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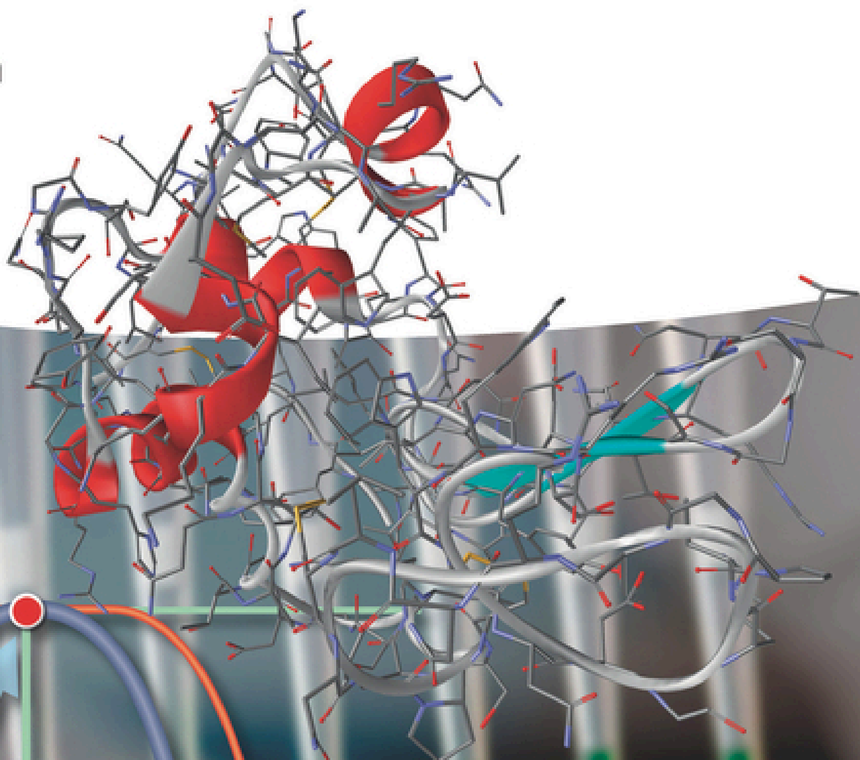
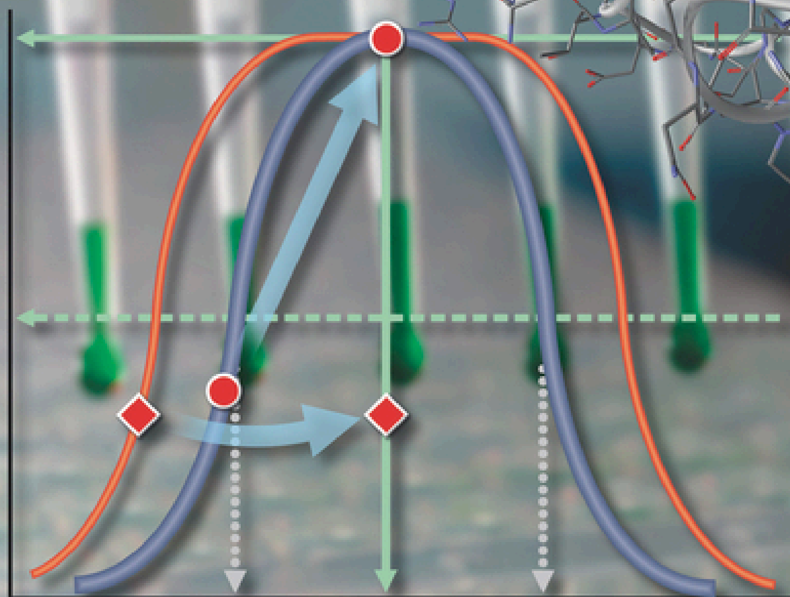
Hans Bisswanger

# Practical Enzymology

Third Edition





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*Hans Bisswanger*

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# Preface to the Third Edition

