzes glycoside cleavage with retention of configuration at the anomeric center concerned. It is specific for the β-anomers of suitable substrates and the products of the reaction are similarly β-glycosides (**3**).[6]



The enzyme also catalyzes glycosyl-transfer reactions, in which a new glycosidic bond is formed (3, R ≠ H) in the presence of a suitable acceptor. Small alcohols compete with water in this reaction, but is is especially favorable with disaccharide acceptors which can bind in the "leaving group" subsites E and F.[6]

The measured enthalpy of activation ranges from 16 kcal mol^{-1} for chitin pentasaccharides[32] to 25 kcal mol^{-1} for NAG_2-*p*-nitrophenyl glycoside.[33] These are high values for enzyme-catalyzed reactions but represent mostly the high enzyme-substrate binding energy.

Much of the mechanistic work has been done using chitin oligosaccharides (NAG_x) as substrates. These show values of k_{cat} and K_m which reach limiting values for the hexasaccharide, similar to those for cell-wall hexasaccharides.[6] Aryl glycosides are convenient substrates for assay purposes, but are hydrolyzed much more slowly, probably because there are no sugar residues in the leaving group to bind to subsites E and F.* For the hydrolysis of β-aryl disaccharides, a Hammett ρ-value (for k_{cat}/K_m) around 1 is obtained.[33,34]

III. PERTINENT CHEMISTRY

A. The Mechanistic Problem

The structural and biochemical studies outlined above pose a notably well-defined problem in mechanistic chemistry: how can one COOH, assisted by a second, COO^-, group catalyze the hydrolysis of the exceedingly unreactive glycosidic linkage of an oligosaccharide? An intriguing supplementary question concerns the relevance of the apparent distortion of the substrate on binding.

The first convincing attempt to answer these questions,[7,35] summarized in Figure 2, is still considered, at least in broad outline, to be correct.

1. "Glu-35 participates as a general acid, donating a proton to" the leaving group oxygen.
2. The negative charge of Asp-52 "favors development of the carbonium ion".
3. "The ring conformation is already close to that required in the transition state."[6]

The chemistry involved in this mechanism was at the time almost completely unprecedented. Much effort since has been devoted to devising model systems which show one or another

* The k_{cat} for the hydrolysis of NAG_3-*O*-(*p*-nitrophenyl) is only a few times smaller than for NAG_4 but some 10^4 times smaller than for NAG_6. For k_{cat}/K_m, the difference is over 10^6-fold.

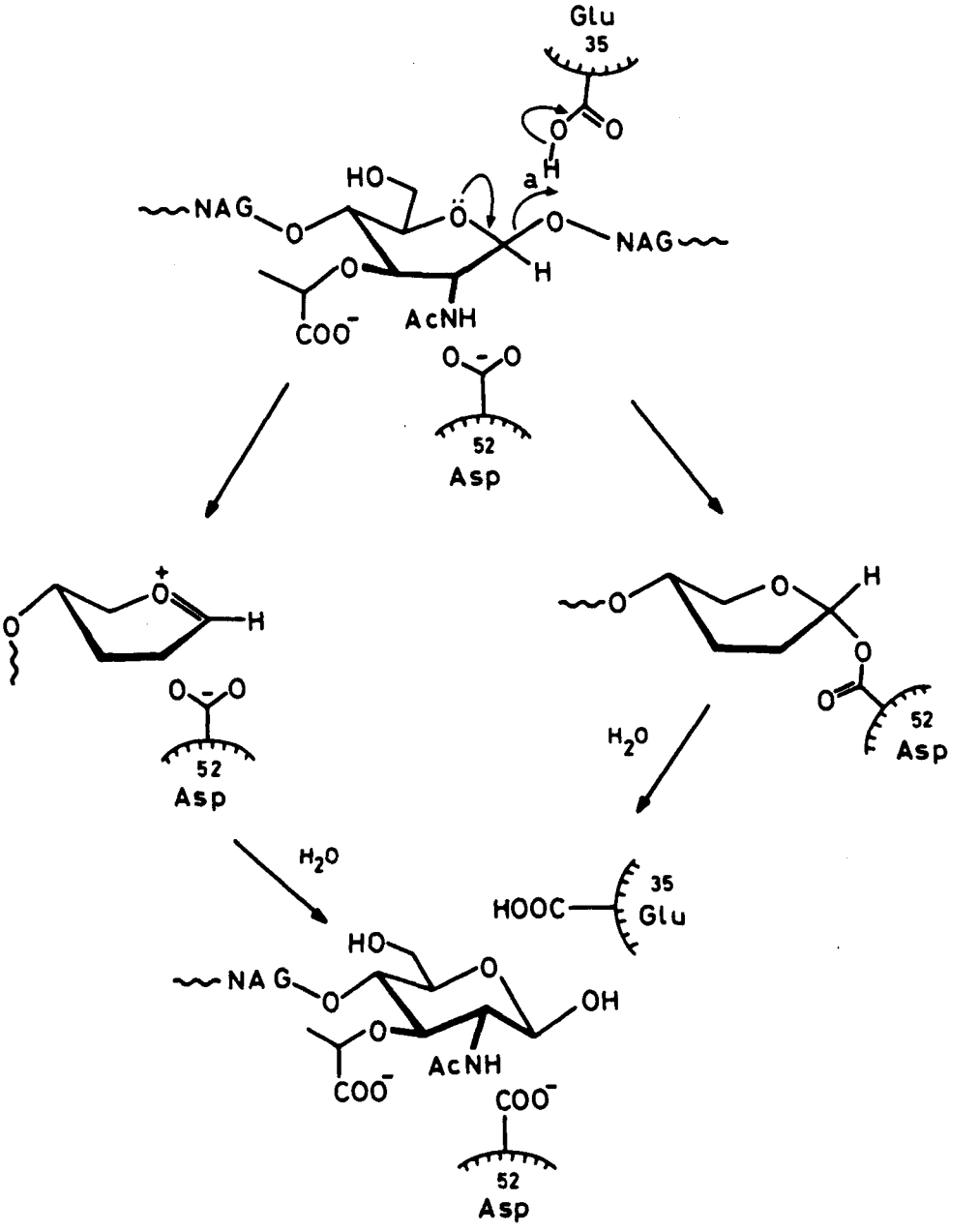


FIGURE 2. Alternative mechanisms for substrate hydrolysis at the active site of lysozyme resulting in retention of configuration at the anomeric center.

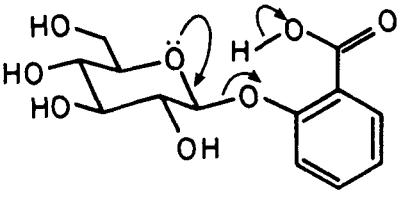
of the three features invoked so that their importance can be assessed. The early work concerned with catalysis by COOH and COO^- has been summarized in two excellent reviews.[2,3] We will discuss the main conclusions of this work and some recent advances to provide the context for an assessment of current ideas on stereoelectronic effects on the lysozyme reaction.

B. Catalysis of Acetyl Hydrolysis

1. Catalysis by COOH

Chemically, the hydrolysis of glycosides, and of acetals in general, was known to require acid.[36,37] The model-building studies on the enzyme-substrate complex placed the COOH group of Glu-35 within hydrogen-binding distance of the leaving group oxygen of the substrate in the active site,[6] making it the logical source of the proton required to complete bond breaking. The proton transfer concerned is thermodynamically highly unfavorable under normal conditions but becomes highly favorable as C–O bond breaking proceeds. It thus fulfills the basic requirement for general acid catalysis suggested by Jencks,[38] and there is general agreement that the role of Glu-35 has been correctly identified (arrow marked *a* in Figure 2). This is not, however, to say that it is properly understood. It is convenient to discuss the various parts of the mechanism separately, but they are in fact interdependent, therefore it is essential that we understand all the chemistry involved as fully as possible.

Carboxyl group participation in acetal hydrolysis was first identified as an intramolecular reaction,[39] with the hydrolysis of the glucoside (**4**) derived from salicylic acid being significantly faster than expected for catalysis by H_3O^+.



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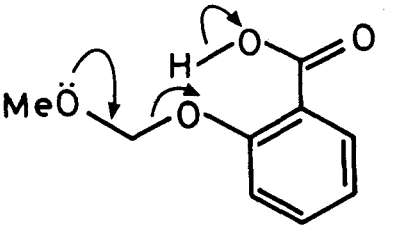

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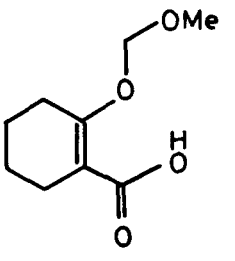
The reaction is relatively easily identified in derivatives of salicylic acid (such as **4** and **5**[39]) because it turns out to be exceptionally efficient in these systems.* Intramolecular general acid catalysis is normally rather inefficient, with effective molarities of the general acid (corresponding to the concentration of the same catalytic group in a separate molecule required to equal the observed rate of reaction)[41] in the region of 1 *M*. In acetals derived from salicylic acid, effective molarities as high as 10^5 *M* have been observed for the COOH group acting as a general acid.[41] It is a reasonable presumption that the same mode of catalysis is relatively efficient in enzyme reactions also, and thus of interest to dissect the mechanism for the model reaction, to try to identify the special factors associated with the salicylic acid system which are relevant to its high efficiency.



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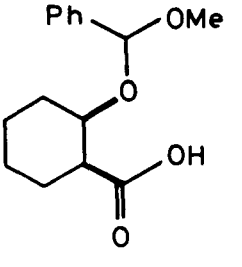

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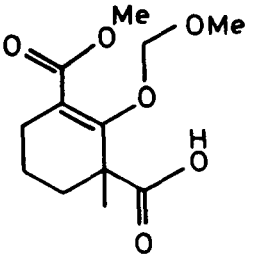

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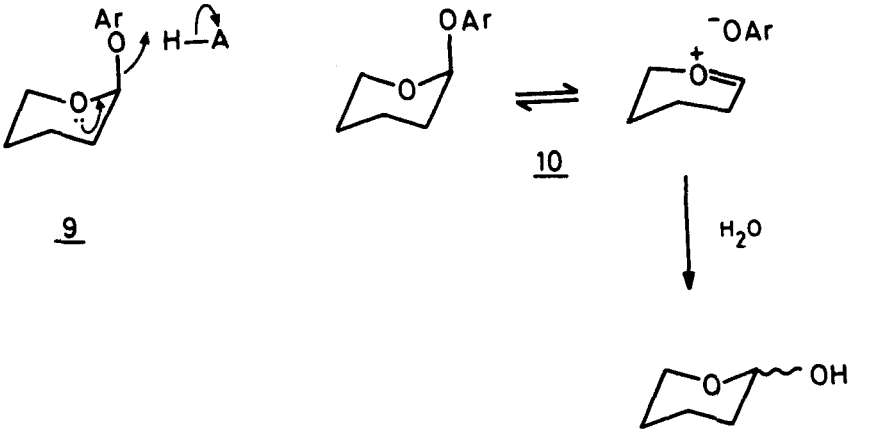
* Structures **4** and **5** are electronically unsymmetrical alkyl aryl acetals and would be expected to be cleaved with C–OAr cleavage even in the absence of efficient intramolecular general acid catalysis.

