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The enantiomeric ratio: origin, determination and prediction

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The enantiomeric ratio $E = (k^R_{cat}/K^R_m)/(k^S_{cat}/K^S_m)$ offers a concise representation of the enantioselective properties of an enzyme in reactions that involve chiral compounds. Both as a measure of the intrinsic selectivity of the catalyst, and as a parameter to model the performance of enzymatic processes for the production of enantiopure fine-chemicals, its merits have been well-recognized.

Several methods for the determination of E exist. The scope and limitations of these methods are evaluated in terms of accuracy and feasibility. There appears to be no single method that is both reliable and readily applicable in all cases. Complementary methods, however, are available.

The outstanding characteristics of the enantiomeric ratio as a quantitative measure of the effects of physical and chemical conditions on the intrinsic enantioselectivity of enzymes are presented in terms of the difference in Gibbs energies of the diastereomeric enzyme-substrate transition states. The prospects of molecular modeling strategies for the prediction of E are discussed. © *1997 Elsevier Science Inc.*

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Introduction

Enantioselective catalysis

In response to the general awareness of the physiological and ecological advantages of the use of single enantiomers, the manufacture of enantiomerically pure compounds has become an expanding area of the fine chemicals industry.[1,2] Annual sales of enantiopure drugs have reached $35 billion.[3] When pharmaceuticals, agrochemicals, food additives, and their synthetic intermediates are marketed as single enantiomers, high enantiomeric purities, typically enantiomeric excess (ee) > 98%, are required. The ee is derived from the concentration of the two enantiomers:

$$ee = \left| \frac{c^R - c^S}{c^R + c^S} \right| \qquad (1)$$

In the development of new process technologies classical resolution, chiral chromatography and enantioselective catalysis are important alternatives.[4] Enantioselective catalytic methods employing enzymes, microbes, or chiral chemocatalysts require appropriate combinations of process type, conditions, and catalyst. Clearly, the enantioselective performance of the catalyst is the single most important factor for the success of such processes. Evaluation of this property has already been greatly facilitated by the use of the enantiomeric ratio, E. Further advancement in the field of enantioselective biocatalysis will benefit from a rigorous application of the concepts underlying its formulation as the prime parameter for the description of enzyme enantioselectivity.

The enantiomeric ratio is the ratio of specificity constants

The apparent second-order rate constant for the reaction of a substrate with an enzyme, k_{cat}/K_m, is called the *specificity constant* of the enzymatic reaction.[5,6] The ratio of specificity constants is the parameter of choice to express the relative rates of competing enzymatic reactions;[7] thus, specificity constants and their ratios are generally used to express enzymatic selectivities.[8]

The first application of specificity constants to characterize the *enantio*selectivity of enzymes as the ratio $(k^L_{cat}/K^L_m)/(k^D_{cat}/K^D_m)$ was reported for the conversion of L- and D-amino acid derivatives by α-chymotrypsin.[9] Since the work of Chen *et al.*[10] ratios of specificity constants for

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enantiomers are commonly expressed as the enantiomeric ratio E.[1]

$$E \equiv \frac{V^R_{max}/K^R_m}{V^S_{max}/K^S_m} \equiv \frac{k^R_{cat}/K^R_m}{k^S_{cat}/K^S_m} \quad (2)$$

By definition, E is an intrinsic property of the enzyme. The intrinsic E value cannot change unless the intrinsic values of K_m or k_{cat} change (see below).

Kinetic resolution

In an (enzyme-)catalyzed kinetic resolution process of racemic substrate, one enantiomer will be converted preferentially. When the catalyst is absolutely selective, 50% of enantiopure product will be obtained while 50% enantiopure substrate will remain at the end of the reaction. For lower enantioselectivity, the required enantiomeric purity of substrate may still be reached at higher conversions; thus, the relationship between enantiomeric excess of substrate and extent of conversion is of practical importance.

Balavoine *et al.*[11] derived this relation for a photodecomposition of a racemate by circularly polarized light. The parameter used in this relation was k^R/k^S, the ratio of rate constants for the two enantiomers, referred to as the "stereoselectivity factor".[12] A similar equation, using k^R/k^S, was derived for the chemocatalytic Sharpless epoxidation of a racemic allylic alcohol.[13] Chen *et al.*[10] showed that this relation also holds for enzymatic catalysis with E as the selectivity parameter; thus, similar equations and parameters are used to describe enantioselective photochemical, chemical, and enzymatic catalysis.[12,14]

The basic enzymatic example is the irreversible conversion of a single racemic substrate, S, into a single chiral product, P, via a Michaelis complex in a homogeneous batch reaction.[10] In the absence of side reactions, Eq. (3) applies:

$$\frac{dc^R_S/dt}{dc^S_S/dt} = E \cdot \frac{c^R_S}{c^S_S} \quad (3)$$

Upon integration with the initial concentrations c^R_{S0} and c^S_{S0} at $t = 0$

$$\ln \frac{c^R_S}{c^R_{S0}} = E \cdot \ln \frac{c^S_S}{c^S_{S0}} \quad (4)$$

Eq. (4) can be transformed into a relationship between the enantiomeric excess of the remaining substrate, ee_S, and the degree of conversion, ξ.[10,13] The latter is defined by

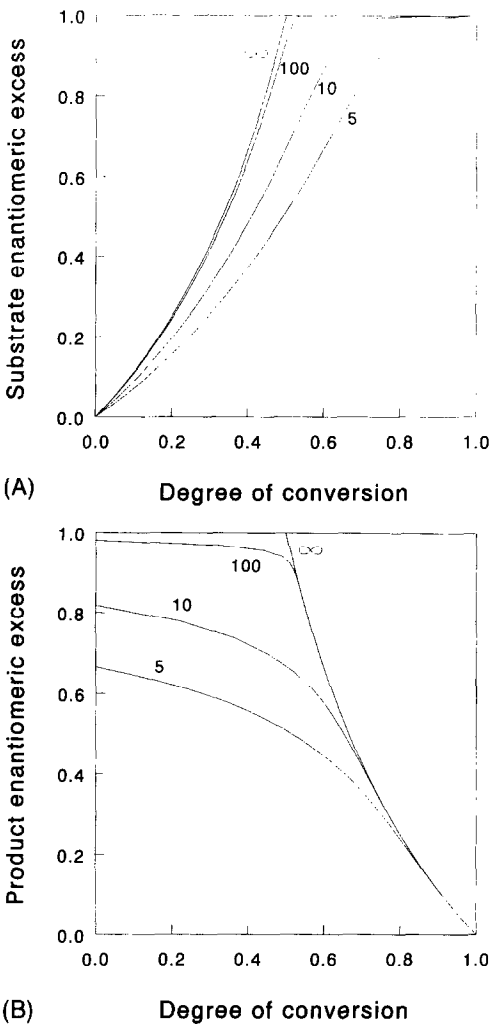

Figure 1 The effect of the enzyme enantioselectivity expressed as the enantiomeric ratio E on the enantiomeric excess of the remaining substrate (A) and the reaction product (B) as a function of the conversion.[10]

