Hans Bisswanger

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Practical Enzymology

Second, Completely Revised Edition
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Preface to the Second Edition

The principal concept of the first edition: general aspects of enzymes and presentation of special enzyme assays and related tests such as protein determination and enzyme immobilization, as well as instrumental aspects, remain conserved in the second edition. Additional enzyme assays and tests, for example, for the determination of glycoproteins and inorganic phosphate, have been included, considering the principles of broad interest and diversity of methods. Features of the enzymes, which are of importance for the assay conditions, such as cofactor requirement, molecular mass, state of aggregation, kinetic constants, and pH optimum, are indicated, but it is not intended to present all known data. Actually, an overwhelming quantity of data accumulated within the last years, owing to the violent progress in gene technology, and features of the same enzyme species can differ extremely, depending on the organisms from where it originates. For practical treatment of an enzyme assay, such a variety of data is more disturbing than helpful; therefore, only the features of one or few representative enzyme species, preferentially from human or mammalian origin, are mentioned. Particular attention has been drawn to frequent pitfalls and error detection for the methods described.

Enzymes applied for molecular biology and gene technology, such as restriction enzymes and polymerases, are not taken into consideration. They are extensively described in text books and manuals of the respective field, but the general rules for handling and for the assay conditions are also valid for these enzymes. This holds also for RNA enzymes (ribozymes), antibody enzymes (antizymes), and artificial enzymes derived, for example, from cyclodextrins or crown ethers, and for enzymes modified by site-specific mutations.

The layout has been improved, for example, by introduction of colors; the structure of text is clearer; and the essential points of the sections are summarized in separated boxes. A companion web site (www.wiley-vch.de/home/enzymology) provides animations for all figures together with supplementary material, for deeper understanding of the partially abstract matter.

Especially emphasized are the valuable contributions of Klaus Möschel and Rainer Figura to the chapter of immobilized enzymes.

Tübingen, January 2011

Hans Bisswanger
Note to the Reader

Animations should assist the comprehension of the text. They are principally self-explaining, but knowledge of the corresponding book chapter and the respective figure legend is presupposed. Most animations are subdivided into sequential steps, which are initiated by the cursor button (→) or a mouse click. A green arrow at the bottom of each figure gives the signal for pressing, during the animation run it disappears. ‘X’ indicates the end of the animation before passing to the next figure.
Abbreviations\textsuperscript{1)}

- A, B, C: specific binding ligands
- \([A]\): ligand concentration
- \([A]\)\textsubscript{0}: total ligand concentration
- A: absorption
- ABTS: 2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid
- ADH: alcohol dehydrogenase
- ANS: anilinonaphthalene sulfonate
- BAPNA: \(N′\)-benzoyl-L-arginine-p-nitroanilide
- BCA: bicinchoninic acid
- BSA: bovine serum albumine
- CDI: carbonyldiimidazol
- CoA: coenzyme A
- CPG: controlled pore glass
- \(d\): density
- DMSO: dimethylsulfoxide
- DPIP: 2,6-dichlorophenolindophenol
- DTE: dithioerythritol
- DTNB: 5,5′-dithio-bis(2-nitrobenzoic acid), Ellman’s reagent
- DTT: dithiothreitol, cleland’s reagent
- E, \([E]\): enzyme, enzyme concentration
- \([E]\)\textsubscript{0}: total enzyme concentration
- \(\varepsilon_{\text{nm}}\): absorption (“extinction”) coefficient at the wavelength indicated
- \(\varepsilon_{r}\): dielectric constant
- EDTA: ethylenediaminetetraacetic acid
- EIA: enzyme immunoassays
- ELISA: enzyme-linked immunoabsorbent assays
- FAD: flavine adenine dinucleotide
- FMN: flavine mononucleotide
- GOD: glucose oxidase

\textsuperscript{1)} Only repeatedly used abbreviations, special abbreviations are defined at the respective section.
Abbreviations

$h$ Planck’s constant

HK hexokinase

$I$ light intensity

IU International enzyme unit (µmol min$^{-1}$)

$k$ rate constant

kat Katal (mol s$^{-1}$)

$k_{\text{cat}}$ catalytical constant

$K_d$ dissociation constant

$K_m$ Michaelis constant

LDH lactate dehydrogenase

MDH malate dehydrogenase

$M_r$ relative molecule mass

$n$ number of subunits

NAD nicotinamide adenine dinucleotide$^2$)

