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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.

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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 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.

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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

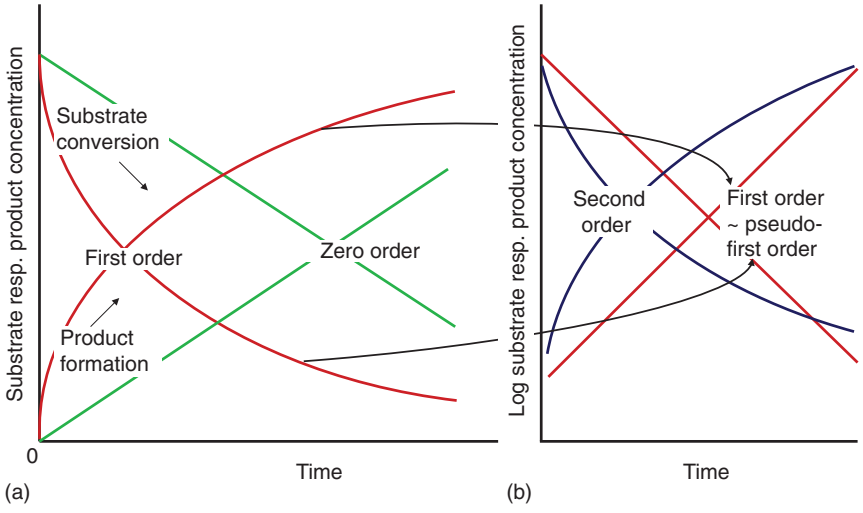

Figure 1.1 Progress curves of various reaction orders. (a) Direct plotting and (b) semilogarithmic plotting.

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

$$A \rightarrow P$$

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

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

t is the time and k is 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 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 ($[A]_0$ is the initial substrate concentration):

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

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

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

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 → P or A + B → 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 ($d[E]/dt = 0$) 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. (1.6)) is taken. Separate equations are derived for the time-dependent change of each component:

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

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

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

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

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 $[E]/dt = [EA]/dt = 0$ 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

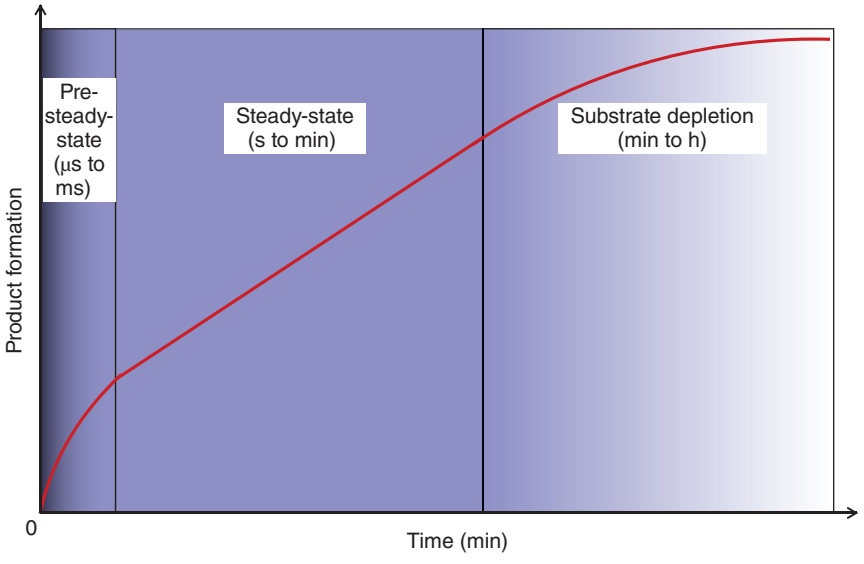

Figure 1.2 Schematic representation of the three phases of a progress curve.

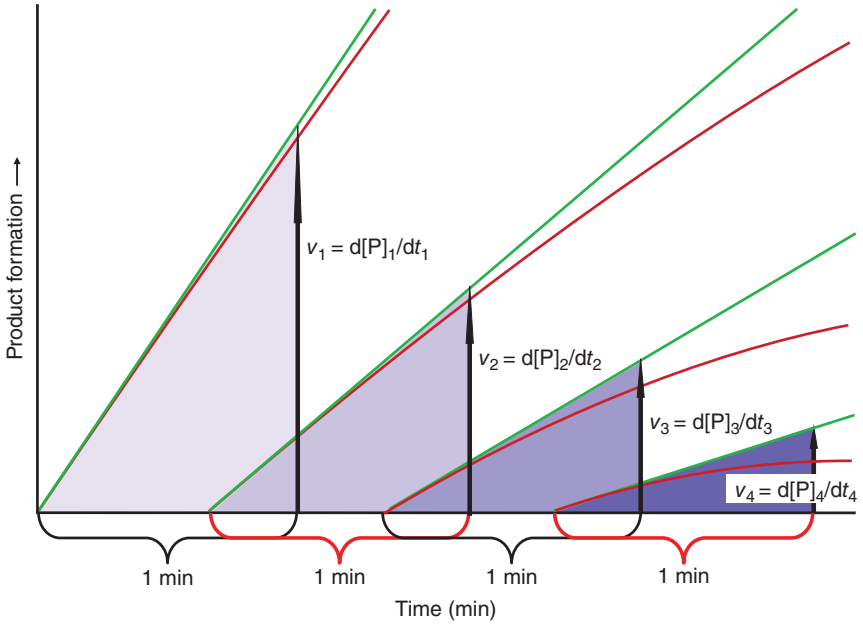

Figure 1.3 Determination of velocities of enzyme reactions from progress curves. The progress curves (———) from left to right are measured with decreasing substrate concentrations. Tangents (———) are aligned to the initial range; one minute is taken as time unit for the evaluation of the velocity *v* according to the definition of IU.

converted to product during the assay. Therefore, strict linearity can only be expected at the start of the reaction, when the substrate concentration is highest. During the progression of the reaction the velocity slows down and deviates from linearity (Figure 1.2). Hence, to determine the reaction velocity not the progress curve itself but rather a tangent aligned to its initial linear range should be applied[8] (**tangent method**, Figure 1.3). Its slope, expressed as concentration of substrate converted or product formed per time unit, gives the actual velocity. If the substrate concentration is reduced within an experimental series, or if higher substrate concentrations cannot be realized (e.g. due to low solubility), the linear range becomes shorter, and the nonlinear range more pronounced (Figure 1.3, curves to the right). Even in such cases, the velocity is obtained from tangents aligned to the initial range. If, however, the linear range becomes very short and is hardly detectable, the prevalence of steady-state conditions and the validity of the Michaelis–Menten equation are no longer verified. It may approximately be assumed that steady-state conditions exist at least at the start of the reaction, but for exact determinations the situation must be regarded more closely.

A serious disturbance in the determination of the initial enzyme reaction velocity is the **dead time**. It is the time interval between the actual start of the reaction and the beginning of detection, i.e. onset of registration, mostly a period of a few seconds. As long as the progress curve is linear this is no problem

8 The extremely short pre-steady-state phase will not affect the adjustment of the tangent.

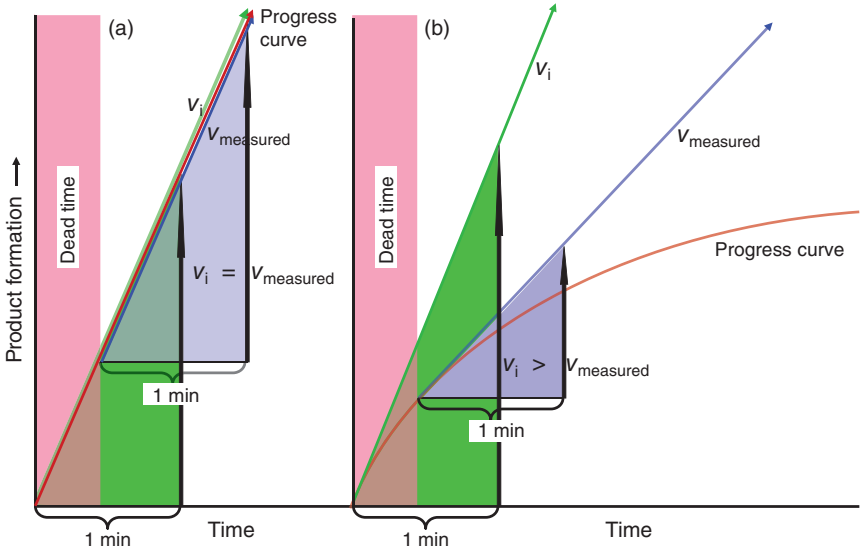

Figure 1.4 Disturbance of velocity determination by the dead time. Progress curve (red); tangent to real initial velocity (green); and tangent to the progress curve after dead time (blue). (a) Linear progress curve and (b) nonlinear progress curve.

(Figure 1.4a), but if it is nonlinear from the beginning, the slope observed at the start of registration is lower than the real initial velocity, and a tangent aligned to this range causes a severe underestimation (Figure 1.4b). Therefore, it should in any case be attempted that the linear range stretches over the dead time into the observed region. A frequent reason for nonlinear progress curves is the presence of too much enzyme. If an enzyme assay does not work immediately, one is tempted to add more and more enzyme. However, too much of enzyme will convert the substrate completely already during the dead time, and no more reaction can be observed when the recording is started. Such a situation is easily misinterpreted as lack of activity, not recognizing that the problem is not too little but too much of activity. As a rule, the enzyme amount should be as low as possible. The lower limit is given by the sensitivity of the method, when the turnover becomes so slow that it cannot be detected within the scatter (Box 1.3). The higher the enzyme concentration the shorter the steady-state range. Figure 1.5 shows a nonlinear progress curve obtained with a high enzyme concentration. Dilution of the enzyme (e.g. 10-fold) reduces the velocity by just this factor and also the steady-state range is extended by this factor. If after enzyme dilution the velocity becomes too slow, a longer assay time can be taken so that the same amount of product is produced in 10 minutes as in one minute by the 10-fold enzyme concentration. Principally, there exists no general rule for the **assay time**. Short times (e.g. one minute) are usually preferred, but assay times of hours or even days can be chosen for low enzyme activities. It must, however, be established that the assay components, especially of the enzyme, remain stable during the whole period.

Box 1.3 Errors in Determination of the Initial Velocity

Error source	Error prevention
Too much enzyme	Reduction of enzyme concentration
	Increase of detection sensitivity
Dead time longer than the linear steady-state range	Immediate recording after reaction start (rapid mixing system)
	Switch on recording before start of the reaction
	Attenuation of reaction velocity (less enzyme, lower temperature)
Measure points outside the linear range (stopped assay)	Shorter time intervals Attenuation of reaction velocity
Strong scattering	Intense mixing, clean cuvettes, and other devices
	Avoidance of dust, air bubbles, turbidity
	Shielding from external interferences (e.g. sunlight)
Blank drift	Assay components of highest purity
	Suppression of oxidative processes (oxygen trapping)
	Correction of the measured reaction velocity for the drift

Particular care on linear progression must be taken with stopped assays. In continuous assays, where the complete progress curve is recorded, any deviation will be recognized. In contrast, with stopped assays only one measuring point is obtained. The velocity is derived from the slope of a line connecting the start of

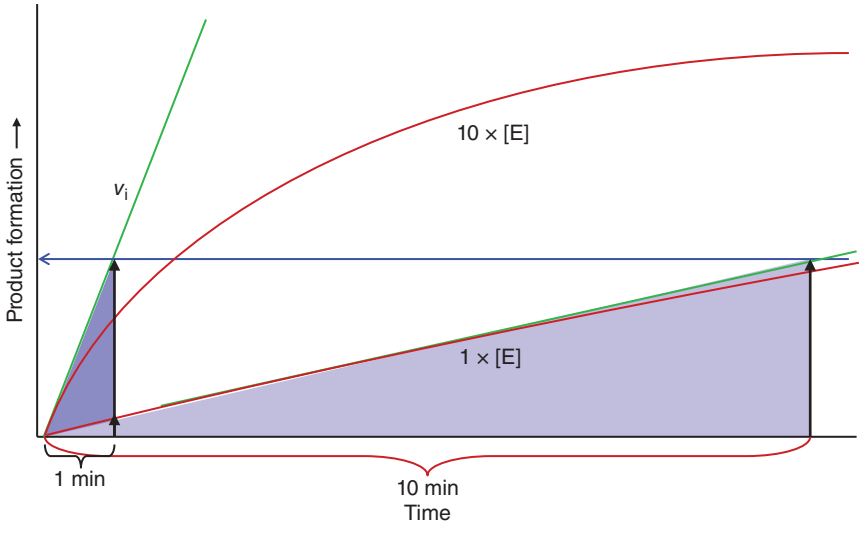

Figure 1.5 Linearity of progress curves depends on the enzyme amount: less enzyme (1 × [E]) yields slower velocities but longer linearity than more enzyme (10 × [E]).

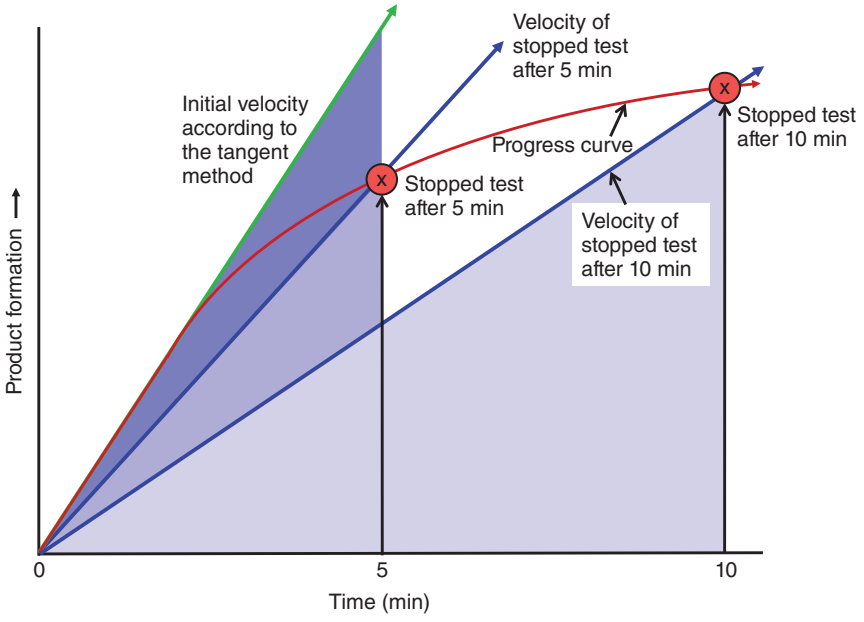

Figure 1.6 Stopped assay performed under nonideal conditions. The measurements ⊗ are carried out beyond the linear range, and the determined velocity is lower than the real initial velocity.

the reaction (usually the blank) with the measure point (Figure 1.6). Any deviations occurring during this time interval cannot be detected. Repeated measurements compensate for the common scatter, but not for accidental or systematic deviations. This problem can be reduced if several measuring points with short time intervals are taken to confirm the expected linearity. However, for such a procedure also more assays and, thus, more time and more material are needed to get finally the same result as with one single measure point, and so the latter procedure is usually favored. At least a representative standard series with sufficient measuring points should be performed to establish that the time interval applied for further single assays lies fairly within the linear range (Figure 1.7). But even if this is the case, it must be considered that this holds only for the actual test conditions. Any change, such as temperature, pH, substrate, or enzyme concentration, can cause a shortening of the linear range so that the chosen time interval is no longer appropriate. In Box 1.3 various error sources in the determination of initial velocities are summarized.

An alternative procedure for evaluation of progress curves is the **integrated Michaelis–Menten equation**, which is obtained by integrating Eq. (1.12) with respect to time:

$$v = -\frac{\mathrm{d[A]}}{\mathrm{d}t} = \frac{V[\mathrm{A}]}{K_\mathrm{m} + [\mathrm{A}]} \tag{1.12}$$

$$K_\mathrm{m} \ln \frac{[\mathrm{A}]_0}{[\mathrm{A}]} + [\mathrm{A}]_0 - [\mathrm{A}] = Vt \tag{1.13}$$

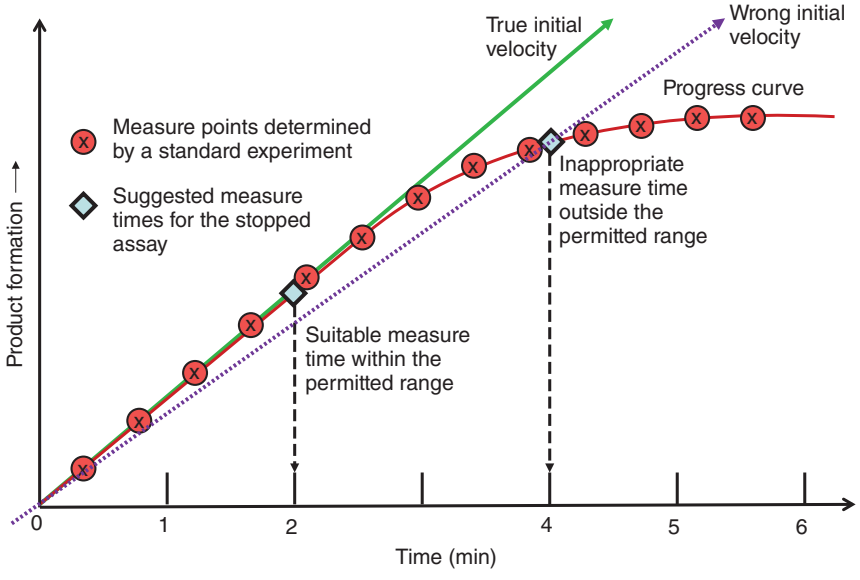

Figure 1.7 Stopped assay performed under ideal conditions. A series of measurements ⊗ allows the control of the linear range. Two different measure times ◇ are suggested for the final procedure; the first one lies within this range and is fairly applicable, and the second one is outside and, thus, inappropriate.

