"AFSTUDEER SCRIPTIE"

ENZYME CATALYSIS IN ORGANIC SOLVENTS

R.J. Mooldijk **november 1990**

Supervisors :

X.M. Shen
Dr. Ir. Th. W. de Loos
Prof. Dr. Ir. J. de Swaan Arons

Delf University of Technology
Faculty for Chemical Engineering and Materials Science
Department for Applied and Technical Chemistry
Laboratory for Applied Thermodynamics and Phase Behaviour

"It is, I think, difficult to exaggerate the importance to biology, and I venture to say to chemistry no less, of extended studies of enzymes and their action"

F.G. Hopkins
(Presidential Address to the Royal Society, 1932)

PREFACE

This work is a part of a final research project performed at Delft University of Technology, faculty for Chemical Engineering and Materials Science, laboratory for Applied Thermodynamics and Phase Behaviour, to obtain an "ingenieurs diploma". The subject of this research project is enzymatic reactions in organic solvents and then especially the role thermodynamics can play in explaining observed behaviour and predicting to what this behaviour would lead in other systems. This report ("afstudeer scriptie") deals with the influence of organic solvents on enzyme behaviour, focusing on stability, activity and stereoselectivity. Another report (" afstudeer verslag") deals with the influence of organic solvents on the equilibrium conversion of the reactions catalyzed. If this work is to be used for solvent selection purposes, both reports should be used since both influences should be taken into account.

Summary

Enzymes are powerful catalysts with many unique properties. With their high activity and (stereo)selectivity they have great potential for many applications as catalysts in organic synthesis. Especially after it was discovered that enzymes are not only active in water, but in organic solvents as well. This is interesting because of the higher solubility of many, possibly interesting, reaction compounds in organic solvents. Enzymes show, however, in an organic solvent a different behaviour then in water. They are in some solvents more stable, in some solvents less, they are less active in organic solvents, they have a different activity in different solvents, and the water content of the organic solvent plays a crucial role. In modern day literature it is believed that all this behaviour is caused by the influence of the solvent on the enzyme molecule, disturbing the active conformation.

This work discusses the behaviour of enzymes in organic solvents and tries to explain this behaviour using a thermodynamical approach. Therefore basic information on thermodynamics and enzyme catalysis is provided.

This thermodynamical approach leads to models that are capable of qualitative explanation of observed behaviour and prediction of enzyme behaviour in other solvents. In these models a solvent does not have a direct influence on the enzyme itself, only on the reaction mechanism. This is the basic difference to which this approach leads.

Index

I. Introduction.

Water is a poor solvent for a great majority of applications in industrial chemistry. Most organic compounds of commercial interest are very sparingly soluble and are often unstable in aqueous solutions. Chemists realized these limitations of aqueous-based catalysis and have long ago replaced water with more suitable organic solvents. Unlike chemical processes, conventional biocatalysis has been performed in aqueous solutions. This is mainly due to the preconceived notion that nature intended enzymes to be catalytically active in water and that organic solvents only destroy the catalytic power of enzymes. However, the use of enzymes in non aqueous environments is not restricted to man made situations, many enzymes function in natural hydrophobic environments, usually in the presence of, or immobilized on a membrane. The concentration of water in the vicinity of these enzymes is significantly less than the 55 M in aqueous solutions. It should not be surprising, then, that enzymes are catalytically active in organic solvent systems.
This use of organic solvents offers some potential advantages, for example an increased solubility of non polar substrates, which makes that enzyme catalysis in organic solvents is a well studied subject these days. A lot of experimental data is available upon how enzymes react to organic solvents, considering the enzyme stability, activity and stereoselectivity. Not so much progress is made, however, in explaining the difference in behaviour of enzymes in water (well studied) and organic solvents. This is of interest because it has been found that organic solvents, for most enzyme properties, (except stability) cannot compare to water. In this work an attempt will be made to explain the difference in this behaviour using thermodynamics. To do this, this work has been split into parts, Hx part where thermodynamics is described (chapters II, III and V), a part where enzyme catalysis (stability, activity, mechanism, stereo-selectivity) in general (= water) is described (chapters IV, VI, VII and VIII), and a part where these two are combined to explain the behaviour of enzymes in organic solvents.

In this part (chapter IX) it turns out that it is necessary to define a parameter that characterizes an organic solvent.
It was observed by Laane et al., 1987, that this had to be a parameter that says something about the hydrophobicity of a solvent. They compared different parameters and found that the logarithm of the partition coefficient of a solvent over a water n-octanol two phase system, log P, was the best parameter. The procedure they followed for the selection of this parameter will be described as well as calculation methods for log P.
Looking at enzyme behaviour from a thermodynamic viewpoint offers the possibility to say something about not so well studied behaviour of enzymes like stereoselectivity (chapter IX).
The last objective is to give the reader a survey on enzyme catalysis in non aqueous solvents. This means not only organic solvent systems (chapter IX), but also supercritical solvent systems (chapter X).

II. Chemical equilibrium

II.1 Introduction

It is widely observed that many chemical reactions, if one wants to be rigorous, all reactions, proceed not to completion but to states of equilibrium in which both reactants and products are present in finite concentrations. It is also observed that the rate at which the equilibrium is reached has no influence on the equilibrium conversion and that the thermodynamic equilibrium constant is temperature dependent only. It should also be possible to calculate the equilibrium conversion from the molecular properties of the reactant and product species. This can be done with statistical thermodynamics using classical thermodynamics (Knox,1978).

II.2 Derivation of the thermodynamic equilibrium equation

There are numerous types of equilibrium reactions but the following model reaction can for example be defined

$$a.A + b.B \quad <==> \quad c.C + d.D \qquad \text{(II.2.1)}$$

The classical equation governing the equilibrium equation is

$$a.\mu_A + b.\mu_B = c.\mu_C + d.\mu_D \qquad \text{(II.2.2)}$$

where a,b,c and d are the stoichiometric coefficients of the reactants and the μ's are the chemical potentials of the species in the equilibrium states.
The statistical thermodynamical equation which expresses the dependence of the chemical potentials upon activities is

$$\mu_i = -kT \ln q^0{}_i + kT \ln a_i \qquad \text{(II.2.3)}$$

where the a_i's are the activities of the species and the $q^0{}_i$'s are the molecular partition functions per unit volume. If these last two equations are combined, rearranged and the logarithms are removed we end up with the definition of the thermodynamic equilibrium constant.

$$K_{TH} = \frac{(a_C)^c\,(a_D)^d}{(a_A)^a\,(a_B)^b} = \frac{(q_C^0)^c\,(q_D^0)^d}{(q_A^0)^a\,(q_B^0)^b} \qquad \text{(II.2.4)}$$

In practice a modification is required for this equation for it is assumed that the chemical potentials are measured from a self-consistent zero of energy. In normal thermodynamic usage they would have to be chemical potentials of formation of the reactants and products. In molecular thermodynamics a common energy zero for the energies of the quantum states used in evaluating the $q_i{}^0$'s must be chosen. A reference state that is often used in this perspective is the energy at 0 K. Since it is conventional to measure the energies of quantum states for any system from that of the ground state of the system, an energy correction factor must be introduced if conventional partition functions are to be used in the expression for the equilibrium constant. The partition function for which the energies are based on some arbitrary zero is related to the conventional partition function by the equation

$$q_i{}^0 = q_i{}^{00} \exp- \frac{\epsilon_i{}^0}{kT} \qquad \text{(II.2.5)}$$

where $\epsilon_i{}^0$ is the energy of the ground state of the molecule above the arbitrary zero. The equilibrium equation thus takes the form

$$K_{TH} = \frac{(a_C)^c\,(a_D)^d}{(a_A)^a\,(a_B)^b} = \frac{(q_C^{00})^c\,(q_D^{00})^d}{(q_A^{00})^a\,(q_B^{00})^b} \exp- \frac{\Delta\,\epsilon_0{}^0}{kT} \qquad \text{(II.2.6)}$$

where there is an extra term for the energy differences in ground states of the products and reactants. This is in principle of course the enthalpy of reaction at absolute zero.

The numerical value is equal to that of the molar enthalpy of reaction, E. We now have the basic thermodynamic equilibrium equation. The partition functions have to be factorized into their component parts and the heats of reaction can be obtained with calorimetric methods.
In the equation for the equilibrium constant we find the molecular partition functions. They can of course be written in terms of the partition functions for different modes of motion.

$$q_i^{00} = q^0_{i,trans}\, q_{i,rot}\, q_{i,vib} \qquad \text{(II.2.7)}$$

It follows that the expression for the equilibrium constant may be similarly split into factors. The Q's used are the quotients of the products of the contributions of the products and the reactants.

$$K_{TH} = Q_{trans}\, Q_{rot}\, Q_{vib}\ \exp-\frac{\Delta\ E^0_0}{RT} \qquad \text{(II.2.8)}$$

The usefulness of this expression is that it enables the various component factors in the equilibrium constant to be evaluated independently once the relevant partition functions are known.

There are general expressions for the various partition function based on statistical thermodynamical considerations for an ideal gas (eq. II.2.9), a harmonic oscillator (eq. II.2.10) and a not hindered fixed rotator (eq. II.2.11).

$$q^\circ_{trans} = \left(\frac{2\pi mkT}{h^2}\right)^{\frac{3}{2}} \qquad \text{(II.2.9)}$$

$$q_{vib} = \frac{\exp\left(-\frac{\Theta_v}{2T}\right)}{1 - \exp\left(-\frac{\Theta_v}{T}\right)} \qquad \text{(II.2.10)}$$

where Θ is the characteristic vibrational temperature, and

$$q_{rot} = \frac{\pi}{\sigma} \left(\frac{8\pi^2 kT}{h^2}\right)^2 (IABC)^{\frac{1}{2}} \qquad \text{(II.2.11)}$$

where σ is the molecule symmetry number, I is the moment of inertia for internal rotation and A,B and C are the moments of inertia about the principle axes of rotation.

The disadvantage of this method is that it requires parameters that are not experimentally available and therefore have to be calculated. These calculations are very complicated and demand sophisticated molecular dynamical software. Why does one want to apply this method then if the equilibrium constant can also be calculated from the Gibbs energies of formation, which are experimentally available ? (This method is described in the "afstudeerverslag") The answer is quite simple, in enzyme catalysis the idea of transition states is generally accepted, which means that the equilibrium constant for a transition state is important and this parameter cannot be measured. The state of the art in statistical thermodynamics is such that it is impossible to calculate the equilibrium constant for a transition state. The theory can, however, be used for qualitative predictions.

III. The rate equation.

The absolute rate equation can be derived using the transition state theory. This theory says that reacting molecules first form a transition state complex, which is not stable,and falls apart into the products. An example in this case can be the complex between an enzyme and a reaction participant in enzyme catalyzed reactions. Let us consider the general bimolecular group exchange reaction, which for complete equilibrium is written

$$A + B \leftrightarrows X^{+} \leftrightarrows C + D \qquad \text{(III.1)}$$

where X^{+} is the activated complex. We initially concentrate on those complexes whose rate in the reaction coordinate lie in the range s to s + ds. Their contribution to the total reaction rate will be

$$R_{s,s+ds} = \frac{1}{2} s C^{*}_{s,s+ds} \qquad \text{(III.2)}$$

The origin of this equation can be found in Knox, 1978.
In the equation R is the reaction rate and C^{*} the equilibrium concentration of complexes. This concentration may be obtained by the methods of molecular thermodynamics as shown in the previous chapter and is for ideal mixtures and concentrations in stead of mole fractions given by

$$\frac{C^{*}_{s,s+ds}}{C_A C_B} = \frac{q^{0*}_{s,s+ds}}{q^{0}_{A} q^{0}_{B}} \exp -\frac{\Delta E^{+}_{0}}{RT} \qquad \text{(III.3)}$$

where $q^{o*}_{(s \text{ to } s+ds)}$ is the partition function per unit volume per unit length of the reaction coordinate of the complexes.
E^{+}_{o} is the difference in ground state energy between the reactants and the complex. By the multiplication theorem, since motion in the reaction coordinate is independent of motions in other directions, the partition function for the reaction mode may be factored out of q^{o+} giving:

$$q^{o*}_{(s,s+ds)} = q^{o+}\, q^{o,rc}_{(s,s+ds)} \qquad \text{(III.4)}$$

where $q^{o,rc}_{(s \text{ to } s+ds)}$ is the partition function per unit length of the reaction coordinate for those systems with velocities in the range s to s+ds, and q^{o+} is the partition function for the remaining modes of motion of the complex. Since the energy in the reaction coordinate, ϵ_{rc}, is the same for all states having velocities in the range s to s+ds, the partition function takes the form:

$$q^{o,rc}_{(s,s+ds)} = 2\frac{\mu}{h} \exp\left(-\frac{\epsilon_{rc}}{kT}\right) ds \qquad \text{(III.5)}$$

where

$$2\frac{\mu}{h}\, ds \qquad \text{(III.6)}$$

is the number of translational states in the speed range s to s+ds.

Inserting equation III.5 into equation III.4, and the result into equation III.3 gives after rearrangement:

$$c^*_{s,s+ds} = \frac{q^{o+}}{q^{o}_{A}\, q^{o}_{B}}\, C_A\, C_B \exp\left(-\frac{\Delta E^{+}_{o}}{kT}\right) 2\frac{\mu}{h} \exp\left(-\frac{\epsilon_{rc}}{kT}\right) ds \qquad \text{(III.7)}$$

If this equation is inserted into equation III.2 and integrated between s=0 and s=∞, the total reaction rate takes the form :

$$R = \frac{kT}{h}\, \frac{q^{o+}}{q^{o}_{A}\, q^{o}_{B}} \exp\left(-\frac{\Delta E^{+}_{o}}{kT}\right) C_A\, C_B \qquad \text{(III.8)}$$

or

$$R = \frac{kT}{h}\, K^{+}\, C_A\, C_B \qquad \text{(III.9)}$$

where K^+ is the equilibrium constant for the formation of the complex.

The rate constant for the reaction will now be

$$k_R = \frac{kT}{h} K^+ \qquad \text{(III.10)}$$

The limiting factor in this theory is the complexity of the calculations necessary to obtain the partition functions. This makes it almost impossible to use it in practice at this time. For qualitative considerations however it may turn out to be quite useful.

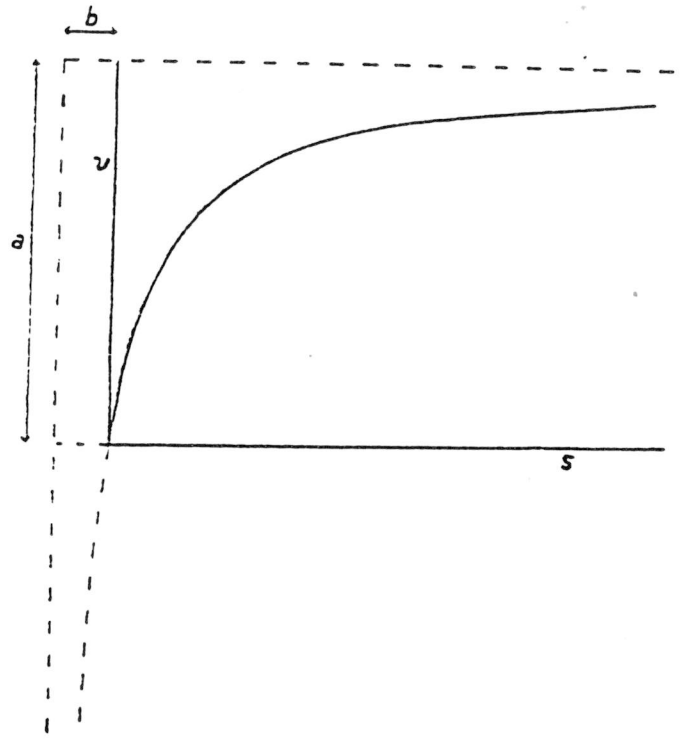


Figure IV.1.1. Hyperbolic form of typical substrate concentration curve

IV. Conventional enzyme kinetics

IV.1 Effect of substrate concentration, the Michaelis theory.

Substrate concentration has turned out to be one of the most important factors which determine the rate of enzyme reactions. In nearly all cases when initial rate is plotted against substrate concentration a section of a rectangular hyperbola is obtained, as shown in figure IV.1.1. The equation describing this curve is :

$$(a - v)\ (b + s) = constant \qquad \text{(IV.1.1)}$$

where s can be the substrate concentration, v the reaction rate and a and b constants. Such a result is obtained whenever a process depends upon simple dissociation; if, for a dissociation XY <==> X + Y, Y is held constant, plotting XY against X will give a curve resembling figure IV.1.1. A theory involving a dissociation of this type was developed by Michaelis and Menten, 1913, and has been the foundation of the greater part of the enzyme kinetics. It adopts the earlier suggestion of Henri, 1902 that enzymes first form a complex with its substrate and this subsequently breaks down giving the free enzyme and the products of the reaction. This theory is known as the transition state theory, the enzyme substrate complex is a transition state. If we write the process in two stages as :

$$E + S \ \text{<==>}\ ES \qquad \text{(IV.1.2)}$$

$$ES\ (+ \text{ any other reactant}) \ \text{==>}\ E + \text{products} \qquad \text{(IV.1.3)}$$

and write s for the concentration (in biochemistry activity coefficients are assumed to be 1 and thus ignored) of free substrate (the amount of enzyme is small so the concentration free substrate is the concentration substrate), e for the total enzyme concentration and p for the concentration of the complex, then since the concentration of free enzyme is e - p,

$$K_s = \frac{(e - p)\ s}{p} \qquad \text{(IV.1.4)}$$

where K_S is the equilibrium constant of the dissociation of ES into E and S and is defined as the substrate constant. This equation applies to equilibrium conditions in which any effect of reaction IV.1.3 on the equilibrium is ignored. If equilibrium IV.1.2 is not attained so rapidly that ES remains always in equilibrium with E and S while the enzyme action is proceeding, the value of p will be less than that given by equation IV.1.4, but for the moment we shall assume with Michaelis that it is that given by rearranging the equation, namely :

$$p = \frac{es}{K_s + s} \qquad \text{(IV.1.5)}$$

The rate of the enzyme reaction is determined by the rate of the brake down by the complex according to chapter III and equation IV.1.3. This will be given by :

$$v = kp \qquad \text{(IV.1.6)}$$

where k is the velocity constant of the breakdown of ES. If a second reaction is involved, its concentration will be included in k. Substituting the value of p from IV.1.5 :

$$v = \frac{k\ e}{1 + \frac{K_s}{s}} \qquad \text{(IV.1.7)}$$

When s becomes large in comparison with K_S, v will become equal to ke; we may write this maximum velocity, obtained when the enzyme is saturated with substrate, as V_m, so that :

$$v = \frac{V_m}{1 + \frac{K_s}{s}} \qquad \text{(IV.1.8)}$$

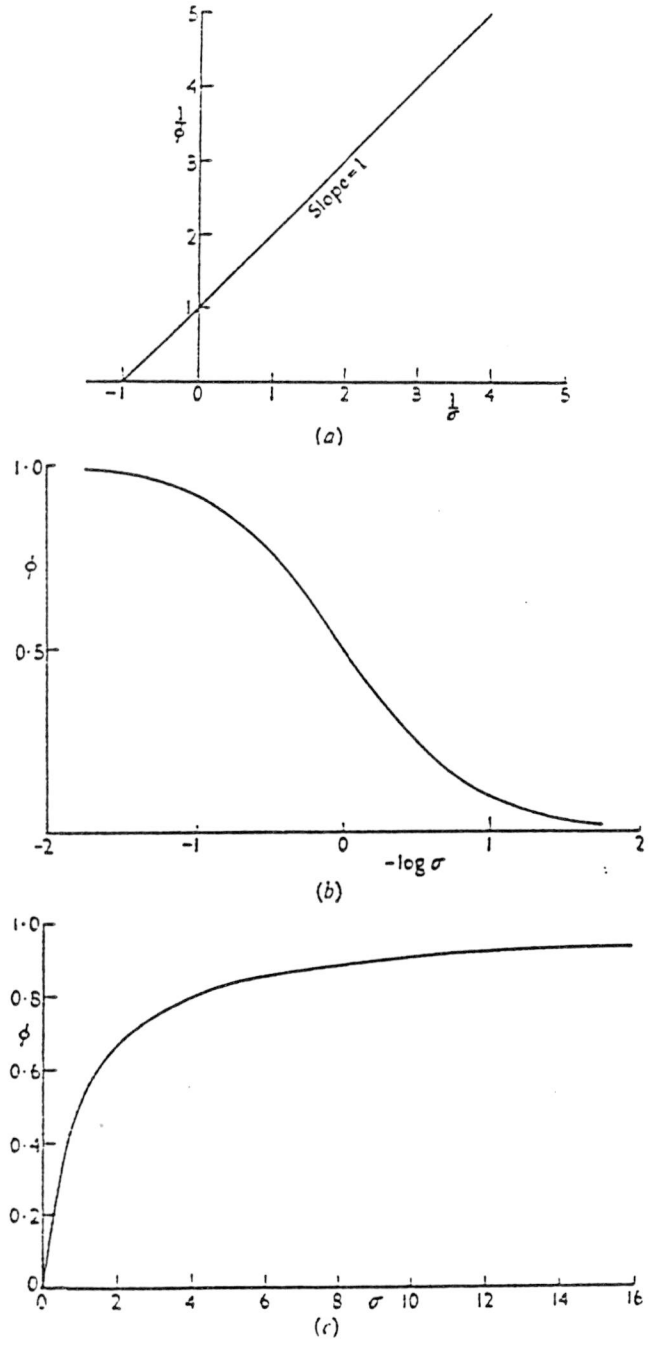


Figure IV.1.2. Different forms of the normalized curves.