NADH nicotinamide adenine dinucleotide$^2$)

NADP nicotinamide adenine dinucleotide phosphate$^2$)

NADPH nicotinamide adenine dinucleotide phosphate$^2$)

ONPG o-nitrophenyl β-D-galactopyranoside

ORD optical rotatory dispersion

P, Q, R products

PAGE polyacrylamide gel electrophoresis

PBS phosphate buffered saline

PEG polyethylene glycol

PLP pyridoxal 5-phosphate

PMSF phenylmethylsulfonyl fluoride

POD peroxidase

$R$ Gas constant (8.3145 J mol$^{-1}$ K$^{-1}$)

RIA radioimmunoassay

RN recommended name

rpm rotations per min

RT room temperature

S substrate

SA specific enzyme activity

SDS sodium dodecyl sulfate

SN systematic name

ThDP thiamine diphosphate

TCA trichloroacetic acid

TRIS tris(hydroxymethyl)aminomethane

$v$ reaction velocity

$V$ maximum reaction velocity

$v_i$ initial reaction velocity

$^2$) For simplicity the charge (NAD(P)$^+$), for the reduced form the free proton (NAD(P)H + H$^+$) is omitted.
1 Introduction

Enzymes are the most important catalysts and regulators indispensably involved in each process in living organisms. Any investigation of the cell metabolism requires a thorough understanding of enzyme action. Enzymes are very sensitive markers for correct function and, consequently, also for dysfunction of the metabolism, serving as indicators both for health and manifestation of diseases. Accordingly, they are used as invaluable tools in medical diagnostics. Beyond that, enzymes are applied in many technical operations. They play an essential role in the environmental processes in the microbial world in waters, rivers, lakes, and soil, and are important for filter plants as well as for fermentation procedures in dairies and breweries.

According to current estimates, about 25 000 enzymes are expected to exist in the living world, where more than 3000 are described in detail, and some hundreds are commercially available. Enzymes are extremely efficient catalysts, enhancing the turnover rates of spontaneous reactions by factors between $10^8$ and $10^{10}$, sometimes even up to $10^{12}$ (Menger, 1993). Orotidine-5′-phosphate decarboxylase is a striking example: the spontaneous reaction proceeds with a half-life of 78 million years and the enzyme increases the velocity by a factor of $10^{17}$ (Radzicka and Wolfenden, 1995). Triosephosphate isomerase accelerates the enolization of dihydroxyacetone phosphate by more than $10^9$ (Alberty and Knowles, 1976).

Even reactions spontaneously proceeding with a considerable rate, such as the formation of water from hydrogen and oxygen in the respiratory chain, are subject to enzyme catalysis: each reaction step in metabolism is controlled by a special enzyme. Thus the role of enzymes in the metabolism is broader than to act only as biocatalysts. The peculiarity of catalysis is not only restricted to the acceleration of spontaneous reactions, but it also allows controlling reactions. Spontaneous reactions, after initiating, run off to the end and cannot be stopped. Catalyzed reactions, in contrast, proceed only in the presence of the catalyst; its activity and amount determines the reaction rate. Consequently, tuning the activity of an enzyme from the outside by activating or inhibiting mediates an exact control of the velocity. In the living cell, a strictly coordinate network of regulation exists, comprising enzymes whose activity is controlled by the concentration levels of metabolites, hormones, and transmitter substances. The precise interaction of all these components is a prerequisite of life.
The protein nature of enzymes is excellently suited for this dual function as catalyst and regulator; it supplies functional groups of amino acids to form specific binding sites and catalytic centers, and it provides flexibility to promote formation and stabilization of transition states and to induce conformational changes for modulation of the catalytic efficiency. The 20 proteinogenic amino acids with their hydrophilic, hydrophobic, acidic, and basic side chains permit most enzymes to realize both functions such as specific binding of substrates and regulator molecules and catalytic conversion. More difficult catalytic mechanisms cannot be brought forth only by the amino acid side chains; rather, nonproteinogenic compounds are included, which can either be dissociable as coenzymes, \(^1\) or nondissociable as prosthetic groups. Dissociable coenzymes are NAD(P), thiamine diphosphate, or coenzyme A, while FAD, cytochromes, porphyrins, pyridoxamine, lipoic acid, biotin, and tetrahydrofolic acid function as nondissociable, partly covalently bound prosthetic groups. Often also metal ions are required, both for catalysis and for stability of the enzyme, Mg\(^{2+}\) serves to neutralize the phosphate groups in compounds such as ADP, ATP, and thiamine diphosphate and mediates their binding to the enzyme. Iron (in cytochromes), cobalt (in the corrin ring system), copper (e.g., in cytochrome oxidase and tyrosinase), zinc (in carboanhydrase and alcohol dehydrogenase), molybdenum (in nitrogenase), manganese (in arginase and xylose/glucose isomerase), and selenium ion (in glutathion peroxidase) support the enzyme reactions.