The integrated Michaelis–Menten equation (1.13) describes the complete progress curve including the nonlinear range, so that there is obviously no necessity to obtain the linear initial range. According to this equation, the Michaelis constant and the maximum velocity can be derived from one single progress curve. By rearranging

$$\frac{[A]_0 - [A]}{t} = V - K_m \frac{\ln \frac{[A]_0}{[A]}}{t} \tag{1.14}$$

the curve is linearized and V and K_m obtained from the ordinate intercept and the slope, respectively. The particular advantage of this procedure is the fact that in computer-controlled instruments the data can be directly transformed according to this equation and the constants can be displayed immediately after the experiment. This method appears tempting, but it is only reliable if the respective progress curve obeys completely the Michaelis–Menten equation. This is, however, often not the case because the product formed inhibits the enzyme and induces the backward reaction. Therefore, the determination of initial velocities from the linear part of progress curves is more reliable.

1.2.3.2 Enzyme Units

Since the velocity is directly proportional to the enzyme concentration, it can be taken as a measure of the amount of active enzyme. For an exact quantification a clear definition of the dimension of the velocity is necessary. In older literature various definitions with respect to the concentration and the time unit can

be found, dependent on the respective enzyme assay. In 1971 the Enzyme Commission of the International Union of Biochemistry (IUB) introduced the **International Unit (IU)** to standardize the enzyme assays: 1 IU is defined as the enzyme activity that converts **1 µmol substrate within one minute** (Box 1.4). But two years later, in 1973, the Commission of Biological Nomenclature adapted the enzyme units to the Système International d'Unités (SI) with mole as concentration and seconds as time units. The enzyme unit that is valid to date is 1 **Katal** (kat), the enzyme activity converting **1 mol substrate s^{-1}**. To compare both enzyme units: 1 kat = 60 000 000 IU, 1 IU = 0.000 000 016 67 kat. Owing to this unpractical dimension Katal could not completely displace the previous IU. For example, for a dehydrogenase assay an enzyme amount generating an absorbance change of about 0.1 min^{-1} is required, corresponding to 0.016 IU, or 0.000 000 000 26 kat. Commercial enzymes are frequently sold in quantities between 100 and 10 000 IU, correspondingly 0.000 001 667–0.000 166 7 kat. Another, but not so conclusive, reason for the preference of IU is the time unit of one minute, while one second is too short for rate determination. In fact, for accurate velocity measurements even one minute is too short; the assay must be observed for a longer period and it is the same whether the value obtained is recalculated to one minute or one second. Nonetheless, in scientific literature and commercial catalogues the IU is still preferred, although the problem is negligible when dealing with nanoKatal (nkat): 1 nkat = 0.06 IU, 1 IU = 16.67 nkat. Different expressions for the enzyme activity are presented in Box 1.4.

Box 1.4 Expressions for the Enzyme Activity

Enzyme units	Measure of the enzyme activity (calculated from the maximum velocity)
– Katal (kat)	Amount of enzyme converting 1 mol substrate s^{-1} (according to the SI system)
– International units (IU)	Amount of enzyme converting 1 µmol substrate min^{-1} 1 kat = 60 000 000 IU ↔ 1 IU = 0.000 000 016 67 kat 1 nkat = 0.06 IU ↔ 1 IU = 16.667 nkat
Maximum velocity	$V = k_{cat}[E]_0$ (concentration per time unit, in Katal or IU); dependent on the enzyme concentration in the assay
Turnover number	$k_{cat} = V/[E]_0$ (s^{-1}) first order rate constant; maximum velocity, divided by the enzyme concentration, independent of the enzyme concentration in the assay
Catalytic constant	k_{cat} (s^{-1}); identical with the turnover number
Michaelis constant	K_m (M); measure of substrate affinity (dissociation constant, extended by the catalytic constant k_{cat})
Catalytic efficiency	k_{cat}/K_m (M^{-1} s^{-1}); specificity constant, higher values indicating higher specificity
Volume activity	enzyme activity per volume unit (enzyme units per ml)
Specific activity	enzyme activity per protein concentration (enzyme units per mg protein)

Some special enzyme units, such as Anson units for proteases (cf. Section 6.25.1) are still in use, especially if the exact product molarities cannot easily be determined. For microbial transformations, instead of enzyme activity a *Productivity Number*, $PN = n_{prod}/m_{dry} \times t$, is applied, where n_{prod} is the amount of product formed, m_{dry} the dry weight of the cells, and t the transformation time.

For the exact determination of enzyme units the enzyme assay must be carried out under *standard conditions*. They will be discussed in detail in the following sections. For special studies, however, such as the investigation of enzyme features, the analysis of the Michaelis constant, the temperature or pH stability, or inhibition mechanisms, distinct parameters, for example substrate concentration, temperature, or pH, must be varied, so that the standard conditions can no longer be maintained. In such cases, the values obtained from velocity determinations do not correspond to defined enzyme units, but the actual enzyme velocity should still be indicated in the respective dimensions, mol s^{-1} or μmol min^{-1}.

1.2.3.3 A Short Discussion About Errors in Enzyme Assays

Errors severely hamper scientific experiments and their elimination would be a desirable goal. However, errors cannot be completely avoided and the only realistic aim is to minimize them as far as possible. Potential sources of errors, their consequences, and possibilities to limit their perturbing influences on enzyme assays should be recognized. At first, the origin of the error should be detected. Generally, it is possible to differentiate between spontaneous and systematic errors. **Spontaneous errors** arise from inexact manipulation (e.g. pipetting). Any handling bears intrinsically a certain inaccuracy, which may be reduced by careful performance, but cannot completely be eliminated. Spontaneous errors produce randomly distributed deviations from the true value of comparable extent both in the positive and negative directions (Figure 1.8a). Such errors will be observed in any experiment, but as long as the deviations are not too large, they can be mastered by common error calculations and regression analysis, presented in the relevant literature of statistics. In contrast, **systematic errors** are not randomly distributed, but rather generate deviations in a distinct direction and distort the results, provoking wrong interpretations. Causes of such errors may be imperfect operation of the instrument (e.g. not correctly adjusted apparatus or a defective pipette, used always for the same step) or faulty manipulation of the operator (making always the same mistake in routine assays).

One crucial argument must be mentioned before going into details with both error types. Errors are regarded as artificial, and, thus, unintended deviations from the real value. The aim of any analysis is to approach the true value, e.g. by calculating a medium value out of a series of repeated measurements, or to adapt a curve, such as a straight line or an exponential function, whereby it is generally accepted that "nature makes no jumps," meaning that physiological processes follow always clear, comprehensible functions. However, it must be borne in mind that any such function, even based on statistical treatment, is solely an *interpretation* of experimental results. The only stringent results are the experimental data and it is the task of the scientist to demonstrate convincingly that the suggested function fits the data. **Outliers**, i.e. values deviating extraordinarily from

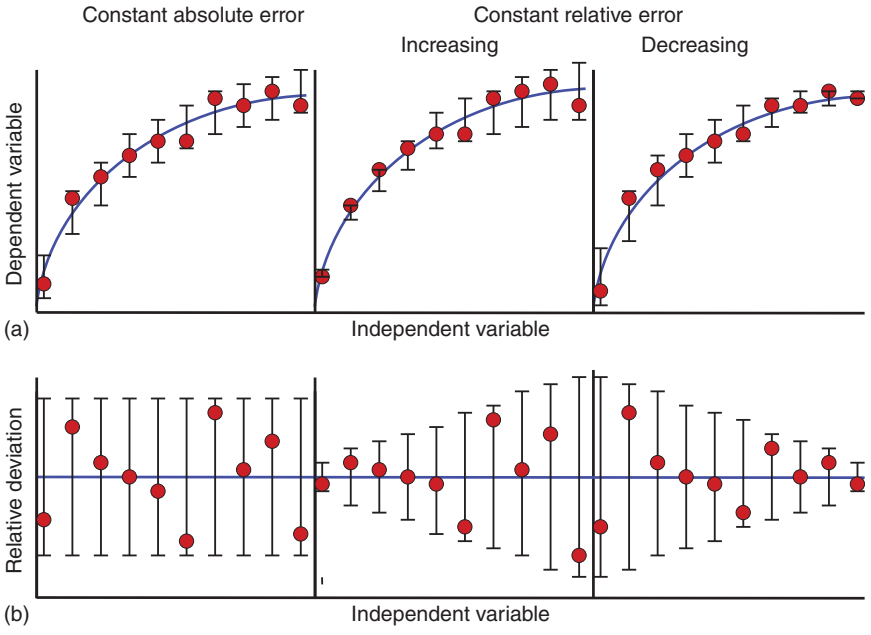

Figure 1.8 Error distributions. (a) Direct plotting of experimental data and (b) residual plots of the same data. Error bars and deviations from the adapted curve are enlarged.

all other measured values, are usually assumed to originate from unusual strong artificial deviations. They distort the regression analysis dramatically in such a way that the calculated function does not fit the majority of data. In such cases one feels authorized to suppress the strongly deviating value. This practice may usually be justified, but principally is the elimination of distinct values out of a data series an arbitrary selection revealing the preference of the scientist for a distinct solution, neglecting any other interpretation. Therefore, it is a strict rule in science to present all original data together with the interpretation (i.e. the fitted curve), and it must be indicated if distinct data points remained unconsidered for regression analysis. This enables an independent observer to judge whether the applied function describes adequately the presented data or whether there exist alternative interpretations.

Even for spontaneous errors there exist different possibilities of expression. During an experimental series the extent of error, i.e. the error limits in both directions, may be *constant* throughout (Figure 1.8a, left diagram). For such a series often one parameter is varied, such as the substrate concentration for enzyme kinetic determinations, and it is a question whether the error remains independent or whether the variation of this parameter influences the error.[9] Different error developments are depicted in Figure 1.8a. In **residual diagrams**, where the deviation of the data from the assumed function is enlarged

9 The parameter varied is regarded as *independent*, and the resulting one, e.g. the reaction rate, as *dependent* variable; it is generally accepted that only the dependent, and not the independent variable is subject to errors, an unfounded but practical assumption.

and plotted around the x-axis, the behavior of the error appears more clear (Figure 1.8b). With equally distributed errors, the error bars are equal throughout (Figure 1.8a,b, left diagrams); otherwise, the error may increase or decrease during the test series (Figure 1.8a,b, middle and right diagrams, respectively).

It must be considered that the dependent variable (velocity) increases with the substrate concentration and if the error limits remain constant throughout (Figure 1.8a, left), they actually increase relative to the velocity in the lower concentration range. The behavior of the error limits depends on the error source. For example, scatter of the instrument will cause a constant error, while pipetting of different volumes influences the extent of the error. Larger volumes can be pipetted with greater accuracy than very small ones, causing the error to decrease with increasing substrate concentration.

Pipetting is one of the severest error sources; careful pipetting improves considerably the accuracy of experiments. Various pipette systems are available, producing different error types. Automatic pipettes with variable volumes are frequently used. Those of good quality work very precisely; nonetheless, some general rules must be regarded (common manipulation is described by the producer's instructions and will not be mentioned here). It is a common time-saving but less accurate usage to pour out the pipette by dipping the tip into the assay solution and mixing the solution with the tip (Figure 1.9I). By extracting an aliquot from the stock solution some liquid will adhere to the outer surface of the tip (Figure 1.9II) and get into the assay solution (Figure 1.9III). Wiping the tip before dipping into the assay solution is not recommended because fibers of the tissue can extract some liquid from inside the tip (Figure 1.10). During dipping

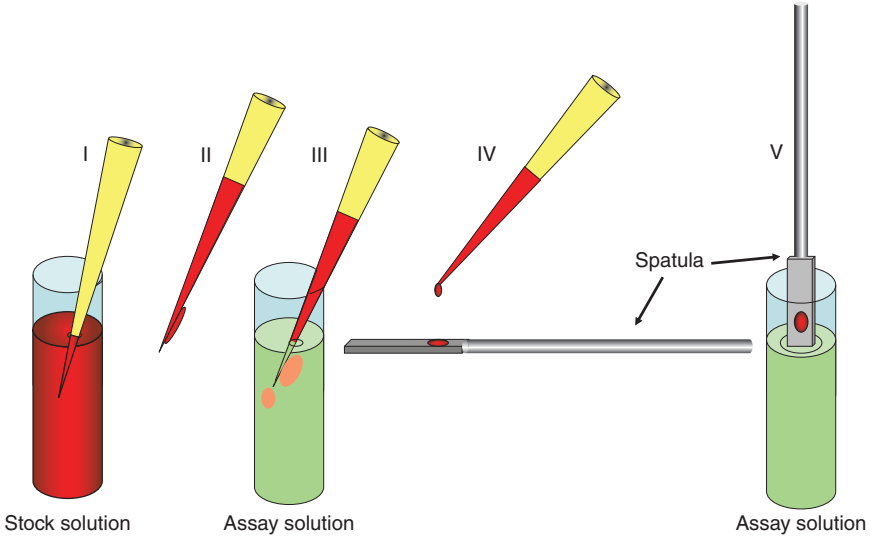

Figure 1.9 Pipetting and mixing in assay solutions. (I–III) Pipetting of a sample from the stock to the assay solution and direct mixing. A drop of the stock solution remains attached at the outside of the tip and gets into the assay solution. (IV, V) Placing the sample on a spatula, inserting the sample and mixing the assay solution.

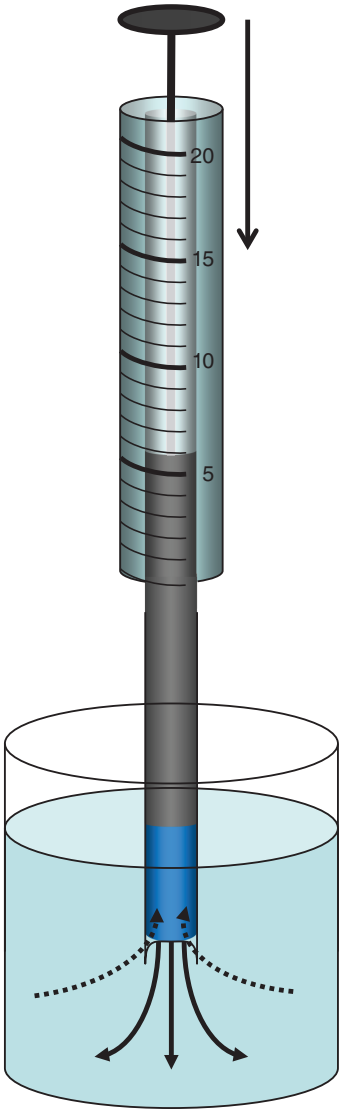

Figure 1.10 Plunging the pipette directly into the assay solution: some solution penetrates into the tip and more of the stock solution gets into the assay solution than intended. A microliter syringe is shown; the inner canal is enlarged.

into the assay solution for ejecting and mixing, the assay solution penetrates into the tip washing out the residual liquid, which usually adheres at the inner tip wall after ejection, and which is already considered for the normal pipetting process (Figures 1.9III and 1.10). Such an error is more severe the smaller the pipetting volume. Therefore, very small volumes (<5 µl) are critical for exact pipetting even if such volumes are within the range of the pipette, although small volumes have the advantage of avoiding significant dilution of the assay solution.[10] Volumes of 10–20 µl are recommended for pipetting; they are easy to

10 For accurate assays, all additions must be accounted to the final assay volume, while in preliminary tests small additions are often neglected.

manipulate and essentially do not change assay volumes of 1 or a few milliliters. The aliquot should not be added directly into the assay solution, but placed on the flat end of a spatula (Figure 1.9IV), which can be used also to mix the assay solution (Figure 1.9V).

