Enzymatic Kinetic Determinations

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Kinetic methods for analytical applications can be divided into noncatalytic and catalytic methods. Enzyme kinetic determinations are a special type of catalytic methods. Enzymatic reactions are used analytically to determine enzyme activities – for instance, in the diagnosis of diseases, substrate concentrations in the food industry or medicine, and concentrations of effectors – for example, in trace analysis. Owing to the high selectivity of enzymatic analysis, the importance of this method in various fields is growing rapidly. The widest use is observed in clinical chemistry and food chemistry. This article gives only a general survey of the high potential of enzymatic analysis, with references to more detailed literature. Enzymatic reactions are characterized by some special features, explained after the introduction. The theoretical background for kinetic determination is the comprehension of the principles on enzymatic kinetics. In Section 3, the simplest mechanism, outlined by Michaelis and Menten, and several transformations of the equation are described. In this section, two kinetic parameters, enzyme activity and the Michaelis constant, are introduced. Section 4 deals with substances, the so-called effectors, influencing the rate of an enzymatic reaction. Simple mechanisms of these effectors are shown. Different methods for determination of substrates, enzymes, and effectors, illustrated by many examples, are represented in Section 5. While substrate concentrations can be determined using both equilibrium or kinetic methods, the determination of enzymes and effectors can be performed only by kinetic approaches. In the last two sections, the application of enzymes in flow systems and biosensor development are shown.

1 INTRODUCTION

Kinetic methods are useful tools in analytical chemistry. The use of enzymes in kinetic-based determinations is widely applied for the analysis of many substances, for instance, in medicine or the food industry, for the analysis of enzymes, especially enzyme activities in clinical diagnostics, and for the determination of inhibitors or activators of enzymes, i.e. trace analysis. The enzymatic analysis method combines the exceptional selectivity with high sensitivity. This article can only attempt a general survey of the enormous potential of the enzymatic analysis, with references to more detailed books or reviews.

At the beginning, the special features of enzymatic reactions are characterized and the theoretical background for the application of enzymatic catalysis and the comprehension of the principles on enzymatic kinetics are described. The rate of the enzymatic reaction can be either increased or decreased by the effect of some substances known as activators or inhibitors, respectively. Simple mechanisms of the effectors are explained in Section 4. Examples of the analytical application of enzymatic catalysis for the determination of substrates, enzymes, and effectors are given. The use of enzymes in flow systems and biosensors is presented in the last, but nonetheless interesting, two sections.

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2 FEATURES OF ENZYMATIC REACTIONS

All enzymes are proteins produced by living cells that catalyze specific reactions with a high degree of efficiency. In the following, the special features of enzymes are represented, including some relevant information for their analytical uses.

2.1 High Specificity of Enzymes

Enzymes are very highly specific in their choice of substrates. Think of the 'key-lock' theory by Fischer or the 'induced fit' hypothesis, suggested by Koshland. Therefore, enzymes are unique analytical reagents. The ideal enzyme for analytical purposes would catalyze only one chemical reaction.

2.2 Temperature

Most enzymatic reactions are carried out within the physiological temperature range of 25–37 °C. As is known from other chemical reactions, there is an increase in reaction rate with temperature. In view of the fact that the enzyme is a protein, normally it becomes denatured above 50 °C. Figure 1 shows a typical curve for the dependence of enzymatic activity on temperature. The optimum operating temperature differs from one enzyme to another. For enzymatic reactions, it is very important to control the temperature during the measurement. Enzymes should be stored at 4 °C or less, as they can lose activity when stored at room temperature. Many producers of enzyme preparations use some substances to stabilize the preparations, but for some analytical purposes (see Section 5.3), these substances can disturb the detection, for example, the detection of inhibitors.

2.3 pH Value

Most enzymes are very sensitive to changes in pH. The effect of pH is due to an alteration in the conformation of the protein structure or the ionization of the active site of the enzyme or substrate. As seen in Figure 2, there is an optimum in pH value (generally in the pH range of 5–7). Therefore, it is very important to keep the pH value constant during the measurement. The use of a buffer is advisable. Extreme pH value also leads to denaturation of the protein. The proper choice of pH is especially important in enzyme assays with enzyme-coupled systems because there can be different pH optima for the coupled enzymes.

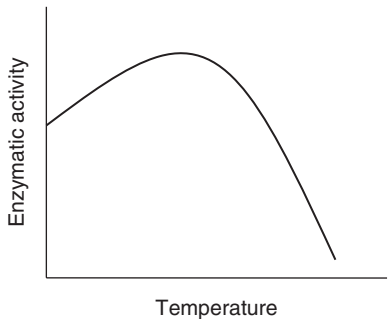

Figure 1 Effect of temperature on the activity of a typical enzyme.

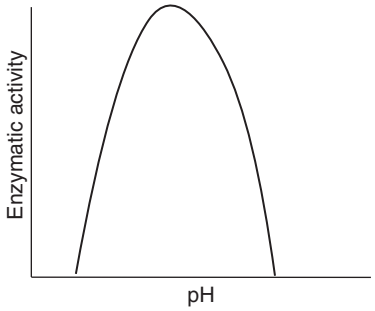

Figure 2 Effect of pH on the activity of a typical enzyme.

2.4 Cofactors

Many enzymes need the presence of a nonprotein component (molecular mass generally between 10^2–10^3 g mol^{-1}), called cofactors, for catalytic activity. In this case, the inactive protein component is termed *apoenzyme* and the active enzyme, including cofactor, the *holoenzyme*. If the cofactor is an organic molecule, it is called *coenzyme*. For analytical purposes, e.g. in photometric analysis, the coenzyme nicotinamide adenine dinucleotide (NAD) is widely used (see Section 5.1). Other cofactors may be metal ions, e.g. zinc or iron ions. Their analytical importance is given in Section 5.3.

2.5 Ionic Strength

Enzymatic reaction rates can be changed by the presence of some salts. The addition of small amounts of neutral salts may increase the solubility of a protein. Salts can cause changes in ionization of amino acid side chains. Therefore, the choice of buffer recipe for any given pH is very important for the reaction rate and the mechanism of an enzymatic reaction.

3 REACTION MECHANISM AND KINETIC EQUATIONS

The following kinetic considerations are restricted to the classical approach by Michaelis and Menten. Deviations

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from the hyperbolic dependence of the reaction rate on the substrate concentration will not be represented. Furthermore, in most cases, only single substrate reactions are significant for the analytical application of enzymes.

3.1 Michaelis–Menten Equation

In the early 1900s, two biochemists, Michaelis and Menten, worked out a theory for the kinetic analysis of enzyme-catalyzed reactions. The simplified mechanism can be written as Equation (1):

$$\mathrm{E} + \mathrm{S} \underset{k\_1}{\overset{k1}{\rightleftharpoons}} \{\mathrm{ES}\} \underset{k\_2}{\overset{k2}{\rightleftharpoons}} \mathrm{P} + \mathrm{E} \tag{1}$$

where E is the enzyme, S the substrate, {ES} the intermediate enzyme–substrate complex, and P the product(s). The *k*s are the rate constants. The substrate concentration is much larger than the enzyme concentration so that the formation of {ES} does not alter the substrate concentration. This reaction scheme has been treated both as pre-equilibrium[1] and as a steady-state case.[2] Then, the Michaelis–Menten equation becomes Equation (2):

$$-\frac{\mathrm{d}[\mathrm{S}]}{t} = \frac{k_2[\mathrm{S}][\mathrm{E}]_0}{K_\mathrm{m} + [\mathrm{S}]} \tag{2}$$

where K_m is the Michaelis constant, [S] the substrate concentration, and $[\mathrm{E}]_0$ the initial concentration of enzyme present. This expression shows the relationship between reaction rate and concentration of both substrate and enzyme. Equation (2) is the base of determination of substrate concentrations and enzyme activities.

Further on, we know that when the substrate concentration is very high, the enzyme is present as the enzyme–substrate complex and the maximum rate v_max is reached (enzyme saturation). Under these conditions, we have Equation (3):

$$v_\mathrm{max} = k_2[\mathrm{E}]_0 \tag{3}$$

We can substitute v_max in Equation (2) and get Equation (4):

$$-\frac{\mathrm{d}[\mathrm{S}]}{\mathrm{d}t} = \frac{\mathrm{d}[\mathrm{P}]}{\mathrm{d}t} = \frac{v_\mathrm{max}[\mathrm{S}]}{K_\mathrm{m} + [\mathrm{S}]} = v \tag{4}$$

Equations (2) and (4) are only precisely valid in the case of the initial reaction rate v_0 (Equation 5):

$$v_0 = \frac{v_\mathrm{max}[\mathrm{S}]_0}{K_\mathrm{m} + [\mathrm{S}]_0} \tag{5}$$

For determinations of substrate concentration and enzyme activities, we have to discuss two experimental possibilities:

1. $[\mathrm{S}] \gg K_\mathrm{m}$, thus the initial reaction rate v_0 is directly proportional to the enzyme concentration (Equation 6):

$$v_0 = \frac{k_2[\mathrm{S}]_0[\mathrm{E}]_0}{[\mathrm{S}]_0} = k_2[\mathrm{E}]_0 \tag{6}$$

   Since $v_\mathrm{max} = k_2[\mathrm{E}]_0$, this attempt is commonly called saturation conditions.
2. $[\mathrm{S}] \ll K_\mathrm{m}$, thus the initial reaction rate v_0 is directly proportional to the substrate concentration, assuming the determination has been carried out under constant enzyme condition ($v_\mathrm{max}/K_\mathrm{m} = \text{constant}$) (Equation 7):

$$v_0 = \frac{k_2[\mathrm{S}]_0[\mathrm{E}]_0}{K_\mathrm{m}} = \frac{v_\mathrm{max}}{K_\mathrm{m}}[\mathrm{S}]_0 \tag{7}$$

When the rate of an enzymatic reaction v is plotted against the substrate concentration [S], a hyperbolic curve is obtained (Figure 3). Enzyme-catalyzed reactions show a first-order dependence of the reaction rate at low concentrations of substrate, and as the substrate concentration rises, the reaction rate approaches a maximum rate v_max, the enzyme saturation. This is the region of zero-order kinetics. At intermediate substrate concentrations, the relationship between the reaction rate and the substrate concentration follows neither first-order nor zero-order kinetics.