$$\xi = 1 - \frac{c^R_S + c^S_S}{c^R_{S0} + c^S_{S0}} \quad (5)$$

From Eqs. (1) and (5), one gets

$$c^R_S = (1 - \xi)(1 - ee_S)(c^R_{S0} + c^S_{S0})/2 \quad (6)$$

and

$$c^S_S = (1 - \xi)(1 + ee_S)(c^R_{S0} + c^S_{S0})/2 \quad (7)$$

Starting from racemic substrate, $c^R_{S0} = c^S_{S0}$, combination of Eqs. (4), (6) and (7) leads to

$$E = \frac{\ln[(1 - \xi)(1 - ee_S)]}{\ln[(1 - \xi)(1 + ee_S)]} \quad (8)$$

A graphic represention of this equation (*Figure 1A*) is very helpful to estimate the amount of enantiopure remaining substrate that can be obtained. When *ee* has increased to the required value, the reaction should be terminated in order to obtain the maximum yield. Although $E = 100$ is

[1] According to Eq. (2), E is routinely taken to represent R-specificity. In cases of S-specificity, however, the use of the reciprocal value may be desirable. Whenever ambiguity arises, it is advised to apply superscripts, *e.g.*, $E^S = 1/E^R$. Future developments may warrant the invocation of pE ($= -\log E^R$), both to emphasize the relation to Gibbs energies and to cover situations where the enantiopreference changes within a set of experiments.[62] Inversion of enantiomeric signature, for example when (R)-substrate is converted into (S)-product merely as a result of ligand priority assignments using the Cahn-Ingold-Prelog rules, is properly covered by the definitions. According to Eq. (2), E concerns the configuration of the substrate; the product configuration does not matter.

almost as effective as $E = \infty$, the difference between $E = 100$ and $E = 10$ is significant; however, even an enantioselectivity as low as $E = 5$ still can be exploited to obtain substrate with high purity ($ee > 98\%$).

Chen *et al.*[10] derived a similar equation for the resolution of product [see Eq. (15)]. In that case, the course of the kinetic resolution process is different (*Figure 1B*). Initially, the enantiomeric excess of the product equals $(E - 1)/(E + 1)$; at $\xi \approx 50\%$, the *ee* value rapidly diminishes, leading to $ee = 0\%$ (racemic product) at $\xi = 100\%$. Values of $ee_P > 98\%$ can only be obtained for $E > 100$. Often appropriate enzymes are not available and then one should aim at obtaining enantiopure remaining substrate rather than at enantiopure product. This is a major conclusion in the field of kinetic resolution.

Asymmetric catalysis

Enantioselective catalysts can be applied to convert prochiral substrates into enantiopure products. In such a case, Eq. (2) reduces to $E = k^R_{cat}/k^S_{cat}$. Both enantiomer concentrations increase simultaneously during the conversion in the ratio $c^R_P/c^S_P = E$.[15] (Hence the term "enantiomeric ratio.") Using Eq. (1), E may be calculated from

$$E = \frac{1 + ee_P}{1 - ee_P} \tag{9}$$

Neither ee_P nor E change during the reaction, and either quantity can be used to express the enantioselectivity. For chemocatalytic processes ee_P is usually employed although this provides less fundamental insight than the use of k^R/k^S, the chemocatalytic equivalent of E.[16] For enzymatic asymmetric catalysis, the use of E has not gained widespread acceptance either. The reason for this is that the magnitude of the enantiomeric ratio is irrelevant when enzymes are virtually absolutely enantioselective as is often the case during enzymatic asymmetric catalysis. For example, in yeast-catalyzed asymmetric reductions, all dehydrogenases involved seem to be absolutely enantiospecific. (Then the enantiomeric excess may be below 100% just because of the presence of several competing enzymes with opposite enantiospecificity.[17]) Because of the relatively low significance of E in the field of asymmetric catalysis, in this contribution attention will be focused on kinetic resolution processes.

Evaluation and improvement of enantioselective processes

Regardless of the method that is used for the determination of E, plots of enantiomeric excess versus conversion are required to evaluate the performance of a kinetic resolution process. If the remaining substrate is the target compound, a plot of ee_S versus ξ should be used (*e.g., Figure 1A*). If the reaction product is the target compound, a plot of ee_P versus ξ is required (*e.g., Figure 1B*).

The shape of the curves depends not only on the intrinsic value of E but also on the reaction conditions. When one wishes to evaluate the potential impact of the reaction conditions, one should be aware that all reaction conditions that are relevant to the determination of E (see below) should be considered to be degrees of freedom to modify the *ee* vs. ξ plot and therefore the performance of a resolution process. A systematic evaluation may point to well-known strategies to improve this performance, for example, substrate racemization, recycling of enriched substrate, and performing sequential reactions. Such strategies have recently been reviewed.[12,14,18,19]

The degrees of freedom to change the intrinsic value of E are discussed below. Temperature, pressure, medium composition, and substrate and enzyme structure appear to be of importance.

Determination of E for (enzyme-)catalyzed kinetic resolutions

Quantities that have to be measured

When a kinetic resolution is performed on a racemic mixture, four different quantities can be monitored:

1. Extent of conversion (ξ)
2. Enantiomeric excess of substrate (ee_S)
3. Enantiomeric excess of product (ee_P)
4. Time (t)

(Instead of *ee*, alternative expressions for the enantiomeric composition may be used.[20])

As will be shown below, all known methods to determine E rely on a combination of measurements of two out of these four quantities. We will discuss the measurement of these quantities first.

The determination of time is unambiguous. Determination of enantiomeric excess values, however, can be performed in various ways. Such measurements are, of course, greatly facilitated because of the rapid development of chiral chromatography.[21] Note that when *ee* is determined from the ratio of concentrations of (*R*- and (*S*)-enantiomer, calibration or quantitative handling of samples is usually not necessary, thus reducing errors;[22] however, accurate measurement of $ee > 98\%$ may require special methods.[23] For nonchiral reaction products (*e.g.*, a ketone obtained by oxidation of a chiral secondary alcohol), determination of ee_P is not applicable for obvious reasons.

When the predominance of one enantiomer is reversed in the course of a reaction, it is useful to modify Eq. (1).[20,24]

$$ee^R = \frac{c^R - c^S}{c^R + c^S} \tag{10}$$

Thus ee^R becomes negative when the (*S*)-enantiomer is in excess. Consequently, $ee^S = -ee^R$. Eq. (1) can be retained when the enantiomer in excess does not alter.

Measurement of the extent of conversion appears straightforward; however, the different characteristics of several methods are worth noting. These differences are important when mass balances are in error as will be shown below.

