tinction seems to be extremely difficult to reconcile with data from small-angle x-ray scattering.

If possible, proposed water models should specify the detail needed for comparison with diffraction data and should do this in a self-consistent way. If this is not done, the diffraction data cannot be invoked as evidence for or against the correctness of the model. Furthermore, any model that claims agreement with the observed diffraction pattern of liquid water should reproduce not only the first peak but all other significant features of the radial distribution function as well. These conditions provide a powerful criterion for the tenability of proposed water models.

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Mechanism of Lysozyme Action

Lysozyme is the first enzyme for which the relation between structure and function has become clear.

David M. Chipman and Nathan Sharon

For years, one of the most intriguing problems in biochemistry has been the mechanism of enzyme action. At the simplest level, without considering the problems of the regulation or coupling of enzyme systems, the aim has been to understand how enzymes achieve their great substrate specificity and to identify the detailed molecular mechanism of a given enzyme-catalyzed reaction. The reactions catalyzed by a number of enzymes, in particular proteolytic enzymes, have been investigated in great detail in an attempt to answer the above questions, and some of these enzymes are now reasonably well understood. It is an ironic testimony to the power of the x-ray crystallographic method, however, that lysozyme, for which there was no substrate of known structure only 12 years ago, and which for a long period of time was a "neglected" enzyme, is now one of the few clearly understood enzymes.

Lysozyme has had a rather peculiar history. In 1922 Alexander Fleming discovered a substance in his own nasal mucus capable of dissolving, or lysing, certain bacteria. The substance, which turned out to be an enzyme, was named "lysozyme" and was found to be widely distributed in nature (*1*). Fleming was quite enthusiastic about the possible therapeutic use of lysozyme—he had found it, in fact, because he believed that some organisms must produce antibacterial substances—but it soon developed that lysozyme was of little clinical value. Heartened perhaps by his initial partial success, Fleming went on to discover penicillin, the first true antibiotic, and interest in lysozyme fell into a relative decline.

Lysozyme was not completely forgotten though. The enzyme from hen egg white is easily isolated and purified, stable, and of rather low molecular weight (14,500), and as such it has become one of the most thoroughly investigated proteins (*2*). In 1963 Jollès and Canfield independently elucidated the complete primary structure

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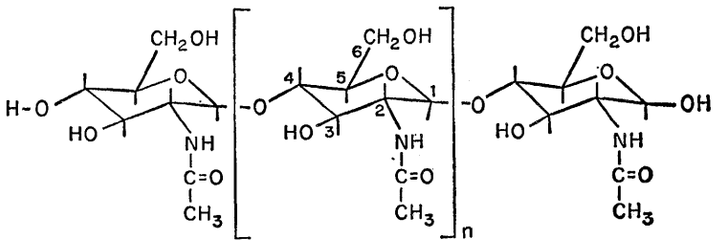


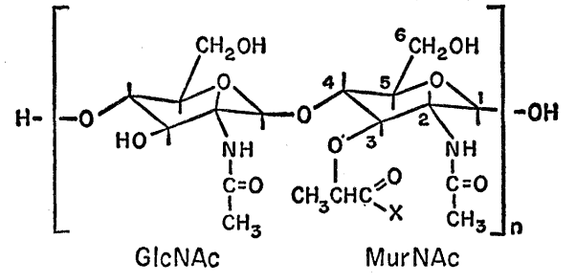


Fig. 1 (left). Structure of chitin or oligosaccharides derived from it. The reducing terminus is to the right. One of the smallest lysozyme substrates, (Glc*N*Ac)$_3$, consists of just three pyranose rings. Fig. 2 (right). Structure of the bacterial cell wall glycopeptide. In native cell walls the group X may be OH (that is, the carboxyl group may be free) or an amino group of a peptide through which the polysaccharide chains are cross-linked (*5*). The products of lysozyme action on the glycopeptide all have this structure; the Glc*N*Ac–Mur*N*Ac bond is not cleaved by the enzyme. The smallest cell wall saccharide which is a substrate is the tetrasaccharide ($n = 2$).

of the enzyme, which is comprised of a single chain of 129 amino acids (*3*), and in 1965 David Phillips and his colleagues described the three-dimensional structure of lysozyme, based on x-ray crystallographic studies which had been initiated in 1960.

Lysozyme also remained of interest to those studying bacterial cell walls. In 1952 Salton showed that the substrate for lysozyme in *Micrococcus lysodeikticus*, the most sensitive bacterial strain, is the cell wall mucopolysaccharide (*4*), a complex material containing both sugars and peptides which is also called cell wall glycopeptide or peptidoglycan. Subsequently, similar insoluble glycopeptides were found in all other bacteria examined, and soluble saccharides and glycopeptides formed by lysozyme action on cell walls were isolated and identified; with this information the structure of cell walls was gradually elucidated (*5*). It should be noted that cell walls of bacteria are solubilized not only by lysozyme, but by action of certain other enzymes as well, either glycosidases or peptidases (*6*).

The first indication of the chemical linkage attacked by lysozyme came in 1957 when Berger and Weiser showed that the hen egg-white enzyme hydrolyzes chitin, a linear $\beta(1 \rightarrow 4)$ polymer of *N*-acetyl-D-glucosamine (Glc*N*Ac) (*7*). The structures of the two classes of lysozyme substrates, (i) chitin and oligosaccharrides derived from Glc*N*Ac and (ii) the cell wall glycopeptide and oligosaccharides derived from it (*8*) are shown in Figs. 1 and 2, respectively. Both structures are made up of $\beta(1 \rightarrow 4)$ linked *N*-acetylhexosaminyl residues, and can be considered as analogs of cellulose. In the cell wall glycopeptide, however, every alternate residue has an *O*-D-lactyl group attached to it; this 3-*O*-D-lactyl ether of *N*-acetylglucosamine is known as *N*-acetylmuramic acid (Mur*N*Ac). The carboxyl group of Mur*N*Ac may be substituted. While lysozyme shows *N*-acetylglucosaminidase activity toward chitin saccharides and certain synthetic substrates (*9*) derived from the saccharides, it acts on the cell wall saccharides which contain alternating Glc*N*Ac and Mur*N*Ac residues exclusively as an *N*-acetylmuramidase, cleaving such compounds only at the glycosidic bond of the Mur*N*Ac residues.

Some 20 closely related lysozymes are now known (*10*). These enzymes, isolated from a variety of sources ranging from avian eggs to phages (*11*), are all basic, low-molecular weight proteins which lyse certain bacteria and have the chemical activity described above (*12*). They have thus been classified as muramidases or *N*-acetylmuramide glycanohydrolases (E.C. 3.2.1.17). Our subject here will be the hen egg white enzyme, which we will briefly call lysozyme.

Serious investigation of the mechanism of lysozyme action did not really begin until the results of the x-ray diffraction studies of Phillips and coworkers were published (*13–15*). The three-dimensional models of lysozyme and of the lysozyme-substrate complex laid the foundation for an understanding of the specificity of lysozyme and of its mechanism of action in terms of the precise arrangement of the individual atoms in the molecule. The publication of this work focused the attention of many scientists on this enzyme, and in a short period of time led to many new studies dealing with lysozyme. It is our aim to review these studies and compare the results of the chemical and crystallographic investigations in an attempt to give strengthened support to the proposed mechanism of action of lysozyme and to indicate what sort of chemical experiments may be useful in the further study of lysozyme and other enzymes.

X-ray Crystallographic Model

The three-dimensional structure of the lysozyme molecule, derived from the 2-Å resolution Fourier map, has contributed to our understanding of protein structure and conformation in general; Phillips, North, Blake, and their co-workers have discussed the implications which may be drawn from it (*13*). It suffices to say here that the molecule is roughly ellipsoidal (45 by 30 by 30 Å) with a deep cleft running up one side. As expected, the polar side chains of the molecule are distributed on the surface in contact with water, while the interior of the molecule by and large contains nonpolar hydrophobic residues. The cleft is also partially lined with hydrophobic residues.

The structure itself could not account for the mechanism by which lysozyme catalyzes the hydrolysis of polysaccharides. However, Phillips and Johnson found that crystals containing various saccharide inhibitors (*16*) bound to lysozyme can readily be prepared, either by allowing the small inhibitor molecules to diffuse into enzyme crystals from a solution, or by growing crystals from a solution containing both enzyme and inhibitor (*14*). The structures of such crystalline complexes can be determined readily once the structure of the pure protein crystal is known (*15, 17*).

