# Principles of Enzyme Assays

# 12

Enzymology is a quantitative and exact science. Therefore it is important to understand how enzyme activity is measured and presented. A robust and reliable measure of the progress of an enzyme-catalyzed reaction is first and foremost requirement. Like with any other chemical reaction, progress of an enzyme-catalyzed reaction can be monitored either by the product formed ($d[P]/dt$) or by the substrate consumed ($-d[A]/dt$). The two rates are of course related by the reaction stoichiometry. It is desirable and often safe to follow the formation of product – a substance is better estimated when it is formed in a background where very little (or none) of it exists. On the other hand, to measure a decrease in the concentration of a reactant as it disappears – a small change in a large background – becomes daunting. In practice, a small decrease in substrate is relatively more difficult to observe than to follow a buildup of product from nothing. This is particularly relevant when we wish to record the initial rate (rate during very early time after the reaction is initiated, abbreviated as "v"), which is given by $d[A]/dt$ when $[P] \approx 0$. This is the rate at the beginning of the reaction or the instantaneous rate extrapolated to time zero.

## 12.1 Detection and Estimation Methods

Reliable methods of detection and estimation, of product formation or substrate depletion, are at the heart of a successful enzyme assay. Designing convenient and reliable assays is the first important step in studying a new enzyme activity. This in turn is limited by the creativity of the investigator alone. In principle, any signal that differentiates the substrate(s) or product(s) from other reaction components can form the basis for an enzyme assay. One usually looks for some physicochemical property of the substrate or product as a handle. Spectral properties (unique to substrate or product) are most often exploited for this purpose. The great majority of enzyme assays are based on absorption measurements. Detection methods available to follow the course of an enzymatic reaction are listed in Table 12.1. For many enzyme



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**Table 12.1 Detection methods used in enzyme assays**

| Technique | Detection of | Enzyme assay for |
|---|---|---|
| *Optical measurements* | | |
| UV spectroscopy | NADH, $A_{340nm}$ | Alcohol dehydrogenase; lactate dehydrogenase; malate dehydrogenase |
| Visible spectroscopy | *p*-Nitrophenol, $A_{405nm}$ | Alkaline phosphatase |
| Polarimetry | Optical rotation, [α] | Invertase |
| Turbidimetry (Nephelometry) | Attenuation of incident light (intensity of scattered light) | Lysozyme |
| Fluorimetry | Fluorescein; ↓at 470 nm and ↑at 510 nm | Cholinesterase; acylase; chymotrypsin |
| Luminometry | Luciferin; ↑at 562 nm | Luciferase |
| *Electrochemical measurements* | | |
| pH meter/pH-stat | $[H^+]$ change | Lipase; cholinesterase; urease; glucose oxidase |
| | Carbon dioxide | Carbonic anhydrase |
| Potentiometry | $Fe^{2+}/Fe^{3+}$ | Oxidase reactions (cytochromes) |
| Amperometry | $O_2$ | Oxygenases; glucose oxidase |
| *Manometric measurements* | | |
| Warburg manometer | $O_2$ consumed, $CO_2$ released | Respiratory enzymes; decarboxylases |
| *Radiotracer measurements* | | |
| Scintillation counter | β-Emission | Dehydrogenases ($^3H$); glutamate decarboxylase ($^{14}C$); protein synthesis ($^{35}S$); kinases; enzymes of nucleic acid metabolism ($^{32}P$) |

assays, these detection methods cannot be directly applied if the method does not discriminate between the substrate, product, or any of the reaction components. Then their prior separation becomes necessary. One of the many separation techniques like chromatography or electrophoresis may be combined with one of the detection techniques. We shall refrain from describing separation techniques any further. However, a detailed treatment on this topic may be found in any text covering analytical biochemistry.

Two very commonly used tools are absorption spectroscopy and fluorescence spectroscopy. Absorption of a molecule at a particular wavelength can be related to its concentration by the Beer–Lambert law:

$$I = I_0 e^{-\varepsilon c l} \quad \text{and} \quad A = \varepsilon c l$$

where **A** is the absorbance ($-\log I/I_0$) of the sample at a fixed wavelength (in nm), $\boldsymbol{c}$ is the sample concentration (in molar units), and $\boldsymbol{l}$ is the path length of the light passing through the sample (usually 1 cm). The intrinsic property of a molecule $\boldsymbol{\varepsilon}$ is a constant known as extension coefficient or molar absorption coefficient. This has typically the units of $M^{-1}$ $cm^{-1}$. The larger the value of $\boldsymbol{\varepsilon}$, the greater is the