It must be realized that ξ should be calculated from the amounts of substance. Only when the reaction volume is constant and one phase is involved, concentrations can be taken instead. Either the total [= (*R*) + (*S*)] substrate concentration or total product concentration of a sample is measured, and the measured concentration is compared with

either the initial concentration of substrate, c_{S0}, or if the reaction goes to completion, the final concentration of product, $c_{P\infty}$. This leads to four alternative ways to express ξ:

$$\xi = 1 - \frac{c_S}{c_{S0}} = \frac{c_P}{c_{P\infty}} = \frac{c_P}{c_{S0}} = 1 - \frac{c_S}{c_{P\infty}} \tag{11}$$

If the substrate is converted in a single product, the ratio of substrate and product concentration of a sample (c_S/c_P) may be used to calculate the conversion and knowledge of c_{S0} or $c_{P\infty}$ is not required.

$$\xi = \frac{1}{1 + c_S/c_P} \tag{12}$$

Alternatively, when the enantiomeric excess of both the substrate and the product are measured, the extent of conversion can also be calculated using the formula of Sih and Wu:[14]

$$\xi = \frac{ee_S}{ee_S + ee_P} \tag{13}$$

This relation is not valid when the initial enantiomeric excess of substrate or the initial concentration of product is not equal to zero, as can be seen from the "chiral balance" on which it is based.[25]

Methods for the determination of E

Considering measurements of two out of the four quantities mentioned above, different combinations are possible. The methods to determine E are arranged according to these (six) combinations. First, they will all be discussed for the "basic case" of an irreversible reaction with one substrate, product, and enzyme in a homogeneous batch reaction. In later sections, other cases are considered.

Method 1. Enantiomeric excess of substrate vs. extent of conversion. This combination of measurements is the basis of the method of Chen *et al.*[10] Application of Eq. (8) allows the determination of E (*Figure 1A*). When multiple data points are available, nonlinear regression analysis of ee_S as an explicit function of E is not possible, because from Eq. (8), only ξ can be obtained as an explicit function of ee_S.

$$\xi = 1 - \left(\frac{1 - ee_S}{(1 + ee_S)^E}\right)^{\frac{1}{E-1}} \tag{14}$$

Appropriate methods for regression analysis will be discussed below.

Method 2. Enantiomeric excess of product vs. extent of conversion. This combination is the basis of the alternative method of Chen *et al.*[10] When ee_s is eliminated from Eq. (8) using Eq. (13), the model equation is obtained:

$$E = \frac{\ln[1 - \xi(1 + ee_P)]}{\ln[1 - \xi(1 - ee_P)]} \tag{15}$$

Explicit expressions for either ee_P or ξ cannot be extracted from Eq. (15), thus obstructing direct regression analysis of multiple data points.

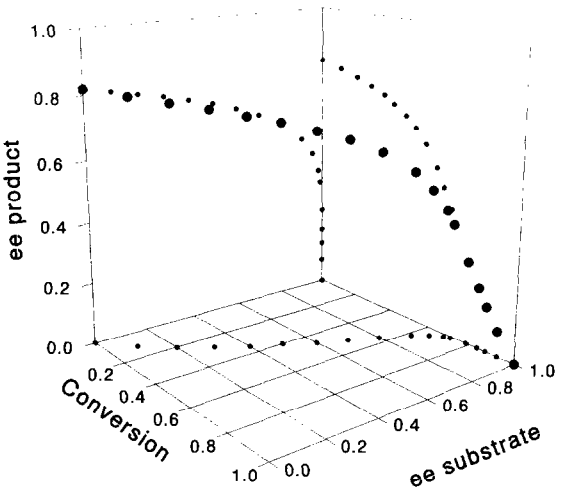

Figure 2 Course of a kinetic resolution for $E = 10$ in three dimensions. The larger dots are hypothetical experimental data. Their projection on the surfaces represent Method 1 (ee_S vs. ξ, bottom surface), Method 2 (ee_P vs. ξ, right surface), and Method 3 (ee_P vs. ee_S, left surface).

Method 3. Enantiomeric excess of product vs. enantiomeric excess of substrate. This combination was introduced by Rakels *et al.*[22] by elimination of ξ from Eq. (8) using Eq. (13). The model equation is

$$E = \frac{\ln[(1 - ee_S)/(1 + ee_S/ee_P)]}{\ln[(1 + ee_S)/(1 + ee_S/ee_P)]} \tag{16}$$

For regression analysis, ee_P can now be written explicitly as a function of ee_S.[22]

Method 3 can be bypassed by calculating ξ from ee_S and ee_P using Eq. (13), and using Method 1 or 2 instead; however, this is not recommended because it obscures the origin of potential errors in the measurements.[22] In general, model equations should be fitted directly to the measured quantities and not to derived data.

The close relation between Methods 1, 2, and 3 is shown in *Figure 2*.

Method 4. Conversion vs. time. The "classical" method (4a) to determine E is by calculation of the ratio of specificity constants using V_{max} and K_m (or V_{max}/K_m) of the pure enantiomers only. When pure enantiomers are available, the latter parameters can be determined by the usual methods[7] by fitting the initial rates to the Michaelis-Menten equation.

It should be noted that when the initial rates of the individual enantiomers are measured separately at a certain substrate concentration, the ratio of initial rates r_0^R/r_0^S depends on the substrate concentration and serves merely as a *qualitative* measure of enantioselectivity.[26]

If $c_{S0}^R = c_{S0}^S = c_{S0}$

$$\frac{r_0^R}{r_0^S} = \frac{V_{max}^R c_{S0}^R/(K_m^R + c_{S0}^R)}{V_{max}^S c_{S0}^S/(K_m^S + c_{S0}^S)} = \frac{V_{max}^R/(K_m^R + c_{S0})}{V_{max}^S/(K_m^S + c_{S0})} \neq \frac{V_{max}^R/K_m^R}{V_{max}^S/K_m^S} \tag{17}$$

Only if concentrations well below the K_m values are used (pseudo first-order kinetics) or if $K_m^R = K_m^S$ the ratio of initial rates will equal E. Many examples where this has not been checked are found in the literature.

In order to deal with practical situations, when samples of single enantiomers of suitable enantiomeric purity are not available, Method 4a has been extended by Jongejan *et al.*[27] In this method (4b), the initial rate of (*R*)/(*S*)-mixtures of different enantiomeric composition, which may include the racemate, should be measured at a fixed overall concentration. (In all other methods, the substrate concentration varies which may be a disturbing effect.) The basic equation is

$$E = \frac{r_0^R(r_0^x - r_0^S)}{r_0^S(r_0^R - r_0^x)} \frac{1 - x}{x} \tag{18}$$

In this equation r_0^R and r_0^S are the initial rates for the pure enantiomers and r^x is the initial rate for a mixture with a fraction x of (*R*)-enantiomer and $1 - x$ of (*S*)-enantiomer. From a plot of r_0^x vs. x the value of E can be determined. One can derive from Eq. (18) the explicit equation suitable for nonlinear regression:

$$r_0^x = \frac{\dfrac{1 - x}{x r_0^S} + \dfrac{E}{r_0^R}}{\dfrac{1 - x}{x} + E} \tag{19}$$

When r_0^R and r_0^S can be evaluated separately, the accuracy of the method is greatly improved. In other cases they are treated as parameters to be estimated in addition to E.

