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# Biocatalysis of the Hydrolysis of the Glycosidic Linkage under the Influence of Lysozyme

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As a result of the advances in the X-ray diffraction analysis of enzymes, it has recently become possible for the first time to describe in a concrete form and quite unambiguously the effective factors in biocatalysis. These factors have been demonstrated by means of biochemical, physicochemical, and physical research techniques. Lysozyme is the first enzyme for which such research has been carried out on an adequate scale. This review deals in fact with such investigations. The ultimate result was not only the solution of the problem of the specific mechanism of the action of this enzyme but also the broad outlines of the mechanisms of biocatalysis were finally established in principle. Instead of the old more or less probable hypotheses, these studies led to quite definite ideas about the mechanism of biocatalysis.
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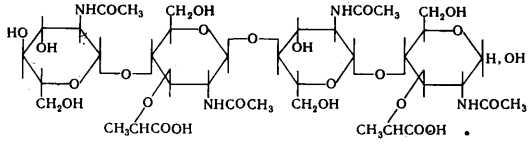

## I. INTRODUCTION

Complete data on the X-ray diffraction analysis of lysozyme and its enzyme–substrate complexes with carbohydrates were published in 1967, the resolution being 2 Å.[1] At that time it was the only structure of an enzyme–substrate complex investigated in all details. It became possible for the first time to obtain for a specific example unambiguous data concerning the structure of the active centre and to put forward on the basis of these direct results a hypothesis about the mechanism of the action of the enzyme.

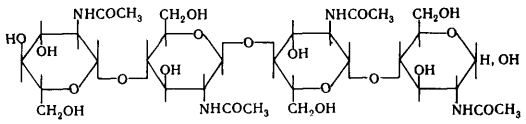
Chemical and physicochemical studies, which developed particularly vigorously after the above publication[1], made it possible to compare the structural data with the catalytic properties of the enzyme and the mechanism of the action of lysozyme has now been elucidated in broad outline. This example is instructive in many respects. Firstly, it shows that a problem as complex as an unambiguous description of the specific mechanism of the action of an enzyme can be solved only as a result of a combined study by various methods: chemical, biochemical, physicochemical, and physical. Secondly, it confirms the earlier view that the usual principles of physical organic chemistry underly the mechanism of biocatalysis. Thirdly, it demonstrates that the effective occurrence of a chemical process (biocatalysis) can be ensured not only by the direct action of the enzyme on the substrate linkages being dissociated but also as a result of a complex reaction pathway via intermediates, each of which is itself a substrate of lysozyme. The aim of the present review is to describe the chemical mechanism of the action of lysozyme and to show how complex the mode of interaction of the individual factors in biocatalysis can be and to attempt thereby to establish its fundamental essential features.

## II. THE EARLY PERIOD IN THE STUDY OF THE ACTION OF LYSOZYME

Lysozyme became widely known as a result of Fleming's publication in 1922 describing the bacteriolytic action of a number of biological liquids and extracts[2]. Nothing was known for a long time about the chemical nature of lysozyme. A number of studies established that lysozyme can act on the polysaccharide components of cells with liberation of *N*-acetyl-D-glucosamine[3–5]. The chemical nature of the lysozyme substrate was definitely established for the first time by Berger and Weiser[6], who showed that chitin may be hydrolysed by lysozyme with formation of *N*-acetyl-D-glucosamine. It was thus shown that the lysis of bacteria takes place as a result of the hydrolysis of the polysaccharide at the β-(1–4)-glycosidic linkage connecting the *N*-acetyl-D-glucosamine residues. The first reports on low-molecular-weight substrates appeared in 1959–1960.[7–10] Salton and Ghysen[7,8] isolated a tetrasaccharide from the products of the lysozyme hydrolysis of bacterial cell walls and showed that the action of lysozyme on this substrate results in only one product—*N*-acetyl glucosamino-β(1–4)-*N*-acetyl muramic acid—a disaccharide the structure of which was finally established later[11,12]. The initial tetrasaccharide has the following structure:



The structure of another tetrasaccharide substrate, a fragment of chitin, is



The structure of the carbohydrate chain in the two cases is the same although the bacterial tetrasaccharide contains residues of D-lactic acid. Direct hydrolysis of the oligosaccharides was suggested in the above investigations[7-10] and also in later studies[13,14].

When lysozyme was allowed to act on a trisaccharide of similar structure, only two products were detected: a monosaccharide and a disaccharide[10,14]. According to Rupley[15], the glycosidic linkage is cleaved preferentially from the non-reducing end[15]. The first measurements of the rate of hydrolysis of oligosaccharides (chitin fragments) showed that the disaccharide is not hydrolysed by lysozyme at all, and the trisaccharide is hydrolysed very slowly, the rate of hydrolysis of oligosaccharides increasing with the length of the oligosaccharide[13].

## III. THE MOLECULAR MECHANISM OF THE HYDROLYSIS OF SHORT OLIGOSACCHARIDES

The study of the mechanism of the action of lysozyme had two starting points. Firstly, it was established that short oligosaccharides are not hydrolysed directly, the reaction proceeding via the intermediate synthesis of longer oligosaccharides (hydrolysis via transglycosylation) and, secondly, it was shown that the hydrolysis goes almost completely to acetyl-D-glucosamine.

### 1. Hydrolysis via Transglycosylation

In 1964 Kravchenko and Maksimov[16] published the first report about the formation of high-molecular-weight water-insoluble products when lysozyme is allowed to act on various oligosaccharides derived from chitin. A similar phenomenon was observed by Sharon and Seifter[17], who employed a bacterial tetrasaccharide as the substrate. A number of communications confirming and extending the knowledge about the synthetic activity of lysozyme and showing that the formation of carbohydrates with a higher molecular weight proceeds via a transglycosylation mechanism (see, for example, Refs. 18–21) were published later.

It was possible to regard the transglycosylation reaction as a side process, which occurs under certain conditions (for example, at high substrate and enzyme concentrations). However, biochemical, X-ray diffraction, and physicochemical studies showed that transglycosylation is a fundamental intermediate stage in the hydrolysis of short oligosaccharides catalysed by lysozyme. Depending on the conditions, the process stops either at the stage of the total hydrolysis of the substrate to the monomer or at an intermediate stage.

The mechanism of hydrolysis via transglycosylation was formulated for the first time in 1965 by Maksimov et al.[19] The conclusion was based on the following experimental facts described for the first time in their report: (1) the induction period in the hydrolysis of short oligosaccharides; (2) the action of the seed†; (3) total hydrolysis of tri- and tetra-saccharides (involving all the glycosidic linkages). A trisaccharide is not split to the monomer and the dimer, as was believed by some investigators[10,14,15], but only to the monomer; (4) a change in the mode of reaction, towards hydrolysis or synthesis, depending on the concentrations, of the enzyme and substrate. Moreover, account was taken of the fact that the rates of the hydrolytic[13] and synthetic[16] lysozyme reactions increase appreciably with increase in the length of the oligosaccharide substrate. On the other hand, it was established that a trisaccharide cannot in principle be hydrolysed by lysozyme directly.