This is the well-known Michaelis equation. It may be noted that when s is equal to K_s, v will be equal to $V_m/2$; the value of s which is experimentally found to give half the maximum velocity (the Michaelis constant) is usually written K_m, so that under these conditions $K_m = K_s$.
This equality depends on the validity of the assumption implicit in equation IV.1.5, that the equilibrium is maintained between ES, S and E, which may not always be the case.

Although the assumptions made in this treatment lead to an equation which fits the experimental facts, this does not prove that the assumptions are correct, for a number of other assumptions also lead to the same form of equation. Among possible alternative mechanisms of which this is true are adsorption mechanisms in accordance with the Langmuir isotherm, chain reaction mechanisms and action at distance.

To compare different enzymes the concept of normalized curves has been introduced. First the relative substrate concentration, σ, is defined

$$\sigma = \frac{S}{K_m} \qquad \text{(IV.1.9)}$$

secondly the relative velocity, Φ, is defined as :

$$\Phi = \frac{V}{V_m} \qquad \text{(IV.1.10)}$$

then the Michaelis equation can be written as :

$$\Phi = \frac{\sigma}{1 + \sigma} \qquad \text{(IV.1.11)}$$

This gives the normalized curves, plotted in three forms in figure IV.1.2, which can be applied to any enzyme.

IV.2 Reactions involving two substrates.

The great majority of enzyme reactions are of the type

$$A + B \Longleftrightarrow C + D \qquad \text{(IV.2.1)}$$

in which two substrate molecules react together. The classical theory developed in the previous section deals with the effect of only one substrate concentration and can be applied only to comparatively uncommon cases where a single substrate molecule is involved. The theory has been extended by Haldane,1930 to the case in which both A and B of equation IV.2.1 combine with the enzyme. Assuming the four equilibria :

$$E + A \Longleftrightarrow EA \qquad \text{(IV.2.2)}$$

$$E + B \Longleftrightarrow EB \qquad \text{(IV.2.3)}$$

$$EA + B \Longleftrightarrow EAB \qquad \text{(IV.2.4)}$$

$$EB + A \Longleftrightarrow EAB \qquad \text{(IV.2.5)}$$

we have four corresponding equilibrium equations

$$(e - p_a - p_b - p)\,a = K_a\,p_a \qquad \text{(IV.2.6)}$$

$$(e - p_a - p_b - p)\,b = K_b\,p_b \qquad \text{(IV.2.7)}$$

$$p_a\,b = K'_b\,p \qquad \text{(IV.2.8)}$$

$$p_b\,a = K_a\,p \qquad \text{(IV.2.9)}$$

where a, b, p, p_a, p_b are the concentrations of A, B, EAB, EA and EB respectively, and K_a, K_b, K'_a, K'_b are the respective dissociation constants of the four equilibria. The velocity will be proportional to the concentration of EAB, thus

$$v = kp \qquad \text{(IV.2.10)}$$

Eliminating the p terms from these equations we get :

$$v = \frac{k\,e}{1 + \frac{K_a}{a} + \frac{K_b}{b} + \frac{K_a\,K_b}{ab}} \qquad \text{(IV.2.11)}$$

This equation contains only three of the four equilibrium constants; it can be expressed in terms of any three of the constants, since the fourth is redundant as it is related to the other three by the equation :

$$K_a\,K_b = K_a\,K_b \qquad \text{(IV.2.12)}$$

In deriving equation IV.2.11, no assumptions have been made as to the effect which combination of the enzyme with one substrate may have on its combination with the other.
If each substrate combines only with its own specific site and there is no effect of one substrate on the affinity for the other, then $K_a = K'_a$ and $K_b = K'_b$. Equation IV.2.11 then reduces to :

$$v = \frac{ke\,\frac{ab}{K_a\,K_b}}{(1 + \frac{a}{K_a})\,(1 + \frac{b}{K_b})} \qquad \text{(IV.2.13)}$$

Making use of relative concentrations as previously defined and writing $\alpha = a/K_a$ and $\beta = b/K_b$, this equation becomes

$$\Phi = \frac{\alpha}{1 + \alpha}\,\frac{\beta}{1 + \beta} \qquad \text{(IV.2.14)}$$

This looks like the product of two Michaelis functions, one for each substrate. As the affinity of each substrate in this case is independent of combination of the enzyme with the other, the true Michaelis constants may be determined by variation of each substrate concentration at any fixed concentration of the other.

It is possible to think of reactions between two substances as occurring without a definite combination of one of them with the enzyme, the second reactant being involved in a bimolecular reaction with a complex of the first reactant with the enzyme, thus :

$$E + A \Longleftrightarrow EA \qquad \text{(IV.2.15)}$$

$$EA + B \Longleftrightarrow E + C + D \qquad \text{(IV.2.16)}$$

In this case equation IV.1.5 must be replaced by

$$v = K'bp_a \qquad \text{(IV.2.17)}$$

where k'is now a bimolecular velocity constant and p_a is as before the concentration of EA. Equation IV.2.14 is then replaced by :

$$\Phi = \frac{\alpha}{1 + \alpha} b \qquad \text{(IV.2.18)}$$

It follows that as the concentration A is increased, the velocity rises to a limit, but as the concentration of B is increased, it rises linearly. This, however, is only true if K_a is the actual dissociation constant of the reaction IV.2.15.

V. Thermodynamics of irreversible processes

Thermodynamics of irreversible processes can be useful for predicting the speed at which an equilibrium reaction reaches its equilibrium and whether coupled reactions can occur or not. (Prigogine, 1955) In these considerations the entropy production and the affinity are the important quantities. What they are, how they can be calculated and used will be described in this chapter.

V.1 Conservation of mass in a closed system

If we have a closed system with a number of components among which a reaction is possible, the variation of masses in this system can only be caused by a reaction. The change of mass of component i during time interval dt can be written as :

$$dm_i = \nu_i M_i \, d\xi \qquad \text{(V.1.1)}$$

where M_i is the molar mass of component i, ν_i its stoichiometric factor and ξ the degree of advancement or extent of the reaction as introduced by De Donder, 1920.
The total mass of the system is given by

$$m = \sum_i m_i \qquad \text{(V.1.2)}$$

The total amount of mass in a closed system has to be constant. This is the principle of the conservation of mass. This leads to the following expression:

$$dm = (\sum_i \nu_i M_i) \, d\xi = 0 \qquad \text{(V.1.3)}$$

Instead of the mass of a component i it is often useful to consider the amount of moles, n_i, of that component.

We then have

$$dn_i = \nu_i \, d\xi \tag{V.1.4}$$

The extent of the reaction per unit time is the reaction rate, v :

$$v = \frac{d\xi}{dt} \tag{V.1.5}$$

This leads to the next expression for the increase on the number of moles of component i:

$$\frac{dn_i}{dt} = \nu_i \, v \tag{V.1.6}$$

These considerations can easily be extended to n simultaneous reactions. For the total change in mass of component i, which is the sum of the changes resulting from the different reactions (index j), this leads to the expression :

$$dm_i = M_i \sum_{j=1}^{n} \nu_{ij} \, d\xi_j \tag{V.1.7}$$

For the number of moles of component i:

$$dn_i = \sum_{j=1}^{n} \nu_{ij} \, d\xi_i \tag{V.1.8}$$

V.2 Entropy production

The second principle of thermodynamics postulates the existence of a function of state, called entropy which possesses the following properties:

- The entropy of the system is an extensive property. This means that entropy is an additive property.
- The change of entropy, dS, can be split into two parts, d_eS is the flow of entropy due to interactions with the exterior and d_iS is the change in entropy due to changes inside the system.

We thus have

$$dS = d_eS + d_iS \qquad \text{(V.2.1)}$$

The change in entropy inside the system is never negative. It is zero for reversible processes and positive for irreversible processes. This is at the same time the definition of irreversibility. For isolated systems there is no interaction with the exterior so :

$$dS = d_iS \geq 0 \qquad \text{(V.2.2)}$$

The entropy is defined by the formula :

$$dS = \frac{dE}{T} + \frac{p}{T}dV - \sum_i \frac{\mu_i}{T}dn_i \qquad \text{(V.2.3)}$$

where T is the absolute temperature, E the internal energy, p the pressure, V the volume and μ the chemical potential.

V.3 Affinity and coupling of reactions

De Donder, 1920 introduced the term affinity, A, for chemical reactions. This affinity is a measure for the driving force of a reaction towards it equilibrium and is defined as :

$$A = - \sum_i \nu_i \mu_i \qquad \text{(V.3.1)}$$

From the equilibrium theory it follows that A is zero if the equilibrium is reached. A is positive for non equilibrium conditions. If there are more than one reaction, each reaction has its own affinity.

If we have a reaction in a closed system, the entropy production, in case of an irreversible process, will only be caused by the reaction :

$$dS = \frac{A\, d\xi}{T} \qquad \text{(V.3.2)}$$

This has of course a positive value, but what happens if we have more than one reaction in this closed system, for example if we have n reactions. As already stated each reaction has its own affinity. This combined with the fact that entropy is an additive quantity leads to the next equation for the entropy production in this system :

$$dS = \frac{1}{T} \sum_{j=1}^{n} A_j\, d\xi_j \qquad \text{(V.3.3)}$$

or, if we want to consider the entropy production per unit time

$$\frac{dS}{dt} = \frac{1}{T} \sum_{j=1}^{n} A_j\, v_j \qquad \text{(V.3.4)}$$

And again, this has to have a positive value.

This equation leads to an interesting conclusions, if the system undergoes for example two simultaneous reactions it may happen that $A_1v_1<0$ and $A_2v_2>0$ provided that $A_1v_1 + A_2v_2 > 0$. This means that a reaction can progress against its own affinity and thus causing a decrease in entropy provided that a coupled reaction compensates for this loss of entropy in such a way that the total system has an increase in entropy. In other words, one reaction can pay for another.
This fact is very important for biological processes.

V.4 Affinity and the reaction rate.

Suppose we have a closed system with the following reaction :

$$A + B \Longleftrightarrow C + D \qquad (V.4.1)$$

The corresponding affinity will in this case be :

$$A = \mu_A + \mu_B - \mu_C - \mu_D \qquad (V.4.2)$$

The chemical potential for component i may be written as

$$\mu_i = \mu^\circ_i + RT \ln a_i \qquad (V.4.3)$$

If this equation is combined with the expression for the equilibrium constant, the expression for the affinity becomes:

$$A = RT \ln \frac{K_{TH}}{Q} \qquad (V.4.4)$$

where

$$Q = \frac{a_C\, a_D}{a_A\, a_B} \qquad (V.4.5)$$

If equilibrium is reached Q is equal to the equilibrium constant and the affinity becomes zero.
The usual expression for the reaction rate of the reaction is given by

$$v = \vec{v} - \overleftarrow{v} = \vec{k}\, a_A\, a_B - \overleftarrow{k}\, a_C\, a_D = \vec{k}\, a_A\, a_B \left(1 - \frac{\overleftarrow{k}}{\vec{k}}\, \frac{a_C\, a_D}{a_A\, a_B}\right) \qquad \text{(V.4.6)}$$

The equilibrium constant is equal to the ratio of the rate constants of the reaction so this equation can be rewritten as

$$v = \vec{v}\left(1 - \exp\left(-\frac{A}{RT}\right)\right) \qquad \text{(V.4.7)}$$

This equation describes the relation between the reaction rate and the affinity. It is clear that initially the reaction rate is equal to the forward reaction rate, is decreased by decreasing affinity and by decreasing reactant activities, eventually ending at zero. This is the nett reaction rate. There is of course a forward and backward reaction but the rates are equal in equilibrium so the nett reaction rate is zero. This theory will be used in a next chapter.

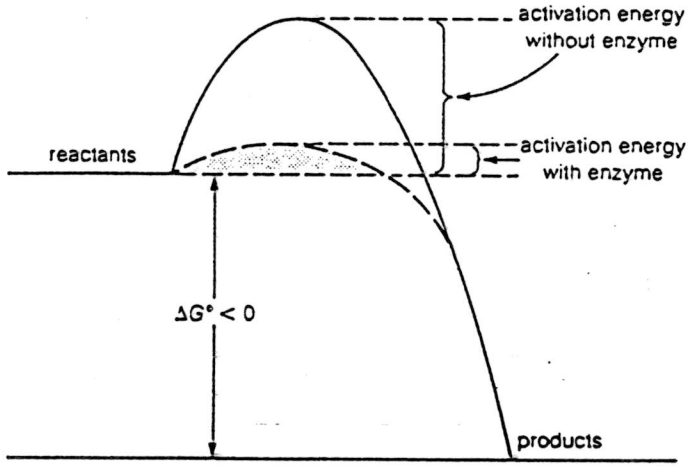


Figure VI.1.1 Principle of activation energy.

VI. The role of enzymes in reactions

VI.1 Introduction

The laws of thermodynamics as described up to this point apply to all chemical reactions. To be feasible and spontaneous a reaction must have a negative change in Gibbs free energy or must be coupled with a reaction in such a way that the total change in Gibbs free energy is negative. What effects do enzymes then have? The answer is simply that enzymes greatly increase the rate at which spontaneous reactions take place. They influence the rate at which the equilibrium is reached and not the equilibrium itself. The next question is then, how do enzymes accomplish that fact? Their basic effect is to reduce the activation energy required for a chemical reaction to occur. The activation energy may be visualized as an energy barrier over which the molecules in a system must be raised for reaction to occur (fig VI.1.1). The requirement for the energy of activation raises the question of why reactions proceed spontaneously at all. In other words, how are some molecules in the system spontaneously raised over the energy barrier? This happens because the molecules of a system held above absolute zero are in constant motion. The kinetic energy is not the same in all molecules of the system, although the statistical average is below the energy required for activation. Some molecules in the system however can have a kinetic energy above average, some below average. The distribution occurs partly as a result of random collisions in which energy is gained and lost by individual molecules, depending on factors such as angles of collision and their number per unit time. Depending on the height of the activation barrier, random collision may raise a number of molecules to the energy level required for the reaction to proceed. This happens when molecules strike each other at the correct angle and with sufficient force for interaction to occur. A high activation barrier indicates that successful collisions of this type are highly improbable.

One way to increase the probability that molecules in the reacting system will pass over the energy barrier is to raise the temperature. At elevated temperatures, both the speed of travel of individual molecules and the frequency of collisions increase, raising the probability that sufficient forceful collisions at the correct angle and place will occur. Once large numbers of molecules begin to pass the barrier, the reaction is frequently self-sustaining. In this case, sufficient energy is released by molecules converted to keep the reaction temperature high. In many cases however the raise of temperature is not a satisfactory approach for pushing the reactants over the energy barrier. It increases the amount of side reactions, sometimes the temperature has to be so high that one or more of the reaction participants becomes unstable and elevated temperatures are expensive.

Enzymes increase the probability that molecules will react by a different mechanism, by reducing the activation energy. The effect is to reduce the force and number of collisions required for interaction, thereby allowing a reaction to proceed at much lower temperatures. As a part of their function in lowering the activation energy, which may involve one or more possible mechanisms (see below), enzymes combine temporarily with the reacting molecules.

Enzyme molecules have several important characteristics. First, all known enzymes are proteins. Second, in catalyzing reactions enzymes combine with the reacting molecules only very briefly and are released unchanged when the reaction is completed. Third, the number of reactions catalyzed by a single enzyme molecule, depending on the enzyme, may vary from one hundred to more than 3 million per minute; therefore, only a small number of enzyme molecules is needed to catalyze a large quantity of reactants. Finally, enzymes are specific in their catalytic activity: Usually they are tailored to catalyze only a single type of reaction and combine only with specific, single molecular types or closely related groups of molecules. The specific molecule or molecular group whose reaction is catalyzed is known as the substrate of the enzyme.

MOLECULAR WEIGHTS OF ENZYMES

Ribonuclease (502)	12,700
Lysozyme (154) (egg white)	17,000
Malate dehydrogenase (298) (ox heart)	15,000–20,000
γ-chymotrypsin (5)	15,000–25,000
Papain (14)	20,700
α-chymotrypsin (5)	21,600–25,000
α-chymotrypsin (dimer)	42,900
Chymotrypsin B (6)	22,500
β-chymotrypsin (5)	23,000
Trypsin (4)	23,800
Carbonic anhydrase (620)	30,000
Polymetaphosphatase (178)	33,000
Carboxypeptidase (33)	34,300
Pepsin (1)	34,500
Thiosulphate transsulphurase (577)	37,000
Peroxidase (379) (horseradish)	40,000
Rennin (3)	40,000
Malate dehydrogenase (298) (pig heart)	40,000
Chymopapain (15)	45,000
α-amylase (138) (pancreas)	45,000
α-amylase (138) (malt)	59,000
L-aminoacid oxidase (223) (snake venom)	61,600
Deoxyribonuclease I (123)	63,000
Inorganic pyrophosphatase (177)	63,000
Enolase (619) (yeast)	64,000
*iso*citrate dehydrogenase (304) (heart)	64,000
Alcohol dehydrogenase (260) (liver)	73,000
Phosphoglucomutase (477)	74,000–78,000
CoII-cytochrome *c* reductase (247) (yeast)	75,000
CoI-cytochrome *c* reductase (246) (animal)	75,000–80,000
Leucine aminopeptidase (38)	75,000–80,000
CoI-diaphorase (242) (pig heart)	81,000
Creatine phosphokinase (472)	81,000
Lactoperoxidase (380)	82,000
Hexokinase (427) (yeast)	96,600
Urate oxidase (377)	100,000
o-diphenol oxidase (369)	100,000
Lactate dehydrogenase (289) (yeast)	100,000
Luciferase (486) (fireflies)	100,000
Lipoxidase (372) (soy bean)	102,000
Phosphoglyceraldehyde dehydrogenase (331) (yeast)	122,000
L-aminoacid oxidase (223) (animal)	*about* 130,000

Table VI.1.1 Molecular weigths of some enzymes.

Thousands of different enzymes have been detected and described. Of these, several hundred have been purified to the extent that they can be crystallized and chemically characterized. These enzymes vary from molecules having about 100 amino acids to large complexes containing several thousands amino acids (see table VI.1.1). In addition, many enzymes are complexed with an inorganic ion or non-protein organic group that contributes to their catalytic function. These non-protein groups are called cofactors.
Enzymes are named and placed in one of six major classes according to their substrate and the type of reaction they catalyze (see table VI.1.2). Within these six classes the enzymes are named by adding -ase to the specific substrate and reaction catalyzed. Some enzymes are still known under their original, trivial, name such as pepsin and tripsin.

VI.2 How enzymes lower the activation energy

The possible mechanisms by which enzymes lower the activation energy have been subject of much research and speculation. There is good evidence that enzymes actually combine with their substrates during catalysis. Many enzymes absorb light at characteristic wavelengths when in solution. The energy of the wavelengths absorbed is taken up by electrons occupying orbitals within the enzyme molecules; as the conformation changes the specific wavelengths absorbed change. Alterations in the wavelengths absorbed by enzymes are noted when they are mixed with their substrates. These changes are interpreted to mean that the enzyme conformation has changed due to substrate binding. Measurement of the duration of the absorption changes indicates that the enzyme-substrate complex lasts only microseconds. Emil Fischer proposed in 1884 the "lock and key" hypothesis of enzyme substrate interaction. The active site of an enzyme was believed to contain a fixed arrangement of chemical groups.

Class and Subclass	General Reaction Type
Oxidoreductases	Removal and addition of electrons or of electrons and hydrogen
Oxidases	
Reductases	
Dehydrogenases	
Transferases	Transferring chemical groups
Kinases	Transferring phosphate groups
Hydrolases	Breakage of chemical bonds by addition or removal of the elements of a molecule of water
Proteases	Hydrolysis of proteins
Ribonucleases	Hydrolysis of RNA
Deoxyribonucleases	Hydrolysis of DNA
Lyases	Formation of double bonds by elimination of a chemical group
Isomerases	Rearrangements of the atoms of a molecule
Ligases or Synthetases	Formation of chemical bonds using ATP or other nucleotides as an energy source
Polymerases	Linkage of identical subunits (monomers) into a polymer

Table VI.1.2 Enzyme classification.