A progression curve method can also be used (Method 4c).[28] Although this method can be performed using the racemic substrate, better accuracy is obtained when ξ is measured separately for both substrate enantiomers. Using initial estimates of V_{max}^R, K_m^R, V_{max}^S and K_m^S, progression curves of conversion vs. time are simulated using differential equations of the following type:

$$-\frac{dc_S^R}{dt} = \frac{\dfrac{V_{max}^R}{K_m^R} c_S^R}{1 + \dfrac{c_S^R}{K_m^R} + \dfrac{c_S^S}{K_m^S}} \tag{20}$$

Subsequently, the simulated curves are fitted to the experimental curves by adjusting the parameter values. E is calculated using Eq. (1).

When an enantiomer has $K_m >> c_S$ (within the experimental range), only the first-order constants $k = V_{max}/K_m$ can be estimated by this procedure, and V_{max} and K_m cannot be determined separately. In that case, the integrated first-order equations can also be used for fitting.[29] Only two independent parameters are involved (k^R and k^S), so it has been argued that the minimum number of data points of conversion vs. time is two;[30] however, one should be aware that this is valid only when the concentrations are in the pseudo first-order range. This range cannot be determined without performing additional experiments, thus limiting the value of this modification[30] of Method 4c. If first-order kinetics cannot be justified, the minimum number of data points is three as shown in an early version of Method 4c.[31]

Method 5. Substrate enantiomeric excess vs. time. For pseudo first-order reactions, the course of the substrate enantiomeric excess with time is known[11] to be the following function of the first-order rate constants k:

$$ee_S = \frac{\exp[(k^S - k^R)t] - 1}{\exp[(k^S - k^R)t] + 1} = \tanh\left[(k^S - k^R)\frac{t}{2}\right] \tag{21}$$

The only parameter in this equation is $(k^S - k^R)$, so this method provides only the **difference** between the first-order rate constants whereas their **ratio** has to be known to determine E: therefore, Method 5 cannot be applied to first-order kinetics. For Michaelis-Menten kinetics, this method has not been evaluated.

Method 6. Product enantiomeric excess vs. time. In this case, the equation has also been derived for first-order kinetics only.[29]

$$ee_P = \frac{\exp(-k^R t) - \exp(-k^S t)}{2 - \exp(-k^R t) - \exp(-k^S t)} \tag{22}$$

Now k^R and k^S are independent parameters that can both be determined from fits of Eq. (22). Calculation of their ratio provides E. This method has only been used in combination with Method 4c.[29]

Because of the limited information on Methods 5–6, the discussion of potential complications will be restricted to Methods 1–4.

Impact of substrate composition

In order to determine E, the use of contaminated substrate is undesirable, but not always avoidable. The determination of E may be affected in various ways as outlined below and summarized in *Table 1*.

If a contamination is not noticed, the value of c_{S0} used in the calculations will be overestimated. Methods using conversion measurements that rely on this value may lead to wrong values of E. A special case occurs when a substrate is contaminated by racemic product. For instance, when the substrate is a racemic ester, decomposition during storage or handling may take place. At the start of an experiment, samples may contain racemic product; thus, methods that depend on the determination of ξ or ee_P are especially sensitive for this problem. Methods 1, 2, and 3 have been modified to deal with this situation.[32] Eqs. (8) and (14)–(15) were extended by introducing the initial conversion ξ_0 as an additional parameter. (For each of these equations, the extension involves that the terms between square brackets are divided by the term "$1 - \xi_0$", both in the numerator and the denominator.) Using these extended equations, E and ξ_0 were determined. A more accurate determination of the enantiomeric ratio by this method is possible when ξ_0 is determined a priori.

Occasionally, the enantiomers of a chiral substrate are present in unequal amounts. This situation may arise when the compound is treated enzymatically several times in a recycling procedure to increase the enantiomeric excess.[10] In those cases, the derivation of Eq. (8) from (4) proceeds differently. Chen *et al.*[10] presented a relationship between the initial enantiomeric excess value of the substrate ee_{S0} and the enantiomeric excess of the product:

Table 1 Comparison of different methods for the determination of E

Method	1	2	3	4a	4b	4c
Equation no.	Eq. (8)	Eq. (15)	Eq. (16)	Eq. (2)	Eq. (18)	Eq. (20), (2)
Required measurements	ξ, ee_S	ξ, ee_P	ee_S, ee_P	ξ, t	ξ, t	ξ, t
Required substrate	Racemate	Racemate	Racemate	Pure enantiomers	Enantiometrically enriched	Anything
Sensitive for substrate contamination	Yes	Yes	Yes	Yes	Yes	Yes
Sensitive for background reaction of substrate	Yes	Yes	Sometimes	Yes	Yes	Yes
Sensitive for background reaction of product	If c_P is monitored	For enantioselective background reaction, if c_p is monitored	For enantioselective background reaction	If c_P is monitored	If c_P is monitored	If c_P is monitored
Sensitive for racemization	Of substrate	Yes	Yes	Of substrate	Of substrate	Of substrate
Sensitive for enzyme inactivation	No	No	No	No	No	Yes
Sensitive for reversibility or product inhibition	No	No	No	Yes	Yes	No
High accuracy	At low E	At low E	At low E	Yes	No	Sometimes
Simple computation	Yes	Yes	Yes	Yes	Yes	No
Additional parameters determined	No	No	No	V^R_{max}, K^R_m, V^S_{max}, K^S_m	No	V^R_{max}, K^R_m, V^S_{max}, K^S_m

$$\left[1 - \xi\left(\frac{1 + ee_P}{1 + ee_{S0}}\right)\right] = \left[1 - \xi\left(\frac{1 - ee_P}{1 - ee_{S0}}\right)\right]^E \qquad (23)$$

When the initial substrate is a racemate ($ee_{S0} = 0$), this equation reduces to Eq. (14). The quantitative treatment of multistep enzymatic resolutions where the product is nonselectively converted into enantiomerically enriched substrate, has been worked out by Guo.[33]

For Method 4a, the enantiomeric impurity that is allowed in order to obtain reliable results depends both on the magnitude of E and the desired accuracy of the determination of E. Large errors readily occur when the slow-reacting enantiomer is contaminated with the fast-reacting enantiomer. As an example, suppose $E = 20$ and the contamination is 2%. Then the fast enantiomer contributes for about $20 \times 2\% = 40\%$ to the initial rate observed for the slow enantiomer. This leads to an error in the value of E of roughly 40%; thus, in general, Method 4a requires the two enantiomeric substrates to be absolutely pure. Otherwise, this method fails and Method 4b should be used instead. In Method 4c, the use of impure enantiomers can be accounted for.[28]

We conclude that application of Method 4 will require additional measurements of ee_S if there are no reliable data about the enantiomeric purity of the substrate samples.

Impact of side reactions

Chemical or enzymatic side reactions of substrate, product, or enzyme may affect the determination of E in various ways (*Table 1*). We assume that the chemical reactions are not enantioselective, although in biological media that are chiral this may be otherwise.