This book is composed of two main parts, a general introduction into working with enzymes and a practical part including special protocols for investigating enzymes, such as enzyme assays, methods for protein determination, and enzyme immobilization. While for the general part fundamental alterations are not required and only corrections and refinements are executed, far-reaching changes are realized for the practical part, which represents the centerpiece of the book. Particularly, numerous enzyme assays and methods for metal determination are supplemented, although this will not completely dispel the principal problem that any special selection of particular assay protocols cannot satisfy all expectations. As far as possible, suggestions of colleagues have been considered. Special thanks in this respect owes to Dr. Apostolos Zarros, University of Glasgow.

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.

Tübingen, June 2019 *Hans Bisswanger*

# List of Abbreviations and Symbols

Only repeatedly used abbreviations, special abbreviations are defined at the respective section.

| | |
|---|---|
| A | absorption |
| [A] | ligand concentration |
| $[A]_0$ | total ligand concentration |
| A, B, C | specific binding ligands |
| 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 albumin |
| 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 |
| $[E]_0$ | total enzyme concentration |
| $\epsilon_{nm}$ | absorption ("extinction") coefficient at the wavelength indicated |
| $\epsilon_r$ | dielectric constant |
| EDTA | ethylenediaminetetraacetic acid |
| EIA | enzyme immunoassays |
| ELISA | enzyme-linked immunoadsorbent assays |
| FAD | flavin adenine dinucleotide |
| FMN | flavin mononucleotide |
| GOD | glucose oxidase |
| *h* | Planck's constant |
| HK | hexokinase |
| *I* | light intensity |

| | |
|---|---|
| IU | International enzyme unit (μmol $min^{-1}$) |
| $k$ | rate constant |
| kat | Katal (mol $s^{-1}$) |
| $k_{cat}$ | catalytical constant |
| $K_d$ | dissociation constant |
| $K_m$ | Michaelis constant |
| LDH | lactate dehydrogenase |
| $M_r$ | relative molecule mass |
| MDH | malate dehydrogenase |
| $n$ | number of subunits |
| NAD | nicotinamide adenine dinucleotide[1] |
| NADH | reduced nicotinamide adenine dinucleotide[1] |
| NADP | nicotinamide adenine dinucleotide phosphate[1] |
| NADPH | reduced nicotinamide adenine dinucleotide phosphate[1] |
| 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 |

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

# 1

# General Aspects of Enzyme Analysis

## 1.1 Introduction and Essentials for Enzyme Assays

The book is intended toward supporting manipulation with enzymes. It starts with a concise presentation of theoretical aspects of enzyme reactions, followed by a description of the general features of enzymes. The observation of these features is indispensable for any manipulation with enzymes. A broad space is assigned to a detailed specification of enzyme assays. They are important because of two reasons: on the one hand, they are the tools to detect and to identify a distinct enzyme; on the other hand, they give a measure of the quantity and activity of the enzyme. First, the general requirements for enzyme assays are described, which must be regarded when performing a special assay and likewise for developing a new assay procedure. This is followed by a presentation of a series of special enzyme tests. The criterion for selection was mainly the frequency of application, but also different techniques and procedures, such as spectroscopic, radioactive, continuous, stopped, and coupled assays. Complementary to enzyme assays is the study of binding processes for the characterization of a distinct enzyme. Its interaction with substrates, products, cofactors, activators, and inhibitors is essential for understanding its mechanism of action. Such studies need special theoretical considerations and distinct methods, and provide different information. Finally, a survey of practical applications of enzymes in technical processes, therapy, and medicine is presented.

Enzymes as very efficient biocatalysts fulfill two essential functions in the living organism. Speeding up of reactions permits even virtually improbably reactions to become accessible to the metabolism, and tuning its catalytic efficiency via inhibition or activation enables precise regulation of the metabolism. The protein nature of enzymes[1] provides the ideal precondition to accomplish this challenge: the keen specificity of the enzymes for their ligands – the substrates, activators, or inhibitors – which is indispensable to perform the multifaceted reactions within the cell and their compartments simultaneously in a controlled manner; the capability to construct distinct structural regions with subtle steric and electrostatic configuration to form an efficient catalytic center; as

1 This book concentrates on protein enzymes; not specially regarded are ribozymes consisting of nucleotides and artificial enzymes (synzymes); they obey principally the same rules as protein enzymes, but are less complicated in both their structure and function.