1.2.3.4 Practical Rules for the Preparation of Dilution Series

If lower concentrations of the added sample are required, instead of reducing the volume the stock solution should be diluted to avoid pipetting of very small volumes. When the concentration of the substrate or cofactor must be varied during a test series, it is suggested and comfortable to remove with an automatic pipette from the stock solution the corresponding volume to yield the desired concentration in the assay mixture (Figure 1.11a). Such a procedure is, however, not very exact due to the inaccuracy of repeated pipette adjustment and additions of different volumes to the assay solution. Even more severe is the varying extent of the error on the pipetted volume; the assays receiving smaller volumes may have a greater error than those getting larger volumes. It is therefore recommended to prepare a dilution series with varying concentrations of the respective components instead of pipetting it directly into the assay mixture. The series should be prepared very carefully to minimize any inaccuracy; it can serve for repeated determinations. Always an equal volume (e.g. 10 µl) is taken from each sample of the series and added to the assay solution so that its final volume remains constant, and the pipette must not be adjusted after each step. A dilution series can be prepared in two different ways. For the stepwise procedure (Figure 1.11b), a defined volume of the stock solution (e.g. 0.1 ml) is added into a cup and filled up with buffer solution to the final sample volume (1 ml, resulting in a 10-fold dilution of the stock solution for our example). After mixing this sample an aliquot of the same volume (0.1 ml) is removed, added to a second cup, and filled up with buffer to the final sample volume (1 ml, yielding again a 10-fold dilution of the previous solution). This procedure is repeated several times until the desired dilution range is covered. This is an easy procedure, but any error occurring in one single step will be carried forward to all following samples and all errors occurring during the procedure accumulate to the end. The second mode of preparing a dilution series avoids this disadvantage. Different volumes of the stock solution are added into a series of cups and they are filled up with buffer solution to an equal final volume (e.g. 1 ml, Figure 1.11c). Here, an error concerns only the particular sample and not the following ones. The disadvantage is that varying volumes are required and the pipette must be adjusted accordingly, but this can be done carefully with higher precision than direct pipetting into the assay mixture. To avoid too small volumes for a broad dilution range one or two diluted stock solutions may carefully be prepared.

For very small volumes microliter glass syringes instead of automatic pipettes should be used (Figure 1.10). The same precautions as discussed for automatic pipettes must be regarded for them also. They are available in different volume ranges and consist of a glass cylinder with a central hole, ending in a steel tubing. A stainless steel piston fitted to the hole serves to draw up the solution. Care must be taken that the pipette is completely filled with liquid without any air bubbles included.

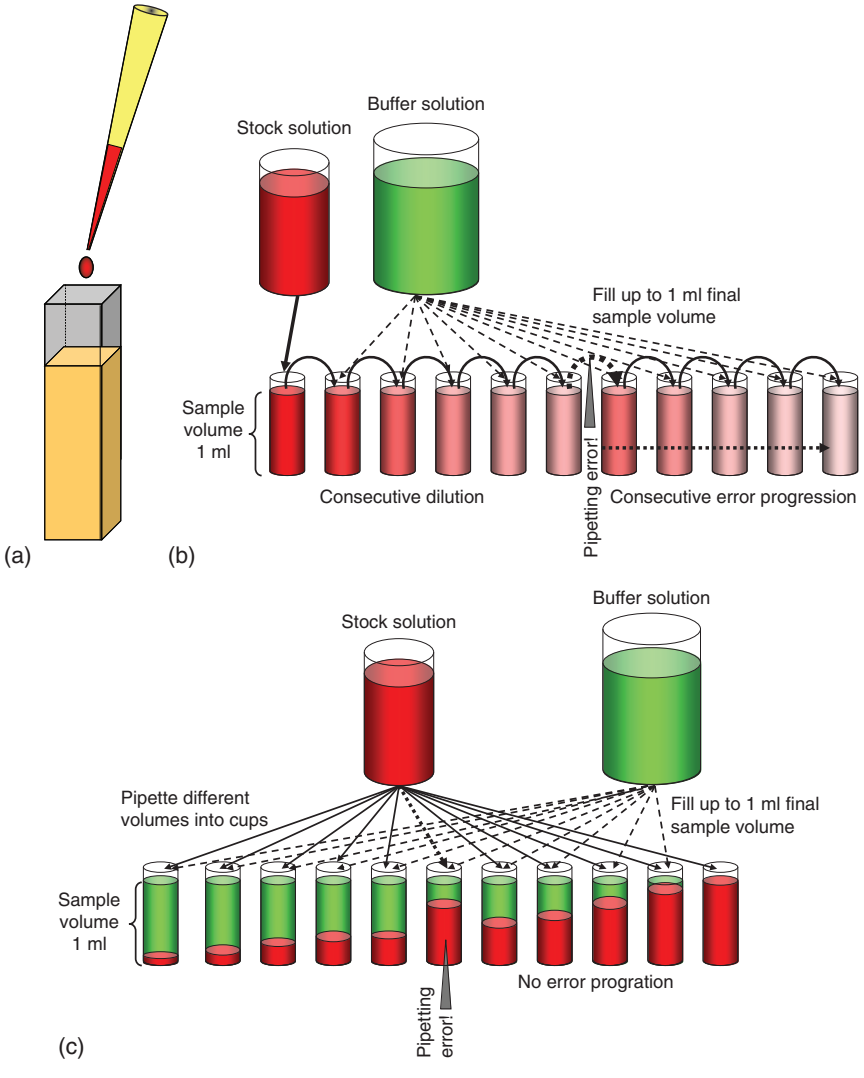

Figure 1.11 Different modes for preparation of a dilution series. (a) Direct pipetting into the assay solution has the lowest accuracy. (b) Stepwise dilution from one cup to the next; a failure will be propagated into the following samples. (c) Dilution from the same stock solution, best method, no failure propagation.

Mixing is a further source of error. Besides the problems discussed above, inappropriate handling can cause two distinct errors. Insufficient mixing, especially with thin tips, produces inhomogeneities in the assay solution with regions of lower and higher concentrations. The result of such an effect in a photometric assay is shown in Figure 1.12, where consecutive areas of higher and lower velocity float through the light path, pretending an oscillating behavior. During the progression of the reaction the oscillation smooths slowly, approaching the actual reaction velocity; however, the real initial rate has already been passed. On the

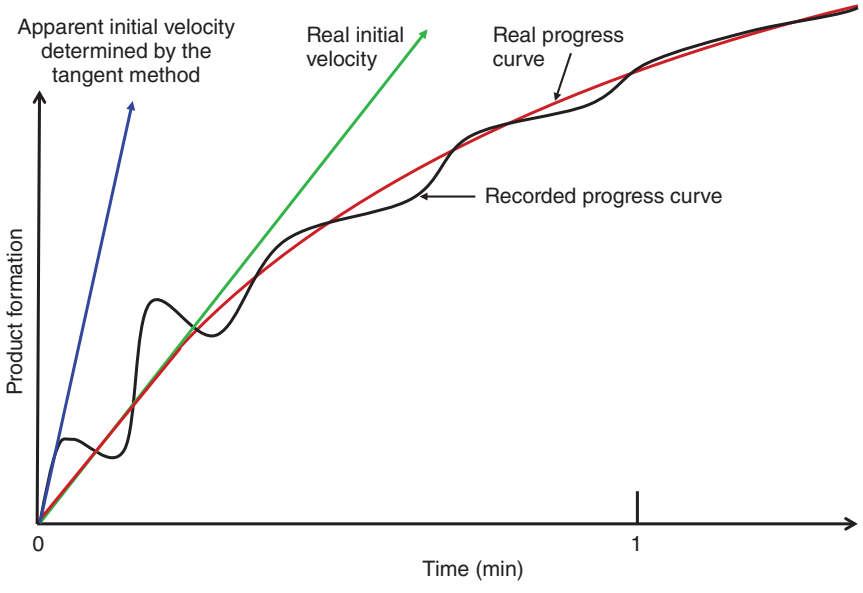

Figure 1.12 Artificial deviations from the progress curve due to insufficient mixing. The initial velocity taken from the recorded curve deviates considerably from the real one.

other hand, too vigorous a mixing causes an enclosure of air bubbles, disturbing especially optical methods, and of oxygen, favoring oxidative processes. Various mixing devices are commercially available, such as the small magnetic stirrer for direct mixing in cuvettes of optical instruments. Special apparatus, e.g. the stopped-flow device, enable rapid mixing within fractions of a second.

1.2.3.5 Statistical Treatment of Enzyme Reactions

To avoid misinterpretations of the final result by errors, always repeated measurements should be performed and a mean value calculated. To decide how many repeats should be done, it may be argued that the more the number of determinations, the more reliable the result, but on the other hand, the principle of parsimony dictates a limit with respect to both material (especially enzyme) and time, and, in fact, accuracy increases not essentially with a higher number of repeats. For routine tests three independent determinations can be regarded as sufficient, while five should be enough for higher accuracy. It can be of advantage to perform repeated assays not under completely identical conditions but to modify one parameter that causes a strictly proportional change. For enzyme assays the amount of enzyme (not of substrate!) and for protein tests that of protein may be modified. The expected linear dependency of the values is a further control for the method. Deviations from linearity, especially when steadily inclining in the same direction, are indications of errors. Any assay is reliable only within a distinct range. Values that are too low disappear in the scatter, while those that are too high fall outside the linearity of the method (due to depletion of assay components or the limited range of the Lambert–Beer law in optical assays). Linear dependency establishes that the assay has been performed completely within the

confidential range of the method. A mean value can be calculated, as regards the different amounts of the varied parameter.

For a test series, such as the determination of the Michaelis constant, various (~10) measurements with increasing substrate concentrations will be required. If each measurement is repeated three times, 30 assays are needed. If each assay needs five minutes, the whole series will require two and a half hours. This is not only the question of time, but also of the stability of the assays, especially of the enzyme. Most enzymes are not stable for longer time in diluted solution, as required for enzyme assays. Therefore, test series must be performed within a time as short as possible to establish equal conditions for all measurements. If, in our example, the enzyme loses 10% of its activity per hour, the value of the last measurement of the test series will be 25% lower than that of the first one under otherwise identical conditions. Obviously, the improvement in statistical reliability by repeated measurements is achieved at the price of a systematic error. Constants derived from such an experiment will seriously be underestimated, although statistical treatment pretends more confidence. If the same series is performed with only single determinations, less than one hour is needed with an activity difference of only 9% between the start and end of the experiment. In this case, it will be better to repeat the whole series three times and to calculate the constants independently for each series and derive finally a mean value for each constant. Such a procedure has the further advantage that systematic deviations, caused either artificially or as a special feature of the system, can be identified more easily by comparison of the independently generated results.

Even if an error source has been discovered, it cannot in any case be avoided (such as scatter of instruments), but can be reduced as far as possible. Sometimes deviations appear without any obvious reason. They may be caused by impurities, such as dust or soiling, wrong handling, or electric disturbances. In such cases, the experiment may carefully be repeated, and only if the deviation appears repeatedly in a similar sense the error source or a special feature of the system may be searched for.

1.2.4 Treatment of the Michaelis–Menten Equation

1.2.4.1 General Considerations

The Michaelis–Menten equation has already been derived in Section 1.2.2, and after the discussion of various aspects of the determination of the enzyme velocity, a prerequisite for treating the Michaelis–Menten equation, this fundamental relationship should be regarded more closely. Although the conditions for a special enzyme assay, such as the concentration of all components, are given in the respective protocols, it is of importance to understand the principles of the enzyme reaction, especially if some modifications are required, as for the determination of the **kinetic constants**. The two kinetic constants K_m and V can be obtained by determining the velocity of the enzyme reaction at various initial substrate concentrations. When plotted in the **Michaelis–Menten diagram,** with the substrate concentration [A] as x coordinate and velocity v as y coordinate, a hyperbolic function results, as shown in Figure 1.13. This establishes that the respective enzyme obeys the Michaelis–Menten equation, which is the case for

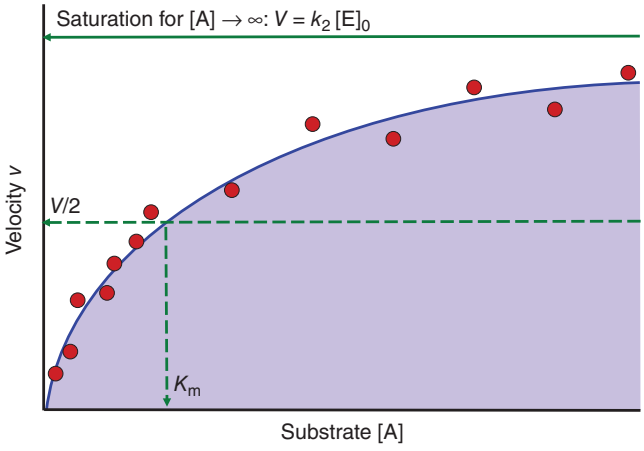

Figure 1.13 Michaelis–Menten presentation of enzyme velocities measured in dependence on substrate concentrations. The data points, the resulting hyperbolic function, and the determination of the kinetic constants are shown.

most enzymes. To obtain such a curve the substrate concentrations must cover the appropriate range around the respective Michaelis constant, i.e. the result must be known before performing the experiment. If this is not the case preliminary experiments should allow a crude estimation, which helps to perform the final experiment (Box 1.5).

Box 1.5 How Much Substrate Is Required for an Enzyme Assay?

- *Saturating amounts* of all substrates and cofactors
- *Saturating amount* means virtually infinite concentration, according to theory
- *Saturation* can practically be related to the K_m value of the respective substrate: 50-fold $K_m \sim 98\%$ saturation is usually sufficient, 10-fold $K_m \sim 91\%$ saturation is not enough
- *However*, saturating concentrations can often not be realized, because of
  - substrate inhibition
  - unspecific inhibition due to high ionic strength
  - limited solubility
- *Example 1*: K_m of NADH is 1×10^{-5} M. A 5×10^{-4} M NADH solution (50-fold K_m) has an absorption of 3.15 at 340 nm, far outside the range of the Lambert–Beer law
- *Example 2*: K_m of the trypsin substrate benzoylarginine *p*-nitroanilide is 1×10^{-3} M, and the maximum solubility in water is 1.3 mg ml^{-1} or 3 mM, corresponding to 75% saturation. Higher concentration solutions can be prepared in dimethylsulfoxide (DMSO), but the solvent influences the enzyme activity

Conclusion: Saturating conditions (50-fold K_m) as far as possible; otherwise, highest possible concentration and extrapolation to saturation in a linear diagram applying the Michaelis–Menten law.

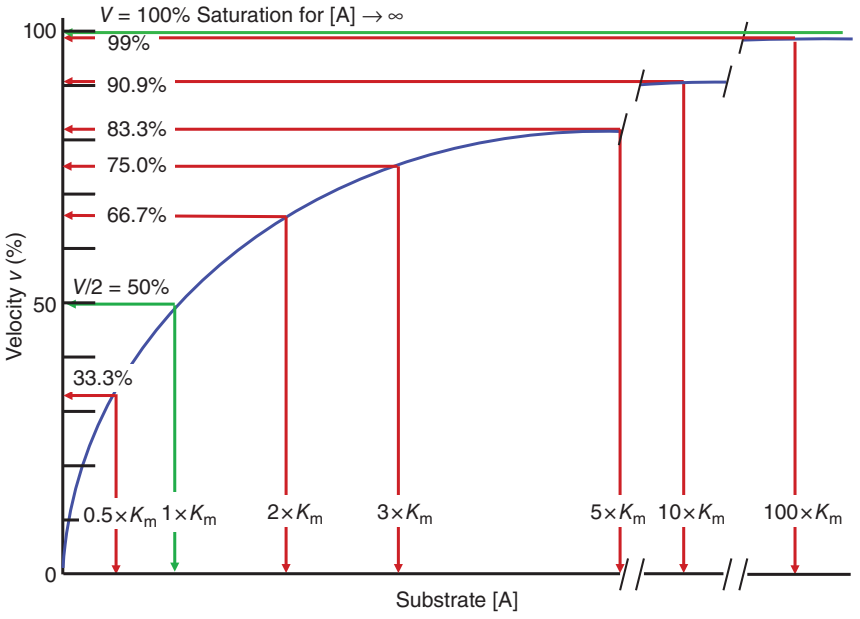

Figure 1.14 Relative saturation (in percentage) of an enzyme in dependence on the substrate concentration, indicated as multiple of the K_m value.

For very high substrate concentrations, $[A] \gg K_m$ (and finally $[A] \to \infty$), K_m can be neglected in the denominator of Michaelis–Menten equation (1.12). The equation reduces to $v = k_2[E]_0$, and all enzyme molecules present in the assay will be involved in the reaction. This is the highest possible reaction rate under this condition, with the **maximum velocity** V. V is a limiting value; the saturation curve approaches it, but can reach it only at infinite substrate concentrations. This aspect is often disregarded and V is taken directly from assays carried out with an apparently high substrate concentration. The hyperbolic curve in Figure 1.14 shows the dependence of the relative degree of saturation on the substrate concentration, indicated as a multiple of the K_m value. The true V value can only be obtained by extrapolation to infinite substrate amounts, as will be discussed below. For $v = V/2$ the substrate concentration becomes equal to the **Michaelis constant** K_m and its value can be directly obtained from the abscissa scale as shown in Figure 1.13. So, knowledge of V is necessary also for the determination of K_m, an unsatisfying situation, since any error of V will be transferred to K_m.