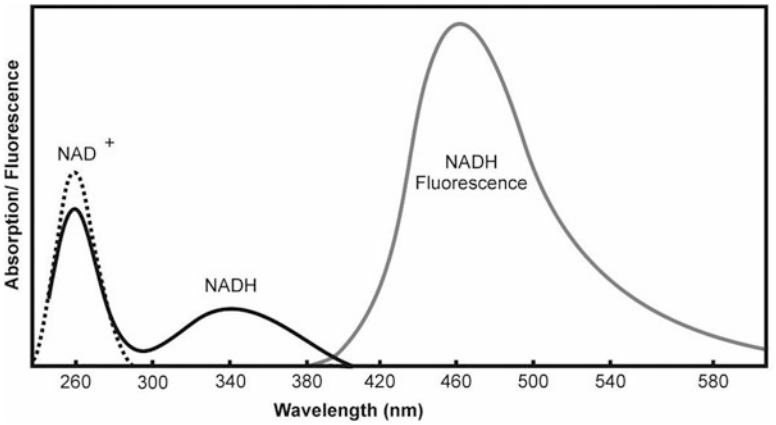


**Fig. 12.1 Ultraviolet absorption spectra of reduced and oxidized NAD**. Corresponding spectra for NADPH and $NADP^+$, respectively, are almost identical

sensitivity of that detection method. Knowing the value of $\boldsymbol{\varepsilon}$ for a given molecule and the path length (commonly the UV-visible spectrophotometer cuvettes have a path length of 1 cm), absorbance values can be directly related to concentration. For instance, many enzymes of biochemical importance involve interconversion of oxidized ($NAD^+$) and reduced (NADH) forms of NAD. We can devise a spectrophotometric assay based on the large absorbance difference at 340 nm ($\boldsymbol{\varepsilon} = 6220\ M^{-1}\ cm^{-1}$) between them (Fig. 12.1). Suppose the absorption at 340 nm decreases (oxidation of NADH) by 0.0622 in a cuvette of 1 cm path length, in 1 min. The reaction velocity may then be expressed and calculated as shown:

$$v = \frac{-d[S]}{dt} = \frac{-\Delta A}{\varepsilon l} \times \frac{1}{\Delta t} = \frac{0.0622}{6220 \times 1} \times \frac{1}{1} = 10^{-5} M\,\text{min}^{-1}$$

The enzyme velocity is expressed here as molar per minute. By accounting for the total volume of the reaction mixture, this can also be given in units of moles per minute.

**Suitability of a Detection Method** Each one of the methods listed in Table 12.1 has its own merits and demerits. Let us analyze them in some detail.

1. A common drawback associated with absorption measurements is deviation from Beer-Lambert law. The linear relationship between absorption of the sample and its concentration holds only over a finite range of absorbance values. This has to be firmly ensured for accuracy of analysis. It is generally more difficult to measure a small absorption change for a sample with high initial (background) absorbance. Sample turbidity is another problem. Turbid samples show light scattering and vitiate the measurements – they need to be filtered beforehand.

2. Fluorimetric assays are relatively more sensitive (at least by a factor of about 100) than the absorption-based methods. While absorption is measured at a single wavelength, fluorescence inherently exploits two distinct wavelengths – excitation wavelength (normally at the absorption maximum of a molecule) and an emission wavelength. Many fluorophores have large Stokes shifts – the fluorescence emission maximum is farther away (toward longer wavelengths) from the excitation maximum. For this reason there will be fewer components that interfere with a unique fluorescence signal. Since not all molecules fluoresce, it may be necessary to attach a fluorescent tag to the substrate in order to develop a fluorescence-based enzyme assay. While most limitations of absorption spectroscopy also apply to fluorescence measurements, there are additional caveats. Sources of interference in accurate fluorescence detection include particulate matter, photodecomposition, polarity of the environment, temperature, and various quenching effects. Lastly, while the quantum yield of the fluorophore is an intrinsic constant for that molecule, the signal obtained from a fluorimeter is relative and cannot be directly compared.
3. Any reaction leading to pH changes can be followed using a pH meter or a pH-stat. Precautions are in order since enzyme reactions are strongly pH-dependent.
4. The oldest but a cumbersome manometric analysis is often substituted by other tools. For instance, decarboxylations are best measured by monitoring the release of $^{14}CO_2$ from a suitably labeled substrate.
5. The use of radioisotopes in enzyme assays invariably makes it a discontinuous method (see below). Since both the substrate and the product are radioactive, an appropriate technique (chromatography or electrophoresis) to separate them becomes mandatory. Most common radioisotopes that find application in enzyme assays are β-emitters ($^{3}H$, $^{14}C$, $^{32}P$, and $^{35}S$). Radiotracer analysis can be very sensitive but requires that suitably labeled substrates are available. Also, handling radioactivity requires experimental rigor and much care. The radioactivity is measured in a scintillation counter; the readout (in counts per min, cpm) can be converted into disintegrations per min (dpm), and this is related to concentration through specific radioactivity (such as μCi/μmol). Since scintillation counting measures light emission problems, and precautions associated with fluorimetry also apply to this technique.
6. Whatever the detection method, before trusting the output provided by any instrument/machine, it is important for an experimenter to be certain that those numbers are properly calculated and are reliable.

