Mechanism of Carbohydrase Action

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The nature of the intermediate complex that is formed between enzyme and substrate during the action of hydrolytic enzymes is one of the oldest problems in biochemistry and one that is still far from being solved. Koshland (*1*) has pointed out the probability that there is more than one type of reaction mechanism and intermediate complex involved and has reviewed the criteria for distinguishing various types of interaction.

Although Koshland and other reviewers have drawn many of their examples from the carbohydrases, it can nevertheless be said that there is less agreement on the reaction mechanisms involved in the action of these enzymes than there is on the reaction mechanisms for esterases, phosphatases, and proteases. This is possibly because the immense variety of readily accessible carbohydrases has precluded the intensive study of more than a handful—those that are most interesting biochemically. It is the purpose of this article (*2*) to correlate some observations (*3*, *4*) on the β-glucoside-splitting enzymes of the mold *Stachybotrys atra* with current theories of enzyme action and to show that these observations can be fitted into a conceptual scheme that will cover most of our present knowledge of the carbohydrases.

It is usual to account for the observed facts (*1*) (competing hydrolase and transferase actions, retention of configuration, rupture of the glycosyl-O bond rather than the O-aglycone bond) about the simpler carbohydrases by some such two-stage reaction scheme (*5*, *6*) as

$$E\text{O-H} + R\text{O-glycosyl} \rightarrow E\text{O-glycosyl} + R\text{O-H} \quad (1)$$

$$E\text{O-glycosyl} + R'\text{O-H} \rightarrow E\text{O-H} + R'\text{O-glycosyl} \quad (2)$$

where E represents the enzyme molecule and R represents the aglycone, and where the enzyme is acting as a hydrolase when $R' = \text{H}$, otherwise as a transferase.

Morton (*7*), in discussing a similar scheme for phosphatases, has pointed out that the binding of R'O-H at the second —"water"—site is probably quite as specific as the binding of the substrate at the site involved in the first stage of the reaction, and he has postulated that the enzymic reaction may be blocked by the binding of substances at the water site which are unable to act as acceptors. This type of inhibition—anticompetitive inhibition, where the inhibitor prevents the dissociation of the enzyme-substrate complex—has recently been discussed with examples by Dodgson, Spencer, and Williams (*8*) and may well be more common than they believe. Anticompetitive inhibition will give straight lines in a Lineweaver-Burk plot (reciprocal of velocity plotted against reciprocal of substrate concentration) which are parallel for the inhibited and uninhibited reactions (*9*). Although, as Ogston has pointed out (*10*), it is impossible to argue unequivocally from enzyme kinetics to any single type of enzyme mechanism—for example, the parallel lines in the Lineweaver-Burk plot do not necessarily imply anticompetitive inhibition—nonetheless, the probability of such a mechanism may be used along with other lines of evidence to frame a working hypothesis.

Beta-glucosidase of Stachybotrys atra

A study of the breakdown of *p*-nitrophenyl-β-glucoside by the β-glucosidase of *Stachybotrys atra,* in which the kinetics were followed by measuring the liberated *p*-nitrophenol, showed that polyhydroxylic organic molecules inhibiting or activating the enzymic reaction could be divided into three classes: (i) typical competitive inhibitors; (ii) substances for which the Lineweaver-Burk plot of the inhibited (or activated) reaction gave a straight line parallel to that for the control reaction and above (or below) it, and (iii) substances showing a behavior intermediate between the behavior of classes i and ii.

When degree of inhibition was plotted against the concentration of inhibitor for inhibitors of the second type, a sigmoid curve was obtained identical in type with the typical dissociation curve that relates reaction velocity and substrate concentration.

The interpretation of these results which is adopted in this article (an enlargement of the point of view presented in an earlier paper, *3*) is that inhibitors of the second type are bound reversibly at the acceptor center without being able to act as acceptors. Furthermore, since the reaction actually observed to be activated or partially inhibited is the liberation of *p*-nitrophenol, it follows that reaction 1 cannot proceed unless a suitable acceptor to complete reaction 2 is also bound to the enzyme.

The simplest way of explaining this observation is to invoke the "double displacement" mechanism of Koshland (*1*), which supposes that substrate, active center, and acceptor are bound into a single complex that does not break up until group transfer from substrate to enzyme to acceptor is complete. To establish this firmly, it will be necessary to check that the β-glucosidase of *Stachybotrys atra* ruptures the bond between the glucosyl carbon and the bridge oxygen with final retention of configuration in the way that has already been demonstrated for another β-glucosidase (*11*).