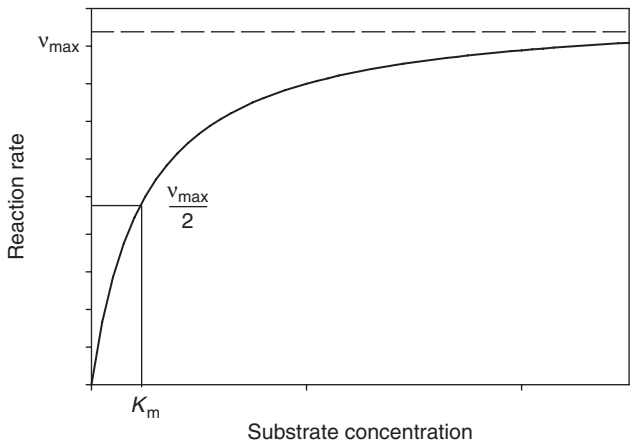

Figure 3 Reaction rate plotted against substrate concentration for an enzyme-catalyzed reaction.

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3.2 Integrated Michaelis–Menten Equation

As mentioned in Section 3.1, the Michaelis–Menten equation is only precisely valid in the case of the initial reaction rate v_0, i.e. when the substrate concentration $[S] = [S]_0$, or when less than 5% of [S] is converted to product [P].[3] Under many experimental conditions, it may not be feasible to restrict the reaction extent to 5% or less. In contrast to this, the integrated form of the Michaelis–Menten equation is valid over the entire course of the reaction. This we can write as Equation (8):

$$[S] = [S]_0 - [P] \tag{8}$$

where [P] and [S] are the product and substrate concentrations at time t.

Insertion of Equation (8) into Equation (4) leads to Equation (9):

$$v = \frac{d[P]}{dt} = \frac{v_{max}([S]_0 - [P])}{[S]_0 - [P] + K_m} \tag{9}$$

Integration of Equation (9) between time $t = 0$ and any time t and between the corresponding two product concentrations 0 and [P] leads to Equation (10):

$$t = \frac{K_m}{v_{max}} \ln \frac{[S]_0}{[S]_0 - [P]} + \frac{[P]}{v_{max}} \tag{10}$$

Equation (10) presents the relationship between product concentration [P] and time t. Moreover, Equation (10) can be rearranged to give Equation (11):

$$t = \frac{K_m}{v_{max}} \ln \frac{[S]_0}{[S]} + \frac{[S]_0 - [S]}{v_{max}} \tag{11}$$

The application of Equation (10) for the determination of K_m and v_{max} (or the enzyme activity) is described in Section 3.4.

3.3 Michaelis Constant and Enzyme Activity

The Michaelis constant, K_m, is defined as the substrate concentration at half the maximum reaction rate (Equation 12):

$$\text{when} \quad K_m = [S], \quad v = \frac{[S]v_{max}}{2[S]} = \frac{v_{max}}{2} \tag{12}$$

Thus, K_m will have the same units as the substrate concentration. Using the two methods for treatment of enzyme-catalyzed reaction (1), one can obtain Equations (14) and (16):

1. Michaelis–Menten assumption of equilibrium between substrate, enzyme, and substrate–enzyme complex (pre-equilibrium or rapid equilibrium), $k_2 \ll k_{-1}$, Equation (13):

$$E + S \overset{Ks}{\rightleftharpoons} \{ES\} \xrightarrow{k2} P + E \tag{13}$$

$$K_m^I = K_s = \frac{k_{-1}}{k_1} \tag{14}$$

where K_s is the dissociation constant for the enzyme–substrate complex.

2. For Equation (15):

$$E + S \underset{k\_1}{\overset{k1}{\rightleftharpoons}} \{ES\} \xrightarrow{k2} P + E \tag{15}$$

Briggs–Haldane steady-state assumption, Equation (16):

$$K_m^{II} = \frac{k_{-1} + k_2}{k_1} \tag{16}$$

The Briggs–Haldane assumption is more general, eliminating the requirement for the enzyme–substrate complex to be in equilibrium with free enzyme and free substrate. Thus, K_m is a dynamic or pseudo-equilibrium constant. K_m is a constant for a given substrate under well-defined experimental conditions such as temperature, buffer, pH, and ionic strength. It represents the affinity between the substrate and the enzyme. A small value of K_m means a high affinity between substrate and enzyme, and the enzyme becomes saturated at small concentrations of substrate. Michaelis constants for enzyme usually range from 10^{-2} to 10^{-5} mol L^{-1}. For enzymatic analysis methods, a small value of K_m results in a very limited linear concentration range in calibration graphs based on initial reaction rate as a function of substrate concentration. If we know K_m, we can adjust the experimental conditions for determining substrate concentration or enzyme activity (see Section 3.1). The activity of an enzyme preparation varies from one source to another as the purification of the enzyme is, in general, not complete. Because of this, in most preparations, the actual molar concentration of the enzyme is unknown, and therefore, the quantity generally used to express the enzyme activity is the *unit*, defined in terms of reaction rate.

One international unit (U) of enzymes is defined as that amount catalyzing the conversion of 1 μmol of substrate to product in 1 min under optimal conditions.

The declaration of the optimal conditions has to include the temperature (usually 25 or 30 °C), pH value, and buffer system. For determining the enzyme activity,

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the substrate concentration should be sufficient for saturation. The Nomenclature Commission of the International Union of Biochemistry has recommended the use of the *katal* (kat), which differs from the definition of the unit through the declaration of 1 mol of substrate and time of 1 s:

$$1\,\text{kat} = 6 \times 10^7\,\text{U}$$

The *specific activity* of an enzyme preparation is the number of units per milligram of protein, or in the case of a solution or suspension, one can declare the number of units per milliliter. If the molecular mass of an enzyme is known, it is possible to express the *molecular activity* in units per micromole of enzyme.

In the study by Palmer and Bonner,[4] one can also find the terms: *turnover number* k_{cat} and *catalytic efficiency* k_{cat}/K_m. The constant k_{cat} is defined in Equation (17):

$$k_{cat} = \frac{v_{max}}{[E]_0} \tag{17}$$

If the enzyme concentration is known, the ratio k_{cat}/K_m is a good measure for catalytic efficiency of an enzyme.

3.4 Determination of Kinetic Parameters

The determination of the kinetic parameters v_{max} and K_m can be carried out by measuring the initial reaction rate as the substrate concentration is varied. If experimentally possible, the substrate concentration should be varied in the range from $K_m/10$ to 10 K_m. In the literature, there are some linearization procedures (Figure 4):

3.4.1 The Lineweaver–Burk Plot

The linearization of the Michaelis–Menten equation (5) by Lineweaver and Burk (Figure 4a) is the most widely and traditionally used method. It is a double-reciprocal plot (Equation 18):

$$\frac{1}{v_0} = \frac{K_m}{v_{max}}\frac{1}{[S]_0} + \frac{1}{v_{max}} \tag{18}$$

From the slope (K_m/v_{max}) or the intercept on the ordinate ($1/v_{max}$) or the intercept on the abscissa ($-1/K_m$), one can obtain the two kinetic parameters. But such a double-reciprocal method has a strong distorting effect on the data.

3.4.2 The Hanes Plot

The plot $[S]_0/v_0$ versus $[S]_0$ by Hanes and Woolf (Figure 4b) is much better with regard to this effect. The data are more evenly weighted over the whole concentration range. Equation (18) may be rearranged to give Equation (19):

$$\frac{[S]_0}{v_0} = \frac{1}{v_{max}}[S]_0 + \frac{K_m}{v_{max}} \tag{19}$$

From this linear plot, the parameters K_m and v_{max} can be calculated.

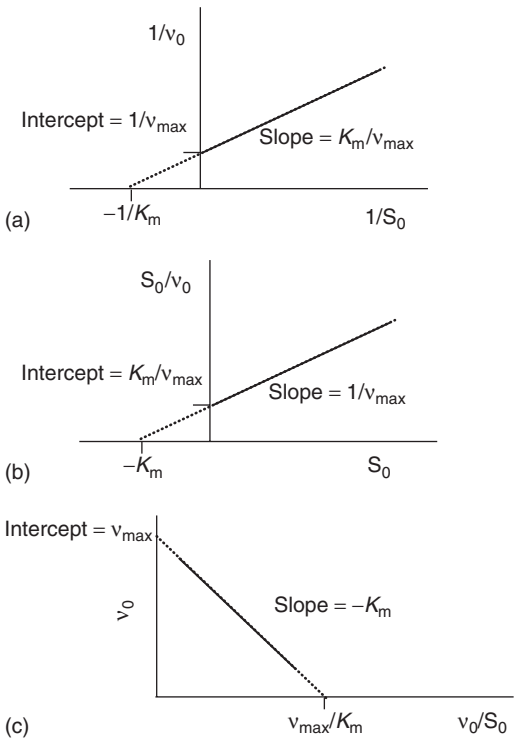

Figure 4 Transformations of the Michaelis–Menten equation to determine v_{max} and K_m. (a) Lineweaver–Burk plot, (b) Hanes plot, and (c) Eadie–Hofstee plot.