The hydrolysis of this substrate is a result of a complex process the mechanism of which was put forward in 1965:[19]

(1) short oligosaccharides

$$+\ \text{short oligosaccharide} \xrightarrow[\text{sylation}]{\text{transglyco-}} \text{long oligosaccharide} + \text{monomer (?)};$$

(2) long oligosaccharides

$$+\ \text{short oligosaccharide} \xrightarrow[\text{drolysis}]{\text{transglyco-sylation hy-}} \text{long oligosaccharide} + \text{monomer (?)}.$$

hydrolysis

This scheme describes two reaction stages: the first is very slow, corresponding to the induction period in the course of which conditions are created for the second reaction stage. In the second stage the bulk of the substrate is digested, the process being cyclic. In a number of subsequent investigations[22-24] these findings were confirmed for other examples. The seed was regarded as a cofactor in the reaction, similar to a coenzyme[22]. It was shown that the substrate and the seed compete for the site where binding to the enzyme active centre takes place[24]. The study of the action of lysozyme on di-*N*-chloroacetylchitobiose showed that under certain conditions only one mole of monosaccharide is formed from one mole of disaccharide[23]. The following reaction mechanism was proposed[23]:

1. dimer + dimer → monomer + trimer;

2. dimer + oligosaccharide ($x$ units) → monomer + oligosaccharide ($x + 1$ units).

According to this scheme, the first stage involves the formation of a seed by a disproportionation mechanism and in the second stage transglycosylation takes place mainly with formation of the monomer.

In 1967 Sharon published data showing that the bacterial tetrasaccharide hydrolyses only after an induction period, the duration of which is inversely proportional to the concentration of the enzyme[25]. Sharon also explains the observed lag period by the complexity of the hydrolytic process and by a molecular mechanism of the hydrolysis via transglycosylation.

In 1968 Chipman et al. published more detailed information about the molecular mechanism of the hydrolysis[26]. In their study the products formed when lysozyme was allowed to act on oligosaccharides from the *Micrococcus lysodeikticus* capsules were investigated by electrophoretic and paper chromatographic techniques and also by ion-exchange chromatography. The use of a tritium-labelled substrate permitted a simple solution of many aspects of the problem of the molecular mechanism. This study demonstrated convincingly that synthesis takes place mainly via a transglycosylation mechanism. The mechanism of the action of the seed was treated by analogy with a chain process, hexa- and octa-saccharides playing the role of the "chain carrier". In Chipman and Sharon's

† For the meaning of the term "seed" see Section IV, 3 (Ed. of Translation).

view[27], hydrolysis via transglycosylation is the most characteristic feature of catalysis by lysozyme. The study of the action of lysozyme on the glycosides of chitin oligosaccharides also led to this molecular mechanism. In 1968 the same view was adopted by Osawa[28] in a study of the hydrolysis of nitrophenyl glycosides of oligosaccharides and by Tsai et al.[29] in a study of the action of lysozyme on the glycosidic isologues of chitin disaccharides. Khorlin et al.[30] arrived at the same conclusion. A kinetic study of the molecular mechanism has been made[31].

## 2. Other Mechanisms

The molecular mechanism of the hydrolysis via transglycosylation has now been proved by many investigators[19,22-31]. However, many of them failed to take into account the fact in both earlier and later studies that the hydrolysis of chitin substrates does not take place directly[7-10,13,14]. The formation of transglycosylation products has been noted recently as a rule. However, the transglycosylation is regarded as a side reaction; assuming that hydrolysis and transglycosylation take place in parallel, Dahlquist and Raftery[32] reported in 1967 that, when lysozyme acts on tri-*N*-acetylchitotriose, a disaccharide and a monosaccharide are formed in molar proportions of 2 : 1 instead of 1 : 1 expected for direct hydrolysis. They suggest that part of the *N*-acetyl-D-glucosamine formed combines with the initial trisaccharide to produce a tetrasaccharide, which they detected among the reaction products. A similar mechanism was also put forward by Raftery and Rand-Mair[33], who believe that the formation of the β-methyl glycoside of *N*-acetyl-D-glycosamine, which they observed, is a result of a direct transfer of the acetylglycosamine residue from di-*N*-acetylchitobiose (the donor in the transglycosylation reaction) to methanol (the acceptor).

Rupley and coworkers also failed to take into account the necessity for the transglycosylation stage in the hydrolysis of short oligosaccharides[13,15,34]. Rupley and Gates considered a molecular mechanism according to which a productive complex of lysozyme and tri-*N*-acetylchitotriose is formed with an enzyme : substrate molar ratio of 1 : 2.[34] According to the authors, direct hydrolysis of one of the adsorbed trisaccharide molecules is then possible. Sharon and coworkers[26] regard such a mechanism as unlikely if only because two trisaccharide molecules contain one oxygen atom more than the hexasaccharide located in the active centre of lysozyme. Direct hydrolysis of tri-*N*-acetylchitotriose is also postulated in later investigations of Rupley and coworkers[35,36]. Rupley and Gates[34] attempted to determine the site of the dissociation of the glycosidic linkage in oligosaccharides in hydrolysis and transglycosylation assuming that the two processes occur in parallel. The reaction was performed with 0.001—0.05 M oligosaccharide and 0.1—0.8 M *N*-acetyl-D-glucosamine. In view of a large excess of *N*-acetyl-D-glucosamine (up to 1000-fold) the reaction must be restricted in this instance to transfer to the monomer. Some results of this investigation are presented in Table 1.

Table 1 shows that the rate of transglycosylation increases so much that the difference in rate for the dimer and hexamer is as much as 7 orders of magnitude. If one takes into account further the fact that, among the products of the reaction of the disaccharide with the monosaccharide in the presence of lysozyme, the authors observed at the end of the reaction a considerable amount of tri- and tetra-saccharides, the process in this system should presumably be self-accelerating owing to the increase of the reaction turnover with accumulation of longer oligosaccharides. Therefore, from our point of view, the mechanism of the cleavage of the dimer and trimer (and tetramer according to Sharon's data) presented in Table 1 corresponds to the induction period of the reaction.

**Table 1.** Characteristics of the cleavage of glycosidic bonds in chitin oligosaccharides in the reaction involving transfer to *N*-acetyl-D-glucosamine.

| Oligosaccharide | Relative rate | Reaction order | Type of action |
|---|---|---|---|
| Dimer | 0.003 | 1 | |
| Trimer | 1 | 1 | $X_1\downarrow X_2\downarrow X_3$ |
| Tetramer | 8 | 1 | $X_1\downarrow X_2\downarrow X_3\downarrow X_4$ |
| Pentamer | 4000 | 0 | $X_1—X_2—X_3\downarrow X_4\downarrow X_5$ |
| Hexamer | 30000 | 0 | $X_1—X_2—X_3—X_4\downarrow X_5—X_6$ |

In 1967 and 1969 Kravchenko and Kuznetsov published papers[37,38] describing the action of lysozyme on tetra-*N*-acetylchitotetraose. Together with all possible hydrolysis products, they observed the formation of a hexasaccharide the amount of which did not vary as long as the substrate remained, disappearing at the end of the reaction. On the basis of the content of hexasaccharide quoted in the figures and assuming it to be equal to 0.1 of an equivalent for the initial 8 equivalents of the tetrasaccharide, calculation of the ratio of the rates of transglycosylation (using the data of Rupley and Gates in Table 1) shows that the transglycosylation of the hexasaccharide should be 50 times faster, despite its low amount, than that of the tetrasaccharide (which behaves as the donor in this reaction). The authors suggest that the hydrolysis and transglycosylation take place simultaneously and the mechanism of hydrolysis via transglycosylation is not mentioned at all.