*Practical Enzymology,* Third Edition. Hans Bisswanger.
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well as their ability to switch between distinct states of different structure and activity. Nevertheless, the protein structure alone cannot accomplish all types of reactions; frequently non-proteinogenic components, *metal ions*, dissociable *coenzymes*, or non-dissociable *prosthetic groups* are included.[2]

The highly developed structure of the enzymes calls for a special differentiated treatment. In this chapter, the principle of enzyme reactions will be examined both from the theoretical and practical viewpoints. By an enzyme reaction, one or more *substrates* are converted into one or more *products*. It is assumed that the reaction runs from substrates to products. However, due to the general principle of reversibility of chemical reactions, both directions are possible, but depending on the energy state frequently one direction is favored and usually, but not in any case, this direction is chosen for testing the enzyme. The task of the operator is to examine the respective compounds both qualitatively and quantitatively. The respective type of the substrate and the product is determining for the special type of the enzyme under study and is a prerequisite for further analysis. The enzyme assay serves to quantify the enzyme with respect to both its concentration and activity. The progress of the enzyme reaction can be observed by the formation of the product, or likewise by the disappearance of the substrate. Owing to the stoichiometric rules, both approaches must yield the same result. This is also valid in the case where more substrates or products are involved in the reaction; it is sufficient to observe only one substrate or product to quantify the reaction.[3] So one reaction partner can optionally be selected to observe the course of the reaction; from the viewpoint of the reaction, it makes no difference which of the respective compounds will be chosen. Therefore, practical aspects determine the choice of the observed component. The most significant aspect is the existence of a specific signal to discern the respective component. The signal should be intense and clear and easily detectable with an appropriate and easily accessible technique. The absolute signal intensity is not only crucial but it must also be different from that of the unobserved reaction components. For example, it is not sufficient if a product shows a high signal when the substrate possesses a similar signal. Therefore, often various assays have been developed for the same enzyme and the assay that can be most easily realized under the conditions of the respective laboratory may be chosen. Considering these arguments, in principle any method can be taken that is appropriate to analyze the compound to be observed, but one crucial aspect must be regarded. Reactions are time dependent and an appropriate detection method should be used to observe the complete reaction course continuously (**continuous assay**). This is possible with various methods, but if none of them can be applied for a special enzyme system, the reaction must be performed unobserved and stopped after a distinct time period. Thereafter, the amount of the substrate remaining or of the product formed during this period can be examined in the assay mixture by a suitable analysis method, such as a

---

2 These components, such as the coenzymes, are part of the catalyst. Within the reaction mechanism they may be transformed, e.g. oxidized or reduced, but, like the enzyme itself, they regain their original state at the end of the reaction, in contrast to cosubstrates, e.g. NAD, which maintain their modified state and must be reconverted by a separate enzymatic reaction.

3 It must be considered that in some cases one compound counts half or twofold if one molecule, such as $O_2$, contributes to two reaction cycles or is split into two identical products.

color-developing detection reaction, thin layer chromatography, high performance liquid chromatography (HPLC), or radioactive labeling (**stopped assay**). This procedure yields instead of a continuous **progress curve** only one single measure point. The complete reaction course can be simulated by combining several measure points, obtained by variation of the reaction time (Box 1.1).

