Organic Chemistry V : Enzyme Mechanisms and Natural Product Biosynthesis

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Recommended Literature :
3) Medicinal Natural Products - A Biosynthetic Approach, P. M. Dewick, J. Wiley, 2002
8) The Organic Chemistry of Biological Pathways, J. E. McMurry & T. P. Begley, Roberts & Co. Publ., USA, 2005

The main goals of these lectures are :
- To provide an introduction into the mechanisms of enzymic reactions, and to introduce the most important coenzymes.
- To provide a mechanistic understanding of how the different classes of complex natural products are constructed in Nature.
- To illustrate the methods and techniques that are used to study biosynthetic pathways.
1. Enzyme Chemistry
1.1. How efficient are enzymes as catalysts?
Essentially all chemical reactions that take place within cells are catalyzed by enzymes. It is, therefore, interesting to investigate how efficient enzymes are as catalysts, and what mechanisms they use to achieve rate enhancements. But how large are the rate enhancements achieved by enzymes? How much faster do enzymic reactions proceed, in comparison to similar non-enzymic processes. Is there an upper limit to the rate enhancements that can be achieved by enzymes, and if so what is this limit? We will attempt to answer these questions in the first part of this lecture course. But quite often this is not so easy to do, because in the absence of an enzyme a reaction might not proceed at a measureable rate at all.

E.g. Hydrolysis of a peptide bond (-CO-NH-):

\[
\text{Ac-G-G-NHMe} \xrightarrow{H_2O} \text{Ac.G-OH} + \text{H-G-NHMe}
\]

\(k\) (150°C) = \(6 \times 10^{-7} \text{ s}^{-1}\); from the temperature dependence of the rate constant we can estimate that \(k_{\text{non}}(\text{RT}) \approx 4 \times 10^{-11} \text{ s}^{-1}\); A first order reaction in water at pH 7 (J. Am. Chem. Soc., 1996, 118, 5498 und 6105), with a half-life of about 500 years at room temperature (RT).

In the case of peptide hydrolysis catalyzed by a typical protease (Carboxypeptidase B):

\[\text{Peptide} = \ldots\text{Gly.Gly}\
\]

\(k_{\text{cat}} \approx 240 \text{ s}^{-1}\) \(k_{\text{cat}}\) = number of substrate molecules converted to product, per enzyme molecule (or active site), per second. This gives \(k_{\text{cat}}/k_{\text{non}} \approx 10^{13}\) (see JACS 1996, 118, 6105; compare JACS 2005, 127, 10828; and JACS 2011, 133, 13821).

Example 2. (J. Am. Chem. Soc. 1999, 121, 11831):

\[
\text{Chorismate mutase} \rightarrow \text{Prephenate}
\]

Example 3. (Science, 1995, 267, 90):

\[
\begin{align*}
\text{Orotidine 5'\text{-phosphate decarboxylase}} & \quad \rightarrow \quad \text{Pyrimidine} \quad + \quad \text{CO}_2
\end{align*}
\]

\(k_{\text{non}}\) (pH independent; 1st order reaction) = \(2.8 \times 10^{-16} \text{ sec}^{-1}\)

\(k_{\text{cat}}\) (yeast OMP-decarboxylase) = \(39 \text{ s}^{-1}\)

In this case the reaction rate is increased by the enzyme by a factor of \(\approx 10^{17}\)!
Enzymes apparently can increase the rate of a chemical reaction by a factor of about $10^9$ up to about $10^{17}$, compared to the same non-catalyzed process under the same conditions in aqueous solution !!


The half-lives of various reactions that proceed spontaneously in water at 25°C in the absence of a catalyst have been estimated and compared (*PNAS* 2003, 100, 5607):

<table>
<thead>
<tr>
<th>$k$ (s$^{-1}$)</th>
<th>Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>$10^{-4}$</td>
<td>23 seconds peptide <em>cis-trans</em> isomerization</td>
</tr>
<tr>
<td>$10^{-4}$</td>
<td>5 seconds CO$_2$ hydration</td>
</tr>
<tr>
<td>$10^{-8}$</td>
<td>7 hours chorismate mutation</td>
</tr>
<tr>
<td>$10^{-12}$</td>
<td>6000 years amino acid racemization</td>
</tr>
<tr>
<td>$10^{-16}$</td>
<td>450 years peptide hydrolysis</td>
</tr>
<tr>
<td>$10^{-20}$</td>
<td>1.1 x $10^{12}$ years phosphomonoester dianion hydrolysis</td>
</tr>
<tr>
<td>$10^{-16}$</td>
<td>1.1 x $10^{9}$ years glycine decarboxylation</td>
</tr>
<tr>
<td>$10^{-12}$</td>
<td>8 x $10^{7}$ years OMP decarboxylation</td>
</tr>
<tr>
<td>$10^{-8}$</td>
<td>1.1 x $10^{6}$ years $\alpha$-O-glycoside hydrolysis</td>
</tr>
<tr>
<td>$10^{-4}$</td>
<td>7 x $10^{5}$ years fumarate hydration</td>
</tr>
<tr>
<td>$10^{-12}$</td>
<td>1.4 x $10^{5}$ years phosphodiester anion hydrolysis (C/O)</td>
</tr>
<tr>
<td>$10^{-4}$</td>
<td>9.8 x $10^{4}$ years mandelate racemization</td>
</tr>
</tbody>
</table>