However, one should note that in certain cases short oligosaccharides can nevertheless hydrolyse directly. For example, the *p*-nitrophenyl glycosides of *N*-acetyl-*D*-glucosaminyl-D-glucose and *N*-acetyl-*D*-glucosaminyl-D-2-deoxyglucose can probably hydrolyse directly to disaccharides without the intermediate formation of longer oligosaccharides, as shown by appropriate techniques[39].

## IV. THE CRYSTALLOGRAPHIC MODEL AND THE MOLECULAR MECHANISM

Fundamental and detailed data on the structure of enzyme—substrate complexes obtained by X-ray diffraction analysis appeared in 1967,[1] although some information had been published even earlier[40]. In these studies the complexes of lysozyme with various carbohydrates are referred to as enzyme-inhibitor complexes. This term probably arose as a result of the view, current at the time, that disaccharides are not substrates of lysozyme and trisaccharides hydrolyse slowly at concentrations corresponding to saturation (see Blake et al.[1], p. 385).

On the other hand, all these carbohydrates are capable of inhibiting the lysis of *Micrococcus lysodeikticus*. Recently the term "inhibitors", in relation to chitin or bacterial oligosaccharides, is used with decreasing frequency.

## 1. The Structural, Thermodynamic, and Functional Characteristics of the Binding Sites

Studies by English crystallographers have shown that the lysozyme molecule is a close-packed globule, close in shape to an ellipsoid of rotation with dimensions of $30 \times 30 \times 45$ Å. Across the globule, there is a deep cleft, which constitutes the active centre of the enzyme. In the formation of an enzyme–substrate complex, the carbohydrate is accommodated in the cleft; for each monomeric unit, there is a separate subsite—six in all. Consequently, the hexasaccharide molecule is capable of completely covering the cleft. Individual sorption subsites have been designated by the letters *A*, *B*, *C*, *D*, *E*, and *F*, starting with the subsite interacting with the non-reducing end of the hexasaccharide. Complexes with tetra-, penta-, and hexa-saccharides could not be detected by X-ray diffraction. The complex of lysozyme with a trisaccharide proved to be very stable and, moreover, the addition of the trisaccharide to the lysozyme did not affect the structure of the enzyme as a whole. Therefore, knowing the structure of lysozyme without the substrate, it was fairly easy to calculate the structure of this complex with the trisaccharide. It was found that the trisaccharide occupies subsites *A*, *B*, and *C* (*C* corresponds to the reducing end of the trisaccharide). Moreover, the retention of a certain rigidity by the enzyme structure on formation of its complexes with short substrates made it possible to obtain data concerning the structure of this enzyme–substrate complex too, by superimposing molecular models of the hexasaccharide onto the molecular model of lysozyme.

The very fact of the extrapolation of the structure of the enzyme–substrate complex to the *D*, *E*, and *F* subsites and confirmation of the validity of this extrapolation by all subsequent chemical and physicochemical data demonstrates quite clearly that the complementarity in the enzyme structure is not generated under the influence of the substrate but exists independently of the substrate. After the addition of the substrate to the lysozyme molecule, only insignificant structural changes take place. For example, a shift of the Trp 62 residue by 0.75 Å was noted.

Other residues also shift slightly, narrowing somewhat the cleft and giving rise to a closer contact between the enzyme and the substrate. However, an induced correspondence between the enzyme and substrate structures cannot be involved here. These changes in the enzyme structure can be more correctly regarded as an improvement of the existing complementarity in the interaction between lysozyme and the trisaccharide.

On the other hand, the substrate undergoes significant changes. Firstly, it loses the freedom of rotation about the C–O bonds joining the monomeric residues. Its structure becomes as rigid as that of the enzyme itself. Secondly, the rigid coupling of the enzyme to the substrate (hexasaccharide) forces the latter to alter the "chair" conformation of the pyrazine ring in one of the six subsites (*D*) and to assume a less stable and more strained "half-chair" conformation. This deformation of the structure of the carbohydrate unit at the *D* subsite is of decisive importance in catalysis by lysozyme. Measurement of the constants for the association of lysozyme with chitin and bacterial oligosaccharides by a wide variety of methods led to the determination of the energy of this interaction as the sum of the free energies of the interaction at all six subsites of the lysozyme molecule.

The results of these measurements are summarised in Chipman and Sharon's review[27] in the form of Table 2. The contributions $\Delta\Delta F_a^\circ$ introduced at each subsite separately are listed at the bottom of Table 2.

**Table 2.** Thermodynamic characteristics of the formation of enzyme–substrate complexes and of individual sorption subsites[27].*

| Sorption subsite | | | | | | | $\Delta F_a^0$, kcal mole$^{-1}$ |
|---|---|---|---|---|---|---|---|
| X | A | B | C | D | E | F | |
| | | | NAG | | | | −4.5(±0.3 |
| | | NAG | — NAG | | | | −7.4 |
| | | NAM | — NAG | | | | −7.9 |
| | NAG | — NAG | — NAG | | | | −9.2 |
| | NAG | — NAM | — NAG | | | | −9.8 |
| | NAG | — NAM | | | | | ⩾−4.2 |
| | NAG | — NAM | — NAG | — NAM | | | ⩾−6.9 |
| | NAG | — NAM | — NAG | — NAM | —NAG | — NAM | −8.6 |
| NAG | — NAG | — NAG | — NAG | | | | −9.2 |
| $\Delta\Delta F_a^0$, kcal mole$^{-1}$ | 0 | −1.8 | −2.9 ⩽−5.7 | | +2.9 | −1.7 | |
| | | | −3.4 | | | (E+F) | |

*NAG = *N*-acetyl-D-glucosamine; NAM = *N*-acetylmuramic acid.