Activators of the second type may be interpreted as being bound with high affinity at the acceptor site and as acting as more efficient acceptors than water. This has now been checked (*12*) by observing the molar ratio of the reducing sugar and the *p*-nitrophenol liberated during hydrolysis of *p*-nitrophenyl-β-glucoside by the β-glucosidase of *Stachybotrys atra.* In control experiments, this ratio is unity, and it has been shown (*3*) that the affinity of the acceptor center for free glucose must be very low; this contrasts with the behavior of the β-glucosidase of *Aspergillus oryzae,* where the ratio of reducing sugar to *p*-nitrophenol is 0.7 to 0.8 at 50 percent hydrolysis, and where oligosaccharides are formed during the hydrolysis (*13*). With the *Stachybotrys atra* enzyme in the presence of 0.1*M* glycerol (type ii activator) the ratio is reduced to 0.5, but in the presence of 0.01*M* phenyl-α-cellobioside (type ii inhibitor) it remains at unity. Thus there is transfer to an activator bound at the acceptor center, but not to an inhibitor. In fact, 1-glyceryl-β-D-glucoside is readily isolated from enzymic digests in which the nitrophenyl glucoside has been hydrolyzed in the presence of glycerol.

Both active centers appear to have some binding power for almost all polyhydroxylic compounds, and the combination of type ii activation and competitive inhibition gives a system in which,

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for a given combination of enzyme and active agent, there is activation at high concentrations of substrate and inhibition at low concentrations of substrate. Similarly, very few weak inhibitors behave as purely competitive inhibitors, and β-glucosides of low affinity for the substrate center show considerable type ii inhibition of *p*-nitrophenyl-β-glucoside breakdown: as might therefore be expected, there is inhibition by excess substrate in the breakdown of all aryl β-glucosides. The elements of specificity at both centers have been worked out, especially at the substrate center where both a structure resembling that of the D-glucose molecule and the possession of an aryl-β-glucoside linkage appear to confer an affinity for the center on the "substrate" molecule that decreases, the further its structure departs from one or both of these feaures. Both must be present before the molecule becomes a true substrate for the enzyme.

These observations are in qualitative agreement with the conclusions of Morita about the normal limit of specificity of a wide variety of glucosidases as he has recently summarized them (*14*). At the acceptor center it seems that both an α-glycosidic linkage and portions of the glucose molecule confer specificity. There is sketchy evidence that specificity at the acceptor center may be directed toward the configuration about carbon atom No. 2 (C-2) of D-glucopyranose in the same way that the specificity at the substrate center is known to be directed to the configuration about C-3 (*3, 15*). L-Arabinose is a fairly good type ii inhibitor and agrees with glucose in the configuration about C-2 (D-arabinose is a weak competitive inhibitor); 2-deoxyglucose (a competitive inhibitor) with no hydroxyl group at C-2 shows no affinity for the acceptor center, nor do other monoses with a reversed configuration. On the other hand, D-glucose itself shows little affinity.

Other Enzymes of Stachybotrys atra

Stachybotrys atra produces at least two other enzymes capable of hydrolyzing the β-glucosidic linkage, both of which seem to be produced adaptively by the mold as a response to growth on cellulose under different circumstances. One, the "cellulase" of Thomas (*4*), splits poly-β-glucose chains at random, reducing cellulose to a mixture of glucose and cellobiose. It does not attack cellobiose even though cellotriose is a substrate, nor does it attack *p*-nitrophenyl-β-cellobioside as the *Irpex* cellulase of Nisizawa (*16*) does. A second, the "cellobiase" of Youatt (*17*), attacks cellobiose and poly-β-glucose chains by removing the terminal nonreducing glucose residues. It is a β-glucosidase but not a β-cellobiosidase, and it will split phenyl-α-cellobioside to glucose and phenyl-α-glucoside. The basic elements of the specificity of the three β-glucosidases of *Stachybotrys atra* at the substrate center for effective enzymic action are fairly clear.

Table 1. Specificity requirements around the β-glucosidic linkage for β-glucosidase, cellobiase, and cellulase.