The protein nature enables enzymes to adapt their specificity to any desired ligand by mutations. This feature is applied in biotechnology using site-directed mutagenesis to modify the specificity and function of enzymes. By the method of molecular modeling (protein design) distinct modifications are simulated and thereafter the respective mutations are executed. An example is hydroxyisocaproate dehydrogenase, an enzyme catalyzing the reductive conversion of \(\alpha\)-oxo acids to chiral hydroxycarbonic acids as hydroxyanalogs of amino acids. Its preferred substrate is \(\alpha\)-oxocaproic acid. \(\alpha\)-Isocaproic acid, an analogous compound, is accepted only with reduced efficiency. By site-directed mutagenesis the catalytic efficiency \((k_{\text{cat}}/K_m)\) for this compound has been increased by four orders of magnitude, as compared to the physiological substrate (Feil, Lerch, and Schomburg, 1994).

Owing to their protein nature, enzymes are very sensitive to environmental influences such as pH, ionic strength, and temperature and, consequently, to attain optimum activity, stringent conditions must be established. In the physiological milieu of the living cell, these conditions are maintained as far as possible, although with respect to temperature, this cannot be permanently guaranteed (with the exception of warm-blooded vertebrates). However, enzymes are remarkably

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\(^1\) The terms coenzyme and cosubstrate are not always clearly differentiated. Coenzymes, in contradistinction to cosubstrates, are supposed to support the catalytic mechanism and should not be converted. For example, pyridoxal phosphate in transamination reactions accepts an amino residue becoming pyridoxamine phosphate, but in the second step of the reaction the amino group is transferred to an \(\alpha\)-oxoacid and the coenzyme regains its original form at the end of the reaction. NAD(P), on the other hand, is reduced in a dehydrogenase reaction and must be reoxidized by a separate enzyme reaction, therefore it is more a cosubstrate than a coenzyme.
able to adapt to extreme conditions. Although proteins are regarded as being very temperature sensitive, distinct microorganisms such as Thermus, Thermotoga, and Thermoplasma, including their complete enzymatic inventory, persist in temperatures up to 100 °C. It must be assumed that during evolution the ancient organisms have had to bear much higher temperatures. The ancient precursors of the present enzymes must have all been thermophilic, but obviously they lost this feature with the decrease in environmental temperature. This can also explain the fact that proteins, instead of the more stable nucleic acids, are preferred by nature as biocatalysts, although some catalytic activities are retained in RNA.

As an introduction to the practical work with enzymes, at least some fundamental theoretical rules must be discussed. They will be addressed in the first part, followed by a description of the general features of enzymes, which must be considered when dealing with them. This is followed by a presentation of the most important techniques. This general part should enable the reader to work with enzymes; for instance, to develop an assay for a newly isolated enzyme without further need to consult the literature. The following special part presents detailed descriptions of enzyme assays and related methods such as protein determination. A multitude of assays corresponding to the immense number of different enzymes exists, which cannot all be considered within the scope of a laboratory manual; rather, only a selection can be presented. Criteria for the selection are not only the frequency of application, but also the broad variety of enzyme types and methods. Certainly, such a selection cannot satisfy all expectations and the choice will sometimes appear rather arbitrary. For further information the reader is referred to standard books and databases of enzymology (see References section below). Procedures for immobilization of enzymes and special aspects of analysis of immobilized enzymes, principles of enzyme reactors, and enzyme electrodes are presented in separate sections of the book.