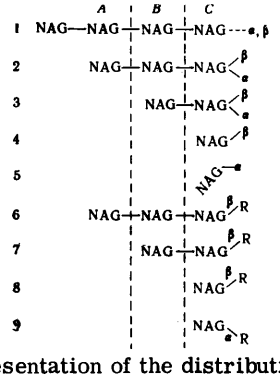
Table 2 shows that these contributions differ so significantly that they must be considered separately. The only subsite where there is a loss of energy is the *D* subsite, which can be readily explained by a change in the conformation of the carbohydrate residue at this site. On the other hand, the adjoining site is distinguished by the highest free energy gain, and hence the strongest binding of the substrate. According to data based on the crystallographic model, the sorbed *N*-acetyl-D-glucosamine is placed, as it were, in a hydrophobic "pocket".[41] In their paper dealing with the characteristics of the lysozyme complexes with various carbohydrates and related compounds, Rupley and coworkers[42] compared the free energies of formation of the lysozyme complexes with *N*-acetyl-D-glucosamine and *N*-methyl; *N*-hydroxyethyl-, and *N*-cyclohexyl-acetamides. They were found to be respectively −4.7, −2.4, −2.8 and, −3.1 kcal mole$^{-1}$. These and other data[42] show that the hydrophobic interaction of the acetamide group of the substrate makes a large contribution to the energy of formation of the complex. Thus a substrate with an *N*-acetamide group should always be sorbed in such a way that the *C* subsite is the first to be occupied.

It is readily seen that short chitin oligosaccharides (the dimer, trimer, and tetramer) should occupy subsites only to the left of *C* in Table 2. Phenomenologically this is shown by the finding that the affinity constant increases on passing from the monomer to the dimer and to the trimer, with very little change on further increase in the length of the substrate. The first quantitative data on this question were published by Dahlquist et al.[43], who obtained the following values for mono-, di-, tri-, tetra-, penta-, and hexa-saccharides: $(4-6)\times10^{-2}$, $1.75\times10^{-4}$, $6.58\times10^{-6}$, $9.45\times10^{-5}$, $9.35\times10^{-6}$, and $6.15\times10^{-6}$. The last two constants are only approximate, since even

before the attainment of equilibrium the substrates were partly hydrolysed. The data for the association constants of enzyme–substrate complexes obtained by different methods are presented in Chipman and Sharon's review[27]. The structures of the lysozyme complexes with chitin oligosaccharides and their glycosides have been investigated by NMR, which established the positions of all the residues[44].

In order to determine the positions of the remaining monomer units of the substrate in the active centre of lysozyme, measurements were made of the chemical shifts of the $CH_3$ protons of the acetamide groups of the chitin oligosaccharides in a free form and in their complexes with lysozyme. On formation of enzyme–substrate complexes, a shift of NMR signals towards higher fields and their broadening were observed. The acetamide groups of the chitin oligosaccharides acquire different magnetic environments on formation of the complex. This makes it possible to characterise each monosaccharide residue of the oligomer in terms of the corresponding resonance. The rule of the distribution of monomer units in the active centre of lysozyme was thereby confirmed. The *C* subsite is the first to be occupied and the remaining sites come into contact with the substrate in the following sequence: *B* first and then *A*. As shown above, this accommodation rule is fully consistent with thermodynamic characteristics. It is interesting that even the tetrasaccharide obeys this preferential accommodation rule. One of its residues is outside the region of the active centre and this disposition is more favourable, since accommodation at the *A*, *B*, *C*, and *D* subsites must lead to a loss of energy of 2.9 kcal $mole^{-1}$.

The situation involving bacterial oligosaccharides is somewhat more complex. According to X-ray diffraction data, the *N*-acetylmuramic acid (NAM) residue cannot be accommodated at the *A* and *C* subsites owing to steric hindrance[1]. For this reason, it is most probable that the NAG–NAM disaccharide is located at the *A* and *B* subsites, while the *C* subsite remains free.




Schematic representation of the distribution of *N*-acetyl-D-glucosamine (NAG) residues of oligosaccharides among the *A*, *B*, and *C* subsites of lysozyme[44].

As already mentioned, the last two subsites, *E* and *F*, were obtained by the extrapolation of X-ray diffraction data. They have been investigated experimentally much less satisfactorily. According to Sharon and coworkers[27,45], the long-lived glycosyl derivatives obtained by the elimination of the "aglycone" are located at the *A*, *B*, *C*, and *D* subsites, while the *E* and *F* subsites contain the acceptor molecules after the elimination of the aglycone residues. The formation of a lysozyme–acceptor complex cannot be recorded directly. However, this was achieved by a quantitative study of the products of the transglycosylation reaction[45]. Using as acceptors a mixture of two substances simultaneously, the authors inferred the proportions of the transglycosylation products formed from the relative rates of their formation. Comparison of two acceptors, glucose and *N*-acetyl-D-glucosamine, showed that hydrophobic interactions in the formation of the enzyme–substrate complex play an active role at the *E* subsite. Their contribution to $\Delta F$ can be estimated as $-0.9$ kcal $mole^{-1}$. It was discovered similarly that these interactions do not occur at the *F* subsite. Thus it was possible to estimate the overall contribution of the *E* and *F* subsites to $\Delta F$, which is $-1.7$ kcal $mole^{-1}$ (Table 2). Evidently it is comparatively small and therefore, in order to drive the process towards transglycosylation, it is necessary that the acceptor concentration should be significantly higher than the donor concentration. The acceptor subsite of lysozyme as well as the donor subsite apparently do not undergo far-reaching changes on formation of the enzyme–substrate complex. Sharon and coworkers[46] tested 40 different monosaccharides and disaccharides as acceptors and showed that only those substances whose geometry corresponds to the surface of the *E* subsite may function as acceptors: only 29 acceptors were found. The remaining 11 substances are not complementary in relation to the *E* subsite and do not react at all. It is most interesting that a geometrical correspondence can be achieved by accommodating the acceptor in different ways. For this reason not only hexoses but also pentoses and even carbohydrates of the L-series can function as acceptors and the glycosidic linkages formed are not only of the $\beta$-(1–4) type but also of the $\beta$-(1–2)- and $\beta$-(1–3)- types. The reactivity of the acceptors could be predicted on the basis of data for the structure of the *E* subsite. For example, D-xylose can be accommodated at the *E* subsite in three ways, which correspond to the formation of products with 1–2, 1–3, and 1–4 glycosidic linkages[49].

## 2. Productive and Non-productive Enzyme–Substrate Complexes

The thermodynamic properties of the sorption subsites in the active centre of lysozyme account for the existence of the so called non-productive complexes. The very name "non-productive" complexes implies that the formation of such enzyme–substrate complexes does not lead to the dissociation or formation of covalent bonds. English crystallographers, who devised models of lysozyme, postulated that the dissociation of the glycosidic linkage in an oligosaccharide can take place only between units at the *D* and *E* subsites[1]. Further experimental studies confirmed this hypothesis and it is now generally accepted[27]. As shown above, the sorption of di-, tri- and tetra-saccharides take place preferentially at the *A*, *B*, and *C* subsites, i.e. outside the region of glycosidic linkages. Consequently all these complexes, the preferential formation of which is observed at substrate concentrations corresponding to saturation, are non-productive. For this reason, they were readily detected by X-ray diffraction analysis and a wide variety of physicochemical methods[27].