**Box 1.1 Major Methods to Determine the Enzyme Activity**

| Measuring method | Principle of measurement | Assay type | Examples |
|---|---|---|---|
| *Optical methods* | | | |
| UV/Vis-spectroscopy | Absorption | Continuous | NAD ↔ NADH |
| Fluorescence spectroscopy | Excitation/ emission | Continuous | Fluorescent and dye-labeled substrates or products |
| Polarimetry, ORD-, CD-spectroscopy | Optical activity | Continuous | Sugars (glucose) |
| Luminometry | Determination of ATP or NAD(P)H | Continuous | Dehydrogenases, kinases |
| Turbidity | Formation/ disappearance of macromolecular or insoluble compounds | Continuous | Degradation of starch |
| Colorimetry | Trapping of the product with color reactions | Stopped | Peroxidase reaction with dianisidine |
| *Electrochemical methods* | | | |
| $O_2$-, $CO_2$-electrodes | Gas release/ consumption | Continuous | Decarboxylase reaction<br>Cytochrome-*c* oxidase |
| pH-stat | pH changes | Continuous | Cleavage of triglycerides |
| *Separation methods* | | | |
| Column chromatography, HPLC, FPLC | Size, polarity | Stopped | Aggregation, Depolymerization |
| Thin layer chromatography | Polarity | Stopped | Phosphodiesterase |
| *Radioactive methods* | | | |
| Radioactive isotopes | Nuclear radiation | Stopped | Kinases, incorporation of phosphate |

ORD, optical rotatory dispersion; CD, circular dichroism; and FPLC, fast protein liquid chromatography.

A further crucial aspect to be considered with enzyme assays is the dimension of the reaction batch, which will be a compromise between two opposite

arguments. Larger volumes guarantee better detection and higher confidence, but require more of the valuable reagents, above all, the enzyme, especially if many assays need to be performed within a short time. Such considerations call for assay volumes as small as possible. Often an accurate result is less important than the general information whether the reaction proceeds at all, i.e. whether the respective enzyme is present or not. In such cases, the assay procedure may be performed as a microassay in 96 well plates, and analyzed with a microplate reader. In the following chapters several microassays are described, but also many other assays can be modified in this sense. Otherwise, the procedures for the enzyme assays described in the following chapters are adapted to a moderate reaction volume of 1 ml, which gives sufficient accuracy for most detection methods.

## Literature

## Standard Books, Series

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Barmann, T.E. and Schomburg, D. (eds.). *Enzyme Handbook*. Berlin: Springer-Verlag.
Bergmeyer, H.U. (ed.) (1983). *Methods of Enzymatic Analysis*, 3e. Weinheim: Verlag Chemie.
*Methods in Enzymology*. San Diego, CA: Academic Press, Elsevier B.V.
Schomburg, D. (ed.) (2000f). *Springer Handbook of Enzymes*. Berlin: Springer-Verlag.

## Databases

BRENDA. *Comprehensive Enzyme Information System*. Germany: Technische Universität Braunschweig www.brenda-enzymes.org/.
*ExPASy Enzyme Database*, Swissport, SIB Swiss Institute of Bioinformatics. www.expasy.org/enzymes.
*IUBMB ExplorEnz Enzyme Database*. www.enzyme-database.
*MetaCyc Metabolic Pathway Database*. www.metacyc.org/.
Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB). *Enzyme Nomenclature*. www.sbcs.qmul.ac.uk/iubmb/enzyme/.

## 1.2 Theoretical Basis of Enzyme Assays

### 1.2.1 Order of Enzyme Reactions

The progression of any chemical reaction is determined by its reaction order. The simplest chemical reaction is the conversion of a substance A (educt in