The crystallographic study showed that the chitin trisaccharide (Glc*N*Ac)$_3$

is bound in the cleft in lysozyme, filling nearly half its length (Fig. 3). The nonreducing terminal Glc*N*Ac unit is at the top of the cleft, in a region or subsite designated *A* (*18*), while the next two units occupy subsites *B* and *C*. A number of interactions between the enzyme and the saccharide are evident in the structure, notably the six hydrogen bonds indicated by dashed lines in Fig. 3, as well as over 40 other van der Waals contacts (*19*). Another observation of considerable interest is that parts of the protein molecule move with respect to one another upon formation of the complex. The "left" side of the cleft moves in such a way as to narrow the cleft, with the indole side chain of tryptophan 62 moving by some 0.75 Å. This change in protein conformation upon binding may be an example of "induced fit," first proposed in 1958 by Koshland (*20*).

N-Acetylglucosamine can exist in two anomeric forms, α and β (Fig. 4), that readily interconvert in water, and it has been shown to bind accordingly in two different modes. The β-anomer is bound in subsite *C* of the enzyme in a manner indistinguishable from the reducing terminal Glc*N*Ac unit of the trimer, and its binding results in a similar change in protein conformation. Binding of the α-anomer of Glc*N*Ac has been observed in the same subsite, but its orientation in the subsite is different from that of the β-anomer. The binding of Mur*N*Ac has been studied only at low resolution (6 Å), and the results are consistent with this sugar being bound as the α-anomer, similarly to α-Glc*N*Ac. The only complex of Glc*N*Ac-$\beta(1 \rightarrow 4)$-Mur*N*Ac observed by x-ray diffraction is apparently that of the α-form of the disaccharide, with the Glc*N*Ac residue lying outside the cleft.

It has not yet been possible to observe complexes of saccharides longer than the trimer by x-ray crystallography because of a number of intrinsic difficulties: it is more difficult for a large saccharide to diffuse into a crystal, longer saccharides are very readily cleaved by lysozyme (see below), and, in the particular crystalline form of lysozyme studied by Phillips' group (tetragonal), the lower part of the enzyme cleft is partially blocked by a portion of a neighboring enzyme molecule. However, Phillips was able to construct a model for such a complex by assuming that the structure of the lysozyme-oligosaccharide complex is superimposable on that of the lysozyme-$(Glc\mathit{N}Ac)_3$ complex as observed by x-ray crystallography. The presence of three additional subsites, designated as *D*, *E*, and *F*, was inferred by fitting molecular models of chitin oligosaccharide substrates to the three-dimensional model of the enzyme, and a lysozyme-$(Glc\mathit{N}Ac)_6$ model was thus obtained. The three sugar residues which were fit by model building are shown in dashed heavy lines in Fig. 3.

The construction of this model led to a number of interesting inferences, some of which we state here briefly (*14*, *15*). (i) The six sugar residues fill the entire length of the cleft, and it is not easy to fit a larger oligosaccharide such that additional sugar residues make contact with the enzyme. This means that sugar residues above subsite *A* or below subsite *F* have no significant interactions with the enzyme. (ii) Because of steric interactions of the enzyme with the CH_2OH group on carbon-6 (C-6) of the sugar unit in subsite *D*, the pyranose ring of this residue must be distorted from the normal chair conformation toward a half-chair conformation. (iii) If the cell wall oligosaccharides interact with the enzyme in the same way as the chitin oligosaccharides, Mur*N*Ac residues cannot be bound in subsites *A*, *C*, or *E*. This is because the 3-hydroxyl group on a saccharide

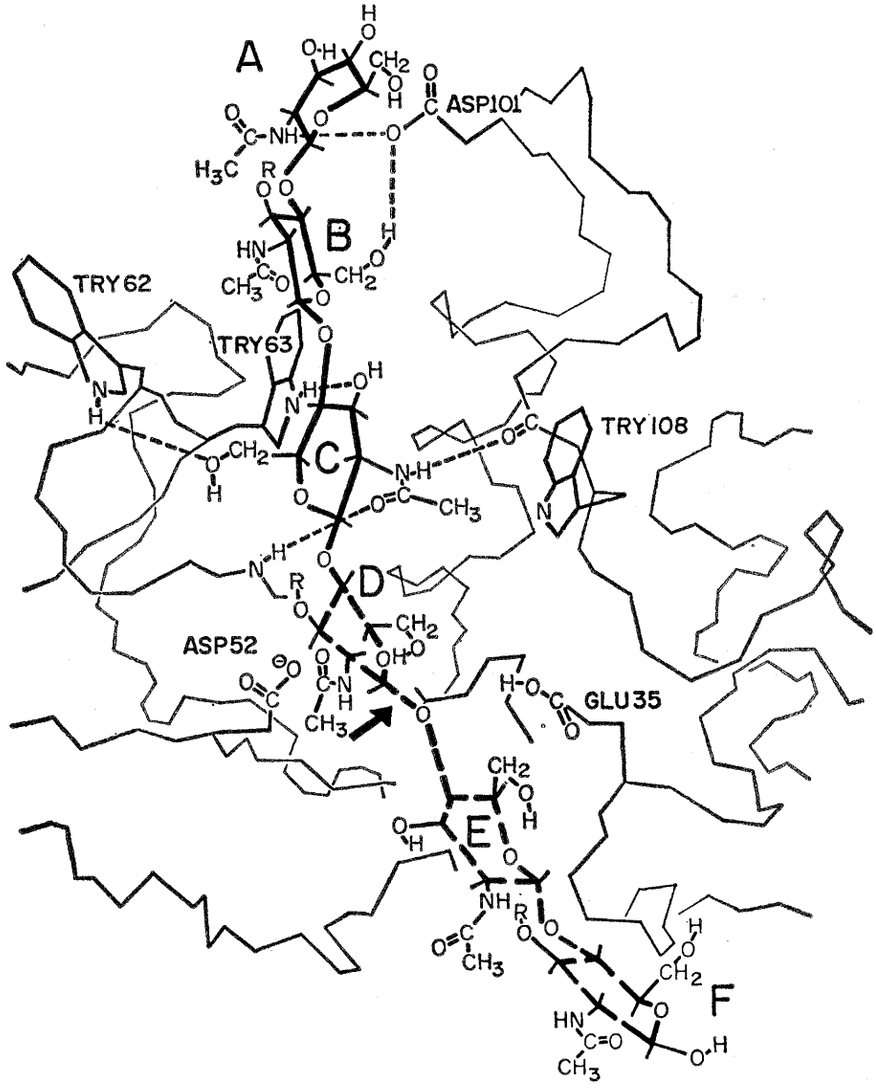


Fig. 3. The three-dimensional model of the area around the active site of the lysozyme-substate complex [after Phillips (*15*)]. The pyranose rings of the substrate are shown in heavy lines, with the monosaccharide units of the lysozyme-$(Glc\mathit{N}Ac)_3$ complex (*A*-*C*) solid and those placed by model building (*D*-*F*) dashed. Except for some groups of particular interest, only the peptide backbone of the protein is shown. The light dashed lines are the six hydrogen bonds between $(Glc\mathit{N}Ac)_3$ and the enzyme. The groups R are hydrogens in the case of chitin substrates, and lactyl groups in cell wall saccharides. The arrow indicates the bond cleaved.

unit in any of these positions points into the cleft. The D-lactyl ether at C-3 in Mur*N*Ac could not fit in any of these subsites. In subsites *B*, *D*, or *F*, on the other hand, the lactyl group would point out of the cleft. (iv) Since cleavage of the cell wall oligosaccharides occurs only between Mur*N*Ac and Glc*N*Ac residues, the linkage affected by the enzyme is either between sugar residues *B* and *C*, or between *D* and *E* (*18*). Sugar residues bound at *B* and *C* form part of the lysozyme-$(Glc\mathit{N}Ac)_3$ complex, which is stable for weeks. In addition there are no reactive groups on the enzyme in the neighborhood of this linkage. Phillips has therefore suggested that the linkage cleaved by lysozyme is between sugar residues *D* and *E*. This means that the observed, stable complex of $(Glc\mathit{N}Ac)_3$ is a nonproductive one, which does not lead to bond cleavage.