In order to form a productive complex, it is necessary to overcome the energy barrier associated with sorption

at the *D* subsite. It is readily seen that the barrier for oligosaccharides produced by the fragmentation of chitin can be overcome only as a result of the simultaneous coverage of almost all the sorption subsites in the lysozyme active centre. Productive complexes with such oligosaccharides can be obtained only with the hexasaccharide or possibly the pentasaccharide. This conclusion is proved, in particular, by the application of a competitive inhibitor such as the dye Biebrich scarlet[36], which is capable of being specifically sorbed in the region of the active centre. The affinity constants for the productive and non-productive complexes of tri-*N*-acetylchitotriose with lysozyme were determined with the aid of this inhibitor. They are respectively $1.0 \times 10^{-2}$ and $1.5 \times 10^{-5}$ mole litre$^{-1}$. The number of non-productive complexes formed is greater by almost 3 powers of ten than that of the productive complexes. The affinity constant for the productive complex of the hexasaccharide was estimated in this investigation as $5 \times 10^{-6}$ mole litre$^{-1}$, which is very close to the affinity constant for the non-productive complex of the substrate.

Cases have been described where short oligosaccharides can form preferentially productive complexes. For example, *p*-nitrophenyl-4-O-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-β-D-glucopyranoside can be slowly hydrolysed by lysozyme without complication by the transglycosylation reaction[39]. The carbohydrate residues of this disaccharide occupy the *C* and *D* subsites and the nitrophenyl glycosidic linkage is in the cleavage zone, which makes possible direct hydrolysis without transglycosylation. A similar result was obtained for trisaccharides with D-glucose, D-xylose, and D-galactose residues at the reducing end, the last being hydrolysed much faster than all others[47]. In this case too only a productive complex of the *CDE* type (*C* = NAG, *D* = NAG, and *E* = galactose) is probably formed.

### 3. Interpretation of the Molecular Mechanism

Without assuming the molecular mechanism of hydrolysis via transglycosylation, it is impossible to combine the data for the structural and thermodynamic characteristics of the sorption subsites of lysozyme. These characteristics and also the hypotheses concerning the productive and non-productive complexes of lysozyme with substrates which follow from them show that in the course of the enzymatic reaction a hexasaccharide or a longer oligosaccharide functions continuously on the surface of the active centre. It is either lengthened as a result of transglycosylation or shortened as a result of hydrolysis and the cyclic process ultimately results in the hydrolysis of the short oligosaccharide involved in the reaction in the transglycosylation stage as an acceptor. The presence of a small amount of a long oligosaccharide is necessary for the effective hydrolysis of short oligosaccharides, the long oligosaccharides behaving as the "seed" in the reaction.

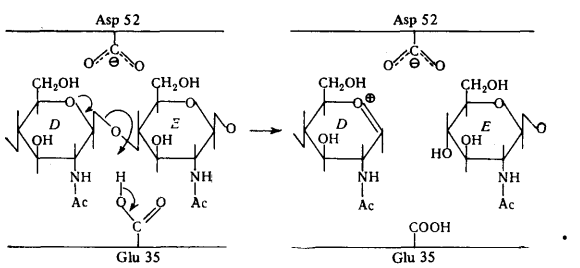
The degree of hydrolysis of the oligosaccharide depends on its structure. Chitin trisaccharides and tetrasaccharides are almost completely cleaved to the monomer[19], while the bacterial trisaccharide is cleaved only to the dimer[26]. This difference can be explained by the fact that the NAM residues in bacterial oligosaccharides cannot be accommodated at the *A*, *C*, and *F* subsites. Moreoever, the inhibiting action of the reaction product on the enzyme is more marked for a disaccharide than for a monosaccharide, since the former is sorbed more strongly.

## V. THE ELECTRONIC MECHANISM

### 1. X-Ray Diffraction Data

Analysis of the structure of lysozyme complexes with substrates also makes it possible to put forward a hypothesis concerning the electronic-conformational principles of catalysis in this specific case. Investigators began with the assumption that the lysozyme complex with tri-*N*-acetylchitotriose (at the *A*, *B*, and *C* subsites), which they detected, is unreactive or non-productive. The complex which is undetectable by this method must be reactive. The most probable site of the dissociation of glycosidic linkages is between the *D* and *E* subsites. The only ionogenic groups located within the cleft and capable of participating in proton transfer are two carboxy-groups (the β-COOH group of Asp 52 and the γ-COOH group of Glu 35), which are located on either side of the β-(1—4)-glycosidic linkage of the substrate. The Glu 35 residue is located preferentially in the non-polar region near the bottom of the cleft, which must hinder the dissociation of the COOH group. Indeed, the p$K_a$ values for these groups are about 6 while the usual value is 4.5—4.6. The Asp 52 residue is in the polar region and is linked by hydrogen bonds to amide groups which behave as proton acceptors. This facilitates the dissociation of the COOH groups of this residue. Their p$K_a$ values are displaced to the acid region compared with the normal value (about 4 and about 4.5 respectively). Thus, by virtue of the rigid fixation of these functional groups and the formation of a stable microenvironment around each, they are ionised to different extents.

A second feature of the conformational-electronic mechanism of the action of lysozyme is the deformation of the glucopyranose ring at the *D* subsite. The stereochemical characteristics of the *D* subsites are such that the carbohydrate residue assumes the half-chair conformation. This is associated with energy consumption (see above), which is compensated by the binding energy at other sorption subsites. However, such deformation promotes the heterolysis of the glycosidic linkage, since it corresponds to a stable conformation of the carbonium ion. The role of the carboxy-groups in this process is that one of them (Glu 35) initiates the heterolysis of the glycosidic linkage, while the other (Asp 52) stabilises the intermediate cation. The mechanism of this process can be represented as follows:




The free carbohydrate residues are desorbed from the *E* and *F* subsites and the reaction is subsequently completed either by the addition of water (hydrolysis) or by sorption of other oligosaccharides at the *E* and *F* sites and the formation of a new glycosidic linkage (transglycosylation). Thus general acid catalysis obtains in the mechanism of the action of lysozyme.

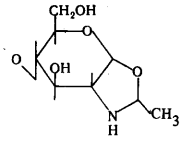
## 2. Chemical and Physicochemical Studies