It is important to note, however, that many enzymes make use of either metal ions or other small organic coenzymes, to catalyze reactions. By including the metal or coenzyme, reaction paths (mechanisms) become possible that would not be reasonable in their absence, e.g.

\[
\begin{align*}
\text{R} & \hspace{0.5cm} \text{H} \hspace{0.5cm} \text{COO}^- \\
\text{H} & \hspace{0.5cm} \text{H} \hspace{0.5cm} \text{H} \\
\text{H} & \hspace{0.5cm} \text{N} \\
\text{H} & \hspace{0.5cm} \text{N} \\
\text{R} & \hspace{0.5cm} \text{H} \\
\text{H} & \hspace{0.5cm} \text{H} \\
\text{H} & \hspace{0.5cm} \text{N} \\
\text{H} & \hspace{0.5cm} \text{N} \\
\text{R} & \hspace{0.5cm} \text{H} \\
\text{H} & \hspace{0.5cm} \text{H} \\
\text{H} & \hspace{0.5cm} \text{N} \\
\text{H} & \hspace{0.5cm} \text{N} \\
\end{align*}
\]

Rate constant at 25°C = $2 \times 10^{-17}$ s$^{-1}$. With PLP (but no enzyme) the reaction rate is greatly enhanced.
Further interesting examples:

- ACC Lyase
  \[ \text{NH}_3 \text{COO}^- \xrightarrow{\text{ACC Lyase}} H_2CCH_2 + \Theta \text{CN} + CO_2 \]

- \[ \text{H}_3\text{N} \text{C}O \text{O} \xrightarrow{\alpha\text{-Ketoglutarate, Fe}^{2+}} \text{Isopenicillin-N synthase} \]
  \[ + \text{Succinate} + \text{H}_2\text{O} \]

- \[ \text{H}_3\text{N} \text{C}O \text{O} \xrightarrow{3 \times \text{P}450} \text{Vancomycin-Aglycon} \]

- \[ \text{Me} \text{C}O \text{SCoA} \xrightarrow{\text{Isobutyryl-CoA Mutase}} \text{Coenzym-B} \_12 \]
  \[ \text{Me} \text{C}O \text{SCoA} \]

- \[ \text{Me} \text{C}O \text{SCoA} \xrightarrow{\text{Taxadiene-Synthase}} \]
  \[ \text{Me} \text{C}O \text{SCoA} \]

- Propionyl-CoA + 6 Methylmalonyl-CoA + 6 NADH
  \[ \text{6-Deoxyerythronolide B} \]

The following coenzymes play important roles in enzymic catalysis. Note that this is not a complete list of all known coenzymes:
1.2. Mechanisms of Chemical Catalysis
How does an enzyme (or any catalyst) accelerate a chemical reaction? Our explanation is usually based on transition state theory, i.e. that the rate of a chemical reaction is determined by the energy difference between the ground state and the transition state of the reaction ($\Delta G^\#$), in other words the transition state is treated as a thermodynamic entity whose concentration is proportional to the rate. When a catalyst is present, the reaction will follow a different mechanism with a lower activation free energy, compared to the process without the catalyst:

It is also important to recognize that for any given reaction, the value of $\Delta G^0$ is identical for both the catalyzed and non-catalyzed reactions; only $\Delta G^\#$ is smaller in the catalyzed process. Hence, both the forward and reverse reactions are accelerated by the catalyst, but the equilibrium constant remains unchanged.

It is often very helpful in the analysis of the structures of transition states to remember the **Hammond postulate**:

In the case of a strongly endothermic reaction step, the structure of the activated complex (transition state) resembles more closely that of the product, whereas in an exothermic reaction the structure of the transition state will resemble more closely that of the starting material. 

From a chemical viewpoint this is reasonable:

In the following discussion, we will start by looking at reactions that proceed without enzymic catalysis, i.e. some normal organic reactions. Nevertheless, organic reactions can be catalyzed; but what sorts of catalyst are used, and what are the mechanisms of these catalyzed reactions? We need to understand this, before we ask how relevant these mechanisms are for an understanding of enzymic catalyzed reactions.
1.2.1. Specific acid and specific base catalysis

There are two types of acid catalysis, and two types of base catalysis. Here we will deal with specific acid and specific base catalysis.

**Specific acid catalysis** involves catalysis by $H^+$ ($H_2O^+$), and so the rate depends upon pH:

In general terms:

$$X + H_2O^+ \rightarrow XH^+ + H_2O \rightarrow \text{products}$$

Rate = $k[XH^+]$

$$= k.K[H_2O^*].[X]$$

In terms of reaction mechanism, a rapid equilibrium protonation of the molecule occurs in the first step, followed by a slow step, which is accelerated in comparison with the uncatalyzed reaction because of the greater reactivity of the protonated molecule. One well known example is ester hydrolysis.

Another example is the diene-phenol rearrangement, which is slow in the absence of acid, but in the presence of acid proceeds rapidly:

The concentration of $[XH^+]$ **depends upon the pH**, and the concentration of X. This mechanism is therefore only important when $pH \leq pK_a$ of $XH^+$.