The most reactive groups in the region of subsites *D* and *E* of the enzyme are the carboxyl groups of glutamic acid (Glu) at position 35 and aspartic acid (Asp) at position 52, which are disposed on either side of the $\beta(1 \rightarrow 4)$ linkage in question. A mechanism has been proposed in which the concerted action of Glu 35 and Asp 52 is involved in catalysis, facilitated by the distortion of the sugar ring occupying subsite *D*; we shall deal with this below.

Binding of Saccharides in Solution

It is interesting to compare the inferences based on the crystallographic model with data on the interaction of lysozyme with saccharides in solution. The association of some 30 different saccharides with lysozyme has been studied by methods as diverse as measurement of the inhibition of lysozyme action on bacterial cells and nuclear magnetic resonance spectroscopy. Although these data have been obtained under varied conditions of *p*H, temperature, and ionic strength, the agreement between equilibrium constants estimated by different workers using different techniques is reassuring. Apparent association constants,

$$K_a = ES/E \cdot S$$

(where E, S, and ES represent the concentrations of the enzyme, substrate, and enzyme-substrate complex, respectively) for 26 saccharides are presented in Table 1. These data can be rationalized if we make the simple assumption

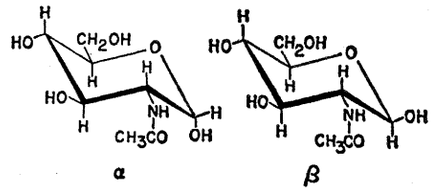


Fig. 4. The α and β anomeric forms of *N*-acetyl-D-glucosamine.

that the active site of the enzyme consists of a series of subsites, each of which makes characteristic contacts with the monosaccharide units of an oligomer, so that various enzyme-saccharide complexes formed are essentially superimposable. This assumption is, of course, founded on the similarity of the complexes of different saccharides with lysozyme, as determined by x-ray crystallography (*15*).

The constants for compounds 1 to 6 in Table 1 indicate that the enzyme has three contiguous subsites, corresponding to *A*, *B*, and *C* of the lysozyme model, in which Glc*N*Ac moieties can interact favorably, but that subsites on either side of this region do not yield favorable interactions. The comparison of trisaccharides 3 and 9, with Glc*N*Ac and Mur*N*Ac, respectively, as the central unit, suggests that the central subsite *B* in this favorable region can accommodate Mur*N*Ac. Since Mur*N*Ac–Glc*N*Ac, compound 8, is bound about as strongly as Glc*N*Ac–Glc*N*Ac, compound 2, while Glc*N*Ac–Mur*N*Ac, compound 7, is bound only very weakly, we must conclude that the strong binding site for a dimer is *B-C* and that subsite *C* cannot accommodate a Mur*N*Ac residue. The decreased binding of Glc*N*Ac–Mur*N*Ac–Glc*N*Ac–Mur*N*Ac, compound 10, compared with Glc*N*Ac–Mur*N*Ac–Glc*N*Ac, compound 9, indicates that subsite *D* is not merely a noninteracting site, but must lead to unfavorable interactions. All of the above conclusions are in striking accord with those derived from the crystallographic data.

The association constants of saccharides 3 to 6, the chitin trimer through hexamer, are very similar (*21, 22*). This suggests that the major mode of binding (that is, that which determines the observed K_a) of each of them is with the reducing terminus in subsite *C*. This arrangement avoids the unfavorable subsite *D*, but is nonproductive (the implications of this for the kinetics of lysozyme-catalyzed reactions will be seen later). On the other hand, the cell wall hexasaccharide 11 is bound more strongly than the cell wall tetrasaccharide 10. Since the region above subsite *A* apparently does not interact at all with an oligosaccharide, the additional interaction observed for compound 11 is probably due to subsites *E* and *F*, and the predominant mode of binding of compound 11 is thus probably *A-F*. Neither cell wall saccharide can bind in the very favorable mode available to chitin saccharides because subsite *C* is blocked to Mur*N*Ac residues. The recent report by Hess (*23*) that there is another mode of binding of $(Glc\mathit{N}Ac)_6$ (proposed as *A-F*) with an equilibrium constant at least within an order of magnitude of that for the dominant complex (with reducing terminus at *C*) also supports the notion that interactions at *E* and *F* can nearly make up for the unfavorable interaction at subsite *D*.

Contributions of Individual Subsites

If one extends the assumption that the various saccharide complexes are superimposable, and assumes that monosaccharide residues contribute additively to the unitary free energy of association of an oligosaccharide with the enzyme [$\Delta F_u = -RT\ln K$, where K is in units of mole fractions (*24*)], the contribution of the enzyme-saccharide interactions at each subsite ($\Delta\Delta F$) can be estimated (*25*). In Table 2 each saccharide has been arranged in the most reasonable set of subsites consistent with the scheme of six subsites described above, and its apparent ΔF_u given.

For saccharide 7 there is an uncertainty involved. The predominant complex may be an "anomalous" one which does not fit this scheme [such a complex is the only one observed in the crystal (*14, 15*)]. This would mean that the association constant for the complex in *AB* shown in Table 2 for this saccharide would be smaller than the observed K_a; the value of ΔF_u for this complex of saccharide 7 is thus given as a lower limit.

By making the appropriate comparisons between saccharides differing by the occupation of only one subsite, we can calculate $\Delta\Delta F$ for each subsite (*26*). One aspect of the values of $\Delta\Delta F$ given in Table 2 may appear puzzling; $\Delta\Delta F$ for subsite *C*, for instance, is found (by comparing saccharides 9 and 7) to be at least -5.7 kilocalories per mole, while ΔF_u for Glc*N*Ac, which is bound in subsite *C*, is only about -4.5 kilocalories per mole. This is not necessarily a discrepancy, however, as ΔF_u

for an enzyme-saccharide complex may also include the energy of any conformational change in the enzyme necessary for binding. By difference, this energy may amount to 1 kilocalorie per mole or more for lysozyme. As mentioned above, a conformational change [involving movement of tryptophan (Try) 62] is observed in each of the lysozyme-saccharide complexes studied crystallographically at high resolution (*17*). The change appears to be of the same type and magnitude in all these complexes, although there may be other small conformational changes in other lysozyme-saccharide complexes (*27*).

Table 3 summarizes the $\Delta\Delta F$'s for the subsites, together with the interactions deduced by Phillips from his model of the enzyme-substrate complex (*28*). The relative magnitudes of $\Delta\Delta F$ for subsites *A*, *B*, and *C* are in good agreement with the numbers of hydrogen bonds and nonpolar interactions implied by the model (*29*). By far the most important interactions observed in the model are those of the acetamido group in subsite *C;* there are two hydrogen bonds to main-chain peptide groups and a number of nonpolar interactions of the methyl group, particularly with Try 108. A comparison of binding of saccharides 9 and 23 (Table 1) shows that the contribution of the acetamido group to binding at subsite *C* is -3 kilocalories per mole (*30*), a value close to that obtained from studies of the binding of *N*-cyclohexylacetamide to lysozyme (*21*). In addition, *N*-acetylhexosamines of different stereochemistry bind about as strongly as Glc*N*Ac, while glucose itself binds with K_a at best 1/100 that of Glc*N*Ac (*21*). The poor binding of *N*-propionylglucosamine, compound 16, presumably reflects steric crowding in the region where the acyl moiety lies in the *N*-acetylglucosamine complex, but the low affinity of lysozyme for *N*-formylglucosamine, compound 17, is somewhat surprising. It must primarily reflect the importance of the hydrophobic interaction of the acetamido methyl group with the enzyme.

The kinetics of the association and dissociation of lysozyme-(Glc*N*Ac)$_2$ and -(Glc*N*Ac)$_3$ complexes sheds further light on these complexes (*31*). Although the binding constants for these saccharides differ by a factor of 30, their bimolecular rate constants for association with the enzyme are the same within experimental error, about 4×10^6 liters per mole per second, and the entire difference in binding constants shows up in the dissociation rates. This result can be explained if the rate determining process in the association in each case is the formation of the closely fitting complex of two saccharide units in subsites *B* and *C*, with the more loosely bound residue in *A* interacting in a second, faster step. Indeed, the crystallographic data on the lysozyme-(Glc*N*Ac)$_3$ complex do not yield very sharp electron-density maximums for the residue in subsite *A*, indicating that this residue is fixed less rigidly in the complex than the other residues (*17*).