Chemical and physicochemical studies should demonstrate the validity of the reaction mechanism put forward on the basis of structural data. Methods involving chemical modification of lysozyme established that the only groups involved directly in catalysis are the carboxy-groups[48,49]. The $\epsilon$-$NH_2$ groups of the lysine residues, the terminal $NH_2$ group of lysozyme[43-50], the single histidine residue[50,52,54], the guanidino-groups of arginine[53], and the phenolic hydroxy-groups of tyrosine[50] can be completely blocked chemically without loss of the entire enzyme activity. In certain cases such modification led to a partial decrease of activity and in others to an increase[51,52,54]. The activity was determined as a rule turbidimetrically on the basis of the solvent action of the enzyme on a suspension of *Micrococcus lysodeikticus* bacteria. Naturally the dissolution of the bacterial suspension (lysis) is influenced by too many factors for a direct relation to be established between the quantitative changes in activity and structural factors. However, when it was possible to modify lysozyme at its carboxy-groups, a completely inactive derivative was obtained in many cases. The lysozyme molecule contains only 11 carboxy-groups (two glutamic acid residues, 8 aspartic acid residues and the *C*-terminal group). When the carbodi-imide method was used, it was possible to join to the carboxy-groups amino-derivatives such as $H_2NCH_2SO_3H$, $H_2NCH_2COOCH_3$, and $H_2NCH_2CONH_2$. One of the COOH groups of the active centre (Glu 35) cannot be amidated, while the other group (Asp 52) can be blocked, the substrate (trisaccharide) hindering the reaction[55,56]. The complete inactivation of the enzyme on blocking Asp 52 and the effect of the substrate on the reaction showed that this group is of critical importance for the activity. Similar results were obtained also on alkylation of lysozyme using triethyloxonium tetrafluoroborate as the alkylating agent[50,57]. This yielded a mixture of products, which was resolved by ion-exchange chromatography. Two monoalkylated lysozyme derivatives were isolated in a pure form, one of which was inactive but could be reactivated spontaneously at pH 2.0.[50] It was shown that this lysozyme derivative contains an alkylated COOH group of the Asp 52 residue[57]. The direct catalytic action of the carboxy-group of Glu 35 and Asp 52 is confirmed also by the finding that the blocking of all other carboxy-groups does not lead to complete loss of activity by lysozyme[55,56].

Both groups have unusual chemical properties. The Glu 35 residue is chemically inert although Lin and Koshland[56] claim that this group is sterically accessible for reaction. Ethyl-lysozyme (ethylated at Asp 52) also has unusual chemical properties. The ester linkage in this compound is incapable of involvement in nucleophilic substitution, is resistant to alkaline hydrolysis and the action of hydrazine and hydroxylamine, and is not reduced by $LiAlH_4$.[57]

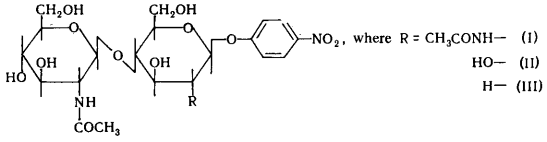
The mechanism of the chemical (non-enzymatic) hydrolysis of glycosides has been described in a review[58]. Therefore, it was natural to suppose that the carboxy-group of Glu 35 exhibits a catalytic activity via the mechanism of general acid catalysis. It protonates the glycosidic oxygen, which leads to the dissociation of the glycosidic linkage and the formation of a carbonium ion. As shown in the formula illustrated above, the dissociation of the glycosidic bridge at the $C_1$—O bond was demonstrated using water with the oxygen-18 isotope.

Raftery and Rand-Mair examined five probable electronic mechanisms of the cleavage of glycosidic linkages[33], two of which led to the formation of $\alpha$-glycosidic linkages. These two mechanisms were rejected, since the authors showed that only the $\beta$-methyl glycoside of *N*-acetyl-D-glucosamine can be detected among the products of the reaction of di-*N*-acetylchitobiose with methanol. If the $\alpha$-glycoside is in fact formed, its amount cannot exceed 0.3% in relation to the total content of methyl glycosides[59]. Consequently the reaction takes place with retention of the configuration at $C_1$ atom. Such a reaction could take place via Koshland's double nucleophilic substitution mechanism[60]. Moreover, there is a possibility of a mechanism involving the acetamide group (anchimeric cooperation) via a stage in which an oxazoline derivative is formed[61].



In experiments with model reactions, it was shown that the involvement of the acetamide group tends to accelerate the hydrolysis of the glycosidic linkage by a factor of 1000.[62] Finally, there is a possibility of a mechanism of the cleavage of the glycosidic linkage via a stage involving the formation of a carbonium ion, but with retention of the configuration at the $C_{(1)}$ atom owing to the oriented addition to the carbonium ion of the appropriate nucleophilic agent (water in hydrolysis or another acceptor in transfer).

For a final solution of this problem by a kinetic method, it is important to choose the substrate correctly, since the reaction investigated must proceed via a unique pathway. For this purpose, three substances of the type




were synthesised and it was shown that, when lysozyme acts on substance (I), a mixture of different carbohydrates is formed, while only the corresponding disaccharides could be isolated chromatographically from substances (II) and (III).[39] The association constant of the complex of lysozyme with (II) was determined by NMR ($K_S$ = $1.5 \pm 0.3 \times 10^{-2}$ mole litre$^{-1}$) and it was found to agree satisfactorily with the Michaelis constant ($K_m$ = $2.3 \pm 0.8 \times 10^{-2}$ mole litre$^{-1}$). Bearing in mind that the NAG residue in (III) is very strongly sorbed on the *C* subsite, one must conclude that (III) forms only a productive complex with lysozyme. This substrate was found to be most suitable for kinetic studies. The method of the secondary kinetic isotope effect ($\alpha$-deuterium) was used for the solution of this problem[63]. Table 3 presents the characteristics of the secondary isotope effect for the hydrolysis of compound (III) under different conditions. Typical $S_N2$ reactions have rate constant ratios $k_H/k_D$ of about unity (hydrolysis by sodium methoxide) and $S_N1$ reactions have a ratio of about 1.14 (hydrolysis by hydrochloric acid). Over a wide pH range, lysozyme has an isotope effect which corresponds to a considerable extent to an $S_N1$ reaction mechanism. For comparison, the authors investigated the mechanism of the hydrolysis of compound (III) catalysed by almond $\beta$-glucosidase and found that the

mechanism of the action of this enzyme is fundamentally different.

Lowe and coworkers[61,64,65] showed in a number of studies that the hydrolysis of oligosaccharides catalysed by lysozyme proceeds via the formation of an oxazoline derivative of the carbohydrate. They found that the kinetics of the hydrolysis of the *p*- and *o*- nitrophenyl glycosides of di-*N*-acetylchitobiose obey the Michaelis–Menten equation. However, despite the extensive investigations which they carried out, their conclusions concerning the involvement of the acetamide group in the catalytic reaction cannot be regarded as reliable, since in this instance the Michaelis–Menten equation was obeyed in a purely formal manner and lacked the physical significance usually attributed to it. In the study quoted above[63], it was shown that, when lysozyme acts on compound (I), a mixture of products is formed and that this substrate is unsuitable for kinetic studies on the electronic mechanism: therefore, the conclusions of Lowe and coworkers are groundless. The digestion of such substrate proceeds via a hydrolytic mechanism involving transglycosylation, as shown for nitrophenyl glycosides by Osawa[28]. The mechanism via the oxazoline derivative is also criticised in Chipman and Sharon's review[27].

**Table 3.**

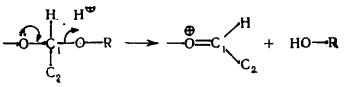
| Conditions of hydrolysis | $k_H/k_D$ |
|---|---|
| 2 *N* HCl, 50° | 1.13 |
| 3 *N* $NaOCH_3$, 70° | 1.03 |
| β-Glucosidase | 1.015 |
| Lysozyme | — |
| pH 5.5 | 1.11 |
| pH 3.1 | 1.11 |
| pH 8.3 | 1.11 |

### 3. Theory of Chemical Conjugation as One of the Preconditions for the Electronic Mechanism of the Action of Lysozyme

A characteristic feature of lysozyme catalysis discussed above is the obligatory action of the enzyme on the structure of the glucopyranose ring at the *D* subsite. Usually this is regarded as confirmation of the earlier hypotheses of the deformation and strain of the bonds undergoing dissociation on the surface of the active centre of the enzyme[1,27,58]. However, it is important to emphasise also the problem of the role of the conjugation of chemical bonds in biocatalysis. It is thereby possible to establish a relation between the classical concepts of organic chemistry and the concepts underlying the mechanism of biocatalysis.