1.2.4.2 Linear Representations of the Michaelis–Menten Equation

The restrictions in the determination of the kinetic constants mentioned above can be overcome by transforming the Michaelis–Menten law into a linear relationship. Three equivalent transformations are derived. The reciprocal form

$$\frac{1}{v} = \frac{1}{V} + \frac{K_m}{V[A]} \tag{1.15}$$

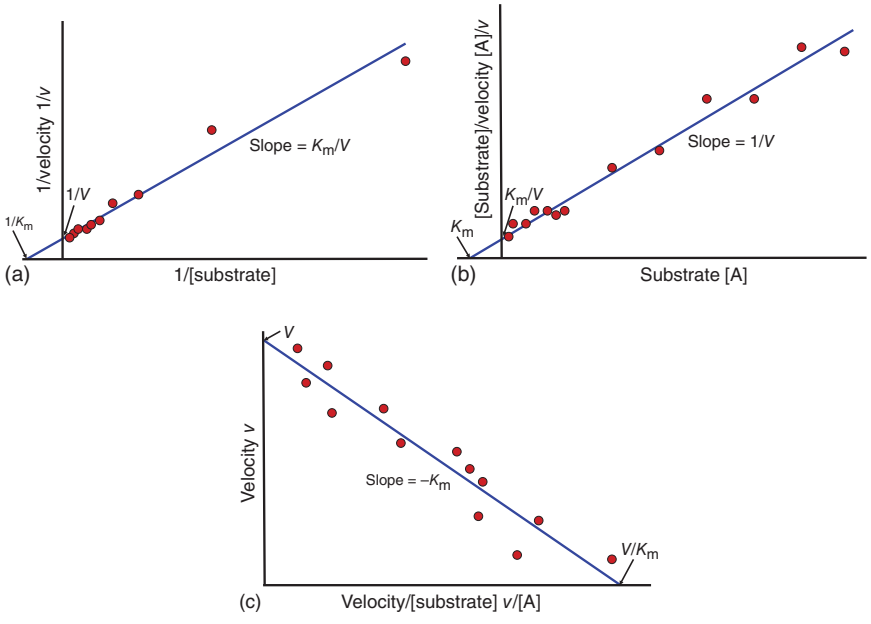

Figure 1.15 Diagrams for linear presentation of the Michaelis–Menten data. Data points, resulting lines, and determination of kinetic constants are shown. (a) Lineweaver–Burk plot; (b) Hanes plot; and (c) Eadie–Hofstee plot.

is known as the **Lineweaver–Burk diagram**.[11] A straight line is obtained by plotting $1/[A]$ against $1/v$ (Figure 1.15a). Extrapolation of the line to the left intersects the ordinate at $1/V$ and the abscissa at $1/K_m$; both constants can be obtained independently. A serious disadvantage of this plot is the distortion of the error limits, which extends from left to right (to lower substrate concentrations!). Therefore, linear regression analysis is not applicable. In this respect the **Hanes diagram** is superior. It is obtained by multiplying Eq. (1.15) with [A]:

$$\frac{[A]}{v} = \frac{[A]}{V} + \frac{K_m}{V} \tag{1.16}$$

Plotting $[A]/v$ against [A] results in a straight line with a positive slope. The intercepts of the ordinate and the abscissa are K_m/V and K_m, respectively (Figure 1.15b). This and Figure 1.15c have the disadvantage that the variables [A] and v are not separated. The same holds also for V when taken from the ordinate intercept, but it can separately be obtained from the slope as $1/V$.

Multiplication with Vv of Eq. (1.15) and rearrangement leads to the third transformation

$$v = V - \frac{vK_m}{[A]} \tag{1.17}$$

For this **Eadie–Hofstee diagram** $v/[A]$ is plotted against v. The slope of the straight line is $-K_m$, the ordinate intercept V, and the abscissa intercept V/K_m (Figure 1.15c).

11 Woolf was actually the first to mention this and the following two transformations.

Owing to the various advantages and disadvantages it is advisable to plot the data in all three figures. Linearization is not only for determination of constants but also to control the experiment and the mechanism; any deviation from linearity indicates also a deviation from the Michaelis–Menten equation, due to an alternative mechanism or artificial influences.

To return to the question about the appropriate substrate range it can now be understood that the K_m value as the substrate concentration at half saturation should be in the middle of the test series and the substrate should be varied from a clearly lower to a higher concentration: as a rule, from 1 order of magnitude below to 1 order of magnitude above the K_m value.

1.2.5 Enzyme Inhibition

Enzyme analysis is limited frequently to the determination of the Michaelis constant and the maximum velocity. For detailed characterization of an enzyme a more thorough analysis must be undertaken. Especially, inhibition and multisubstrate reactions should be examined. Both mechanisms show various similarities. A second variable, an inhibitor or a cosubstrate, must be considered besides the one substrate regarded in the hitherto discussed simple enzyme reaction.

An **inhibitor** is a substance binding specifically to the enzyme and reducing its activity. The enzyme activity can be reduced also by other influences, such as temperature, pH, ionic strength, or unspecific interactions with various substances, but inhibition in its strict sense requires specific interaction of the inhibitor with the enzyme. Several modes of interaction exist, and the main types will be discussed.

The initial step of any inhibitor action is its binding to the enzyme. The binding strength or **affinity** is characterized by a binding or **dissociation constant** $\boldsymbol{K_i}$[12] (dimension M^{-1}). Its value indicates the amount of inhibitor required to achieve half saturation, similar to the K_m value with respect to the substrate concentration. However, while K_m can be determined with the substrate alone, for the determination of K_i besides the inhibitor the substrate must be present to promote the enzyme reaction. This fact complicates the analysis, because the substrate influences the binding of the inhibitor, frequently by attenuating the inhibitory effect. Therefore, to estimate the degree of inhibition in a preliminary test the substrate concentration should not be saturating; the inhibitory effect will become clearer at half saturation of the substrate. At first the uninhibited reaction should be measured as a reference (100%). Thereafter, the reaction is measured in the presence of a defined amount of the inhibitor. The extent of inhibition can be controlled by varying the inhibitor concentration, and the amounts required to reduce the uninhibited reaction to 25%, 50%, and 75% can be found out. To explore the inhibition mechanism, test series are performed in the presence of these three inhibitor concentrations and also in the

12 Although the term "dissociation" expresses the opposite of binding, the dissociation constant can be taken as a measure of affinity; the lower its value, the higher the affinity. In physical chemistry literature the reciprocal function, the **association constant** (M), often designated as true binding constant, is preferred, higher values indicating higher affinity. Enzyme kinetics deals with dissociation constants; the Michaelis–Menten constant is derived from them too.

absence of the inhibitor. Each series comprises about 10 independent assays with substrate concentrations varying around the K_m value. The data are plotted in a linear diagram, such as the Lineweaver–Burk plot. As a proof for the universal validity of the Michaelis–Menten law even in the presence of inhibitor, all four series should yield straight lines, arranging in the diagram in a pattern that is characteristic of the respective inhibition mechanism. The special arrangement of the lines depends on the linearization method and is mostly discussed by means of the double reciprocal or Lineweaver–Burk plot. Increasing steepness with higher inhibitor concentration and a common ordinate intercept in this plot is indicative of a **competitive inhibition** (Figure 1.16a). Since the ordinate intercept indicates the reciprocal maximum velocity $1/V$, the maximum velocity is not modified by the inhibitor. Such a mechanism prevails if the inhibitor binds to the substrate site of the enzyme displacing the substrate. Competitive inhibitors are usually structurally similar to the substrate. They cannot be converted to the product (*substrate analogs*), but can block the active site of the enzyme. Since both the substrate and the inhibitor compete for the same binding site, the inhibitor is completely displaced by very high (infinite) amounts of the substrate. Therefore, the maximum velocity is reached even in the presence of (limiting amounts of) the inhibitor. Conversely, high amounts of inhibitor displace the substrate completely.

Several methods for the determination of the binding constant of the inhibitor, the **inhibition constant** $K_{ic} = [E][I]/[EI]$, exist. A favorable method is the **secondary diagram** (or replot), a graphical method, where the inhibitor concentrations (the uninhibited reaction being zero) are plotted against the slopes of the respective lines in the primary (Lineweaver–Burk) diagram. A straight line should result in intersecting the abscissa just at the value of $-K_{ic}$ (Figure 1.17a). Deviation from linearity in this plot (if error can be excluded) is an indication of a more complicated inhibition type, while strict linearity is a further confirmation for the competitive mechanism.

If the lines in the Lineweaver–Burk diagram differ in their slope without common ordinate intersection, the inhibition is **noncompetitive**. But also in this case, all lines *must* share a common intersection point, which now is left of the ordinate. The relative position of the intersecting point gives further information about the inhibition type; it can be either directly at the abscissa, or above or below it. According to the expression "noncompetitive" the inhibitor does *not* bind to the site of the substrate and cannot displace it. An additional site for the inhibitor must exist and if both substrate and inhibitor bind to their respective sites completely independent from one another, the lines intersect directly on the abscissa (pure noncompetitive inhibition; Figure 1.16a, middle diagram). If both compounds influence one another in their binding the lines intersect apart from the abscissa. In most cases this is an impeding effect; binding is better in the absence than in the presence of the other component. Consequently, two different inhibition constants must be considered, one for binding to the free enzyme (K_{ic}) and the other for binding to the enzyme–substrate complex (K_{iu}), the former constant being smaller (higher affinity) than the latter: $K_{ic} < K_{iu}$. In this case, the lines intersect above the abscissa. If, in a rarer case, the binding to the free enzyme is weaker, i.e. already bound substrate helps the inhibitor in its binding

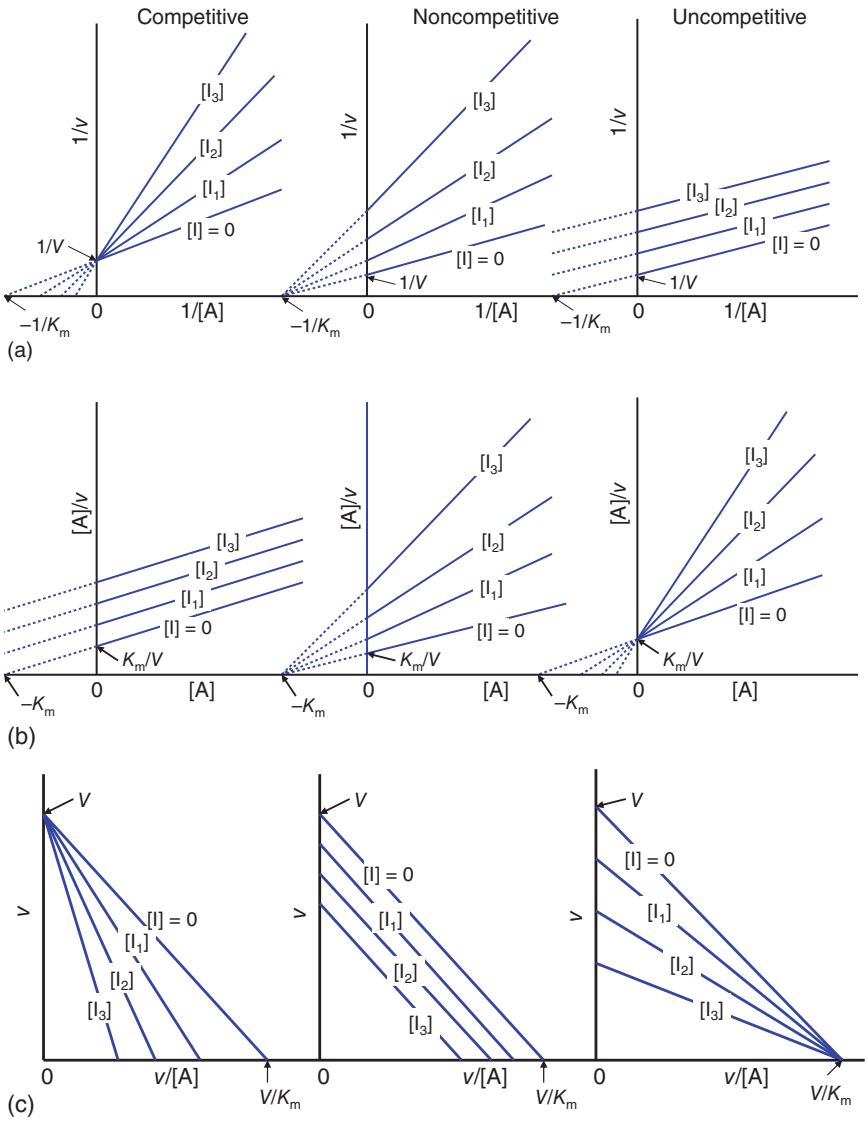

Figure 1.16 Three main mechanisms of enzyme inhibition in the linear presentations. (a) Lineweaver–Burk plot; (b) Hanes plot; (c) Eadie–Hofstee plot; ———, lines in the experimental region, ... lines in the extrapolated region.

(and *vice versa*), $K_{ic} > K_{iu}$, the intersection is below the abscissa. The types of inhibition with unequal inhibition constants are sometimes called *mixed* inhibition.

Since the inhibitor does not bind to the substrate site, a special site must exist. This can be a regulatory site, as is the case with **allosteric enzymes**. Usually these enzymes class obeys not the Michaelis–Menten law, showing nonlinear curves in the linearized plots. Inhibition experiments can be performed as described above. Extrapolating the nonlinear curves should yield a common intersection point left

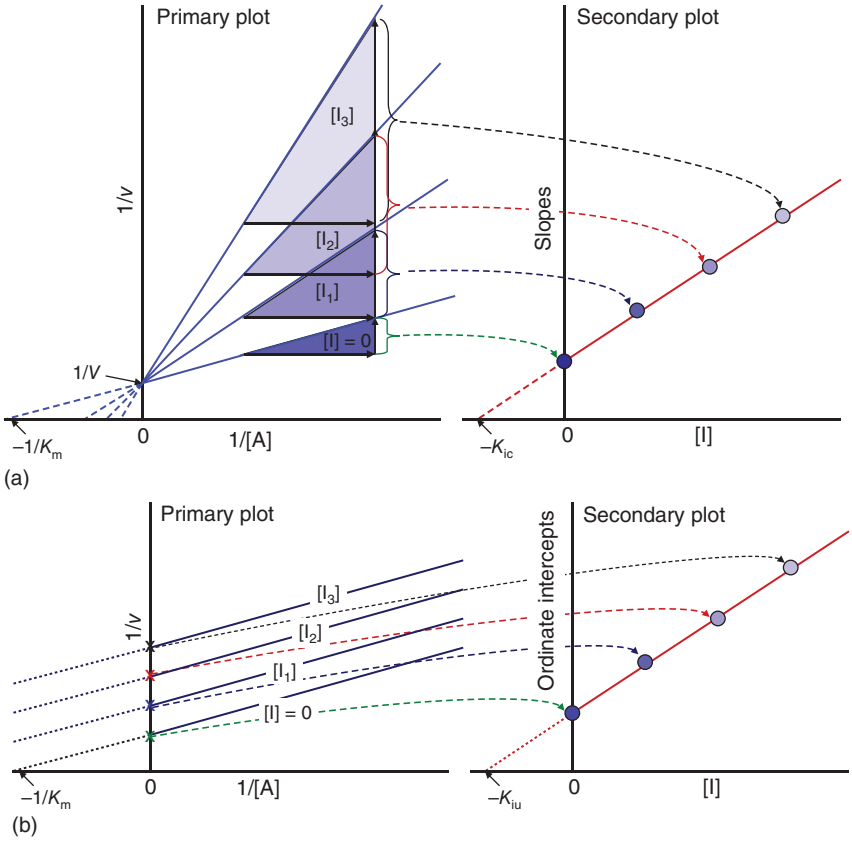

Figure 1.17 Secondary plot (right), derived from the slopes (a) and the ordinate intercepts (b) of the lines in the primary plot (Lineweaver–Burk plot, left). In the case of multisubstrate reactions on the abscissa 1/cosubstrate concentration (1/[B]) is plotted instead of [I], both in (a) and (b).

of the ordinate. More typical for the noncompetitive inhibition are reactions with two or more substrates. The inhibitor may compete with one substrate for its binding site, but this inhibitor will not compete for the binding site of the other substrate. The second substrate cannot displace the inhibitor from the binding site of the first substrate and cannot restore the enzyme activity even at high concentrations. The inhibition will be competitive with respect to the first substrate but noncompetitive with respect to the second substrate.