References


Standard Books, Series, and Databases

2
General Aspects of Enzyme Analysis

2.1
Basic Requirements for Enzyme Assays

The task of enzymes as biocatalysts is to render feasible reactions, which cannot proceed in their absence. Therefore, the first requirement when dealing with a special enzyme is to study its reaction. In a simple generalization it can be stated that one compound (or more than one), designated as the substrate, gets converted into another compound, the product, with the aid of the enzyme. Thus, to identify an enzyme its reaction must be demonstrable or measurable, that is, a method must be developed for the quantitative detection of the reacting components. The prerequisite for this is a detectable signal for the reacting components. But a signal alone is not sufficient; rather, a clear distinction between the substrate and the product is necessary. Absorption ultraviolet and visible (UV/Vis) spectroscopy may be an illustrative example. It is an easy and convenient detection method and in fact, each biological substance shows absorption at least in the UV region. Therefore, this method is principally suited for the quantification of every substance and, thus, may be the method of choice for any enzyme assay. However, in most cases, the substrate and the product of the same reaction show similar absorption features. So, even if the compounds possess pronounced absorption spectra, they are not useful to detect the reaction. This is the case with sugars, such as glucose and fructose, which cannot be distinguished by absorption spectroscopy and so this method is not applicable here.

Hence, the first step is to find a clear signal for detection of the substrate and/or the product, and the second step is to uncover differences between both compounds. The compound showing the clearer difference signal will be used. Principally, this is irrespective of whether the substrate or the product will be detected, as it can be assumed, that the amount of substrate converted corresponds exactly to the amount of product formed. Observing the decay of the substrate or the formation of the product must give the same result, only changing the sign. However, if possible, product formation is preferred because of practical reasons. At the start of the reaction the product concentration and, consequently, its signal, is zero and any increase is a direct indication of the progressing reaction. Conversely, the concentration of the substrate and thus its signal is highest at
the beginning of the reaction. This can influence the detection method. Each method shows some scatter and usually higher signals cause stronger scatter, and small changes, for example in the case of slow reaction rates are difficult to detect. Some substrates are unstable and decay spontaneously appearing to be an enzyme-catalyzed reaction; an effect, which is also more pronounced at high concentrations.

Various methods are available to search for an appropriate difference signal for an enzyme assay. Any analytic method for identifying substances such as the substrate and the product can be considered and usually the method yielding the clearest difference signal will be chosen; however, other criteria must also be considered. A simple, but practical aspect, is the availability of an appropriate instrument. For enzyme analysis, frequent assays series must be performed and the appropriate instrument should be permanently accessible. Accordingly, it must be affordable and handling should be easy. Such demands limit the kind of methods that can be employed. One important aspect concerns the mode of registration. As discussed in detail later, a progressive reaction should be pursued continuously (continuous assay) as far as possible, while various methods allow only detection of single points of the reaction after defined time periods (stopped assay, Box 2.1). In fact, often a method enabling continuous registration is superior to a method allowing only stopped assays even if a weaker signal must be accepted.

**Box 2.1: Fundamental Demands for Enzyme Assays**

- *Product* formation (increasing reaction) or *substrate* consumption (decreasing reaction) can be detected, alternatively
- *Enzyme activity* is defined as the amount of product formed – substrate consumed – within a distinct time unit (second, minute, hour). During this time unit the reaction must proceed *strictly linearly* (zero order)
- *Continuous* monitoring of the reaction (e.g., spectral change, pH change)
- *Stopped assay* (if continuous monitoring is not possible): stop of the reaction after a defined time and subsequent analysis of the amount of product formed – substrate consumed – within the time unit

The most frequently used methods for enzyme assays are summarized in Box 2.2. All spectroscopic methods (absorption, fluorescence, circular dichroism (CD), optical rotatory dispersion (ORD), turbidimetry) and electrochemical techniques such as pH stat, can principally be performed as a continuous test (but only if the substrate or the product can be identified directly), while trapping and separation methods allow only stopped assays. Therefore, such methods will be used only if the other methods do not work. For routine assays, simple devices and instruments belonging to the standard equipment of an analytical
2.1 Basic Requirements for Enzyme Assays

Box 2.2: How to Determine the Enzyme Activity?

\[ E + A \xrightarrow{k_1} EA \xrightarrow{k_2} E + P \]

Substrate A and product P must differ in at least one detectable feature.