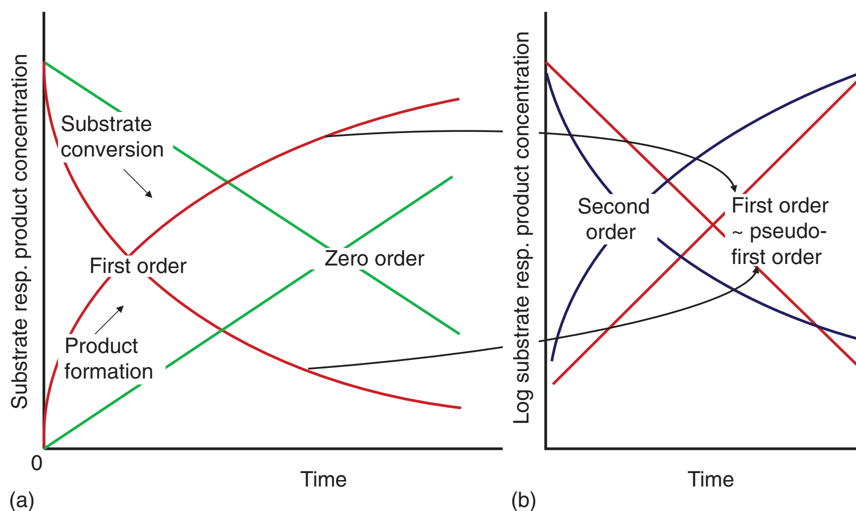
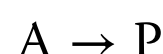



**Figure 1.1** Progress curves of various reaction orders. (a) Direct plotting and (b) semilogarithmic plotting.

chemical terms, **substrate** in enzyme reactions) into the **product** P, such as the spontaneous decomposition of instable substances, e.g. radioactive decay:



$$\mathrm{A} \rightarrow \mathrm{P}$$

The velocity $v$ of this reaction depends on the initial concentration of A and is expressed as

$$-\frac{\mathrm{d[A]}}{\mathrm{d}t} = \frac{\mathrm{d[P]}}{\mathrm{d}t} = k[\mathrm{A}] = v \tag{1.1}$$

$t$ is the time and $k$ is the rate constant with the dimension of $\mathrm{s}^{-1}$. It is obvious that the higher the amount of A, the faster the reaction. Because A decays permanently during the reaction, the velocity declines steadily, and the reaction follows a curve, which is steepest at its beginning and decreases continuously (Figure 1.1a). A similar curve, only with a positive sign, is obtained, when the formation of P is observed. Mathematically, this curve is described by an exponential relationship ($[\mathrm{A}]_0$ is the initial substrate concentration):

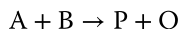
$$[\mathrm{A}] = [\mathrm{A}]_0 \mathrm{e}^{-kt} \tag{1.2}$$

This is the equation for a **first order reaction**, because only one substrate is involved. Hence, an exponential curve is indicative for a first order reaction. Reactions of higher order follow no simple exponential relationship but their nonlinear curves have similar shapes and cannot easily be differentiated from a pure exponential progression. In cases of ambiguity the nonlinear relationship should be transformed into a linear function. Only dependencies obeying the original relationship will yield straight lines, while others show characteristic deviations. Transformation of the first order Eq. (1.2) into a half logarithmic form

$$\ln[\mathrm{A}] = \ln[\mathrm{A}]_0 - k_1 t \tag{1.3}$$

yields linear curves if a logarithmic ordinate scale is applied (Figure 1.1b).

First order reactions start spontaneously, and they cannot optionally be initiated. Therefore, they will not occur independently but rather as successive reactions – for example, spontaneous isomerization or decay of a just formed product from a preceding reaction. In contrast, reactions with two or more reactive substrates can be controlled by removal of one substrate and can be initiated by its addition. The number of substrates involved determines the reaction order.[4] The reaction



$$A + B \rightarrow P + Q$$

is of **second order**.[5] The velocity of a second order reaction depends on two variable components. As shown in Figure 1.1a, nonlinear behavior will be observed and even in the half logarithmic plot no straight line appears (Figure 1.1b). This feature allows the distinction of first and second orders (and similarly of higher orders, which are not treated here). In experiments the dependency of the second order reaction on two variables is impracticable. Under common conditions both substrates may be present in comparable amounts, but this is not a necessary condition. If one component (e.g. B) is present in a large surplus in comparison to the other one (A), then conversion of the very small amount of A will not essentially change the high amount of B; its concentration can be regarded as constant. Under this condition the reaction depends only on one, the minor component (A) and equals a first order reaction. It follows an exponential time course, which now becomes linear in the half logarithmic plot (Figure 1.1b). As this reaction is only formally first order, in reality still being second order, it is designated as **pseudo-first order**.