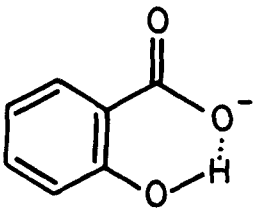
A study of ring-substituted derivatives of **5** showed that the transition state for acetal cleavage (**5**, arrows) involves little proton transfer,* but has a significant amount of C–O bond breaking,[42] resulting in a buildup of negative charge on the leaving group oxygen. Catalysis remains efficient in the reduced system **6**[43] but reverts to the normal inefficient mode for the fully reduced analog **7**.[44a] This is the case for **all** derivatives of saturated acids studied and suggests that in simple systems at least C–O bond breaking needs to be well underway before the intramolecular proton transfer can become clearly thermodynamically favorable and thus efficient. Work with this type of catalysis in related phosphate[45] and sulfate diester systems,[46] where bond breaking cannot be far advanced in the transition state, is consistent with this conclusion. Furthermore, one of the two situations[2,3] in which intermolecular general acid catalysis can be observed is exemplified by the hydrolysis of unsymmetric acetals like **9**,[47] which are also subject to spontaneous hydrolysis (**10**).[48]



This reaction (**10**) is characterized by a very high sensitivity to the nature of the leaving group ArO^-, which denotes a large buildup of negative charge on the leaving group oxygen in the transition state. In both inter- and intramolecular general acid catalyzed reactions, the developing negative charge is neutralized by the proton transfer, which inhibits the reverse reaction (**10**) and leads to an earlier transition state of lower energy.

If this development of negative charge on the leaving group oxygen is necessary for catalysis, it is clearly not sufficient since the exact relationship of the leaving group oxygen to the catalytic COOH group is also crucial. This is shown by the very inefficient catalysis observed with **8**,[43] which has the same type of leaving group as **6**, but with the carboxyl group on an adjacent saturated carbon. What **8** lacks, but salicylic acid derivatives (such as **4** and **5**) and acetal **6** have in common, is a strong hydrogen bond from the catalytic COOH group to the leaving group oxygen in the anion produced. Salicylate is well known to be characterized by an intramolecular H bond (**11**), so strong that it persists in water,

* The extent of proton transfer in the transition state in this class of reaction is a matter of some dispute and may in fact depend on the substituent at the acetal center.[3,44b]



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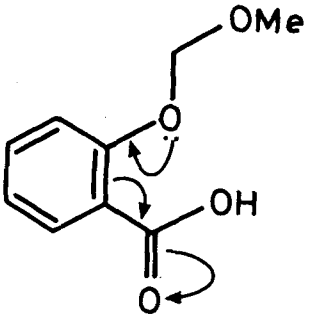

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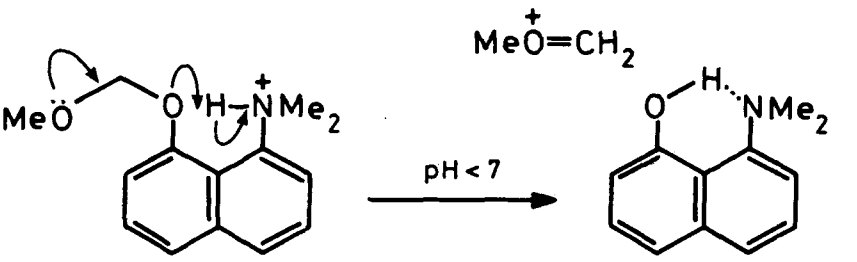
as shown by the exceptionally high pK_a (12.95) of the phenolic OH group.[49] This is unusual, particularly where one of the groups is OH, because it is generally more favorable for two neighboring groups to be solvated separately by water and is observed only when rather precise geometrical requirements are fulfilled; particularly an optimum OH . . . X (X is the H-bond acceptor) distance of approach.

One other obvious property that **4**, **5**, and **6** have in common is conjugation (**12**) between the leaving group oxygen and the COOH group. This is clearly not a factor which could be relevant to any related enzyme-catalyzed reaction, so it is important to decide whether it is essential for efficient catalysis in the model system, as might be suggested by its disappearance when this conjugation is interrupted in **7** and **8**.

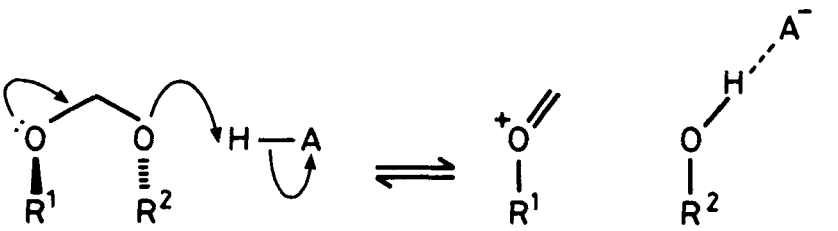
This possibility is effectively eliminated by recent work with the model system **13**.[50] Using Me_2NH^+ as the general acid eliminates any possibility of conjugation with the oxygen atoms of the leaving group. The product (**14**) is also characterized by a strong intramolecular hydrogen bond which persists in water (pK_a of OH group 14.9).[51]



13 14

Efficient catalysis of the hydrolysis of the acetal group of **13** is observed: it is not possible to calculate an effective molarity because the intermolecular reaction is too slow to measure, but the rate of hydrolysis is comparable with efficient COOH-catalyzed reactions. Since the pK_a of the Me_2NH^+ group is almost 7, this means that catalysis is observed up to pH 7. The ratio k_o/k_Ha_H (k_o is the observed rate constant for the reaction **13** → **14**, k_H that for the corresponding specific acid catalyzed hydrolysis of **13**) is over 10^6 at the pK_a, compared with a ratio of a few hundred for typical reactions of unreactive salicylic acid acetals like **4** and **5**.[50] (High ratios, of the order of 10^5 to 10^6, are observed for very reactive systems such as alkyl salicyl acetals of substituted benzaldehydes[44b,52] and salicyl tetrahydropyranyl[44b] acetals.)

To summarize, efficient intramolecular general acid catalysis of acetal hydrolysis (**15a**) appears to have very specific requirements. One is for a strong hydrogen bond between the OH group produced and the conjugate base of the general acid. The second is for an electronic imbalance in the acetal group (**15b**) such that the bond to the leaving group oxygen is already the weaker of the two C–O bonds at the acetal center, with a tendency to break even in the absence of the general acid. In model systems, this is easily arranged. In the glycoside substrate of lysozyme, the acetal group is essentially symmetrical (however, see Section III.C.1) so that any electronic imbalance has to be created by the interaction with the enzyme.



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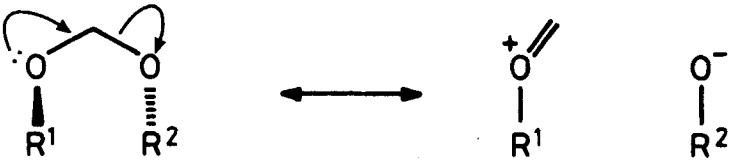


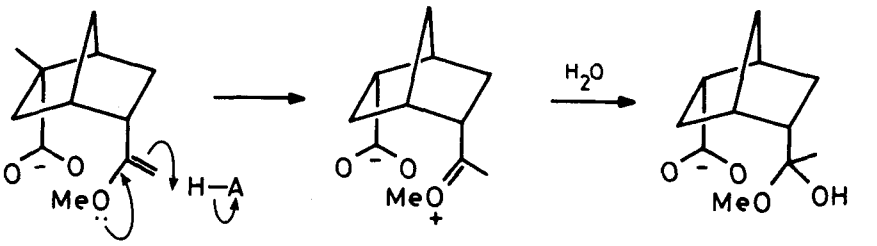
15b

2. Catalysis by COO^-

In contrast to the situation with Glu-35, which is generally agreed to act as a general acid, there is no consensus regarding the role of Asp-52 in the lysozyme reaction. There are two "extreme" possibilities, both of which find some support, plus a third, that the truth may lie somewhere in between.

The two "extreme" possibilities are illustrated in Figure 2. In the left-hand channel, the COO^- group, which in the model of the enzyme-substrate complex lies on the opposite side of the glycosidic center from Glu-35, provides electrostatic stabilization of the developing positive charge associated with the formation of the oxocarbonium ion.[7] This role is supported by calculations, particularly by Warshel,[53-54] who stresses the importance of electrostatic interactions in the stabilization of charged species by proteins in general and uses the lysozyme reaction as an example.

It is difficult to assess the quantitative importance of electrostatic stabilization since the results of attempts to demonstrate its effects experimentally have been almost totally negative. The most elaborate model system devised so far (**16**) was designed[55] to avoid the ambiguities inherent in related acetal reactions by generating the oxocarbonium ion (**17**) by protonation of a vinyl ether. The COO^- and vinyl ether groups are close enough (2.5 to 3 Å) in **16** to compare with the lysozyme system and to exclude the possibility of a bridging solvent molecule, therefore a direct through-space interaction is expected. Only small effects of carboxyl group ionization were observed, and electrostatic stabilization was considered insignificant in the predominantly aqueous solvents used.



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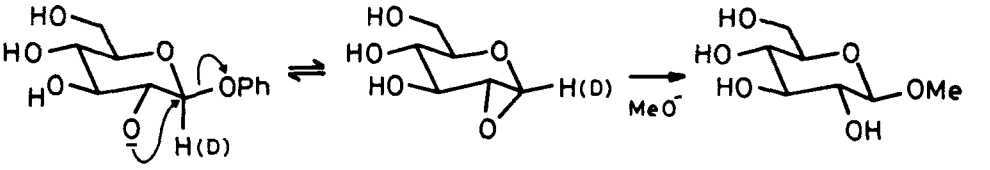
Other systems give results that are ambiguous, in the same way as the lysozyme reaction itself (Figure 2).