The third and more seldom encountered mechanism, the **uncompetitive inhibition**, is characterized by strictly parallel lines without common intersection points in the Lineweaver–Burk plot (Figure 1.16a). The inhibitor binds only to the enzyme–substrate complex, but not to the free enzyme. The substrate helps the inhibitor to bind, for example, by creating or completing a binding site for the inhibitor. Its affinity is quantified by the uncompetitive inhibition constant $K_{iu} = [EA][I]/[EAI]$, which describes the binding exclusively to the enzyme–substrate complex, in contrast to the competitive inhibition constant

K_{ic}, which describes the inhibitor binding to the free enzyme. A secondary plot of the ordinate intercepts from the Lineweaver–Burk diagram plotted against the inhibitor concentration should yield a straight line intersecting the abscissa at K_{iu} (Figure 1.17b). In the noncompetitive inhibition, where the inhibitor binds to the free enzyme and the enzyme–substrate complex, both constants, K_{ic} and $K_{iu,}$ are involved. Their values can be obtained by applying the two different secondary plots, plotting the slopes form the Lineweaver–Burk diagram for K_{ic} and the ordinate intercepts for K_{iu}. For independent binding both constants are equal.

Two other frequent inhibition types, the substrate and the product inhibition, are related to the already mentioned main types. **Substrate inhibition** (also called *substrate surplus inhibition*) is observed when the reaction rate decreases with higher substrate concentrations instead of approaching a saturation value (Figure 1.18a). This happens when a second substrate molecule binds to the enzyme after the first one. It occupies a distinct inhibitory site, such as the product binding site, so that the substrate can no longer be converted into product. This is formally an uncompetitive inhibition type, since the inhibitory substrate molecule binds only to the EA complex. As the same compound acts as substrate and inhibitor, both effects overlay causing a deviation from the

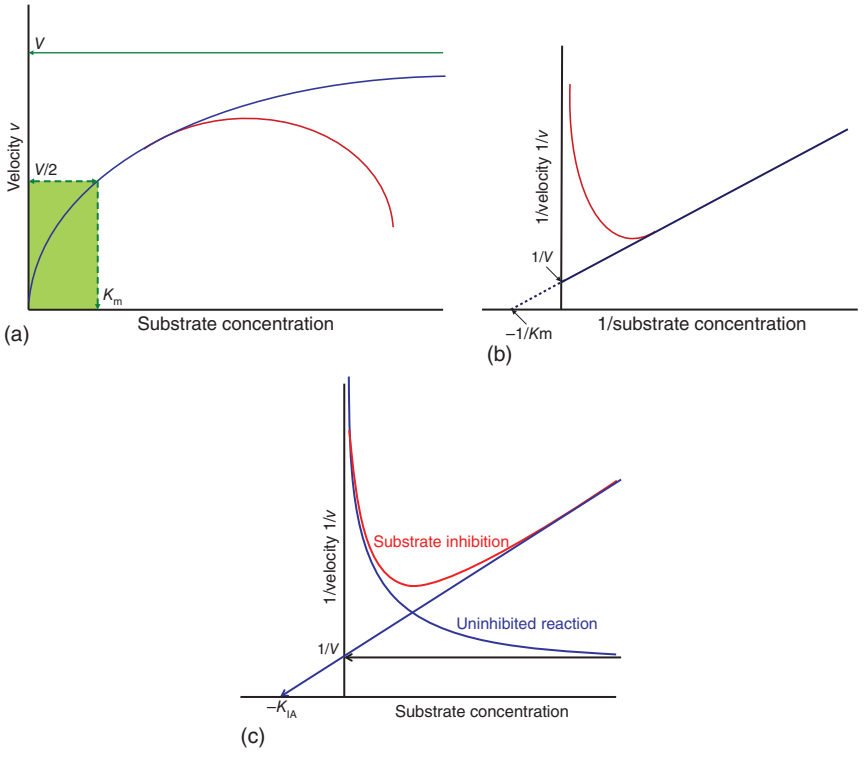

Figure 1.18 Substrate inhibition. (a) Michaelis–Menten plot; (b) Lineweaver–Burk plot; (c) Dixon plot. Lines in the experimental region (solid, blue); deviation due to substrate inhibition (red); lines in the extrapolated region (dotted, blue). The determination of kinetic constants is shown.

Michaelis–Menten behavior. In the low substrate range the catalytic function prevails, while the inhibitory function predominates at higher concentrations. Linear plots show characteristic deviations. In the Lineweaver–Burk diagram two regions can be observed (Figure 1.18b). The right part of the diagram (low substrate concentrations) reflecting the substrate function is still linear and $-1/K_m$ and V can be obtained from this part by extrapolation to the x- and y-axis, respectively. The curve deviates drastically upwards to the left part of the diagram, due to the inhibitory effect at high substrate concentrations. A similar curve is obtained, when [A] instead of 1/[A] is plotted at the abscissa (Figure 1.18c). Here, the linear range in the right part (high concentrations!) represents the inhibitory effect and extrapolation to the abscissa yields the dissociation constant K_{iu} for the inhibitory substrate.

Product inhibition is based on the general principle of reversibility of chemical reactions and is, therefore, a feature of all enzyme reactions. The product formed during the catalytic process remains bound to the enzyme for a short time before being released. To its free binding site another product molecule from the medium can bind, especially when larger amounts of product have been accumulated. Because substrate and product interact with the same region of the catalytic center, binding of the product and the substrate is exclusive; both displace one another, and the inhibition is of the competitive type. If product is added to the assay already before starting the reaction, it behaves like a competitive inhibitor, and the corresponding patterns are observed in diagrams; in the Lineweaver–Burk diagram straight lines with a common ordinate intercept at the position of V (Figure 1.16).

Besides these main inhibition types, there exist some more special mechanisms. If the observed inhibition pattern does not fit into the rules described here the special literature for enzyme kinetics should be consulted.

1.2.6 Multisubstrate Reactions

In the majority of all enzyme reactions two, sometimes even three, different substrates are involved, and any general treatment must take notice of this fact. As already mentioned, enzyme assays should contain all components, substrates, cofactors, and essential ions, in saturating amounts so that the maximum velocity will be obtained, and no interfering influence from any component should occur. For a more detailed analysis, the dependence of the reaction rate on *all* substrates involved must be considered. The treatment of multisubstrate reactions is essentially similar to that of inhibition mechanisms, both depending on two components. Also, three main mechanisms can be discerned. The main difference is that an inhibitor reduces the velocity, while the second substrate, the *cosubstrate*, accelerates it. The **random mechanism** corresponds to noncompetitive inhibition. The alcohol dehydrogenase reaction should serve as an example. The presence of two substrates, ethanol (A) and NAD^+ (B), is required to promote the reaction. The substrate with the lower concentration will control the velocity. A test series can be performed varying A in the presence of saturating amounts of B, and, *vice versa*, a series varying B at saturating concentrations of A. A hyperbolic curve obeying the Michaelis–Menten law

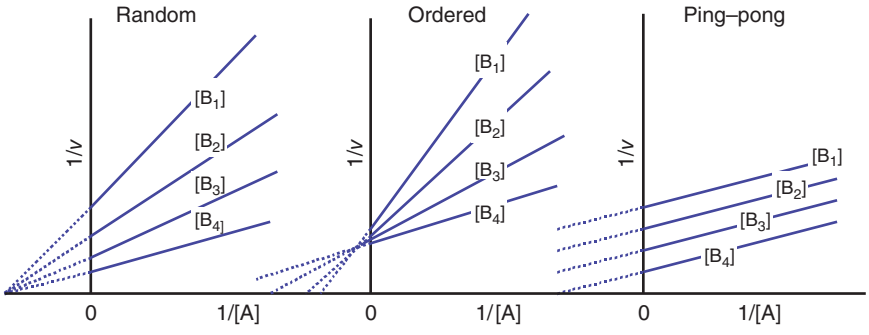

Figure 1.19 Multisubstrate mechanisms in the Lineweaver–Burk diagram. ———, lines in the experimental region; ..... lines in the extrapolated region.

should result and the kinetic constants can be determined as already described, e.g. by linearization, and separate Michaelis constants and maximum velocities are obtained from each series. K_m is characteristic of the respective substrate, while the maximum velocity V at saturating conditions must be the same for both. This simple approach is acceptable for a crude estimation, but for an exact analysis, which should also provide information about the respective mechanism, it must be considered that the reaction velocity depends now on two substrates. The procedure is similar as described already for inhibition; one substrate (e.g. A) is varied in the presence of a constant (but not necessarily saturating) concentration of B. Several (e.g. four) such series will be performed, modifying the constant amount of B between them. The outcome plotted in a linearized diagram, such as the Lineweaver–Burk plot, should yield a pattern of straight lines with different slopes intercepting left of the ordinate, directly at, above, or below the abscissa (Figure 1.19). The actual position of the intercept depends on the mode of binding. For independent binding of both substrates the intercept will be on the abscissa; if both substrates impede one another in binding, the intercept is above and if they help one another it is below the abscissa, similarly as discussed for the noncompetitive inhibition. In the case of independent binding both substrates will bind in random order; the one reaching the enzyme first will bind first (random mechanism). If both substrates interact with one another, an **ordered mechanism** results, the first substrate (A) binding to the free enzyme, and the second one (B) to the EA complex. The common intercept of the straight lines will appear left of the ordinate above the x-axis (Figure 1.19).

Also in similarity to the noncompetitive mechanism two binding constants for each substrate are defined. One, called **inhibition constant**, K_{iA} for substrate A, K_{iB} for substrate B, stands for binding to the free enzyme E; the other, the **Michaelis constant**, K_{mA} and K_{mB}, stands for binding to the enzyme substrate complexes EA or EB, respectively. These constants can also be determined from secondary plots by plotting the *reciprocal* concentrations (in contrast to direct plotting of the inhibitor concentration) of the (constant) cosubstrate against the slopes and ordinate intercepts of the respective lines in the primary plot, as already described for the inhibition mechanisms (cf. Figure 1.17).

Parallel lines in the Lineweaver–Burk diagram are characteristic of the **Ping–Pong mechanism** (Figure 1.19). The first substrate forms an intermediate with the enzyme, which reacts with the second substrate under restoration of the original enzyme form. Transaminations are typical examples for this mechanism. An amino acid transfers its amino group to the active site of the enzyme, being released as an α-oxoacid. Another α-oxoacid removes this amino group, becoming an amino acid. Such a mutual exchange of a functional group resembles a ping-pong game.

Multisubstrate reactions are more difficult to analyze, but they provide a lot of information about the respective enzyme. The coordinate intercepts of the linearized diagrams are complex expressions and the Michaelis constants and the maximum velocity cannot be obtained by simple extrapolation, but must be calculated by applying the rate equation of the respective mechanism.

1.3 Essential Conditions for Enzyme Assays

1.3.1 Dependence on Solvents and Ionic Strength

In the previous sections the dependence of enzyme reactions on the enzyme, the substrates, and the inhibitors was described, but the velocity is also influenced by environmental conditions, especially pH, temperature, and the solvent. For the enzyme activity the solvent plays a decisive role. Enzymes bound to or connected with the membrane prefer an apolar environment, such as lipases, which are active in organic solvents. The great majority of enzymes are instable and denature in organic solvents; they prefer the polar aqueous milieu of the cell. For all these enzymes water is exclusively used as solvent during the assays. In some cases, the presence of organic solvents cannot be completely avoided. Several substrates and metabolites are water insoluble, especially in higher concentrations and must be dissolved as stock solution in less polar, but water mixable solvents such as ethanol, acetone, tetrahydrofuran, or DMSO. For the assay, aliquots from such stock solutions are added to the aqueous assay mixture in such a quantity that the diluted compound will remain resolved in the water phase and the enzyme can tolerate the small amount of the added apolar solvent. Both preconditions must be established for each test, especially when the concentration of the respective compound should be varied, implying a corresponding variation of the added organic solvent. Organic solvents, such as ethanol, are sometimes added to prevent microbial attack or to decrease the freezing temperature.

When different components are added to the enzyme assay, care must be taken that they are compatible with one another, and do not form precipitates or complexes (e.g. divalent cations in phosphate buffer) or influence the redox state (e.g. NAD, NADH, thiols).

Most enzymes are sensitive against high as well as very low ionic strength (the measure of the concentration of electrolytes). This applies to all components and substrates, as well as to components not directly involved in the reaction, such as protectives for thiol groups, or counterions for neutralization of acid or basic compounds. Mono- and divalent ions are often necessary for the catalytic

mechanism or for stabilization of the enzyme, but they may cause unspecific effects, e.g. promoting oxidative processes. To avoid such effects often chelate forming substances, mostly ethylenediaminetetraacetic acid (EDTA), are added providently, but they can also withdraw the stabilizing ions.

The ionic strength tolerated by the enzyme depends on its special requirements and must individually be examined. Most enzymes prefer moderate ionic strength (~0.1 M) and will denature at considerably lower or higher values. Distinct enzymes, especially from organisms growing in brine or at high temperatures prefer high ionic strength up to 1 M.

1.3.2 pH Dependency

Enzymes depend strictly on the pH in the medium. Two different aspects are responsible for this pH dependency: (i) charged groups are involved in the catalytic mechanisms and their state of protonation is decisive for the catalytic efficiency; (ii) enzymes are amphoteric substances with positive and negative charges on their surface and in the inner core, which stabilize the native structure. The influence on the catalytic mechanism can be investigated by an experiment, where the enzyme assay is carried out under otherwise identical conditions, changing pH values. A pH range between 3 and 12 should be covered in steps of about 0.5 pH units. Usually an optimum curve results, with the enzyme activity increasing from the acid region, passing a maximum and decreasing to the alkaline region (Figure 1.20). The maximum, designated as **pH optimum**, is a characteristic value for each enzyme and is mostly in the neutral range between pH 6.5 and 8.5. Certain enzymes show extreme pH preferences, such as acid and alkaline phosphatases or pepsin, the latter with a pH optimum near 1. The whole optimum curve can be regarded as a combination of titration

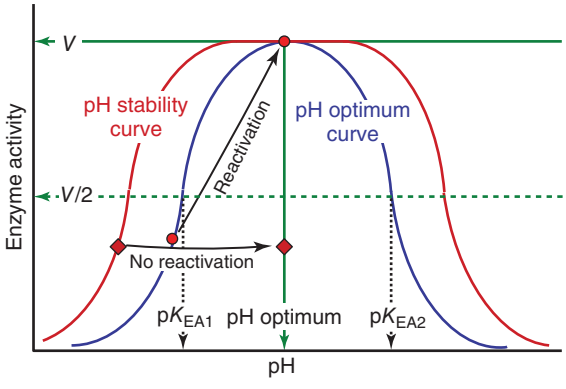

Figure 1.20 pH behavior of the enzyme activity. pH optimum curve: the enzyme is tested at the pH indicated. pH stability curve: the enzyme is preincubated for a distinct time (e.g. one hour) at the pH indicated and tested at the pH optimum. The p*K* values can be obtained from the inflexion points of the optimum curve. Shift of an enzyme sample from a marginal pH region of the optimum curve to the optimum pH restores the maximum activity (●), while no regain of activity occurs for a similar shift within the stability curve (♦).

Table 1.1 pK_a values of functional groups of various amino acids.

Amino acid	pK_a
Aspartic acid	3.86
Glutamic acid	4.32
Histidine	6.09
Cysteine	8.30
Serine	9.15
Tyrosine	10.10
Threonine	10.40
Lysine	10.53
Arginine	12.30

curves of charged groups, which are essential for catalysis, mostly amino acid residues, but also cofactors can be involved. One type of groups will be active in the protonated state, and others in the deprotonated state. Their respective titration curves form the sites of the pH optimum curve. For example, a carboxy group may be active in its charged state and the transition from the protonated ($-COOH$) to the deprotonated ($-COO^-$) state in the acid region will form the left side of the optimum curve, while deprotonation of an amino group ($-NH_3^+ \rightarrow -NH_2$) determines the right, declining site. If for each site only one single group is responsible, a pure titration curve results with an inflexion point corresponding to the pK_a value (the pH, where the respective group is just half protonated) of the titrated residue. Thus the pK_a value can serve to identify the respective group involved in the catalytic mechanism (Table 1.1), but it must be considered that pK_a values can be markedly changed ($\pm$1–2 pH units) by the integration of the respective amino acid into the three-dimensional protein structure and it must also be established that only a single residue is observed.

The pH-dependent catalytic protonations are usually reversible. When the enzyme is incubated at a marginal pH with lower activity, it will regain its full activity when shifted to its pH optimum (Figure 1.20). In contrast to this, pH-dependent processes concerning the three-dimensional enzyme structure are mostly irreversible. A **pH stability curve** can discern between reversible and irreversible pH changes. Aliquots of the enzyme are preincubated for a distinct time (e.g. one hour) at various pH values. The activity is tested thereafter at the optimum pH. Identical activities will be obtained as long as the pH-dependent changes are reversible, but after an irreversible change the enzyme cannot return to its optimum activity (Figure 1.20). In comparison to the pH optimum curve the pH stability curve expands more to the extreme pH values and has a broad plateau of equal activities around the pH optimum. As the enzyme has its highest activity at its pH optimum, usually this is taken as the actual pH for enzyme assays. In special cases, other aspects demand a deviation from the optimum

pH. An alkaline pH is applied to push the reaction of the alcohol dehydrogenase in the direction of the product against the reaction equilibrium, which favors the substrate.