Remember:

$$HA + H_2O \rightleftharpoons H_3O^* + A^-$$

$$K_{eq} = \frac{[H_3O^*][A^-]}{[AH][H_2O]} \quad K_a = \frac{[H_2O^*][A^-]}{[AH]} \quad pK_a = - \log K_a$$

The pK$_a$ of HA corresponds to the pH where it is half dissociated in aqueous solution.

In summary:

- $H_3O^+$ is the catalyst (is the only effective catalyst)
- the protonation step is not rate limiting (fast pre-equilibrium)
- means a rate determining reaction of the protonated species
- is effective only at or below the pKa of the protonated species.
- the rate depends on pH
- Usually only simple uni- and bi-molecular RDSs.
- Typically an inverse isotope effect is seen : $k(H_2O) < k(D_2O)$

* $D_2O^*$ in $D_2O$ is a stronger acid than $H_2O^*$ in $H_2O$. Water ($H_2O$) is a better solvating agent for $H_3O^+$ than $D_2O$ for $D_3O^*$ because it forms stronger H-bonds; due to the greater O-H vibration frequency.
In specific base catalysis, the rate also depends on pH, i.e. on $[\text{OH}^-]$. This typically involves a reaction with a first order dependence on $[\text{OH}^-]$. It may involve the direct attack by $\text{OH}^-$ in the RDS, or the rapid removal of a proton from the substrate by hydroxide ion (OH$^-$) in a fast pre-equilibrium, followed by a rate-limiting reaction of the anion. For example:

\[ \text{R-SH} + \text{OH}^- \rightleftharpoons \text{OH}^- \text{R-SH} \]

The hydrolysis of esters is catalyzed by both acids and bases. The specific base OH$^-$ also functions as a nucleophile, and the rate equation involves $[\text{OH}^-]$:

\[ k \text{ rate} = \frac{[\text{EtOAc}]}{[\text{AcOH}][\text{EtOH}]} \]

Another example is the aldol reaction:

\[ \Theta \text{HO} : \overset{\text{HO}^- / \text{H}_2\text{O}}{\text{MeO\text{H}}} \overset{\text{fast}}{\rightleftharpoons} \overset{\text{RDS}}{\text{MeO\text{H} + H}_2\text{O}} \overset{k_{\text{obs}}}{\longrightarrow} \overset{\text{H}_2\text{O}}{\text{MeO\text{H}}} \]

In D$_2$O, D is rapidly incorporated into the aldehyde (faster than the aldol reaction).

In summary:
- $\text{HO}^-$ is the catalyst
- the rate depends on pH
- often means a rate-determining reaction of a deprotonated species
- usually only simple uni- or bi-molecular steps

Specific acid and specific base catalysis are mechanisms that are not used by enzymes, since the pH of an enzymatic reaction must remain close to 7.

### 1.2.2. General acid catalysis and general base catalysis

This is the second important type of catalysis by acids and bases. Here at constant pH the rate depends on the concentration of a general acid (not H$_3$O$^+$) or a general base (not OH$^-$). In such cases, the proton transfer is not complete before the rate-determining step, but occurs during the rate-determining step. The mechanism of the reaction involves a proton transfer during the RDS.

e.g. in catalysis by a general acid HA, or a general base A:
For example, acetate accelerates the formation of esters from alcohols and acetic anhydride:

Why does acetate accelerate this reaction? Acetate ($pK_a 4.7$) is far too weak a base to deprotonate the alcohol ($pK_a \approx 16$). If it acted as a nucleophile there would be no net reaction. But acetate can remove a proton from the alcohol as the reaction occurs:

Acetate would be more than basic enough to deprotonate the intermediate, and at the transition state, the $pK_a$'s become matched, and the proton transfer occurs very rapidly:

A major disadvantage of this mechanism is that the RDS is termolecular, so the entropy of activation tends to be large and negative. This can be overcome if one component is in large excess (e.g. the alcohol is used as solvent).

Another example:

Summary of general base catalysis:
- At constant pH the rate is dependent upon the concentration of the general base.
- Proton transfer occurs at the transition state of the RDS.
- Is effective at neutral pH, even if below the $pK_a$ of the substrate (see above).
- Often a termolecular RDS (large $-\Delta S^\ddagger$).
- A normal solvent isotope effect ($k(H)/k(D) \approx 2-4$) is seen, since an $X-H$ ($X = O, N$) bond is broken in the transition state of the RDS.

In the case of **general acid catalysis**, a reaction is catalysed by the presence of a general acid (HA a non-dissociated acid, but not $H_3O^+$). The general acid is usually not strong enough to protonate the substrate in a rapid pre-equilibrium before the RDS. Rather, the proton is transferred during the RDS (at the transition state). The rate of the reaction increases with increasing concentration of HA, at constant pH, e.g.
Normally, ester formation and ester hydrolysis require specific acid/base catalysis. The weaker general acid/base catalysis is not effective enough. However, in some special cases GA/GB catalysis is seen, e.g.

![Ester formation and hydrolysis](image)

Normally, the formation and hydrolysis of acetals requires SAC catalysis, since alcohols are poor leaving groups; GA/GB catalysis is not very effective.