Enzyme	Specificity requirements around the β-glucosidic linkage	
	Glucosyl moiety	Aglycone moiety
β-Glucosidase	One glucose residue	Aryl group
Cellobiase	One glucose residue	Aryl group, alkyl group, one or more glucose residues
Cellulase	At least one glucose residue	At least one glucose residue

In Table 1 it is clear that, for cellulase, either the specificity of one moiety of the molecule will eventually have to be amended to "at least *two* β-linked glucose residues" or else there is an overriding requirement that the sum of the two moieties shall be at least three glucose residues. It is here proposed to classify enzymes of the type of the β-glucosidase and the cellobiase with a requirement for a specific number of monose residues in the glycosyl moiety as "exoglycosidases." Enzymes that can hydrolyze nonterminal glycosidic linkages—that is, enzymes for which the number of monose residues in the glycosyl moiety is restricted to a minimum only—are classified as "endoglycosidases."

Other pairs of polysaccharidases similar to the cellulase pair from *Stachybotrys atra* are known—for example, α-amylase and "gluc-amylase" (*18*) and the polygalacturonases I and II of Ozawa and Okamoto (*19*). There is at present no knowledge about the specificity involved in binding at the substrate center of the cellulase and cellobiase without effective enzyme action; for exoglycosidases, at least, it is obvious that the labor involved in synthesizing the compounds needed to test even the simplest hypothesis will preclude a ready answer to such questions.

There is some preliminary evidence that the cellobiase of *Stachybotrys atra* is a transferase, but no knowledge for either cellobiase or cellulase of the specificities involved in binding at the acceptor center.

Hypothesis about Carbohydrases

The hypothesis about carbohydrases which is proposed may be briefly stated as follows. The activity of all carbohydrases that act as transferases with retention of configuration (β-amylase is the only well-studied exception to this type) depends on the simultaneous binding at contiguous active sites of two suitable substrate molecules. In principle, the over-all reaction

$$\text{Gly-O}R + POQ \rightleftharpoons \text{Gly-O}P + Q^+ + RO^-$$

where Gly represents a glycosyl radical and *P*, *Q*, and *R* represent hydrogen atoms or suitable organic radicals, is reversible, but in practice the free energy change of the reaction drives the reaction sufficiently far to the right in most cases for Gly-O*R* to be labeled "substrate" and *POQ* "acceptor." The blocking of one or both active centers will inhibit the enzyme; the substrate centers are occupied by competitive inhibitors, and the acceptor centers by anticompetitive inhibitors.

Both active centers have specificity directed toward the nature and configuration of the groups about the susceptible bonds, but this is not absolute, and a wide variety of compounds can generally be bound to some degree. As the number of kinds of molecule that can be accommodated to these specificities diminishes, there is a change of enzyme type from rather general hydrolases with some transferase activity to narrowly specific transferases.

The work of Whitaker (*20*) with the cellulase of *Myrothecium verrucaria* suggests that, when these specificities are directed toward sufficiently large molecules, they may not be absolutely constant. Brief exposure of the enzyme protein to denaturing conditions changes the activities toward different substrates to different degrees, and Whitaker believes that this is because the slightly altered shape of the enzyme surface—the "lock"—may allow various substrate molecule "keys" to fit into it better or worse than before.

In any case, as the complex net of forces involved is directed toward larger molecules, it must become increasingly liable to disturbance, and the difference between enzymes of apparently widely different specificities may amount to very little in terms of the configuration of the enzyme surface. The way in which many fungi can produce a number of enzymes of very similar specificity, or can be switched from producing one enzyme to producing another related one by small changes in the environment, suggests

that these enzymes are variants of a few basic patterns.

We may (with some reservations) draw the postulated reaction scheme diagrammatically as shown in Fig. 1. The nature of the active center E which catalyzes the nucleophilic attack on the bonds cannot as yet be specified. The important question left undecided is whether the reaction is one of metathesis or not—that is, is ROQ a product of the reaction, or are the final products of the reaction besides Gly-OP due to the solvolysis of Q^+ and RO^-.

So far as I am aware, direct metathesis of the type

$$\text{Gly}_1\text{-O-aglycone}_1 + \text{gly}_2\text{-O-aglycone}_2 \rightleftharpoons \text{gly}_1\text{-O-aglycone}_2 + \text{gly}_2\text{-O-aglycone}_1$$

has never been observed, and isotope experiments have been done under conditions where $POQ = HOH$ (or POH in equilibrium with water), although it is obviously possible to devise critical experiments if the necessary labeled substrates can be synthesized. Although direct metathesis may be stereochemically and thermodynamically unlikely, it is possible to imagine arrangements of E such that the required electronic mechanism is too bizarre. It is suggested that a watch should be kept for this type of mechanism in suitable cases such as the branching enzymes.