The theory of bond conjugation and the effect of conjugation on reactivity were developed in organic chemistry a long time ago. One of the principal postulates concerning the conjugation of single bonds, which were developed by Nesmeyanov[68], is that specific stereochemical requirements must be met. The large class of organic reactions which occur as a result of the conjugation of single bonds, has been called "fragmentation reactions", which were studied on a wide scale both theoretically and experimentally by Grob and coworkers[68]. We have already mentioned that Grob's theory[67] of the fragmentation of organic substances is consistent in its main postulates with Nesmeyanov's theory of the conjugation of single bonds[66]. The differences between these theories concern mainly the terminology. Grob uses terms such as "stereoelectronic factor" and "frangomeric acceleration",[68,69] which reflect essentially the influence of molecular conformation on the reaction rate. It has also been suggested that the above frangomeric acceleration may be one of the factors in biocatalysis (Kossower[70]).

In the present author's studies on the fragmentation of α-aminoacids with a tertiary nitrogen atom, it was concluded that, by virtue of steric hindrance, the α-aminoacid molecule can assume a fixed conformation which is the most favourable for the conjugation of single bonds[71,72]. In this case the rotation in the aminoacid molecule about the N–C bond is hindered and the conformation is fixed in consequence of steric hindrance due to the bulky substituent at the nitrogen atom. The conformation of the substrate molecule is also fixed on formation of an enzyme–substrate complex. One cannot conclude that the fixation of the conformation is favourable for *p*, σ conjugation of the following type:



As a result of this conjugation, the transition state exists partly in the oxonium form with a double bond. The present author called this factor in biocatalysis "inclusion of conjugation" or the "steric-orientation factor".[73]

The molecular and electronic mechanisms of the action of lysozyme are interrelated. In order that the energy of the "chair–half-chair" transformation should be compensated by the energy of the formation of the enzyme–substrate complex, the involvement of six (or five) sorption centres is essential.

## VI. CERTAIN UNSOLVED PROBLEMS

The study of the mechanism of the action of lysozyme constitutes an example of a combined investigation of a large research problem. For many reasons, lysozyme has proved convenient for study by X-ray diffraction. However, at the time of the completion of this review there were very few chemical and physicochemical data concerning the mechanism of its action on a substrate. Substrates with specific chemical characteristics became known only in 1960. The published X-ray diffraction data (1965–1967) on the structure of lysozyme and its complexes with substrates stimulated chemical and physicochemical research. In the course of approximately 5–6 years (1964–1969), almost all the details of the mechanism of the reaction of lysozyme were elucidated in a number of laboratories in the USSR, USA, England, Japan, France and other countries. The complete agreement between the chemical and physicochemical data on the one hand and the X-ray diffraction data on the other suggests that the mechanism of the action of lysozyme has been largely established correctly and fully.

However, there are still certain problems which require further investigation. These are related, in particular, to the nature of the structural changes in lysozyme in the pH range 6–7, which are reflected in its activity. The dissociation constants of both the imidazole group, the single histidine residue which is not involved directly in catalysis, and of the anomalous carboxy-group of Glu 35, which plays a key role in catalysis, have p*K*

values corresponding to the pH range. The dissociation of the carboxy-group, which occurs on increase of pH to 6—7 and above, is probably accompanied by changes in the details of the enzyme structure which increase the tendency towards aggregation of both native[74,75] and irradiated[76,77] protein in this pH range. It has been shown by NMR that the dimerisation of native lysozyme in solution takes place via the surface of the enzyme adjoining the region of the active centre[78]. The nature of the interaction of lysozyme with chitin is also obscure.

A detailed study showed in the first place that this interaction takes place as a result of a specific enzyme—substrate affinity. In the second place it was established that there exist two complexes between lysozyme and chitin, differing in a number of properties, particularly in the fact that one is formed preferentially at a pH below 6—7 and the other is formed under more basic conditions[79-82]. Since no other methods (differential ultraviolet spectroscopy, fluorescence, NMR spectroscopy, etc.) indicated the existence of two different lysozyme complexes with water-soluble substrates, the data obtained for the interaction of lysozyme with water-insoluble chitin can be interpreted as due to a characteristic feature of chitin itself, since, in contrast to low-molecular-weight and water-soluble substrates, chitin sorbs lysozyme molecules not only via an enzyme—substrate interaction but also as a result of forces acting on the interface between the solid (chitin) and liquid (solution) phases.

The study of the catalytic activity at high pH is also by no means complete. When lysozyme was allowed to act on *Micrococcus lysodeikticus* bacteria, it was noted that the dependence of the activity on the pH has a second maximum at pH 9.2.[83-85] Since it is unlikely that the carboxy-group of Glu 35 is protonated in this pH range, it was suggested that under these conditions general acid catalysis is not due to Glu 35 but to the $\epsilon$-amino-group of Lys 97 located at the outlet from the cleft[84]. Later it was concluded[85] that the sorbed substrate (bacterial cell) affects the dissociation of Glu 35 in such a way that, owing to electrostatic interaction, it remains in an undissociated form even at pH 9.2. According to these data, tetra-*N*-acetylchitotetraose (an uncharged substrate) is not hydrolysed by lysozyme at pH in excess of 8.[85]

It has also been observed[86] that the pH optima for the hydrolase and transferase activities of lysozyme do not coincide: hydrolysis takes place preferentially at a lower pH and the synthetic activity is shown to a greater extent at an elevated pH (particularly at pH 8—9). The change in the type of catalytic activity of lysozyme on altering the pH may be attributed to the dissociation of the carboxy-group of Glu 35 and a correspondong change in the state of aggregation of the enzyme. The present author suggested[86] that the hydrolysis is due to the monomeric lysozyme molecule and transglycosylation is due to the dimeric molecule. One must emphasise that, over the entire region of the active centre, the *E* and *F* subsites have been most conclusively investigated; as stated above, the role of acceptor subsites is attributed to them.

However, the interrelations between the sorption of the substrate, catalysis, and the degree of aggregation of the enzyme, proved to be more complex than might have been expected. Many data show that the degree of activation of native[87-90] and irradiated[91,92] lysozyme is reduced in the presence of a substrate. Moreover, an impression has been created that the inhibiting action of the substrate on the dimerisation of lysozyme is shown even after its complete elimination. If this is so, the substrate induces structural changes in lysozyme, which persist for a long time[93]. In this case, one may be dealing with the so called subtle conformational changes. One should probably include among them those induced by the substrate at the opposite end of the enzyme molecule, which have been detected by the paramagnetic probe method[95].