The contribution of subsite *D* to saccharide binding, as pointed out above, is unfavorable; when a Mur*N*Ac residue is bound at this subsite, $\Delta\Delta F$ is at least $+$ 2.9 kilocalories per mole. Binding data also suggest that subsites *E* and *F* can interact with saccharides, with a combined contribution of ≤ -1.7 kilocalories per mole. From the number of interactions implied by the model of the lysozyme-substrate complex, one would expect a much more negative value; we will consider the properties of the three important subsites *D*, *E*, and *F* in greater detail later.

Table 1. Apparent association constants, K_a, of some saccharides with lysozyme at approximately 25°C, with the *p*H in the plateau region for binding (*p*H 5 to 6) and at ionic strength of 0.1*M*. All saccharides listed here are $\beta(1 \rightarrow 4)$ linked, with reducing terminus written to the right. Abbreviations are: Glc*N*Ac, *N*-acetyl-D-glucosamine; Mur*N*Ac, *N*-acetylmuramic acid; Glc, D-glucose. The methods, designated by lower-case letters, are as follows: a, ultraviolet difference spectrum (*54*); b, ultraviolet difference spectrum or inhibition of action on cell walls, or both (*21*); c, fluorescence spectra (*22*); d, fluorescence spectra (*26, 30, 43*); e, equilibrium dialysis (*26*); f, nuclear magnetic resonance chemical shift difference (*66, 67*); g, difference spectropolarimetry (*68*); h, kinetics of association-dissociation processes (*31*); and i, inhibition of action on cells (*69*).

	Saccharide	K_a (M^{-1})	Methods
1	Glc*N*Ac	20 — 50*	abdgi
2	(Glc*N*Ac)$_2$	5×10^3	abdhi
3	(Glc*N*Ac)$_3$	1.0×10^5	abcdh
4	(Glc*N*Ac)$_4$	1.0×10^5	ab
5	(Glc*N*Ac)$_5$	1.0×10^5	ab
6	(Glc*N*Ac)$_6$	1.0×10^5	ab
7	Glc*N*Ac-Mur*N*Ac	20	e
8	Mur*N*Ac-Glc*N*Ac	1.0×10^4	d
9	Glc*N*Ac-Mur*N*Ac-Glc*N*Ac	3×10^5	d
10	Glc*N*Ac-Mur*N*Ac-Glc*N*Ac-Mur*N*Ac	2×10^3	d
11	(Glc*N*Ac-Mur*N*Ac)$_3$	3.5×10^4	d
12	(Glc*N*Ac-Mur*N*Ac)$_2$ dimethyl ester	2×10^3	d
13	(Glc*N*Ac-Mur*N*Ac)$_2$ pentapeptide	7×10^3	d
14	*N*-Acetylmannosamine	70	b
15	*N*-Acetylgalactosamine	30	b
16	*N*-Propionylglucosamine	< 1	i
17	*N*-Formylglucosamine	< 1	i
18	Glucose	< 0.1	b
19	Cellobiose [Glc-$\beta(1 \rightarrow 4)$-Glc]	~ 1	b
20	Glc*N*Ac α-methylglycoside	20	fi
21	Glc*N*Ac β-methylglycoside	27	fi
22	Glc*N*Ac β-ethylglycoside	~ 5	i
23	Glc*N*Ac-Mur*N*Ac-Glc	1.8×10^3	d
24	Glc*N*Ac-Mur*N*Ac-Glc-Glc	1.7×10^3	d
25	Glc*N*Ac-Mur*N*Ac-Glc-Glc-Glc-Glc	5×10^2	d
26	Glc*N*Ac-Glc*N*Ac-Glc*N*Ac-Glc-Glc	3×10^4	d

* Values of *K* for this weakly bound saccharide show considerable variation from one worker to another.

Correlation between Structure in Crystal and in Solution

One might digress at this juncture and wonder whether there is any point in pressing the correlation between the properties of lysozyme-saccharide complexes in solution and the crystallographers' model, when it is not obvious, a priori, that the conformation of the enzyme is the same in solution as in the crystal. Although the environment of a protein in a crystal is not very different from that in solution (lysozyme crystals contain 30 percent water by weight), this criticism of x-ray crystallographic studies of protein structure has been raised many times in recent years.

Studies on the chemical properties of proteins in crystals, notably ribonuclease (*32*), show that the conformations of protein molecules that are observed in crystals correspond closely to the conformations that are important in solution. The differences that have been observed appear to arise from limitations that are imposed upon conformational changes and access to reactive groups by contacts between neighboring molecules in the crystals. Other types of evidence come from x-ray crystallographic studies (*33*). For crystals of different myo- and hemoglobins examined, the conformations of the individual globin chains are closely similar, although these proteins crystallize in different forms. The conformation of their polypeptide chains is therefore little affected by crystal lattice forces. As a corollary it may be safely presumed that the conformation is unchanged when there are no crystal lattice forces, that is, when the molecule is free in solution. In the case of lysozyme, two different crystal forms (the tetragonal and the triclinic) have been compared at low resolution, and no significant differences between the conformations of the peptide chains have been observed (*17*).

If such doubts in the case of lysozyme are not quieted by the above observations and by the remarkable agreement between the conclusions which can be drawn from binding data and from crystallography, other types of data are reassuring. In a very elegant group of experiments, Rupley showed that the constants for association of Glc*N*Ac and $(\text{Glc}NAc)_2$ with lysozyme crystals are nearly identical to those for association with dissolved lysozyme (*34*). Nuclear magnetic resonance spectra of some saccharides in the presence of lysozyme also agree with the crystallographic model. The signal due to the acetamido methyl group of Glc*N*Ac is shifted upfield and broadened in the presence of lysozyme, as expected if the acetyl methyl group lies over the indole ring of tryptophan 108 (see Fig. 3). On the other hand, only one of the acetamido methyl groups in $(\text{Glc}NAc)_2$–β-methyl glycoside is shifted upfield in the complex with lysozyme, and Thomas has shown that this is the one adjacent to the methyl glycoside group (*35*). The crystallographic model suggests that while the acetamido group in ring C makes hydrophobic contacts with Try 108, the one in B points out of the cleft.

The results of chemical modification studies of lysozyme (*36*) and of tritium exchange experiments with lysozyme and a lysozyme-saccharide complex (*37*) also indicate that the conformation in solution is the same as in the crystal.

Position of Bond Cleavage within the Complexes

Let us now consider the reactions catalyzed by lysozyme. We have mentioned above the deduction by Phillips that the position of bond cleavage in the lysozyme-substrate complex is the glycosidic bond of the sugar unit in subsite *D*. The very slow cleavage of $(\text{Glc}NAc)_3$ or Glc*N*Ac–Mur*N*Ac–Glc*N*Ac–Mur*N*Ac compared to the related hexasaccharides (*38, 39*) strongly suggests that the *B-C* bond cannot be the reactive one; the smaller saccharides are strongly bound across this position, and it would appear that these complexes are nonproductive. $(\text{Glc}NAc)_6$ is cleaved nearly quantitatively between the second and third residues from the reducing terminus, and $(\text{Glc}NAc)_5$ is cleaved either between the first and second or second and third (*38, 40*). This is also consistent with cleavage between subsites *D* and *E*, if there are a total of six subsites.

The location of the site of cleavage for an enzyme is a very exciting step forward, making it possible to try to explain the catalytic effect of the enzyme on the basis of its structure, but we will first use this conclusion to complete our analysis of the interactions between lysozyme and saccharides.

Transglycosylation Reaction

In 1964 Sharon and Seifter reported that the action of lysozyme on the cell wall tetrasaccharide 10 leads to the formation of products of higher as well as lower molecular weight (*41*), and Kravchenko and his co-workers reported

Tabel 2. Analysis of saccharide binding data in etrms of contributions of subsites. See Table 1 for names of saccharides as indicated by numeral designations.