**Direct or Indirect Detection** In many enzyme assays the detection techniques described in Table 12.1 can be directly applied. For example, lactate dehydrogenase catalyzes the oxidation of NADH while stoichiometrically reducing pyruvate to lactate. Concomitantly, NADH absorption (at 340 nm) decreases as a function of reaction time. Such assays are called *direct assay*s because NADH (substrate) disappearance is directly measured. We may not always be lucky to devise such a direct enzyme assay. Neither the substrate nor the product of an enzymatic reaction

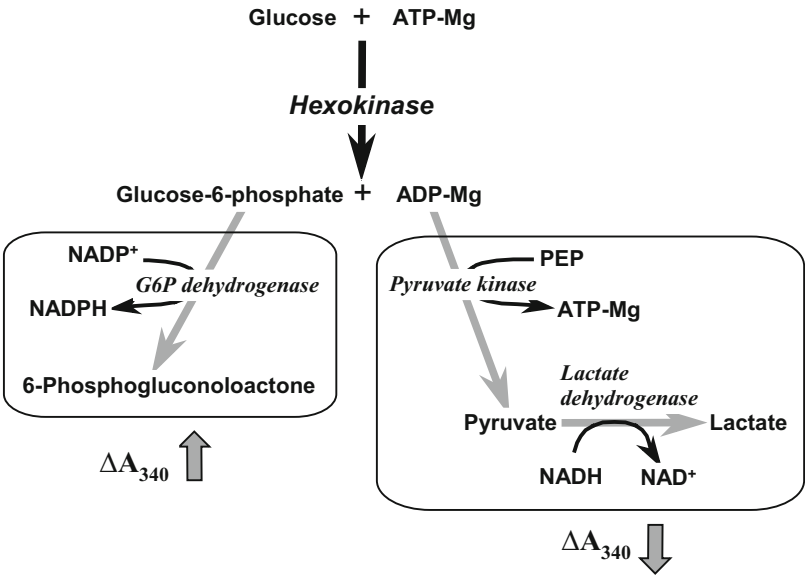


**Fig. 12.2 Coupled-enzyme assays to monitor hexokinase reaction**. (1) Glucose-6-phosphate is detected indirectly as an increase in $A_{340nm}$ due to NADPH formed. (2) Mg-ADP is converted to Mg-ATP, while phosphoenolpyruvate (PEP) forms pyruvate. In the second coupled step, pyruvate is detected indirectly as a decrease in $A_{340nm}$ due to NADH oxidized

may provide a distinct signal, convenient for measurement. It may, however, be possible to chemically convert the product into a convenient signal. Such a detection strategy is known as an *indirect assay*. Monitoring arginase and glutamate dehydrogenase activities provides two such examples. Firstly, urea, a product of arginase reaction, is converted to a yellow-colored complex (and measured at 478 nm) by reacting with dimethylglyoxime reagent. Secondly, the electrons generated through glutamate oxidation (captured as NADH and transferred via phenazine methosulfate) are made to stoichiometrically reduce 2,6-dichlorophenolindophenol (DCPIP). Decrease in blue color (DCPIP absorbs at 600 nm, but its reduced product does not!) with time is thus a good, indirect measure of glutamate dehydrogenase activity.

The above examples of indirect assays generate a detectable signal from the product by coupling to a nonenzymatic, chemical reaction. However it may be possible to couple a second (sometimes even a third!) enzyme to the reaction to be observed. In such *coupled-enzyme assays*, the second enzymatic reaction is chosen for its convenience of measurement. Monitoring hexokinase reaction provides a succinct example of how coupled assays are designed. These approaches are shown in Fig. 12.2. Neither glucose-6-phosphate nor Mg-ADP provides any direct means of detecting them in the background of the hexokinase assay. However, glucose-6-phosphate is a substrate for glucose-6-phosphate dehydrogenase (G6P dehydrogenase); $NADP^+$ is reduced to NADPH in the presence of this coupling enzyme, and

indirectly glucose-6-phosphate can be monitored at 340 nm. At times, it may be desirable to follow the other product of hexokinase reaction. Mg-ADP can be detected by coupling to two enzymes – pyruvate kinase and lactate dehydrogenase – and oxidation of NADH to $NAD^+$ (Fig. 12.2).

Because they involve multiple enzymes in the same reaction mixture, coupled-enzyme assays are tricky to perform and require utmost care when high kinetic rigor is necessary. The following conditions have to be met for a successful coupled-enzyme assay: (a) it is necessary to identify conditions (such as a common pH) that are compatible for all the enzymes involved, (b) the reaction to be monitored should be the sole rate limiting step and not the subsequent steps used for coupling, and (c) effects of inhibitors and other assay conditions to be tested on the enzyme of interest (like hexokinase above) should not interfere with the coupling reactions. For these reasons, rigorous kinetic analysis with coupled-enzyme reactions is difficult. However coupled assays are convenient and simple to routinely follow enzyme activity when high accuracy is not needed – say during stages of purification. A final aspect of using coupled-enzyme assays is the quality and cost of coupling enzyme (s) used. While it may be required in high amounts (this adds to cost) for an efficient assay, its purity cannot be compromised. Simply stated, an enzyme chosen for coupling cannot have certain impurities that catalyze unwanted reactions. For instance, estimation of glucose by glucose oxidase–peroxidase pair involves $H_2O_2$ as the stoichiometric intermediate. Presence of catalase as an impurity severely compromises this assay.