The apparent reversal of the reaction of some carbohydrases—for example, the formation of alkylglucosides by almond emulsin in the presence of high concentrations of alcohols and glucose (*21*)

$$\text{Glu-O}R + \text{H-OH} \rightleftharpoons \text{Glu-OH} + R\text{-OH}$$

would be most readily explained by metathesis if the double-displacement reaction mechanism is accepted—that is, if Glu-OR + H-OH and Glu-OH + R-OH give the same intermediate state.

The primary attachment of the substrate molecule to the enzyme appears to be through the oxygen of the glycosidic linkage; by analogy, a similar attachment of the acceptor groups appears likely. For the β-glucosidase of *Stachybotrys atra,* attachment through S and NH groups also occurs (*2*), and some enzymic activity against S-glucosides can be demonstrated. Since affinities of O-, N-, and S-glucosides are of the same order of magnitude, it does not appear that direct coordination to a metal is involved; indeed, no carbohydrase is known to be a heavy-metal enzyme.

Furthermore, the more easily electrons can be withdrawn from the bond between the glucosyl carbon and the bridge atoms, the more readily this bond should be split by nucleophilic agents; in fact, the expected order of ease of withdrawal of electrons from this bond with variations in the bridge atom (NH > S > O) is found to be the same as the order of ease of alkaline hydrolysis. Yet it is the opposite of the order of ease of enzymic hydrolysis. It must therefore be supposed that there is some other property of a bridging oxygen atom that allows it to take part in the formation of an intermediate complex with at least four groups bound to the active center and that is crucial for enzymic action.

This interpretation of the matter is supported by the observation that aryl glycosides are more readily hydrolyzed than alkyl glycosides by both simple glycosidases and alkalis. The electron-attracting properties of the aromatic ring provide a simple explanation for both cases. Since the observed effects are here in the direction predicted by the ordinary electronic theory of reaction mechanisms, the effects of replacing O by NH and S cannot be the result of electronic effects on the labile bond.

It has recently been recognized (*22*) that at least one "carbohydrase" (bacterial hyaluronidase) can act by an unsaturating rather than by a replacement mechanism. Such a mechanism demands an enzyme that can form an activated complex without involving an acceptor molecule, the complex then decomposing to unsaturated products by well-known electronic rearrangements.

The Nature of Specificity

It has come to be realized that many observations on the relative lability of the glycosidic linkage in different classes of compounds cannot be explained if the glycopyranose ring is visualized in the form of the flat ring by which it is conventionally represented. The ring in fact shares with the cyclohexane ring the property of potentially existing in "boat" and "chair" configurations, with one chair form being preferred both in the solid state and in solution (*23*). By considering the distribution of axial and equatorial bonds in glycosides of various types, it can be shown (*24, 25*) that the glycosidic oxygen is sometimes readily accessible for attack by the proton during acid hydrolysis and that it is sometimes heavily masked by screening groups.

Similar considerations apply to enzymic hydrolysis, and Gottschalk (*15*) predicted the critical importance of the hydroxyl group on C-3 of the glucopyranose ring for attachment at the substrate center of β-glucosidases, by considering the distribution of groups in the chair form of β-glucopyranose. Even when the glycose ring is attached to the enzyme surface, it is apparent that free movement of the aglycone portion of the molecule allows an infinite number of configurations of the glycosidic linkage;

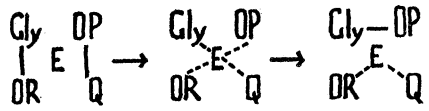

Fig. 1. Postulated reaction scheme.

it is here suggested that in endoglycosidases the active center and the susceptible bond can be in only one of their possible relative orientations if the active complex is to be formed, and that this is achieved by a highly specific binding of both aglycone and glycose. In simple glycosidases, there is considerable latitude in orientation about the preferred configuration and no need for a specific binding of aglycone.

For the enzymic hydrolysis of aryl glycosides, it is known that ortho-substitution is often inhibitory, even though acid hydrolysis is speeded up by the same substitution (*26*). It appears that this effect is due to hindered rotation about the glycosidic linkage. The β-glucosidase of *Stachybotrys atra* is almost completely inactive against derivatives of phenyl-β-glucoside with one or two ortho positions substituted by heavy groups, and examination of molecular models of such a substrate as 2,6-diiodo-4-methyl-phenyl-β-glucoside shows that it is impossible to rotate the glucosyl and aglucone portions of the molecule past each other. It is apparent that enzyme action can take place only when the glucosidic linkage can be suitably oriented. There seems to be, however, no valid evidence that there is for any simple glycosidase a specific enzymic affinity toward any particular nonpolar aglycone group such as has been suggested by some authors (*27*).