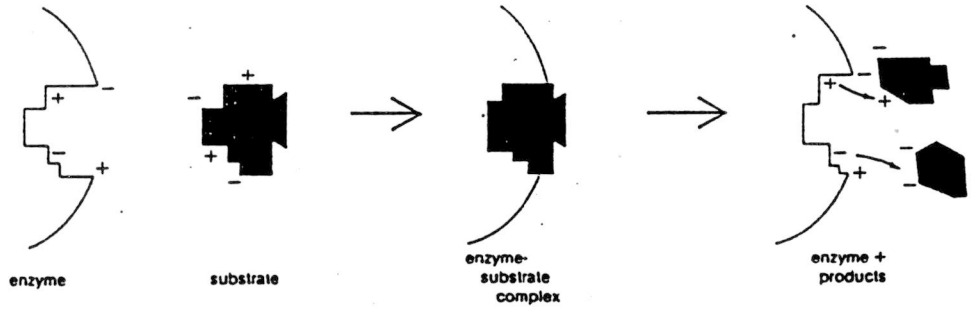


Figure VI.2.1 Lock and key model for enzyme substrate interaction.

These groups, according to this proposal, are held in a surface conformation that precisely matches and complements chemical groups on the substrate molecule (fig VI.2.1). Amino acid side chains, held in the active side by the three dimensional structure of the enzyme protein, contribute to the binding groups in the active site. These groups, including charged, polar, and nonpolar regions of the amino acid side chains, form a "lock" that only a specific substrate "key" can fit.

The contemporary view of the active side still maintains that its binding sites are shaped to fit the substrate. However, the observations made in light absorption and X-ray diffraction studies suggests that, rather than being rigid, the enzyme site is flexible and changes in conformation during binding. These conformational changes, according to D.E. Koshland, 1973, who first hypothesized the flexible nature of the active site, contribute to the catalytic function of the enzyme (fig VI.2.2). Conformational changes in the active site induce an intermediate condition of the reactants known as the transition state. During any chemical reaction the reactants briefly enter a state in which the "old" chemical bonds are incompletely broken and the "new" chemical bonds are incompletely formed. What this means for the reaction rate has been shown in a previous chapter.

According to the current hypotheses of enzymatic catalysis, the active site, rather than fitting only the reactant molecule in a rigid lock and key fashion, has additional conformational arrangements that can also fit the transition state and the products. Of these, the tightest binding and the most precise fit is to the transition state. Substrate binding and catalysis would then proceed as follows. The initial conformation of the active site exposes chemical groups tailored to fit the reactants closely but not precisely. Binding of the reactants induces changes in the conformation of the enzyme molecules, changing the active site to a conformation that precisely matches the transition state. This tight fit induces the substrate to assume the transition state.

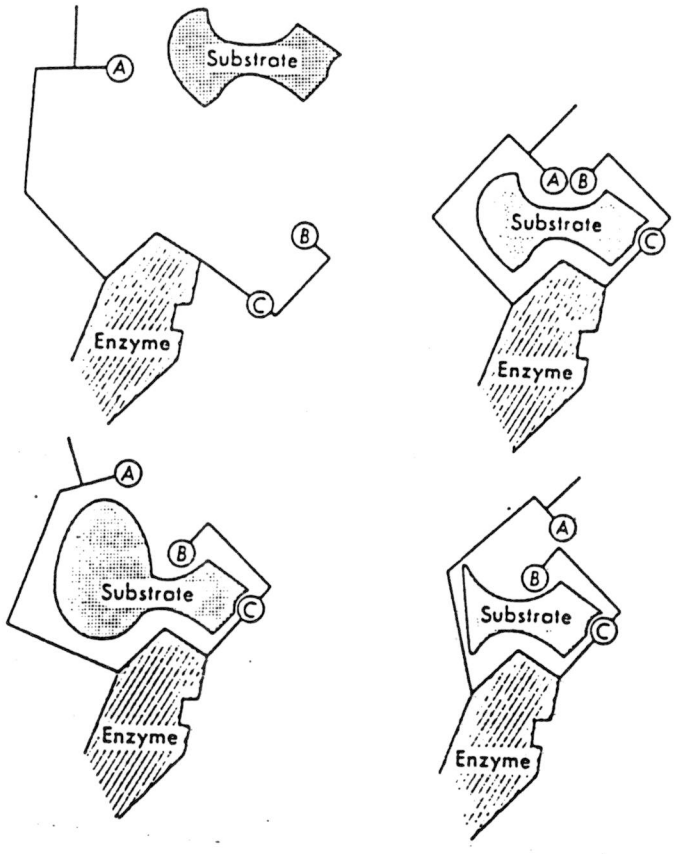


Figure VI.2.2 The flexible active site model.

Once in this unstable state, the reacting molecules are easily pushed in the direction of the products by catalytic groups in the active site that are brought into position to further weaken the old bonds in the substrate and favour formation of the new ones.

The idea that the active site also contains conformations that fit the products, which at first seems very surprising helps explain the role of enzymes in catalyzing reversible reactions, a capability never adequately explained by the rigid lock and key hypothesis. Depending on the relative concentration of reactants and products and the position taken by the equilibrium constant for a reaction, enzymes may either combine with reactants, speeding their conversion to products, or with products, speeding their conversion to reactants.

This capability is easily accommodated by the new model for the active site. According to the new hypothesis, which of the two molecular groups, reactants or products, actually binds to the active site depends on its activity in the medium. Once either is bound, the enzyme undergoes its conformational changes in response, altering the active site to the conformation that fits the transition state. Once in the unstable transition state, the reaction can proceed in either direction.

VI.3 Catalytic mechanisms at the active site

Much speculations currently centres on the precise nature of the molecular interactions taking place at the enzyme active site to induce formation of the transition state. These interactions are ultimately reflected in the reduction of the activation energy for a given reaction, thus increasing the reaction rate.

Four possible interactions are believed to contribute to the transition state, either singly or in combination:

- positioning substrate molecules in an alignment promoting the transition state,
- inducing "strain" in substrate molecules by distorting them towards the transition state,
- placing proton donors or acceptors on positions promoting disturbance of substrate bonds towards transition state,
- exposing substrates to nonpolar environments that alter their conformation towards the transition state.

Alignment of substrate molecules. Precise alignment of substrate molecules within the active site has several important effects in inducing the transition state and reducing the energy of activation. Bringing reactant molecules closer together raises their effective concentration and greatly increases the probability of a collision. Alignment also places the reactants in an arrangement in which they collide at the correct angles and positions.

Inducing "strain" in substrate molecules. Distortion of substrate molecules through binding to the active site may directly bend constituent atoms towards angles approximating the transition state. In addition, binding may increase the distance between substrate atoms, thus weakening the attractive force of the bonds and making them easier to break.

Placement of proton donors or acceptors. Certain chemical groups carried on amino acid side chains, when brought into the proximity of the substrate molecule, can act as donors or acceptors of protons. This creates localized conditions of acidity or basicity different from the pH of the medium. The altered pH of the active site can promote conversion of the substrate to the transition state and products.

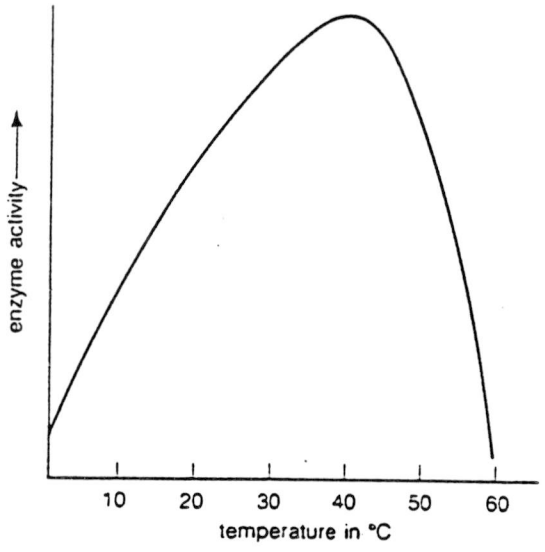


Figure VI.4.1 Dependence of enzyme activity on temperature.

Exposure of substrate groups to nonpolar environment. Regions within the enzyme active site may also contain nonpolar amino acid side chains. These nonpolar groups, unexposed in the unbound form of the enzyme, could be brought into the proximity of the substrate as a part of the conformational changes occurring in response to enzyme-substrate binding, creating a nonpolar or hydrophobic environment around the reacting molecules. This environment enhances reactions that take place more easily in nonpolar environments, reducing the energy barrier imposed for these reactions by polarity of the medium.

VI.4 The effects of the temperature on the enzyme activity

Enzymatic catalysis depends on the three dimensional structure of the active site. As might be expected, exposing enzymes to conditions that alter their three dimensional structure seriously interferes with catalysis. Proteins are held in their final structure by interactions among their component amino acid side chains. The pattern and strength of two types of these interactions, hydrogen bonding and electrostatic attractions, are highly sensitive to changes in temperature and pH of the medium surrounding the enzyme. In case of an organic solvent, dealt with in a special chapter, the polarity of the solvent has a major effect too.

Gradual increases in temperature increase the kinetic motion of both the component amino acid chains forming an enzyme molecule and the molecules bombarding the enzyme from the surrounding solution. At elevated temperature, these disturbances become strong enough to overcome the attraction of hydrogen bonds, which are individually relatively weak, causing the enzyme gradually to unfold and lose its "correct" three dimensional form.

These changes affect the enzymatic activity in a characteristic way (fig VI.4.1).

Rises in temperature over the range from 0 to about 40 °C increase the activity of most enzymes along the lines followed by all chemical reactions; each 10 °C rise in temperature approximately doubles the reaction rate. This effect is due to increases in the force and frequency of collisions of enzyme and reactant molecules in the solution, reflecting the increased kinetic motion of all molecules in the solution. However, in enzymatically catalyzed reactions, rates begin to fall off at temperatures above approximately 40 °C. The drop observed in activity for most enzymes becomes precipitous at 55 °C and falls to zero at 60 °C. At these temperatures, disturbance in hydrogen bonding causes the enzyme to unfold into an inactive form, counteracting the positive effects of increased kinetic motion at elevated temperatures. (Tanford, 1968, 1970; Ahern et al., 1985; Zale et al., 1986)
Denaturation caused by temperature is irreversible. An enzyme molecule is in principle a meta stable molecule.

As a result of these two opposing effects of increased temperatures, all enzymes exhibit an activity optimum representing the highest temperature at which no significant disturbance of internal hydrogen bonding and three dimensional structure occurs. This transition temperature of protein defolding can be measured by differential calorimetry (Donovan, 1984). For most enzymes, this optimum lies between 40 and 50 °C. A few organisms, such as bacteria living in hot springs, possess enzymes with an elevated temperature optimum, some of which remain active at temperatures of 85 °C or more.
All these results have been obtained using water as the solvent for the enzyme and this water plays an important role as a catalyst for the denaturating reactions. This will turn out to be important if enzymes are used in organic solvents. Solid dry enzymes have shown to be quite thermostable. (Mulhaney, 1966)

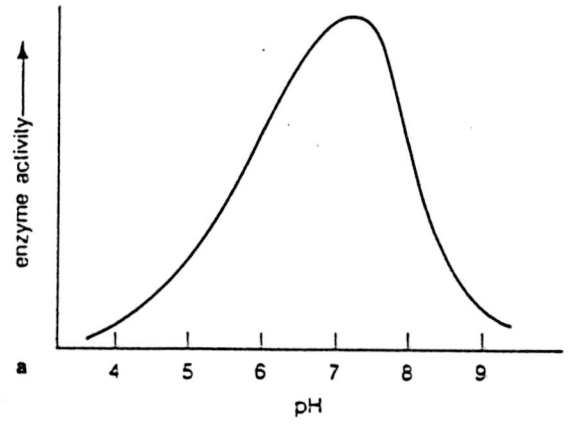


Figure VI.5.1 Optimum enzyme activity at intermediate pH

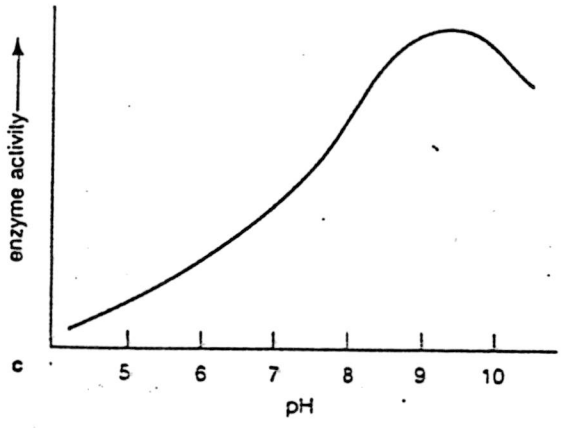


Figure VI.5.2 Optimum enzyme activity at high pH.

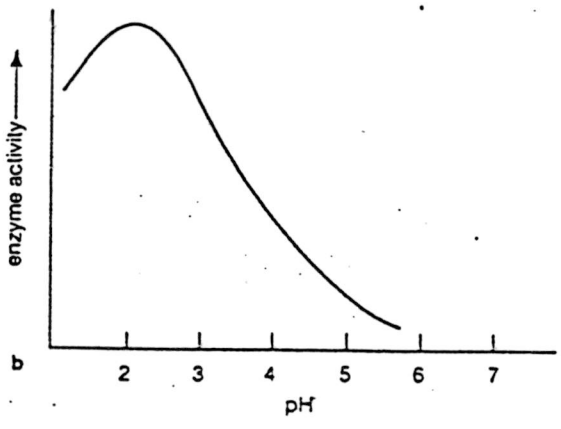


Figure VI.5.3 Optimum enzyme activity at low pH.

VI.5 The effects of the pH on the enzyme activity.

Changes in the pH of the medium surrounding the enzymes affect enzyme structure and activity by altering the charge of groups carried on the amino acid side chains. Particularly important among the affected groups are those, such as the -COOH and $-NH_2$ groups, capable of releasing or accepting protons and converting to a charged form. Each of these groups, depending on their location in a protein molecule, undergoes conversion from uncharged to charged at a characteristic pH. Changes of this type affect both the charge of groups holding enzymes in their final three dimensional shape and the activity of proton donors and acceptors as catalytic groups within the active site. As a result, changes in the pH affect the activity of enzymes in characteristic ways. Most enzymes reach an optimum pH level at intermediate pH (fig VI.5.1). Others show optimum catalytic activity at high (fig VI.5.2) or low pH (fig VI.5.3).

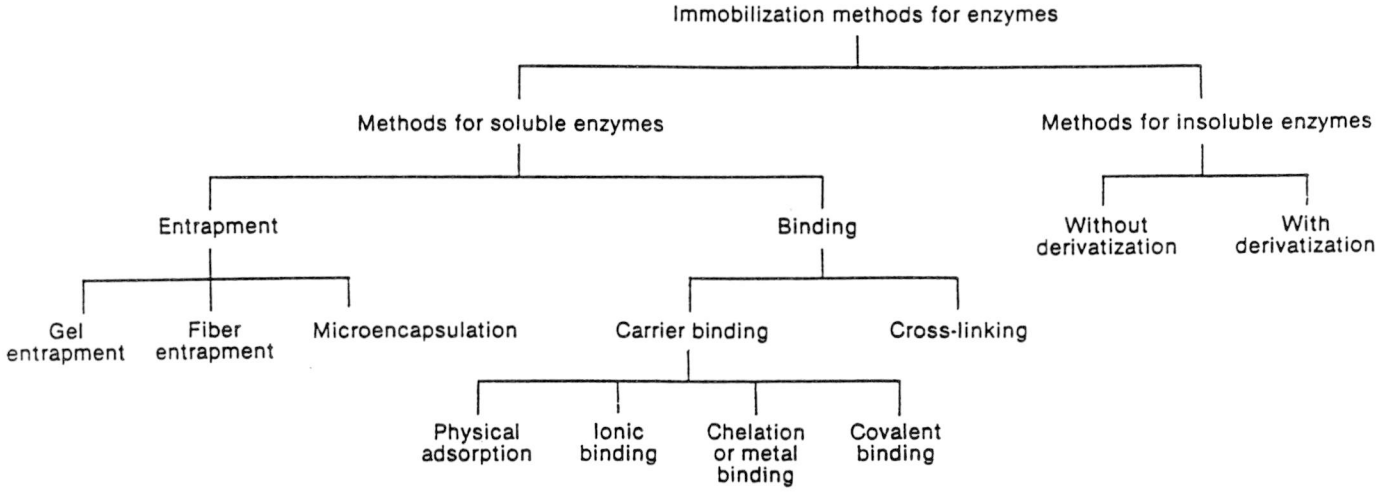


Figure VII.2.1 Possible classification method for immobilized enzymes.

Characteristic	Cross-linking	Physical Adsorption	Ionic Binding	Metal Binding	Covalent Binding	Entrapping
Preparation	Intermediate	Simple	Simple	Simple	Difficult	Difficult
Binding force	Strong	Weak	Intermediate	Intermediate	Strong	Intermediate
Enzyme activity	Low	Intermediate	High	High	High	Low
Regeneration of carrier	Impossible	Possible	Possible	Intermediate	High	Intermediate
Cost of immobilization	Intermediate	Low	Low	Intermediate	High	Intermediate
Stability	High	Low	Intermediate	Intermediate	High	High
General	No	Yes	Yes	Yes	No	Yes
Protection of enzyme from microbial attack	Somewhat	No	No	No	No	Yes

Figure VII.3.1 Possible classification method for immobilized enzymes

VII. Stabilization of enzymes, immobilization.

VII.1 Introduction

Enzyme specifity is clearly an important consideration when choosing catalysts for reactions of commercial interest. The cost of producing the necessary enzyme is, however, often prohibitive. In this context immobilized enzyme preparations can be more effective because immobilized enzymes are easy recoverable and more stable than free enzymes (reviews : Martinek et al., 1977; Koch-Schmidt et al., 1977; Schmid et al. 1979; Mozhaev, 1984). For this reason the prospects for immobilized enzymes are fundamentally bright although there is always a difficulty in introducing a new technique in well established processes.

VII.2 Immobilized enzymes

Enzymes that are physically confined or localized in a micro-environment with retention of their catalytic activities and that can be used repeatedly and continuously are defined as immobilized enzymes. The various types of immobilized enzymes can be classified in several ways. A possible way is shown in figure VII.2.1.

VII.3 Methods of immobilization

A large number of methods for enzyme immobilization has been developed during the last decades, and the number continues to expand rapidly. Using the classification of table VII.3.1 the various methods will be described.

VII.3.1 Cross-linking

This method is based on the formation of covalent bonds either between enzyme molecules in the absence of a solid support or between enzyme and a support by means of bi- or multifunctional reagents. The cross-linking between enzyme molecules leads to three-dimensional cross-linked aggregates that are completely insoluble in water but do not require water insoluble supports. This method can also be applied with a carrierbinding method to allow cross linking between adjacent enzyme molecules on the support surface, which increases the strength of immobilization. The use of reagents with two identical functional groups or two or more different functional groups is necessary for a cross-linking reaction, the latter being more frequently used in binding enzymes to insoluble carriers than in intermolecular cross-linking reactions.
The major difference between cross-linking and covalent binding methods, however, is the bridge between enzyme molecules or between the enzyme and carrier in cross-linking while in covalent binding the enzyme is bonded directly to the support. As a result, much of the enzyme used in the cross-linking method acts only as a support and is not available for catalysis.

VII.3.2 Physical Adsorption

This is the oldest and simplest method of enzyme immobilization. Adsorption is based on the contact between the enzyme and the surface of the support. Depending on the nature of the support, the enzyme binding may be the result of ionic interactions, physical adsorption, hydrophobic bonding, or Van Der Waals attractive forces or a combination of these. The procedure is based on mixing the enzyme and the support material under appropriate conditions and, following a period of interaction, post-separation of the insoluble enzyme derivative from the material by centrifugation or filtration.

Because no reactive species are involved, there is little or no conformational change in the enzyme on immobilization; therefore, a derivative with an activity similar to that of the original enzyme can be obtained. The dependence of the enzyme adsorption involves experimental variables, such as pH, the nature of the solvent, the ionic strength, the concentration of the enzyme and carrier and the temperature.
The advantage in using adsorption to fix an enzyme to a support materials lies in the simplicity, the wide variety of adsorbents that can be used and the reusability of the adsorbent. The major disadvantage is that desorption can occur because of the generally limiting strength of the binding forces.

VII.3.3 Ionic binding

Like the Van Der Waals binding method, this is an old and simple way of immobilizing enzymes. In this method the enzymes are fixed onto solid supports containing ion-exchange residues. Physical adsorption may also take place. The main difference between physical adsorption and ionic binding is the strength of the linkage between the enzyme and the support. The binding forces in the case of ionic binding are stronger than those in the case of physical adsorption. This method of immobilization depends on the pH and the ionic strength of the surrounding medium; changes in pH and ionic strength may, in some situations, result in leakage of the enzyme from the support.
Ion-exchange materials are used as a solid support for the ionic binding. Organic supports are most frequently used with ion-exchange residues, although there are reports of inorganic supports being used. According to the type of ion-exchange residues, these supports can be classified as anion or cation exchangers.