Nowadays, developing a method of detection from scratch is rarely required. Assays for almost every known enzyme may be found in the dedicated series *Methods in Enzymology* (Academic Press). Simplicity of operation is an important criterion for choosing a method of detection. After that, techniques that permit continuous monitoring of an enzyme reaction are desirable (see below).

## 12.2 Enzyme Reaction Time Course

Armed with a convenient detection method, time courses for an enzymatic reaction are easy to conduct. Progress curves for the product formation may be generated in two distinct modes (Fig. 12.3). Reaction progress could be monitored continuously with a suitable signal and an automatic recorder. For example, formation (or disappearance) of NADH is easily followed in a recording spectrophotometer at 340 nm. Such *continuous assay*s are very desirable as they provide the safest mode of determining initial reaction rates. We may not always be lucky to establish a continuous assay for the enzyme of interest. Then the reaction/assay has to be quenched (stopped) at preselected time intervals to allow for subsequent product measurement. This second strategy of generating a progress curve is called *discontinuous assay* (or *end-point assay*). An enzyme assay based on radioactivity invariably makes it a discontinuous assay. One has to separate the product from the remaining substrate, as both of them will contain the label.

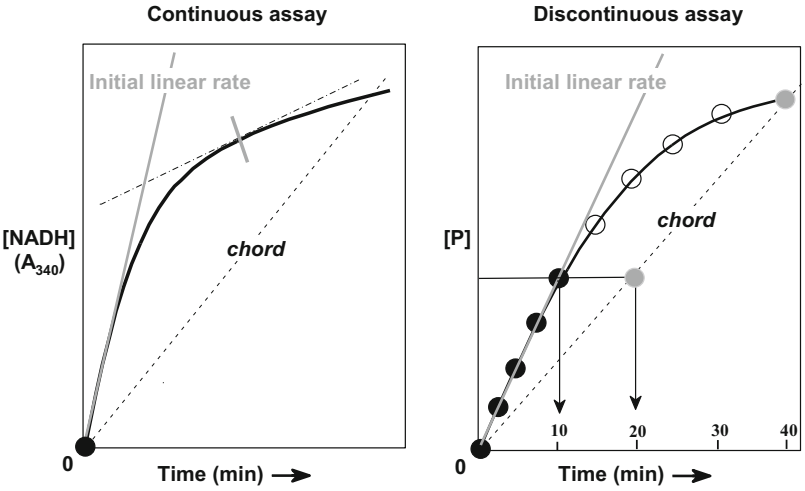


**Fig. 12.3 Progress curve for an enzyme-catalyzed reaction**. Estimation of initial rate from a continuous assay is possible by aligning a tangent to the early phase of the progress curve. Tangents at any other point on the curve or a chord always underestimate the true initial velocity. In discontinuous assays any data point beyond linear portion of the plot (such as data beyond 10 min; open circles in the right panel) is unsuitable for rate measurements. Velocity estimates from a single-point assay after 40 min (data point in gray; as defined by the chord) are just half of the true initial velocity

Whenever used, the method of stopping (quenching) the reaction should be such that it completely stops the reaction. And it must not interfere with the subsequent product determination step.

A reliable progress curve is particularly relevant when we wish to record the initial rate, i.e., $-d[A]/dt$ when $[P] \approx 0$. This is the rate at the beginning of the reaction or the instantaneous rate extrapolated to time zero. It is possible to evaluate reaction velocity from the slope of a plot of signal versus time (Fig. 12.3). Obtaining perfectly linear initial velocity for an enzyme-catalyzed reaction is a challenge. Progress curves are often *nonlinear*. This is because the reaction rate changes – usually decreases – due to consumption of substrate(s), accumulation of product(s), loss of enzyme activity with time, etc. An uninterrupted monitoring (a continuous assay) gives a clear picture of the extent of nonlinearity. One attempts to find the initial rate (and not the average rate!) from such progress curves (Fig. 12.3). If there is nonlinearity, then precise extrapolation to zero time is the only way out. This is done by drawing a tangent (and not a chord!) as close to the origin as possible. Manually, the tangent for a curve is best drawn using a glass rod. A straight line at right angles to the curve can be drawn (by aligning the glass rod such that when seen through it, the curve appears continuous and without the two breaks), and then the required tangent is obtained as a line perpendicular to the first one. However, most recording spectrophotometers are equipped with programs to analyze the progress curves and provide best estimates of initial velocity.

While continuous assays are desirable, discontinuous assays can be resorted to with due precautions. Ensuring linearity of a reaction progress curve is much more demanding with discontinuous assays. The reason being product measurements are made at preselected time points, and this data is interpolated/extrapolated to generate the entire progress curve. The problem is severe when single end-point assays are used. It is therefore necessary to exercise great care and attention – to ensure that the assay is indeed linear for the entire period. For example, proper initial velocity will not be estimated for any data point beyond 10 min in Fig. 12.3 (right panel)! If attempted, almost invariably this will be an underestimation of true initial velocity (i.e., $-d[A]/dt$ when $[P] \approx 0$). It is therefore mandatory to establish the extent of linearity before extracting rate information from such data.