3.4.3 The Eadie–Hofstee Plot

The Eadie–Hofstee plot (Figure 4c) is the straight line graph of v_0 versus $v_0/[S]_0$, Equation (20):

$$v_0 = -K_m\frac{v_0}{[S]_0} + v_{max} \tag{20}$$

Both plots (Eadie–Hofstee plot and Hanes plot) are less frequently used, but they are better suited for kinetic determination than the Lineweaver–Burk plot.

3.4.4 Integrated Michaelis–Menten Equation

All the above-mentioned methods require the measurement under initial conditions. If it is not possible to fulfill

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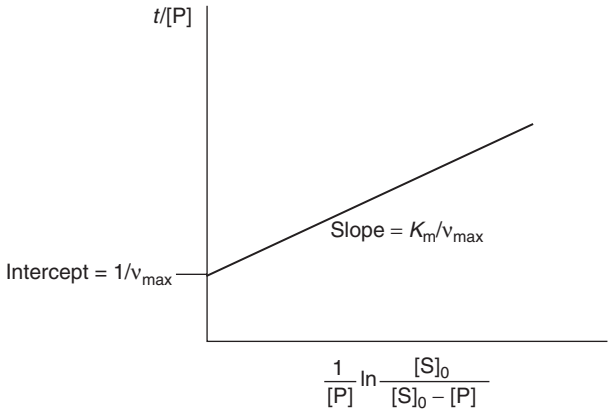

Figure 5 Plot of integrated Michaelis–Menten equation.

this requirement – for example, it may be difficult to determine very low concentrations of product – one can use the integrated Michaelis–Menten equation (see Section 3.2) for the determination of v_{max} and K_m. The linear transformation of Equation (10) gives Equation (21):

$$\frac{t}{[P]} = \frac{K_m}{v_{max}} \frac{1}{[P]} \ln \frac{[S]_0}{[S]_0 - [P]} + \frac{1}{v_{max}} \qquad (21)$$

The Michaelis constant and maximum rate can be determined by measuring the concentration of product several times during the reaction and plotting the respective values as shown in Figure 5.

4 MODIFYING THE KINETIC BY EFFECTORS

The rate of the enzymatic reaction can be either increased or decreased by effectors. While activators accelerate the enzymatic reaction, inhibitors reduce the rate of the enzymatic reaction. For this reason, all enzymatic analyses of effectors involve the determination of reaction rates (different kinetic methods are described in Section 5 or in the study by Pérez-Bendito and Silva[5]).

4.1 Activators

An activator increases the catalytic effectiveness or the activator transforms the inactive apoenzyme (see Section 2.4) to the active holoenzyme. Various substances can activate the same enzyme. In consequence, there is a lack of specificity for determination of individual ions.

The course of the initial rate in the presence of an activator is similar to that of substrate concentration, discussed in Section 3.1. At low activator concentrations, one can find that the initial rate is directly proportional to the concentration of the activator, but at higher concentrations, the enzyme is maximally activated (similar to the enzyme saturation at high substrate concentrations) and the initial rate becomes independent of the concentration of the activator. Consequently, the determination of effectors has to be carried out at low levels of activator concentrations. Examples for the analytical application, e.g. the determination of metallic ions, are given in Section 5.3.

4.2 Inhibitors

Inhibitors are substances that cause a decrease in the rate of a catalytic reaction. The enzyme inhibition can be either reversible or irreversible. The characteristic of the reversible inhibition is the equilibrium between enzyme and inhibitor. In contrast, the irreversible inhibitor binds covalently to the enzyme. Seen from Figure 6, the enzymatic reaction rate can be influenced by several substances. We distinguish

- substrate inhibition
- product inhibition
- competitive inhibition (formation of enzyme–inhibitor complex EI)
- uncompetitive inhibition (formation of enzyme–inhibitor–substrate complex EIS)
- noncompetitive inhibition (equal affinity of formation of EI and EIS).

4.2.1 Competitive Inhibition

The inhibitor competes with substrate (or coenzyme) for the active center of the enzyme. As the inhibitor forms the enzyme–inhibitor complex, the proportion of free enzyme reduces and the rate of the enzymatic reaction decreases. Hence, the competitive inhibition can be made ineffective by an excess of substrate. In most cases, we find that the chemical structure of the substrate is similar to that of the competitive inhibitor. Figure 7a shows the Lineweaver–Burk plot of the competitively

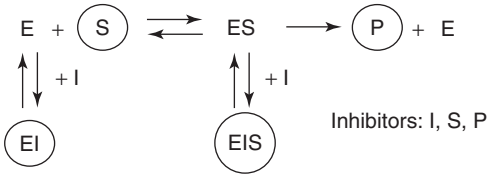

Figure 6 Schematic representation of the various forms of inhibition.

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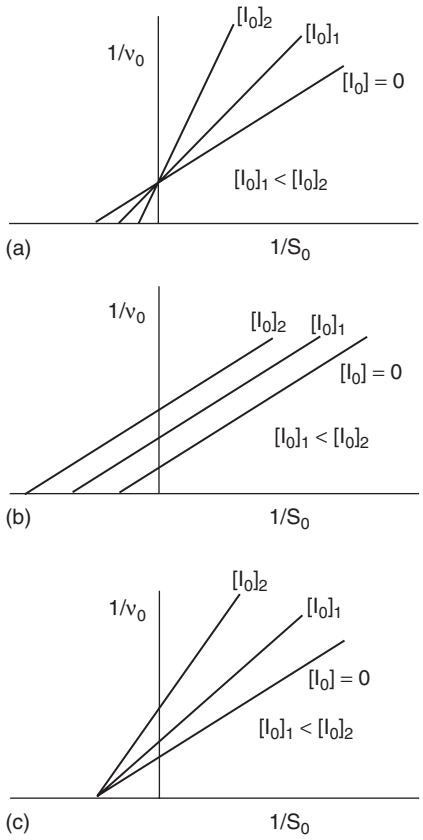

Figure 7 Lineweaver–Burk plots for (a) competitive inhibition, (b) uncompetitive inhibition, and (c) noncompetitive inhibition.

inhibited enzyme reactions. The curves increase in slope (increase in K_m), while the intercept on the $1/v_0$ axis remains constant. The maximum rate in the presence of a competitive inhibitor is always equal to v_{max} of the uninhibited reaction.

4.2.2 Uncompetitive Inhibition

The inhibitor binds only to the enzyme–substrate complex ES. Figure 7b shows the Lineweaver–Burk plot of the uncompetitively inhibited enzyme reaction. The uncompetitive inhibitor decreases v_{max} and K_m. The decrease in K_m means an apparent increase in the affinity of the enzyme for its substrate.

4.2.3 Noncompetitive Inhibition

The inhibitory mechanism, where the inhibitor binds with equal affinity to both the enzyme and the enzyme–substrate complex, is called noncompetitive inhibition. Noncompetitive inhibitors bind to the enzyme at a site that is distinct from the substrate binding site.[6]

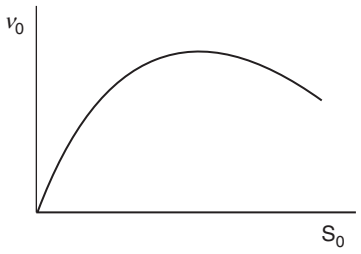

Figure 8 Substrate inhibition.

Hence, this type of inhibition cannot be overcome by increasing the substrate concentration. Figure 7c illustrates that the noncompetitive inhibition leads to an apparent decrease in v_{max}, while the Michaelis constant is not altered.

4.2.4 Substrate Inhibition

As represented in Section 3.1, the initial rate of an enzyme reaction increases with increasing initial substrate concentration to a maximum value v_{max}, but in some cases, we can find a decreased initial rate at high substrate concentrations, as seen in Figure 8. There, an excess of substrate can inhibit its own conversion to product.

4.2.5 Product Inhibition

The product of some enzyme reactions can also be an inhibitor. The product may bind to the active site of the enzyme. Consequently, the active site is blocked and cannot bind further substrate molecules. Thus, the rate of the enzyme reaction decreases.

The represented types of enzyme inhibition are not complete. More detailed information can be found in the study by Segel.[7] Examples for analytical applications are given in Section 5.3.

5 ENZYMATIC DETERMINATIONS

The methods to be used in monitoring enzyme-catalyzed reactions are dictated by the chemistry and physical chemistry of the reaction. We can distinguish three main classes:

- spectrophotometric
- electrochemical
- calorimetric.

In the following, only some typical examples of the determination of substrates, enzymes, and effectors, using different monitoring techniques, are represented. In the

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literature, one enzyme plays an exceptional role – glucose oxidase (GOD) as an analytical reagent is the most widely used enzyme.[8,9]

5.1 Determination of Substrates

The substrate determination can be carried out by both equilibrium and kinetic approaches. The *equilibrium method*, also called *end-point method*, means the measurement of the total change, almost all of the substrate being converted to product. The analysis can be performed by measuring the appearance of product or the disappearance of substrate. To ensure rapid progress toward equilibrium, the concentration of the used enzyme should be high. Any other substances necessary for enzyme-catalyzed reaction, such as cofactors (coenzyme or metal ions), should be in excess, so that they do not limit the reaction. Because of the possible side reactions or instability of products or reactants, the enzyme reactions are often not stoichiometric with respect to the substrate concentration. Therefore, the calibration curves must be prepared under the selected experimental conditions. There, the measured quantity is related to known concentrations of substrates.