The alternative "extreme" mechanism involves Glu-52-COO^- acting as a nucleophile so that the overall reaction is a double displacement (Figure 2, right-hand channel). Like electrostatic stabilization, this role for Glu-52 was unprecedented when the lysozyme mechanism was first discussed on the basis of the crystallographic results and was considered unlikely on the grounds that the COO^- group of Asp-52 would need to move by some 1.5 Å. In light of more recent data on the conformation flexibility of the active site region and of much larger movements of groups in other enzymes, this seems now to be a less powerful objection. A second objection was that the secondary isotope effects for the lysozyme-catalyzed hydrolysis of β-D-phenyl-NAG-glucoside labeled with deuterium at the anomeric center (k_H/k_D 1.11)[56] and for the corresponding tritium-labeled NAG_3 (k_H/k_T 1.19)[57] were close to those observed for the specific acid-catalyzed hydrolysis of phenyl glucoside, considered undoubtedly to go by way of an oxocarbonium ion. For the alkaline methanolysis of phenyl glucoside, probably a rare case of intramolecular nucleophilic participation by the ionized 2-OH group (**18**), only a very small isotope effect (k_H/k_D 1.03) was observed.

The problem with this evidence is that, necessarily, it uses an unusual, and very likely atypical, model for the nucleophilic reaction. Substitution reactions at acetal centers generally involve dissociative processes,[2,3] and the intramolecular reaction (**18**) was the best available. But the transition state for the formation of the acetal epoxide (**19**) must be very different from normal for a substitution reaction, both in timing and geometry, and the observed α-deuterium isotope effect will reflect this.



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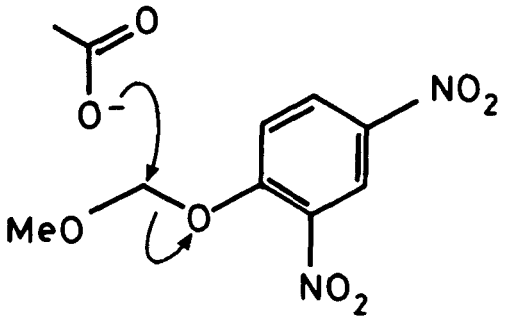
What was needed was information on a simple nucleophilic reaction of an acetal, and more recently such information has become available.

Intermolecular nuelcophilic catalysis of acetal hydrolysis is not detectable for the hydrolysis of pyranosides;[58] however, in the most favorable systems (e.g., **20**) with a primary center, expected to be the most accessible to nucleophilic attack, the reaction is observed and can be characterized.[58,59]



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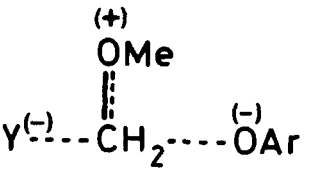
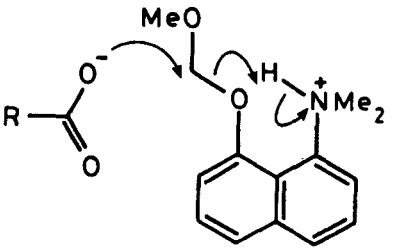


21

The evidence shows that substitution involves a very loose transition state (**21**), with weak bonding to both nucleophilic and leaving group, and hence a substantial buildup of positive charge at the acetal center. As a result, the secondary α-deuterium isotope effect (k_{CH_2}/k_{CD_2}) is similar to that for the hydrolysis reaction: for the reaction with acetate, a figure of 15% per deuteron is found, the same as the value found for the lysozyme-catalyzed hydrolysis of NAG_3.[57]

In fact, the conclusion that S_N2 reactions of acetals should go through S_N1-like transition states is logical since substitution reactions of acetals like (**20**) are much faster than those of ethers, where the second oxygen atom (e.g., the OMe of **20**) is absent; its role must therefore be to stabilize the transition state, which it can only reasonably do by stabilizing, and thus encouraging, the developing of a positive charge at the acetal center.

Recent work suggests that substitutions at glycosidic centers resemble those at the primary center of (**20**). Even hydrolysis appears to involve weak participation by solvent water as a nucleophile,[58-60] and it is suggested that this is a consequence of the instability of methoxymethyl and glycosyl cations,[60,61] which have predicted lifetimes in water shorter than the period of a bond vibration.[61] The lifetime of a glycosyl carbonium ion could of course be longer in the active site of an enzyme, from which water is excluded, but it is difficult to imagine what sort of conformational restrictions could be strong enough to prevent the oxocarbonium ion-carboxylate pair (Figure 2) collapsing to neutralize each other by forming a covalent bond. Glu-52-COO^-, in other words, must be at least as good a nucleophile as water towards the glycosyl carbonium ion. In the only model (**22**) which combines intermolecular nucleophilic and general acid catalysis, for example, the second-order rate constant for attack by acetate is at least 42 times greater than that for attack by water solvent.[50]



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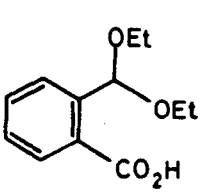

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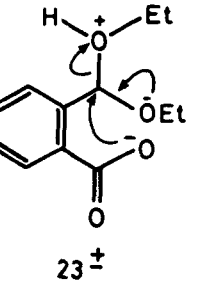
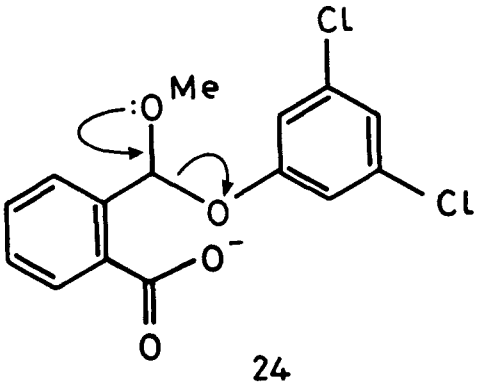


23±

Intramolecular nucleophilic catalysis by carboxylate has been implicated in the hydrolysis of a handful of acetals, mostly derivatives of phthalaldehydic acid, although the ambiguity

running through all this work, already illustrated in Figure 2 for the lysozyme reaction, is still unresolved. The hydrolysis of **23** in 82% dioxan, **but not in water**, is 3000 times faster than that of the *p*-COOH compound[62] and leads to the product of nucleophilic addition of carboxylate to the neighboring acetal carbon. Nucleophilic participation by CO_2^- (**23**) was suggested. On the other hand, the spontaneous cleavage of the mixed acetal **24**, which is 100 times faster in 50% aqueous dioxan than that of the isomer with the COOH group in the *para* position, has been explained[63] in terms of electrostatic participation. The same authors prefer the same explanation for perhaps the most interesting of all the model compounds studied so far. The introduction of a second 2-carboxycyclohexanol into the mixed acetal **7** (above) gives a (benzaldehyde) acetal center (**25**),



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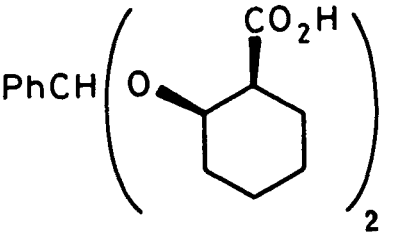
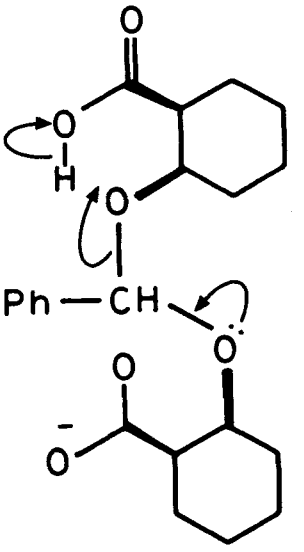


25

flanked by two carboxyl groups, where the leaving group is the oxygen atom of an alcohol rather than a phenol. The hydrolysis of this compound in 50% aqueous dioxan is up to 4 × 10^4 times faster than that of the diethyl ester and shows a bell-shaped pH-rate profile with a rate maximum between pH 6 to 7, indicating that the monoanion is the reactive species. Thus the presence of the second COO^- group elicits a previously undetectable catalytic contribution from the COOH group, and a concerted mechanism (**26**) is suggested.[44] (A similar, much more efficient reaction was



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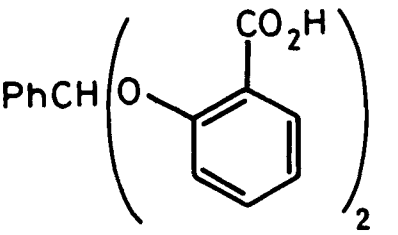


27

observed previously for the bis [salicylic acid] acetal **27**, where the contribution of the COO^- group could be estimated, and turns out to be small.[64])

There is no conformational or other constraint on the COO^- groups of **24** or **26** which would prevent them from acting as nucleophiles, and covalent bond formation is by far the most effective way of stabilizing the oxocarbonium ion intermediate in these reactions and thus the transition states leading to them. What the models tell us is that efficient catalysis of acetal hydrolysis by two carboxyl groups requires one to be ionized to COO^- and the other to be in the COOH form and that the two groups must work very precisely together. The COOH group appears not to act as a general acid unless C–O bond breaking is facilitated by electronic or other means, which is the function of the neighboring COO^- group. Similarly, measurable nucleophilic (or electrostatic) participation by the COO^- group requires C–O bond breaking to be facilitated, as it can be by general acid catalysis by COOH.

The third essential component of all these reactions, without which no C–O bond breaking would occur, is the second oxygen atom at the acetal center, which stabilizes the developing oxocarbonium ion directly by electron donation. We now go on to consider the factors involved in this process.

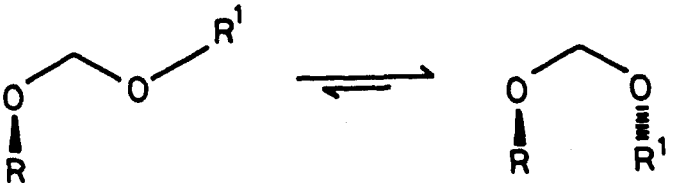
C. Stereoelectronic Effects at Acetal Centers

1. The Anomeric Effect

The anomeric effect[65] is the well-known preference of glycosides, and generally of tetrahydropyrans with electronegative groups in the 2-position (**28**), for the axial conformation (**29**).