**Reasons for Nonlinearity** Time course of an enzyme-catalyzed reaction is initially linear, but the rate (slope of the curve!) starts to decline at later time points (Fig. 12.3). Since true initial velocity is best obtained from linear time courses, it is important to understand the reasons for departure from linearity. Many of these were succinctly listed by Haldane in his early classic (Haldane 1930). If the velocity falls off during the reaction, then one or more of the following may be occurring:

1. As the substrate is continuously consumed, the actual [*S*] falls with time. The period of linearity would therefore be expected to be longer at higher initial [*S*] values. Nonlinearity is more pronounced at low [*S*] and with high $S \rightarrow P$ conversion. In order to obtain linearity at lower [*S*], however, highly sensitive assays are required so that smaller values of [*P*] can be detected.
2. Increasing [*P*] with time leads to increase in backward reaction ($P \rightarrow S$) rates. The net forward rate will continue to fall until the forward and backward rates become equal, and the equilibrium is reached.
3. Product, by virtue of being derived from the cognate substrate, often interacts and reversibly binds to the enzyme to form inactive species. For instance, when NADH is a substrate for an enzyme, the corresponding product $NAD^+$ retains significant affinity to bind and inhibit the enzyme.
4. The assay pH may change during the course of the reaction. For instance, pH will decrease when an esterase will continuously liberate an acidic product from a neutral substrate. Unless the assay system is adequately buffered, this change of pH results in nonlinearity of the reaction time course.
5. Irreversible loss of one of the assay components may be occurring during the assay. It could be an unstable enzyme or a less stable substrate. By isolating the effect of one component at a time, the probable cause of nonlinearity can be identified and excluded.

Increasing the straightness of a progress curve may be attempted by addressing the above issues. Fortunately, in many cases, the reaction initial velocity remains constant for a relatively long period and hence can be measured accurately. It is desirable to observe the progress curve for a longer time period to discern the curvature properly. This helps better estimate the initial linear rate.

Despite taking precautions to overcome the different reasons for nonlinearity listed above, some enzymes show either a burst or a lag in the product formation rate before the linear phase is attained. When they are not artifacts of the assay, such a *burst phase* or a *lag phase* can give valuable information about the enzyme reaction mechanism. An excellent example of burst kinetics is the hydrolysis of *p*-nitrophenyl acetate by chymotrypsin. When *p*-nitrophenol formation is monitored, there is an initial rapid formation (burst phase) followed by a slower linear (steady-state) rate. The slower linear rate is governed by the rate of hydrolysis of the acetyl-enzyme. The burst kinetics here provides useful kinetic evidence for the occurrence of an acyl-enzyme reaction intermediate during catalysis.

## 12.3 Precautions and Practical Considerations

Reliable kinetic data is a direct outcome of clean experimental design and good kinetic practices. In order to obtain meaningful results without artifacts and/or interferences, certain practical considerations are important. Although not meant to be exhaustive, some of these are listed below with suitable examples.

**Purity of Assay Components** The purity of substrates, buffers, and other assay components plays a crucial role in the final outcome of an enzyme assay. At the least, unaccounted impurities result in overestimating the concentration of that component. For example, samples of $NAD^+$ purchased from suppliers often contain extraneous matter like buffer salts, moisture, etc. Obviously the substance is not 100% $NAD^+$. A sample of $NAD^+$ from a vendor may be 95% $NAD^+$ and the rest made up of alcohol, water, and phosphate buffer (carried over from the method of its preparation). While making stock solutions of such components, one should exercise precaution. $NAD^+$ stocks may be routinely calibrated in a spectrophotometer directly at 259 nm and also by reducing the same to NADH ($\varepsilon = 6220\ M^{-1}\ cm^{-1}$ at 340 nm). Obtaining accurate concentrations may require the use of primary standards – just the same way we make primary standards for acid-base titrations.

At times the impurity may be a potential inhibitor or activator. Vanadate ($VO_4^{3-}$) present in ATP samples was identified as an inhibitor of ATPase activity. It is thus desirable to use vanadate-free ATP for unbiased ATPase assays.

Mg-ATP is the true substrate of most kinases. Many of them are discriminatory and respond to sub-micromolar concentrations of $Mn^{2+}$ in the presence of millimolar concentration of $Mg^{2+}$ ions. Magnesium salts quite often contain low levels of $Mn^{2+}$ – this trace impurity may significantly interfere in $Mg^{2+}$ studies.

**Stability of Assay Components** Instability of substrate, product, or the enzyme itself can vitiate the outcome of an assay. If the substrate is unstable and is destroyed during the course of the assay, then the effect [*S*] would be significantly different from what is actually added. Accurate representation of the actual concentration is

difficult with inherently labile molecules like superoxide anion ($O_2^-$), carbamyl phosphate, etc. Ferrous ions are inserted into porphyrins by ferrochelatase; $Fe^{2+}$ species is susceptible to oxidation under assay conditions, while $Fe^{3+}$ is not a substrate. Measuring oxaloacetate reduction rates can be affected by its instability and loss due decarboxylation. Similarly if the product is labile, we may underestimate the true reaction rate. Many molecules like inorganic pyrophosphate (PPi), $H_2O_2$, etc. are unstable in the conditions of the assay – their actual concentration may be underestimated unless this loss is accounted for.