<table>
<thead>
<tr>
<th>Feature</th>
<th>Detection method</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absorption spectrum</td>
<td>UV/Vis spectroscopy</td>
<td>NAD/NADH</td>
</tr>
<tr>
<td>Fluorescence spectrum</td>
<td>Fluorescence spectroscopy</td>
<td>Umbelliferone-coupled substrates</td>
</tr>
<tr>
<td>Formation of ATP or NAD(P)H</td>
<td>Luminometry</td>
<td>Kinases, dehydrogenases</td>
</tr>
<tr>
<td>Optical rotation</td>
<td>Polarimetry, ORD, CD</td>
<td>Sugars (glucose)</td>
</tr>
<tr>
<td>pH</td>
<td>pH stat</td>
<td>Cleavage of triglycerides</td>
</tr>
<tr>
<td>Gas release or consumption</td>
<td>O₂ and CO₂ electrodes, manometry</td>
<td>Decarboxylase reaction</td>
</tr>
<tr>
<td>Turbidity</td>
<td>Turbidimetry</td>
<td>Degradation of starch</td>
</tr>
<tr>
<td>Chemical reactivity</td>
<td>Trapping reactions, colorimetry</td>
<td>Peroxidase reaction with diaminidin</td>
</tr>
<tr>
<td>General features (size, polarity)</td>
<td>Separation methods, HPLC, FPLC</td>
<td>Aggregation, depolymerization (cellulose, starch)</td>
</tr>
</tbody>
</table>

or a biochemical laboratory are preferred. These criteria are fulfilled in the best manner by absorption (UV/Vis) spectroscopy, which is an easy method with various applications, for example, determinations of proteins, nucleic acids, and phosphate (cf. Box 2.15). Suitable apparatus are available at moderate prices and computer-controlled instruments with monitoring and calculation modes make the evaluation of reactions easy so that photometric enzyme assays are the first choice. Other spectroscopic methods, such as fluorescence, CD, and ORD are for special applications and thus usually not present as standard equipment in laboratories. Manipulation is more difficult, intense knowledge for appropriate operation is required, and high-quality instruments are rather expensive; all these are aspects not supporting their application. Nevertheless, these spectroscopic methods possess significant advantages. They are more selective and, especially for fluorescence, much more sensitive compared with absorption spectroscopy, a feature important especially for enzyme studies. If such an instrument is not available, the work must be done in specialized laboratories, which usually provide not only the appropriate apparatus, but also a thorough knowledge of the technique, indispensable to avoid inappropriate procedures and misinterpretations. These considerations hold also for other instruments such as the pH stat, a very useful device, if demanded by the type of assay, such as the digestion of lipids, but superfluous if not really required. The main instruments applied for enzyme tests are described in detail in Section 2.3.
2.2
What Must Be Observed for an Enzyme Assay?

As already mentioned, for an enzyme assay the progression of the reaction (progress curve) is decisive and should be carefully observed. For normal enzyme reactions this curve should obey a common pattern, that is, it should be a straight line reflecting linear progression of the reaction proportional to time. In reality, however, nonlinear behavior is often observed, which is frequently a smooth curvature, but sometimes even irregular deviations. Evaluation of such behavior requires some knowledge of the theoretical background; the essential rules are discussed in the following chapter.

2.2.1
Order of Reactions

The progression of a reaction is determined by its order. The simplest chemical reaction is the conversion of a substance A (in chemical terms: educt) into the product P, as the spontaneous decomposition of instable substances, for example, the radioactive decay:

\[ \text{A} \rightarrow \text{P} \]

The velocity \( \nu \) of this reaction depends on the initial concentration of A and is expressed as:

\[
\frac{-d[A]}{dt} = \frac{d[P]}{dt} = k[A] = \nu
\]

(2.1)

\( t \) is the time, \( k \) the rate constant with the dimension of \( s^{-1} \). It is obvious that the higher the amount of A, the faster the reaction. Because A decays during the reaction, the velocity declines permanently, and the reaction follows a curve, which is steepest at the start and decreases steadily (Figure 2.1a). A similar curve, only in a positive sense, is obtained, when the formation of P is observed. Mathematically, this curve is described by an exponential relationship ([A]_0 is the initial substrate concentration):

\[
[A] = [A]_0 e^{-kt}
\]

(2.2)

This is the equation for a first-order reaction, because only one substrate is involved. Hence, an exponential curve is indicative of a first-order reaction. However, an exponential progression of a reaction is not easy to recognize unequivocally, because other reaction types (higher orders) show similar nonlinear curves. Although they follow no simple exponential relationship, in practice they are often difficult to discern from real exponential curves. In such cases of ambiguity it is a good principle to transform the nonlinear relationship into a linear form, where only dependencies obeying the original relationship will yield straight lines, while others show characteristic deviations. By transformation of the first-order equation (2.2)
2.2 What Must Be Observed for an Enzyme Assay?