![Acetal formation and hydrolysis](image)

In some cases GA catalysis can be observed, for example, when there is additional stabilization of the carbocation intermediate:

![Acetal hydrolysis](image)

In summary, in general acid catalysis:
- any undissociated acid can function as a GA catalyst
- the acid is too weak to protonate the substrate in a rapid pre-equilibrium
- the proton is transferred in the rate-determining step (in the transition state)
- this mechanism of catalysis can be effective even at neutral pH
- the rate increases with increasing [HA], at constant pH
- normal solvent kinetic isotope effects are seen
- often termolecular with large $\Delta S^*$

Another interesting example where GA catalysis has been observed during acetal hydrolysis (*Tet.Lett*. **1963**, *4*, 911-3):
Concerted general acid-base catalysis

Of special interest in the context of enzymic reactions is the concept of concerted general acid-base catalysis. Model systems have long been sought, to investigate whether concerted general acid-base catalysis could make a significant contribution to the rate enhancements seen in enzymic reactions. Examples of such enzyme model reactions have been difficult to find. One example:

When acids and bases are not present, the solvent can have a large influence on the rate of mutarotation:

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Rel. rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzene</td>
<td>very slow</td>
</tr>
<tr>
<td>Benzene + cat. H₂O</td>
<td>faster</td>
</tr>
<tr>
<td>Pyridine</td>
<td>very slow</td>
</tr>
<tr>
<td>Cresol</td>
<td>very slow</td>
</tr>
<tr>
<td>Pyridine + Cresol</td>
<td>fast</td>
</tr>
<tr>
<td>α-Pyridon</td>
<td>best catalyst (although a weaker base than pyridine and a weaker acid than phenol)</td>
</tr>
</tbody>
</table>

Mechanism:

A similar effect was not observed in aqueous solution. In this case water can act as a proton donor/acceptor, and concerted acid/base catalysis is not observed.

Organocatalysis - A new concept in asymmetric synthesis

Organocatalysis has emerged recently as a new and powerful tool in chemical synthesis, in particular, the asymmetric version in enantioselective synthesis. Here, small organic molecules are designed to catalyze organic reactions. The catalysts are often chiral, so enantioselective catalysis can be observed. One class of organocatalysts uses chiral phosphoric acids:

Chiral phosphoric acids (*ACIE, 2006, 45, 3909; ACIE 2008, 47, 4638*)

The aim is to design chiral acid catalysts. If these catalysts can accelerate a reaction by general acid catalysis, then proton transfer in the RDS would occur in a chiral environment, and so in principle the
reaction must be stereoselective. But how good is the stereoselectivity? e.g. (Chem. Comm. 2008, 4097):

So far few detailed mechanistic studies on these catalysts have been reported. However, it seems clear that the chiral catalyst acts either as a general acid, and donates a proton to the electrophile within a chiral environment (rather like an enzyme) or keeps the protonated substrate bound as an ion pair in a chiral environment for subsequent reaction.

1.2.3. Covalent Catalysis - Nucleophilic Catalysis
Here the catalyst does not simply act as an acid or base, but rather provides catalysis by acting as a
nucleophile. The catalyst attacks the substrate and so opens a mechanistic path, which is not possible in the absence of the catalyst. As a result new intermediates will be generated on the reaction pathway, and in some cases these intermediates can be observed spectroscopically, or trapped, so providing evidence for the proposed mechanism. One example is the benzoin condensation:

\[
\text{PhCHO} + \text{CN}^-, \text{H}_2\text{O} \rightarrow \text{PhCHO} + \text{CNH}_2\text{CO}_2\text{H}
\]

CN\(^-\) is a catalyst for this reaction because of its special properties; 1) it is a good nucleophile and attacks the carbonyl group rapidly, and 2) it can stabilize a negative charge in the \(\alpha\)-position by resonance.

Another well known example:

\[
\text{MeO}_2\text{C} + \text{R}^-\text{OH} \rightarrow \text{MeO}_2\text{C} + \text{AcOH}
\]

Here:
- The catalyst (pyridine) is a stronger nucleophile than the alcohol in the non-catalysed reaction (without pyridine).
- The pyridinium group in the intermediate is a much better leaving group, and
- This intermediate is less stable than the product (the ester).

The powerful catalytic effects of 4-(dimethylamino)pyridine (DMAP) in acyl transfer reactions is well known, and DMAP is frequently used for this purpose (see *Angew. Chem. Int. Ed.* 2004, **43**, 5436).

For the coupling of carboxylic acids and amines with carbodiimide, the catalytic effect of N-hydroxybenzotriazole (HOBt) is frequently exploited in the synthesis of amides:

\[
\text{R}^1\text{-COOH} + \text{R}^2\text{-NH}_2 \rightarrow \text{R}^1\text{HN} + \text{R}^2\text{NH}_2
\]

- the rate is independent of [HOBt]; the RDS is formation of the O-acylisourea intermediate (*JOC*, 2007, **72**, 8863).
- the rate falls sharply at high pH consistent with a second order reaction between protonated diimide and the carbonylate group
- the O-acylisourea reacts rapidly and selectively with HOBt to form an -OBt active ester. This reacts selectively with the amine to form product.