**Nature of the True Substrate** The true substrate for a number of enzymes is a complex of the substrate and a divalent metal ion. Most widely studied example is Mg-ATP, the true substrate of most enzymes that are ATP-dependent. Similarly, other chelating substrates like citrate, isocitrate, etc. can exist in free or complexed form – the two forms may be differently accepted by an enzyme. Citrate binds $Mg^{2+}$ much more tightly than isocitrate. The presence of $Mg^{2+}$ ions alters the apparent aconitase equilibrium since only the uncomplexed forms serve as substrates.

A compound in solution may exist in more than one form, and only one of these is an effective substrate for the enzyme. The substrate aldehyde, of glyceraldehyde-3-phosphate dehydrogenase, also occurs as a hydrate. In fact most of it in solution is in the form of the hydrate – the remaining 3% free aldehyde is the true substrate. Glutamate γ-semialdehyde (GSA) – an intermediate in the biosynthesis and catabolism of glutamate, proline, and ornithine – is another interesting example of this kind. Glutamate γ-semialdehyde exists in unfavorable equilibrium (Fig. 12.4) with its intramolecular cyclization product pyrroline-5-carboxylate (P5C). The aldehyde form itself suffers another equilibrium between the free and hydrated state. So, for an enzyme interacting with glutamate γ-semialdehyde, only a small fraction (0.05%) of the total is available in solution (Beame and Wolfenden 1995). Oxidation of glucose by glucose oxidase provides yet another illustration of substrate inter-conversions. In solution three forms of D-glucose exist in equilibrium – linear chain (traces), the α-anomer (36%), and the β-anomer (64%). Only the β-anomer is acted upon by glucose oxidase.

Many enzymes act on substrates that are optically active. Racemic mixtures cannot be treated as the true substrate – only one stereoisomer (either D(*R*) or L(*S*) substrate) may be acted upon by the enzyme. It cannot be just assumed that effective substrate concentration is half of the total; in some cases the wrong stereoisomer may inhibit the enzyme and complicate the kinetic data.

Heterogeneity and nonspecificity of substrates should be carefully considered. Many protein kinases are routinely assayed by their capacity to phosphorylate casein; in almost all such cases, the true physiological substrate is unknown.

As long as the nature of the true substrate (or inhibitor) for an enzyme is known, the actual concentration can be and should be evaluated for accurate representation of data.

**Contribution by Nonenzymatic Rates** A number of substrates are inherently unstable and hence disappear with time. As long as the reaction rate is measured

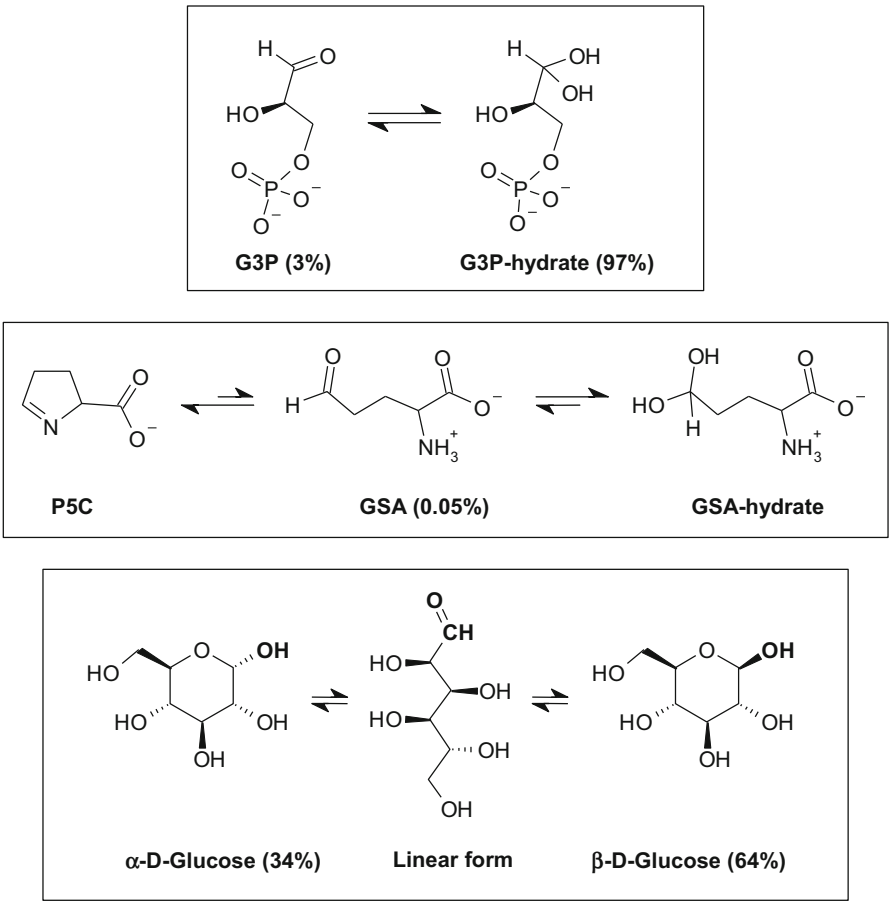


**Fig. 12.4 Compounds that show inter-converting forms in solution**. The true substrate for glyceraldehyde-3-phosphate dehydrogenase (G3P), glutamate γ-semialdehyde dehydrogenase (GSA), and glucose oxidase (β-anomer of D-glucose) forms only a fraction of the total concentration present

