Spectrophotometric enzymatic stop-time assays:
Applications to VHR phosphatase, alkaline phosphatase, and β-galactosidase

Introduction and scope

This document primarily address enzymes which are assayed via a stop time (also known as a fixed time or endpoint) assay performed spectrophotometrically, by means of a chromogenic substrate and a chromophoric product. However, many of the points are germane to other kinds of enzymatic assays. The primary example used is VHR phosphatase, which hydrolyzes 4-nitrophenylphosphate. The term "endpoint assay" is also used (perhaps more frequently in clinical chemistry) to refer to an assay in which the reaction goes to completion or reaches equilibrium, but that is not the meaning of the term in this document. Another document "Basics of enzyme initial velocity and inhibition studies" is closely related to this one; however, it treats other kinds of enzyme assays.

Absorbance measurements

Another laboratory document discuss spectrophotometry in general on a basic level, and it covers many of the problems typically encountered when measuring absorbance. The essay by R. A. John covers enzymatic assays using spectrophotometry and spectrofluorimetry.

Introduction to control experiments

A blank that has no enzyme should always be run, and its absorbance value should be subtracted from all others (see below under Blanks). Checking for linearity versus time and versus the amount of enzyme are other important and related control experiments discussed in more detail below. Checking for the possible loss of enzyme activity in the stock solution (below) is also a necessary control experiment. Some of the control experiments must be performed every time an assay is run, whereas others need to be performed each time the conditions of the assay are changed.

Assays when following protein purification steps

Sometimes this work does not require the same degree of quantitative accuracy as kinetic studies on the final enzyme. However, if good values for the specific activity and total activity of the enzyme are needed, then the assays should be as quantitatively accurate as possible. Another consideration in performing assays on crude enzyme preparations is that the presence of other enzymes may deplete substrate or product (Reference 225 in Scopes).

Initial velocity conditions and the choice of concentration of substrate

Although a universal definition of what is meant by initial velocity conditions may not be possible, it is generally taken to mean that the reaction is no more than 10% complete, preferably 5%. For a reaction that proceeds to completion, a reaction that proceeds 10% toward completion is equivalent to consumption of 10% of the substrate. It is straightforward and very useful to estimate how much substrate has been consumed for a particular value of the absorbance, given the initial concentration of substrate and the molar absorptivity of the product.

Where practical, the substrate concentration should be larger than K_m, inasmuch as this tends to make the rate linear in time for as long as possible by minimizing the effects of substrate

depletion and possibly by stabilizing the enzyme (Cornish-Bowden). However, cost or solubility may become limiting, and a few enzymes are inhibited by large concentrations of substrate.

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)."

Unlike continuous assays, endpoint (stopped-time) assays may be nonlinear in time without there being obvious evidence of it. This can be a serious drawback when one is performing a calculation of specific activity or performing an enzyme inactivation (see below). H. J. Fromm recommends using a minimum of four time periods to establish linearity, and R. A. John recommends using two. Fromm also implies that one should do this at low, medium, and high substrate concentrations when one is determining kinetic parameters such as, K_m and V_{max}. John writes, "The necessity to make measurements at multiple time-intervals may justifiably be avoided in routine measurements if the conditions of the assay system, such as substrate concentration, are kept constant and the amounts of enzyme measured are well within already established bounds of linearity."

Enzymes vary greatly in the length of time that the reaction remains linear. *The three main factors that decrease rate are substrate depletion (see above), product inhibition, and enzyme inactivation (see below).* Careful choice of the type of assay and the solution conditions can greatly influence the length of this period. Again, the severity of substrate depletion is related to the ratio of concentration of substrate to K_m. Product inhibition may occur even when the reaction as a whole is thermodynamically favorable (Allison and Purich)

Enzymes are often found to denature at the very low concentrations at which they are used in kinetic assays. This may occur for a variety of reasons, including adsorption of the enzyme onto surfaces. Sometimes glycerol stabilizes the enzymes. Sometimes bovine serum albumin at 1 mg/mL is used to minimize the adsorption process, but commercial BSA typically contains fatty acids and may bind to other hydrophobic substances. Other proteins could substitute for BSA in this role, such as egg albumin, but the possibility of other interferences should be considered. Peroxide-free, nonionic detergents may prevent adsorption. EDTA and a reducing agent are often used when an enzyme has a thiol group whose oxidation would inactivate the enzyme.

Stability of the stock solution of enzyme
Typically one uses a small aliquot of a concentrated stock solution of enzyme to initiate an assay. Some of the same factors that affect the rate of denaturation during an assay also affect the enzyme stability in the stock solution, although some enzymes denature more rapidly at low concentration. However, it can be convenient to prepare the stock solution under different conditions, especially pH, from the assay. When a number of assays are performed over several hours, it is prudent to determine whether or not the enzyme in the stock solution loses activity over this time period (Suelter, p. 233). If it loses activity, then the rates must be corrected.

