

Basics of enzyme initial velocity and inhibition studies

Introduction and Scope

This document is intended to introduce a new worker to some issues in setting up and interpreting initial velocity enzyme kinetics. Other laboratory documents cover enzyme inactivation planning, enzyme inactivation, endpoint (stop time) assays, theory or practice of regression, buffers, and desalting, among other topics. The purpose of this document is to provide general guidance and tips to help a worker avoid some common pitfalls. Some references are given at the end. Another document “Spectrophotometric enzymatic stop-time assays” is closely related to this one; however, it treats only end-point assays.

General Considerations

Control of pH and ionic strength

It is vitally important to minimize variation in pH or in ionic strength from one assay to the next. Many enzymatic substrates or inhibitors are salts, and when they are varied in concentration, the pH and the ionic strength may be affected.

Stability of stock solutions of reagents

It is generally best to make solutions of substrates close in time to when they are used or to freeze them in small aliquots. Thawing and refreezing a single aliquot of a reagent multiple times may lead to degradation. It is generally better to prepare the substrates or inhibitors without buffer, so that the conditions established by the buffer of the assay are not altered. For some reagents, it may be necessary to make stock solutions in *dilute* buffer, to minimize its degradation or to adjust the pH of the stock solution with small amounts of acid or base.

Stability of stock solutions of enzymes

Enzymes may be much more susceptible to loss of activity by freeze/thaw cycles than other components of the assay. Each enzyme must be treated on a case-by-case basis in terms of maximizing. In some cases, the enzyme is first prepared as a stock solution in a dilute buffer that may be very different from the assay buffer and may contain stabilizing agents not found in the assay. Often this stock is stored on ice over the period in which the assays are performed, often several hours. The stability of the enzyme in the stock solution must be assessed separately from the stability of the enzyme during the assay.

When performing a large pH or inhibition study, it is often necessary to combine the results of enzyme assays performed on different days. This may require the experimenter to normalize the rates in some way.

Purification of substrates

Sometimes substrates are purchased with small amounts of impurities that may interfere with the assay. For example 4-nitrophenyl phosphate may have small amounts of 4-nitrophenol and inorganic phosphate. Dual specificity phosphatases may be harder to assay in the presence of 4-nitrophenol than in its absence (owing to a high background reading), and alkaline phosphatase is

inhibited by free phosphate. Therefore, different applications may require different purification schemes even of the same substrate.

Purification of enzymes

Commercially prepared enzymes may have other enzyme activities that interfere with the assay. Another kind of problem often encountered is that salts interfere with some assays. For example, ammonium sulfate (found in high concentration in some suspensions of enzymes) inhibits some phosphoryl-group transfer enzymes. There are several ways to remove ammonium sulfate and other salts, which are discussed in a different document.

Preparation of solutions of substrates or inhibitors

It is often preferable to prepare the substrates as an unbuffered solution (by dissolving in pure water, not buffer). This tactic has at least two advantages. One, it allows a single stock solution to be used when pH is varied by changing the assay buffer. Two, it minimizes the change in ionic strength when the concentration of this species is varied. In this situation the assay buffer is added as a concentrated stock; for example if one wished to attain a final buffer concentration of 0.100 M in a 1-mL assay, one might use 500 μ L of a 0.200 M stock solution of buffer.

The alternative is to dissolve all of the components of the assay in the same buffer used in the assay. This tactic has some disadvantages. For example, NADH is stable at high pH but one may wish to use it at pH 5. Dissolving NADH in a pH 5 buffer risks degradation.

Sometimes a low concentration of buffer at a different pH from the assay is needed for the stability of an enzyme or a reagent. For example, NADH is sometimes dissolved in dilute Tris base. As long as the dilution factor for this component of the assay is sufficiently large, the presence of buffer at a different pH should not pose a problem. Sometimes several of the components of the assay are mixed into a “cocktail.” This tactic may minimize run-to-run variation in composition of the assay, but the experimenter must be certain that the compounds are unreactive toward each other or the buffer.

Choice and preparation of buffer

The choice of the correct buffer is often critical to the success of the experiment. When preparing a buffer, the experimenter should record the temperature of the pH measurement, the type of counterion present, and the lot number of the buffer. Use of a pH meter is covered in a separate document.

Running assays

Condition of initial velocity

Generally initial velocity is taken to mean that less than about 5% of the substrate is consumed by the time that the assay is terminated. For spectrophotometric assays it is possible to assess whether or not this condition is fulfilled by comparing ΔA_{assay} versus ΔA_{max} , where the former term is the largest experimental change in absorbance and the latter is the theoretical (calculated) change in absorbance if 100% of the substrate were consumed.

Initiating the reaction

The reaction is usually started by adding a small aliquot of enzyme solution to a mixture of substrate, buffer, and any other necessary components. However, it may occasionally be advantageous to initiate a reaction with substrate. The volume and temperature of the initiating solution should not be so great as to change the temperature of the mixture significantly.

Stability of the enzyme during the assay

Loss of the activity of an enzyme is a common reason why reactions slow down a few seconds or minutes after they are initiated. In some cases the use of an additive (glycerol, reducing agents, inert proteins) can decrease the rate of inactivation.

Linearity of rate with respect to enzyme concentration

This is an extremely important control experiment, yet the reasons why an assay might deviate from linearity are different for different types of assays. Among the most thorough discussions of the reasons for deviations from linearity is that found in Dixon and Webb's *Enzymes*, pp. 54-63.