Saccharide No.	Subsite							ΔF_u (kcal/mole)
	X	*A*	*B*	*C*	*D*	*E*	*F*	
1				Glc*N*Ac				−4.5 (±.3)
2			Glc*N*Ac	—Glc*N*Ac				−7.4
8			Mur*N*Ac	—Glc*N*Ac				−7.9
3		Glc*N*Ac	—Glc*N*Ac	—Glc*N*Ac				−9.2
9		Glc*N*Ac	—Mur*N*Ac	—Glc*N*Ac				−9.8
7		Glc*N*Ac	—Mur*N*Ac					≥ −4.2
10		Glc*N*Ac	—Mur*N*Ac	—Glc*N*Ac	—Mur*N*Ac			≥ −6.9
11		Glc*N*Ac	—Mur*N*Ac	—Glc*N*Ac	—Mur*N*Ac	—Glc*N*Ac	—Mur*N*Ac	−8.6
4	Glc*N*Ac	—Glc*N*Ac	—Glc*N*Ac	—Glc*N*Ac				−9.2
$\Delta\Delta F$*	0	−1.8	−2.9†	≤ −5.7	+2.9‡	−1.7‡ (*E* and *F*)		

* Calculated contribution of a residue in the given site to the free energy of association of a saccharide with lysozyme, in kilocalories per mole. † For a Mur*N*Ac residue in *B*, $\Delta\Delta F$ is −3.4 kcal/mole. ‡ Other evidence suggests other values for *D*, *E*, and *F* (see text).

Table 3. Summary of subsite interactions. Abbreviations are: Arg, arginine; Asn, asparagine; Asp, aspartic acid; Glu, glutamic acid; Gln, glutamine; Try, tryptophan. In the polar contacts column, the left side refers to the substrate moiety and the right side to the enzyme moiety.

Subsite	$\Delta\Delta$F* (kcal/mole)	Polar contacts†	Total van der Waals contacts†
A	−1.8	(NH)—Asp 101	7
B	−2.9	(O-6)—Asp 101	11
C	≤ −5.7	(O-6)—Try 62	30
		(O-3)—Try 63	
		(NH)—CO 107	
		(CO)—NH 59	
D	+3 to +6?	(O-6)—CO 57	35
		(O-1)—Glu 35	
E	−4?	(O-3)—Gln 57	45
		(NH)—CO 35	
		(CO)—Asn 44	
F	−1	(O-6)—CO 34	13
		(O-6)—Asn 37	
		(O-5)—Arg 114	
		(O-1)—Arg 114	

* From various chemical experiments. † From D. C. Phillips (*28*) and personal communication. The van der Waals contacts are those at less than 4 angstroms as calculated from atomic positions in the crystallographic model (*19*).

Table 4. Some relative acceptor efficiences. See Table 1 for names of substrates as indicated by numeral designations.

Acceptor A	Acceptor B	Substrate No.	Relative efficiency, A/B	References
Glucose	Glc*N*Ac	10	0.22	*(44)*
$(Glc\mathit{N}Ac)_2$	Glc*N*Ac	10	2.0	*(44)*
Glc*N*Ac-Mur*N*Ac	Glc*N*Ac	10	3.25	*(44)*
Cellobiose (Glc-$\beta(1\rightarrow4)$-Glc)	Glc*N*Ac	10	0.67	*(44)*
Cellobiose	Glucose	10	2.8	*(44)*
$(Glc\mathit{N}Ac)_2$	Glucose	10	9	*(44)*
Glc*N*Ac	H_2O	3	2200	*(38, 40)*
Glucose	H_2O	3	28	*(38, 40)*
Glc*N*Ac-Mur*N*Ac-Glc*N*Ac-Mur*N*Ac	H_2O	10	$\sim 10^4$	*(44)*

similar observations with chitin saccharides (*42*). Both groups proposed that these products arise by "transglycosylation," that is, glycoside transfer to acceptor saccharides, in these cases the substrates themselves. It was soon realized that lysozyme can catalyze transfer to a number of different acceptors, and that the enzyme exhibits specificity toward them. Rupley and his co-workers (*38, 40*) and our group at the Weizmann Institute (*43, 44*) have used the transfer reaction to study the properties of the region of the enzyme beyond the cleavage point, arguing that the specificity of transfer must be due to interactions between this region of the enzyme and the acceptor molecule.

The new bond formed is of the β-configuration; this was shown conclusively for Glc*N*Ac–Mur*N*Ac–Glc*N*Ac formed by the lysozyme-catalyzed transfer of a Glc*N*Ac–Mur*N*Ac moiety of the cell wall tetrasaccharide to Glc*N*Ac (*44*) and for the $(Glc\mathit{N}Ac)_n$-Glc-Glc saccharides (where $n = 1$, 2, or 3) formed by transfer from $(Glc\mathit{N}Ac)_5$ to cellobiose (*45*). The formation of a new bond of the β-configuration is not due only to the interactions of an acceptor saccharide with the enzyme, however. Transfer from $(Glc\mathit{N}Ac)_2$ to methanol yields only the β-methyl glycoside of Glc*N*Ac (*46*). We have also shown that the reaction in question is indeed a transglycosylation and not simply the reverse of hydrolysis (*39*).

These results suggest that the reaction of a saccharide with lysozyme occurs in several steps. The saccharide is cleaved at the glycosidic bond of the residue in subsite *D*, and the portion of the substrate lying in *E* and beyond is released from the cleft, leaving an intermediate glycosyl enzyme. The glycosyl enzyme may then either react with water (hydrolysis) or with an acceptor molecule which comes into the cleft (transfer). The postulation of an intermediate is necessary, of course, because the stereochemistry of the reaction demands that the leaving saccharide and acceptor saccharide moieties occupy the same region—and they certainly cannot do so at the same time.

Properties of Subsites E and F

Data on the relative acceptor abilities of various compounds have been obtained by determining the amounts of various products formed in competitive experiments. However, if any product of interest is readily destroyed by a subsequent reaction or can be formed by a route other than direct transfer, such results will not accurately reflect the relative rates of reaction of the acceptors with the intermediate. Furthermore, the possibility of the formation of linkages other than $1\rightarrow4$ should be taken into consideration (*47*).

The data of Table 4 suffer from these ambiguities, but are still rather interesting. The data indicate that a residue in subsite *F* can indeed interact favorably with the enzyme. It can also be deduced that the acetamido group plays an important role in the interaction of a saccharide at subsite *E*, but not at *F*. This result is predicted by Phillips' model (Fig. 3), in which the acetamido groups make good contacts with the enzyme in subsites *A*, *C*, and *E*, but have fairly few contacts in *B*, *D*, and *F*. Other, qualitative observations of acceptor ability all seem to suggest that a good acceptor must be capable of fitting this model.

It is not obvious how to relate acceptor data quantitatively to enzyme-saccharide interactions. Relative acceptor abilities are likely to depend both on the strengths of glycosyl enzyme-acceptor complexes and on the rates of bond formation in the complexes. It is reasonable to assume that such rates are equal only if the hydroxyl groups of the acceptors in question are chemically similar, and are located in the same position in the various complexes. Thus, one may conclude (Table 4) that the saccharide-enzyme interaction at *F* amounts to about -1 kilocalorie per mole, and that the difference between Glc*N*Ac and glucose at *E* amounts to about -1 to -2 kilocalories per mole, but one concludes with less assurance that the total interaction of a Glc*N*Ac residue at subsite *E* amounts to -4.0 kilocalories per mole.

There is yet another way to estimate the magnitude of various enzyme-saccharide interactions—to compare the rates of certain lysozyme-catalyzed hydrolyses. The data given in Table 5 probably represent true hydrolysis rates (uncomplicated by transglycosylation) of lysozyme-saccharide complexes. If we assume that the rates of cleavage of all productive complexes (that is, across *D-E*) are the same, the differences in rates in Table 5 can be explained in terms of relative amounts of various productive and nonproductive binding modes involved in each enzyme-saccharide complex. The relative rates of cleavage of two different bonds in $(Glc\mathit{N}Ac)_5$ (Table 5, a and b) suggest that binding at subsite *A* is stronger than at *F* by -0.4 kilocalorie per mole; $\Delta\Delta F$ for subsite *F* is then about -1.4 kilocalories per mole. Comparison of b and c (Table 5) leads to a similar conclusion.

Table 5. Rates of some lysozyme-catalyzed hydrolyses, at enzyme saturation. The reactions are probably uncomplicated by transglycosylation, *p*H 5.0 to 5.5. The rate is given as moles hydrolyzed per mole of enzyme per minute. The arrow indicates the point of cleavage in the saccharides. The saccharides shown are all $\beta(1\rightarrow4)$ linked, with the reducing end written to the right; G, *N*-acetyl-D-glucosamine residue; M, *N*-acetylmuramic acid residue.