**Chiral amines in organocatalysis**
The use of chiral secondary amines to catalyze a wide variety of organic reactions enantioselectively has grown enormously in importance in recent years (for reviews see: *Drug Discovery Today* 2007, **12**, 8-27; *ACIE* 2008, **47**, 4638). One of the first reported examples is the enantioselective aldol reaction catalysed by the amino acid proline:
The mechanism has been well studied, and begins with the 2'-amine acting as a nucleophilic catalyst to generate an iminium-ion. Iminium-ions are much more reactive than neutral imines; they are formed and can be hydrolyzed more rapidly, and can be rapidly and reversibly converted into enamines. An aldol-like reaction can then occur, with the carboxylic acid acting as a general acid catalyst:

Some other proline-catalyzed aldol, Mannich, and related reactions:

(a) Proline-catalysed intermolecular aldol reaction

(b) Proline-catalysed manich reaction

(c) Proline-catalysed 'dihydroxylation'

(d) Proline-catalysed aldehyde cross aldehyde

Other secondary amines can also catalyze interesting organic reactions:
1.2.4. Covalent catalysis - Electrophilic catalysis

Here the catalyst functions as an electrophile, for example, by withdrawing electron density from the reaction center. In general, electrophilic catalysts may include Lewis and Bronsted acids, metal ions, as well as other organic "cofactors" (compare later coenzymes like pyridoxal phosphate). Two important effects of metal ions are:

An interesting model reaction was reported several years ago, which demonstrates the catalytic effects of metal ions on amide hydrolysis (*J. Am. Chem. Soc.*, 1993, 115, 1157):

The metal ion facilitates the formation of hydroxide ion, as seen also in the following complexes:
The metal can also act as a Lewis acid to activate the carbonyl group towards nucleophilic attack:

The hydrolysis of phosphate monoesters is especially slow in water. However, phosphatases can catalyze this reaction, and are some of the most efficient enzyme catalysts known. How do they do this? This is still a topic of intense research. But some phosphatases are known to possess an active site containing two metal ions. The structure of the ligated metal ions in the purple phosphatase I (PAP) is known from X-ray crystallography. Model complexes have also been synthesized with similar structures:

The catalyst shown left can catalyze the hydrolysis of p-nitrophenylphosphate by a factor of $10^{12}$. By $^{18}$O-labelling it was shown that Co-bound OH groups act as a nucleophile (c.f. JACS, 2004, 126, 11864).

**Organic electrophilic catalysis**

Organic groups that can act as a $\pi$-acceptor, may also be classified as electrophilic catalysts. For example, the decarboxylation of $\beta$-ketoacids is catalyzed by amines ($1^\circ > 2^\circ > 3^\circ$):

We have already seen that amines can catalyze aldol reactions (and retro-aldol reactions). Catalytic mechanisms such as these play a very important role in many enzymic reactions.

**1.2.5. Preorganisation**

When 2 molecules ($A + B$) react with one another, they first have to collide and associate, and for this an entropy barrier must be overcome before they can cross the transition state (TS). Enzymic reactions are fundamentally different, in that the first step is always the binding of the substrate(s) ($S$) at the active site of the enzyme ($E$), to form a so-called Michaelis complex (ES). Only after the substrates have bound (and perhaps after complex conformational changes have occurred in the enzyme) does a reaction take place. The entropic barrier, therefore, is now encountered in the formation of the ES complex, and not in the
process leading to the TS. The reaction at the active site, therefore, is rather analogous to an intramolecular process, in which all the participating functional groups are not only collected together, but also preorganized for a reaction to occur, i.e. bound in the correct conformations, and in the correct relative positions and orientations, both with respect to groups in the substrates but also catalytic groups in the enzyme. It is even conceivable, that the atomic motions that have to occur along the reaction pathway can be coordinated with the natural breathing (i.e. dynamic motions) of the protein. But in terms of catalytic efficiency, how much are these effects worth; how much do they contribute?

This is a fundamentally important question that has still not been completely answered. However, some important insights have come from studies of simpler model systems, in particular, from comparisons of inter- vs. intra-molecular reactions.

For example, intramolecular reactions can be considered as models for enzymic reactions, in which the reacting groups are held together, and perhaps also correctly oriented for the reaction to occur. Such “proximity-effects” can have a dramatic influence upon reaction rates:

Before a nucleophilic attack can occur, the attacking nucleophile must approach from the correct position and trajectory; this is strongly influenced by the preferred conformation of the molecule:
The efficiency of intramolecular catalysis can be estimated with the so-called "effective molarity" - The ratio of the first-order rate constant of an intramolecular reaction involving two functional groups within the same molecular entity, to the second-order rate constant of an analogous intermolecular elementary reaction. This ratio has the dimension of concentration. In other words, the EM-value corresponds to the concentration of the catalytic group required in the intermolecular process in order that it proceeds as fast as the equivalent intramolecular process.

In early studies it proved difficult to find such model reactions with EM values larger than 10-100 M, wherein a proton transfer (and not a nucleophilic attack, as above) was involved in the RDS. Does this mean that acid catalysis is not so important? e.g.

In later studies, however, much larger EM values were observed in systems where efficient intramolecular proton transfer occurs, and a feature of such cases is a strong H-bond (preorganization) in the reactant and/or product:

The formation of a strong hydrogen bond in the product appears to characterize the high EM values obtained in these systems. They also indicate that very efficient proton transfer occurs in the TS. Finally, such efficiency in the proton transfer step seems to require a precise positioning (preorganization) of the reactive groups (see *Angew. Chem. Int. Ed.* 1996, 35, 707).

This very precise positioning of reactive groups is something that can be anticipated in an enzymic reaction, where the spatial interactions between functional groups can be precisely controlled by substrate binding at the active site.