Unstable substrates may decompose by a nonenzymatic background reaction. This is often the case for carboxylic esters. Sometimes the product is the same as for the enzymatic reaction (see below). If other products are formed, *ee* values are unaffected, but all ξ values obtained from the analysis of product concentrations will be wrong. Methods that rely on such conversion measurements result in erroneous E values.

An enzymatic background reaction of substrate to other compounds may be undesired, but it may also be used to increase the value of ee_P that may be obtained.[34] The E value cannot be determined by any of the methods unless the kinetic parameters of the second enzymatic reaction are incorporated to the model.[35]

If a chemical background reaction leads to the same product as the enzymatic reaction all methods will lead to wrong E values, because only ee_S is unaffected. For Method 4c such a spontaneous reaction was incorporated to the model to circumvent this problem.[28] When Methods 1–3 are used, an apparent enantiomeric ratio (E_{app}) is obtained. To correct for the background reaction, it was assumed that the enzymatic reaction obeys first-order kinetics for both enantiomers,[36] hence the first-order rate constants for the spontaneous and the overall reaction should be measured separately. The overall first-order rate constant, k_{ov}, consists of the sum of first-order rate constants for the enzymatic and spontaneous reaction for (R)- and (S)-enantiomer ($k_{ov} = k^R_E + k^S_E + k^R_{sp} + k^S_{sp}$). Using $E_{app} = (k^R_E + k^R_{sp})/(k^S_E + k^S_{sp})$, and $k^R_{sp} = k^S_{sp} = k_{sp}$, the equation that is the basis of this method[36] can be derived:

$$E = \frac{\dfrac{E_{app}}{E_{app} + 1} - \dfrac{k_{sp}}{k_{ov}}}{\dfrac{1}{E_{app} + 1} - \dfrac{k_{sp}}{k_{ov}}} \qquad (24)$$

Enzymatic background reactions of substrate to product are catalyzed in the presence of isoenzymes. This is a common disturbing effect and well-known for *Candida rugosa* lipase.[37] Isoenzymes may have different E values, and one should use pure enzymes to determine kinetic parameters such as E, because none of the methods is able to correctly predict E values using a mixture of enzymes. According to Chen *et al.*,[10] Method 1 and 2 can be used

when the K_m values of the different enzymes are in the same range. An apparent E value is obtained which is a function of the kinetic constants and proportions of the different enzymes. For mixtures of enzymes with different K_m values, the situation becomes much more complicated.[38]

When the substrate racemizes (either chemically or enzymatically), the course of the resolution process changes considerably.[39] All methods to determine E require an additional parameter to be incorporated to the model, i.e., the ratio of the rate constant of racemization and the rate constant of the kinetic resolution reaction for one of the enantiomers.[40]

If racemization of the product occurs,[39] only the enantiomeric excess of the product will be wrong.

If the chemical background reaction of the product to other compounds occurs, only conversion measurements that involve the determination of product concentration will be wrong. Consequently, methods for E determination relying on such a determination will lead to erroneous values.

Also, for enzymatic background reactions of the product to other compounds conversion measurements that involve the determination of the product concentration will be wrong. In this case, also ee_P will be wrong unless the enzymatic background reaction is not enantioselective. So the E value can only be determined by a few methods unless the kinetic parameters of the second enzymatic reaction are incorporated in the model.[35] Although a background reaction usually will not be favored, in some cases a sequential enzymatic reaction of the product has been used to raise the obtainable *ee* value.[18,41]

If the enzyme becomes inactivated, the rate of the resolution reaction slows down, but the ratio of reaction rates of both enantiomers does not change. Only the progression curve technique (Method 4c) is sensitive to enzyme inactivation. It is possible to account for this.[28]

Impact of kinetics and thermodynamics of the main reaction

The equations in the above sections have been derived for the "basic case", *i.e.*, reactions where one (chiral) substrate is irreversibly converted into one (chiral) product; however, in most cases of interest, enzymatic reactions involve more than one substrate and/or product. In addition, while irreversible reactions are usually the goal, one may have to deal with an equilibrium. This implies that the progress of the kinetic resolution process may be affected by the concentration of the other (nonchiral) substrate(s), of the chiral product, and of the other (nonchiral) product(s); therefore, the kinetics and thermodynamics of the reaction have to be taken into account whenever appropriate (*Table 1*).

Reversible reactions

For a reversible uni-uni reaction, Eq. (8) can be modified in order to account for the effects of reversibility.[42]

$$E = \frac{\ln\left[1 - \left(1 + \frac{1}{K_{eq}}\right)(\xi + (1 - \xi)ee_S)\right]}{\ln\left[1 - \left(1 + \frac{1}{K_{eq}}\right)(\xi - (1 - \xi)ee_S)\right]} \qquad (25)$$

This equation reduces to Eq. (8) for $K_{eq} = \infty$. It should be noted that a disturbing definition of the equilibrium constant has been used by a several authors, *i.e.*, not the equilibrium ratio of product over substrate concentrations, but its reciprocal value.

For Methods 2 and 3, similar equations are available to deal with reversibility in uni-uni reactions.[22,42] The initial rate Methods 4a and 4b are unaffected by reversibility whereas the progression curve Method 4c will require that Eq. (20) is replaced by a rate equation in which the reverse reaction rate is incorporated.

Although Eq. (25) is widely cited, the effect of reversibility of the reaction is seldom taken into account. This may have led to numerous erroneous conclusions. For example, organic solvents will affect the equilibrium conversion. Only when this effect has been properly accounted for may conclusions be drawn concerning the influence of the solvent on the intrinsic enantiomeric ratio.

The reason for not considering reversibility may be that the determination of two parameters (E and K_{eq}) requires the processing of multiple samples which complicates the experiments and/or computations. In addition, for many reactions, *e.g.*, for the hydrolysis of an ester, a uni-uni equilibrium constant has no formal meaning because two products are obtained, and the reaction should be treated as a uni-bi or bi-bi reaction instead.[43]

(Product) inhibition of the enzyme occurs

Inhibition will slow down the reaction and has to be accounted for in methods where time is measured. But also methods that depend on measurement of conversion or enantiomeric excess may lead to the wrong value of E, because inhibition may be enantioselective.[44] The mechanism of reactions that are overall irreversible may contain reversible parts where the products may affect the enantioselectivity. The actual kinetic mechanism of the enzymatic reaction will determine how the concentrations of these products have to be incorporated in the equations that describe the kinetic resolution process.[43] Accordingly, the enantiomeric ratio of the hydrolysis of glycidyl butyrate catalyzed by porcine pancreas lipase (PPL) was determined by correcting for product inhibition by glycidol.[45] For the carboxylesterase-catalyzed hydrolysis of 2-chloropropionic esters, such a correction for product inhibition became significant when the concentration of liberated alcohol approached the inhibition constant (about 0.5 mol/l).[46]

If a dilute substrate solution is used, the E value usually can be determined without accounting for product inhibition because it will be negligible. The effect of product inhibition may become significant at process conditions, however, when concentrated substrate solutions are used. In those cases, substrate inhibition may also occur and affect Method 4. Methods 1–3 will only be affected if the substrate inhibition is enantioselective. No clear examples of the latter case are known to us.