as disappearance of substrate, this contributes to the enzymatic rate. Substrates like NAD(P)H, tetrahydrofolate, $O_2^-$, $H_2O_2$, thiol (RSH), p-nitrophenylacetate, p-nitrophenylphosphate, etc. are unstable in solution. They get converted to the same end product as that formed by the corresponding enzymatic reaction. Reaction between different components of an assay mixture can also contribute to blank rates. For example, the nonenzymatic reaction of thiols with hydrogen peroxide is significant even in the absence of the peroxidase. Such nonenzymatic rates have to be accounted for and suitably subtracted from the measured values to obtain true enzymatic rates. For instance, significant hydration of carbon dioxide occurs ($CO_2 + H_2O \rightleftarrows HCO_3^- + H^+$) in water; only after subtracting this rate from the

rates obtained in presence of carbonic anhydrase, actual enzyme-catalyzed rates are obtained.

**Careful Examination of Interferences** Apart from their direct action on the enzyme, substrate, or product, reagents of an indirect assay can interfere in the measurements. Enzyme assay strategies based on indirect (and discontinuous) methods necessitate many controls. For instance, glucose assay using glucose oxidase–peroxidase coupled-enzyme system is sensitive to many reducing/oxidizing compounds. Redox-active compounds, other than glucose, may interfere in a peroxidase reaction. Thiourea drastically reduces the color yield of Chinard's ninhydrin method (of ornithine estimation) thereby appearing as if it is an arginase inhibitor (Sudarshana et al. 2001). Therefore, a careful examination of all possible interferences in the chosen assay method becomes important.

**Control of Assay pH, Temperature, and Ionic Strength** Activities of most enzymes are sensitive to changes in the assay parameters like pH, temperature, ionic strength, etc. Unless these are strictly maintained, the results of such enzyme assays are useless. We will have more to say later (Chap. 13, Good Kinetic Practices) on how to control these parameters and design good enzyme experiments.

**Nature of Enzyme Preparation** Several aspects of the enzyme sample used in the assay influence the measured rates. Some component may be inadvertently carried in to the assay along with the enzyme – this may be an activator or inhibitor. One possible outcome of their interference is nonlinear enzyme concentration curve. Few illustrative examples of how the nature of enzyme sample matters are listed below:

1. Ammonium sulfate used to precipitate the enzyme protein may affect the enzymatic rates. While ammonium is a substrate/product of some enzymes, the high ionic strength (μ) contributed by it may activate/inhibit the enzyme activity.
2. The enzyme may have been so prepared that significant fraction of it is in the apoenzyme form. Such subsaturated enzymes show sub-optimal activity in the assay. For instance, yeast pyruvate decarboxylase loses thiamine pyrophosphate during purification, and addition of this cofactor is required to reconstitute full enzyme activity. It is not uncommon to find an enzyme which binds loosely to other cofactors like pyridoxal phosphate, divalent metal ion, etc.
3. A bound activator/inhibitor may be associated with or have co-purified with the enzyme. Despite extensive studies on glycolysis and phosphofructokinase over the century, an important allosteric effector (fructose-2,6-bisphosphate) was discovered only recently (in early 1980s). While the loss of activator by dilution may decrease the measured enzymatic rate, removal of an inhibitor from the enzyme preparation results in perceptible increase in observed rates.
4. Activities of contaminating enzyme(s) at times interfere in the assay of enzyme of our interest. There may be other activities in the preparation that compete for the same substrate or product. For instance, a nonspecific ATPase in a kinase preparation contributes to excess ATP hydrolysis. Similarly, assay of

dehydrogenases in crude tissue preparations becomes difficult because of the presence of nonspecific NADH oxidase activity. Monitoring glucose through glucose oxidase–peroxidase coupled system requires that the two enzymes used are devoid of catalase contamination. Otherwise the $H_2O_2$ formed is destroyed by catalase. In some cases the contaminating enzyme may simply exploit the assay conditions and add its own rate to the true rate. Pyruvate decarboxylase activity is determined by measuring the reduction of acetaldehyde (coupled to alcohol dehydrogenase and conversion of NADH to $NAD^+$). Since reduction of pyruvate by NADH can also occur, this assay system does not distinguish pyruvate decarboxylase from lactate dehydrogenase. The observed activity therefore should be corrected for controls performed in an identical manner but omitting alcohol dehydrogenase.