Reaction	Rate
(a) G—G—↓G—G—G	0.5*
(b) G—G—G—↓G—G	1*
(c) G—G—G—↓G—G—G	10*
(d) G—M—G—↓M—G—M	30†
(e) G—M—G—↓M—↓G—M—G—M	30† (each)

* As calculated from Rupley (*38*) at 40°C.
† Our data (*39*) at 37°C.

Magnitude of Strain in Subsite D

In light of Phillips' conclusion that the saccharide unit in subsite *D* must be distorted from the stable chair con-

formation in order to avoid steric hindrance in the enzyme-substrate complex, the value of $\Delta\Delta F$ for subsite *D* is of enormous interest. Unfortunately, an unequivocal way of determining this value is not yet available.

From binding data (compare saccharides 9 and 11 in Table 2) and from Hess's observations (*23*), we can conclude that the sum of the interactions in subsites *D*, *E*, and *F* is about +1 kilocalorie per mole. The $\Delta\Delta F$ for subsite *F* is about −1 kilocalorie per mole, from transglycosylation data and relative cleavage rates. Thus, *D* and *E* must account for a net contribution of +2 kilocalories per mole. The problem is in dividing this sum between them. One approach is to assume that the competition between water and Glc*N*Ac as acceptors gives a proper estimate for the contribution of subsite *E*, despite the difficulties mentioned above. If this value of −4 kilocalories per mole is accepted for subsite *E*, then $\Delta\Delta F$ for *D* must be +6 kilocalories per mole (*38*). On the other hand, we suggest (*26*) a value of +2.9 kilocalories per mole on the basis of a direct comparison of binding constants (compare saccharides 9 and 10 in Table 2). It is possible, however, that the terminal Mur*N*Ac residue in saccharide 10 is not quite so strained as a residue in subsite *D* would be in a saccharide filling the entire cleft, and that the total strain in such a case might indeed be as much as +6 kilocalories per mole.

Since the model suggests that some favorable contacts are made at this subsite, the distortion of the pyranose ring in *D* must require even more energy than is implied by $\Delta\Delta F$ for the subsite. It has been estimated that distortion of a cyclohexane ring all the way to the half chair, the conformation suggested by Phillips for the sugar residue bound at subsite *D* (Fig. 5), involves a ΔF of about 10 kilocalories per mole (*48*). The limited data available indicate that distortion of a pyranose ring requires about the same energy (*49*).

Hydrolysis by Way of Transglycosylation

As might well be imagined, transglycosylation greatly complicates the study of lysozyme-catalyzed hydrolyses. In addition to leading to complex reaction mixtures in which it is not clear by what route any given product arises, transglycosylation causes peculiarities in the kinetics of hydrolyses as well. Kravchenko noted that the hydrolyses of the chitin dimer, trimer, and tetramer have induction periods, and that the induction period for the dimer could be considerably shortened by addition of small amounts of the tetramer (*50*). Rupley found that the rates of hydrolysis of these saccharides continue to increase with substrate concentration beyond the point where the enzyme is saturated with 1 mole of substrate (*38*). We have observed all of these phenomena in the hydrolysis of cell wall oligosaccharides, and have been able to show that they can be explained in terms of reaction pathways involving transglycosylation.

We studied the course of the reaction of the cell wall tetrasaccharide 10 with lysozyme by using uniformly tritium-labeled tetrasaccharide and by analyzing the saccharide composition of the reaction mixture by a combination of paper chromatography and electrophoresis (*39*). The course of a typical reaction is shown in Fig. 6. It can be seen that large concentrations of higher oligomers [which have been shown to be $\beta(1 \rightarrow 4)$ linked saccharides of the general structure

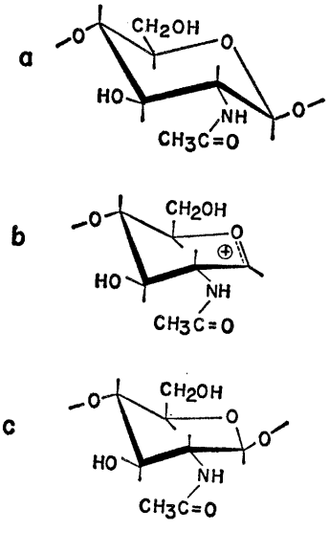
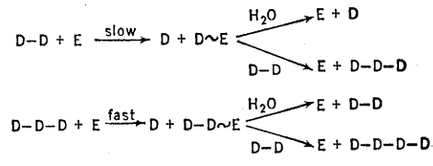


Fig. 5. Conformation of Glc*N*Ac and intermediate in hydrolysis of its glycosides. (a) The preferred chair conformation of free Glc*N*Ac; (b) the half-chair conformation of the carbonium ion formed from a Glc*N*Ac glycoside in the course of acid-catalyzed hydrolysis; (c) the presumed conformation of a Glc*N*Ac unit bound in subsite *D* of lysozyme, distorted toward the half-chair. Note that this distortion rotates C-6 upward into an approximately axial position, and is thus promoted by steric hindrance at the position C-6 and oxygen-6 (O-6) would take up in the chair conformation. In (b) and (c) C-1, C-2, C-5, and the ring oxygen are coplanar.

$(\text{Glc}NAc\text{–Mur}NAc)_n$] build up during the reaction, accounting for more than one quarter of the substrate at maximum. The period of maximum higher oligomer concentration more or less coincides with the maximum rate of production of the disaccharide; during the initial induction period the oligomers seem to build up, and, as the reaction slows down, the higher oligomers disappear. It would seem that these higher oligomers catalyze the reaction of the tetrasaccharide; indeed it can be shown that addition of a small quantity of octasaccharide to the tetrasaccharide abolishes the induction period and greatly speeds up the hydrolysis of the tetrasaccharide (*39*).

We can explain these and other observations by a fairly simple scheme, illustrated in part in the following.



D represents a Glc*NAc*–Mur*N*Ac disaccharide unit and D~E a glycosyl enzyme. Cell wall tetrasaccharide 10 is a rather poor substrate for lysozyme because it is preferentially bound in a nonproductive manner. From Table 2 one can calculate that at saturation of the enzyme with tetrasaccharide, less than 1 percent will be in the form of the productive complex in subsites *CDEF*. When the productive complex is cleaved, however, the intermediate glycosyl enzyme can react with a second molecule of tetrasaccharide to form hexasaccharide. The hexasaccharide (or any larger cell wall saccharide) should be bound preferentially along the entire cleft of lysozyme, and is a much better substrate—indeed, the cell wall hexa- and octasaccharides are the best well-defined substrates known to date, and each is hydrolyzed with a turnover number of about 30 per minute.

Once some of these higher oligosaccharides is present, most of the reaction will proceed with these saccharides as substrate. Tetrasaccharide acts as transglycosylation acceptor and is thus consumed; disaccharide is produced by cleavage of successive Glc*N*Ac–Mur*N*Ac units from the reducing end of larger saccharides. However, as disaccharide builds up in the reaction mixture, it competes with the tetrasaccharide as an acceptor. Since transfer to disaccharide does not build up longer

saccharides, the higher saccharide pool decays by hydrolysis, and the reaction slows down. Eventually only di- and tetrasaccharide remain in any quantity, and only the very slow hydrolysis of tetrasaccharide continues.

This scheme also explains why the rate of cleavage of the tetrasaccharide increases with its concentration beyond the point where the enzyme is saturated with 1 mole of saccharide. The overall rate will depend on the level of higher oligosaccharide concentration reached, which in turn depends on the concentration of tetrasaccharide competing with water for the glycosyl-enzyme intermediates.

The same analysis clearly can explain the similar properties of the lysozyme-catalyzed hydrolyses of the chitin di-, tri-, and tetrasaccharides (*38, 50*). There are three differences. (i) Any of the bonds in a chitin saccharide is in principle susceptible to hydrolysis, as the restriction against Mur*N*Ac residues in subsite *C* has no effect. (ii) Because the very favorable nonproductive mode of binding with the reducing terminus at subsite *C* is available to the chitin saccharides, nonproductive binding is even more overwhelming than for cell wall saccharides. One can estimate that at saturation less than 0.3 percent of the $(GlcNAc)_4$ and less than 0.05 percent of the $(GlcNAc)_3$ complexes involve productive modes of binding. (iii) Very large chitin oligosaccharides, unlike the charged cell wall saccharides, are insoluble in water. At high chitin saccharide concentrations ($> 0.01M$), a chitin-like precipitate forms during the lysozyme reaction (*42*).