**1.2.6. Electrostatic effects**

Water possesses a rather high dielectric constant $\varepsilon = 79$. The electrostatic interaction between two point charges $e_1$ and $e_2$ depends on their distance apart, and on $\varepsilon$:

$$E = \frac{e_1 \cdot e_2}{\varepsilon \cdot r}$$

Water as solvent reduces dramatically the energy of electrostatic interactions:

- Electron ° ° Proton $r = 3.3\,\text{Å}$
  - in Vacuo ( $\varepsilon = 1$) $E = -100\,\text{Kcal/mol}$
  - in water ( $\varepsilon = 79$) $E = -1.3\,\text{Kcal/mol}$

The interior of a protein is a very heterogeneous environment, where the dielectric constant is certainly smaller than in bulk water. So electrostatic interactions will be more important at the active site of an enzyme than in water. Unfortunately, it is rather difficult to exactly measure (or calculate) the precise dielectric in the interior of a protein. But the importance of such effects must not be overlooked, e.g. what is the energetic value two H-bonds? $\rightarrow$ depends on the environment!

Enzymes can also use the protein architecture to impose specific electrostatic fields onto their bound substrates, but the magnitude and catalytic effect of these electric fields have proven difficult to quantify. One interesting case is the active site of the enzyme ketosteroid isomerase, which exterts an extremely large electric field onto a carbonyl C=O bond, thereby increasing catalysis (*Science* 2014, 346, 1510).
1.3 Enzyme kinetics

Although enzymic reactions take place in aqueous solution, the substrate is not transformed into product in free solution, but rather whilst bound at the active site of the enzyme. So the substrate must bind to the active site of the enzyme before a reaction can be catalyzed. Note that the process of substrate binding quite often causes large-scale conformational changes in the enzyme itself, such as the closure of loops over the active site, which can for example exclude water from the vicinity of the active site during catalysis. To a first approximation, the structure of the active site should be complementary (i.e. bind tightest) to the transition state of the reaction:

In the simplest case:

We are normally interested first in so-called steady state kinetics, which represents the changes in product concentration (the initial rate) during the first few seconds-to-minutes of the reaction (i.e. <10% of the substrate has reacted). An important assumption is that \([S] >> [E_o]\), so that the concentrations of all enzymic species can be neglected. If this is not the case, the kinetic treatment is more complicated. However, under these conditions \([S] >> [E_o]\), at any given enzyme concentration, the rate \(v\) depends on \([S]\) and the parameter \(K_m\) (Michaelis constant):

This type of kinetic behavior is described by the Michaelis-Menten equation:

\[
v = \frac{k_{cat} [E_o] [S]}{K_m + [S]} \quad \text{(Michaelis-Menten equation)}
\]

Thus, as \([S]\) becomes larger and finally \([S] >> K_m\), then:

\[
v = k_{cat} [E_o] = V_{max} \quad \text{(often just written as } V)\]

Also:

\[
v = \frac{V_{max} [S]}{K_m + [S]} \quad \text{when } [S] = K_m \quad \text{then } v = \frac{1}{2} V_{max}
\]

Note also when \([S] << K_m\) the Michaelis-Menten equation can be simplified:

\[
v = \frac{k_{cat} [E_o] [S]}{K_m + [S]} = \frac{k_{cat} [E_o] [S]}{K_m}
\]
But what is the significance (the meaning) of \( k_{\text{cat}} \), \( K_m \) and \( k_{\text{cat}}/K_m \) ?

1. **\( k_{\text{cat}} \) is a first order rate constant** (s\(^{-1}\)). This is called the catalytic constant or turnover number. It corresponds to the maximum number of substrate molecules that can be converted into product per second per enzyme molecule. Typical values are in the range from 1-10\(^5\) s\(^{-1}\). In order to determine this constant, the enzyme concentration must be known.

\( k_{\text{cat}} \) is the sum and weighted property of the ES and all other E-intermediate complexes up to the EP complex, that are formed along the reaction path. Only in the simplest case (see below) can \( k_{\text{cat}} \) be identified with one specific step in the reaction path. **In the most general case, it describes how quickly all enzyme-bound species are converted into free product (P).** If one of the steps is much slower than all the others, then this will dominate the \( k_{\text{cat}} \) value. Of course, \( k_{\text{cat}} \) cannot be larger than any one of the first order steps along the reaction pathway (\( k_1, k_2 \) etc.).

2. The **\( K_m \) is the substrate concentration needed to achieve half-maximal velocity**, i.e. \( \frac{1}{2} V_{\text{max}} \). Typical values are in the range 10\(^{-1}\)-10\(^{-7}\) M. **However, the \( K_m \) value expresses how tightly all enzyme-bound species interact with the enzyme**, or in other words, it can be viewed as a global dissociation constant for all enzyme bound species. A high value means weak binding, a low value means tight binding. Again, if there is one intermediate that binds much more tightly to the enzyme than all others, this will have a higher weighting in determining \( K_m \).

3. As shown above, \( k_{\text{cat}}/K_m \) is itself an important constant that is equivalent to the second order rate constant for the reaction between E and S (s\(^{-1}\) M\(^{-1}\)). This constant is a property of the free E and the free S. It is frequently used as a measure of the efficiency or specificity (when comparing two or more substrates) of an enzyme. Typical values are 10\(^6\)-10\(^9\) M\(^{-1}\) s\(^{-1}\).