### 1.2.2 Importance of the Reaction Order for Enzyme Reactions

As will be discussed in the following section, enzyme reactions proceed ideally in a linear manner. This fact may be surprising, as in the preceding section it was shown that even the simplest chemical reaction, the first order reaction, advances in a nonlinear, exponential manner. 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 the substrate is present in low or high substrate concentration:

$$-\frac{d[A]}{dt} = \frac{d[P]}{dt} = k = v \tag{1.4}$$

$$[A] = [A]_0 - k_1 t \tag{1.5}$$

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

$$A + E \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EA \xrightarrow{k_2} E + P \tag{1.6}$$

---

4 Substrates are written in alphabetic order A, B, C, …, and products as P, Q, R, … .

5 The reaction order is defined by the number of substrates; the number of products formed is of significance only if the reverse reaction is regarded. Accordingly, reactions such as $A + B \rightarrow P$ or $A + B \rightarrow P + Q + R$ will be treated equally.

which is obviously a second order reaction.[6] However, there is an important difference to the second order reactions described above: although the enzyme is involved in the reaction, it does not get converted. As a catalyst it appears unchanged at the product site and the same enzyme molecule enters into a new reaction cycle. So the rate Eq. (1.1) is not valid for the enzyme; rather it must be written as $d[E]/dt = 0$, because the amount of the enzyme remains unchanged during the reaction. Also the expression $-d[A]/dt = k[A]$ for the substrate does not hold. It cannot be expressed as a first order reaction, because the substrate can only be converted in the presence of the enzyme, and only the portion of 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 solely on the amount of enzyme. While the substrate becomes converted by one reaction cycle, the same enzyme molecule takes part in many cycles; therefore, in comparison to the substrate, very low amounts of the enzyme (*catalytic amounts*) are sufficient: $[E] \ll [A]$ (Box 1.2). Since the reaction depends only on the – constant – enzyme concentration, and also since the amount of product formed per time unit is constant, the reaction proceeds in a strictly linear manner (Figure 1.1a). Such reactions are called **zero order**. To answer the above question about reactions to be simpler than simple, the progression of the enzyme reaction looks simpler, but its mechanism is more complicated than first order reactions. The linear progression is characteristic of catalytic reactions, but this feature 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 above discussion, the duration of the steady-state phase varies, depending on the relative amounts of both the substrate and the enzyme.

### Box 1.2 How Much Enzyme Is Required for an Enzyme Assay?

The velocity $v$ of enzyme-catalyzed reactions is strictly proportional to the enzyme amount (cf. Eq. (1.10)):

$$v = k_{cat}[EA]$$

For substrate saturation

$$V = k_{cat}[E]_0$$

*(Continued)*

6 For each partial reaction a rate constant $k$ is defined with consecutive positive digits in the forward and negative digits in the backward directions.

**Box 1.2 (Continued)**

This calls apparently for high enzyme amounts to speed up the reaction, but high speeds are not a goal for enzyme assays

Ideal conditions are only guaranteed in the linear steady-state range

Deviation from linearity is an indication for nonideal conditions

The enzyme concentration $[E]_0$[7] in the assay must adhere to the following rules:

- it should be as low as possible, according to the steady-state theory [enzyme] ≪ [substrate] (theoretical aspect)
- it must be just sufficiently high to detect the initial velocity (practical aspect)