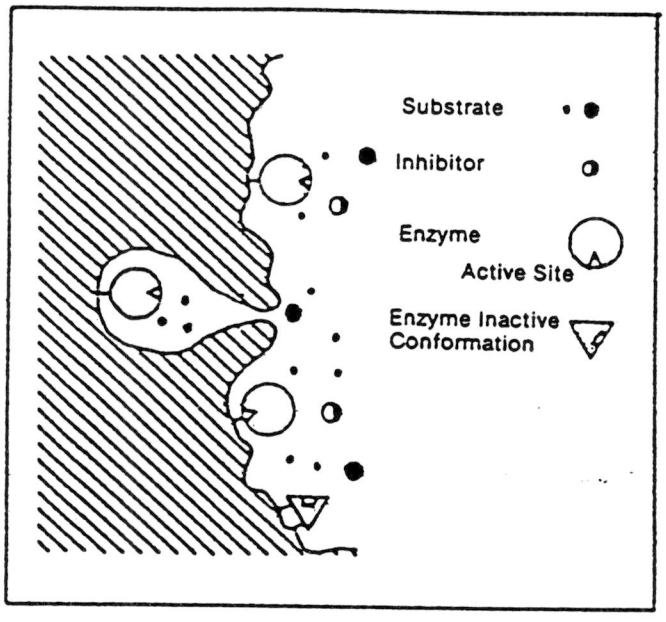


Figure VII.3.5.1 Influence of polymer matrix on immobilized enzyme.

VII.3.4 Metal binding

This relatively new technique involves the use of transition metal compounds as a means of activating the surface of a support and allowing direct coupling of the enzyme with no prior derivatization of the activated support through the formation of chelates. The activating transition metals are normally titanium, iron, zirconium, vanadium and zinc. Organic support material can be cellulose, chitin and alginic acid, inorganic carriers can be on silica bases, celite and glass.

VII.3.5 Covalent binding

Covalent binding for the immobilization of enzymes is based on the covalent attachment of enzymes to water-insoluble supports. The selection of conditions for immobilization by covalent binding is more difficult than in other carrier binding methods. Only a few supports contain reactive groups for direct coupling enzymes. The reagents used must not affect the binding or the essential amino acid residues of the active side of the enzyme in order to avoid steric effects of the polymer matrix on immobilized enzyme. (fig VII.3.5.1) After covalent bonds have been formed, stable immobilized enzyme preparations can be obtained.
The strong nature of the covalent binding does not allow the enzyme to leak into the solution containing substrate.

VII.3.6 Entrapping

The techniques for immobilization reviewed involve modification of the enzyme molecule and/or its microenvironment with a subsequent alteration of its kinetics and, sometimes, pH and temperature profiles. Reduced activity often results from these alterations.

Enzymes have been immobilized by physically confining them with semipermeable membranes in hollow fibres or ultrafiltration membrane devices in order to utilize an enzyme in its native state continoulsy over a long period of time. This has the advantage that chemical alterations of the enzyme do not occur which makes the method especially suitable for high-molecular-weight substrate. Other advantages include the simplicity of the immobilization method, the simultaneous immobilization of different enzymes, selective control of substrate and products through membrane selectivity and the absence of enzyme leakage. The disadvantages are inherent in this method; possible loss of reaction velocity due to the permeability resistance and the difficulty of working at low concentrations due to substrate absorption by membranes.

VIII. Stereoselective catalysis

It has been known for a long time that enzymes convert different enantiomers with a different speed. This can possibly be of commercial interest for producing optically pure substances. The difficult part is the fact that the equilibrium conversion of the enantiomers are equal. This means that the reaction has to be stopped before equilibrium is reached. Chen and coworkers, 1982, formulated effective expressions with three key parameters that are generally used since then. The key parameters are the extent of conversion of racemic substrate (c), the optical purity, expressed as enantiomeric excess (ee) of the product or the remaining substrate, and the enantiomeric ratio (E), which is an enzyme specific term.

Suppose that A and B are the fast and the slow reacting enantiomers that compete for the same site on the enzyme. For a simple three step kinetic mechanism assuming the reaction is virtually irreversible,

$$A + E \underset{k_2}{\overset{k_{1A}}{\rightleftarrows}} EA \xrightarrow{k_3} EP \xrightarrow{k_5} E + P \qquad \text{(VIII.1)}$$

$$B + E \underset{k_2'}{\overset{k_{1B}'}{\rightleftarrows}} EB \xrightarrow{k_3'} EP \xrightarrow{k_5'} E + P \qquad \text{(VIII.2)}$$

the ratio of the two partial reaction rates (v_A and v_B) may be shown by steady state kinetics to be :

$$\frac{V_A}{V_B} = \frac{V_A}{V_B}\,\frac{K_B}{K_A}\,\frac{A}{B} \qquad \text{(VIII.3)}$$

where V_A, K_A and V_B, K_B denote maximal velocities and Michaelis constants of the fast and slow reacting enantiomers, respectively.

Integration of this equation leads to an equation that shows that the discrimination between two competing enantiomers, A and B, by enzymes is dedicated by E, the ratio of specificity constants, V/K.

$$\frac{\ln(\frac{A}{A_o})}{\ln(\frac{B}{B_o})} = \frac{(\frac{V_A}{K_A})}{(\frac{V_B}{K_B})} = E \qquad \text{(VIII.4)}$$

In instances where kinetic resolution experiments are conducted by the selective destruction of one of the antipodes e.g., D-amino acid oxidases have been used to oxidize D-α-amino acids preferentially, leaving most of the L acids unchanged, the relationship between the extent of conversion (c) and the enantiomeric excess of the recovered substrate fraction (ee(S)) for various values of the enantiomeric ratio (E) is governed by :

$$\frac{\ln[(1 - c)\ (1 - ee(S))]}{\ln[(1 - c)\ (1 + ee(S))]} = \frac{(\frac{V_A}{K_A})}{(\frac{V_B}{K_B})} = E \qquad \text{(VIII.5)}$$

where

$$c = 1 - \frac{A + B}{A_o + B_o} \qquad \text{(VIII.6)}$$

and

$$ee(S) = \frac{B - A}{A + B} \qquad \text{(VIII.7)}$$

In kinetic resolution experiments and possibly in practical applications it is also desirable to relate c, the extent of conversion, and ee(P), enantiomeric excess of the product fraction, to various values of E.

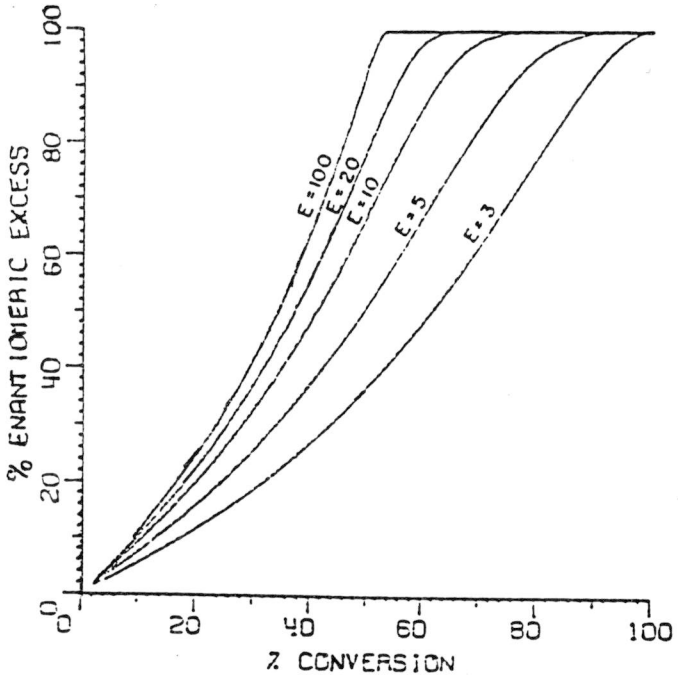


Figure VIII.1 Plot of the enantiomeric excess of the substrate as a function of the conversion for various enantiomeric ratios.

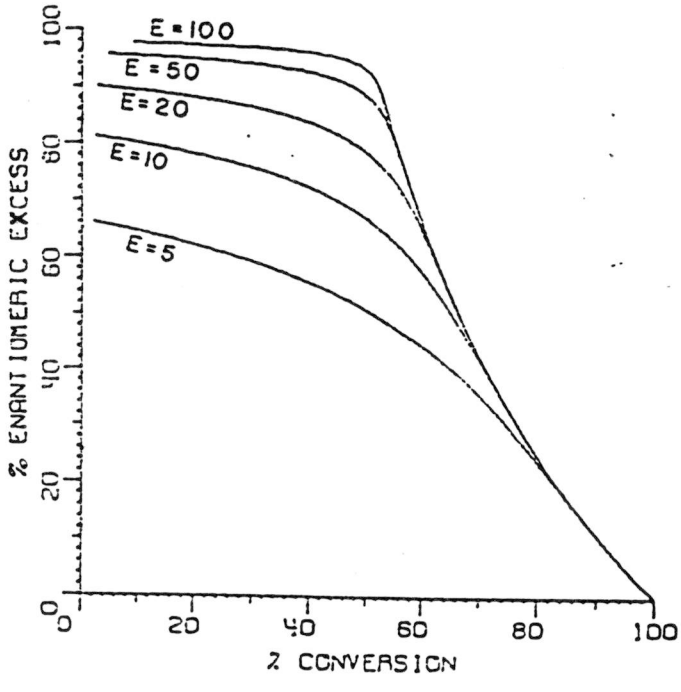


Figure VIII.2 Plot of the enantiomeric excess of the product as a function of the conversion for various enantiomeric ratios.

This correlation may be achieved by adapting equation VIII.3 into :

$$\frac{\ln[1 - c(1 + ee(P))]}{\ln[1 - c(1 - ee(P))]} = \frac{(\frac{V_A}{K_A})}{(\frac{V_B}{K_B})} = E \qquad \text{(VIII.8)}$$

where

$$c = 1 - \frac{A + B}{A_o + B_o} \qquad \text{(VIII.9)}$$

and

$$ee(P) = \frac{P - Q}{P + Q} \qquad \text{(VIII.10)}$$

The figures VIII.1 and VIII.2 show plots of the equations VIII.3 and VIII.8. Figure VIII.2 shows the abrupt decrease in ee(P) for values of c beyond 0.5. Consequently, one should not carry out the conversion beyond 50 % to obtain an enantiomer, irrespective of the value of E.

The graphs also show that it is also practically impossible to purify enantiomers in one step. Enzymes however also catalyze the reaction in the opposite direction. So the resulting products of the first step, P and Q (P > Q) may be reacted back to A and B respectively. The optical purity of this enriched fraction (A > B) may be further enhanced by its reincubation with the same system. In recycling studies, ee_o (the enantiomeric excess of the initial antipodal mixture), is always greater than zero.

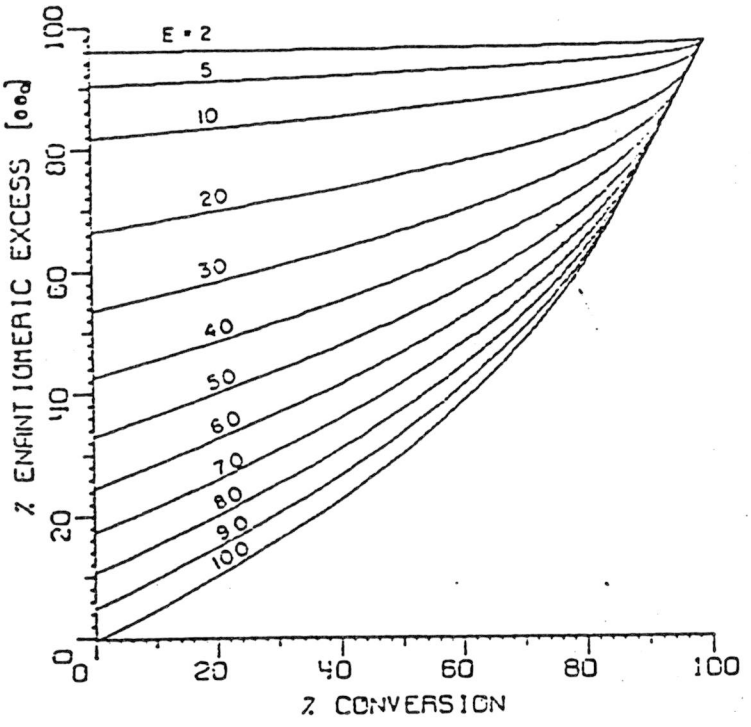


Figure VIII.3 Plot showing the interrelationship if the initial enantiomeric excess, the conversion and the enantiomeric ratio with ee'= 0.98.

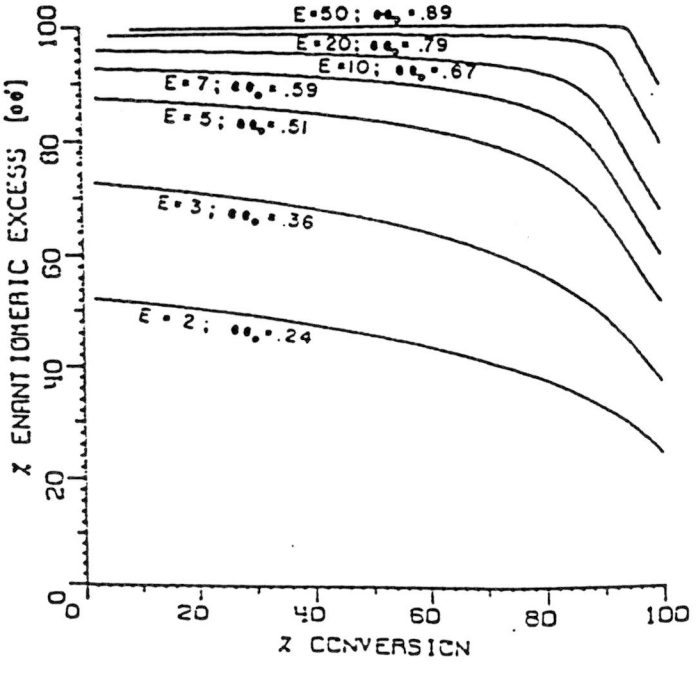


Figure VIII.4 Expression of the final enantiomeric excess as a function of the conversion for various values of initial enantiomeric excess and enantioric ratio

Hence, a new expression is needed to relate the variables: c, the extent of the conversion; e, the enantiomeric ratio; ee_o; and ee', the enantiomeric excess of the recycled product fraction. If we consider 1 mole of an antipodal mixture with an initial enantiomeric excess of ee_o, this would contain $(1 + ee_o)/2$ moles of A_o (fast reacting) and $(1 - ee_o)/2$ moles of B_o (slow reacting). Hence,

$$A = \frac{(1 + ee_o)}{2} - c\,\frac{(1 + ee')}{2} \qquad \text{(VIII.11)}$$

$$B = \frac{(1 - ee_o)}{2} - c\,\frac{(1 - ee')}{2} \qquad \text{(VIII.12)}$$

Substitution of these terms into equation VIII.3 leads to equation VIII.13 :

$$1 - c\,\frac{1 + ee'}{1 + ee_o} = \left(1 - c\,\frac{1 - ee'}{1 - ee_o}\right)^{E} \qquad \text{(VIII.13)}$$

A plot of this equation is presented in figure VIII.3. It provides an overview of the relationships between the variables ee_o, E, and c for a fixed value of ee', which was set at 0.98. For example, let us suppose that an antipodal mixture with an ee_o of 0.40 is exposed to an enzyme possessing an E value of 50. To obtain the product fraction with a ee'of >0.98, it is necessary to terminate the reaction when c<0.21. For an enzyme with an E value of 60, the reaction may be extended until c<0.38. In recycling work, the values of E and ee_o are known and ee' is usually fixed at 0.98. Figure VIII.3 enables one to predict the maximum conversion allowed to obtain a product fraction with an ee' value of 0.98. Thus the graph indicates when the reaction should be terminated.

When enzymes with modest E (5-10) values are used, it is perhaps more advantageous to arrange the before mentioned variables into the format shown in figure VIII.4.

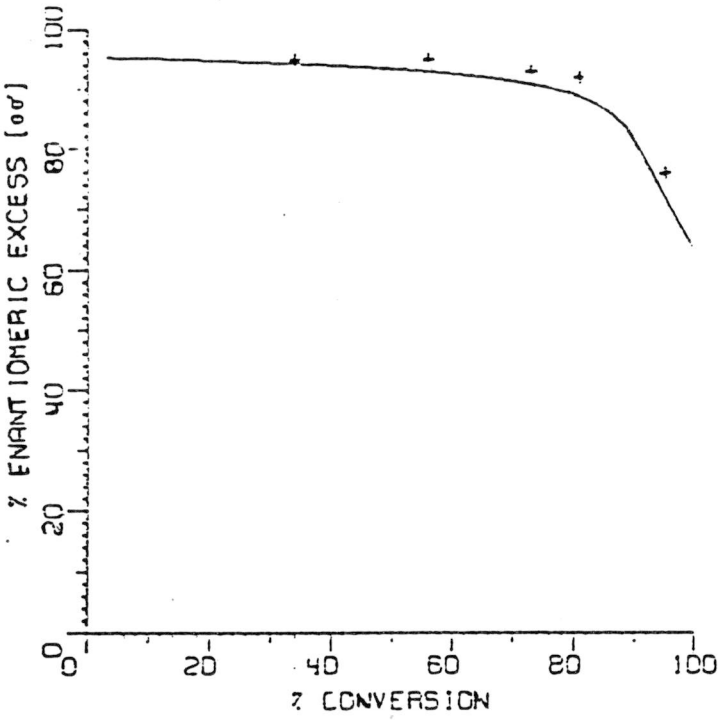


Figure VIII.5 Dependence of enantiomeric excess on the conversion. Experimentally and calculated.
E = 9.7

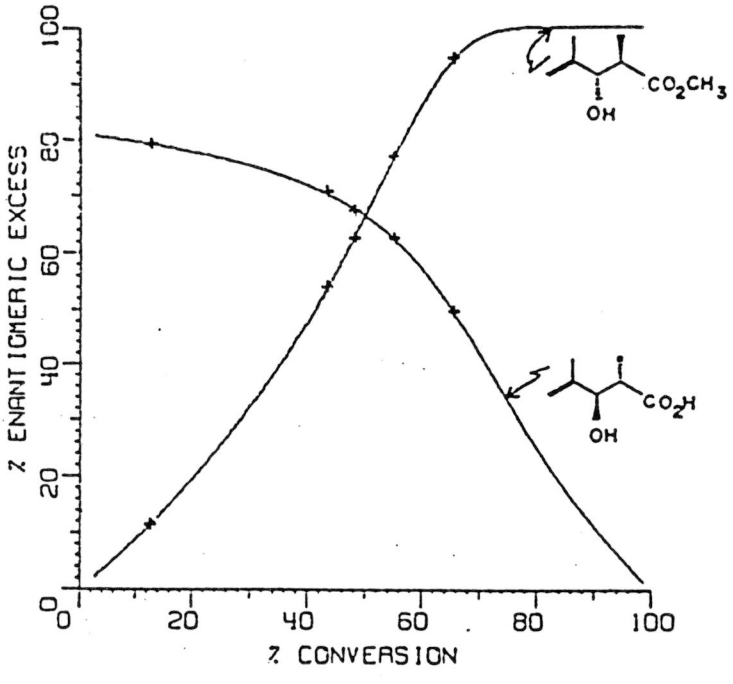


Figure VIII.6 Final enantiomeric excess as a function of the conversion. Experimentally and calculated.
E = 9.7, ee_o = 0.64

This graph allows one to estimate the relationship between ee' and c at various fixed values of E and ee_o. For example starting with an antipodal mixture with an ee_o of 0.67 and E of 10, the ee' obtainable after 80% conversion is 0.91. When c=1, ee'= ee_o. In principle, the product could be recycled an infinite number of times to achieve the ultimate goal of absolute optical purity. In reality, to prepare enantiomers with ee'values of > 0.98, it would be more convenient to select enzymes with E values larger than 10 and subject the product of recycling not more than two times.

The validity of the equations were tested with pig liver esterase and the results are shown in figure VIII.5 and VIII.6. A more extensive check was impossible because of the lack of experimental data.

Solvent	Thermal inactivation rate constant ($10^{-4}s^{-1}$)
Water	4.2
Glycerol	1.0
Ethanediol	1.7
Propane 1, 3-diol	2.5
Methanol	3.4
Propane 1, 2-diol	2.2
butane 1, 4-diol	6.3
Ethanol	4.9
Propan-1-ol	20.0

Table IX.2.1 The denaturation rate constant for different solvents when 2 mole added to water.