The *kinetic determinations* of substrates should be carried out under conditions, where the concentration of substrate is in the range to fulfill the first-order circumstances ($[S]_0 \ll K_m$, see Equation (7) and Section 3.1). In the study by Christian,[10] one can find three forms:

- measurement of the time required for the reaction to convert a certain amount of substrate or form a certain amount of product;
- measurement of the amount of substrate converted or product generated in a given time; and
- continuous measurement of substrate or product concentration as a function of time, or by initial slope method.

The initial slope method means that the change in some physicochemical parameters (absorption, pH, or temperature), related to the change in concentration, are plotted as a function of time. Kinetic methods are generally more rapid than equilibrium methods and also more sensitive to enzyme effectors.

Analytical applications of enzyme-catalyzed reactions for determining substrates are widely used in many fields. Table 1 gives some examples for applications in food industry, medicine, and environmental analysis; further pertinent information can be found in the study by Bergmeyer.[11]

As seen from Table 1, many reactions with dehydrogenases need the coenzyme NAD. The reduced form of nicotinamide adenine dinucleotide (NADH) and the oxidized form (NAD^+) differ in their ultraviolet absorption spectra. NAD does not adsorb photons in the 340-nm region of spectra, whereas its reduced form does.

Such differences in absorption spectra of products, substrates, or other reactants are widely used in the *spectrophotometric method* to follow the progress of enzyme reactions.

As an example, the amount of NADH produced according to Equation (22) is a measure of the amount of acetaldehyde present in wine or beer:[12]

$$\text{Acetaldehyde} + NAD^+ + H_2O \xrightarrow{\text{A1-DH}} \text{acetic acid} + NADH + H^+ \quad (22)$$

(Al-DH, aldehyde dehydrogenase, EC 1.2.1.3, 9028-86-8.)

Moreover, NADH is used as a coupling agent. Two or more reactions occur in sequence. At the end of such a sequence, NAD^+ is generated or consumed. For example, the determination of creatinine or creatine is represented by Equations (23)–(26):

$$\text{Creatinine} + H_2O \xrightarrow{\text{CAH}} \text{creatine} \quad (23)$$

$$\text{Creatine} + ATP \xrightarrow{\text{CK}} \text{creatine phosphate} + ADP \quad (24)$$

$$ADP + \text{phosphoenolpyruvate} \xrightarrow{\text{PK}} ATP + \text{pyruvate} \quad (25)$$

$$\text{Pyruvate} + NADH + H^+ \xrightarrow{\text{LDH}} \text{L-lactate} + NAD^+ \quad (26)$$

The amount of NADH consumed is stoichiometric with the amount of creatine or creatinine.[13]

(CAH, creatinine amidohydrolase, EC 3.5.2.10, 9025-13-2; CK, creatinine kinase, EC 2.7.3.2, 9001-15-4; PK, pyruvate kinase, EC 2.7.1.40, 9001-59-6; LDH, lactate dehydrogenase, EC 1.1.1.27, 9001-60-9; ADP, adenosine diphosphate; ATP, adenosine triphosphate.)

The coupling of enzyme-catalyzed reactions with further reactions is commonly applied in enzymatic analysis, chiefly by using spectrophotometric or electrochemical methods. In contrast, the *calorimetric determination (enthalpimetry, thermal sensors)* of the substrate is much more general, because each enzyme reaction with a defined product can be used. There is no demand for colored products or electrochemically active reactants. The measured heat is proportional to the substrate concentration.[14] Especially the *ITC (isothermal titration calorimetry)* is well established for enzyme kinetic investigations.[15]

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Table 1 Typical determinations of substrates using enzymes

Substrate	Enzyme	EC number	CAS registry number	Reaction	Examples for application
A. Food industry					
Ethanol	Alcohol dehydrogenase	1.1.1.1	9031-72-5	$Ethanol + NAD^+ \rightarrow acetaldehyde + NADH + H^+$	Alcoholic drinks
Sucrose	Invertase	3.2.1.26	9001-57-4	$Sucrose + H_2O \rightarrow glucose + fructose$	Fruit juices, syrups
Glutamic acid	Glutamic dehydrogenase	1.4.1.3	9029-12-3	$Glutamate + NAD^+ + H_2O \rightarrow 2-oxoglutarate + NADH + NH_4{}^+$	Spices, meat
Ascorbic acid	Ascorbate oxidase	1.10.3.3	9029-44-1	$Ascorbate + 12O_2 \rightarrow dehydroascorbate + H_2O$	Fruit juices, drinks, meat
Sorbitol	Sorbitol dehydrogenase	1.1.1.14	9028-21-1	$Sorbitol + NAD^+ \rightarrow fructose + NADH + H^+$	Foodstuffs for diet, fruits
B. Medicine					
Cholesterol	Cholesterol oxidase	1.1.3.6	9028-76-6	$Cholesterol + O_2 \rightarrow 4\text{-}cholesten\text{-}3\text{-}one + H_2O_2$	Blood analysis of risk factors
Glucose	GOD	1.1.3.4	9001-37-0	$Glucose + O_2 + H_2O \rightarrow gluconic\ acid + H_2O_2$	Diagnosis of diabetes
	Glucose dehydrogenase	1.1.1.47	9028-53-9	$Glucose + NAD^+ + H_2O \rightarrow gluconic\ acid + NADH + H^+$	
Lactic acid	D-LDH	1.1.1.28	9028-36-8	$(R)lactate + NAD^+ \rightarrow pyruvate + NADH + H^+$	Sports medicine
Uric acid	Uricase	1.7.3.3	9002-12-4	$Urate + O_2 + 2H_2O \rightarrow allantoin + CO_2 + H_2O_2$	Diagnosis of gout
Penicillin	Penicillinase	3.5.2.6	9001-74-5	$Penicillins + H_2O \rightarrow penicilloic\ acid$	Diagnosis of allergy
C. Environment					
Phenol	Tyrosinase	1.14.18.1	9002-10-2	$Phenol + 12O_2 \rightarrow o\text{-}benzochinone$	Drinking water, waste water
Urea	Urease	3.5.1.5	9002-13-5	$Urea + 2H_2O + H^+ \rightarrow HCO_3{}^- + 2NH_4{}^+$	Bathwater
Nitrate	Nitrate reductase	1.6.6.1	9013-03-0	$Nitrate + NADH + H^+ \rightarrow nitrite + NAD^+ + H_2O$	Water

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Furthermore, Table 1 shows that the reaction of oxidases consumes oxygen. That makes them accessible to an *electrochemical method*, the amperometric detection. For the *amperometric determination* one can also use the production of hydrogen peroxide, Equation (27):

$$\text{Glucose} + O_2 + H_2O \xrightarrow{\text{GOD}} \text{gluconic acid} + H_2O_2 \quad (27)$$

The hydrogen peroxide is electrochemically oxidized at the platinum cathode, Equation (28):

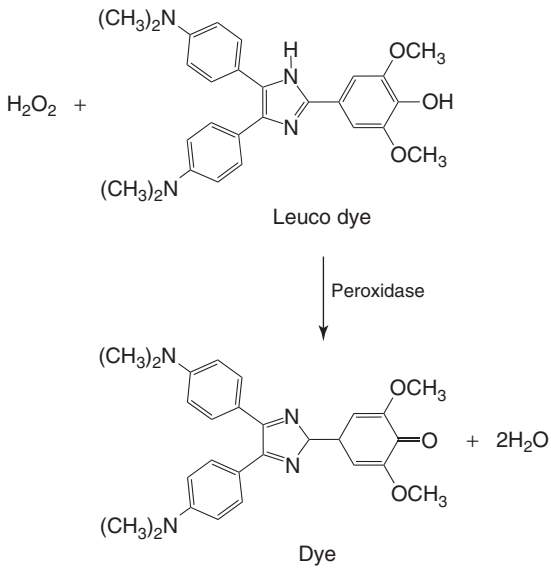
$$H_2O_2 \rightarrow O_2 + 2H^+ + 2e^- \quad (28)$$

The current obtained is proportional to the glucose concentration (principle of Clark oxygen electrode).

The substrate concentration of such an oxidase-catalyzed reaction (Equation 27) can also be measured with an *iodide-selective electrode*[16], Equation (29):

$$H_2O_2 + 2I^- + 2H^+ \xrightarrow{\text{Mo(VI)}} I_2 + 2H_2O \quad (29)$$

The decrease in iodide concentration is proportional to the substrate concentration, for instance glucose.



(30)

In H_2O_2-generating systems, substrate concentrations can also be determined by using *indicator reactions*.[17] One example is the determination of glucose with indicator reactions based on the peroxidase (EC 1.11.1.7, 9003-99-0) catalyzed oxidation of single chromogen (Equation 30).

An interesting way to increase the sensitivity of enzymatic substrate determinations is *amplification by cycling of enzyme-catalyzed reactions*. The principle is based on the following scheme.

The substrate to be determined is converted by an initial enzyme to a product. This product is the substrate of a second enzyme, which regenerates the substrate. Therefore, it can be converted by the first enzyme again and thus, the cycle is reinitiated.