Figure 2.1 Progress curves of various reaction orders. (a) Direct plotting and (b) semilogarithmic plotting.

Into a half logarithmic form

\[ \ln[A] = \ln[A]_0 - k_1 t \]  

The curves in Figure 2.1a become linear if a logarithmic ordinate scale is chosen (Figure 2.1b).

In nature, spontaneous decays are rather seldom.\(^1\) More frequent are reactions initiated by collisions of two or more reactive substances. The number of substrates involved determines the reaction order,\(^2\) for example, a reaction:

\[ A + B \rightarrow P + Q \]

is of second order.\(^3\) The velocity of a second-order reaction depends on two variables. As shown in Figure 2.1a, nonlinear behavior is observed and no straight line results in the half logarithmic plot (Figure 2.1b). This feature allows the distinction of first and second orders (and similarly for higher orders, which are not dealt with here). When performing experiments, the dependency of the second-order reaction on two independent variables is impracticable. Under normal conditions both substrates may be present in comparable amounts, but this is not a necessary condition. If we assume that one component (e.g., B)

---

1) With the exception of radioactive decay, it can be assumed that spontaneous reactions already came to their end.
2) Substrates are written in alphabetic order A, B, C, ..., products as P, Q, R, ....
3) The reaction order is only defined by the number of substrates; the number of products formed is only of significance if the reverse reaction is considered. Accordingly, the second order reaction can also be written as A + B → P or A + B → P + Q + R.
is present in surplus in comparison with the other one (A), conversion of the lesser amount of A will not essentially change the higher amount of B, so that its concentration can be considered as constant. Under this condition, the reaction depends only on one, the minor component (A), and becomes similar to a first-order reaction, following an exponential time course, which now becomes linear in the half logarithmic plot (Figure 2.1b). As this reaction is only formally first order, but second order in reality, it is designated as pseudo-first order.

2.2.2 Significance of the Reaction Order for Enzyme Reactions

It has already been mentioned that enzyme reactions should, ideally, proceed in a linear manner, while we now see that the simplest chemical reaction, the first-order reaction, is already exponential. Are enzyme reactions simpler than simple? Linear progression can only be expected if the reaction rate is completely independent of the substrate concentration, so that the amount of product formed per time unit remains constant, irrespective of whether low or high substrate concentrations are present:

\[
- \frac{d[A]}{dt} = \frac{d[P]}{dt} = k = \nu
\]  

(2.4)

\[
[A] = [A]_0 - k_1 t
\]  

(2.5)

To explain this apparent contradiction let us turn to enzyme reactions. The simplest enzyme reaction is the conversion of one substrate catalyzed by the enzyme

\[
A + E \stackrel{k_1}{\rightleftharpoons} EA \stackrel{k_2}{\rightarrow} E + P
\]  

(2.6)

obviously, a second-order reaction.\(^4\) However, there is an important difference compared with the second-order reaction described above: the enzyme takes part in the reaction, but does not become converted. It appears unchanged at the product site, according to its function as catalyst, and enters again into the reaction cycle from the substrate site. So the rate Eq. (2.1): \(\frac{d[A]}{dt} = k[A]\) is not valid for the enzyme, rather it must be written \(\frac{d[E]}{dt} = 0\), because the amount of the enzyme remains unchanged during the reaction. Also the expression \(-\frac{d[A]}{dt} = k[A]\) for the substrate is not true. It cannot be written as a first-order reaction, because substrate can only be converted in the presence of the enzyme, and only that portion of the substrate actually bound to the enzyme reacts. Therefore, the reaction rate depends not on the substrate concentration, as for a first- or higher-order reaction, but only on the amount of enzyme. As the same

\(^4\) For each partial reaction, a rate constant \(k\) is defined with consecutive positive digits in the forward direction and negative digits in the backward direction.
2.2 What Must Be Observed for an Enzyme Assay?