IX. Enzyme catalysis in organic solvents.

IX.1 Introduction.

This chapter is one of the key chapters of this report. In the previous chapters thermodynamic "tools" and enzymological "tools" have been provided, and in this chapter they will be combined to try to describe the behaviour of enzymes in organic solvents. For this purpose this chapter will be divided in paragraphs dealing with a specific enzyme property, like activity, stability and stereoselectivity.

IX.2 Enzyme stability.

Before going into detail it is perhaps wise to describe first what is meant by enzyme stability. Enzymes denaturate, they loose their initial activity, which is greater than zero. That is a well known fact, and it has been described in chapter VI.
Enzymes thus have a life time that is far from infinity and, it is dependent on the medium they are in, think at temperature and pH. pH, one can imagine, will not be a big problem in an organic solvent. This is a big advantage, if one wants to conduct a reaction in which for example one of the reactants is an acid, an organic solvent is the only alternative. In water the acid will dissociate, thus inhibiting the reaction. It may be possible if the enzyme has a very high pH optimum, but that is normally not the case. Thermal stability is something else, Rodgers et al, 1987 found for example that at 85 °C the addition of 2 mole organic solvent to 1 litre water influences the thermal inactivation rate constant (first order). In some cases an increase and other cases a decrease was found. (Fig IX.2.1)
The effect organic solvents have on enzymes is that they adsorb to (interact with) the hydrophobic parts. In doing this they lower the Gibbs energy compared to the situation where these sites were occupied by water.

During unfolding (denaturation) the Gibbs energy decreases, mainly caused by the entropy term in the Gibbs energy, thus by increasing temperature this effect grows. If the Gibbs energy of the unfolded enzyme molecule system is lower than in the original system the enzyme will unfold. If organic solvents are adsorbed the temperature at which this situation is reached may be higher, the enzyme is then a bit more thermostable. This leaves the question left why not all organic solvent are thermostabilizers. In the previous hypothesis it was assumed that the Gibbs energy of the enzyme + organic solvent was lower than the Gibbs energy of the enzyme + water situation. It is however possible that this is just the other way around and the organic molecule is still adsorbed to the enzyme. This situation is possible if the Gibbs energy of the system where the water is adsorbed to the enzyme and the organic solvent is in the water phase has a higher Gibbs energy than the system where the organic solvent is adsorbed to the enzyme. The Gibbs energy of the enzyme with organic molecules adsorbed can then be higher than the Gibbs energy of the enzyme with the water molecules. The situation for the total systems is than however reversed. This principle is the same as the principle of the coupled reaction of the irreversible thermodynamics. It is now clear to see that such an organic solvent destabilizes the enzyme compared to water. If the temperature increases the Gibbs energy of the unfolded system will at a lower temperature be lower than the original situation. Thermodynamics is also able to explain this phenomena but to predict it is more difficult. It is at this time impossible to calculate the Gibbs energy of the enzyme with or without molecules adsorbed to it, but that may be possible in the future. For enzymes in pure organic solvents the same theory is applicable, only now an enzyme/solvent in solvent system has to be compared with an enzyme/water in water system. For catalysis there always has to be some water in the system (next paragraph) and this influences the thermal stability of enzymes in organic solvents in a negative way if it is too much.

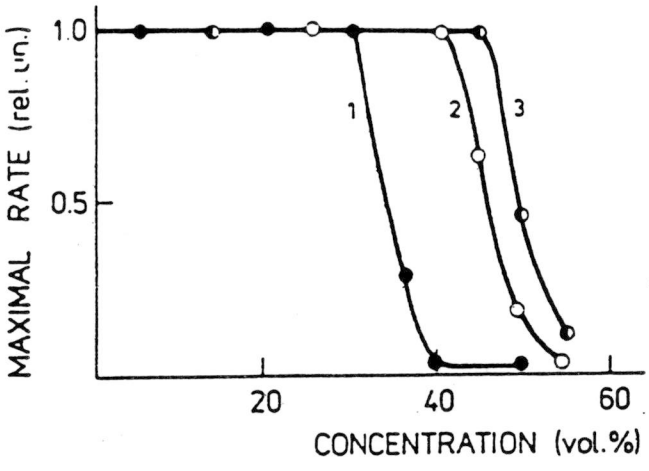


Figure IX.2.2 Dependence of the maximum rate (expressed in relative units) of α-chymotrypsin-catalysed hydrolysis of *N*-benzoyl-L-tyrosine *p*-nitroanilide on the concentration of ethanol: (1) free enzyme, and (2,3) enzyme attached to polyacrylamide gel by 5 and 12 covalent bonds, respectively.

This makes sense, a small amount of water adsorbs to the polar parts of the enzyme, thus lowering the total Gibbs energy of the system and increasing the thermostability but too much water forces water molecules in the organic bulk raising the total Gibbs energy of the system and decreasing the thermostability. It is also possible to stabilize enzymes with immobilization, which is described in chapter VII. It is of course possible to use immobilized enzymes in organic solvents. It can be expected that the response to the amount of water in the system is roughly the same and that is indeed observed by Khmelnitsky et al, 1988. They also observed that the number of covalent bonds between the carrier and the enzyme have a big influence, the more covalent bonds, the more stabile the enzyme. This is not to difficult to understand, the more bonds the more energy it takes to unfold (break these bonds), the higher the temperature can be. (Figure IX.2.2)

IX.3 Enzyme activity.

IX.3.1 Introduction.

Concluding the previous paragraph you can say that organic solvents can stabilize enzymes against denaturation. This is however but one part that is important in enzyme catalysis, the other part is activity. These two are quite often mixed up. You see graphs presented in activity studies where the activity is followed in time. This is however not an activity experiment but a joint activity and stability experiment. The initial activity is the activity caused by the solvent and the decrease of the activity in time is the stability effect. In this paragraph the initial activity will also be considered. The activity is in principle the rate constant of the reaction catalyzed.

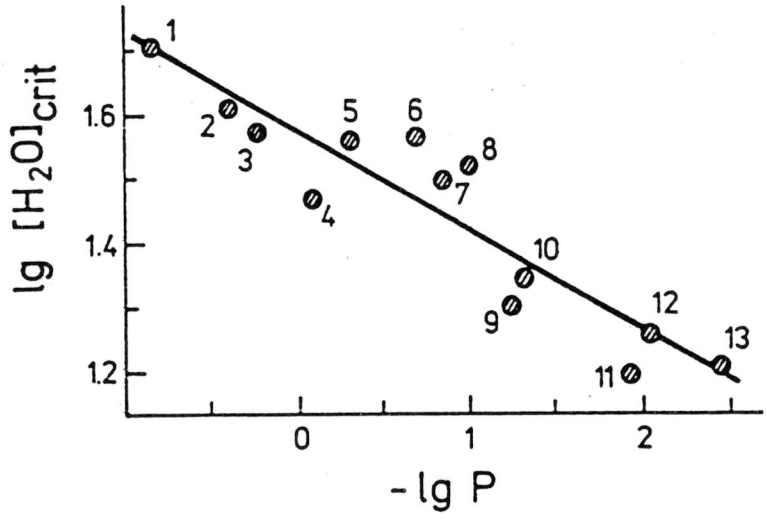


Figure IX.3.2.1 Dependence of the critical value of water concentration in water–organic cosolvent mixture (cf. *Fig. 3*) on the hydrophobicity (−lg P) of nonaqueous components: (1) 2-methyl-1-propanol, (2) 2-propanol; (3) 1-propanol, (4) 2-methyl-2,4-pentanediol, (5) ethanol, (6) methanol, (7) 2,3-butanediol, (8) 1,3-butanediol, (9) 1,2-butanediol, (10) 1,2-propanediol, (11) 1,2-ethanediol, (12) 1,2,6-hexanetriol, (13) 1,2,3-propanetriol. Hansch's parameter *P* represents the partition coefficient of the organic component in the two-phase system of octanol–water. From ref. 101

In chapter III a relation for this rate constant has been derived and that equation is dependent on the equilibrium constant of the transition state. To be able to say something about this quantity the mechanism (participants) of the transition state formation has to be known. Of course the reaction compound and the enzyme are participants and another possible one is water.
The solvent has no direct influence. In the next paragraphs the influence of water on the activity will be discussed and an attempt will be made to explain the influence of organic solvents on the activity by applying the thermodynamics described in the chapters II. and III.

IX.3.2 The influence of water on the enzyme activity.

That water has an influence on the activity of enzymes in organic solvents has been observed experimentally on a large scale. The explanation why is not given yet. Of course there are a lot of ideas about it but there is no proof yet. The most generally accepted ideas are by Klibanov, 1986, 1989, Halling, 1987 and Cassels, 1988 and their ideas will be presented now and sometimes extended based on thermodynamic knowledge.
Enzymes need a layer of essential water around them. This layer keeps the active site in its good conformation. Layer is not quite a correct name because for some enzymes 70 molecules per enzyme molecule are enough and that is not enough for a monolayer but it is sure that completely dry enzymes are not active. This explains why enzymes are not active in water miscible organic solvents, they strip the water of the enzyme disturbing the essential layer of water. This means that water has to be added to water miscible organic solvents in such an amount that all the essential water remains around the enzyme. This is called the critical water concentration and is of course different for all water miscible organic solvents (fig IX.3.2.1). From a thermodynamic viewpoint this means of course that the water around the enzyme has to have a lower activity than the water in the bulk.

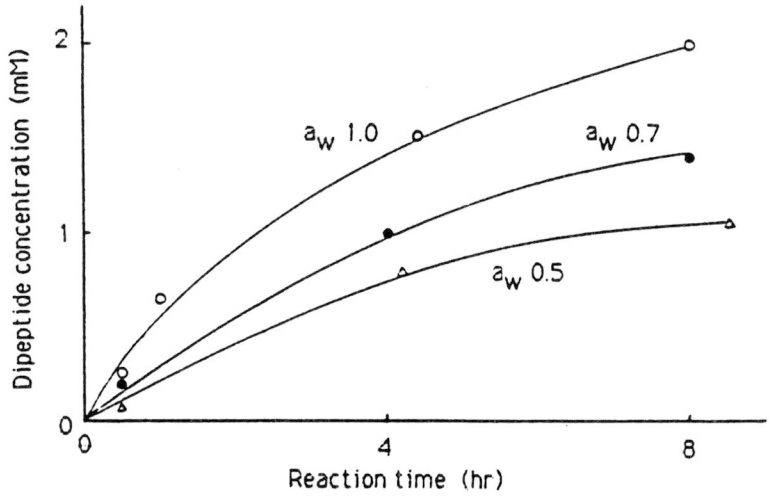


Figure IX.3.2.2 Enzymatic activity as a function of the water activity in the solvent.

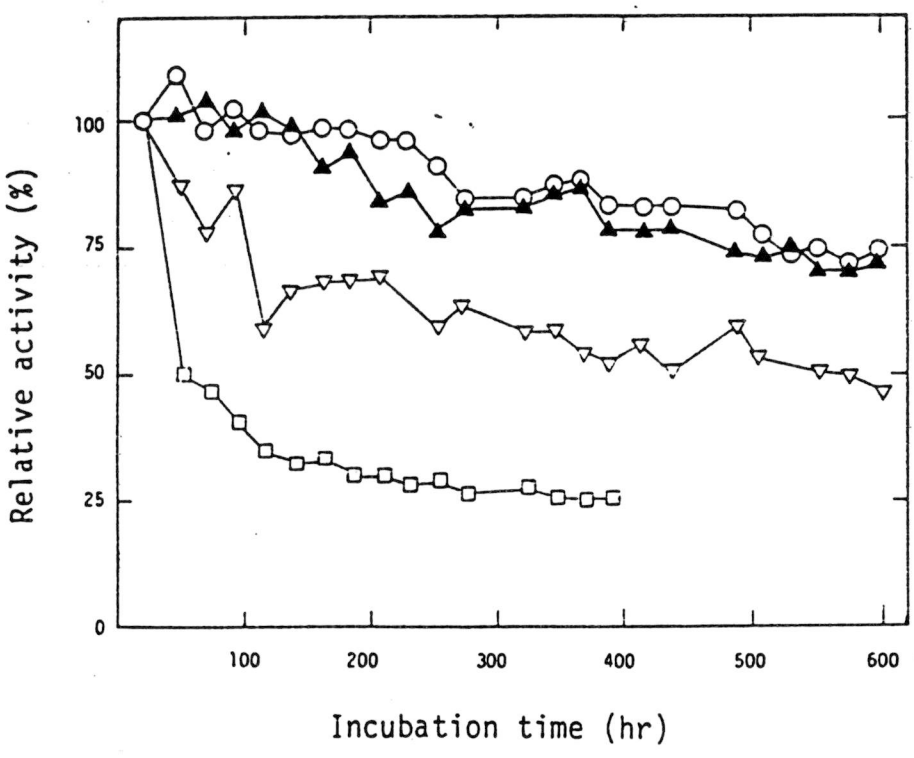


Figure IX.3.2.3 Repeated use of free and entrapped *Rhodotorula minuta* cells in hydrolysis of *dl*-menthyl succinate. Each reaction was carried out for 24 hr. (□), Free cells; (o), PU-3 entrapped cells; (▲), PU-6-entrapped cells; (▽), ENT-4000-entrapped cells.

The critical water concentration represents the situation where the activity of water in the bulk is equal to the activity of water in the essential layer around the enzyme. This is called the critical activity. The enzyme activity at the critical water activity is not immediately the maximum obtainable activity.

The complete layer is not formed yet but is starting to be formed so the activity is raising until the total layer is formed. This occurs at the optimum water activity.
The critical activity is enzyme dependent, one enzyme binds its necessary water stronger than another.
It is now easy to understand that enzymes are active in water immiscible organic solvents. A little water is enough to provide the enzymes with their essential layer of water. This is also observed. In this case however there is an other factor that can play a role, if there is too much water a distinct water mantle is formed around the enzyme causing diffusion limitation problems, especially when water is a reaction product. In water immiscible organic solvents with a limiting solubility of water you have a maximum enzyme activity at the optimum water activity. (Fig IX.3.2.2) This plot is of course universal for an enzyme, just like adsorption isotherms, so once one is made, it can be used for reactions in other solvents. One has to be sure that the activity of water in the solvent stays equal to the optimum water activity.
Immobilization on a hydrophillic carrier can decrease the critical water activity a bit because now the carrier decreases the activity of the water adsorbed to the enzyme a bit so water from the bulk will at a lower activity (smaller amount) adsorb to the enzyme. In water miscible organic solvents you thus can have a higher concentration organic solvent in the system.
(Fig IX.3.2.3)

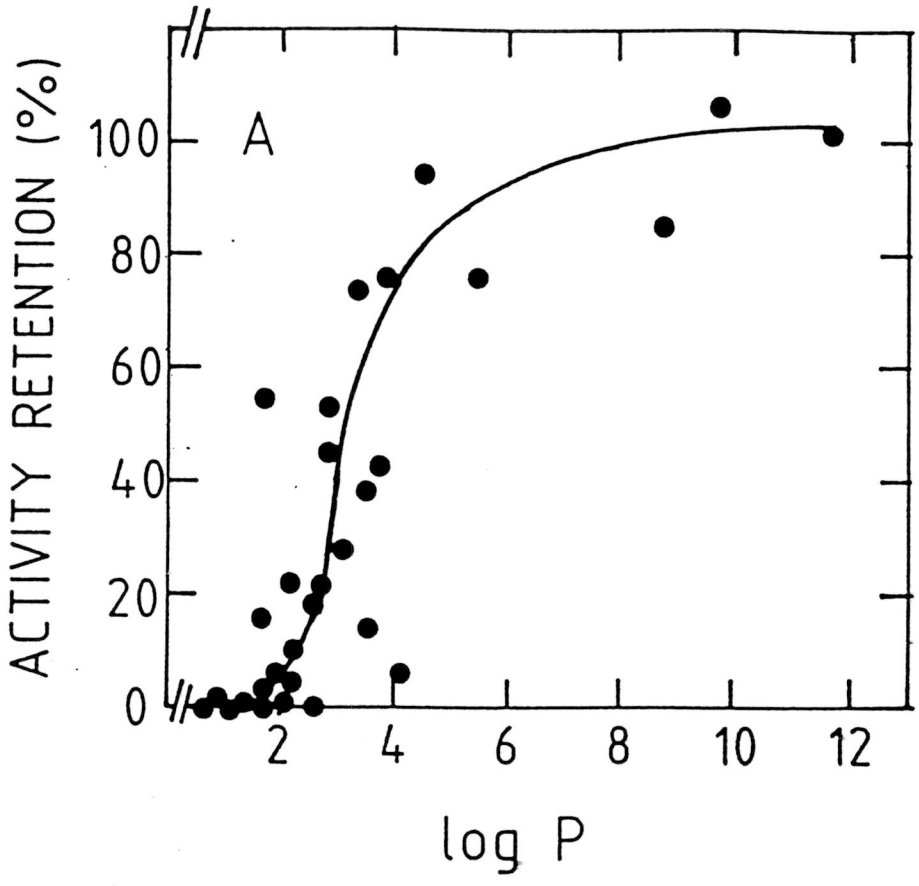


Figure IX.3.3.1 Activity retention as a function of log P

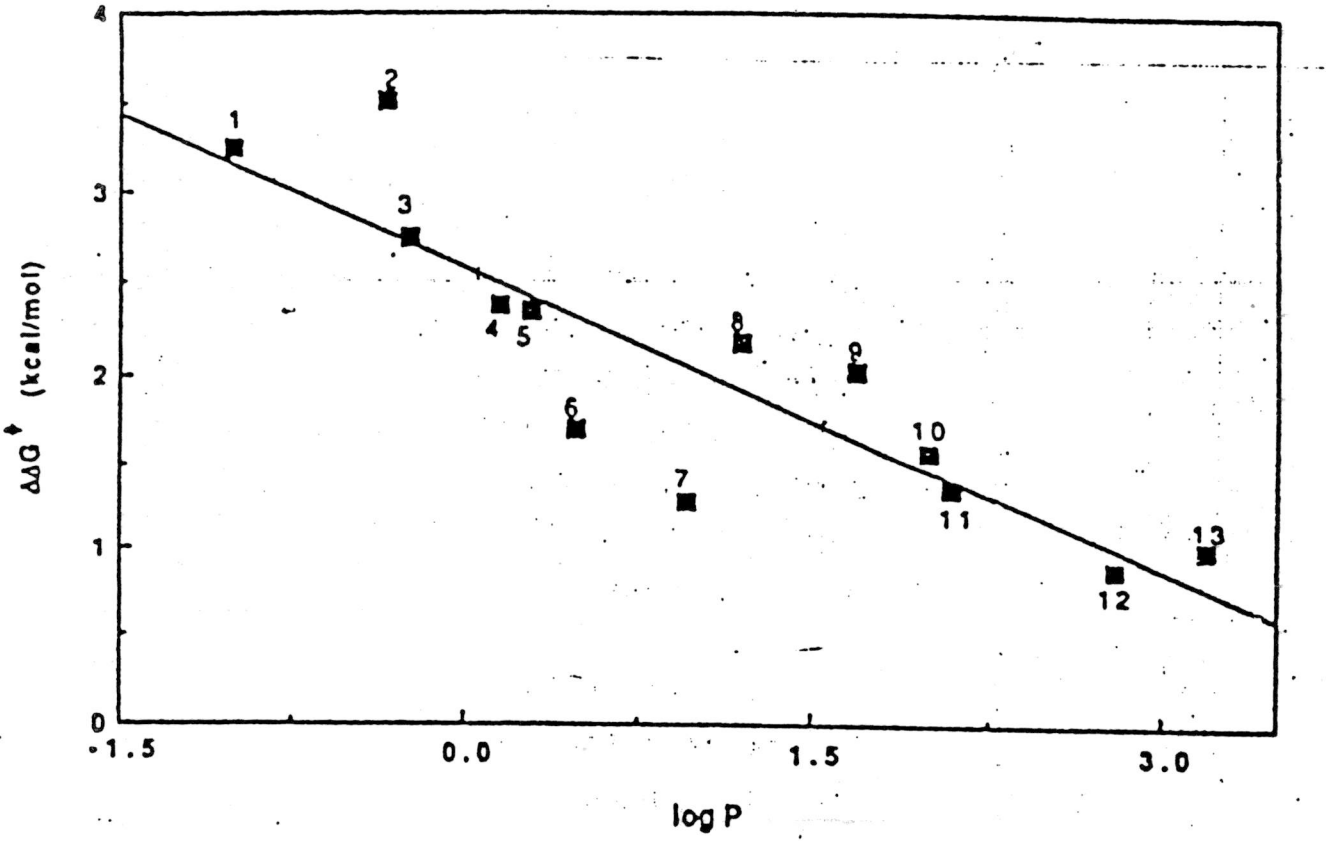


Figure IX.3.3.2 Enantiomeric ratio as a function of log P

IX.3.3 The influence of organic solvents on enzyme activity.