At low \([S]\), then \([E] \approx [E_0]\) so:

\[
\frac{v}{K_m} = \frac{k_{\text{cat}} [E] [S]}{K_m}
\]

Thus, when \([S] \ll K_m\) the ratio \( k_{\text{cat}}/K_m \) is an "apparent" second order rate constant (M\(^{-1}\) s\(^{-1}\)) for the enzymic reaction. But note that the value cannot be higher than the diffusion-controlled rate of a reaction in aqueous solution (the upper limit is ca. 10\(^9\) M\(^{-1}\) s\(^{-1}\)).

We can represent these constants on one typical reaction energy diagramm:

How was the Michaelis-Menten equation derived (see *Biochemistry*, 2011, 50, 8264)?
Important contributions to the theory of enzyme kinetics were made by A. J. Brown and V. Henri (Univ. Zürich). However, it is usually the later contributions of Michaelis and Menten that are taken as a starting point in discussions of enzyme kinetics (for an historical account: FEBS Lett 2013, 587, 2725 & 2753).

Michaelis and Menten assumed that the first step was a rapid equilibrium, and the substrate concentration is in excess over the enzyme concentration (i.e. so that \([S_0] \approx [S]\)), where then:

\[
\begin{align*}
E + S & \overset{K_S}{\rightleftharpoons} ES \\
& \overset{k (=k_{cat})}{\longrightarrow} E + P
\end{align*}
\]

\[
\text{conc}^a = \frac{(E_o - x)}{x}
\]

where \(E_o = \text{total enzyme conc.}\)

\[
K_S = \frac{[E][S]}{[ES]} = \frac{[E_o - x][S]}{[x]}
\]

\[
x = \frac{[E_o][S]}{K_S + [S]}
\]

so the rate \(v = k \cdot x = \frac{k \cdot [E_o][S]}{K_S + [S]}
\)

Michaelis and Menten assumed that many enzymic reactions could be described by equation-1, and that the formation of ES would be rapid compared to the subsequent "chemical" step.

Later Briggs and Haldane suggested that it was not necessary for the binding of substrate to be a rapid pre-equilibrium step. Rather, they assumed that a steady state was quickly established, in which the concentrations of all forms of E did not change during the initial velocity phase (when \([S] \gg [E]\)), and their concentration was determined by how fast they were formed and how fast they break down.

The steady state assumption made by Briggs and Haldane is a more general mechanism in which the binding of S is not assumed to be reversible, nor necessarily at equilibrium during the reaction:

\[
\begin{align*}
E + S & \overset{k_1}{\leftrightarrow} ES \\
& \overset{k_2}{\longrightarrow} E + P
\end{align*}
\]

\[
\text{conc}^a = \frac{(E_o - x)}{x}
\]

Briggs and Haldane postulated that very rapidly (within milliseconds of mixing everything) a steady state would be established (again, when \([S] \gg [E]\) should be true), where:

\[
\frac{dx}{dt} = 0 = k_1 [E_o - x][S] - k_{-1}[x] - k_2 [x]
\]

Rearranging:

\[
x = \frac{k_1 [E_o][S]}{k_{-1} + k_2 + k_1 [S]}
\]

so

\[
v = x k_2 = \frac{k_1 k_2 [E_o][S]}{k_{-1} + k_2 + k_1 [S]}
\]
or, dividing all terms by $k_1$:

$$v = \frac{k_{\text{cat}} [E_0] [S]}{K_m + [S]}$$

where $k_{\text{cat}} = k_2$ and $K_m = \frac{k_{-1} + k_2}{k_1}$

This again is the Michalis-Menten equation.

**How can $V_{\text{max}}$, $k_{\text{cat}}$ und $K_m$ be determined?**

The $K_m$ und $V_{\text{max}}$ (or just $V$) are determined by measuring the initial rate ($v$) as a function of the concentration of one substrate ([S]), while keeping all other concentrations constant, and then repeating this at several additional fixed levels of a second substrate (if required). The data are then displayed graphically using linear transforms of the Michaelis-Menten equation:

$$v = \frac{k_{\text{cat}} [E_0] [S]}{K_m + [S]} = \frac{V [S]}{K_m + [S]}$$

$$\frac{1}{v} = \frac{1}{V} + \frac{K_m}{V} \cdot [S]$$

oder

$$\frac{[S]}{v} = \frac{K_m}{V} + [S]$$

Nowadays, a lap-top computer and a commercial software package can be used to directly fit by non-linear regression the experimental data to the Michaelis-Menten equation to determine the constants $K_m$ and $k_{\text{cat}}$.

Note, however, that in order to determine $k_{\text{cat}}$ the value of $[E_0]$ must be known, and this assumes that the enzyme is available in pure form and that its concentration can be measured. This is not necessary when the aim is to measure $K_m$ and $V_{\text{max}}$ - this can be done without knowing the value of $[E_0]$, and so can also be done using crude cell extracts containing the enzyme activity of interest.
1.4 The role of general acid-base catalysis and nucleophilic catalysis in enzymic reactions: glycosidases and glycosyl-transferases

Glycosidases catalyze the hydrolysis of oligosaccharides. But how stable are glycoside derivatives under physiological conditions? The rate of hydrolysis of β-methyl-D-glucopyranoside has been investigated, and compared to that of some other molecules related to important biopolymers (J. Am. Chem. Soc. 1998, 120, 6814):

When compared to the rate of phosphodiester hydrolysis (oligonucleotides) and amide bond hydrolysis (peptides and proteins), the rate of glycoside hydrolysis under comparable conditions is slower: oligosaccharides are the most stable of the three biopolymers.