A typical example is the determination of NADP by a cycling of NADPH between two enzyme reactions.[18]

5.2 Determination of Enzymes

As enzymes are catalysts affecting the rate of the reaction, not the equilibrium, their activity must be measured by a *kinetic* method. The kinetic determination of enzymes should be carried out under conditions where the substrate concentration is in excess and all other substances, which influence the rate of reaction, are present in fixed and nonlimiting concentrations. Then the value of K_m can be neglected, and the rate of the enzyme reaction depends only on the enzyme concentration (zero-order conditions, $[S]_0 \gg K_m$, see Equation (6) and Section 3.1). The three possible forms of determination are identical to the *kinetic* determination of substrates (see Section 5.1). Because of the influence on the enzyme activity (see Section 2), the pH value and the temperature should be kept constant during the measurement.

Enzyme determinations are chiefly applied in clinical diagnostics, food industry, and monitoring biotechnological processes. Table 2 shows a selection of interesting enzymes; further information is found in the study by Bergmeyer.[11]

As seen in the Table 2, *spectrophotometric monitoring* of NADH consumption as a function of time can be used to determine the enzyme activity of various enzymes.

The determination of amylase activity by measuring the produced reducing sugar as a function of time is an example of the application of the *colorimetric method*. An alkaline solution of 3,5-dinitrosalicylic acid and K-Na-tartrate is used as a chromogenic reagent.[19] Another, even more simple, case of *colorimetric method* is the determination of the AP activity. There, the formation of the yellow *p*-nitrophenol under alkaline conditions is measured.

Furthermore, the *pH-stat method* is used to determine enzyme activities. Monitoring enzyme reaction by pH-stat method means that the pH is maintained at constant value by frequent addition of alkali. The rate at which the base is added is proportional to the enzyme activity. The determination of lipase activity in serum (Equation 31) is a typical example.[20]

$$\text{Triglyceride} + H_2O \xrightarrow{\text{lipase}} \text{diglyceride} + \text{fatty acid} \quad (31)$$

The produced fatty acid is neutralized by counter titration with NaOH. Normally olive oil is used as the

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Table 2 Typical examples for enzyme determinations

Enzyme	EC number	CAS registry number	Reaction	Application
Glutamic-oxaloacetic transaminase (GOT)	2.6.1.1	9000-97-9	Asparate + α-ketoglutarateoxaloacetate $\xrightarrow{\text{GOT}}$ glutamate + oxaloacetateoxaloacetate + NADH + $H^+ \xrightarrow{\text{MDH}}$ malate + NAD^+(MDH malate del, 9001-64-3)	Clinical diagnostics, fresh meat control
Glutamic-pyruvic transaminase (GTP)	2.6.1.2	9000-86-6	L-Alanin + α-ketoglutarate $\xrightarrow{\text{GPT}}$ glutamate + pyruvatepyruvate + NADH + $H^+ \xrightarrow{\text{LDH}}$ lactate + NAD^+	Clinical diagnostics (liver diagnostic, bilious attack)
LDH	1.1.1.27	9001-60-9	Pyruvate + NADH + $H^+ \xrightarrow{\text{LDH}}$ lactate + NAD^+	Clinical diagnostics (cardiac infarction)
CK	2.7.3.2	9001-15-4	Creatinine phosphate + ADP $\xrightarrow{\text{CK}}$ creatine + ATP	Clinical diagnostics (cardiac infarction)
Alkaline phosphatase	3.1.3.1	9001-78-9	*p*-Nitrophenylphosphate + $H_2O \xrightarrow{\text{AP}}$ *p*-nitrophenol + phosphate	Food industry (milk products)
α-Amylase	3.2.1.1	9000-90-2	Polysachharide containing alpha-(1 - 4) - linked glucose units + H_2O ® maltooligosaccharides	Biotechnological processes
Lipase	3.1.1.3	9001-62-1	Triglyceride + $H_2O \xrightarrow{\text{lipase}}$ diglyceride + fatty acid	Biotechnological processes detergent industry
Catalase	1.11.1.6	5/2/9001	$H_2O_2 \rightarrow H_2O + O_2$	Food stuffs
Alanine aminopeptidase	3.4.11.2	9054-63-1	Aminoacyl-peptide + $H_2O \rightarrow$ amino acid + oligopeptide	Waste water
Peroxidase	1.11.1.7	9003-99-0	Donor + $H_2O_2 \rightarrow$ oxidized donor + H_2O	Food stuffs

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substrate. More examples for enzyme determinations and automated pH-stat systems can be found in the study by Gul et al.[21] and a recent application in the food industry is given in Ref. 22.

Among the pH-stat systems, other *electrochemical methods* such as *conductometry* (change in overall ionic mobility is measured as a function of time), *amperometry* (O_2 consumption or H_2O_2 production is measured as a function of time), or *ion-sensitive electrodes* (see Section 5.1) are also usable.

The *calorimetric determination* of invertase (INV) activity is described.[23] The sucrose hydrolysis shown in Equation (32) was performed in an isoperibolic calorimeter (EC 3.2.1.26, 9001-57-4). The kinetic evaluation of the calorimetric curves qualified to determine the enzymatic activity.

$$\text{Sucrose} + H_2O \xrightarrow{\text{invertase}} \text{glucose} + \text{fructose} \qquad (32)$$

Finally, *electrophoresis* is an important method for determining the catalytic activity of nucleases.[24]

5.3 Determinations of Effectors

The rate of the enzyme reaction can be influenced by various substances (see Section 4). This characteristic feature of enzyme reactions can be used for analytical purposes, the determination of *activators*, and the determination of *inhibitors*. These methods are especially important in trace metal determinations, as in most cases only a single oxidation state of metal is determined. The enzymatic analysis based on activator effects is not as frequently used as the determination based on inhibitor effects. Because effectors influence the kinetic of the enzyme reaction, their determination requires the monitoring of enzyme reactions as a function of time, i.e. kinetic determinations.

5.3.1 Activators

For their catalytic activity, many enzymes require *cofactors* (see Section 4.1) or the 'true substrate' (SA), where A is generally a metal ion.[25] For example, reactions involving ATP require Mg^{2+} ions to form $MgATP^{2-}$ ($A = Mg^{2+}$).

For instance, the amounts of magnesium can be determined according to Equation (1):

$$\text{Luciferin} + O_2 + \text{ATP} \overset{\text{luciferase}}{\underset{Mg^{2+}}{\rightleftharpoons}} \text{oxyluciferin} + \text{ADP} + PO_4^{3-} \qquad (33)$$

The formation of oxyluciferin is combined with green *chemiluminescence*. Trace amounts of magnesium (parts per billion levels) can be determined.[26]

Other inorganic substances combine with the enzyme at a specific activator site. This activation may be essential or nonessential. Table 3 shows some examples of activation by inorganic substances. Pertinent information on the requirement or effectiveness of some substances for the catalytic activity of a multitude of enzymes can be found in the 'Braunschweig Enzyme Database' (BRENDA).[27] These details are important for the application of activator effects for analytical purposes.

The experimental procedure of trace element analysis, using *metallo-enzymes* (specific metal ions are located at the active centers of the enzyme), is characterized by the removal of the metal ion from the holoenzyme (see Section 4.1) by treatment with chelating agents (for instance, EDTA). The resultant *apoenzyme* is catalytically inactive. The activator assay consists of the respective apoenzyme, its natural substrate, and a sample containing the metal ion. Then the restoration of enzyme

Table 3 Examples for activation of enzymes by inorganic substances[27]

Enzyme	EC number	CAS number	Activators
Arginase	3.5.3.1	9000-96-8	Mn^{2+}, Co^{2+}
Citrate lyase	4.1.3.6	9012-83-3	Mg^{2+}, Mn^{2+}, Fe^{2+}, Zn^{2+}
Enolase	4.2.1.11	9014-08-8	Mg^{2+}, Mn^{2+}, Zn^{2+}
Cytochrome oxidase	1.9.3.1	9001-16-5	Cu^{2+}
Carboxypeptidase A	3.4.17.1	11075-17-5	Zn^{2+}, Co^{2+}, Ni^{2+}, Mn^{2+}, Cd^{2+}
Creatinine kinase	2.7.3.2	9001-15-4	Mg^{2+}, Mn^{2+}
Glycerol kinase	2.7.1.30	9030-66-4	Mg^{2+}, Mn^{2+}, Co^{2+}, Zn^{2+}
Isocitrate dehydrogenase	1.1.1.41	9001-58-5	Mn^{2+}, Zn^{2+}, Co^{2+}, Mg^{2+}
Phosphofructokinase	2.7.1.11	9001-80-3	K^{+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, Zn^{2+}
AP	3.1.3.1	9001-78-9	Zn^{2+}, Mg^{2+}, Mn^{2+}, Co^{2+}, Ca^{2+}
Collagenase	3.4.24.3	9001-12-1	Ca^{2+}, Li^{+}, Mg^{2+}, Mn^{2+}, Sr^{2+}, Zn^{2+}
Luciferase	1.13.12.7	61970-00-1	Mg^{2+}

Created using data from Ref. 27.