The Active Center

The picture of carbohydrase action presented here still leaves the nature of the active center unclear. About it lie two molecules held in such orientation that both simultaneously undergo the same attack—electron withdrawal from the glycosidic oxygen and nucleophilic attack on C-1 (or their equivalents for the acceptor molecule). There is very little evidence on the action of group-specific inhibitors on most carbohydrases, which are in general inactivated only under conditions that denature most protein molecules in any case. The only well-studied exception to this statement, β-amylase, is by definition excluded from the type of carbohydrase to which the generalizations of this paper apply.

Myrbäck (*6*) has postulated from the results of inhibition experiments that the functioning of yeast sucrase depends on one or more carboxyl groups on the enzyme molecule being present in an un-

charged, uncomplexed form. As with similar demonstrations for other enzymes, it still remains uncertain what part, if any, the carboxyl groups play in formation of the actual enzyme complex.

It is perhaps significant that many carbohydrases seem to be stabilized or activated by macromolecules of rather unspecific type—for example, *Myrothecium* cellulase is activated by a number of proteins (*28*); yeast sucrase is stabilized by a mannan that seems to be identical with the structural mannan of the yeast cell (*29*) and *Stachybotrys* β-glucosidase (*30*) and cellulase are both stabilized by a compiex polysaccharide that seems to be a normal metabolic product of the mold. A large area of enzyme surface must be masked in some way to keep the molecule in the active state. The functioning of the enzyme may involve the configurations of equally large areas of enzyme surface rather than small "active centers" or prosthetic groups.

When even such an enzyme as testicular hyaluronidase—an endoglycosidase restricted to alternate glycosidic linkages along the chain—has been shown (*31*) to be a transferase with apparently much the same specificity at substrate and acceptor centers, it is apparent that the range of facts that can be explained by the hypothesis is very large. Thus, the cyclizing enzymes that produce the Schardinger dextrins are carbohydrases that can bind one end of an amylose chain at an acceptor center and the same chain at a point six or seven glucose residues away at the substrate center. From the results of Morton (*7*), it seems that many sugar phosphatases are indistinguishable in reaction mechanism from the carbohydrases, and indeed there is no difference in principle between the hydrolysis of, say, glucose-1-phosphate and an arylglucoside.

The postulate of a high specificity for the acceptor center, water being a rather inefficient competitor with other potential acceptors, means that the problem of the relatively high efficiency of transferase action as against hydrolase action can be solved without further special hypotheses. Although the acceptor molecules are often transient intermediates of the enzymic hydrolysis, present at very low concentrations at any time, the fact that quite a large portion of the reaction proceeds by transfer in these cases is readily explained by assuming suitable values of the affinity constants. In the event that affinity for a complex molecule as acceptor is high and for water nearly or quite negligible, the enzyme will be a pure transferase, and a "primer" will have to be added to allow its action to begin.

Takano (*32*) has shown that four nominally closely related enzymes (the β-glucosidase and β-galactosidase of apricot and elder) have each perfectly distinct acceptor specificities. The simple method of naming a carbohydrase by adding *-ase* to the name of its "substrate" is seen to be inadequate when the dual specificity is so strongly marked.

Oparin and Bardinskaya (*5*) have protested against the postulation of mechanisms for the transferase action of potential hydrolases that do not involve the intervention of water on the ground that these reactions do not take place in nonaqueous media. Since most active proteins are not in their normal physicochemical state in these media in any case, and since they would not be expected to exhibit their characteristic behavior, the question of whether the presence of water is necessary to activate transferases is an academic one. By suitable definition of the "active complex," its presence can be made essential or irrelevant. Carbohydrases can be activated or stabilized by large excess of light-metal cations in a way strongly reminiscent of the stabilizing effect of macromolecules; like water, they seem to maintain the necessary conditions for enzymic transfer rather than to take part in it, and their inclusion as part of the reaction mechanism is likewise a matter of definition.

Conclusion

The hypothetical structure erected here depends on a single crucial observation, the effect of anticompetitive inhibitors and activators on aglycone liberation by *Stachybotrys atra* β-glucosidase, an enzyme which apparently acts by the "double displacement" mechanism. Such behavior should be readily observed in other related enzymes, if it exists there.

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