In the previous paragraph it is shown that enzymes have an optimum activity in an organic solvent. This, however, does not say anything about the absolute activity that belongs to the maximum activity. The optimum activity is caused by the amount of water around the enzyme and it can fairly reasonable be explained why this is so. This is something else with the absolute activity, which depends on the type of organic solvent.

The only researchers that made a serious attempt to explain this phenomena were Laane et al., 1987. They collected data about activities in different organic solvents. The first interpretation was that the activity retention, which is the activity of the enzyme in an organic solvent after a few hours relative to water, was dependent on the hydrophobicity of the solvent. They found an, at first sight, suitable parameter, log P. A graph of the activity retention versus log P is given in figure IX.3.3.1. How they came to this parameter and what the log P is will be described in the next paragraphs. The weak parts in this attempt are the definition of the activity retention and the definition of the medium. They say for example that the activity retention of enzymes in solvents with log P's smaller than two is about zero. A solvent with a log P smaller than two is water miscible and in the previous paragraph it is shown how important the amount of water is for the enzyme activity in such solvents. If in the experiments used by Laane et al. water miscible organic solvents with to small an amount of water are used it makes sense that no activity is observed. The activity retention is based on the relative activity after a few hours. This fact combined with the fact that denaturation happens at different rates in different solvents leads to the conclusion that the method of Laane et al. is probably not very reliable. Their method, however, caused log P to become an important parameter. For example in stereoselectivity experiments as shown in figure IX.3.3.2.

Because of the widely spread use of log P in modern enzymology in the next paragraphs the definition of this quantity, the reasons of its selection and various methods of estimation are described. In the last paragraph of this chapter an attempt will be made to find out whether this parameter can be used to describe the activity retention and stereoselectivity.

IX.4 Solvent characterization

IX.4.1 Introduction

For selecting solvents it is important to know a quantity that characterises a certain solvent. In case of enzyme catalyzed reactions the hydrophobicity of the solvent seems to be a useful quantity both for solvent influence on the catalytic activity and for solvent influence on the equilibrium conversion. Laane et al. (1987) have taken several different parameters as indicators for the solvent hydrophobicity like the Hildebrand solubility parameter, the dye E_T (see IX.4.3), the dielectric constant, the dipole moment and log P and correlated them with biocatalytic activity. In this chapter their results will be shown.

IX.4.2 The Hildebrand solubility parameter

The Hildebrand solubility parameter (δ) has first been used by Brink et al. (1985) to relate biocatalytic activity and solvent hydrophobicity. They studied the influence of various water-immiscible solvents on biocatalysis in general and on the microbial epoxidation of propene and 1-butene in particular. The δ- values can be obtained from available literature (eg Shinoda et al. (1978)) or by means of the formula :

$$\delta = \frac{\sqrt{\rho \ (\ \Delta H_v - RT \ )}}{M} \qquad \text{(IX.4.1)}$$

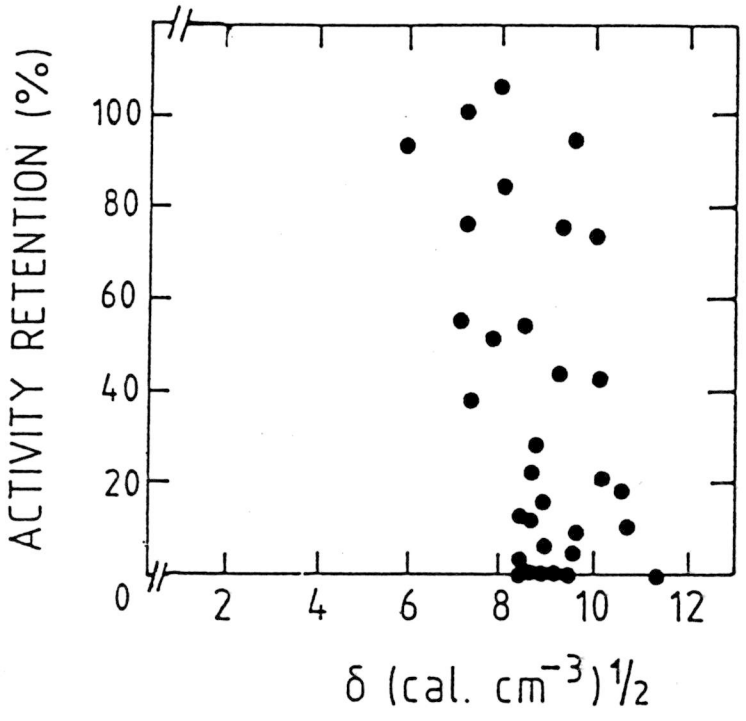


Figure IX.4.2.1 Activity retentions of epoxidizing cells exposed to different organic solvents versus the Hildebrand solubility parameter.

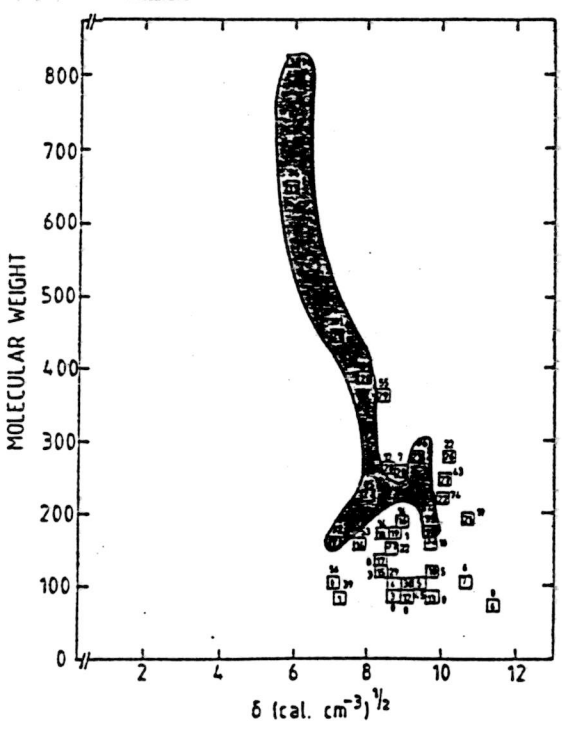
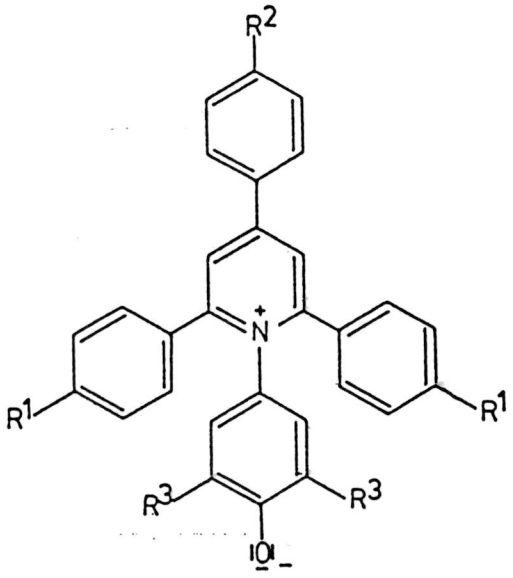


Figure IX.4.2.2 The Hildebrand solubility parameter as a function of the molecular weight.

where H_V is the enthalpy of vaporisation, the specific gravity of the solvent, the only parameters that have to be determined experimentally, M is the molecular weight of the solvent, R the gas constant and T the temperature. By plotting these δ- values against the rates of epoxidation, expressed as percentage activity after a few hours relative to the activity observed in water, the results presented in figure IX.4.2.1 were obtained. It can be seen that high activities can be found over the complete δ range but that low activities were observed only in the region $\delta > 8$. This means that more hydrophobic solvent (smaller δ value) are better solvents for enzyme catalyzed reactions. Figure IX.4.2.1 also shows that the Hildebrand solubility parameter is not a particularly useful parameter. The scale is too narrow and there are still a lot of solvents with a $\delta > 8$ that show quite a high activity. The fact that the scale is so narrow is easy to understand, the heat of vaporisation is most strongly dependent on polar interactions between solvent molecules so organic molecules, not having a lot of polar interactions, will all have a comparable heat of vaporisation and so a comparable δ- value.

Brink et al. have tried to improve their method by introducing the molecular weight, reasoning that a high molecular weight solvent might be less harmful to the epoxidizing microbes due to the fact that they cannot penetrate into the cell. The results are shown in figure IX.4.2.2, where the molecular weight of the organic solvent is plotted against δ. Every box represents an organic solvent which has been numbered by Brink et al. in their original paper. The number closest to the box is the activity retention found for that particular solvent.
The shaded area is the area including the solvents with an activity retention of 75% and higher. Brink et al. came to a final conclusion: High biocatalytic activity is found in solvents with a $\delta < 8$ and a molecular weight > 150. However, the shape of the shaded area shows that this concept cannot be very useful because of the numerous exceptions.



$E_T(30)$: $R^1=R^2=H$: $R^3=C_6H_5$

Figure IX.4.3.1 Structure of dye E_T.

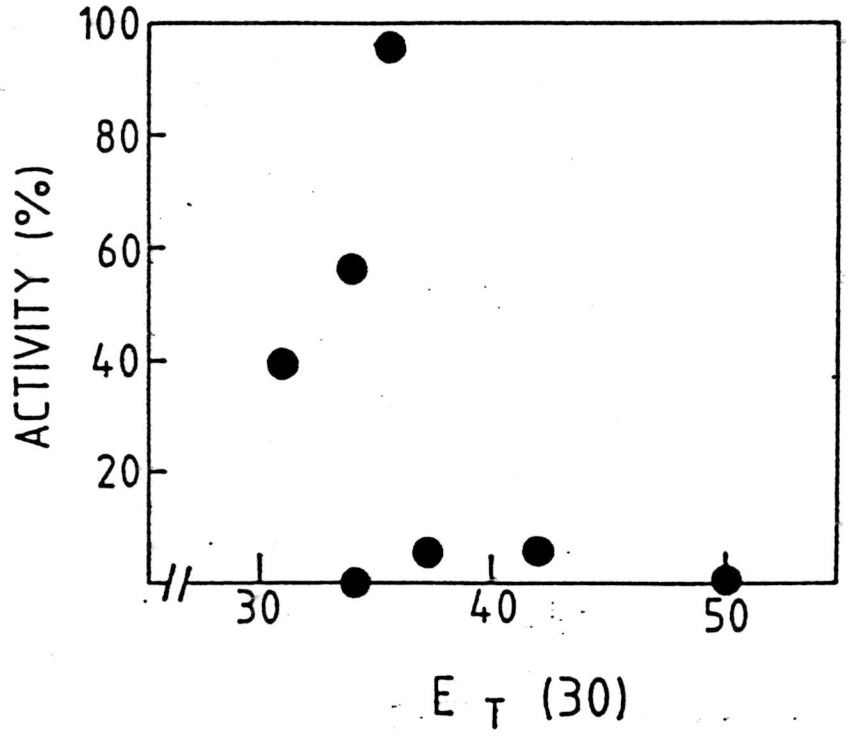


Figure IX.4.3.2 Activity retention of epoxidizing cells exposed to organic solvents versus the E_T value.

IX.4.3 The E_T value.

The E_T value stands for the difference in transition energy between the ground state and the first excited state of a probe, whose structure is depicted in figure IX.4.3.1. (Reichardt 1979) Among physical organic chemists E_T is a rather popular indicator of polarity. This dye is a remarkable one, because its colour depends strongly on the hydrophobicity of the solvent in which it is dissolved. In figure IX.4.3.2 the data of Brink et al. were plotted against E_T values by Laane et al. Although few E_T values could be found in the literature, the amount is sufficient to illustrate that there is a poor correlation between E_T and the activity retention. This is again caused by the insensitiveness of the method for the more hydrophobic solvents.

IX.4.4 The dielectric constant and dipole moment.

Although the dielectric constant (ϵ) is not a direct measure of the hydrophobicity of a solvent it reflects to some extent differences in hydrophobicity. Laane et al. have plotted the data of Brink et al. against ϵ. The results are presented in figure IX.4.4.1. The ϵ values were taken from Hildebrand et al. (1970). Again poor correlations are found and again problems arise with the more hydrophobic solvents. The differences in ϵ for the hydrophobic solvents are so small that ϵ is not a useful parameter for characterising solvents.
Another method that should be tried is the dipole moment. Again correlations are poor and to avoid falling into repetitions no results will be shown.

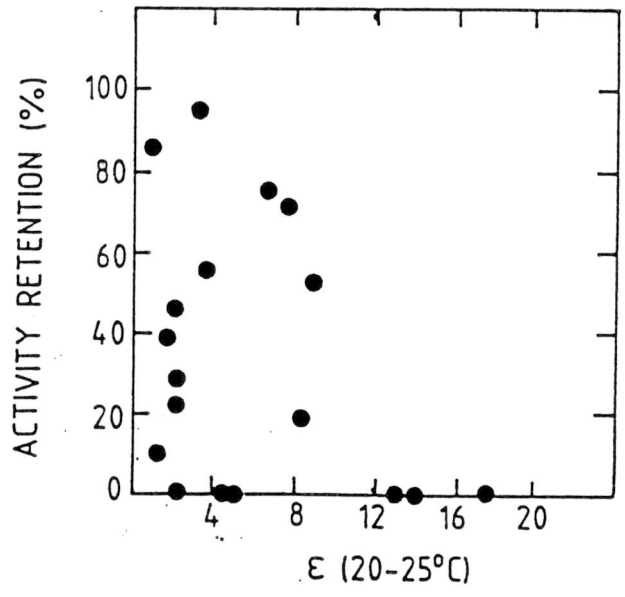


Figure IX.4.4.1 Activity retention of epoxidizing cells exposed to organic solvents versus ϵ.

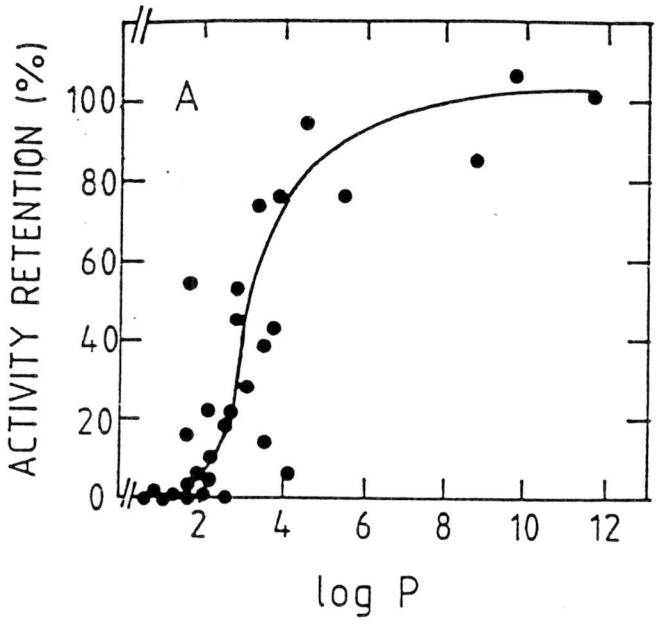


Figure IX.4.5.1 Activity retention of epoxidizing cells exposed to organic solvents versus log P.

IX.4.5 The partition coefficient.

The meaning of the partition coefficient (P) will be described separately (next chapter) because there is a lot to say about this parameter. Basically log P is used and log P is defined as the logarithm of the partition coefficient of a solute in a standard octanol-water two-phase system. How the values can be obtained will also be discussed in the next chapter.
Again Laane et al. have plotted the data of Brink et al. against log P and the results are shown in figure IX.4.5.1. A clear sigmoidal-shaped correlation exists between the activity retention and log P which means that log P is a useful quantity for describing the solvent hydrophobicity.

IX.4.6 Conclusions.

Log P is the most useful measure for describing the solvent hydrophobicity. It is the only one of the indicators tested that shows correlations with the activity retention of hydrophobic as well as hydrophillic solvents. As already mentioned the activity of the enzyme has been taken as a quantity where of course the equilibrium conversion could have been taken. Nowhere in the literature can be found whether this difference has a big influence on the indicator choice or not. However, because of uniformity, log P will also be applied for equilibrium matters although it may not be the best hydrophobicity indicator for this purpose.

IX.5 The partition coefficient

IX.5.1 Introduction

Laane et al. (1987) have shown that the partition coefficient (P) or better, the logarithm of the partition coefficient, (log P) is a better indicator of the hydrophobicity of a solvent than the Hildebrand solubility, the E_T value, the dielectric constant and the dipole moment, which are more or less well known physical properties. This is not the case with the partition coefficient, it is not a physical property in the same way as the others.

What it is and how it can be computed will be discussed in this chapter.

IX.5.2 The history of the partition coefficient.

The distribution of a solute between two phases in which it is soluble has been an important subject for experimentation and study for many years. In one form or another this technique has been used since earliest times to isolate natural products such as the essences of flowers.
The first systematic study of distribution between two immiscible liquids which led to a theory with predictive capabilities was carried out by Berthelot et al. (1872)
These investigators accurately measured the amounts present at equilibrium of both I_2 and Br_2 when distributed between CS_2 and water. They also measured the amounts of various organic and inorganic acids when distributed between ethyl ether and water. From these early investigations came the first appreciation of the basic fact that the ratio of the concentrations of solute distributed between two immiscible solvents was a constant and did not depend on the relative volumes of solutions used.

It was concluded from these early observations that there was a small variation in partition coefficient with temperature, with the more volatile solvent being favoured by a temperature decrease. It was also evident that some systems did not obey their simple rule but they intuitively felt the rule would be justified nonetheless. The next significant contribution to the subject was made by Nernst (1891). He stressed that the fact that the partition coefficient would be constant only if a single molecular species were being considered as partitioned between the two phases. Considered in this way partitioning could be treated by classical thermodynamics as an equilibrium process where the tendency of any single molecular species of solute to leave one solvent and enter another would be a measure of its activity in that solvent and would be related in the usual fashion to the other commonly measured activity functions such as partial pressure, osmotic pressure and chemical potential. The partition coefficient (P) was now defined as the ratio of concentrations of a solute in two solvents :

$$P = \frac{[solute]_{solvent\ 1}}{[solute]_{solvent\ 2}} \qquad \text{(IX.5.2.1)}$$

The reason for choosing the P as symbol for the partition coefficient is that P stands out from the variety of other equilibrium constant values and so it can be followed easier in discussions.

The reason that not only physical chemists were interested in this parameter was the work of Meyer (1899) and Overton (1901) who showed that the relative narcotic activities of drugs often parallelled their oil water partition coefficients. However, their work did not lead to useful correlations so the interest in the partition coefficient greatly declined for quite a long period, until Hansch (1969) and Helmer et al. (1968) used partition coefficients as extra thermodynamic reference parameters.

for hydrophobic bonding in biochemical and pharmacological systems. Now especially biochemists and pharmacologists got interested in the partition coefficient.

In the following period the partition coefficient was extensively used in the molecular design of drugs and the scientist involved wanted to have the ability of calculating the P value of a certain molecule. The methods that were developed do not have a solid thermodynamic basis, partly because thermodynamists did not show to much interest and partly because they were not able to calculate P values because the calculations involved were to complicated. The first methods were completely empirical, the newer methods have a semi-empirical background and are based on quantum chemical calculations.
Of the first group the two most widely used methods will be described and the second group is so small that it will be described completely.

IX.5.3 The Hansch method.

This was the first model that offered the possibility of predicting the partition coefficient of solutes. It was published by Hansch et al. (1971) They proposed to chose water and octanol as reference solvents. That was necessary because then it would be possible to compare and use the P values found by different authors, which is of course very important.
Hansch et al. assumed addivity of group contributions and defined their π-substituent :

$$\Pi_{RX} = \log P_{RX} - \log P_{RH} \qquad \text{(IX.5.3.1)}$$

where P_{RH} is the partition coefficient for the parent compound and P_{RX} is the partition coefficient for an analoque in which an H-atom has been exchanged for an X-atom or group.
This means that it is now possible to calculate the log P value for a certain compound if the basic structure and group π-substituents are available. In formula :

$$\log P_{RX_1X_2\ldots X_n} = \log P_{RHH\ldots H} + \sum_{1}^{n} \Pi(X_n) \qquad \text{(IX.5.3.2)}$$

With these last two equations and with experimental data a lot of π-substituents were determined and it turned out that the it did not work in all cases so correction factors had to be introduced for branched molecules, for aromats and other rings, for different double bonds, for multiple halogenation and for polar effects. This makes the method complex to work with because for most molecules different approaches leading to different results are possible. A computerized version is available (CLOGP) which makes it easier to work with but not more reliable, still the deviations from the experimental values can be quite large, especially for multiply substituted aromats.
As already mentioned the method is extensively used either from the book the authors published (Hansch and Leo, 1979) or from the updated version stored in computer at the Pompona College Medicinal Chemistry Database. (Pompona,CA)

IX.5.4 The Rekker method.