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Table 4 Some examples of coenzymes

Coenzyme	Abbreviation	Apoenzyme	Function
Adenosine triphosphate	ATP	Kinases, transferases	Transphosphorylation
Coenzyme A	CoA	Acyltransferases, thioligases	Acyl group transfer
Flavine adenine dinucleotide	FAD	Oxidases	Proton transfer
Nicotinamide adenine dinucleotide	NAD	Dehydrogenases	Proton transfer
Pyridoxal phosphate	PAL	Transaminases	Amino group transfer

activity is proportional to the amount of metal ion in the sample. In this way, trace amounts of metal ions can be determined. An example is the determination of zinc ions by using AP (Equation 34):

$$\text{4-Nitrophenyl phosphate} + H_2O \xrightarrow{\text{AP}} \text{4-nitrophenol} + \text{phosphate} \quad (34)$$

The enzyme activity is determined by measuring the rate of release of 4-nitrophenolate ion (alkaline pH) at 410 nm in a recording spectrometer. The method is so sensitive that 0.5 ng of zinc can be determined in small volumes (0.5 μl) of plasma.[28]

Many enzymes require a specific *coenzyme* for their catalytic activity (Table 4). At low concentrations of enzyme, the degree of activation of such an enzyme is proportional to the concentration of the coenzyme. That is in contrast with the assay for determining substrate concentrations (see Section 5.1), where the coenzyme should be in excess.

For instance, ATP, an important intermediate in energy metabolism, can be determined by the following reaction scheme (Equations 35 and 36):

$$\text{ATP} + \text{glucose} \xrightarrow{\text{HK}} \text{glucose-6-phosphate} + \text{ADP} \quad (35)$$

$$\text{Glucose-6-phosphate} + \text{NADP}^+ \xrightarrow{\text{G6PDH}} \text{glucono-1,5-lactone 6-phosphate} + \text{NADPH} \quad (36)$$

(HK, Hexokinase, EC 2.7.1.1, 9001-51-8; G6PDH, glucose 6-phosphate dehydrogenase, EC 1.1.1.49, 9001-40-5.)

The reaction can be monitored by the formation of NADPH spectrophotometrically at 340 nm.

5.3.2 *Inhibitors*

In the literature, one can find a variety of enzyme inhibitors. Table 5 shows a selection of well-known inorganic and organic inhibitors. The relative effect on enzyme activity of different substances is characterized by the IC_{50} value. The IC_{50} value is the inhibitor concentration giving 50% inhibition.[29] Several analytical methods have been developed based on the inhibition of enzyme reactions.

As seen from Table 5, one enzyme often has various inhibitors. Therefore, investigations of inhibitor effects are not as practicable for single-component analysis as for determination of summary or group-specific parameters. An example is the screening of pollutants in waste water. Furthermore, enzyme inhibition tests are suitable to preselect polluted samples.

Table 5 Some examples for inhibitors

Enzyme	Inhibitor	EC number	CAS number	Ref.
GOD	Cd^{2+}, Co^{2+}, Cu^{2+}, Ni^{2+}; Hg^{2+}, Ag^{+}, Pb^{2+}, Fe^{3+}, Zn^{2+}, Ni^{2+}, CrO_4^{2-}	1.1.3.4	9001-37-0	[30,31]
Dihydrofolate reductase	Methotrexate (MTX)	1.5.1.3	9002-03-3	[32]
Tyrosinase	Pesticides	1.14.18.1	9002-10-2	[31]
Butyrylcholinesterase	Pesticides	3.1.1.8	9001-08-5	[31]
AP	Pesticides, Ag^{+}, CN^{-}	3.1.3.1.	9001-78-9	[30]
Arylsulfatase	Phosphate, sulfate	3.1.6.1	9016-17-5	[33]
β-Galactosidase	Organic sulfur compounds	3.2.1.23	9031-11-2	[34]
Invertase	Ag^{+}, Hg^{2+}, Pb^{2+}, Cd^{2+}	3.2.1.26	9001-57-4	[30]
Urease	Hg^{+}, Hg^{2+}, Cu^{2+}, Cd^{2+}, Ni^{2+}, Pb^{2+}, Co^{2+}, Zn^{2+}	3.5.1.5	9002-13-5	[30,31]
δ-Aminolevulinate dehydratase	Pb^{2+}	4.2.1.24	9036-37-7	[35]

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In the following, some examples for analytical application (see also Sections 6 and 7) are represented. There is a variety of methods for determination of inhibitors, dictated by the physicochemistry of the enzyme reaction to be monitored.

Heavy metal and pesticide pollution in water is a serious problem, especially for drinking water quality. Therefore, the World Health Organization has set limits for highly toxic substances such as Hg^{2+} in drinking water. For quality monitoring, simple and rapid determination of Hg^{2+} at very low concentrations and with the option of high-throughput analysis are required. Deshpande et al.[36] proposed a bi-enzymatic approach based on alcohol oxidase (AOD, EC 1.1.3.13, 9073-63-6) and horseradish peroxidase (HRP, EC 1.11.1.7, 9003-99-0). The principle of the applied chemiluminescence technique in short: methanol is oxidized to formaldehyde and hydrogen peroxide by AOD. Afterward, the formed hydrogen peroxide is converted to water, nitrogen, oxygen, and light ($h\nu$). The presence of Hg^{2+} inhibits AOD and hence, reduces the emitted light. On applying the assay on a 384-well plate, a detection limit of 1 pg mL^{-1} was achieved.

The acetylcholinesterase (AChE) inhibition test is a well-known method for the determination of organophosphorus acid esters and *N*-methyl carbamates. *Photometric* tests are widely used. The hydrolysis of acetylthiocholine by AChE (EC 3.1.1.7, 9000-81-1) is measured (Equations 37 and 38):

$$\text{Acetylthiocholine} + H_2O \xrightarrow{\text{ACHE}} \text{thiocholine} + CH_3COOH \quad (37)$$

$$\text{Thiocholine} + \text{dithiobisnitrobenzoate} \rightarrow \text{thionitrobenzoate (yellow dye)} \quad (38)$$

The entire inhibition effect, in comparison with the uninhibited AChE, is given in paraoxon equivalent.[37]

This inhibition effect can also be measured by *amperometric* detection (Equations 39–41):

$$\text{Acetylcholine} + H_2O \xrightarrow{\text{ACHE}} \text{choline} + CH_3COOH \quad (39)$$

$$\text{Choline} + 2O_2 + H_2O \xrightarrow{\text{choline oxidase}} \text{betaine aldehyde} + 2H_2O_2 \quad (40)$$

$$H_2O_2 \xrightarrow{\text{anotic oxidation}} O_2 + 2e^- + 2H^+ \quad (41)$$

(Choline oxidase, EC 1.1.3.17, 9028-67-5.)

The inhibition of AChE is proportional to pesticide concentration and is measured through the decrease in current.[38]

Inhibition studies are also suitable to distinguish between oxidation states of metals. As an example, *calorimetric investigations* of urease inhibition of heavy metals show that As(III) ions inhibit the urea hydrolysis by competitive mechanism, while As(V) ions do not inhibit the enzyme in the comparable concentration range.[39] For the evaluation of the potential of toxicological hazard, the differentiation of arsenic content should be relevant.

Owing to the demand for high-throughput techniques at low cost, the development of enzyme arrays for simultaneous determination of multiple analytes increased recently. As examples, screen-printed biosensors[40,41] and integrated sensors combining electrochemical and optical transducers[42] as well as arrays with encapsulated enzymes based on sol–gel techniques[43,44] have to be mentioned (see also Section 7).

6 ENZYMATIC DETERMINATIONS IN FLOW SYSTEMS

As an alternative to the discussed batch systems, the tendency to perform analytical determinations under flow conditions has increased in the last few decades. Ružička and Hansen[45] introduced the flow injection analysis (FIA) in the analytical chemistry, and the recent developments are favored by the progress in electronics, microfluidics, and the numerous possibilities for miniaturization of flow systems. Furthermore, the simple integration of such systems in process analysis, the high potential of automation, and the shortening of analysis time lead to the broad application. FIA and its extensions SIA (sequential injection analysis) and LOV (lab-on-valve) can be coupled with various types of detectors, for instance, electrochemical, chromatographic, thermal, or spectrometric. The analytical objectives of enzymatic determinations in flow systems are primarily substrates, enzyme activities, enzyme inhibitors, or enzyme cofactors.[46]

Enzyme kinetics studies in flow systems are based on various techniques, especially continuous, stopped, or segmented flow. In the review of Hartwell and Grudpan,[47] the benefit of using automatic flow-based analytical systems for enzyme kinetics versus manual operation for kinetic studies are discussed. By comparison, the authors point out the rapid mixing of enzyme and substrate, and the facility for real-time measurement of the initial rate in flow systems.

The arrangements for FIA differ by the usage of dissolved or immobilized enzymes, which means the construction of homogeneous or heterogeneous systems. By using enzymes in solution, it is possible to ensure a

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fresh portion of it for every determination, avoiding in this way the deactivation effects from previously analyzed samples.[48] This is of great importance, in particular, for the kinetic determination of inhibitors or cofactors.

6.1 Flow Systems Using Immobilized Enzymes

The term *immobilized enzymes* stands for a preparation in which an enzyme is confined or localized in a relatively well-defined region. Enzymes may be immobilized by covalent bindings, crosslinking, and adsorption or entrapment within various matrices, for instance, membranes or polymeric gels. Different techniques used for the immobilization of enzymes are described extensively.[49,50]

Enzyme immobilization is an economical requirement as immobilized enzymes can be easily separated from the reaction solution and thus, become reusable. Besides, immobilization frequently leads to an improvement of thermal and/or chemical stability of enzyme preparation in comparison to the soluble or native enzyme. On the other hand, the immobilization often causes a reduction in catalytic activity. Immobilized enzyme systems always differ from the soluble counterparts by the K_m value or the optimal pH condition. A further advantage of immobilization is the possibility to prepare *multienzyme systems*, where two or more enzymes are immobilized on the same carrier. Immobilized multienzyme systems are suitable to catalyze sequential sets of reactions.