An enzyme molecule can repeatedly take part in the reaction, very low amounts of the enzyme compared with the substrate (catalytic amounts) are sufficient: \([E] \ll [A]\) (Box 2.3). Since the reaction depends only on – constant – enzyme concentration, and the amount of product formed per time unit is also constant, the reaction proceeds in a strictly linear manner (Figure 2.1a). Such a reaction is called a zero-order reaction. To answer the above question, the course of the enzyme reaction is more simple, but the reaction mechanism is more complicated compared with first-order reactions. This is an essential feature of catalytic reactions and it must be kept in mind that this condition holds only as long as the catalyst is clearly limiting. When during the reaction course the amount of substrate declines (or when the reaction is started with low amounts of substrate and/or high amounts of enzyme), this condition no longer prevails and the reaction course becomes nonlinear (first order). The linear zero-order range is called steady state. It can be regarded as a time-dependent equilibrium, existing only as long as the condition \([E] \ll [A]\) predominates, in contrast to a true time-independent equilibrium. Linearity of the progress curve is a clear indication for the presence of the steady state phase. As follows from the upper discussion, the duration of the steady-state phase depends on the relative amounts of both the substrate and the enzyme.

**Box 2.3: How Much Enzyme Is Required for an Assay?**

The velocity of enzyme-catalyzed reactions is strictly proportional to the enzyme amount:

\[
v = k_{\text{cat}}[E][A]
\]

For substrate saturation:

\[
V = k_{\text{cat}}[E]_0
\]

Deviation from linear relationship is an indication for nonideal conditions.

The enzyme concentration \([E]_0\) in the assay must adhere to the following rules:

- concentration should be as low as possible, according to the steady-state theory \([E] \ll [A]\)
- however, it must be sufficient to detect the initial velocity

The central relationship of enzymology, the Michaelis–Menten equation, is based on this steady-state assumption. As already mentioned, under steady-state conditions, the reaction proceeds in a strictly linear manner.
conditions the enzyme concentration remains constant \((d[E]/dt = 0)\) and, consequently, also the amount of substrate bound to the enzyme, the Michaelis–Menten complex, \(d[EA]/dt = 0\). Therefore, the reaction rate \(v\) is determined solely by the concentration of \(EA\). The derivation of the Michaelis–Menten equation is based on this assumption. For simplicity the one-substrate reaction (Eq. (2.6)) is taken. Separate equations are derived for the time-dependent change of each component:

\[
\frac{d[A]}{dt} = -k_1[A][E] + k_{-1}[EA] \tag{2.7}
\]

\[
\frac{d[E]}{dt} = -k_1[A][E] + (k_{-1} + k_2)[EA] \tag{2.8}
\]

\[
\frac{d[EA]}{dt} = k_1[A][E] - (k_{-1} + k_2)[EA] \tag{2.9}
\]

\[
\frac{d[P]}{dt} = k_2[EA] = v \tag{2.10}
\]

The overall reaction velocity \(v\) is defined as the rate of product formation (Eq. (2.10)). In addition to these four equations the mass conservation relationships

\[
[A]_0 = [A] + [EA] \tag{2.11}
\]

\[
[E]_0 = [E] + [EA] \tag{2.12}
\]

are considered. However, even these six relationships yield no simple solution. But recalling the above stated steady-state condition, Eqs. (8) and (9) can be simplified by \(d[E]/dt = d[EA]/dt = 0\) and combined to yield the Michaelis–Menten equation

\[
v = \frac{k_2[E]_0[A]}{k_{-1} + k_2} + [A] = \frac{V[A]}{K_m + [A]} \tag{2.13}
\]

which shows the dependence of the reaction velocity \(v\) on the substrate concentration \([A]\). The equation is directly derived in the form of rate constants (left term), which is usually simplified and presented in the form of the right term, where \(k_2[E]_0 = V\) is defined as a new constant, the maximum velocity. It is obtained from the rate constant \(k_2\), the so-called catalytic constant \(k_{cat}\) for the conversion of the Michaelis–Menten complex to product (and enzyme), and the total enzyme amount \([E]_0\), which is assumed to remain constant during the reaction. The highest possible rate under the given conditions, the maximum velocity \(V\), is attained when all enzyme molecules present in the assay \(([E]_0)\) contribute at the same time to the reaction. The three rate constants of the denominator term