This method was introduced by Rekker shortly after Hansch published his method. (Rekker,1974) It was in some ways an improved Hansch model. Rekker too assumed additivity of the group contributions and defined a parameter similar to the π-substituent, the hydrophobic fragment constant (f).

The difference is that Rekker did not base the f-value of a group on the change in log P by substituting one group for another starting with a molecule, he determined real group contributions with all groups together forming a molecule. This makes the method more consistent than the Hansch method which is an improvement. The expression for log P is :

$$\log P = \sum_{1}^{n} a_n f_n \qquad \text{(IX.5.4.1)}$$

where a is the number of times a certain group is involved and n is a certain group. This method also needed some correction factors, for the proximity effect (distance between effective groups), for polarity and for conjugated systems. The values for all the parameters were obtained by fitting with experimental data. This method is just as widely used as the previous one and a complete description is given in a book published on it. (Rekker,1976)

IX.5.5 Quantum chemical methods.

The methods described before are, as far as calculations are involved, very simple to use. The more difficult computations, the calculation of the parameters, have already been done by the authors of the methods. These calculations of course need computers but they are still relatively simple because the models are linear in the parameters that were fitted. The coming of stronger computers offered the possibility for more complex computations. Quantum chemical methods are like that and in the last years two methods were published that were based on quantum chemical calculations. The first one is by Klopman et al. (1981) who used a molecular approach to calculate log P values.
They developed a linear regression model which included the total number of hydrogen, carbon, oxygen, and nitrogen atoms in the molecule and the sums of squared charges (quantum chemical calculation) for carbon, nitrogen and oxygen atoms.

no.	compd	log *P* expt[a]	log *P* est[b]	CLOGP expt[c]	CLOGP est[d]	no.	compd	log *P* expt[a]	log *P* est[b]	CLOGP expt[c]	CLOGP est[d]
1	propane	2.36	2.20	2.31	2.281	60	*N*,*N*-dimethylacetamide	−0.77	−0.26	−0.77	−0.802
2	isobutane	2.76	2.75	2.76	2.680	61	butyramide	−0.21	−0.25	−0.21	−0.176
3	pentane	3.31	3.28	3.39	3.339	62	furan	1.34	1.14	1.34	1.348
4	neopentane	3.11	3.30	3.11	3.079	63	pyrrole	0.75	0.36	0.75	0.758
5	cyclohexane	3.44	2.64	3.44	3.354	64	pyrrolidine	0.46	0.44	0.46	0.004
6	benzene	2.10	2.43	2.13	2.142	65	pyridine	0.67	0.89	0.65	0.665
7	toluene	2.74	2.73	2.73	2.791	66	chloroform	1.96	2.20	1.97	1.952
8	ethylbenzene	3.15	3.15	3.15	3.320	67	dichloromethane	1.25	1.50	1.25	1.249
9	propylbenzene	3.63	3.55	3.72	3.849	68	difluoromethane	0.20	0.53	0.20	0.369
10	methanol	−0.71	−0.69	−0.74	−0.764	69	methyl chloride	0.91	0.89	0.91	0.936
11	ethanol	−0.28	−0.22	−0.31	−0.235	70	methyl fluoride	0.51	0.39	0.51	0.496
12	propanol	0.28	0.29	0.25	0.294	71	nitromethane	−0.34	−0.74	−0.35	−0.284
13	butanol	0.88	0.84	0.88	0.823	72	ethylene oxide	−0.30	−0.26	−0.30	−0.792
14	isobutyl alcohol	0.75	0.87	0.76	0.693	73	ethyl chloride	1.43	1.20	1.43	1.465
15	*sec*-butyl alcohol	0.61	0.84	0.61	0.603	74	carbon tetrachloride	2.73	3.00	2.83	2.875
16	*tert*-butyl alcohol	0.36	0.83	0.35	0.473	75	crotonic acid	0.72	0.41	0.72	0.690
17	pentanol	1.48	1.34	1.56	1.352	76	adenine	−0.13	−0.36	n/a	−0.561
18	isopentyl alcohol	1.29	1.39	1.42	1.222	77	2-aminopyridine	0.52	0.55	0.49	0.345
19	neopentyl alcohol	1.34	1.38	3.11	3.079	78	2,4-dinitrophenol	1.52	1.54	1.54	1.915
20	*tert*-amyl alcohol	0.89	1.40	0.89	1.002	79	*m*-chlorophenol	2.50	1.78	2.50	2.485
21	cyclohexanol	1.23	1.67	0.81	0.805	80	nitrobenzene	1.84	1.60	1.85	1.885
22	1-hexanol	2.03	1.88	2.03	1.881	81	*m*-nitroaniline	1.37	1.35	2.45	2.534
23	1-octanol	3.15	2.80	2.97	2.030	82	phenol	1.49	1.36	1.46	1.475
24	dimethyl ether	0.10	−0.27	0.10	−0.188	83	hydroquinone	0.55	0.90	0.59	0.808
25	diethyl ether	0.83	0.80	0.89	0.870	84	aniline	0.90	1.12	0.90	0.915
26	dipropyl ether	2.03	1.86	2.03	1.928	85	*m*-aminophenol	0.18	0.29	0.17	0.248
27	butyl ethyl ether	2.03	1.89	2.03	1.928	86	*o*-aminophenol	0.62	0.18	0.62	0.648
28	methylamine	−0.57	−0.80	−0.57	−0.664	87	*p*-aminophenol	0.04	0.38	0.62	0.648
29	isopropylamine	−0.03	0.14	0.26	0.174	88	benzonitrile	1.56	2.04	1.56	1.575
30	butylamine	0.87	0.77	0.97	0.923	89	benzimidazole	1.37	0.92	1.46	1.547
31	*tert*-butylamine	0.40	0.70	0.40	0.573	90	benzaldehyde	1.45	1.46	1.48	1.495
32	cyclohexylamine	1.49	1.52	1.49	1.367	91	benzoic acid	1.95	1.57	1.87	1.885
33	diethylamine	0.53	0.74	0.58	0.540	92	2-acetylpyridine	0.84	0.73	0.85	0.438
34	piperidine	0.76	0.94	0.84	0.555	93	*p*-aminobenzoic acid	0.77	0.42	0.83	1.004
35	butylmethylamine	1.33	1.18	1.33	1.069	94	phenylurea	0.87	1.26	0.83	0.845
36	dipropylamine	1.62	1.77	1.67	1.598	95	anisole	2.08	1.81	2.11	2.061
37	dibutylamine	2.76	2.68	2.83	2.656	96	*o*-methoxyphenol	1.33	1.43	1.32	1.294
38	trimethylamine	0.22	0.08	0.16	0.048	97	*m*-toluidine	1.42	1.49	1.32	1.564
39	butyldimethylamine	1.70	1.70	n/a	0.946	98	2-(trifluoromethyl)benzimidazole	2.39	2.05	2.67	2.677
40	triethylamine	1.45	1.74	1.45	1.395	99	acetophenone	1.66	1.87	1.58	1.581
41	tripropylamine	2.79	3.10	2.79	2.822	100	phenylacetic acid	1.46	2.05	1.41	1.414
42	acetone	−0.24	−0.07	−0.24	−0.268	101	vanillin	1.26	1.33	1.21	1.354
43	2-butanone	0.35	0.45	0.29	0.261	102	phenoxyacetic acid	1.29	1.72	1.34	1.326
44	2-hexanone	1.78	1.55	1.38	1.319	103	*o*-vanillin	1.35	1.37	1.37	1.984
45	cyclohexanone	0.81	1.32	0.81	0.805	104	acetanilide	1.21	1.03	1.16	1.161
46	formic acid	−0.54	−0.67	−0.54	error	105	ethyl nicotinate	1.34	1.24	1.32	1.497
47	acetic acid	−0.24	−0.37	−0.17	−0.234	106	caffeine	−0.02	0.45	−0.07	0.260
48	propionic acid	0.29	0.19	0.33	0.295	107	quinoline	2.04	2.22	2.03	2.049
49	butyric acid	0.79	0.70	0.79	0.824	108	2-phenylimidazole	1.88	1.55	1.88	2.051
50	hexanoic acid	1.90	1.76	1.92	1.882	109	propionanilide	1.63	1.37	1.61	1.690
51	methyl acetate	0.18	0.05	0.18	0.142	110	naphthalene	3.35	3.66	3.30	3.316
52	ethyl acetate	0.70	0.59	0.73	0.671	111	2-ethylphenoxyacetic acid	2.59	2.48	2.42	2.749
53	propyl formate	0.83	0.61	0.83	0.794	112	4-phenylpyridine	2.55	2.69	2.59	2.553
54	ethyl propionate	1.21	1.15	1.21	1.200	113	biphenyl	4.06	4.26	4.09	4.030
55	isobutylene	2.37	1.61	2.34	2.136	114	diphenylamine	3.45	2.91	3.50	3.620
56	cyclohexene	2.86	2.51	2.86	2.810	115	diazepam	2.80	2.52	n/a	2.466
57	acetonitrile	−0.34	0.06	−0.34	−0.394	116	atropine	1.81	2.04	1.83	1.319
58	propionitrile	0.16	0.10	0.16	0.135	117	methadone	2.43	2.38	2.93	2.969
59	*N*-methylacetamide	−1.05	−0.61	−1.05	−1.078	118	tetracycline	−1.31	−1.29	n/a	−2.711

[a] From ref 21. There are very slight differences from the CLOGP experimental set in some cases. [b] Present method. [c] CLOGP file. [d] Calculated CLOGP.

Table IX.5.5.1 Comparison of log P values obtained in different ways.

The latter parameters characterize the interaction of the solute and the solvent according to a simple electrostatic model. They also included some indicator variables to show if ester, acid, amide or nitrile functionalities are present. This idea was further exploited by Bodor et al. (1989) who stressed that the charge densities alone cannot be sufficient, molecular volume and surface are just as important for calculating the partition coefficient. If they are not known for a certain molecule they can be calculated with a molecular building program. The model they developed is nonlinear and has as variables the molecular surface, the ovality of the molecule, an alkane indicator, the molecular weight, the dipole moment, the atomic charges on nitrogen and oxygen atoms and the squared charges on nitrogen atoms and oxygen atoms.
In table IX.5.5.1 a comparison between experimental data, values calculated with Hansch and values calculated with this method is presented. As can be observed the results are not more accurate although the method is more complicated to use and experimental data is still needed. It has however a more solid basis and because the possibilities with it are limited by computer capacity it can probably be improved since computer capacities grow by the day.

IX.6 Activity, Stereoselectivity and thermodynamics.

In chapter III an equation for the reaction rate constant for a reaction of one component has been derived. Let us apply this strategy now to enzyme reactions in organic solvents. First we adopt a suggestion made by Klibanov, 1986, that if a substrate adsorbs to an enzyme a number of water molecules enter the bulk, thus for one substrate, A and enzyme, E:

$$E.yH_2O + A \leftrightarrows EA + yH_2O \qquad \text{(IX.6.1)}$$

This is the equilibrium that forms the transition state product EA.

The equilibrium constant for the transition state is now

$$K^{+}_{TH} = \frac{a_{EA}\, a_{H_2O}{}^{y}}{a_{E.yH_2O}\, a_{A}} \qquad \text{(IX.6.2)}$$

Activities are the products of mole fractions and activity coefficients. The thermodynamic equilibrium constant, as defined above, is only temperature dependent. For our considerations it is also constant. The reaction rate of a possible following reaction is proportional to the equilibrium constant, according to chapter III. Here not the thermodynamic equilibrium constant is meant (independent of solvent) but the quotient of the mole fractions of the products and reaction components, which is then

$$K^{+}_{X} = \frac{x_{EA}\, x_{H_2O}{}^{y}}{x_{E.yH_2O}\, x_{A}} = K^{+}_{TH}\, \frac{\gamma_{E.yH_2O}\, \gamma_{A}}{\gamma_{EA}\, \gamma_{H_2O}{}^{y}} \qquad \text{(IX.6.3)}$$

Since the molecular weight and thus the size of an enzyme is very big compared to the components that adsorb to it (factor 1000) we assume that the activity coefficient of the enzyme/water complex and the enzyme/substrate complex are equal and thus cancel out of the equation. This leaves the equation

$$K^{+}_{X} = K^{+}_{TH}\, \frac{\gamma_{A}}{\gamma_{H_2O}{}^{y}} \qquad \text{(IX.6.4)}$$

Applying equation III.10 the next expression for the rate constant, k, results

$$k = C(T)\, \frac{\gamma_{A}}{\gamma_{H_2O}{}^{y}} \qquad \text{(IX.6.5)}$$

The constant is a product of the thermodynamic equilibrium constant for the transition state formation, the temperature, the Boltzman constant and the Planck constant.

The constant is also for a given reaction temperature dependent only. It is now possible to define the activity retention, AR, as the quotient of the rate constant of the reaction in an organic solvent and in water. The activity coefficient of water in water is one so the equation becomes

$$AR = \frac{k^{o}}{k^{w}} = \frac{\gamma_A{}^{o}}{\gamma_A{}^{w}} \frac{1}{(\gamma^{w}_{H_2O})^{y}} \qquad \text{(IX.6.6)}$$

The activity coefficient of the substrate in water is for a given substrate constant, so basically the activity retention is caused by three parameters, the amount of water molecules that have to enter the organic bulk, y, the activity coefficient of the substrate in the organic solvent and the activity coefficient of water in the organic solvent.

This equation is based on simple kinetics and it is of course possible to derive a similar equation for other possible mechanisms like the ones mentioned in chapter IV. They will however always lead to expressions with as only variables the activity coefficients of the substrates in the organic solvent and in water, y and the activity coefficient of water in the organic solvent.

The consequence of this equation is that the activity retention is dependent on the type of substrate, water and the type of organic solvent only. This model roughly leads to a decreasing with increasing hydrophobicity. The assumption made that the water has to enter the organic bulk is however not very realistic. Probably it will stick to the enzyme, or adsorb to a different part of the enzyme. This lowers the activity coefficient of water in the model by an unpredictable factor. The value can practically not be calculated. It can however be assumed that the value will be nearly constant in different organic solvent since it interacts mainly with the enzyme, which is the same. In that case the activity retention is only dependent on the activity coefficient of the substrate in the solvent.

This too is of course a measure for hydrophobicity of the solvent if the substrate is known, like the log P of Laane et al.. Why do they find a correlation with log P and no disturbance by the substrate. Their experiments are based on the epoxidation of propene and 1-butene, two substrates that are quite alike, and the small difference can cause their points not to be on a nice line. The log P values are 1.6 and 2.3 respectively. In solvents with a log P in the same range they feel best, which means that their activity coefficient in that solvent will be small and according to our model the activity retention will be small. It will raise if the activity coefficients raise and that is what they do at higher log P values. The method can thus account for the activity retention curve determined by Laane et al.. The reason they found the best correlation with log P is that log P has the widest scale of the parameters they tested for the solvents they used. Laane et al., however, failed to see that the activity retention is substrate dependent and that their curve consequently is only useful for the substrates they used.
The method can also be applied to stereoselective catalysis. In that case the fit of the substrates to the active site of the enzyme is different and thus the y value is different for both enantiomers. The activity coefficients of the both enantiomers in water and in the organic solvent are assumed to be equal. The enantionmeric ratio, E, is nothing else then the ratio of the rate constants of the both enantiomers. The thermodynamic equilibrium constants are not equal in this case since different amounts of water are involved. Leaving out all the activity coefficients that cancel out, the expression for the enantiomeric ratio becomes

$$E = \frac{k_1}{k_2} = \frac{K^+_{TH,1}}{K^+_{TH,2}} \frac{\gamma^{y_2}_{H_2O,O}}{\gamma^{y_1}_{H_2O,O}} = \frac{K^+_{TH,1}}{K^+_{TH,2}} \gamma^{y_2-y_1}_{H_2O,O} \qquad \text{(IX.6.7)}$$

The ratio of the thermodynamic equilibrium constants is constant for two enantiomers.

The enantiomeric ratio is also dependent on the difference in the number of water molecules that play a role in the mechanism and on the activity coefficient they have once the substrate is adsorbed to the enzyme. E values are always bigger than one, which means that $y_1 > y_2$. This means that if the activity coefficient of the water gets bigger the enantiomeric ratio gets smaller. This has been observed by Dorovskaya et al., 1972 (Fig IX.3.3.2). From the curve the difference in y's could be calculated. It can be observed that water as a solvent will always lead to the maximum E value. In other solvents the water E value, which is the ratio of the thermodynamic equilibrium constants, has to be multiplied by something that is smaller than 1, since the activity coefficient of the water molecules will be bigger than 1.

The graph shows that the activity coefficient increases roughly by a factor 3 if log P increases by a factor 4. This means that the assumption made before that this activity coefficient is equal in al solvents is not valid over a large scale. In the expression for the activity retention the activity coefficient of water should be taken into account.

The effect is however not very big compared to the increase the activity coefficient would have if the water had to enter pure organic solvent. This would cause an increase by a factor 10000. This proves that, if the method is right, the water molecules do not enter the organic bulk. The assumption is thus not correct but for qualitative considerations acceptable.

All considerations so far have been about the initial enzymatic activity. If one, however, wants to do something with enzymes not only the inial activity is of interest. An equilibrium reaction will be carried out until the equilibrium conversion or a conversion close to it. The speed at which a reaction reaches it equilibrium is however not constant.

In chapter V relations for the rate of an equilibrium reaction have been derived.

It turned out that the reaction rate could be described with the equations V.4.4, V.4.5 and V.4.7 , applied to reaction IX.6.1

$$v = \vec{v}\,(1 - \exp(-\frac{A}{RT})) \qquad \text{(IX.6.8)}$$

$$A = RT \ln \frac{K_{TH}}{Q} \qquad \text{(IX.6.9)}$$

$$Q = \frac{x_{EA}\, x_{H_2O}{}^{y}}{x_{E.yH_2O}\, x_A} \; \frac{\gamma_{EA}\, \gamma_{H_2O}{}^{y}}{\gamma_{E.yH_2O}\, \gamma_A} \qquad \text{(IX.6.10)}$$

if the assumption that the activity coefficients of the enzyme substrate complex and the enzyme water complex are equal is made this equation will be

$$Q = \frac{x_{EA}\, x_{H_2O}{}^{y}}{x_{E.yH_2O}\, x_A} \; \frac{\gamma_{H_2O}{}^{y}}{\gamma_A} \qquad \text{(IX.6.11)}$$

The affinity changes with the conversion (x's). If one wants to compare affinities in different solvents it is possible to compare the affinities at a given conversion. This means that the term with the x's is constant. Just like before we also assume that the activity coefficient of water is constant, which leads to the equation

$$Q = C \frac{1}{\gamma_A} \qquad \text{(IX.6.12)}$$

This mean if the activity coefficient of the substrate molecule in the solvent increases the value of Q decreases and the affinity increases. If the affinity increases the reaction rate increases. A solvent in which the substrate has a high activity coefficient means that the reaction rate will at a given conversion be close to the initial, maximal reaction rate.
It can be convenient to define the affinity retention, AfR, which is the affinity of a reaction in an organic solvent at a given conversion divided by the affinity of the same reaction under the same conditions in water.

If we change the solvents the affinity of the reaction in water will be constant, at a given conversion (x's). Define the reciprocal of this affinity as C_{1x}. The term with the x's in the affinity of the reaction in the organic solvent is constant too, and has the same value as in water. The activity coefficient of the water is assumed to be constant too. Define constant C_{2x} as

$$C_{2x} = \frac{X_{EA}\, X_{H_2O}{}^{y}}{X_{E.yH_2O}\, X_A}\, \gamma_{H_2O}{}^{y} \qquad \text{(IX.6.13)}$$

This leads to the following equation for the affinity retention for a reaction at given conversion in an organic solvent

$$AfR = \frac{A^{o}}{A^{w}} = \frac{\ln C_{2x}\, \gamma^{o}{}_{A}}{\ln C_{1x}} \qquad \text{(IX.6.14)}$$

This implies that the reaction rate at a given conversion, which is the enzymatic activity, is proportional to the natural logarithm of the activity coefficient of the substrate in the solvent. The maximum activity is the initial activity.
This equation too predicts a curve of the shape found by Laane et al.. They said that they measured the activity of the enzyme in the organic solvent after a few hours in a batch reactor. This means that in solvents were a low conversion is favourable (see afstudeer verslag), in which the solvent has a small activity coefficient, the reaction has a small affinity because the value of Q is close that of K_{TH}, the enzymatic activity is low, and that is what they have found. It thus appears that the in biotechnology well accepted activity retention versus log P graph is a mixture of different effects and definitely not reliable or useful.