High enzyme-substrate concentration ratios

Usually the molar ratio of enzyme to substrate is much smaller than 1:100, and the enzyme-substrate complexes may be assumed to reach a pseudo steady-state; however,

for a hypothetical example with an enzyme-to-substrate concentration ratio of only 1:2 considerable deviations result when applying Eq. (8) (Method 1) because the steady-state assumption is not valid.[47] Practical situations where this effect might lead to an incorrect determination of E are not very likely to occur.

Effect of phase inhomogeneity

In a heterogeneous system, the local concentrations will be different from the average concentrations. The observed reaction rate will be a function of the local concentrations rather than of the average concentrations. If this is not taken into account, the enantiomeric ratio in a heterogeneous system may appear to differ from the value obtained for a homogeneous solution.

Concentration differences may be caused by thermodynamic effects (partitioning between liquid phases, incomplete solubility) or by kinetic effects (diffusion limitation, incomplete mixing).

The effects of phase inhomogeneity on the intrinsic enantioselectivity of the enzyme will be discussed later. Effects on the resolution process are discussed here.

Equilibration

In a kinetic resolution process, there will be no *reaction* equilibrium and the concentrations of the enantiomers in the reaction phase will not be equal ($ee \neq 0$). In practice, opposite enantiomers will have identical partition coefficients and solubilities; thus, when there is *phase* equilibrium, *ee* in the reaction phase will be identical to *ee* in the other phases. As shown above, the course of a kinetic resolution is dependent on the equilibrium constant of the reaction. In a multi-phase system, the apparent equilibrium constant in the reaction phase will be influenced by the presence of other phases.[48] This effect could be detected when E was determined for the hydrolysis of glycidyl butyrate by PPL.[45] In this case, the change in volume ratio of aqueous and glycidyl butyrate phases during the conversion formed an additional complication. The influence of this transient equilibrium on the course of the kinetic resolution was small, however.

Diffusion limitation (immobilized enzymes)

Immobilization of an enzyme in a spherical particle or on a membrane is a common procedure. We consider the simple situation that the substrate partition coefficient between bulk and enzyme support phase is unity. If diffusional limitation occurs, the concentration of substrate within the support will be lower than in the bulk solution. Both substrate enantiomers will have the same diffusion coefficient, but the diffusion limitation will be more severe for the more reactive enantiomer. The result is that the enantiomeric selectivity of the enzyme will be lowered to some degree by the relative availabilities of the two enantiomers to the enzyme. The *apparent* enantiomeric ratio, as estimated from the bulk phase process, will be lower than the *intrinsic* enantiomeric ratio of the enzyme, and depend on the Thiele modulus. If the diffusional resistance is very large, the apparent enantiomeric ratio approaches the square root of the intrinsic enantiomeric ratio, leading to a significant decrease.[49,50]

Similar to intraparticle mass transport limitation, external limitations give rise to a decrease in enantiomeric purity.[50]

Incomplete mixing

Local deviations from the average phase composition may occur when the mixing time is not much shorter than the reaction time. Then the determination of the E value may be incorrect; however, on a laboratory scale, mixing problems usually do not occur. Examples of mixing problems in industrial-scale kinetic resolutions have not been reported.

Effect of reactor type

Enzymatic kinetic resolution has mainly been studied in batch reactors. In recent years, flow reactors have also been applied. In a batch reactor, the accumulation of the product equals its production rate and Eq. (3) applies. In a flow reactor, other relations may exist.

For an ideal continuous stirred-tank reactor (CSTR), the macroscopic balances for (*R*)- and (*S*)-enantiomer lead to the following ratio:

$$\frac{c_{S0}^{R} - c_{S}^{R}}{c_{S0}^{S} - c_{S}^{S}} = E\,\frac{c_{S}^{R}}{c_{S}^{S}} \tag{26}$$

This equation has been used to express E as a function of ξ and ee_S, ξ and ee_P, or ee_S and ee_P.[51] Subsequently, E was determined from a plot of ee_S vs. ξ (Method 1) for an esterase-catalyzed hydrolysis in a CSTR. The enantiomeric ratio thus determined was found similar to that in a batch reactor, so the reactor type does not affect the intrinsic kinetic properties of the enzyme. The method for determining E has to be adapted, however.

For an ideal (continuous) plug-flow reactor, the ratio of macroscopic balances of both enantiomers is identical to the ratio for a batch reactor; thus, the equations given in the previous sections are equally valid for this reactor.[47]

Real continuous flow reactors behave nonideally and may be modeled by a series of n CSTRs. The single CSTR and the plug-flow reactor are the limiting cases, for $n = 1$ and $n \to \infty$, respectively.[52,53] The equations that relate E to ξ, ee_S, ee_R and/or time also have to be adapted for the presteady state operation period of a continuous flow reactor[52] and for fed-batch reactors.

Evaluation of the different methods

Like any parameter estimation method, the determination of the enantiomeric ratio requires the combination of an appropriate experimental set-up and a correct interpretation of the experimental data. Many effects may lead to incorrect values of E, as has been described earlier. There are two strategies to deal with these situations:

a) Prevention. Proper selection of the two quantities that are to be measured (ξ, ee_S, ee_P and/or time) may exclude a potential bias. For example, if product racemization is suspected, it is desirable to exclude methods relying on ee_P measurements.
b) Correction. The model equations that are used for the

parameter estimation may be corrected for the disturbing effect. For example, if some product racemization takes place while a method relying on ee_P measurements is used, the rate of racemization should be incorporated to the model. Subsequently, the extra model parameter (the rate constant of racemization) may be estimated from the data, together with the enantiomeric ratio, or it may be determined by independent measurements (evaluating product racemization). It is highly advisable to carry out such independent experiments since models that contain only E as the unknown parameter are to be preferred for reasons of accuracy of parameter estimation. Eqs. (8), (15), and (16) fulfill this demand.

In general, prevention is the method of choice; however, in some cases correction cannot be avoided.

As a result of the logarithmic relationships underlying Eqs. (8) and (15), the accuracy of E value estimates based on a single-point fitting to these equations is inherently poor.[54] Although averaging of E values from multiple data points has been performed,[55] there is a clear need for customized nonlinear least-squares fitting procedures. When combined with experimental measurements that are easily performed for multiple samples, the accuracy of the determination of E will be increased, and in addition, systematic errors will be detected more readily.

The method of choice for the determination of E thus depends on

a) The availability, reliability, accuracy, and ease of experimental methods to measure ξ, ee_S, ee_P, and/or t,
b) The availability and chemical and enantiomeric purity of substrate,
c) The extent to which the method is insensitive or can be modified to become insensitive for disturbing effects,
d) The availability, reliability, accuracy, and ease of the fitting method

Table 1 summarizes some characteristics of Methods 1–4 for the determination of E. There is no single method that will be both simple and safe in all cases. The choice will depend on the actual conditions. The evaluation and comparison of the accuracy of the different methods is cumbersome. The strategy which should be followed is outlined by van Tol *et al.*[54] The accuracy will depend on the actual conditions. In general, low E values can be determined more accurately than high values. Values cited in the literature usually have to be considered with caution.