Linearity of the rate with respect to time

On p. 5 Mehler wrote, "One of the most common analytical errors is the measurement of a single value after a period of incubation of enzyme with substrate. It cannot be overemphasized that single-point assays are not valid until a reaction has been studied thoroughly, and it is known that the points measured fall on a meaningful part of the curve (Fig. 3)." Other kinds of assays besides the stop-time assays Mehler discussed above may be nonlinear in time but for different reasons. For example, coupled enzyme assays (see below) have a finite lag period.

Temperature control

Along with control of pH/ionic strength, controlling the temperature of the assay is critical to running reproducible assays. The preincubation period (the period prior to the start of the assay) must be demonstrated to be sufficiently long for temperature equilibration to occur. Any temperature disequilibrium that occurs after this must be small. The two most obvious sources of disequilibrium are removal of a spectrophotometric cuvette from a thermostatted cell holder to make an addition and the addition of a small volume of enzyme at a different temperature from the assay. The time should be as brief as possible, and the volume should be as small as possible.

Comments on rate calculations and preparing the data for fitting to an equation

When running continuous assays, a background rate is sometimes observed prior to the initiation of the reaction with enzyme. This rate is usually subtracted from the observed rate.

For endpoint assays, a blank (a reaction with no enzyme) is sometimes run. The blank value may be subtracted from the various runs with enzyme, as shown below. See the handout on endpoint assays for more discussion of controls.

It is often convenient to calculate the rate in μmol per minute. This allows one to calculate activity and specific activity most easily. One must be careful to use the correct volume and the

appropriate molar absorptivity at the wavelength of the assay when performing this calculation. An equation to calculate rate from absorbance data is:

$$\text{Rate } (\mu\text{mol/min}) = \text{Rate } (\Delta A/\text{min}) \times (\text{Volume}/\epsilon) \times (1,000,000 \mu\text{mol}/1 \text{ mol})$$

Where ΔA is $A_{\text{assay}} - A_{\text{blank}}$, ϵ is the molar absorptivity of the chromophore, and V is the volume of the assay in liters. When the reaction is assayed using a stop-time (endpoint) assay, the volume used in this calculation is the volume of the assay *after* the addition of the stopping solution.

It is often the case that different runs use different amounts of enzyme. Before fitting to the Michaelis-Menten or other equations, the rate data from individual runs must be made comparable; in other words, the rates must be corrected for differences in the amount of enzyme. A good way to do this is to divide the rates by the number of milligrams of assay enzyme, so that the rates are now in units of $\mu\text{mol min}^{-1} \text{ mg}^{-1}$. *It is very important to realize that this calculation assumes that the rates are linearly proportional to the concentration of enzyme in the assay.* See the discussion of this above.

It is sometimes the case that data from different days must be combined before the rates can be fit to determine kinetic parameters. The problem faced by the experimenter is how to account for the inevitable variation of enzymatic activity on different days, or when a new lot of enzyme is used. A paper from the Cleland lab on glycerol kinase (Knight and Cleland, 1989) briefly describes this normalization procedure, which uses a standard assay under conditions of pH and ionic strength that may be different from the conditions in the experimental study.

Some of these ideas are developed more fully in the course packs for CHM 467 and CHML 365, especially the meanings of and calculations of activity and specific activity.

Tips on performing coupled enzyme assays

A detailed discussion of how to set up and perform coupled enzyme assays is outside of the scope of the present document. However, a few guidelines are presented, inasmuch as they reinforce concepts discussed here and suggest ideas for the experimentalist to explore.

Checking for linearity in the concentration of the assay enzyme is an essential control experiment.

The conditions of the experiment (pH, ionic strength, nature of the counterions) must be chosen in such a way that both the assay enzyme and the coupling enzymes maintain some activity.

Coupled assays involving NAD and NADH must take into account the pH stabilities of these compounds, which is opposite. NAD is labile to basic hydrolysis, and NADH undergoes acid-catalyzed epimerization. For this reason stock solutions of NADH are sometimes prepared in very dilute base, such as 10 mM Tris. If the assays themselves are short, the labilities of these compounds in the assay cuvette itself will not be a concern.

Some coupling enzymes are stored as suspensions in ammonium sulfate, which inhibits some enzymes.

Occasionally a coupling enzyme must be repurified to remove a contaminating activity.

An example of how to set up a coupled enzyme assay for ATP-dependent kinases is given in the CHM 467 course pack.

Basic Considerations in finding K_m and V_{max}

The unweighted form of Michaelis-Menten equation may be used to fit data if the errors are constant with respect to the magnitude of the velocity. The Lineweaver-Burk equation can be used, as long as weights proportional to $(\text{velocity})^4$ are applied. If the errors in the velocity are proportional to the velocity, then the weighting scheme should be changed appropriately. The unweighted Lineweaver-Burk equation should not be used.

The basic theory of regression is treated in the CHML 365 course pack, and there is a discussion of weighting and curve fitting for the Michaelis-Menten and Lineweaver-Burk equations. There are more advanced discussions by Athel Cornish-Bowden and by Peter J. F. Henderson in Enzyme Assays: A Practical Approach, among other authors.

For Further Reading

Athel Cornish-Bowden, Analysis of Enzyme Kinetic Data (1995) Oxford Science

Malcolm Dixon and Edwin C. Webb Enzymes (1964) Academic Press

Robert Eisenthal and Michael Danson, Enzyme Assays: A Practical Approach

Herbert Fromm, Initial Rate Enzyme Kinetics

W Blaine Knight and W. Wallace Cleland, *Biochemistry* 1989, 28, 5728-5734.

Alan Mehler Introduction to Enzymology, Academic Press (1957).

Clarence Suelter, Practical Enzymology