28 **29**

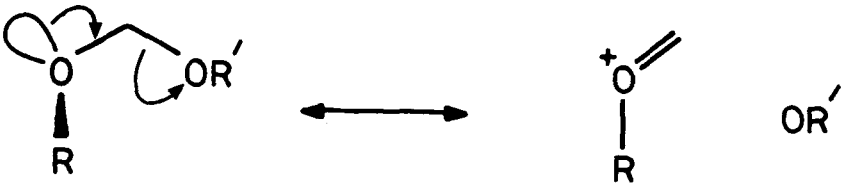
The same effect is observed in acyclic systems, where the geometrical consequences vis à vis the lone pair electrons are perhaps easier to appreciate (**30** and **31**).*



30 **31**

The most satisfactory explanation of the effect is in terms of a stabilizing (two-electron bonding) interaction between a lone pair orbital on one oxygen and the antibonding orbital of the C–O bond to the other. This n-σ interaction, simply represented as **32**, is most efficient when the two orbitals concerned are parallel and thus antiperiplanar.

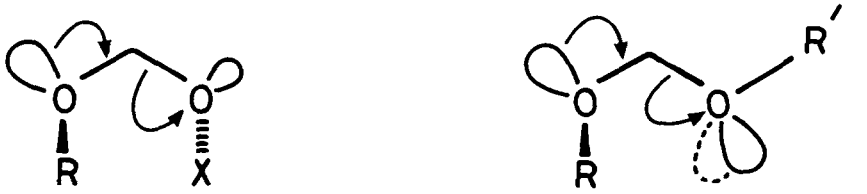
* The resulting preference for the O–R bond of **28** and **29** to adopt a conformation with a lone pair antiperiplanar to the endocyclic C–O bond is known as the exo-anomeric effect.



32

Evidently it should lead to some double bond character, thus strengthening one C–O bond at the acetal center, at the expense of lengthening and weakening the other. In the preferred conformation (**31** and **29**), the geometry is favorable for *n*-donation by **both** oxygen atoms. The overall effect in a symmetrical acetal is net stabilizing: **both** C–O bonds are shorter, and thus stronger, than they would be if the second oxygenation were absent.

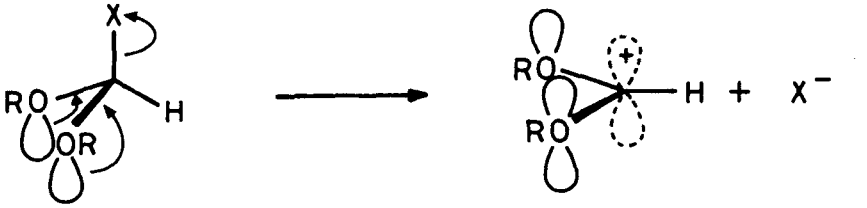
If the acetal is unsymmetrical, either because the R groups are different or because the **conformation** is different from that preferred (**31**), one C–O bond is longer, and thus weaker, than the other. In the former case, this is because the oxygen atom attached to the more electron-withdrawing group (X in **33**) is intrinsically a less effective *n*-donor. In the latter case, exemplified by the conformation (**30**), with the O–R′ bond antiperiplanar to C–OR, it is because only the C–OR′ bond is antiperiplanar to a lone pair on (the other) oxygen (**34**).



33 34

2. Deslongchamps' Theory of Stereoelectronic Control

Deslongchamps has suggested that the orientation of lone pair electrons can control reactivity in appropriate systems.[66-68] Specifically, it is suggested that the C–X bond of a system like **35**, with three carbon-heteroatom bonds, will break more easily when lone pairs on both remaining heteroatoms are antiperiplanar to it.

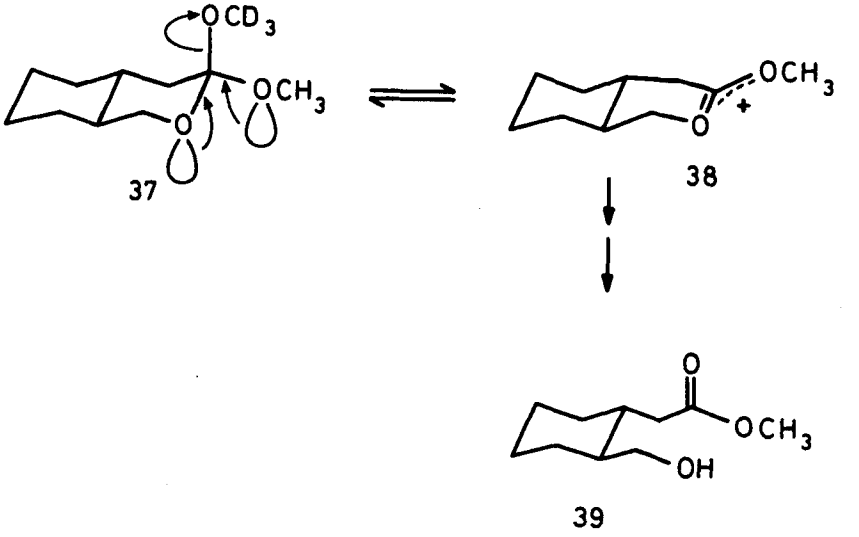


35 36

The n-σ^*_{C-X} overlap, which leads to the extended delocalized system in the carbocation produced (**36**), is thus maintained at optimum efficiency through the bond-breaking process, which therefore follows the minimum energy pathway. In fact, of course, it is important

only that overlap should be optimal at the transition state, so only systems prevented by significant conformational barriers from attaining the required geometry (**35**) are expected to show effects on reactivity. (The stabilizing effect of n-σ^*_{C-X} overlap [a generalized anomeric effect] on the ground state of a compound like **35** means that it will adopt the conformation shown in the absence of overriding conformational restrictions.)

For example, orthoester **37**, which is formed stereospecifically by the addition of CD_3O^- to the cation **38** under aprotic conditions, is hydrolyzed in acid specifically to the methyl (CH_3) ester **39**.[69] Compound **37** is also formed stereospecifically when the dimethyl $(CH_3)_2$ orthoester undergoes acid-catalyzed exchange with CD_3OH in methanol-d_4-dichloromethane.[70] The exchange of the equatorial OCH_3 group can be observed but is so much slower (of the order of 100 times) that it is difficult to measure relative rates under the same conditions.



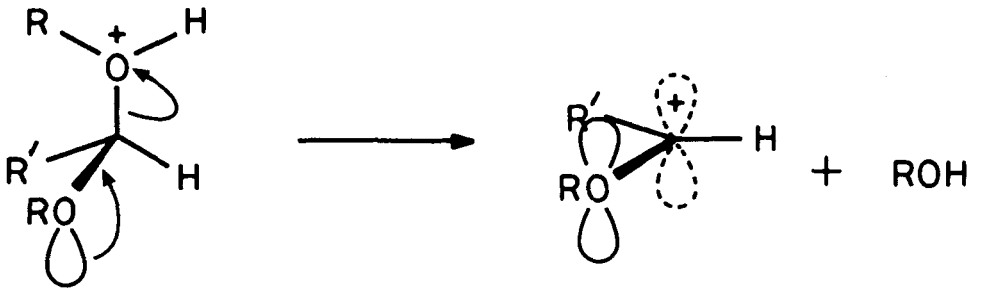
This is consistent with Deslongchamps' theory because the geometry of the tetrahydropyran chair precludes either lone pair on the ring oxygen from getting antiperiplanar to the equatorial C–OMe bond, whereas lone pairs on both ring and equatorial oxygen atoms are antiperiplanar to the axial C–O bond. This illustrates a clear prediction of the theory, that axial should be lost more easily than equatorial leaving groups from systems like **37**.

The basic tenets of Deslongchamps' theory are widely accepted, and it has had an important influence on the way organic chemists think about reactions around heteroatom centers. It is important, however, to bear in mind that stereoelectronic effects of this sort are just one, often not the most important, of the many factors which control the reactivity of organic compounds.

3. Stereoelectronic Effects On Acetal Cleavage

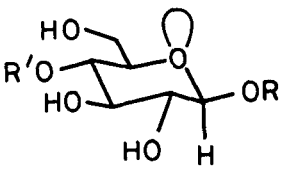
a. In Theory

Deslongchamps' theory is simply extended to acetal cleavage. The cleavage of an acetal like **40** must be more dependent on the stabilization it receives from the single oxygen donor atom than that of an orthoester derivative (**35**), so it should be more dependent also on any factor, including the orientation of its lone pairs, which affects the donor capability of that oxygen.



40

This conclusion has immediate application for the reactions of glycosides. In the ground state conformation of a β-glycoside, such as the lysozyme substrate, the aglycone (OR in **41**) is equatorial, antiperiplanar only to ring bonds, so that C–OR cleavage, the key step in normal glycoside hydrolysis, is predicted to be stereoelectronically unfavorable. For an α-glycoside (**42**), on the other hand, a lone pair of electrons in the ring oxygen is available antiperiplanar to the C–OR bond, so C–OR cleavage is stereoelectronically favorable in the ground state conformation.