Chemical Mechanism

Up to this point we have said nothing about the chemical mechanism by which lysozyme catalyzes the cleavage of glycosidic bonds. On the basis of what is known about the chemistry of glycosides, the possible mechanisms can be narrowed down to a few classes (*51*). Nonenzymatic solvolyses of ordinary glycosides (not aryl or tertiary alkyl glycosides) occur by cleavage of the C-1 oxygen bond, as do reactions catalyzed by many glycosidases. Rupley has shown by means of ^{18}O-labeling experiments that lysozyme-catalyzed reactions involve cleavage between C-1 of one pyranose ring and the oxygen joining it to C-4 of the next ring. The

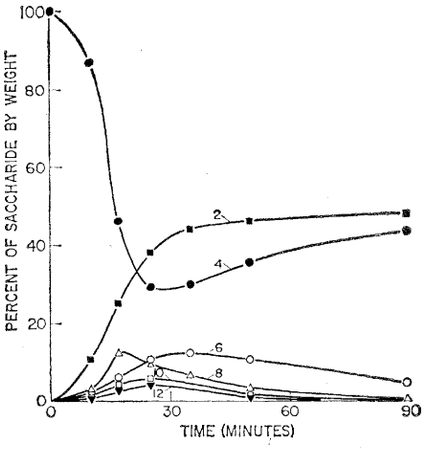


Fig. 6. Course of a typical digestion of the cell wall tetrasaccharide Glc*N*Ac-Mur*N*Ac-Glc*N*Ac-Mur*N*Ac by lysozyme (*39*). The reaction mixture consists of $7.5 \times 10^{-3}M$ saccharide and 0.3 milligram per milliliter of lysozyme in an $0.1M$ acetate buffer at *p*H 5.25 and 37°C. The percent of the initial saccharide (by weight) in the form of each of the oligomers is given as a function of time; curve 2 represents (Glc*N*Ac–Mur*N*Ac), curve 4 represents $(GlcNAc–MurNAc)_2$, curve 6 represents $(GlcNAc–MurNAc)_3$, curve 8 represents $(GlcNAc–MurNAc)_4$, curve 10 represents $(GlcNAc–MurNAc)_5$, and curve 12 represents $(GlcNAc–MurNAc)_6$.

reaction may proceed either by formation of a carbonium ion at C-1 or by bimolecular attack of a nucleophile at this carbon, although bimolecular attack usually does not occur in hydroxylic solvents unless the nucleophile is either very powerful or intramolecular. Glycosides, like all acetals, are especially susceptible to acid-catalyzed carbonium ion formation because the neighboring ring oxygen stabilizes the positive charge, giving a species with partial oxonium ion character. However, in glycopyranosides a conformational change—distortion of the pyranose ring from its normal chair conformation toward the half-chair, with C-1, C-2, C-5, and the ring oxygen in a plane (see Fig. 5)—is necessary for this interaction to occur, and hydrolysis is slower than for simple acetals, for which the planar conformation is unstrained (*52*).

Upon examination of the region near subsite *D* in the crystallographic model for the lysozyme-substrate complex, two mechanistic features become evident. First, there are only two functional residues of the enzyme near the cleavage site, the carboxyl groups of glutamic acid 35 and aspartic acid 52 (*15*). The carboxyl of Glu 35 appears to be in a good position to protonate the D-E bridge oxygen, that is, to act as a general acid catalyst for the cleavage of the glycosidic bond. It also is in a nonpolar environment, which makes it likely that it is the one carboxyl group of abnormally high pK_a (approximately 6.3) in the enzyme (*53*). Saccharide binding shows a dependence on the protonation of a group in the enzyme of pK_a about 6.1 to 6.3 (*21, 22, 26, 54*). This can only be an abnormal carboxyl group or a histidine residue, but the single histidine in lysozyme is far from the active site (*15, 17*) and is unaffected by inhibitor binding (*55*). Thus it seems that the carboxyl group of Glu 35 has a pK_a high enough so that it would be protonated in the *p*H region of maximum lysozyme activity, *p*H 5 to 6, and could act as a general acid catalyst for the hydrolysis.

The second striking feature of the enzyme-substrate complex is, of course, the distortion of the pyranose ring toward the half-chair conformation. We have seen that some interaction in subsite *D* raises the free energy of the complex by 3 to 6 kilocalories per mole; if this distortion is such that it brings the substate part way toward the transition state (see Fig. 5), it may accelerate the cleavage of the complex by 10^2- to 10^4-fold. This is, of course, an example of the often-proposed strain or rack mechanism (*56*). It is important to note that the distortion of the substrate proposed by Phillips is toward the conformation of the transition state rather than that of the product, which is also preferentially a chair conformation. The induction of strain in a substrate is only catalytically useful if this strain is relieved in going toward the transition state for the reaction (*57*).

What other factors besides acid catalysis and distortion of the substrate may play a role in accelerating the cleavage of a glycosidic bond by lysozyme? A slightly different question whose answer must be related to this is the nature of the glycosyl enzyme intermediate. The enzyme-substrate complex has two additional features whose significance has not yet been clearly demonstrated: the carboxylate group of Asp 52 (which would presumably be in the form of the anion at the *p*H's at which lysozyme is active) and the acetamido group of the natural substrate itself, on the ring in subsite *D*. The carboxylate anion might conceivably act as a nucleophile

(Fig. 7a) or even a general base, or simply stabilize the carbonium ion by means of its negative charge (Fig. 7b). Unfortunately, there does not yet exist any clear evidence that Asp 52 plays any role at all in lysozyme action (*58*). The Asp 52 probably has an abnormally low pK_a (*59*), so that its protonation cannot be observed in the *p*H range of lysozyme activity. The *p*H-activity curve for the hydrolysis by lysozyme of (Glc*N*Ac)$_3$ [as well as of some aryl glycosides (*46*)] seems to indicate an absolute dependence of the activity on the same two groups (an ionized carboxylate of normal pK_a and a protonated carboxyl of high pK_a) implicated in the binding of (Glc*N*Ac)$_3$ (*40*), but, as Rupley has pointed out, this may reflect an effect on the relative strengths of productive and nonproductive complexes, rather than on rates of reaction within productive complexes.

Role of the Substrate Acetamido Group

It has been suggested that the acetamido group of the substrate itself can act as a nucleophile, to give the intermediate shown in Fig. 7c (*60*). Bruice has shown that the acetamido group can participate in the rate determining step for nonenzymatic hydrolysis of glycosides when the acetamido group is *trans* to the leaving group (*61*). Thus the β-*N*-acetylglucosaminide of *o*-nitrophenol cleaves rapidly at neutrality, about 10^4 times faster than the α-*N*-acetylglucosaminide or the β-glucoside. There is no clear evidence for or against participation of a substrate acetamido group in a lysozyme-catalyzed reaction, but it is significant that the enzyme does not have an absolute requirement for an acetamido group on the ring at which cleavage occurs. Various experiments suggest that lysozyme cleaves bonds at *N*-acetylhexosamine residues from two to over twenty times faster than at glucose residues (*45, 46, 62*), but lysozyme may cleave glycosidic bonds at 2-deoxyglucose even faster (*46*). No experiment to date involves an unambiguous comparison of the rates of cleavage of productive complexes, however. Even with cleaner experiments, interpretation would be difficult since the hydrolysis of glycosides is notoriously sensitive to subtle and complex conformational effects (*52*), and the proposed role of a specific conformational distortion in the lysozyme mechanism further accentuates the problem. Thus, it may be that differences in cleavage rates at *N*-acetylglucosamine, glucose, and 2-deoxyglucose are primarily due to strain and conformational effects.