The application of immobilized enzymes in flow systems is a very convenient way for rapid and automated enzymatic determinations. The most common configurations in such flow systems are reactors (microreactors) with enzymes immobilized on beads (supporting material) or on the interior surface of microfluidic channels. The incorporation of such enzyme reactors in FIA or SIA systems allows outstanding repeatability and reproducibility.[51]

Figure 9 shows a practicable arrangement of such a flow system for enzymatic determinations in a simplified representation. Generally, FIA is based on the injection of the liquid sample into a nonsegmented continuous buffer stream. The injected sample forms a zone in the stream, flowing into the enzyme reactor. There, the enzyme reaction takes place and the resulting change in a physicochemical parameter (e.g. absorption, temperature, and pH) is registered by the detector.

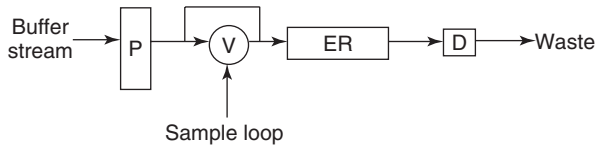

Figure 9 Simplest arrangement of enzymatic flow system. P, pump; V, valve; ER, enzyme reactor; D, detector.

The detector signal during the passage of the sample (substrate zone) has the form of a peak. The analytical information is given by the peak height, the peak area, or the width of the peak, which is analogous to the evaluation of chromatographic measurements. Enzymatic flow systems are calibrated with standard solutions before their application. The operating conditions, such as pH, buffer system, or buffer flow stream, have to be optimized. In FIA systems, kinetic controlled reactions take place. Besides this basic approach in continuous flow, there are other techniques (see preceding paragraphs) and the options are extremely diverse, assuring perfect adaptation to the particular analytical problem.

Enzymatic microreactors for the determination of ethanol by an automatic SIA system using the *stopped flow technique* are presented by Alhadeff et al.[52] The use of two microreactors with immobilized AOD and HRP allowed an important saving of enzymatic reagents in the described spectrophotometric detection system. The authors immobilized AOD and HRP separately on aminopropyl glass beads. The system was applied to the analysis of alcoholic beverages and bioprocess medium samples.

The *enzyme thermistor* (ET) is a well-known example for the combination of the specific enzymatic reaction with a universal transducer principle. The ET was first described by Danielsson and Mosbach.[53] Generally, the ET consists of immobilized enzymes and a flow system with *calorimetric* detection. The immobilized enzymes are arranged in columns, and the temperature is measured at the top of the column with a thermistor. As a result of the enzyme-catalyzed reaction, a temperature peak can be detected. The height of the peak is proportional to the enthalpy change, corresponding to a definite substrate concentration. Normally, the ET device is calibrated with known concentrations of an analyte. Applications of the ET for determination of various analytes over the last 30 years are presented in Ref. 54.

Immobilized enzyme reactors in *high performance liquid chromatography (HPLC)* and its operation in inhibitor screening are summarized in Ref. 55. With the reactor placed between the HPLC pump and the detector, various immobilization techniques are possible and inhibition studies can be performed by injecting both substrate or drugs and substrate simultaneously. In the publication 'Fundamentals and applications of immobilized microfluidic enzymatic reactors',[56] the utility of the so-called IMERs is compiled impressively.

The combination of enzymatic reactions with electrochemical methods can be used to improve the selectivity of many reactions using, for instance, amperometric detection of reducing reagents in complex media.[57] The

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authors present an amperometric detection of ascorbic acid in honey using ascorbate oxidase (AAO, EC 1.10.3.3, 9029-44-1). The combination of FIA and a tubular reactor containing AAO immobilized on Amberlite IRA-743 permits ascorbic acid determination at low concentrations as well as in colored samples.

Vlakh and Tennikova[58] give a review about kinetic studies and applications of flow-through immobilized enzyme reactors based on *monoliths*. Different classes of enzymes such as hydrolases, lyases, and oxidoreductases have been immobilized and employed in proteomics, biotechnology, and pharmaceutics. AChE encapsulated in monolithic silica for usage as an immobilized enzyme reactor in combination with tandem mass spectrometry is presented by Forsberg et al.[59] This system is suitable for screening of AChE inhibitors in complex mixtures and by varying the pump configuration also for the determination of kinetic parameters such as K_I and IC_{50}.

Finally, a recent example for *miniaturization or chip configuration* is given in Ref. 60. The authors show a solid-state sensor incorporated in a microfluidic chip for creatinine and glucose detection in serum. The special approach for immobilization of enzymes for this sensor is the use of *magnetic beads* to immobilize the enzymes. For this purpose, the magnetic beads were modified with carboxyl functional groups for immobilization of GOD, whereas magnetic beads with amino functions bind to creatinine deaminase.

6.2 Flow Systems Using Soluble Enzymes

Despite the one-time use of enzymes in homogeneous systems, in the last few years, application of enzymes in solution has gained more attention, mainly due to expensive immobilization processes, enzyme inactivation during the analytical cycles, and developments in microfluidics.[48] In the following, some interesting examples of application of soluble enzymes in flow systems are presented.

In the field of *miniaturization*, droplet-based microfluidics has emerged as an efficient way for realization of chemical or biological experiments in pico- to nanoliter range. A *droplet-based microfluidic flow injection* system with large-scale concentration gradient by a single nanoliter-scale injection is described by Cai et al.[61] This system was applied for drug screening by the inhibition of β-galactosidase (EC 3.2.1.23). The authors demonstrated that, with a single injection of 16 nL of drug solution, more than 240 in-droplet enzyme inhibition reactions could be performed with analysis time of 2.5 min. The group of Kennedy analyses such *segmented samples* in combination with capillary electrophoresis or electrospray ionization mass spectrometry for high-throughput determinations of enzyme activities or enzyme inhibitors.[62,63]

Micro-structured heat power detectors are examples for the miniaturization trend in enzymatic analysis using the thermal detection principle.[64] Label-free determination of enzyme activities with a sample volume of 10–20 μL is presented by Lerchner et al.[65] The authors estimate the activity of dissolved INV and point out, that by applying a special version of the chip module immobilized enzymes are usable.

A *sequential injection lab-on-valve (SI-LOV)* method for the enzymatic determination of hydrogen peroxide by spectrophotometric detection was depicted by Vidigal et al.[66] The quantification of hydrogen peroxide rests on the HRP-catalyzed reaction between H_2O_2 and ABTS (2,2′-azino-bis(3-ethylbenzothiazoline 6-sulfonic acid, 30931-67-0). This arrangement represents an expedient tool for the monitoring of the disinfection–neutralization process for the cleaning of contact lenses. A review about SI-LOV as the 'third generation of flow injection analysis' is given by Wang and Hansen, who is one of the two inventors of the FIA technique.[67]

The use of enzymes in uncommon solvents is presented by Araujo et al.[68] for kinetic investigations of reactions with poorly water-soluble substrates in *ionic liquid systems*. Different 1-butyl-3-methylimidazolium tetrafluoroborate ([bmim][BF4])/buffer mixtures were applied as reaction media for the mushroom tyrosinase (EC 1.14.18.1)-catalyzed oxidation of the phenolic compound, caffeic acid. This determination was realized by the spectrophotometric measurement of the depletion rate of the poorly water-soluble substrate caffeic acid. Furthermore, the authors use this SIA arrangement for *inhibition studies* of tyrosinase by cinnamic acid and benzoic acid.

The high stereoselectivity of enzyme utilizes the *chiral analysis* and can be combined with an *electrophoretically mediated microanalysis (EMMA)* method for fast online discrimination and determination of substrate enantiomers.[69] The presented method was successfully applied to determine the L-/D-lactate in several yogurt and wine samples.

7 IMMOBILIZED ENZYMES IN BIOSENSOR DEVELOPMENT

One of the first applications of immobilized enzymes in analytical chemistry was the construction of an enzyme electrode for glucose determination. An oxygen electrode, Clark type, was combined with a thin layer of GOD entrapped in a polyacrylamide gel.[70] Such enzyme electrodes form a major category of the so-called biosensors.

Generally, a biosensor is a compact analytical device incorporating a biological or biologically derived sensing

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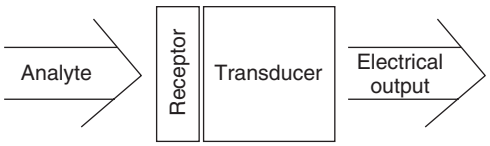

Figure 10 General principle for a biosensor.

element either integrated within or intimately associated with a physicochemical transducer.[71] The biological or biorecognition element may be an enzyme, antibody, or microorganism system. Besides the biological element, or in sensor development called *receptor*, the biosensor consists of a transducer, which interprets the interaction of the analyte with the receptor as an electrical signal, related to the concentration (or activity) of a chemical or biochemical analyte (Figure 10). For instance, an enzymatic catalyzed reaction may produce ions, electrons, heat, color or changes in mass, which can be used in the transducer, to provide an electrical output. The receptor as a biorecognition element imparts the required high analyte selectivity to the transducer.