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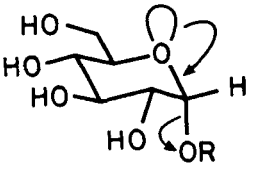

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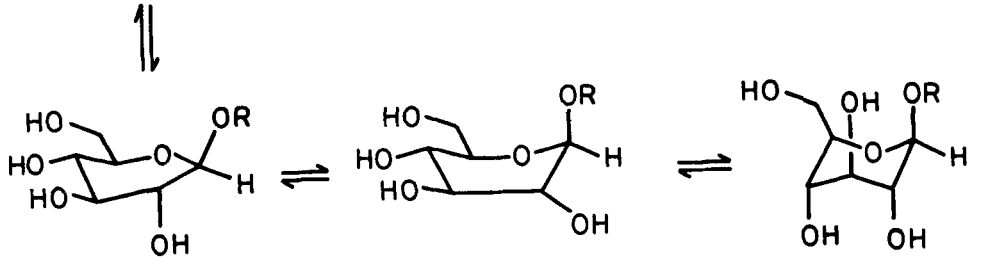


43 44 45

Stereoelectronic effects of this sort are conformation dependent, therefore the predicted stereoelectronic barrier to the cleavage of the C–OR bond of a β-glycoside (**41**) could be "got round" by ring inversion (**41** → **45**). However, D-glycopyranosides do not invert readily (the equatorial 5-CH_2OH substituent being the most important in determining the conformation[65]) even though the invertomer (**45**) would then be stabilized by the anomeric effect. If the reaction is indeed blocked by a stereoelectronic barrier in the ground state (**41**) and unfavorable through **45** because of the conformational barrier, then the reaction might be expected to go through an intermediate conformation. A continuum of these exists on the pathway between **41** and **45**, some of which retain the chair geometry at C (4, 5, and 6) but have relaxed at the anomeric center in the first stage of the conversion to **45**. (Recognizable intermediate forms are the half-chair [**43**] and the boat [**44**].) As a result,

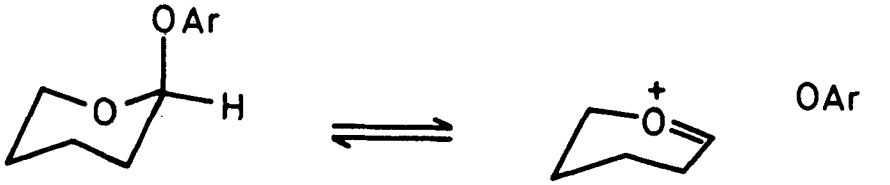
lone pair donation (n_o-σ^*_{C-OR} overlap) can become stereoelectronically favorable at minimal conformational cost.

The half-chair conformation (**43**) is immediately recognizable as similar to that suggested by the model-building studies of the Phillips groups (discussed previously) for the NAM residue bound in subsite D at the active site of lysozyme. Hence, the intriguing suggestion[71] that lysozyme distorts its substrate on binding specifically in order to avoid the inherent stereoelectronic barrier to the cleavage of a β-glycoside in its ground state conformation.

b. Experimental Evidence

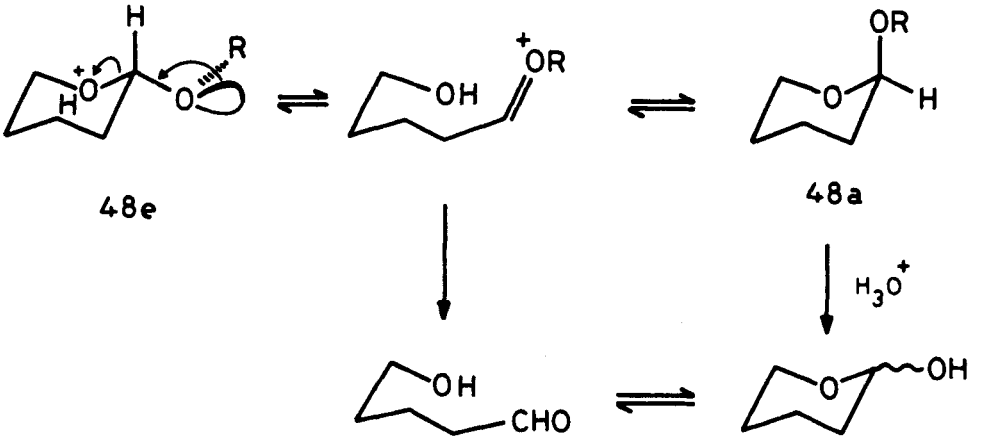
The theory makes the simple prediction that there is a stereoelectronic barrier to the cleavage of an equatorial glycoside, which is absent for the axial isomer. It is important to test such a theory experimentally. It turns out that finding unambiguous evidence for primary stereoelectronic effects on reactivity at acetal centers is not a simple matter. The evidence is summarized in this section: for more detail a recent review is available.[72]

In solution, β-glycosides are not hydrolyzed significantly less rapidly than their α-isomers, presumably because of the conformational flexibility of the single-ring system. So the test systems necessarily have conformational restrictions built in. A second essential feature is that test systems should be electronically unsymmetrical acetals, such as the aryltetrahydropyranyl system (**46**). These undergo spontaneous cleavage (**46** → **47**) without the need for acid if the leaving group ArO^- is good enough, with no tendency for cleavage of the bond to the ring oxygen.



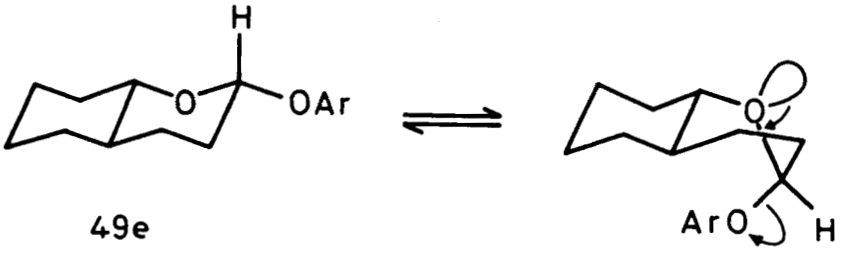
46 47

In an electronically more symmetrical system, such as **48e**, acid-catalyzed ring-opening could compete with exocyclic C–O cleavage, leading to hydrolysis either directly or by way of the axial anomer (**48a**).



48e 48a

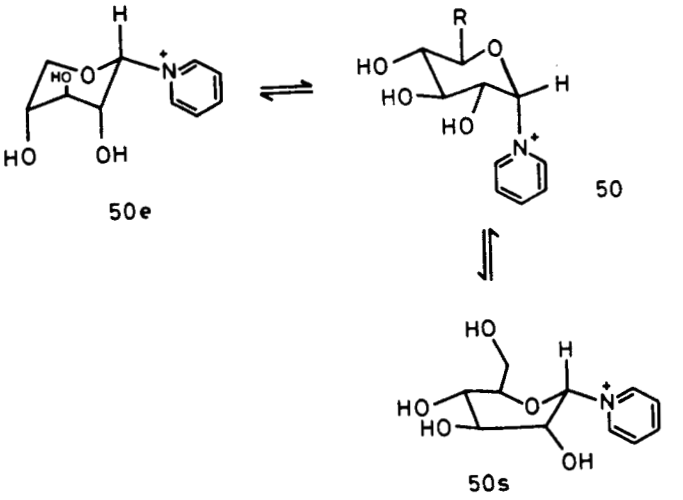
The simple oxadecalin acetal (**49e**) shows no evidence of a stereoelectronic barrier to the loss of the equatorial leaving group, being hydrolyzed three times more rapidly than the axial anomer. A simple explanation is that, like the glucoside (**41**) discussed previously, the conformation of the tetrahydropyran ring, although fixed at C (4, 5, and 6) is flexible at the acetal center and so can react by way of conformations like the boat (**49b**), which have a lone pair on the ring oxygen antiperiplanar to the C–OAr bond.[73]



49e 49b

The transition state for spontaneous cleavage of an acetal of this type is very late, close in energy to the oxocarbonium ion formed from both axial and equatorial anomers, so their relative rates of hydrolysis are controlled by ground state energies. Since the axial isomer is more stable (the anomeric effect), it is hydrolyzed more slowly.

A similar result was found by Sinnott and co-workers[74] for the hydrolysis of α-D-xylopyranosyl and α-D-glucopyranosyl pyridinium salts (**50**, R=H and CH_2OH, respectively).



50e 50 50s

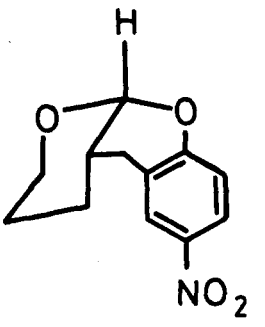
Amino-acetals and glycosides of this sort, with N^+ replacing the exocyclic oxygen at the anomeric center, exhibit the "reverse anomeric effect":[65] the $^+NR_3$ substituent exhibiting a larger than expected preference for the **equatorial** position. The axial anomer is thus **less** stable in these systems and expected to be hydrolyzed more rapidly than the equatorial isomer if reactivity is indeed controlled by ground state energies.

This is observed. A glycosyl derivative (**50**, R=CH_2OH) is hydrolyzed 80 times more rapidly than the β-anomer, and factors of 8 to 23 (at 25°C, depending on the leaving pyridine) are observed for the xylose derivatives. (These data refer to spontaneous cleavage: since the leaving group is N^+ in the ground state, acid catalysis is not possible.)

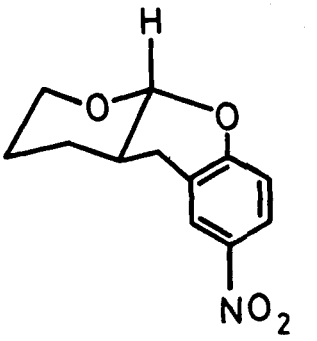
As Sinnott points out,[74,75] the faster hydrolysis of the α-anomers is not evidence for stereoelectronic control since this is another reaction controlled by ground state energies. In fact, the conformations adopted by the two series (**50**, R=H,CH_2OH) in solution do not even have axial pyridinium groups. The xylosyl derivatives are ring inverted to the $^1C_4$ conformation (**50e**), with the reverse anomeric effect of the N^+ driving the three OH groups into axial positions. The equatorial 5-CH_2OH group prevents complete ring inversion in the glucosyl system, (cf. **43**, above) but the preference of N^+ for an equatorial disposition distorts the conformation around the anomeric center to something close to the twist-boat (or skew) conformation $^1S_3$ (**50s**).[74]

Evidently the uncritical application of stereoelectronic theory can be misleading. If an antiperiplanar lone pair is necessary for C–O bond cleavage, it is not essential that it is available in the ground state conformation. As long as the stereoelectronic requirements can be satisfied in some accessible alternative conformation, reaction can still occur without impediment. The criterion is simply that the conformational barrier involved should be insignificant compared with the activation energy for bond cleavage.

When the conformation is sufficiently firmly fixed at the acetal center, the predicted effects do appear. Thus **51e** is hydrolyzed 60 times more rapidly that its axial anomer (**51a**) in 0.1 *M* HCl[76] (although this system suffers from complications arising from the rapid intramolecular return of the leaving group).