The central relationship of enzymology, the Michaelis–Menten equation, is based on this steady-state assumption, which was originally derived by G.E. Briggs and J.B.S. Haldane. As already mentioned, under steady-state conditions the enzyme concentration remains constant ($\mathrm{d}[E]/\mathrm{d}t = 0$) and, consequently, also the amount of substrate bound to the enzyme, the **Michaelis–Menten complex**, $\mathrm{d}[EA]/\mathrm{d}t = 0$. 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. (1.6)) is taken. Separate equations are derived for the time-dependent change of each component:

$$\frac{\mathrm{d}[A]}{\mathrm{d}t} = -k_1[A][E] + k_{-1}[EA] \tag{1.7}$$

$$\frac{\mathrm{d}[E]}{\mathrm{d}t} = -k_1[A][E] + (k_{-1} + k_2)[EA] \tag{1.8}$$

$$\frac{\mathrm{d}[EA]}{\mathrm{d}t} = k_1[A][E] - (k_{-1} + k_2)[EA] \tag{1.9}$$

$$\frac{\mathrm{d}[P]}{\mathrm{d}t} = k_2[EA] = v \tag{1.10}$$

The overall reaction velocity $v$ is defined as the rate of product formation (1.10). To derive a general rate equation the mass conservation relationships

$$[A]_0 = [A] + [EA] \tag{1.11a}$$

$$[E]_0 = [E] + [EA] \tag{1.11b}$$

are considered, but even these six relationships Eqs. (1.7)–(1.11) yield no simple solution. However, referring to the steady-state condition Eqs. (1.7)–(1.10) can be simplified by $[E]/\mathrm{d}t = [EA]/\mathrm{d}t = 0$ and combined as

$$v = \frac{k_2[E]_0[A]}{\frac{k_{-1} + k_2}{k_1} + [A]} = \frac{V[A]}{K_m + [A]} \tag{1.12}$$

to the **Michaelis–Menten equation**. It describes the dependence of the reaction velocity $v$ on the substrate concentration [A]. Equation (1.12) is represented in

7 For exact calculation of the enzyme amount, see Box 2.5.

two forms, first the directly derived form of rate constants and, to the right, the usually applied form. The term $k_2[E]_0$ is defined as a new constant, the **maximum velocity** $V$, consisting of the rate constant $k_2$, designated also as **catalytic constant** ($k_{cat}$) for the conversion of the Michaelis–Menten complex to the product (and enzyme), multiplied by the total enzyme amount $[E]_0$, which is assumed to remain constant during the reaction. The maximum velocity $V$ is the highest possible rate under the given conditions. It is attained when all enzyme molecules present in the assay ($[E]_0$) are simultaneously involved in the reaction. Furthermore, the three rate constants of the denominator term are combined to one single constant, the **Michaelis constant** $K_m$. The ratio between the two rate constants $k_{-1}/k_1$ represents the **dissociation constant** $K_d$, a thermodynamic equilibrium constant for the binding equilibrium between substrate and enzyme (first part of Eq. (1.6)). The Michaelis constant contains a third rate constant in addition to the two rate constants of the dissociation constant, the catalytic constant $k_2$. So it consists of both the equilibrium constant of the binding process and the kinetic constant for the conversion of substrate to product. Since the chemical conversion is usually slower than the fast binding equilibrium, the value of the Michaelis constant is mainly dominated by the dissociation constant, with the contribution of the catalytic constant remaining small. Actually, the early derivations of this equation by A. Brown, A.V. Hill, L. Michaelis, and M. Menten considered only the dissociation constant without regarding $k_2$. The modification based on the steady-state theory better describes the real situation.

The derivation of the Michaelis–Menten equation on the basis of constancy of the EA-complex accentuates its strict limitation to the linear zero order range. Nonlinear deviations are indications for nonvalidity of this relationship and it can now be understood that linear progress curves are a prerequisite for analyzing enzymes.