Usually, the enzyme is immobilized directly at the surface of the transducer or entrapped in a gel or rather in membranes, fixed immediately at the transducer. Pertinent information about the immobilization of biological components for biosensor application is summarized in Ref. 72. The optimization of the enzyme immobilization is one of the critical issues of biosensor development. Problems with long-term stability of enzyme layers and long response times of biosensors have to be solved. Frequently, membranes are used to separate the enzyme layer and the medium. Because of this, additional barriers, not only the enzyme kinetics but also the diffusion effects, have to be taken into account. A synopsis in mathematical modeling of enzymatic sensors is given by Bănică.[73] Furthermore, the biosensor signal is also influenced by temperature, inhibitors, and pH value. In many practical applications, the highest efficiency can be achieved in combination with FIA systems. In this context, nanomaterials, such as carbon nanotubes, titanate nanotubes, and nanoparticles[74] or mesoporous materials,[75] disclose promising biosensor constructions.

Advantages of biosensors are the detection of biological and chemical substances without the need for complex sample processing, simple method for use outside of a laboratory, and the high potential of miniaturization in combination with the microsystem technique. Biosensors excel by a high selectivity and sensitivity.

The most commonly used transducers for biosensors are electrochemical and optical. An extensive representation of the different transducer principles is given in the monograph by Yoon[76] or the *Handbook of Biosensors and Biochips*.[77]

Amperometric biosensors monitor currents in the transducer generated when electrons are exchanged between a biological system and an electrode. Many blood glucose sensors are based on this principle. A prominent representative is the glucose analyzer Yellow Springs Instrument for the measurement of glucose (according to Equations 27 and 28, Section 5.1) in whole blood.

A review of enzymatic uric acid biosensor with amperometric detection is given by Erden and Kılıc.[78] According to reaction (42), uricase (urate oxidase, UOX, EC 1.7.3.3, 9002-12-4) catalyzes the oxidation of uric acid to allantoin, CO_2, and H_2O_2. Analogous to the glucose determination, the produced H_2O_2 is measured amperometrically:

$$\text{Uric acid} + 2\,H_2O + O_2 \xrightarrow{\text{UOX}} \text{Allantoin} + H_2O_2 + CO_2 \quad (42)$$

Uric acid determinations in biological fluids are significant for diagnosis and treatment of numerous renal and metabolic disorders. The authors[78] note that *screen-printed electrodes* provide an important approach toward disposable, selective, and sensitive uric acid biosensors for routine analysis.

For the construction of enzyme electrodes, the electron transfer from the active site of the redox enzyme to the electrode plays an important role.[73] A recent example of *mediated electrochemical enzyme sensors* is shown by Schuhmann et al. The authors coupled osmium complexes containing amine functional groups to epoxy-functionalized polymers to provide mediated enzyme electrodes for glucose oxidation.[79]

The usage of *enzyme-cascades* or *sequences*, analogue Section 5.1, is actually reported in Ref. 80 for the detection of sucrose. There, an integrated amperometric sucrose biosensor involving a 3-mercaptopropionic acid (MPA) self-assembled monolayer (SAM)-modified gold disk electrode (AuE) and coimmobilization of the enzymes INV and fructose dehydrogenase (FDH) as well as the redox mediator tetrathiafulvalene (TTF) by means of a dialysis membrane is presented.

Additionally, developments are based on the application of *novel enzymes*. Scheller et al.[81] use the unspecific aromatic peroxygenase (APO; EC 1.11.2.1, 93229-67-5) for the determination of aromatic compounds as an overall parameter, relevant in environmental analysis and drug monitoring.

The design of a cytochrome *c* electrochemical biosensor using *nanoparticles* for the immobilization of cytochrome *c* reductase is depicted by Pandiaraj et al.[82] The authors use two kinds of nanomaterial-based biosensor platforms: (i) *carbon nanotubes* incorporated polypyrrole (PPy)

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matrix on Pt electrode and (ii) SAM functionalized *gold nanoparticles* in PPy-Pt.

An innovative *conductometric* biosensor for determination of surfactants based on AChE inhibition is characterized in Ref. 83. According to Equation (39), acetylcholine (ACh) is hydrolyzed by AChE in choline and acetic acid, which dissociates, and the ion concentration in the working enzyme membrane increases. The resultant change in conductivity is registered by the transducer. Surfactants reduced the reaction rate and their concentration was evaluated by measuring the initial rate of the hydrolysis.

Potentiometric devices measure the accumulation of charge density at the surface of electrodes, especially pH-sensitive electrodes are used. Here, any enzyme reaction affecting the acid–base equilibrium can be applied. Potentiometric enzyme sensors based on the ion-sensitive field effect transistors (ISFETs) are also called *enzyme field effect transistors* (ENFETs). A potentiometric enzyme electrode or ENFET will be a good choice, if no redox enzyme is available for amperometric sensing, and an enzyme is applicable to produce or consume H^+, HCO_3^-, or NH_4^+ ions. A recent example is the industrial fabrication of urea-ENFET for online monitoring of urea in dialysate solutions.[84] The authors describe the development of urea-ENFET/pH-ISFET differential analysis technique and the realization of the enzymatic layer by ink jet printing.

A comparison of the two *semiconductor-based chemical imaging sensors*, a light-addressable potentiometric sensor *(LAPS)* and a pH imaging sensor based on a charge-coupled device *(CCD)* for developing of an ACh imaging sensor for neural cell-activity observations, is presented in Ref. 85. Both sensors rest on the field effect in an electrolyte–insulator–semiconductor (EIS) structure; however, the readout mechanism is different. AChE was immobilized in a polyion-based membrane. Especially, the higher long-term stability of the ACh-LAPS, in comparison to the CCD-type sensor, makes this sensor interesting to estimate the prolonged cell degradation for investigating diseases, such as Alzheimer and myasthenia.[85]

Besides the above-envisaged electrochemical biosensors, the optical transducer principle in combination with enzymes has to be mentioned. Optical biosensors are sensitive techniques that are based on the change in the phase, amplitude, polarization, or frequency of the input light in response to the biorecognition processes. They can be classified into colorimetric, fluorescence, luminescence, surface plasmon resonance (SPR), and fiber-optics/bio-optrode based biosensors.[86] Gauglitz[87] gives an overview of direct optical detection in bioanalysis, including the measurement of kinetics.

An enzymatic *fiber-optic biosensor* for the determination of halogenated hydrocarbons is presented in Ref. 88. The enzyme haloalkane dehalogenase (EC 3.8.1.5, 95990-29-7) and a *fluorescence* pH indicator (5(6)-carboxyfluorescein) were coimmobilized on the tip of an *optical fiber*. Owing to the enzyme-catalyzed hydrolytic dehalogenation, the local pH changed and affected the fluorescence of the indicator.

Changes in the *phosphorescence* intensity are used for the construction of toluene biosensor.[89] Oxygen-sensitive ruthenium-based phosphorescent dye served as the transducer for consumption of oxygen, which accompanied the enzymatic reaction catalyzed by toluene *ortho*-monooxygenase, expressed by *Escherichia coli* TG1 carrying pBS(Kan)TOM. Some more recent examples of fiber-optic enzymatic biosensors enclose the review given by Wang and Wolfbeis.[90]

An *SPR* biosensor-based assay for membrane-embedded full-length BACE1 (b-site amyloid precursor protein cleaving enzyme 1), a drug target for Alzheimer's disease, is introduced by Christopeit et al.[91] This approach is characterized by the possibility of analysis of interactions with the protein in its natural lipid membrane environment, which is significant for the discovery of clinically efficient BACE1 inhibitors.

Finally, brief remarks on the status of implantable enzymatic sensors. The requirements for miniaturization, biocompatibility, or, for instance, sensor stability of these biosensors are particularly high. The review[92] outlines the challenges to successful development of implantable amperometric enzyme sensors and accents the emerging technological progress. For continuous glucose monitoring, the integration of nanostructured surface or nanomaterials improves the performance of the biocomponents and hence, the construction of implantable devices.[93]

ABBREVIATIONS AND ACRONYMS

NAD	Nicotinamide Adenine Dinucleotide
EI	Enzyme–inhibitor complex
EIS	Enzyme–inhibitor–substrate complex
GOD	Glucose Oxidase
NADH	Nicotinamide Adenine Dinucleotide
Al-DH	Aldehyde Dehydrogenase
CAH	Creatinine amidohydrolase
CK	Creatinine Kinase
PK	Pyruvate Kinase
LDH	Lactate Dehydrogenase
ADP	Adenosine Diphosphate
ATP	Adenosine Triphosphate
INV	Invertase

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AP	Alkaline Phosphatase
BRENDA	Braunschweig Enzyme Database
HK	Hexokinase
G6PDH	Glucose 6-phosphate dehydrogenase
AOD	Alcohol Oxidase
HRP	Horseradish Peroxidase
AChE	Acetylcholinesterase
FIA	Flow injection Analysis
SIA	Sequential injection Analysis
LOV	Lab-on-valve
ET	Enzyme Thermistor
HPLC	High Performance Liquid Chromatography
AAO	Ascorbate Oxidase
SI-LOV	Sequential injection Lab-on-valve
ABTS	2,2′-azino-bis(3-ethylbenzothiazoline 6-sulfonic acid
EMMA	Electrophoretically Mediated Microanalysis
UOX	Urate oxidase
MPA	3-mercaptopropionic Acid
SAM	Self-assembled monolayer
FDH	Fructose Dehydrogenase
TTF	Tetrathiafulvalene
APO	Aromatic Peroxygenase
ACh	Acetylcholine
ISFET	Ion-sensitive Field Effect Transistor
ENFET	Enzyme field effect transistor
LAPS	Light-addressable potentiometric sensor
CCD	Charge-coupled device
EIS	Electrolyte–insulator–semiconductor
SPR	Surface Plasmon Resonance

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