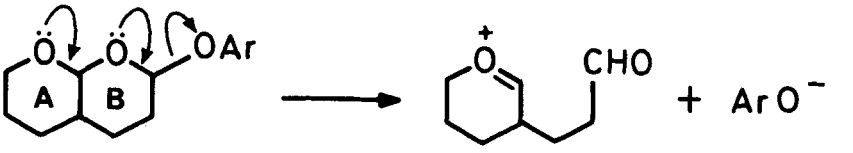


51e



51a

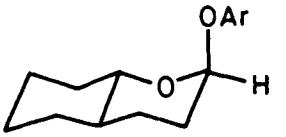
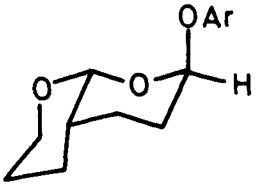
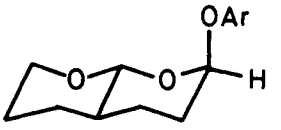
The most clear-cut comparison of isomers is the reaction of the double acetal **52**.[77] This is expected to be a concerted process (**52**, arrows), so that the rate of loss of ArO^- should depend on the *n*-donor capability of the remote (A-ring) oxygen.

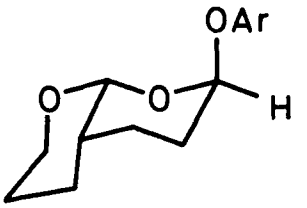


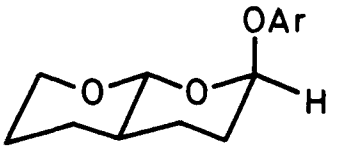
52

This in turn should depend on the geometry of the acetal center at the ring junction. In **52a**, with a *cis* ring junction the ring B oxygen is axial on ring A, so that a reaction is favored stereoelectronically. In **52e**, the (leaving group) oxygen of ring B is equatorial on ring A.

Table 1
RELATIVE REACTIVITIES OF CONFORMATIONALLY RESTRICTED TETRAHYDROPYRANYL ACETALS: DATA FOR SPONTANEOUS HYDROLYSIS AT 39°C AND IONIC STRENGTH 1 *M* IN WATER[75,78]

Compound	k_{rel}	ΔH^{2+} (kcal mol^{-1})	ΔS^{2+} (eu)
 49a	1.0	25.1	1.4
 52a	1.36	26.9	8.2
 52e	6.8×10^{-3}	34.1	20.4

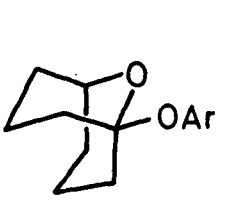

52a


52e

The effect of the A-ring on the reactivity of the tetrahydropyranyl acetal (**46**) is striking. For the same leaving group (Ar= 4-nitrophenyl), **52a** is hydrolyzed 6 to 7 times more slowly than the parent compound **46**, but for **52e** spontaneous cleavage is 1570 times slower. The equatorial isomer is hydrolyzed 200 times more slowly than the axial. This factor derives entirely from a less favorable enthalpy of activation, as expected for an electronic effect (offset to some extent by a very favorable entropy of activation for the reaction of **50e**) (Table 1).

All these cases involve isomers reacting through common oxocarbonium ions. What

happens in the extreme case, where the leaving group of the acetal is rigidly fixed in the equatorial conformation, is illustrated by results with the bicyclic acetal (**53**).[78]



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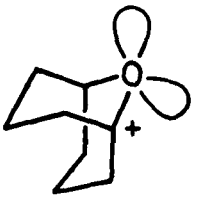

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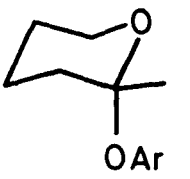


55

Here *n*-donation by the ring oxygen is prevented even in the carbocation (**54**) because the *p*-electron pair of an sp^2-hybridized oxygen would be perpendicular to the vacant *p* orbitals on C^+. As a result, oxygen acts more or less exclusively by σ-electron-withdrawal (inductive effect) to **destabilize** the carbocation and the transition state leading to it. The compound (**53**, Ar=2,4-dinitrophenyl) is exceedingly unreactive, hydrolyzing very slowly at 100°C in water, whereas an unconstrained (and thus axial) tetrahydropyranyl acetal with the 2,4-dinitrophenoxide leaving group is too reactive to prepare. The best analog (**55**), with a tertiary anomeric center, is even more reactive: by suitable extrapolations, it can be shown to be hydrolyzed 1.2×10^{13} times more rapidly than **53** at 39°C.[78]

Of course, an enzyme cannot fix conformations rigidly in this way, and this enormous potential barrier to the cleavage of an equatorially fixed tetrahydropyranyl acetal, such as a β-glycoside, is only indirectly relevant to the lysozyme problem. In practice, the barrier will be avoided. It is indeed exactly how it is avoided that becomes the crux of this particular problem. The alternatives are (1) that the productive conformation of the bound substrate differs from the ground state conformation so that reaction becomes stereoelectronically favorable and (2) that binding the substrate in the ground state conformation is not in fact a stereoelectronic cul-de-sac because the conformation can relax as part of the bond-breaking process.

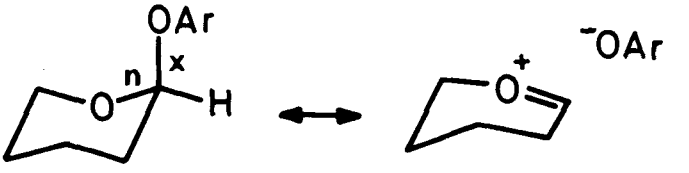
In practice, conformational barriers are relatively small, therefore conformational changes occur on a substantially longer time scale than bond vibrations. As a result, the two processes are not normally tightly coupled, and there would not appear to be time for an important change in conformation as part of a productive vibration which results in bond breaking. Direct evidence on this point is difficult to obtain, but recent work using crystal-structure correlations has shed some light on the reaction pathway for the cleavage of some relevant acetals.

4. Evidence from Crystal-Structure Correlations

Accurate crystal-structure determinations of a series of aryltetrahydropyranyl acetals (**46**) reveal a striking and systematic pattern of changes in the bond lengths at the anomeric center.[79] The exocyclic C–O bond (x) is longer for better leaving groups ArO^- (derived from more strongly acidic phenols) and is, in fact, a linear function of the pK_a of ArOH (and thus of **reactivity** since there is a linear free-energy relationship between the rate of hydrolysis of these compounds and the pK_a of the leaving group).[80] This turns out to be a general property of the C–O single bond: for a given series of compounds R–OX, where X is varied, the C–O bond is longer for better leaving groups XO^-.[81] It can be shown that this is accompanied by increasing polarization of the bond in the sense R–OX, leading to significant charge separation (R–OX ↔ R^+ ^-OX) in the ground state. By varying the

"probe" OX, it is therefore possible to examine the way the rest of the molecule adapts to accommodate the developing positive charge.

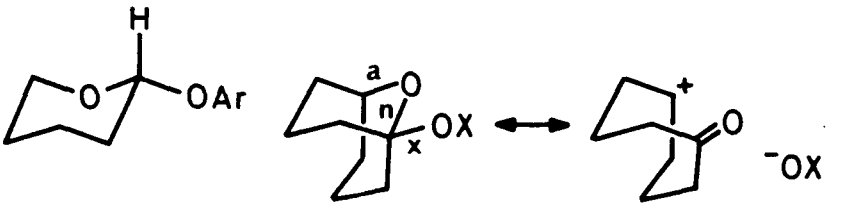
For the acetals **46**, this charge is primarily delocalized onto the ring oxygen by n-σ^*_{C-OX} overlap as described previously. The lengthening of the exocyclic bond x is accompanied by a (lesser) shortening of the endocyclic bond n. The process is conveniently described in terms of increasing contributions from the ion-pair valence bond tautomer **47** to the structure of the ground state and is thus a "visible" manifestation of the stereoelectronic origin of the anomeric effect.



46 **47**

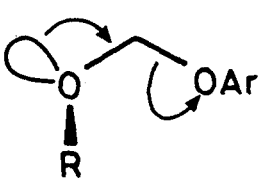
Now the **cleavage** of bond x is the rate-determining step for the hydrolysis of these acetals (**46** → **47**), so the connection between bond length and reactivity is simply explained. Thus each crystal structure can be seen as representing a stage in the bond-breaking process, a series of compounds "mapping out" the reaction coordinate[82] in the way described by Bürgi and Dunitz.[83] We can therefore "see" what happens during at least the early stages of the bond-breaking process: in fact the range of compounds **46** for which data are available now covers over 60% of the energy coordinate for the cleavage of an alkyl tetrahydropyranyl acetal.[82]

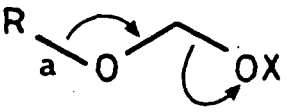
For a series of axial acetals **46**, the only important changes are the variations in bond length described in **46** and **47**; only for the two most reactive compounds do small amounts of flattening at the anomeric center become apparent. In the corresponding equatorial series (**56**), similar but significantly smaller changes of bond length are observed



56 **57** **58**

the origin of these becomes clear when a much wider variety of leaving groups are examined, possibly because of the great stability of the bicyclic system (**57**) discussed previously. In this series, the (reduced) lengthening of the C–OX bond and the concomitant shortening of the endocyclic bond n is accompanied by a significant lengthening of the remote C–O bond **a**. This is readily interpreted in terms of an increasing contribution from the fragmentation product structure **58** to the ground state of **57**.[77] In both axial and equatorial systems **46** and **56**, the bond length variations reveal a significant interaction between σ^*_{C-OX} and the donor orbital antiperiplanar to it. In the axial acetal (**46**), this is a lone pair on oxygen; in the equatorial system, it is the σ-bonding orbital of the remote C–O bond a (**59**).





59

This process also represents a real reaction: when the methanesulfonate (**57**, X=SO_2CH_3) is dissolved in an inert solvent, it does indeed fragment to give products derived from the carbocation (**58**).

These bond length changes are almost entirely suppressed in α-glucosides,[79] reflecting their much reduced reactivity compared with the unsubstituted tetrahydropyranyl acetals. This is attributed to destabilization of the oxocarbonium ion (corresponding to **47**) by the inductive effects of the four OH groups of the sugar. Otherwise, stereoelectronic effects on bond lengths are as predicted. In particular, the exo- and endocyclic bonds (n and x in **46**) at the anomeric center have similar lengths in axial alkyl tetrahydropyranyl acetals[84] and α-glycosides, reflecting competing anomeric and exo-anomeric effects. But in the equatorial isomers, the exocyclic C–O bond x is significantly shorter;[79] the anomeric effect is blocked because the equatorial σ^*_{C-OX} cannot act as an acceptor, so that only the exo-anomeric effect operates.