The Michaelis–Menten equation describes the dependency of the substrate concentration on the reaction velocity $v$. This appears contradictory to the above statement, the zero order range being independent of the substrate concentration, depending only on the enzyme amount. This apparent contradiction can be understood considering the term **saturation**. If a very small amount of enzyme is given to a high surplus of substrate to establish the condition $[E] \ll [A]$, it may intuitively be assumed that the enzyme must be saturated; the large substrate surplus should occupy all available catalytic centers. This, however, will not be the case. In fact, even with a very high surplus of the substrate only a fraction of the enzyme molecules will bind the substrate and this fraction, not the high substrate amount, determines the reaction rate. The other part of the enzyme remains unoccupied and does not contribute to the reaction. The ratio between both parts is determined by the binding affinity, expressed by the dissociation constant $K_d$. Its value indicates just the concentration of substrate required for half saturation of the enzyme. Lower substrate concentration causes a weak degree of saturation, while higher substrate concentration causes a strong degree of saturation. The following example should demonstrate this situation. Assuming a $K_d$ value of $10^{-5}$ M, the substrate is added just in this concentration to a $10^{-9}$ M enzyme solution. As the substrate concentration is the same as the $K_d$ value, the enzyme is only half saturated – in spite of a 10 000-fold surplus of substrate. Any variation

of the enzyme concentration will not influence the degree of saturation, and it remains always half saturated; only the reaction velocity will change corresponding to the actual enzyme amount. On the other hand, change of the substrate concentration at constant enzyme amount alters the degree of saturation corresponding to the Michaelis–Menten law and the velocity changes accordingly. This demonstrates the mutual dependence of the velocity from both the enzyme and the substrate concentration, the first one being strictly linear, while the second one depends on the Michaelis–Menten law. For our example, increase of the enzyme concentration by a factor of 10 increases the velocity 10-fold. In contrast, a 10-fold increase of substrate concentration raises the degree of saturation, and consequently the reaction velocity, from 50% to 90.9%, less than twofold!

### 1.2.3 The Reaction Velocity, Significance, and Practical Aspects

#### 1.2.3.1 Determination of the Reaction Velocity, the Progress Curve

The prerequisite for any application of the Michaelis–Menten equation is the accurate determination of the velocity of the respective enzyme reaction.

The general progression of an enzyme reaction is schematically depicted in Figure 1.2. Three phases can be discerned: initially a steep, nonlinear *pre-steady-state phase*, which is too short (~μs) to be detected in normal enzyme assays. Immediately thereafter the linear *steady-state phase*, lasting usually several seconds to some minutes, follows, until the reaction comes to its end during the nonlinear *phase of substrate depletion*.

Linear progress curves are an indication for the prevalence of steady-state conditions and the validity of the Michaelis–Menten equation; they appear most clearly at substrate saturation and become shorter when the substrate becomes

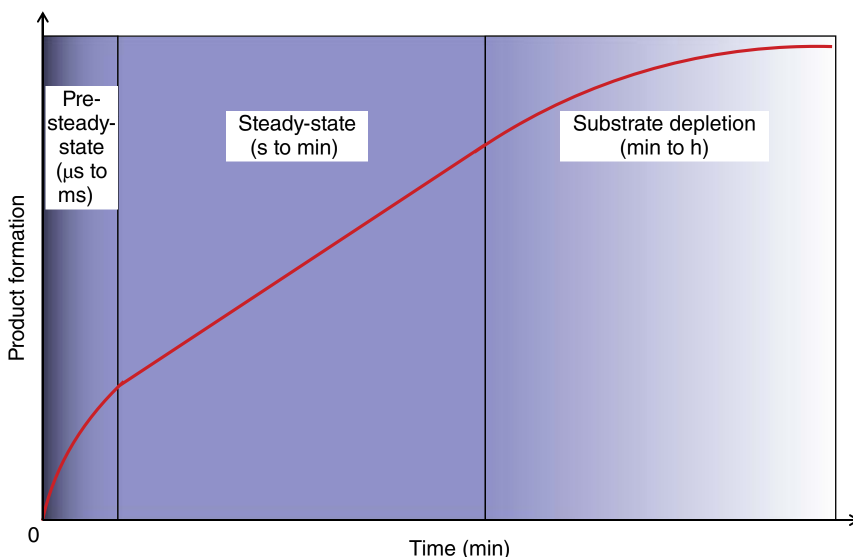



**Figure 1.2** Schematic representation of the three phases of a progress curve.