1.3.2.1 Isoelectric Point

Besides the pH optimum, the **isoelectric point** (IP) is a characteristic value for enzymes. It is the pH value where the positive and the negative charges of the enzyme or protein are just counterbalanced; the protein is neutral without positive or negative surplus charges. At this pH, the enzyme possesses its lowest solubility in water and does not migrate in the electric field. This feature is used to determine the IP.

1.3.2.2 Buffers: What Must Be Regarded?

According to the importance of the pH for enzyme activity and stability the enzyme should always be kept at its optimum pH. With water alone as solvent this cannot be ensured. Additions, such as substrate or cofactors, if not strictly neutral, can modify the pH drastically. Therefore, buffers, commonly consisting of a weak acid and a strong basic component, are used to stabilize the pH. The relationship between the concentration of the buffer components and the pH is described by the **Henderson–Hasselbalch equation** (Box 1.6). The efficiency to stabilize the pH, the **buffer capacity**, depends on the concentration and on the interval between the actual pH and the pK_a value of the buffer. As a rule it can be assumed that the buffer capacity is, at most, within one pH unit below and above the pK_a value, covering two pH units. This is not a very broad range, but there exist various buffer systems such that for each pH a suitable buffer can be found (Table 1.2, Box 1.7). As long as the same pH is always required for the same assay there is no problem, but when, for example, for a pH optimum or

Box 1.6 Henderson–Hasselbalch Equation

Definitions

Mass action law for the acid (Ac):

$$K_a = \frac{[H^+][Ac^-]}{[HAc]}$$

K_a: ionization constant ~ dissociation constant for the acid

$-\log[H^+] = pH$ = negative logarithm of hydrogen concentration

Rearranging:

$$[H^+] = K_a \frac{[Ac^-]}{[HAc]}$$

$-\log K_a = pK_a$

Transforming into the logarithm

$$-\log[H^+] = -\log K_a - \log \frac{[Ac^-]}{[HAc]}$$

Final form of the Henderson–Hasselbalch equation:

$$pH = pK_a - \log \frac{[Ac^-]}{[HAc]}$$

Table 1.2 Biological buffers (pK_a values and pH range refer to 25 °C).

Short name	Full name	pK_a	pH range
Acetate	Acetic acid/sodium acetate	4.76	4.0–5.5
MES	2-(*N*-Morpholino)ethanesulfonic acid	6.15	5.5–6.7
Cacodylat	Cacodylic acid	6.27	5.4–7.0
Citrate	Citric acid/sodium citrate	6.4 (pK_{a3})	5.7–7.1
BIS–TRIS	Bis(2-hydroxyethyl)iminotris(hydroxymethyl)-methane	6.5	5.8–7.2
ADA	*N*-(2-Acetamido)-2-iminodiacetic acid	6.6	6.0–7.2
PIPES	Piperazine-*N,N′*-bis(2-ethanesulfonic acid)	6.88	6.1–7.5
ACES	*N*-(Carbamoylmethyl)-2-aminoethanesulfonic acid	6.9	6.2–7.6
MOPSO	3-(*N*-Morpholino)-2-hydroxypropanesulfonic acid		
BIS-TRIS PROPANE	1,3-Bis(tris[hydroxymethyl]methylamino) propane	6.8 (pK_{a1}) 9.0 (pK_{a2})	6.3–9.5
BES	*N,N′*-Bis(2-hydroxyethyl)2-aminoethane sulfonic acid	7.15	6.4–7.8
MOPS	3-(*N*-Morpholino)propanesulfonic acid	7.20	6.5–7.9
P_i	Phosphate	7.21 (pK_{a2})	6.5–8.0
TES	*N*-Tris(hydroxymethyl)methyl-2-aminoethane-sulfonic acid	7.50	6.8–8.2
HEPES	*N*-(2-Hydroxyethyl)piperazine-*N′*-(ethanesulfonic acid)	7.55	6.8–8.2
DIPSO	3-(*N,N′*-Bis[2-hydroxyethyl]amino)-2-hydroxy-propanesulfonic acid	7.6	7.0–8.2
MOBS	4-(*N*-Morpholino)butanesulfonic acid	7.6	6.9–8.3
TAPSO	3-(*N*-Tris[hydroxymethyl]methylamino)-2-hydroxypropanesulfonic acid	7.7	7.0–8.3
TEA	Triethanolamine	7.74	7.3–8.3
POPSO	Piperazine-*N,N′*-bis(2-hydroxypropane sulfonic acid)	7.85	7.2–85
HEPPSO	*N*-(2-Hydroxyethyl)piperazine-*N′*-(2-hydroxy-propanesulfonic acid)	7.9	7.2–8.6
EPPS (HEPPS)	*N*-2-Hydroxyethylpiperazine-*N′*-3-propanesulfonic acid	8.0	7.3–8.7
TRIS	Tris(hydroxymethyl)aminomethane	8.1	7.0–9.0
TRICINE	*N*-Tris(hydroxymethyl)methylglycine	8.15	7.4–8.8
Gly–amide	Glycinamide, hydrochloride	8.20	7.4–8.8
BICINE	*N,N*-Bis(2-hydroxyethylglycine)	8.35	7.6–9.0
Gly–Gly	Glycylglycine	8.40	7.7–9.1

(*continued*)

Table 1.2 (Continued)

Short name	Full name	pK_a	pH range
HEPBS	*N*-(2-Hydroxyethyl)piperazine-*N*′-(4-butanesulfonic acid)	8.3	7.6–9.0
TAPS	*N*-Tris(hydroxymethyl)methyl-3-aminopropane-sulfonic acid	8.4	7.7–9.1
AMPD	2-Amino-2-methyl-1,3-propanediol	8.8	7.8–9.7
TABS	*N*-Tris(hydroxymethyl)methyl-4-aminobutane-sulfonic acid	8.9	8.2–9.6
AMPSO	3-[(1,1-Dimethyl-2-hydroxyethyl)amino]-2-hydroxy-propanesulfonic acid	9.0	8.3–9.7
CHES	2-(*N*-Cyclohexylamino)ethanesulfonic acid	9.3	8.6–10.0
EA	Ethanolamine, hydrochloride	9.5	8.9–10.2
CAPSO	3-(Cyclohexylamino)-2-hydroxy-1-propanesulfonic acid	9.6	8.9–10.3
AMP	2-Amino-2-methyl-1-propanol	9.7	9.0–10.5
CAPS	2-(Cyclohexylamino)-1-propanesulfonic acid	10.4	9.7–11.1
CABS	4-(Cyclohexylamino)-1-butanesulfonic acid	10.7	10.0–11.4

stability curve a broader range should be tested, different buffer systems must be combined. This is not without problems, since unequal buffer systems differ in the ionic strength and the nature of ions and both can have a considerable influence on the enzyme activity even at the same pH values. Such deviations may be approximated by determinations at overlapping pH values, but this is not really satisfying. Instead, it is recommended to use universal buffers consisting of more components, such as the Teorell–Stenhagen or the Britton–Robinson buffer, both covering a broad pH range (Box 1.8).

Even in the presence of buffers, addition of acid or basic compounds can cause considerable pH changes. Some compounds, e.g. NAD^+, are available in an acid form and must be neutralized before usage. Also, the enzyme reaction itself can change the pH, as do lipases by releasing fatty acids.

For a distinct buffer system besides the desired pH other criteria must be considered. Some buffers, such as phosphate and especially diphosphate buffers, possess complexes forming capacities that may withdraw essential metal ions from the solution and from the enzyme and can form precipitates with them. Phosphate buffers are, on the other hand, inexpensive and compatible with most enzymes. Distinct buffer ions show activating or inhibiting effects with some enzymes. Also for the frequently used Tris/HCl buffer detrimental effects and even covalent reactions with proteins have been reported. Recommended for special enzymes are "biological buffers," such as MOPS, HEPES, and TES ("Good buffers," Table 1.2). Most buffers are stable for months or years, and only microbial attack, perceptible by a progressing turbidity, limits their stability. For longer

storage, addition of a preservative (e.g. EDTA) or freezing is recommended.

Owing to its strong temperature dependency the pH of the buffers must be adjusted at the actual working temperature, as regards the respective correction function of the pH meter. Instructions for buffer preparation refer usually to room temperature (25 °C), while enzyme studies are often carried out in the cold (~4°).

Box 1.7 How to Prepare Buffers?

Common Procedure

Example: 1 l 0.1 M Tris/HCl pH 8.1

Step 1: Dissolve the basic component in part of the final volume:
12.11 g Tris base (tris(hydroxymethyl)aminomethane, M_r = 121.14) in 600 ml H_2O

Step 2: Add the acid component (1 N HCl) dropwise under pH control until the desired pH (8.1) is reached

Step 3: Fill up to 1 l with H_2O

Phosphate Buffer

Example: 0.1 M potassium phosphate pH 7.5

Step 1: Prepare 1 M stock solutions:
- 136.1 g KH_2PO_4, fill up to 1 l with H_2O (acid solution)
- 228.2 g $K_2HPO_4 \cdot 3H_2O$, fill up to 1 l with H_2O (basic solution)

The concentrated solutions are stable for months

Step 2: Dilute aliquots of both solutions 10-fold (fill up 100 ml to 1 l H_2O)

Step 3: Adjust the acid solution at the pH meter to pH 7.5 by adding the basic solution under permanent stirring

1.3.2.3 How to Prepare Buffers?

Box 1.7 describes the general procedure for preparation of buffers. The weak component (e.g. Tris) is dissolved in a smaller (60–80%) volume than the final volume, and a (~10 times) concentrated solution of the strong component (e.g. HCl) is slowly added under permanent stirring until the desired pH is reached. Now the solution is filled up with water to the final volume. If large quantities of the same buffer are needed, e.g. for routine tests, it may be favorable to prepare a concentrated stock solution and to dilute just before usage. This also has the advantage that concentrated solutions are less susceptible to microbial growth. Since the pH of the buffer depends on its concentration the actual pH must be controlled after dilution. Therefore, it is preferable to store instead of the concentrated buffer the separate stock solutions of each component and dilute and adjust the pH immediately before usage. Special buffer systems and their preparation are described in Box 1.8.

Box 1.8 Preparation of Special Buffers

Phosphate Buffered Saline (PBS, Physiological Salt Solution)

0.02 M sodium/potassium phosphate pH 7.2, 0.9% NaCl
Dissolve 1.09 g KH_2PO_4, 2.14 g $Na_2HPO_4 \cdot H_2O$, and 9.0 g NaCl in 1 l H_2O

Borate Buffer, pH 8.2

Boric acid pK_{a1}: 9.2; tetraboric acid pK_{a2}: 7.8
Solutions:

- 50 mM sodium tetraborate (borax, $Na_2B_4O_7 \cdot 10H_2O$, $M_r = 381.4$; dissolve 3.84 g in 200 ml H_2O)
- 50 mM boric acid ($M_r = 61.83$; dissolve 1.24 g in 800 ml H_2O)

Combine the two solutions; the pH should be 8.6; adjust with 1 N HCl

Universal Buffer According to Teorell and Stenhagen, Buffer Range: pH 3.0–12.0

Solutions:

- 100 ml 0.33 M citric acid monohydrate ($M_r = 210.1$; 7 g in 100 ml H_2O)
- 100 ml 0.33 M phosphoric acid, 85% ($M_r = 98.0$; 2.2 g in 100 ml H_2O)
- 100 ml boric acid ($M_r = 61.8$; 3.54 g in 343 ml 1 M NaOH)

Mix the solutions and fill up to 900 ml with H_2O; adjust to the desired pH with 1 N HCl; fill up to 1 l with H_2O

Universal Buffer According to Britton and Robinson, Buffer Range: pH 2.6–11.8

Dissolve in 800 ml:

- 6.004 g citric acid monohydrate (28.6 mM)
- 3.89 g KH_2PO_4 (28.6 mM)
- 5.263 g barbital (28.6 mM)
- 1.77 g boric acid (28.6 mM)

Adjust to the desired pH with 1 N NaOH and fill up to 1 l with H_2O

1.3.3 Temperature Dependency

According to the Van't Hoff rule, the reaction velocity accelerates with increasing temperature by a factor of 2–3 per 10°. This is valid also for enzyme reactions. Thus, the activity of enzymes depends essentially on the assay temperature and it is an important question as to which temperature should be used. As enzymes are valuable compounds, assay conditions that generate the highest activities are preferred. This holds for the already discussed pH optimum and in comparison to this often a temperature optimum is mentioned,[13] although no such optimum

exists. According to the Van't Hoff rule the enzyme activity should increase with the temperature without passing any optimum. However, practical reasons, such as boiling of water, limit the assay temperature. A more severe limitation is the thermosensitivity of the protein structure. At higher temperature enzymes become irreversibly denatured, precipitate, and lose their catalytic capacity. Denaturation occurs not at the same temperature for all enzymes; rather it depends on various factors determining the enzyme stability. Some enzymes are very thermosensitive, while others, especially from thermophilic organisms, maintain their stability even at the temperature of boiling water. It is difficult to predict the relative temperature stability of a distinct enzyme from its structure and the same type of enzymes can be thermosensitive in one and thermostable in another organism, with only minor structural modifications. It is argued that thermostability is not really a problem for proteins. In the early time of evolution, when the temperature on the earth was considerably higher, proteins must have been resistant against high temperatures throughout. Concomitant with the decreasing earth temperature thermostability was no longer generally required and became lost. Owing to the diverse temperature preferences of enzymes a single standardized assay temperature cannot be defined.

The temperature dependency of the enzyme activity is determined by both effects, the increasing reaction velocity according to the Van't Hoff rule in the lower temperature range and the decrease due to thermal denaturation in the higher temperature range; together they form an apparent optimum curve (Figure 1.21). This apparent optimum is not at a constant temperature, but rather it depends on the incubation conditions. When incubated at a higher temperature the enzyme denatures in a time-dependent process, which usually follows exponential first order kinetics; it can be linearized in a half logarithmic representation (Figure 1.22). Only at low and moderate temperatures the enzyme will be stable. This behavior appears clearer in a diagram based on the Arrhenius equation (Box 1.9). Plotting $\ln \nu$ against $1/T$ (absolute temperature, Kelvin, $0\,°C = 273.15\,K$) yields a straight line indicating the range of stability of the enzyme and establishing the validity of the Van't Hoff rule (Figure 1.21). At higher temperature, when the enzyme becomes destabilized, the curve deviates from linearity, the reaction rate slows down, and finally reaches zero. This denaturation process is time dependent and more pronounced, the longer the enzyme remains exposed to the high temperature. Therefore, it makes a severe difference whether the assay is carried out immediately after addition of the enzyme or whether the enzyme has already remained for a distinct time at this temperature, such as the time needed after addition of the enzyme to start the assay. The actual velocity will decrease and the activity maximum in Figure 1.21 shifts to lower temperatures. This occurs only in the higher temperature range of denaturation; preincubation of the enzyme in the lower temperature range should not reduce its activity (if other reasons for destabilization of the enzyme do not exist; cf. Section 1.3.4).

13 Even in the BRENDA database temperature optima are indicated for each enzyme.

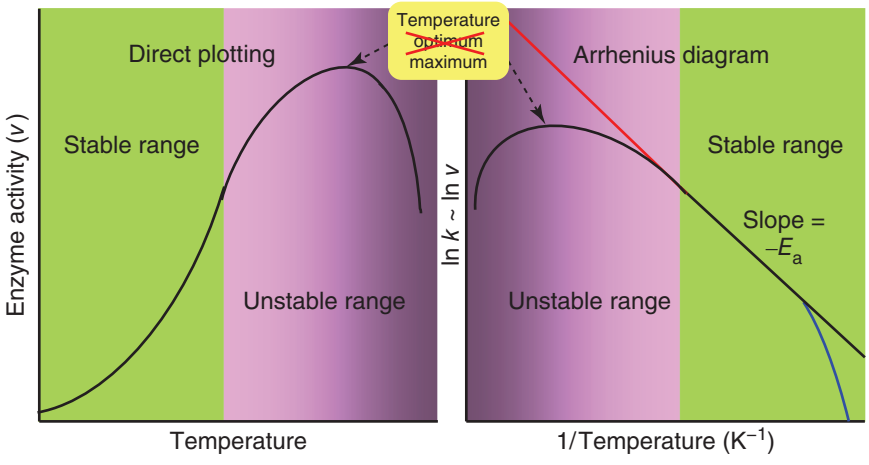

Figure 1.21 Temperature behavior of the enzyme activity in direct plotting and the Arrhenius diagram. Experimental region (black); hypothetical progression in the absence of enzyme denaturation (red); example for a physiological, temperature-dependent transition of the enzyme (blue).

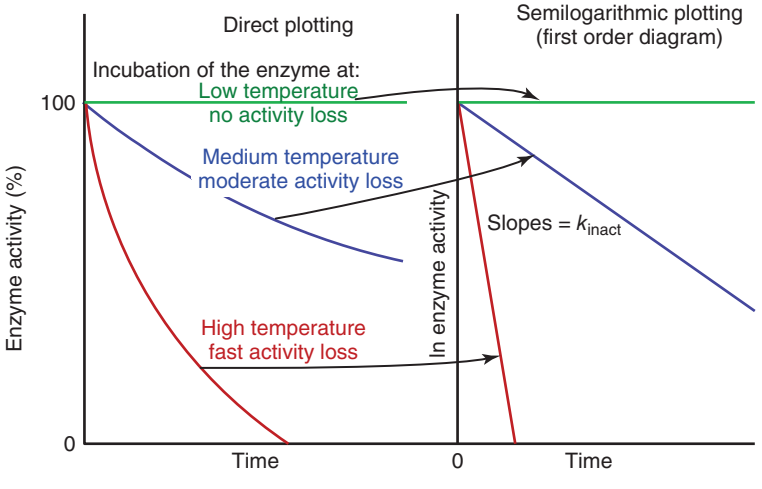

Figure 1.22 Temperature stability of the enzyme activity in direct and semilogarithmic diagrams. The enzyme is preincubated in different temperatures and tested after distinct time intervals at the normal assay temperature.

Box 1.9 Arrhenius Equation

The empirical equation of Svante Arrhenius

$$k = A e^{\frac{-E_a}{RT}}$$

is transformed into the logarithm for plotting

$$\ln k = \ln A - \frac{E_a}{RT}$$

$$\ln k = \ln A - \frac{E_a}{RT}$$

$$\log \frac{k_2}{k_1} = \frac{E_a}{2.3R}\left(\frac{T_2 - T_1}{T_1 T_2}\right)$$

According to this equation a plot of $\ln k$ against $1/T$ should yield a straight line

Definitions:

E_a: Activation energy
A: Collision factor
T: Absolute temperature
R: Gas constant
k: Catalytic rate constant (can be replaced in the plot by the velocity v)

Sometimes, deviations from linearity in the Arrhenius plot are observed even in the lower (stable) range, caused by transitions between different active states of the enzyme. Also, the presence of isoenzymes with similar activities but differing in their structure can be responsible for such inhomogeneities (Figure 1.21).

Owing to these reasons the thermal activity maximum is not useful as assay temperature. Rather a temperature in the stable, linear range should be chosen, evidently the highest temperature just before onset of denaturation. Because this temperature depends on the respective enzyme a universal standard temperature cannot be defined, but at least a preferential temperature suitable for the majority of the enzymes may be taken. Three such standard temperatures have been suggested, 25, 30, and 37 °C. The most convenient one may be chosen for the assay. The advantages and disadvantages of the respective temperatures are summarized in Box 1.10. To maintain constant temperature for the assay, tempering is inevitably required. Room temperature cannot be taken as constant; it depends not only on the actual room climate, but also on influences from the outside, such as solar radiation, open windows, or doors. Distinct enzymes, such as thermophilic or thermosensitive enzymes, have special temperature requirements. Some enzymes are even cold sensitive and become destabilized at very low temperatures. The most significant consequences of the temperature dependency of enzymes are summarized in Box 1.11.

Box 1.10 Standard Temperatures for Enzyme Assays

Temperature (°C)	Reasons in favor	Reasons against
25	Slightly above room temperature Easy to maintain No need for preincubation of the assay mixture Suited especially for thermosensitive enzymes	Low enzyme activities More enzyme required for the assay
30	Only modest tempering necessary Closer to physiological conditions Medium enzyme activity Reasonable enzyme amounts required	Preincubation of assay mixture required
37	Physiological temperature High enzyme activities Low enzyme amounts required for the assay	Preincubation of assay mixture inevitable Long warming up intervals Risk of incipient inactivation

Box 1.11 Consequences of the Temperature Dependency of Enzymes

- Enzymes are stable within a defined, mostly lower temperature range, discernible at the linear part of the Arrhenius diagram
- Activation energy calculated from the slope of this line (mostly between 40 and 60 kJ mol^{-1}) is a measure of the catalytic efficiency of the enzyme
- Deflections from the linear part can be indications for conformational changes or isoenzymes
- At high temperatures enzyme becomes instable
- Thermal inactivation counteracts the steady increase of the reaction rate with rising temperature according to the Van't Hoff rule
- These two counteracting effects form a temperature maximum, falsely designated as temperature optimum
- The maximum temperature is not a constant; it depends on the time the enzyme is exposed to this temperature; the longer the exposure, the lower the temperature maximum
- The actual assay temperature should be within the linear part of the Arrhenius diagram and not at the temperature maximum

1.3.4 Stability of Enzymes

1.3.4.1 Why Are Enzymes Unstable?

It is generally observed that the stability of enzymes is limited. In aqueous solution they lose their activity within weeks or even days; some become inactivated already within hours. The chemical nature of enzymes, their protein structure, gives no direct indication for such instability. Proteins can be considerably stable; active enzymes can even be found in Egyptian mummies. Therefore, knowledge of the reasons for the instability can help prolong the lifetime of a distinct enzyme. In the previous chapters, instabilities due to extreme temperatures, pH values, or ionic strength have been mentioned, and such conditions must be avoided. Keeping the enzyme at low temperature (+4 °C) in buffered solution at its pH optimum is generally recommended. Besides influences due to inappropriate conditions, especially proteolytic attack, chemical modifications caused by oxidative processes or reactive components in the solution are frequent reasons for inactivation (Box 1.12).

Box 1.12 General Reasons[14] for the Inactivation of Enzymes

Effect	Conditions of inactivation	Protection
Temperature	High temperature Very low temperature (seldom)	Low temperature (~4 °C)
pH	Extreme (low and high) pH values	pH optimum
Ionic strength	Very high (>1 M) and very low (<10 mM) electrolyte concentrations[15]	Medium electrolyte concentration (~0.05–0.2 M)
Proteolysis	Contamination with proteases not completely removed by the purification procedure	Protease inhibitors (cf Table 1.3) Use of recombinant enzymes
Chemical modification	Contamination with reactive reagents (SH-active compounds)	High purity of all components Avoidance of oxidative conditions Chelating reagents
SH poisoning	Oxidative conditions, promoted by heavy metal ions	SH reagents: dithiothreitol (DTT), dithioerythritol (DTE) mercaptoethanol

Proteolytic attack is mostly due to contamination with proteases from the same source from which the enzyme was isolated. Even very low amounts, not detectable in electrophoresis, can have a detrimental effect; proteases are themselves enzymes acting in catalytic amounts. The action of a protease becomes evident by the disappearance of the enzyme band in the electrophoresis,

14 Certain enzymes can have different requirements.
15 Enzymes from halophilic and thermophilic organisms prefer concentrated milieu.

concomitant with the appearance of new, smaller bands. Protease inhibitors can prevent proteolysis. Some special inhibitors are efficient against most proteases, others only against one type of proteases (Table 1.3). To avoid unintended co-purification of proteases with the desired enzyme the use of recombinant enzymes is recommended.

1.3.4.2 How Can Enzymes Be Stabilized?

Enzymes exist best in their natural environment in the cell. The cell medium is highly concentrated especially with proteins and, accordingly, enzymes prefer high protein concentrations, and correspondingly dislike strong dilution. This can be achieved by a high concentration of the enzyme itself (10–20 mg ml^{-1}). If high amounts of the enzyme are not available, addition of inert proteins, mostly serum albumin, has a similar effect. Serum albumin tempers also proteolytic or oxidative effects.

When treating enzymes, for example, during purification procedures, unsuitable conditions cannot always be avoided, causing temporary structure deformations and partial loss of enzyme activity. Structural areas from the inside, especially hydrophobic regions, become exposed and can either be a target for proteases (while native proteins are mostly protected, at least against proteases of the own cell), or promote aggregation with other proteins, producing insoluble, irreversible precipitates.

To avoid inactivation of the enzyme various additives can be added to the assay solution, depending on the special features of the respective enzyme. If not disturbing for the assay it is generally advantageous to add substrates and/or cofactors. Their binding stabilizes the three-dimensional enzyme structure. Mono- and bivalent metal ions are frequently added, act as counterions of surplus charges, and have stabilizing effects on the structure of distinct enzymes, especially the bivalent cations. On the other hand, heavy metal ions support oxidative attack, especially of thiol groups, and must be trapped with chelate forming substances such as EDTA, but also functional cations can be removed by such substances. To avoid poisoning of SH groups, thiol active reagents are added. Oxygen can be removed from the assay solution by applying a vacuum or degassing with a nitrogen stream. Sodium dithionite is very efficient in capturing traces of oxygen, but it is a strong reducing reagent that can damage the enzyme. A powerful oxygen trap is the coupled enzyme system glucose oxidase (GOD) and peroxidase (POD) or catalase (cf. Section 4.13). Additives for stabilizing enzymes are summarized in Table 1.3.

1.3.4.3 How to Store Enzymes?

Storage, especially for longer periods or for shipping, is a further problem due to the limited stability of enzymes. Several methods are summarized in Box 1.13. Their application depends on the compatibility with the respective enzyme. As already mentioned, enzymes are not very stable in dilute solutions, but even in concentrated solutions at low temperature their stability is limited due to proteolytic attack or growth of microorganisms, for which the enzyme serves as

Table 1.3 Frequently applied additives for enzyme assays.

Additive	Mostly applied substance	Concentration range	Application and remarks
Monovalent cations	K^+, Na^+	Dependent on special enzyme requirements, ~0.1 M	Essential for several enzyme reactions and for protein structure (counterions of surplus charges) Components of several buffers
Bivalent cations	Mg^{2+}, Ca^{2+}	Dependent on special enzyme requirements, ~1 mM	Cofactors of several enzyme reactions (metalloproteases, kinases, neutralization of di- and triphosphates [e.g. ATP, GTP, ThDP]) Stabilization of protein structure
Chelate former	EDTA, EGTA	1–2 mM	Capture divalent cations to protect from oxidative processes; however, also removal of cations essential for catalysis and structure
Thiol reagents	DTT, DTE mercapto-ethanol	0.1–0.2 mM	Protects SH group from oxidation and formation of —S—S-bridges
Protease inhibitors	α_2-Macroglobulin, leupeptin	~0.5 µg ml^{-1}	General protease inhibitors
	TCLK TPCK	~0.05 µg ml^{-1} 0.1 µg ml^{-1}	Inhibitors for serine and cysteine proteases
	PMSF	0.1 mg ml^{-1}	Inhibits serine proteases, instable in aqueous solution (0.5 hour half life!)
	E-64, calpain inhibitor I	~0.01 mg ml^{-1}	Inhibits cysteine proteases
	EDTA	1 mM	Inhibit metalloproteases by complexing of divalent cations
Proteins	Serum albumin	1 mg ml^{-1}	Protects against denaturation especially in diluted solutions
Oxygen traps	GOD/catalase, sodium dithionite	cf. Section 4.13 ~0.1 mM	Prevent oxidative modifications strong reducing reagent

Calpain inhibitor I, *N*-acetyl-leu-leu-norleucinal; DTE, dithioerythritol; DTT, dithiothreitol; E-64, *N*-[*N*-(L-3-trans-carboxirane-2-carbonyl)L-leucyl]-agmatine; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol-bis(ß-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; GOD, glucose oxidase; PMSF, phenylmethylsulfonyl fluoride; TCLK, L-1-chloro-3-(4-tosylamido)-7-amino-2-heptanone; ThDP, thiamine diphosphate; and TPCK, L-1-chloro-3-(4-tosylamido)-4-phenyl-2-butanone.

a nutriment. A simple preservation method is freezing. In the frozen state the enzyme is stable for long time, but a severe problem is the freezing and thawing processes; both are stressful for the enzyme, especially due to the shearing forces of the ice structure. Additives such as glycerol or sucrose moderate this effect, but repetitive freezing and thawing of the same enzyme sample should be avoided. It is advisable to divide the enzyme preparation in small samples before freezing. Similar problems of conversion of the enzyme into another state exist with other methods, such as precipitation and lyophilization. The latter is a practical method, if tolerated by the enzyme. The lyophilized samples can be stored in the refrigerator (4 °C) and be easily shipped in this state. For each special enzyme the appropriate method must be tried out.

Box 1.13 How to Store Enzymes?

Method	Conditions[16]	Advantages	Disadvantages
Aqueous solution	Buffered solution (pH optimum, 4 °C) Protectives (protease inhibitors, chelating and SH reagents)	Gentle method No special treatment required	Risk of proteolysis, oxidative processes, and microbial grown For short-term storage (days) only
Freezing	−20 to −80 °C Buffered solution, containing 20–30% glycerol or sucrose	Fast and simple method	Shearing forces (water crystals) are harmful for protein structure Permanent freezing required (risk of freezer defects, difficulties during shipping) Avoid repetitive freezing
Lyophilization	Solutions with volatile buffers to avoid high ionic strength Addition of stabilizers (glycerol) recommended	Stable preparation, easy to handle (e.g. shipping) Tolerate moderate temperature fluctuations	Often harmful for protein structure Risk of denaturation Accumulation of contaminant substances (buffer components)
Crystallization	Special crystallization conditions (ammonium sulfate, polyethylene glycol)	Stable preparation for long-term storage (years)	Laborious method, applicable only for enzymes easy to crystallize
Precipitation	Ammonium sulfate or polyethylene glycol precipitation	Easy method for long-term storage	Crude method, applicable only for stable enzymes

16 High protein concentrations (10–20 mg ml^{-1}) recommended for all methods.

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1.4 Theory of Coupled Enzyme Reactions

1.4.1 Two Coupled Reactions

If an enzyme reaction is not accessible to convenient detection methods it can be coupled to an easily measurable reaction. A prerequisite is that the coupled reaction must either accept the product of the test reaction as its own substrate, or the product of the coupled reaction must be accepted as substrate by the test reaction. If no such reaction exists, a third reaction can be included.

A typical example for coupling is hexokinase (HK) catalyzing the phosphorylation of D-glucose:

$$\text{D-Glucose} + \text{ATP} \xrightarrow{\text{HK}} \text{glucose-6-P} + \text{ADP}$$

Substrates and products exhibit no significant absorption difference. The product glucose-6-P is the substrate of glucose-6-phosphate dehydrogenase (G6PDH):

$$\text{Glucose-6-P} + \text{NADP}^+ \xrightarrow{\text{G6PDH}} \text{gluconate-6-P} + \text{NADPH} + \text{H}^+$$

$NADP^+$ becomes reduced during this reaction so that the absorption increase at 340 nm can be followed. The coupled reaction is called **indicator reaction** (G6PDH in our example), and the reaction under study (HK) **test** (or helper) **reaction**.

Some crucial aspects must be regarded for coupled assays. Optimum conditions should be maintained for both enzymes, but this can be problematic if they differ in essential features, such as pH optimum or temperature behavior. For instance, if one enzyme is thermophilic it will show its highest activity at a temperature where the other one denatures. Of importance is also the state of equilibrium of the reactions involved. At least the equilibrium of the final reaction must favor the product site to guarantee quantitative conversion. If this is not the case, the final reaction cannot be a reliable measure for the test reaction, and such a combination is inappropriate. It is, otherwise, no problem if the equilibrium of the first reaction favors its substrate, as long as the subsequent reaction proceeds quantitatively to the product site. It eliminates all the product of the first reaction and forces it also to proceed quantitatively against its own equilibrium.

Thus, the first requirement to develop a coupled enzyme assay is to determine test conditions compatible for all involved enzymes. If such conditions cannot be found, the assay can only be conducted stepwise, running the first reaction separately until it comes to an end, and thereafter conducting the second reaction.

The indicator reaction should never become limiting. Its enzyme, the cofactors, and cosubstrates, such as NADP in the above example, must be present in a large surplus. One should also be aware of the fact that any change of the test conditions during the experiment (e.g. substrate or cofactor concentration, addition of inhibitors) may alter the conditions in a manner that the indicator reaction becomes rate limiting, yielding incorrect results.

Two distinct arrangements requiring different treatments are possible with coupled enzyme assays. In most cases, the indicator reaction follows the test reaction and the detectable component arises at the end of the reaction sequence, as in the above example. The reverse case, where the indicator reaction precedes the test reaction, is seldom.

After starting a coupled assay with the indicator reaction at the end of the reaction sequence, a distinct time is needed to reach a stationary phase where formation and conversion of the intermediate substrate (P_{int}) are constant:

$$S \xrightarrow{E_{test}} P_{int} \xrightarrow{E_{in}} P_{end}$$

This stationary phase is the optimum state for the coupled assay and should be reached as fast as possible. To realize this, the test enzyme E_{test} must be limiting and must work under conditions of substrate saturation $[S] \gg K_m$, so that $v_{test} \cong V_{test}$. The indicator reaction works with a surplus of the indicator enzyme E_{in}. When the reaction starts a lag phase[17] is observed, during which the steady-state concentration of P_{int} increases (Figure 1.23). As P_{int} is supplemented by the primary reaction at constant velocity, the indicator enzyme reacts also with constant velocity v_{in}, which obeys the Michaelis–Menten equation and with $[P_{int}]$

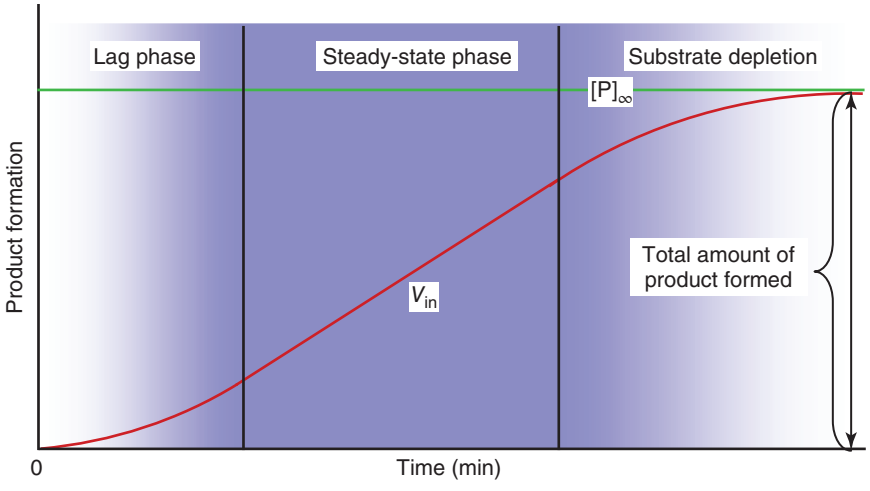


Figure 1.23 Progress curve of a coupled enzyme assay. For substrate determination the concentration of the product at the end of the reaction is measured, as indicated.

17 Lag phases occur sometimes even with normal reactions, when the enzyme is subject to a slow activation process, but can have also artificial reasons, such as warming up of the assay solution.

as substrate concentration. Because $v_{test} \cong V_{test}$, the effective velocity of the indicator reaction must be equal to the maximum velocity of the test reaction V_{test}:

$$v_{in} = \frac{V_{in}[P_{int}]}{K_{m/in} + [P_{int}]} = V_{test} \tag{1.18}$$

The concentration of the intermediate P_{int} under these conditions is

$$[P_{int}] = \frac{K_{m/in} V_{test}}{V_{in} - V_{test}} \tag{1.19}$$

Obviously, the intermediate concentration $[P_{int}]$ behaves in a reverse manner to the maximum velocity of the indicator reaction and thus to the amount of the indicator enzyme $V_{in} = k_{in}[E_{in}]$. Large amounts of the indicator enzyme thus reduce $[P_{int}]$ and improve the conditions for the coupled assay for linearity. Because of the versatile connections between the two reactions under various relationships of the components involved, there are different possibilities of order of the resulting reaction. Zero order, i.e. linearity, is achieved if the test reaction proceeds with its maximum velocity V_{test}. In the stationary state, if $[P_{int}] = \text{constant}$

$$v_{in} = \frac{V_{in}[P_{int}]}{K_{m/in} + [P_{int}]} = \frac{V_{in}}{\frac{K_{m/in}}{[P_{int}]} + 1} \tag{1.20}$$

The denominator $K_{m/in}[P_{int}]^{-1} + 1$ will also be constant and the reaction proceeds in a linear manner with constant velocity. It is important that the stationary (zero order) state is reached fast, since the initial lag phase approaches asymptotically and the substrate concentration decreases constantly so that the time period, during which substrate saturation prevails, is limited.

Coupled reactions with two substrates are treated in a similar manner. As long as both substrates can be regarded as saturating, the condition $v_{test} \cong V_{test}$ should hold. But it is often difficult to fulfill conditions of saturation for both substrates simultaneously, e.g. with dehydrogenases, NADH cannot be added in too high concentration due to its high absorbance.

In special cases, for example, when the substrate of the test enzyme is unstable and must be provided by the preceding reaction, the indicator reaction can precede the test reaction. No principle problem exists if the preceding reaction serves only as provider for the substrate and the test reaction can directly be measured, as long as the first enzyme is present in surplus so that the test reaction becomes rate limiting. This is the case for the malate dehydrogenase (MDH) reaction:

$$\text{Oxalacetate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{MDH}} \text{malate} + \text{NAD}^+$$

when the unstable oxalacetate is supplemented by the aspartate aminotransferase (AAT) reaction:

$$\text{Aspartate} + \text{2-oxoglutarate} \xrightarrow{\text{AAT}} \text{oxalacetate} + \text{glutamate}.$$

More difficult is the situation where the provider reaction is at the same time the indicator reaction to be determined, as in the case of citrate synthase (CS), where oxalacetate becomes supplemented by the MDH reaction:

$$\text{Malate} + \text{NAD}^+ \xrightarrow{\text{MDH}} \text{oxalacetate} + \text{NADH} + \text{H}^+$$

$$\text{Acetyl-CoA} + \text{oxalacetate} \xrightarrow{\text{CS}} \text{citrate} + \text{CoA}$$

This reaction sequence is used for the determination of acetate (respectively acetyl-CoA). However, the amount of NADH formed in the first reaction must not be proportional to the acetate turnover in the second reaction. This is only the case when the equilibrium of the first (indicator) reaction favors the substrate. Under this condition, only small amounts of NADH will be formed, until the CS reaction captures oxalacetate and thus forces its formation, and consequently also that of NADH. To enable this, the equilibrium of the CS reaction must favor the end product. This is a general rule for coupled assays with initial indicator reactions: the equilibrium of the first reaction should favor the substrate and that of the test enzyme should favor the product.

1.4.2 Three Coupled Reactions

The situation with three coupled enzyme reactions is principally similar to that with two reactions. The first reaction carried out by the test enzyme is followed by a second enzyme reaction (E_{con}), connecting the test reaction with the indicator reaction:

$$S \xrightarrow{E_{test}} P_{int1} \xrightarrow{E_{con}} P_{int2} \xrightarrow{E_{in}} P_{end}$$

here, both intermediate products P_{int1} and P_{int2} must be kept very small to reduce the initial lag phase. The effective velocities of both the connecting and the indicator enzyme should be similar and equal to the maximum velocity of the test reaction:

$$V_{test} = \nu_{con} = \nu_{in}$$

1.5 Substrate Determination

The high specificity of enzymes can be used for precise determination of concentrations of compounds such as metabolites even in crude cellular extracts. The respective compound must be accepted by an enzyme as its substrate and converted to product. The relative change due to the enzyme reaction indicates the presence and the amount of the respective compound. The fact that the respective compound is not identified by its absolute absorption but by its relative change enables detection out of a variety of other metabolites. Care must be taken, especially in crude extracts, that the determination will not be disturbed by side reactions. The reaction will either be directly followed if substrate or product can be detected by an appropriate measuring method, or coupled to a further enzymatic

reaction with a detectable product, such as a dehydrogenase. Two principally different methods for enzymatic substrate determination are applied, the end point and the kinetic method.

1.5.1 End Point Method

The end point method is the best and simplest procedure, provided that the substrate or product can directly be determined and the reaction proceeds irreversibly. The reaction is started by addition of enzyme, employing the respective test conditions. The course of the reaction must be followed, either continuously (e.g. photometrically) or by removing and analyzing samples after distinct time intervals. When the turnover ceases and the reaction comes to its end, a positive plateau for product formation (Figure 1.23) and a negative one for substrate consumption are obtained. The plateau value is directly related to the amount of product formed, which can be calculated from the absorption coefficient or from a calibration curve of a known standard solution. As long as the reaction proceeds irreversibly, substrate and product concentrations should be identical. It must, however, be considered that the real plateau value will be reached only *ad infinitum*; if the experiment is terminated too early the value will be underestimated. Since slow enzyme reactions require a long time to reach the plateau, high enzyme amounts should be taken, which can also serve to avoid disturbing influences, such as inactivation or side reactions.

With two-substrate reactions, when the substrate to be measured is difficult to identify, the easier detectable cosubstrate reacting with the same stoichiometry can be determined (e.g. NADH). In this case, the cosubstrate (as well as all other components) must be present in a higher concentration than the substrate; otherwise, the actual amount of the cosubstrate and not of the substrate will be measured.

For substrate determination linear initial velocities are not essential as for the determination of the enzyme activity, and thus steady-state conditions must not be regarded. More of interest is the final phase of the reaction, which, in its simplest case, obeys first order (or pseudo-first order with two-substrate reactions, with the cosubstrate being present in surplus):

$$\ln[\mathrm{A}] = \ln[\mathrm{A}]_0 - k_1 t \tag{1.21}$$

Table 1.4 shows that the time required for a nearly complete conversion of substrate is 10-fold compared to that needed to reach 50% turnover. Real conditions can severely deviate, since the actual reaction must not obey first order and also other influences may be considered, especially product inhibition, which can essentially extend the time required to reach equilibrium.

Many enzyme reactions do not convert the substrate completely to product, but rather approach an equilibrium state with a defined substrate–product ratio. In such cases, two different modes for substrate determination can be applied. From the amount of product formed at the end of the reaction the total amount can be calculated with the aid of the equilibrium constant of the reaction. Likewise, in a reference experiment the percentage of product formed from a certain initial

Table 1.4 Time required for the conversion of substrate to product assuming an irreversible first order reaction and a half-life time of 10 minutes.

Product formed (% of $[A]_0$)	Reaction time (min)
50	10.0
80	23.3
90	33.3
95	43.4
98	56.5
99	66.7
99.9	100.0

substrate amount is determined and from the result the amount of an unknown sample can be estimated.

Alternatively, quantitative conversion of the substrate can be enforced even in the case of an unfavorable equilibrium state by trapping the product with the aid of chemical or enzymatic reactions. In the alcohol dehydrogenase reaction aldehydes or ketones formed are trapped with semicarbazide or hydrazine. Protons can be withdrawn from the equilibrium in the presence of alkaline pH. When inorganic phosphate in the glyceraldehyde-3-phosphate dehydrogenase reaction is substituted by arsenate in place of the 1,3-bisphosphoglycerate, the unstable 3-phosphoglycerate-1-arsenate is formed. Its rapid decay drives the reaction quantitatively to the product site.

1.5.2 Substrate Determination by Coupled Enzyme Reactions

Coupled enzyme reactions can also be applied for substrate determination. Here the conditions are less stringent as for determination of enzyme activity, since only the value at the end of the reaction must be obtained. The essential prerequisite is that the final indicator reaction must react quantitatively to the product. One or more reactions can precede the indicator reaction. They must not necessarily be irreversible, since the indicator reaction removes the intermediate from the equilibrium to form the final product. If two or more enzymes are coupled in a reaction sequence, only one must be completely specific for its substrate. An example is the relatively unspecific conversion of glucose by HK:

$$\text{D-Glucose} + \text{ATP} \xrightarrow{\text{HK}} \text{ADP} + \text{D-glucose-6-phosphate}$$

Besides D-glucose the enzyme accepts also other hexoses, such as fructose and mannose. For selective determination of glucose, instead of HK, the more

specific, but also more (about 250-fold) expensive, glucokinase may be taken. But since the following indicator reaction of the G6PDH

$$\text{D-Glucose-6-P} + \text{NADP}^+ \xrightarrow{\text{G6PDH}} \text{gluconate-6-P} + \text{NADPH} + \text{H}^+$$

is highly specific for D-glucose-6-phosphate, by-products of HK originating from other substrates do not affect the quantitative determination of glucose in the coupled assay.

1.5.3 Kinetic Method for Substrate Determination

The initial substrate concentration of an enzyme-catalyzed reaction is related to the reaction velocity according to the (rearranged) Michaelis–Menten equation (1.12):

$$[\text{A}] = \frac{K_\text{m} v}{V - v} \tag{1.22}$$

Thus, knowing the kinetic constants K_m and V, the actual substrate concentration can be derived from the initial velocity. Likewise, a standard curve following the hyperbolic Michaelis–Menten curve can be prepared by determining the reaction velocity at different substrate concentrations. With this method, the substrate can be determined only in the lower concentration range ($[\text{A}] \leq K_\text{m}$), while in the higher range (nearer to saturation) even strong variations in the substrate concentration cause only slight changes in the velocity.

A more stringent relationship holds for first order reactions. Here, the substrate conversion within a defined time period $\Delta t = t_2 - t_1$ is directly proportional to the initial substrate concentration $[\text{A}]_0$:

$$-\frac{\text{d}[\text{A}]}{\text{d}t} = k_1[\text{A}] \tag{1.23}$$

$$[\text{A}] = [\text{A}]_0 \text{e}^{-k_1 t} \tag{1.24}$$

$$-\frac{\text{d}[\text{A}]}{\text{d}t} = k_1[\text{A}]_0 \text{e}^{-k_1 t} \tag{1.25}$$

$$[\text{A}]_0 = -\frac{\Delta[\text{A}]}{\text{e}^{-k_1 t_1} - \text{e}^{-k_1 t_2}} \tag{1.26}$$

If k_1 is known a concentration change between a defined time interval $t_2 - t_1$ is measured, from which [A] can be determined. For this fixed-time procedure the time interval $t_2 - t_1$ must be the same for all measurements. With the Michaelis–Menten equation first order conditions can only be achieved at low substrate concentrations ($[\text{A}] \ll K_\text{m}$). Therefore, the amounts of substrate to be determined by this procedure must be rather low. Alternatively, the Michaelis constant should be high. This condition can be achieved by addition of a competitive inhibitor, which increases the apparent Michaelis constant. An example of substrate determination with the kinetic method is the glucose determination with the coupled assay of GOD and POD (cf. Section 4.13).

1.5.4 Enzymatic Cycling

Very low amounts of metabolites can be detected by enzymatic cycling. The metabolite to be determined functions as an intermediate within a reaction

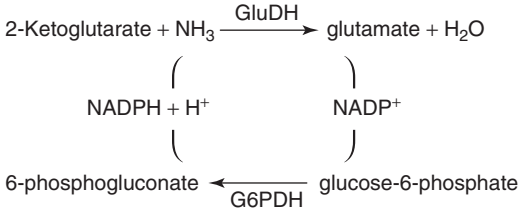
sequence and remains constant in its concentration. Coenzyme A (CoA) is an example of a metabolite occurring in the cell at very low concentration. In a coupled reaction it can be formed from acetyl phosphate by phosphate acetyl-transferase (PTA). MDH serves as the indicator reaction and provides oxaloacetate for citrate formation by the CS:

$$\text{Acetylphosphate} + \text{CoA} \xrightarrow{\text{PTA}} \text{acetyl-CoA} + P_i$$

$$\text{Malate} + \text{NAD}^+ \xrightarrow{\text{MDH}} \text{oxaloacetate} + \text{NADH} + \text{H}^+$$

$$\text{Acetyl-CoA} + \text{oxaloacetate} + \text{H}_2\text{O} \xrightarrow{\text{CS}} \text{citrate} + \text{CoA}$$

Low amounts of NAD or NADP can be determined by enzymatic cycling, e.g. the regeneration of NADPH by coupling of the glutamate dehydrogenase (GluDH) and G6PDH.



In this case, the constant remaining NADPH intermediate cannot be used as indicator reaction. Instead the reaction is stopped after a defined time, e.g. 30 minutes. The total amount of 6-phosphogluconate formed is analyzed separately with the 6-phosphogluconate dehydrogenase reaction applying the end point method. By a large number of passages through the cycle a more than million fold increase in sensitivity can be achieved. Since the amount of the intermediate that should be determined remains constant during the reaction, it can be regarded like a catalyst.

Generally, the reaction sequence for enzymatic cycling can be formulated as

$$\text{A} + \text{B} \xrightarrow{\text{E1}} \text{P} + \text{Q}$$

$$\text{P} + \text{C} \xrightarrow{\text{E2}} \text{A} + \text{R}$$

The sum of the reaction sequence is

$$\text{B} + \text{C} \rightarrow \text{Q} + \text{R}$$

A is the substance to be determined, either a cosubstrate or a coenzyme. The concentration of A must be limiting and must be smaller than its own K_m value, and the concentrations of the other two substrates B and C must be large. Also, the activities of both enzymes E1 and E2 should be high. When the cyclic system reaches the steady state, the rate of formation of P must be equal to the back reaction for the reformation of A:

$$k_1[\text{A}] = k_2[\text{P}]$$

The first order rate constant for the overall reaction k is

$$k = \frac{V}{K_m} = \frac{k_1 k_2}{k_1 + k_2} \tag{1.27}$$

V is the velocity with saturating levels of A, the substrate or cofactor to be determined.

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