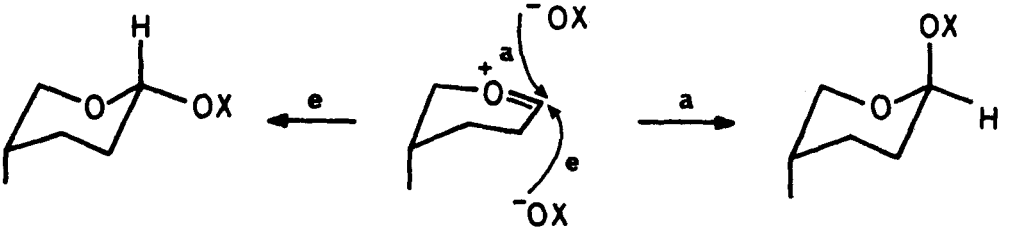
5. Conclusions

There is ample evidence for stereoelectronic effects at acetal centers involving the lone pairs of the oxygen atoms. Observed effects are consistent with n_O-σ^*_{C-OX} -bonding interactions involving tetrahedral lone pairs, which are most efficient when the interacting orbitals are antiperiplanar and disappear when they are orthogonal. They are apparent as the preference for axial conformations and as effects on the length, and therefore strength, of the C–OX bond, and, in compounds with appropriate restricted conformations, on their reactivity.

Effects on bond lengths in equatorial systems differ from those in the axial series. They are significantly smaller and, in particular, indicate progress along a different reaction channel, leading to fragmentation rather than simple acetal cleavage.

Equatorial leaving groups are lost more slowly than the same groups from axial compounds, but only in systems where the conformation is significantly restricted at the acetal center. When these restrictions are relaxed, the effects disappear and, even in favorable cases, they are not large. In two cases (**51** and **52**), where anomers react via a common oxocarbonium ion, the enthalpy of activation is lower by 7 kcal mol^{-1} for the hydrolysis of the axial compound.[76,77]

Large effects are not, in fact, expected. The difference in transition state energies for the loss of axial and equatorial leaving groups XO^- from a tetrahydropyranyl acetal of appropriate constrained conformation (**60**) can conveniently be analyzed in terms of the reverse reaction:



60e 60a

This difference arises because the equatorial isomer must be generated in a high energy conformation if it is to have a lone pair generated antiperiplanar to the developing C–OX bond. But the transition state for this reverse reaction is early, so differences reflecting different product energies cannot have developed very far by the time it is reached.*

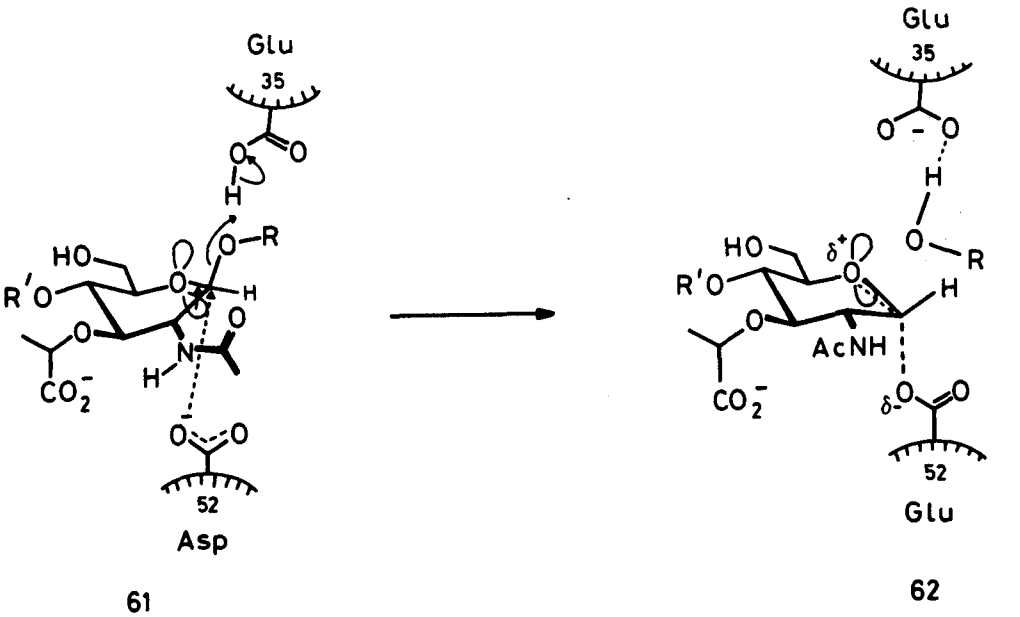
IV. THE ENZYME MECHANISM

A. Detailed Proposals

The recent advances in our understanding of the chemistry of substitutions at acetal centers described in Section III allow us to make more sense of the lysozyme reaction. We begin to see how the two carboxyl groups of Glu-35 and Asp-52 can act cooperatively to effect a catalysis which is more efficient than the sum of its parts. And we can identify good stereoelectronic reasons why cleavage of the glycosidic bond would be easier with a substrate conformation different from the ground state.

An increasingly popular view is that if enzyme binding takes care of the thermodynamics of a reaction then the kinetic advantages will follow. Insofar as this refers to the stabilization of high energy intermediates, it has some merit, but the key feature of enzyme catalysis is the stabilization of the rate-determining transition state for the chemical reaction. If an enzyme can do this so effectively that a diffusion process becomes rate determining, it has reached limiting efficiency[85] (''perfection''[86]).

Lysozyme is not ''perfect'': the well-defined secondary α-deuterium and tritium isotope effects on the hydrolysis of reasonably good substrates show that a chemical step (bond making or breaking at the glycosidic center) is rate determining. But it is nevertheless very efficient (rate enhancement of the order of 10^{10}),[6] and we can assume that the enzyme-substrate interaction in the rate-determining transition state takes advantage of, indeed maximizes, all available stabilizing factors. How this might be done is outlined in Scheme 1.



Scheme 1

* Sinnott[74,75] disagrees strongly with interpretations of reactivity differences involving stereoelectronic effects of oxygen lone pairs. Where differences consistent with a requirement for an antiperiplanar lone pair are observed, he prefers to explain them in terms of the principle of least motion.

1. Substrate Conformation

Stereoelectronic effects on reactivity at a glycosidic center are to be expected in the presence of conformational restrictions which are significant compared with activation energies for reaction. This is a combination of circumstances very likely to arise in an enzyme active site. A substrate with the full hexasaccharide binding available to the natural substrate of a β-glycosidase-like lysozyme is bound rather strongly before undergoing a rapid catalytic reaction. Therefore, the detailed proposed mechanism (Scheme 1) must take the stereoelectronic factor into account.

The suggested productive conformation (**61**) of the bound substrate, probably not the same as in the Michaelis complex, is similar to that in the transition state. If the substrate is bound initially in the chair conformation at the active site, the necessary change in conformation brings the leaving group (OR) oxygen closer to Glu-35 by about 1 Å, moves the anomeric center (C[1]) away from Asp-52 by 0.3 to 0.4 Å, and presumably brings the orbitals involved at these three centers into optimum alignment for reaction. It also brings the C–OR bond to within about 60° of antiperiplanar to one of the lone pairs on the ring oxygen atom (compared with 120° away from both in the chair conformation), allowing the beginnings of the n_O-σ^*_{C-OR} overlap necessary for C–OR bond breaking.

2. Bond Breaking and Making

All the chemical evidence suggests that efficient general acid catalysis by Glu-35 cannot occur without substantial further assistance to C–OR bond breaking. This is available both from Asp-52 and from lone pair donation from the ring oxygen. We have already discussed reasons why assistance from the aspartate-CO_2^- group might be expected to be most effective if it is primarily nucleophilic, rather than electrostatic, at C(1). Lone pair donation by the ring oxygen, on the other hand, would be potentiated by the proximity of a negative charge. A **minimum** requirement is that the donor oxygen should not act as a hydrogen-bond acceptor: H bonding is known to affect bond lengths and strengths at acetal centers,[79,87] and an oxygen with its lone pair electrons tied up in a H bond is clearly a weaker donor. (It has been suggested that desolvation of donor oxygens, i.e., breaking of H bonds to solvent water, may account for the very large entropies of activation found for some acetal hydrolyses.)[77] So the ring oxygen in **61** is expected to be in an at least a slightly negative environment, raising the donor capability of the lone pairs and favoring the development of positive charge at oxygen during the reaction.

With the development of positive charge taken care of, thereby facilitating C–OR bond breaking at the otherwise highly unreactive anomeric center, general acid catalysis by the COOH group of Glu-35 becomes efficient also. When proton transfer and C–OR cleavage are complete (Scheme 1, **62**), this group has become COO^- and might also assist in creating the negative environment for any positive charge on the ring oxygen. The geometry is favorable in **62** but not optimal because the two systems are separated by the OH bond of the leaving group. As the reaction proceeds, ROH departs and is replaced by H_2O, but any interim improved interaction with Glu-35-CO_2^- is irrelevant to catalytic efficiency because the rate-determining transition state is already past.*

The product of this step is a glycosyl-enzyme intermediate (**62**), which is hydrolyzed to the final product by the reverse mechanism, (**62** → **61**) with ROH=H_2O. The same process (with R′OH) accounts for glycosyl-exchange reactions (see later). There is no direct evidence for the existence of an intermediate in the lysozyme reaction. For example, it cannot be trapped by borotritiide, as might have been expected for an oxocarbonium ion.[88] (Although

* The conformational changes involved in this sequence need be little more than the minimum necessary for a double displacement reaction. Sinnott considers that a variant of this pathway involving a full covalent bond to Asp-52-COO^- and a twist-boat conformation for (**61**) would require "extraordinary conformational subtlety" on the part of the enzyme.[75]

perhaps not for one sandwiched by two negatively charged groups, which could prevent access for BH_4^- while assisting the entry of an H-bond donor, ROH.) But negative evidence in this situation often means simply that the lifetime of the intermediate is very short.