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Lysozyme proved to be the first enzyme the most important details of the mechanism of whose action have been elucidated. It has also become clear that this mechanism cannot be extended to all related enzymes (glycosidases). Therefore, it is important to be able to isolate correctly the most general principles of biocatalysis constituting the basis of the mechanisms of the action of enzymes in general. In order to stay close to lysozyme, we shall consider as an example the hydrolysis of the glucosidic linkage. In order that the hydrolysis should follow the reaction pathway via the carbonium ion, it is necessary that (1) the glucosidic oxygen should be protonated, (2) that appropriate changes should occur in the conformation of the glucopyranose ring, and (3) that the negatively charged ion in the vicinity of the carbonium ion should assume an appropriate orientation. Each of the three conditions enumerated above is achieved with a certain degree of probability. The probability of the simultaneous coincidence of all three conditions is expressed by the products of the above three probabilities. We enumerated only the most important conditions responsible for the catalytic activity of lysozyme. In the general case the number of conditions may be even greater. The probability of the occurrence of a chemical reaction is equal to the product of the probabilities $f_i$ of the occurrence of each condition:

$$f = f_1 \cdot f_2 \cdot f_3 \cdots f_n, \text{ where } f_i < 1 \quad .$$

The greater the number of conditions involved in the mechanism of the process the smaller its overall probability. By altering the external parameters of the medium it is possible to achieve a sharp increase of the individual components of the probabilities $f_i$. For example, the hydrolysis of glycosides by acids implies a sharp increase of the probability of the protonation of the glycosidic oxygen ($f_1$) by collisions with protons (hydronium ions). An increase of temperature increases the probability of the occurrence of the transition state conformation ($f_2$). However, a simultaneous increase of the probabilities of the protonation ($f_1$) and orientation of the negative ion ($f_3$) in chemical systems is virtually unattainable. Only in certain individual cases, known as concerted action mechanisms, is this situation realised in non-enzymatic reactions. The familiar catalytic action of 2-hydroxypyridine on the mutarotation of glucose may serve as an example. This reaction is in fact usually regarded as one of the models of the enzymatic process. The orienting effect of the enzyme, which according to Koshland[96] ensures the mutual approach and appropriate orientation of the reacting sites in the substrate molecules, essentially reduces to an increase of the probability of occurrence of the conditions governing the transition state.

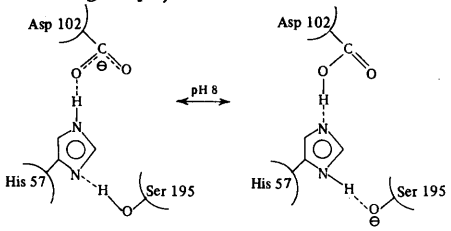
In lysozyme catalysis, the probability of each of the three conditions is very high, owing to the relatively rigid fixation of the "working" functional groups and their rigorous stereochemical correspondence to the substrate. A weak acid such as Glu 35 with $pK_a = 6—7$ protonates the glycosidic oxygen with the same high probability as, for

example, hydrochloric or sulphuric acid. The conformation of the transition state is determined by the stereochemical characteristics of the *D* subsite and its probability is probably very close to unity. The probability of the required orientation of the negative ion (of Asp 52) is just as high. The behaviour, which in a chemical process is a consequence of a random favourable combination of several conditions for the occurrence of the reaction, in an enzymatic process is programmed in the structure of the protein molecule. This in fact ensures a high efficiency of enzymatic catalysis.

The function of the enzyme is to organise the chemical process, drive it along a specific reaction pathway, and ensure a high probability of the necessary reaction conditions. This directing influence of the enzyme is possible only if the enzyme itself has a fairly rigid fixed structure. In low-molecular-weight compounds the rigidity of structure can be achieved mainly via covalent bonds, as, for example, in polycyclic steroid systems. In high-molecular-weight compounds the rigidity of structure is ensured by the compact packing of the polypeptide chain into globules and by non-covalent interactions. X-Ray diffraction analysis has shown that a wide variety of proteins have a close-packed globular structure with rigidly fixed positions of the polypeptide chains[97]. Therefore, when one speaks of the induced correspondence between the enzyme and the substrate, widely known as Koshland's hypothesis, such induction must be restricted to very small displacements of the atoms in the enzyme molecule. In lysozyme these shifts do not exceed a distance of 1 Å. Such slight structural changes in the protein can hardly serve as an adequate basis for the assertion of conformational flexibility, particularly since deformations of valence angles and distances are usually involved and not conformational changes.

The high molecular weight of the enzyme and its compact and rigid globular structure ensure the fixation of the individual functional groups, which leads to the appearance of new properties and phenomena unknown in organic chemistry. As a result of their fixed position, these groups remain in an almost unchanged microenvironment and their properties depend sharply on each specific microenvironment. In organic chemistry the functional group of any molecule is subject to the influence of a constantly varying microenvironment and the properties observed experimentally are as a rule macroproperties averaged with respect to the properties of the numerous microenvironments of the given group. Moreover, as a result of the fixation of the functional group in the rigid globule of the enzyme, there is also a possibility of the formation of structures which are unusual for organic chemistry. The above ideas can be illustrated by a number of examples.

The Asp 52 residue in the lysozyme molecule is fixed in a zone of enhanced nucleophilicity. It can be readily esterified by triethyloxonium tetrafluoroborate, but the resulting ester does not enter into nucleophilic substitution reactions (see above). The serine hydroxyl, which enters into the active centre of chymotrypsin, has unusual chemical properties. In this instance the enzyme exhibits a type of conjugation of chemical bonds which is unknown in organic chemistry. It would probably be correct to call it transannular conjugation, since it resembles most closely the transannular interaction in macrocyclic compounds. As a result of the rigid fixation of the functional groups in chymotrypsin, the geometrical disposition of Asp 102, His 57, and Ser 195 ensures chemical conjugation of their functional groups, as illustrated below:




The His 57 and Asp 102 residues are immersed in the globule and are not in contact with the solution. The Ser 195 residue projects onto the surface. As a result of this conjugation, the usual aliphatic hydroxy-group is capable of dissociation at pH 8, i.e. as regards this property it resembles the hydroxy-group of nitrophenol. As in catalysis, the probability of transannular conjugation in a mixture of the above three aminoacids is negligibly small, whereas in a protein it occurs with a probability of unity as a result of steric proximity.

The chemistry of proteins provides in addition examples of unusual structural formations. For instance, in lysozyme crystals one of the contacts between neighbouring enzyme molecules has a structure resembling the sandwich structures familiar in the chemistry of organometallic compounds. This structure is formed with participation of the benzene ring of phenylalanine in one lysozyme molecule and the phenolic ring of the tyrosine residue in another molecule. The guanidino-group of arginine in the form of a cation linked to a $Cl^-$ anion is located between the parallel rings. Such a structure is stabilised by additional hydrogen bonds.

Biocatalysis is merely a special manifestation of the characteristics of the chemistry of proteins arising by virtue of their organised and almost time-invariant molecular structure.

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