X. Solvents in a supercritical state as reaction media.

Application of supercritical gases as solvents for enzyme catalyzed reactions could be very interesting. The reaction rates are probably higher due to the more rapid diffusion of the reagents, denaturation caused by a to high temperature will not occur if solvents with a low critical temperature like carbon dioxide (31°C) or ethane (32°C) are chosen and the separation of the reaction compounds and the solvent is very simple. A disadvantage will be the limited solubility of most of the interesting reaction compounds.

The number of articles published on this subject is still very limited. So far three different authors have reported on the use of four different enzymes in supercritical fluids. Mostly their work just concerns the fact that enzymes catalyze reactions in supercritical fluids. Nakamura et al. (1986) have demonstrated that lipases catalyze transesterifications in supercritical carbon dioxide, Hammond et al. (1985) have demonstrated that polyphenyl oxidase is active in supercritical carbon dioxide and fluoroform and Randolph et al. (1985) have shown that alkaline phosphatase and cholesterol oxidase are active in supercritical carbon dioxide. Furthermore two authors published on mechanistic investigations; Randolph et al. (1988) have examined some effects concerning the cholesterol oxidase activity in supercritical carbon dioxide and Erickson et al. (1990) have examined the effect of pressure on a lipase catalyzed transesterification reaction in supercritical carbon dioxide and ethane. Of these last two articles the results and some of the figures will be shown.

Randolph et al. investigated a few parameters that could be of interest in the cholesterol oxidation and published it in the two articles mentioned above.

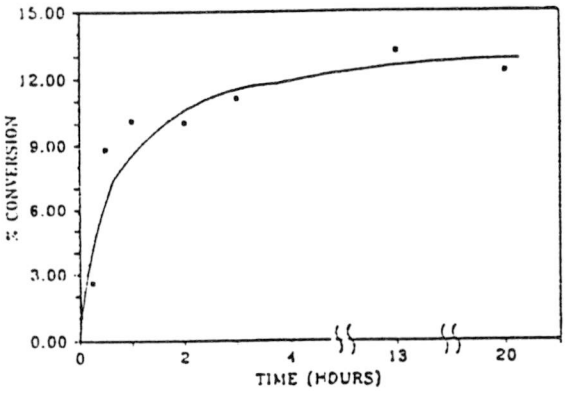


Figure X.1 Batch reaction kinetics of cholesterol oxidation with cholesterol oxidase from *Streptomyces* in supercritical CO_2 at 308 K, 101 bar.

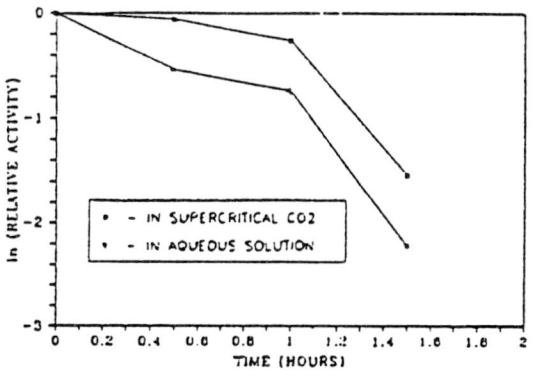


Figure X.2 Thermal denaturation of cholesterol oxidase from *Streptomyces* sp. in supercritical carbon dioxide (101 bar) and water (50-mM phosphate, pH 7.0) at 40°C.

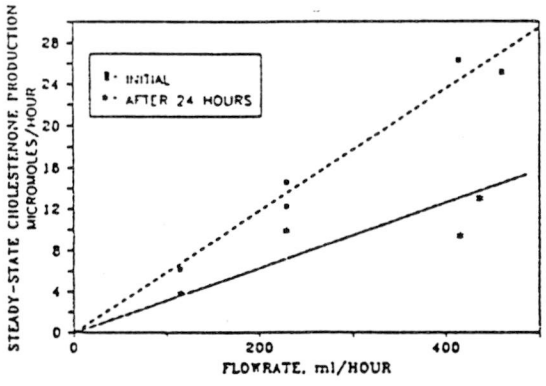


Figure X.3 Effect of carbon dioxide flow rate on packed-bed kinetics of cholesterol oxidation using cholesterol oxidase from *Streptomyces*.

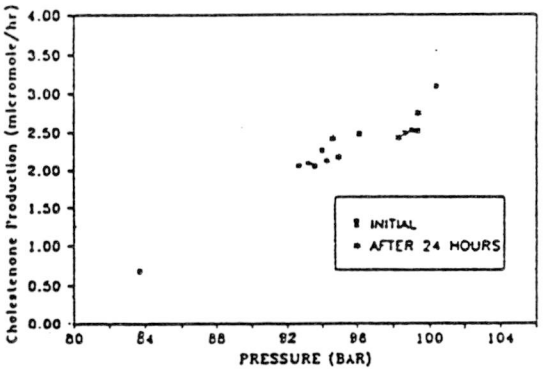


Figure X.4 Effect of pressure on the rate of cholesterol oxidation in supercritical carbon dioxide.

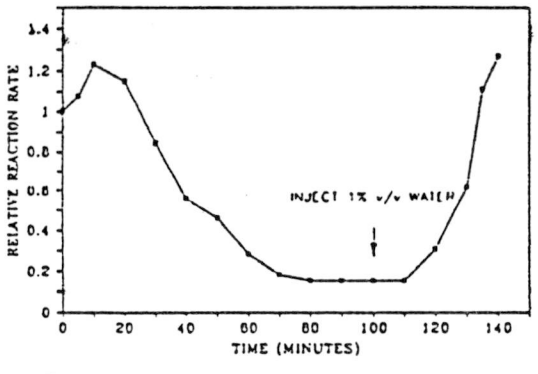


Figure X.5 Effect of water concentration on the rate of enzymatic oxidation of cholesterol in supercritical carbon dioxide using cholesterol oxidase from *Gloeocysticum chrysocreas*.

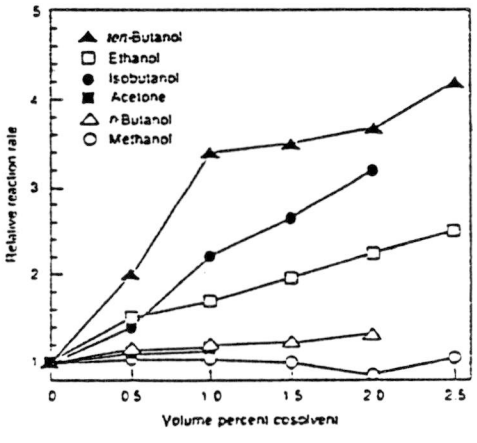


Figure X.6

The parameters they have looked at were :

- batch reaction kinetics of cholesterol oxidation with cholesterol oxidase from Streptomices in supercritical carbon dioxide at 308 K and 101 bar, (figure X.1)
- thermal denaturation of cholesterol oxidase from Streptomices in supercritical carbon dioxide and water with 50 mM phosphate and pH 7.0 at 101 bar and 40 °C, (figure X.2)
- the effect of carbon dioxide flow rate on packed bed kinetics of cholesterol oxidation using cholesterol oxidase from Streptomices, (figure X.3)
- effect of pressure on the rate of cholesterol oxidation in supercritical carbon dioxide, (figure X.4)
- the effect of the water concentration on the rate of enzymatic oxidation of cholesterol in supercritical carbon dioxide using cholesterol oxidase from Gloeocysticum Chrysocreas, (figure X.5)
- the effect of cosolvents on the rate of enzymatic oxidation of cholesterol using cholesterol oxidase from Gloeocysticum Chrysocreas. (figure X.6)

Their conclusions based on the figures and table mentioned are the following :

- stability under supercritical conditions varies according to species but the one used in the experiment is more stable than in water. (Fig X.2, upper line is in CO_2)
- diffusion of cholesterol to the enzyme is not a rate-limiting step in the range of flow rates used,
- at least a trace of water is needed to maintain catalytic activity in dense carbon dioxide,
- reaction rates are comparable to or greater than those found in aqueous solutions.

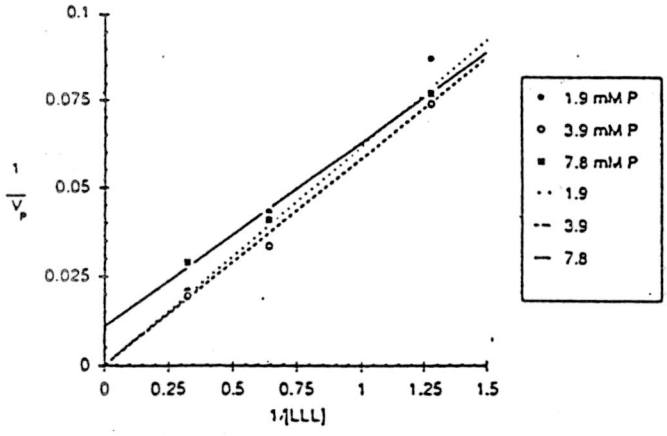


Figure X.7 Double reciprocal plot of reaction rate in CO_2 at 40°C and 15 MPa vs. [LLL]

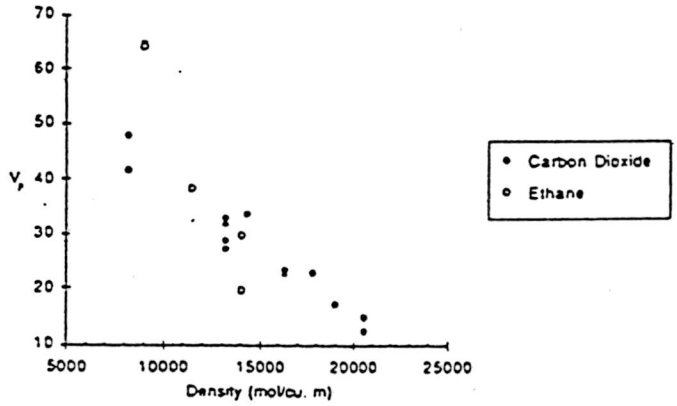


Figure X.8 Reaction rate vs. density for both CO_2 and ethane at 40°C.

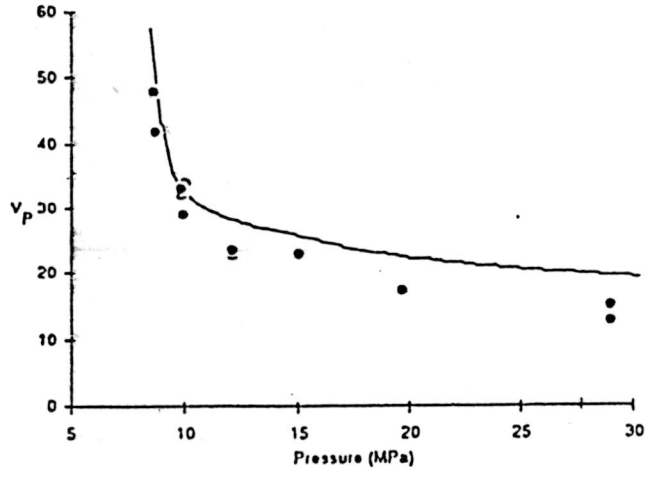


Figure X.9 Effect of pressure on reaction rate in supercritical CO_2 at 40°C

They suggest that cholesterol formes aggregates in supercritical carbon dioxide with increasing pressure (solubility) or by addition of small amounts of cosolvents.
Aggregates cause a higher activity because of high local concentrations. Branched butanols and to a lesser degree ethanol promote aggregation, methanol, acetone and 1-butanol do not promote aggregation. Cosolvent that promote aggregation also increase the reaction rate as does the pressure.

Erickson et al. studied the effect of the pressure on the transesterification of trilaurin (LLL) and palmitic acid (P) catalyzed by lipase from Rhizopus arrhizius. Initial reaction rates were measured in supercritical carbon dioxide at 15 MPa and 40 °C. Data of the initial rate versus [LLL] at constant [P] different pressures is presented in a double reciprocal form in figure X.7. The influence of the reaction medium has been studied by changing the carbon dioxide for ethane. Because ethane has a higher critical pressure, the initial rates were determined versus the density which makes the results comparable. (figure X.8) Furthermore the effect of the pressure on the reaction rate in supercritical carbon dioxide at 40 °C has been determined, presented in figure X.9. The initial rates all fall along the same line except at lowest densities, which are near the critical pressures. The authors develop a model based on the mole fraction of reactants and the molar volume of the solvent at 15 MPa. This model is the line in figure X.8.

It is funny to see that the experiments of Randolph et al. show an initial rate increasing with pressure while Erickson et al. find an initial rate decreasing with pressure. They both cannot completely explain the phenomena they have observed so the conclusion that supercritical solvents indeed seem to be good solvent for enzyme catalyzed reactions but that there still has to be done a lot of research on this topic appears to be the right conclusion.

XI. Conclusions.

The objective of this work was to try to explain and possibly predict enzyme behaviour in organic solvents, focusing on stability, activity and stereoselectivity and present an overview on these topics.

The experimentally found fact that some organic solvents stabilize enzymes and some do not, can be explained by considering the Gibbs energy of the enzyme/organic molecule complex, the enzyme/water molecule complex and the organic/water bulk. If the Gibbs energy of the enzyme/organic complex is lower than the enzyme/water complex the enzyme will be more thermostable compared to water, if this is not the case, normally this complex would not exist. This is, however, still possible if it is favourable for the Gibbs energy of the total system. In this case it will destabilize the enzyme.

The theory developed for the influence of the organic solvent on the enzyme activity can explain the fact that enzymes are not equally active in all organic solvents. The reason for this is, however, not the influence of the solvent itself on the enzyme, but the fact that the equilibrium between enzyme and substrate is influenced by the solvent properties. If the substrate feels comfortable in the solvent, the equilibrium will shift towards the substrate side, thus slowing the reaction down, which means a low enzyme activity is determined. The same theory can be applied to stereoselectivity and here too, it is possible to describe the decrease in enantiomeric ratio by the equilibrium constant for the enzyme substrate complex. In this case involved water is responsible for the decrease in enantiomeric ratio. It is also possible to describe and explain observed behaviour and thus predict qualitatively the behaviour in other solvents. The solvent does not have a direct influence on the enzyme and that is in contradiction with what is believed so far. It is a pity that for quantitative calculations the theory is still too complicated.

Experimentally it has been observed that enzymes need a certain amount of water to maintain there active conformation.

This has been called a critical water concentration. This is of course proportional to a critical water activity. In other words, an enzyme is active in an organic solvent if the water activity in the solvent is at least as high as the water activity of the water molecules that form the necessary layer of water around the active sites of the enzyme. This is also a restriction for the use of enzymes in water miscible organic solvents, there the water fraction will sometimes have to be in the order of 70% . Once the critical water activity is known, it can be used for the enzyme in all solvents possible. Each enzyme however has its own critical water activity.

XII. References

- Ahern, T.J., Klibanov, A.M., Science, 228, 1280 (1985)
- Ayala, G., de Gomez-Puyou, M.T., de Gomez-Puyou, A., Darszon, A., FEBS Lett. 203, 41 (1986)
- Berthelot, Jungfleisch, Ann. Chim. Phys., 4, 26 (1872)
- Bodor, N., Gabanyi, Z., Chu-Kuok Wong, J. Am. Chem. Soc., 111, 3783 (1989)
- Brink, L.E.S., Tramper, J., Biotechnol. Bioeng., 27, 1258 (1985)
- Butler, L.G., Reithel, F.J., Arch. Biochem. Biophys., 178, 43 (1979)
- Cassels, J.M., Halling, P.J., Enzyme Microb. Technol., 10, 486 (1988)
- Chen, C.S., Fujimoto, Y., Girdaukas, G., Sih, C.J., J. Am. Chem. Soc., 1982, 104, 7294 (1982)
- De Donder, T., Lecons de Thermodynamique et de Chimie-Physique, Gauthier-Villars, Paris (1920)
- Donovan, J.W., Trends Biochem. Sci., 9, 340 (1984)
- Dordick, J.S., Enzyme Microb. Technol., 11, 194 (1989)
- Dorovskaya, V.N., Varfolomeyev, S.D., Kazanskaya, N.F., Klyosov, A.A., Martinek, K., FEBS Lett., 23, 122 (1972)
- Erickson, J.C., Schyns, P., Cooney, C.L., AIChE Journal, 36(2), 299 (1990)
- Fujita, Y., Miyanga, A., Noda, Y., Bull. Chem. Soc. Japan, 52, 3659 (1979)
- Fujita, Y., Izumiguchi, S., Noda, Y., Int. J. Peptide Protein Res., 19, 25 (1982)
- Haldane, J.B.S., Enzymes, Longman, Green & Co, London (1930)
- Halling, P.J., Biocatalysis in Organic Media, Elsevier Science Publishers BV, Amsterdam (1987)
- Hammond, D.A., Karel, M., Klibanov, A.M., Krukunis, V.J., Appl. Biochem. Biotech., 11, 123 (1985)
- Hansch, C., Accounts Chem. Res., 2, 232 (1969)
- Hansch, C., Leo, A., Elkens, D., Chem. Rev., 71, 525 (1971)
- Hansch, C., Leo, A., Substituent Constants for Correlation Analysis in Chemistry and Biology, Wiley New York (1979)

- Helmer, F., Kiehs, K., Hansch, C., Biochemistry, 7, 2858 (1968)
- Henri, V.C.R., Acad. Sci. Paris, 135, 916 (1902)
- Hildebrand, J.H., Prausnitz, J.M., Scott, R.L., Regular and Related Solutions, Van Nostrand Reinhold Company (1970)
- Ingalls, R.G., Squires, R.G., Butler, L.G., Biotechnol. Bioeng., 17, 1627 (1975)
- Kennedy, J.F., Melo, E.H.M., Chem. Eng. Prog. Des. Dev., July, 81 (1990)
- Khmelnitsky, Y.L., Levashov, A.V., Klyachko, N.L., Martinek, K., Enzyme Microb. Technol., 10, 710 (1988)
- Klibanov, A.M., Anal. Biochem., 92, 1 (1979)
- Klibanov, A.M., Adv. Appl. Microbiol., 29, 1 (1983)
- Klibanov, A.M., Chemtech, June, 354 (1986)
- Klibanov, A.M., TIBS, April, 141 (1989)
- Klopman, G., Iroff, L.D., J. Comput. Chem. 2, 157 (1981)
- Knox, J.H., Molecular Thermodynamics, John Wiley & Sons, New York (1978)
- Koch-Schmidt, A.C., Mosbach, C., Biochemistry, 16, 2105 (1977)
- Koshland, .D.E., Sci. Amer., 229, 52 (1973)
- Laane, C., Boeren, S., Hilhorst, R., Veeger, C., Biocatalysis in Organic Media, Elsevier Science Publishers B.V., Amsterdam (1987)
- Martinek, K., Klibanov, A.M., Berezin, I.V., J. Solid-Phase Biochem., 2, 343 (1977)
- Meyer, H., Arch. Exptl. Pathol. Pharmakol., 42, 110 (1899)
- Michaelis, L., Menten, M.L., Biochem. Z., 49, 333 (1913)
- Mozhaev, V.V., Martinek, K., Enzyme Microb. Technol., 6, 50 (1984)
- Mullaney, P.F., Nature, 210, 953 (1966)
- Nakamura, K., Chi, Y., Yamada, Y., Yano, T., Chem. Eng. Commun., 45, 207 (1986)
- Nernst, W., Z. Phys. Chem., 8, 110 (1891)
- Overton, E., Studien uber die Narkose, Fischer, Jena, Germany (1901)
- Prigogine, I., Thermodynamics of Irreversible Processes, Interscience Publishers, New York (1955)

- Randolph, T.W., Blanch, H.W., Prausnitz, J.M., Biotech. Lett., 7(5), 325 (1985)
- Randolph, T.W., Blanch, H.W., Prausnitz, J.M., Sci., 238, 378 (1988)
- Reichardt, C., Solvent Effects in Organic Chemistry. Monographs in Modern Chemistry, 3, Verlag Chemie, Weinman-New York (1979)
- Rekker, R.F., Chemisch Weekblad, 12th april, 1974
- Rekker, R.F., The Hydrophobic Fragment Constent, Elsevier Amsterdam (1976)
- Reslow, M., Aldercreutz, P., Mattiasson, B., Appl. Microbiol. Biotechnol., 26, 1 (1987)
- Rodgers, P.B., Durrant, I., Beynon, R.J., Biocatalysys in Organic Media, Elsevier Science Publishers BV , Amsterdam (1987)
- Schmid, R.D., Adv. Biochem. Eng., 12, 41 (1979)
- Shinoda, K., Becher, P. Principles of Solution and Solubility, Marcel Dekker, New York (1978)
- Tanford, C., Adv. Protein Chem., 23, 121 (1968)
- Tanford, C., Adv. Protein Chem., 24, 1 (1970)
- Zaks, A., Klibanov, A.M., Science, 224, 1249 (1984)
- Zaks, A., Klibanov, A.M., J. Bio. Chem., 263, 8017 (1988)
- Zale, S.E., Klibanov, A.M., Biochemistry, 25, 5432 (1986)