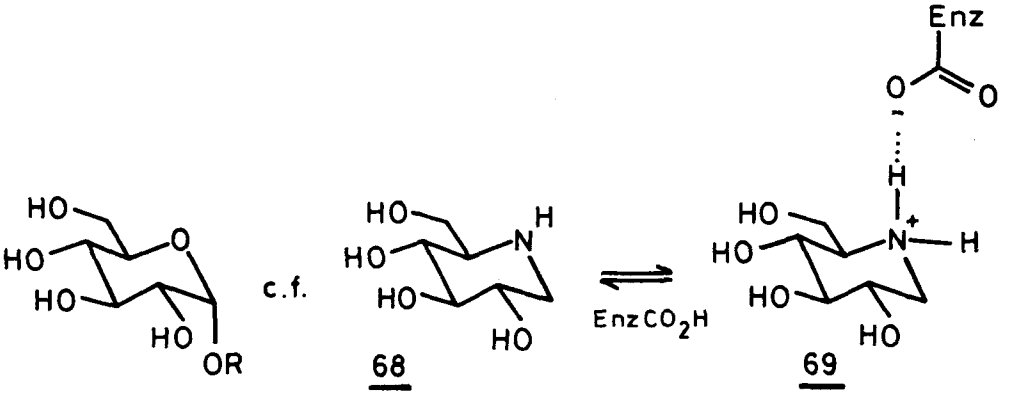
An acyl-glycoside is a relatively stable compound (which may be hydrolyzed in solution at the ester rather than the acetal center: this clearly does not happen to the putative intermediate [**62**] in the lysozyme reaction, where a double displacement at the glycosidic center is required to explain the observed retention of configuration). The same factors that stabilize the transition state for the formation of the intermediate are also available to assist in its hydrolysis, but an intermediate that is too stable (a thermodynamic pit, in Fersht's terminology[85]) is no less a bar to efficient catalysis than one which is not stabilized enough. A compromise that avoids that problem is less-than-complete covalent bond formation between the reactivity glycosidic center and Asp-52-COO^-.

This could arise primarily from poor orbital alignment or from a simple distance effect (C[1] and O^- held too far apart to form a full covalent bond). But C–O bond formation would need to be rather far from completion; bond dissociation curves are steep in the region of moderately large extensions, and the sort of forces an enzyme can exert on a substrate are unlikely to be strong enough to prevent C–O bond formation going to completion once it is well advanced. (This requirement may appear to run counter to the need for nucleophilic assistance from Asp-52-CO^- to C–OR bond cleavage, but the transition state for this sort of reaction is known to involve very weak bonding to both nucleophile and leaving group.) Alternatively, **62** might not collapse to give a full C–O bond in the absence of ROH because of the similar long-range interaction possible with the carboxylate group of Glu-35.

3. An Alternative Proposal

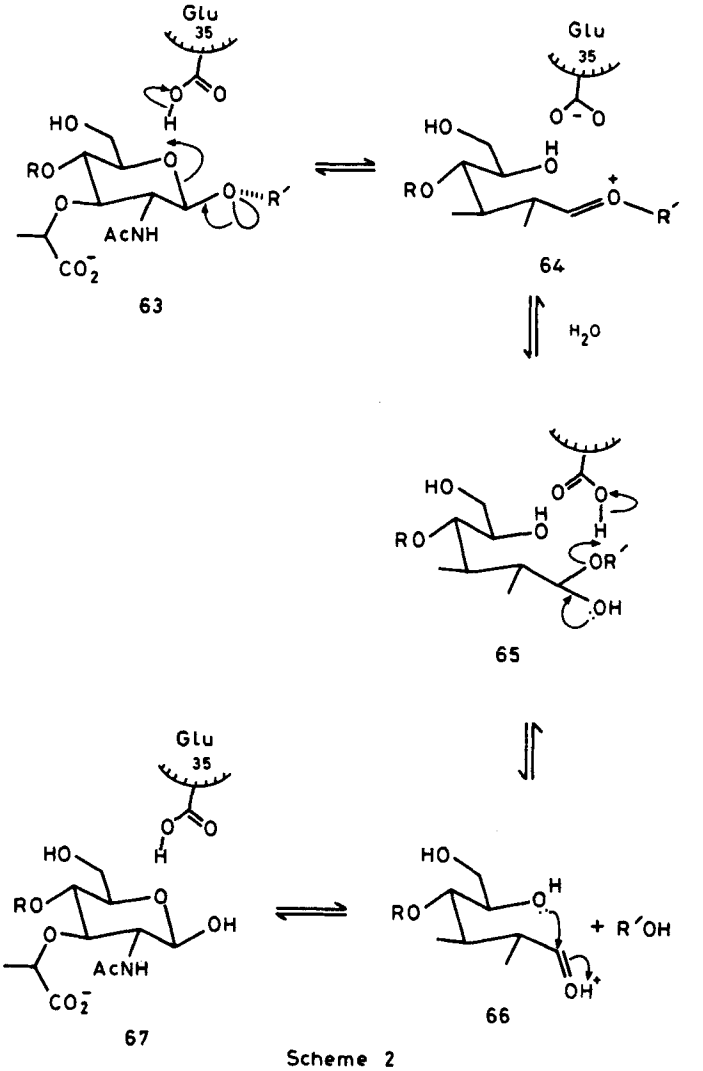
An alternative solution to the stereoelectronic problem has been proposed recently by two different authors, on quite different grounds. The 55-pS molecular dynamics simulation of the active-site NAG_6 complex by Post and Karplus,[17] as discussed previously, allowed favorable substrate binding without perturbing the chair conformation of the sugar residue bound at the active site (subsite D). Furthermore, the motion of the carboxyl group of Glu-35 brought it to within H-bond contact with the ring oxygen of this residue, while the exocyclic (what we have called the "leaving group") oxygen appeared to be both too far away and wrongly placed for H-bond formation. Post and Karplus propose that Glu-35 in fact protonates the ring oxygen (**63** and **64**), thus avoiding the stereoelectronic barrier to the loss of an equatorial leaving group. (The exo-anomeric effect, controlling the conformation about the exocyclic C–O bond at the anomeric center, ensures a lone pair on the exocyclic oxygen antiperiplanar to the endocyclic C–O bond.)

Fleet[89] arrived at a similar conclusion for both α- and β-glycosidases from considering their inhibition by sugar analogs in which the ring oxygen is replaced by nitrogen. A typical competitive inhibitor of several α-glycosidases is deoxynojirimycin (**68**), probably acting as the free base.



In the active site, the conjugate acid (**69**) looks a better mimic of a protonated ring oxygen than the planar oxygen of an oxocarbonium ion (**62** in Scheme 1). There is no conclusive evidence about the initial bond-breaking event in glycosidase reactions, although there seems no doubt that simple glycopyranosides react by exocyclic C–O protonation and cleavage.[90]

Glycoside hydrolysis via initial ring-opening requires several extra steps (Scheme 2)



Scheme 2

compared with simple double displacement of the exocyclic oxygen substituent. This is not in itself a powerful objection, but some of the details do pose problems; for example, the leaving group oxygen has to be protonated eventually (**65** → **66**) and there is still only Glu-35 available. And the mechanism of Scheme 2 cannot explain the results, constituting a majority of kinetic measurments, obtained with substrates such as aryl and pyridinium glycosides, which as unsymmetrical acetals can only hydrolyze with exocyclic bond cleavage. Some of these reactions are quite efficient, remarkably so if they represent the enzyme catalyzing unnatural reactions.

Most difficult of all to explain are glycosyl transfer reactions. These are most favorable when the incoming nucleophile is bound in the leaving group site (R′OH replacing ROH).[6]

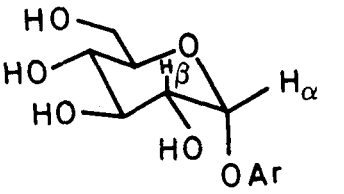
Since by the mechanism of Scheme 2 ROH departs only when hydrolysis is complete, glycosyl transfer must involve the complete reverse sequence, with the hydrolysis product (**66** → **67**) an obligatory intermediate. Yet an analysis of glycosyl transfer reactions in the hydrolysis of natural substrates indicates that transfer to a specific $(NAG–NAM)_x$ acceptor occurs several thousand times faster than to water.[91] Scheme 1 therefore still appears to account best for all the facts.

B. Implications for Other Glycosidases

It is a specific prediction of the stereoelectronic theory that α-glycosides, with axial aglycones, can be cleaved in the ground-state chair conformation even under significant conformational restraint, whereas β-glycosides cannot. In principle, therefore, any β-glycosidase or transferase must adjust the conformation of its substrate before the C–O bond is broken, whereas an α-glycosidase need not. This applies equally to enzyme reactions involving retention of configuration, as found for lysozyme, and to (the currently smaller number of) reactions going with inversion. There are some indications that the lysozyme mechanism is a useful model for other retaining glycosidases, which seem generally to possess active-site carboxylate groups corresponding to Asp-52 (for references see Hosie and Sinnott[92]).

This is the sort of question which might usefully be tackled by comparing the many enzymes which catalyze reactions of α- and β-glycosides, but very few relevant data are available, and in any case the conformational changes necessary do not have to be kinetically detectable, so negative evidence for β-glycosidase reactions is not conclusive.

Recent work by Hosie and Sinnott[92] graphically illustrates the problems of interpretation involved in kinetic studies with individual enzymes. These authors measured k_{cat} and K_m for the hydrolysis of isotopically labeled (α- and β-deuterated, ^{18}OAr) aryl α-glucosides (**70**) catalyzed by yeast α-glucosidase and found no significant isotope effects on the reaction. C–O bond cleavage is therefore not rate determining.



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The authors suggest that a rate-determining conformation change precedes bond breaking, which is clearly not a stereoelectronic requirement. For similar substrates with substituted pyridine leaving groups (**50**), α- and β-deuterium isotope effects are observed, so this change, whatever it is, is faster. Since these compounds exist in a twist-boat conformation (**50s**), as discussed in section III.C.3.b, the inference is drawn[92] that the glucosides (**70**) are being converted to a boat-type conformation before C–O cleavage.

This is slender evidence on which to base such a specific conclusion, although the original paper should be consulted to follow the detailed argument. In particular, there are bound to be significant differences in binding between **50** and a more normal substrate since the oxygen, but not the nitrogen, leaving group needs to be brought in contact with the active-site general acid.

To sum up, mechanisms proposed for enzyme reactions at glycosidic centers should take into account the stereoelectronic requirements for overlap between the electron-deficient

center, which invariably develops in these reactions, and the lone pair electrons of the (generally ring) oxygen which remains attached. These requirements are reflected in the productive conformation of the bound substrate, which will represent a compromise between the competing demands of several factors. It is not necessary to take literally the earliest formulation of the stereoelectronic theory, i.e., that β-glycosides "must first assume a boat conformation" before reacting, but it is important to apply the general principle involved.

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