It is our experience that the different methods are complementary. When they are used in combination, unexpected phenomena may be revealed, in particular in cases where E values suggested by the erroneous use of a single method may go unnoticed.[56]

Simulation and regression

Analytical integration of the differential equations that are used to describe the kinetic resolution is only feasible for a highly limited number of kinetic schemes. In addition, explicit relations of the variables can be obtained only in a few simple cases. Numerical integration, on the other hand, can be routinely performed using standard algorithms (for example Runge-Kutta methods). Application to $dc_S^R/dc_S^S = f(c_S^R, c_S^S)$ will generate a collection of points (c_S^R, c_S^S) from which plots of ee_S viz. ee_P vs. ξ ("Chen plots") or ee_P vs. ee_S ("Rakels plots") can be obtained by calculating the corresponding values of the enantiomeric excess and extent of reaction. This strategy is not always convenient. In particular, application of Runge-Kutta methods with fixed steps leads to nonuniformly distributed sets of points. Plots of ee_S vs ξ obtained in this way show severe clustering of points in the relatively uninteresting high ξ region. The area $0 < \xi < 0.5$, where discrimination between various settings of E is optimal, is scarcely covered. Adaptive stepsize control hardly improves this situation while prevention of (abortive) overshoot at $\xi = \xi_{eq}$ for reversible reactions requires fancy programming.

We found that these problems can be circumvented by direct numerical integration of $dee_S/d\xi$, viz. $dee_P/d\xi$ or dee_P/dee_S. As an example, we give the modification of Eq. (3). For initial values $c_0^R = c_0^S = c_0/2$ and using Eqs. (6)–(7), we obtain:

$$\frac{dc_S^R}{dc_S^S} = \frac{d[(1 - ee_S)(1 - \xi)]}{d[(1 + ee_S)(1 - \xi)]} \tag{27}$$

which can be transformed into:

$$\frac{dee_S}{d\xi} = \frac{(1 + ee_S)(1 - ee_S)}{(1 - \xi)(E^* - ee_S)} \tag{28}$$

with $E^* = (E + 1)/(E - 1)$.

This equation is highly recommended for numerical integrations, *e.g.*, simulation of plots of ee_S vs ξ, and regression analyses. Application to other cases of interest is straightforward, although somewhat cumbersome.

The nonlinear character of the relations from which E has to be determined places high demands on the regression methods used. The ubiquitous presence of (logarithms of) ratios of variables leads to highly nonsymmetric distributions of errors while the accuracy of the independent variable, ξ, cannot normally be taken for granted. A nonlinear regression program based on the method of Levenberg-Marquardt has been developed (SimFit[54]). The versatility is, however, limited to MS-DOS operating systems. Similar software for MacIntosh has been announced (H. Anthonsen, personal communication). We feel that the further development of customized programs of good compatibility is badly needed.

Towards the prediction of the intrinsic enantiomeric ratio

By definition, the intrinsic E value is dependent on the values of k_{cat}/K_m of both enantiomers [Eq. (2)]. The fundamental background of the k_{cat}/K_m values and their ratio will be treated on two levels: 1. Identification of the relevant elementary rate constants in the catalytic pathway; and 2. Treatment of these rate constants according to the transition state theory.

The elementary rate constants involved in enantioselectivity

When Michaelis-Menten kinetics are obeyed, the enzyme and substrate reversibly form a Michaelis complex (rate constants k_1 and k_{-1}) which is subsequently converted into enzyme and product (rate constant k_2). For this case, the specificity constant is related to the elementary rate constants by[7]

$$\frac{k_{cat}}{K_m} = \frac{k_1 k_2}{k_{-1} + k_2} \tag{29}$$

This relation can only be readily evaluated in terms of fundamental molecular properties if the first step is rate limiting ($k_{-1} << k_2$).[57] Then $k_{cat}/K_m = k_1$ and consequently $E = k_1^R/k_1^S$. If the second step is rate limiting ($k_{-1} >> k_2$), $k_{cat}/K_m = k_1 k_2/k_{-1}$, and E will be a more complex function of elementary rate constants. For the sake of argument, we discuss the situation where the first step is much slower, so E depends on two rate constants, k_1^R and k_1^S. This implies that the kinetics are first order with respect to the enantiomeric substrates. Note that there is no indication that this assumption is generally valid. For example, in studies of the pH or temperature dependance of k_{cat}/K_m in non-enantioselective enzymatic catalysis, the pH or temperature dependence of k_1, k_{-1} as well as k_2 had to be taken into account.[7,57]

Transition state theory

Consider an elementary second-order reaction via transition state *TS*:

$$A + B \rightarrow TS \rightarrow \text{product(s)}$$

According to the *TS* theory, the microscopic kinetic constant, k, can be expressed as[7]

$$k = \kappa \frac{k_b T}{\hbar} \exp\left(\frac{-(\Delta G_{TS}^0 - \Delta G_A^0 - \Delta G_B^0)}{RT}\right) \tag{30}$$

where ΔG^0 is a standard Gibbs energy, T is the temperature, and the other symbols are constants.

Although this equation has been derived for ideal gas phase reactions only, the close analogy with empirical correlations formulated by Arrhenius (and Van't Hoff) has motivated its use for condensed (dilute liquid) systems as well. Within the formalism of *TS* theory, nonideal behavior can be accounted for by introducing the thermodynamic activities (γ) of the participating species:[58–60]

$$k = \kappa \frac{k_b T}{\hbar} \exp\left(\frac{-(\Delta G_{TS}^0 - \Delta G_A^0 - \Delta G_B^0)}{RT}\right) \times \frac{\gamma_A \cdot \gamma_B}{\gamma_{TS}} \tag{31}$$

The *TS* theory can be applied[61] to the conversion of a substrate by an enzyme displaying minimal kinetics:

$$E + S \rightarrow TS \rightarrow \text{product(s)}$$

assuming the rate to be first order in substrate and in free enzyme. If the enantiomeric ratio is the ratio of the first order rate constants of the enantiomers, both according to Eq. (31), one can readily derive

$$E = \frac{\exp\left(\frac{-(\Delta G_{TS}^{0R} - \Delta G_S^{0R})}{RT}\right) \frac{\gamma_S^R}{\gamma_{TS}^R}}{\exp\left(\frac{-(\Delta G_{TS}^{0S} - \Delta G_S^{0S})}{RT}\right) \frac{\gamma_S^S}{\gamma_{TS}^S}} \tag{32}$$

This equation provides an attractive starting point for the analysis of temperature effects, molecular modeling strategies, and solvent effects on the value of E. In particular, modifications of E by protein-, medium-, or substrate engineering may eventually be evaluated against this background.