5. The discovery of DNA replication (and of DNA polymerase) is an interesting historical case of the nature of enzyme preparation leading to discovery. While the [$^{14}$C]thymidine incorporation into an acid-insoluble form led Severo Ochoa to polynucleotide phosphorylase, a similar approach with an *E. coli* extract steered Arthur Kornberg to DNA polymerase I. A purified enzyme and his commandment V (– *Do not waste clean enzymes on dirty substrates* (Kornberg 2000)) eventually resulted in the discovery of RNA priming for DNA synthesis.

Finally, one should be aware that in some cases the same enzyme may exhibit additional catalytic activities. This can be experimentally confirmed, however (see Chap. 14, Quantification of Catalysis and Measures of Enzyme Purity). For instance, the oxygenase activity of RuBP carboxylase is not due to a contamination; but the two reactions are catalyzed at the same active site.

**Enzyme Stability** Proteolysis often leads to enzyme inactivation over time. Apart from inactivation due to contaminating endogenous proteases, an enzyme preparation may lose its activity for other reasons. For instance, presence of heavy metal ions ($Hg^{2+}$, $Ag^+$, or redox-active metal ions like $Cu^{2+}$, $Fe^{2+}$, etc.) may inhibit/inactivate the enzyme. Activation of fructose-1,6-bisphosphatase by EDTA (chelating agent) could be ascribed to its ability to chelate inhibitory heavy metal ions. Enzyme instability during the time course of the assay interferes and complicates initial velocity measurements. Whatever be the reason, it is of interest to know whether enzyme activity is being lost during the assay. Such enzyme inactivation can be detected by a simple test described by Selwyn (see Chap. 13).

## 12.4 Summing Up

A robust and reliable assay method is fundamental to the measurement of enzyme activity. It is often necessary to use different assay methods for characterizing the behavior of a single enzyme. The choice of a method also has to take into account key features like sensitivity, convenience, economy, and reliability. Students are often initiated into an enzyme study by providing them a published assay procedure.

In most cases the literature is so presented as to highlight on the strengths of a method. The *controls* are taken for granted, and on rare occasions explicit mention is made of interfering factors. Careful controls therefore are "not extra" but absolutely essential in the use of an existing assay procedure as well as those being newly established.

While arriving at a suitable method to quantify enzyme activity, the following points are borne in mind. (a) It is convenient to measure precisely a finite increase (in the product concentration) from zero than a small decrease (in the substrate concentration) from a large initial concentration. (b) Continuous assay methods score over stopped assays. They provide a continuous readout as the reaction proceeds, thereby enabling one to detect any deviations from linearity. For single-time point assays, it is mandatory to establish reaction linearity with time and enzyme concentration (c) A detection method that directly measures the changing reactant concentration is desirable. Alternative assay strategies based on indirect measurements necessitate many more controls.

Developing an assay to make reliable initial velocity measurements is a prerequisite for scientific and meaningful characterization of any enzyme activity. It is critical to collect good enzyme data in the first place; because any degree of sophisticated analysis later will not transform bad data into good data. Keeping in view all aspects described in this chapter (and the next!), a robust assay should be chosen. The next most logical step is to generate a reliable time course for the enzyme-catalyzed reaction. From this data, at as early time points as possible, initial velocity (linear rates) may be obtained. Collecting initial velocity data at low [*S*] is always a challenge – as nonlinearity sets in early under these conditions. A plot of initial velocity versus [*E*] (the enzyme concentration curve) should then be constructed (Fig. 12.5). A linear relationship between initial velocity and [*E*] is a

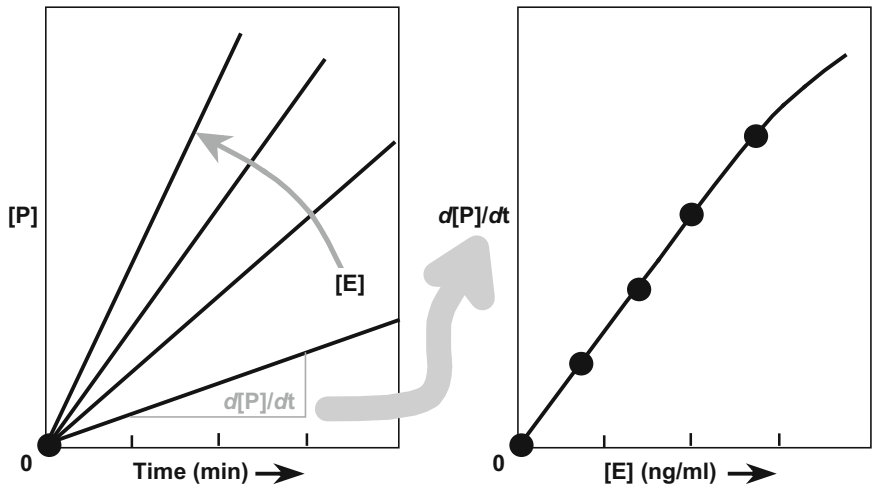


**Fig. 12.5 Enzyme concentration curve**. This may be constructed by plotting linear initial velocity ($d[P]/dt$) data at different [*E*] values

good control and a measure of the reliability of the assay. From all the iterated information, excellent data can be gathered for enzyme kinetic analysis.

Additional experimental measures that constitute good kinetic practices are described in the next chapter.

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