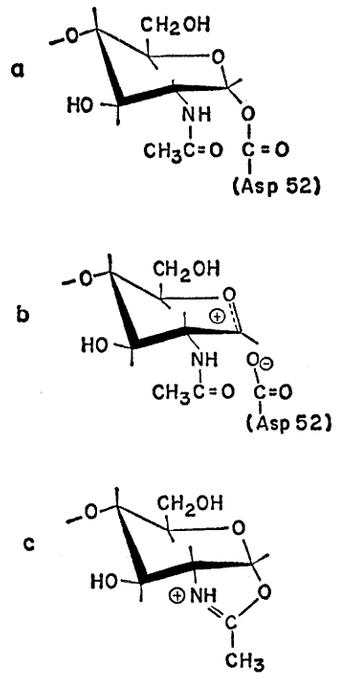


Fig. 7. Possible structure of the glycosyl enzyme: (a) with bonding between the carboxyl oxygen of Asp 52 and C-1 of ring D; (b) with the carboxylate anion of Asp 52 and carbonium ion at C-1 as an ion pair; (c) with nucleophilic participation by the oxygen of the neighboring acetamido group.

Rupley (*63*) has recently presented some evidence that suggests that the glycosyl enzyme intermediate is a carbonium ion. The relative rates of attack of several nonsaccharide acceptors (for example, alcohols) on the intermediate are not as expected for nucleophilic displacement at a tetrahedral carbon. It thus seems likely that any bonding between the positive carbon of the intermediate (C-1) and an oxygen of either Asp 52 or the neighboring acetamido group is very weak.

If the intermediate is in fact very much like a carbonium ion, the great stereospecificity of its reaction with nucleophiles (to yield β-glycosides) must be due to a restriction on the approach of the nucleophile. The three-dimensional model of the lysozyme-substrate complex shows that acceptors can only approach the postulated enzyme intermediate from the same side as the leaving group, resulting in retention of configuration (*17*).

Conclusion

On the basis of all the chemical and crystallographic data available today, a convincing picture of the mechanism of lysozyme action can be presented. The enzyme binds its oligo- or polysaccharide substrate in a cleft running the whole length of the protein molecule, stabilizing the complex by means of a large number of hydrogen bonds and nonpolar interactions with as many as six monomer units of the substrate (*64*). The substrate is distorted from its most stable conformation on binding; this distortion raises the energy of the complex, possibly by as much as 6 kilocalories per mole or even more. All the remaining favorable interactions make up for this strain, however, so that the association constant for a cell wall saccharide six or more units long is still well over 10^4 liter per mole. This situation leads to a difficulty, however. The strain is associated with the region of the catalytic site (the *D-E* bond), while a large part of the favorable interactions are in sites *A*, *B*, and *C*. A nonproductive mode of saccharide binding with the reducing terminus in substite *C* is thus stronger than productive binding—indeed, this can clearly be seen in the reactions of chitin saccharides—but for cell wall saccharides terminating in a Mur*N*Ac residue, this most favorable mode of binding is eliminated by the restriction of subsite *C* to Glc*N*Ac residues only. This restriction, of steric origin, also gives the enzyme its *N*-acetylmuramidase specificity in the cleavage of cell walls.

In the enzyme-substrate complex, the pyranose ring in subsite *D* is apparently forced into a conformation—toward a half-chair—which raises the energy of the ground state, and presumably lowers the energy of the transition state for bond cleavage. This lowers the activation energy of the process, and must play a considerable role in the enzymic rate enhancement. The oxygen bridging D and E is protonated by the carboxylic acid group of Glu 35, and the glycosidic bond is cleaved. The "aglycone" can then diffuse away from *E* and *F*, leaving a glycosyl enzyme intermediate. Water can attack the intermediate to complete the hydrolysis, and the second product saccharide can then also dissociate. However, because the enzyme has a site (subsites *E* and *F*) for favorable interaction with the "aglycone" part of the intermediate—necessary for productive binding—saccharide acceptors

can associate with the intermediate glycosyl enzyme, and lead to transglycosylation (*65*).

Although more work is clearly needed to prove the mechanism described above, the most serious remaining question about the mechanism is what additional factors accelerate the bond-cleavage step—that is, what is the nature of the glycosyl enzyme intermediate? The most likely possibilities seem to be the three shown in Fig. 7: a carbonium ion stabilized by the negative charge of Asp 52 (b), a species with some bonding between the Asp 52 carboxyl group and C-1 (a), and, perhaps least likely at this point, an ion with intramolecular bonding between the acetamido group and C-1 (c).

There is another puzzling problem which most discussions of lysozyme mechanism do not touch upon. What, after all, is lysozyme's biological role? There do not appear to be many bacteria pathogenic to vertebrates which are susceptible to lysis by lysozyme, so a role as a bacteriocide per se seems unlikely. On the other hand, nearly all bacteria do have a layer in their cell walls which can be digested by lysozyme (*5*). Various aspects of the action of lysozyme suggest that it is "designed" specifically to work on polysaccharides with alternating Glc*N*Ac and Mur*N*Ac residues, and that it is intended not merely to cleave a large polymer in a few places, but to hydrolyze the cell wall oligosaccharides all the way down to the di- and tetrasaccharide level. We would suggest, then, that lysozyme's function in vivo might be the digestion of the glycopeptide debris from cell walls of bacteria killed in other ways.

Whatever lysozyme's function might be in vivo, it has played a vital role in vitro—for recent studies of lysozyme action have added greatly to our understanding of the mechanism of enzyme action in general. The study of lysozyme has provided the first clear evidence for some aspects of enzyme action previously suspected: induced fit, nonproductive substrate binding, and the strain or rack mechanism, in particular. The research described above also has indicated a number of useful approaches to the study of enzyme mechanisms in general, and we hope that these will be applied to many more enzymes in the future.

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Some Ecological Benefits of Woody Plant Control with Herbicides

Productivity of range and pastureland can be increased through use of agricultural chemicals.

Keith C. Barrons

In recent months, several ecologists have expressed concern that the use of herbicides as defoliants in Vietnam (*1*), for the control of undesirable woody plants in pastures, and on right-of-ways might result in soil erosion and soil laterization. These fears would indeed be well founded if the herbicides employed for the control of woody vegetation killed all kinds of plants and kept them from growing. The selective nature of these herbicides has apparently not been fully recognized, and, as a result, the misconception has been advanced that all higher plants disappear after their use.

Those responsible for the extensive application of these materials during the quarter century since 2,4-dichlorophenoxyacetic acid (2,4-D), the first of them, became available fully recognize the tolerance of grasses and some nongrass species. They have seen how grass increases in treated rangeland and pastures as the sprayed weeds and brush die back. They have observed how a sod tends to develop on right-of-ways following treatment. They have further observed that, even where grasses are originally limited in the flora, there is no prolonged soil sterility and tolerant nongrasses soon begin to reestablish and provide soil cover. The need for the subsequent seeding of grasses in some situations where woody growth was very dense, particularly in pasture improvement programs, is well recognized.

Selective herbicides are of tremendous value to agriculture in controlling weeds among, and thereby increasing productivity of, small grains, corn, rice, sorghum, and sugar cane, all members of the grass family. In addition, they are making more animal protein available at a lower cost through improved pastures and rangeland. In forest management, they are useful for site preparation and the selective control of weed trees and shrubs. Apart from being a benefit to crops, their other uses provide easier inspection, better visibility, and increased safety along railroad, public utility, and highway right-of-ways.

The control of unwanted trees, brush, and woody vines with herbicides provides labor economy as compared with cutting, particularly as a result of root and crown kill. The tendency of most woody plants to resprout from underground structures is well recognized by all who have had experience in their removal by mechanical means. Selective chemical control results in far less soil erosion than grubbing or bulldozing and less upset to the ecology in general than fire.

We have an obligation to see that debate on the use of selective herbicides for improving visibility in a theater of war does not hamper continuing use where they can be of great benefit to mankind; however, ecological effects as well as immediate benefits must be understood.

What is the ecological aftermath of woody-plant control with the herbicides 2,4-D (2,4-dicholophenoxyacetic acid), 2,4,5-T (2,4,5-trichlorophenoxyacetic acid), silvex (2,4,5-trichlorophenoxypropionic acid), and Tordon (picloram) (4-amino-3,5,6 trichloropicolinic acid), the major chemicals in current use for this purpose? Obviously, there is need for further studies, but experimentation and commercial use over many years have provided a great deal of information.

Range and Pasture Management

Mitich (*2*) measured the grass and nongrass vegetative growth in a pasture improvement program including plots in 22 counties in North Dakota. After two annual applications of 2.24 kilograms of 2,4-D per hectare, the vegetative cover changed from 67 percent

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