Temperature and pressure effects

Temperature-dependent variations in E values have recently been reviewed.[62] It has been observed that they range from virtually none to as much as 4–5-fold. Pressure effects (according to a study up to 300 bar)[63] seem to be in the same range; thus, significant improvements in E values can be obtained which deserves further study.

In an isotropic solvent, the standard Gibbs energy for both enantiomers will be equal, and Eq. (32) reduces to:

$$\ln E = \frac{\Delta G_{TS}^S - \Delta G_{TS}^R}{RT} \tag{33}$$

The influence of temperature (T) and pressure (p) on changes in Gibbs energies is given by the following general equation:

$$\Delta G = \Delta U + p\Delta V - T\Delta S \tag{34}$$

where ΔU, ΔV, and ΔS are the changes in internal energy, volume, and entropy. Under isobaric conditions, this equation can be simplified using the change in enthalpy, $\Delta H = \Delta U + p\Delta V$. For the evaluation of temperature effects on the enantiomeric ratio, the following relation has been derived from Eq. (33)[62]:

$$\ln E = \frac{\Delta H_{TS}^S - \Delta H_{TS}^R}{RT} - \frac{\Delta S_{TS}^S - \Delta S_{TS}^R}{R} \tag{35}$$

This relation predicts that Eyring plots of ln E vs. $(1/T)$ will be linear. In several cases, this has indeed been observed;[62] moreover, it has been predicted and observed that there is a racemic temperature, T_r, at which there is no enantioselectivity ($E = 1$) and where an inversion of R- to S-preference occurs.[62]

$$T_r = \frac{\Delta\Delta H_{TS}}{\Delta\Delta S_{TS}} \tag{36}$$

It should be possible to describe pressure effects on enantioselectivity in a similar manner.[64]

Effects of media

Effects of organic (co)solvents on the intrinsic enantioselectivity have been reviewed in the literature.[18,65–69] For different solvents, variations in the E value of about an order of magnitude have frequently been observed. In extreme cases, complete reversal of the enantioselectivity oc-

curred,[70] whereas in other cases no significant changes were shown.[71–73] For individual cases, it has been possible to explain the effects by correlating E values with solvent properties such as log P, but this has not led to a general theoretic framework. We will again use Eq. (32) as the starting point.

In isotropic solvents, ΔG^0 will be similar for both enantiomers. Also, at the thermodynamic standard state (*e.g.*, dilute aqueous solution), the activity coefficients will be unity; thus, at these conditions the enantiomeric ratio at the standard state (E^0) can be derived:

$$\ln E^0 = \frac{\Delta G^{0S}_{TS} - \Delta G^{0R}_{TS}}{RT} \tag{37}$$

In isotropic solvents that are different from the standard state, the activity coefficients of the diastereomeric enzyme-substrate transition states do not have to be equal and, consequently, the E value may change:

$$E = E^0 \frac{\gamma^S_{TS}}{\gamma^R_{TS}} \tag{38}$$

Thus, effects on E will be restricted to a possible change in the ratio of γ^R_{TS} and γ^S_{TS}. Such a change implies that the solvent stabilizes the (*R*)- and (*S*)-transition states to a different extent. It may be expected that this effect will not be very important for enzymes with relatively "closed" active sites where the solvent cannot readily influence the structure of the transition state, *e.g.*, for some ester hydrolysis reactions catalyzed by PPL and α-chymotrypsin, the E value did not depend on the solvent type.[71–73]

Supporting evidence for this model comes from the observation that in many cases, K_m values depend on the solvent composition according to predictions that also rely on this treatment of the transition state theory.[74] When additional variations in K_m values occur or when E values vary,[26,70] there will be a solvent-induced change in transition states.

Other medium components that affect the instrinsic E value must act in a similar manner. Reported examples include crown ethers,[75] phase interfaces,[76] immobilization matrices,[77] and surface active compounds.[31] Also, effects of anisotropic solvents on E have been studied.[78]

Molecular modeling of enzyme enantioselectivity

Changes in the transition states (and consequently in the ratio $\gamma^R_{TS}/\gamma^S_{TS}$) will occur when the structure of the substrate or enzyme is varied. Changes in enantioselectivity that must be due to such effects have been well documented.[8,14,18,79] Improvement of the E value by modification of the substrate or enzyme structure is a major research target. This includes pH changes,[18] cofactor engineering,[80] site-directed mutagenesis,[81] and chemical modification of enzymes.[82]

In the past, interesting (substrate) structure-enantioselectivity correlations have been derived for porcine liver esterase.[83] In the absence of a crystal structure of this enzyme, the results have been more or less elegantly summarized in the form of ligand boxes. More recently, similar data for several lipases of known 3D structure have allowed interpretation in terms of enzyme active-site structure.[84]

Molecular modeling strategies employing molecular mechanics and molecular dynamics calculations have been performed for some serine hydrolases.[85] So far, these studies have focused on the calculation of force field potentials for the diastereomeric tetrahedral intermediates that are formed during serine hydrolase-catalyzed reactions. Protocols involve semi-empirical quantum mechanical calculations of the diastereomeric complexes in order to estimate bond lengths, hydrogen bonding networks, and charge distributions of the oxyanionic species. Next, the derived parameters are used to generate an appropriate force field for molecular mechanics minimization while global minima are searched for by molecular dynamics. Interesting results have been obtained showing a clear correlation of energy differences and E values. It must be emphasized, however, that these calculations give an indication for the enthalpic contribution to the enantiomer differentiation only. More elaborate simulations are required to provide estimates of the entropic components involved. In particular, effects of solvent displacement should eventually be explained. Preliminary calculations have been performed.[86]

One should notice that an increase in the enantiomeric ratio from 1 to 1,000 corresponds to a difference in $\Delta\Delta G$ of 17 kJ mol^{-1}. This is less than the energy of a normal hydrogen bond which is about 20 kJ mol^{-1}.[7]

Analyses of the factors involved in chiral discrimination have been performed by (visual) inspection of enzyme-substrate interactions.[87] Since these approaches do not yield quantitative results, their value appears to be limited.

Conclusions

A large number of methods is currently available for accurate determinations of E values. In the past, these methods have not always been used adequately. Several published reports of E values are based on a single measurement. This may be sufficient for screening purposes in synthetic organic chemistry; however, these values should be used with caution when conclusions are drawn about molecular effects on the intrinsic E value unless there is no doubt about the correctness of the assumptions on which the methods are based. When one has to choose between alternative methods for determining E, *Table 1* may be helpful.

Presently, the models to predict E values are only of limited accuracy. In general, knowledge of the elementary catalytic steps that determine E is lacking. According to the transition state model, all effects on the E values must be the result of changes in transition state structures of these elementary steps. Only in a few cases these structures are known in sufficient detail, so that molecular modeling strategies can be used to calculate an E value. Modification of substrate structure can be modeled much better than changes in medium composition. Changes in E value, however, are best understood in cases where they are relatively small, *i.e.*, for temperature effects.

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