Glycosidases are very efficient catalysts. They can accelerate the rate of oligosaccharide hydrolysis by factors up to $10^{17}$ compared to the rate of spontaneous hydrolysis under comparable (physiological) conditions.

The glycosidases are a very large class of enzymes, with over 2000 from different origins now known. These enzymes can be divided mainly into two large classes, that have different mechanisms of action, and different stereochemical courses (see: Curr. Opin. Chem. Biol., 2000, 4, 573; Accts. Chem. Res. 2000, 33, 11):

One family is called the “inverting glycosidases” - they catalyze hydrolysis with inversion of configuration at the anomeric center:

Here the distance between the precisely positioned general acid and general base in the enzyme's active site is optimal (~10.5Å) to allow a more-or-less concerted hydrolysis mechanism. In such cases no
covalently bound E-S intermediates have been detected. This is not the case with the second major family, the retaining glycosidases, which proceed with retention of configuration at the anomeric center:

The “retaining glycosidases” - Here the two sides of the active site are only ~5.5Å apart:

![Diagram of retaining glycosidase mechanism]

An important difference is that a covalent enzyme-intermediate complex is now formed. Support for this mechanism can be obtained by demonstrating that this intermediate is indeed formed. But how?

Through subtle modification of the substrate this was possible by X-ray crystallography (Vgl. *Biochemistry* 1998, 37, 11707):

- replacing 2-OH by -F leads to:
  - loss of H-bonding interactions in the active site, which are important in TS stabilization, and
  - inductively destabilizes an oxo-carbenium ion-like TS, so breakdown of the intermediate is slow.
The first crystal structure of an enzyme was that of lysozyme, first published in 1965. However, only in the past few years was the mechanism of the lysozyme reaction proven. This enzyme catalyzes the hydrolysis of peptidoglycan, an important component of the cell walls of bacteria. Lysozyme therefore kills bacteria and is produced to help defend organisms from bacterial attack. The structure and biosynthesis of peptidoglycan are shown below:

Molecules that block (inhibit) the biosynthesis of peptidoglycan act as antibiotics. Well known examples include penicillin and vancomycin:

The crystal structures of various complexes formed between lysozyme and oligosaccharide fragments showed how the substrate binds at the active site of the enzyme. These structures also revealed two key groups in the enzyme that are essential for catalysis Glu35 and Asp52, which lie on opposite sides of the substrate binding cleft. One (Glu35) exists in a neutral (undissociated) form, and the other (Asp52) as a carboxylate anion:
The reaction proceeds with retention of configuration at the anomeric center. For a long time, there was great uncertainty about the mechanism, because a covalent ES complex (as intermediate) had never been detected. Either a carbocation is formed which reacts further to product, or a covalent intermediate is generated:

Only recently were experiments described, which proved that a covalent ES complex can be formed. In order to achieve this the chemical properties of the substrate were altered to allow the intermediate to accumulate; Glu35 was mutated to Gln35 (deactivates the enzyme) and the substrate used was a glycosyl fluoride (more reactive):

The activated glycoside substrate can still react, but breakdown of the covalent intermediate is slowed by a factor of $10^5$ (half life now 64 h) due to destabilization of the oxo-carbenium ion-like TS by 2-F.
In this way, the concentration of the stationary concentration of the intermediate could be raised enough to allow detection by MS and X-ray crystallography (Nature, 2001, 412, 835).

Inhibitors of glycosidases are also important in medicinal chemistry. For example, neuraminidase (NA) is a membrane bound enzyme on the influenza virus, which is important for the life cycle of the virus. NA catalyzes the removal of terminal sialic acid linked to glycoproteins and glycolipids on the cell surface. The sialic acid is the ligand on the cell surface that binds to the other major protein on the influenza virus, the hemagglutinin (HA). The reaction catalyzed is:

\[
\text{influenza virus} \xrightarrow{\text{NA}} \text{HA}
\]

NA appears to be crucial in the release of progeny virion from infected cells, and in the movement of virus through the mucus of the respiratory tract, thus reducing the propensity of the virus to aggregate.

Two inhibitors of NA are Tamiflu and Relenza, which each bind with high affinity to the active site of the enzyme because they mimic the geometry of the transition state in the hydrolysis reaction:

The inhibition of enzymes active in carbohydrate metabolism is also valuable in the treatment of diabetes. For example, the following molecules are inhibitors of α-glucosidases used to digest carbohydrates. They are all currently used to treat type-2 diabetes:
Also of great importance are **glycosyl transferases**, which add sugar residues to (for example) proteins (to make glycoproteins) or small molecule natural products, e.g. antibiotics (see later). Glycosylation is the most frequently observed type of post-translational modification of proteins. Glycosyl transferases are therefore of great interest in biotechnology.

**Activated sugar residues** are required as glycosyl donors (not free sugars), and one typical example proceeding with inversion ($\alpha \rightarrow \beta$) is shown below: