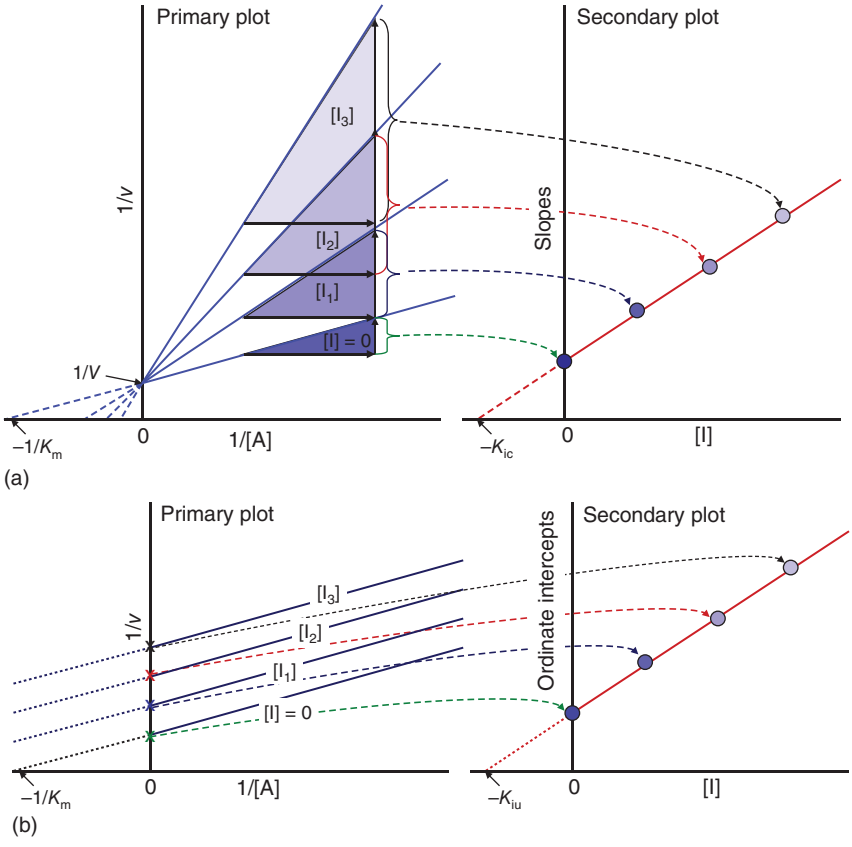

Figure 1.17 Secondary plot (right), derived from the slopes (a) and the ordinate intercepts (b) of the lines in the primary plot (Lineweaver–Burk plot, left). In the case of multisubstrate reactions on the abscissa 1/cosubstrate concentration (1/[B]) is plotted instead of [I], both in (a) and (b).

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

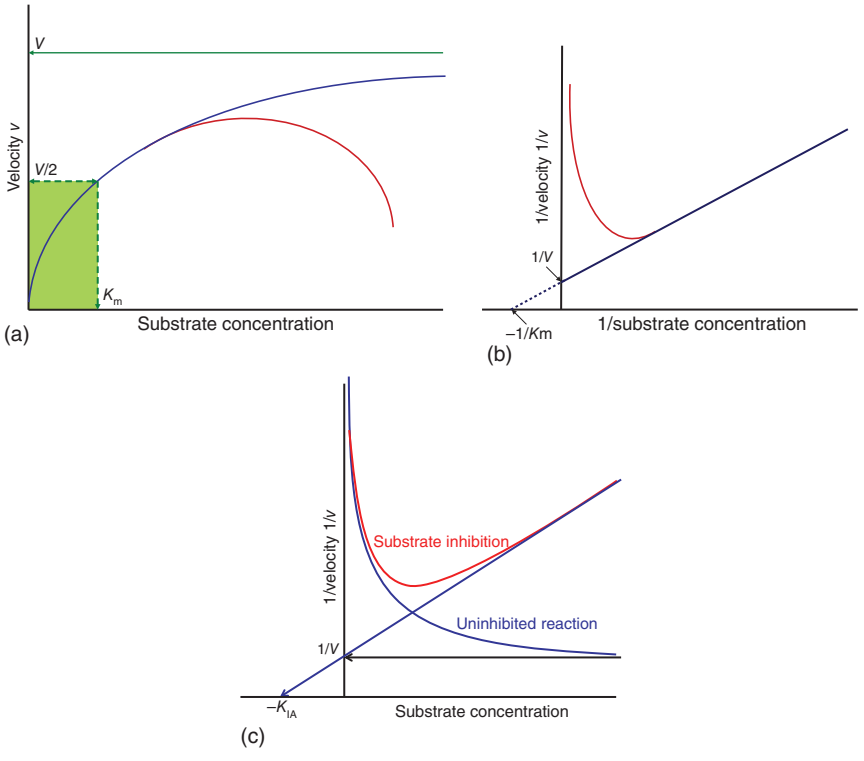

Figure 1.18 Substrate inhibition. (a) Michaelis–Menten plot; (b) Lineweaver–Burk plot; (c) Dixon plot. Lines in the experimental region (solid, blue); deviation due to substrate inhibition (red); lines in the extrapolated region (dotted, blue). The determination of kinetic constants is shown.

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

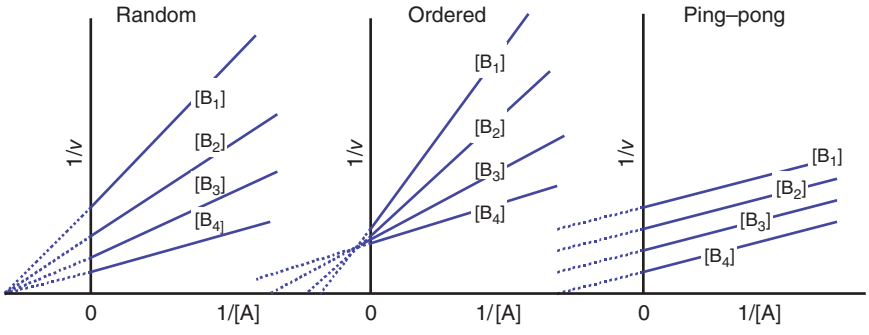

Figure 1.19 Multisubstrate mechanisms in the Lineweaver–Burk diagram. ———, lines in the experimental region; ..... lines in the extrapolated region.

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

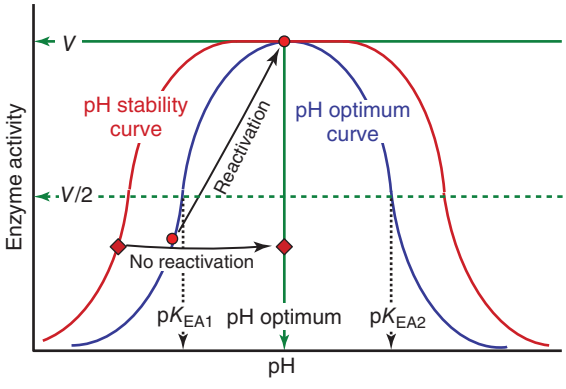

Figure 1.20 pH behavior of the enzyme activity. pH optimum curve: the enzyme is tested at the pH indicated. pH stability curve: the enzyme is preincubated for a distinct time (e.g. one hour) at the pH indicated and tested at the pH optimum. The p*K* values can be obtained from the inflexion points of 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.

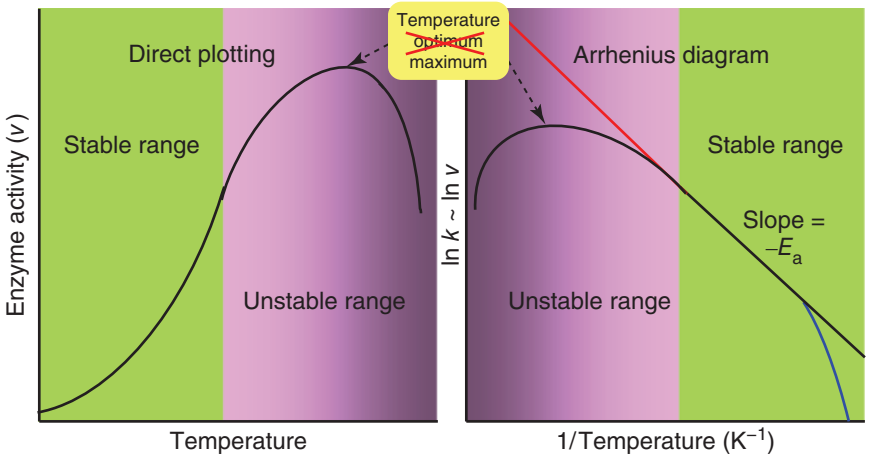

Figure 1.21 Temperature behavior of the enzyme activity in direct plotting and the Arrhenius diagram. Experimental region (black); hypothetical progression in the absence of enzyme denaturation (red); example for a physiological, temperature-dependent transition of the enzyme (blue).

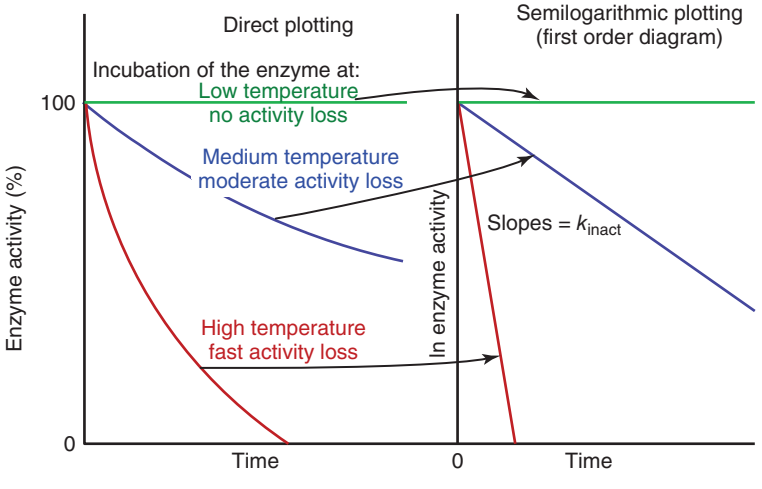

Figure 1.22 Temperature stability of the enzyme activity in direct and semilogarithmic diagrams. The enzyme is preincubated in different temperatures and tested after distinct time intervals at the normal assay temperature.

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.

Literature

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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 $[P_{int}]$

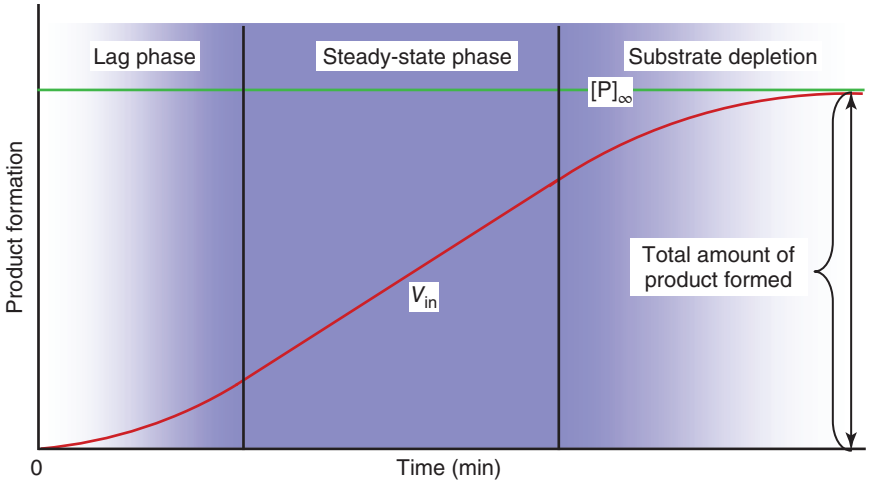


Figure 1.23 Progress curve of a coupled enzyme assay. For substrate determination the concentration of the product at the end of the reaction is measured, as indicated.

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

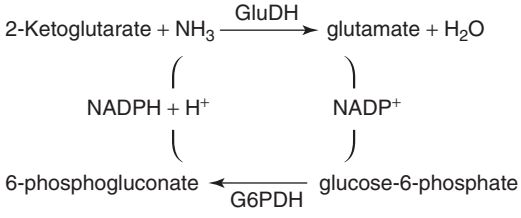
sequence and remains constant in its concentration. Coenzyme A (CoA) is an example of a metabolite occurring in the cell at very low concentration. In a coupled reaction it can be formed from acetyl phosphate by phosphate acetyl-transferase (PTA). MDH serves as the indicator reaction and provides oxaloacetate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