There are times when a substance present in the stock solution of an enzyme may interfere with the rate measurement in some way. Sulfate from an ammonium sulfate suspension is one example. If differing volumes of the stock solution are used in a set of assays, then the interfering substance will change in concentration. In some cases the interfering substance must be removed. Some examples of how to do so are treated in a separate document.

Linearity of rate with respect to concentration of enzyme
Most authors recommend varying the rate as a function of the concentration enzyme, an extremely important control experiment. This control is important when performing enzyme inactivation experiments, which depend upon a linear relationship between the measured rate and concentration of active enzyme (Parsons and Gates). When varying the concentration of substrate or inhibitor, one may vary the concentration of enzyme to keep the rate within certain limits. This variation of enzyme concentration can only be correctly taken into account in one's calculations if the relationship between velocity and concentration is truly linear. Thus the graph of velocity versus concentration of enzyme should be linear (Montalibet; Parsons and Gates, Figure 8.1). This control can help the experimenter choose the ideal range of enzyme concentrations and rates in which to work (see below).

R. A. John points out that the issues of linearity in concentration of enzyme versus linearity in time (above) are sometimes related, a point also made by K. F. Tipton (p. 22). Imagine using a relatively large amount of enzyme. This will create a situation where initial velocity conditions (see above) will be over relatively soon. In this situation checking for linearity in time and linearity in [E] should show the problem of the rate decreasing due to substrate depletion. However, one can imagine problems that are detected only by one or the other type of experiment. Let us assume that the rate is linearly proportional to the enzyme concentration, and that one is attempting to increase the length of time that the reaction remains linear. Cornish-Bowden writes, "the curvature of the progress curve cannot be altered by using more or less enzyme: this just alters the scale of the time axis and any apparent change in curvature is an illusion; indeed, this property forms the basis of Selwyn's test for enzyme inactivation…" Varying the concentration of the assay enzyme is also a very important control when performing coupled enzyme assays, and some of the same considerations apply in this case.

On p. 5 Mehler notes an interesting caveat: "Finding that the amount of product formed or substrate removed is proportional to the amount of enzyme added is essential for a meaningful assay, but it is not sufficient to establish that the assay is valid, since a contribution of the enzyme preparation may be a substrate that determines the extent of a reaction." It may be that this point is more of a concern for crude enzyme preparations that pure enzymes. Among the most thorough discussions of the reasons for deviations from linearity is that found in Dixon and Webb's Enzymes, pp. 54-63.

Tips and tricks

Absorbance measurements
Try to avoid very large or very small values of absorbance. Very large absorbance values (roughly > 1.50-2.00) may fall into a region where Beer's law does not hold due to stray light effects or where substrate depletion has taken place (Allison and Purich; John). Very small

values may be poorly determined (roughly < 0.2), especially if the blank value is large. If the time of the assay is several minutes long and if multiple assays are being run, it may be convenient to start each assay 30 seconds apart, then do the same with the stopping solution.

Temperature control
This is an often-overlooked source of error when doing quantitative work. Lack of complete temperature equilibration may show up when checking for linearity with time (John). Wherever possible, one should check how long it takes for a solution to reach temperature equilibrium. This can be done using phenolphthalein in glycine buffer (pH ~9) and monitoring the change in absorbance near 550 nm. As temperature rises, the absorbance will fall. Other combinations of buffer and indicator may also be used, such as phenol red in Tris pH 7.8 (Figure 8 in John). The equilibration time for a cuvette in a spectrophotometer depends upon whether the cuvette is quartz or glass and the design of the cuvette holder. When a commercial cuvette holder was replaced with a custom holder, the time to achieve temperature equilibration dropped threefold in one instance. If a sample needs to be removed from a cooling or heating bath, the time it spends out of the bath must be as small as possible. Some solutions may need to be preincubated at a temperature close to that of the assay, if the volume of this solution is large, relative to the volume of the assay.

Substrate
If the substrate is chromogenic, it is sometimes contaminated with its associated chromophoric product. This issue will manifest itself in nonzero values of blanks. Phosphate esters may also be contaminated by inorganic phosphate, which is a strong inhibitor of alkaline phosphatase.

Some substrates are labile to degradation from light or via hydrolysis. If a substrate is stored at a different pH from the assay, the buffer must have sufficient capacity to control the pH despite this. If the substrate is stored at a reduced temperature to the assay, the solution may change temperature over the course of the measurement. Preincubation of the substrate with the buffer prior to the start of the assay may alleviate this problem.

Different salt forms of 4-nitrophenylphosphate (PNPP) have different solubilities, and at least one salt form (cyclohexylammonium) is not soluble enough to produce a good stock solution, given the high concentration needed for VHR phosphatase. Some commercial preparations of PNPP have large amounts of 4-nitrophenol (PNP), which will show up as a large blank value. There are several ways in which this might be removed. These include extraction of PNP into ethyl acetate, recrystallization of PNPP from acetone, and ion exchange chromatographic purification using a gradient of triethylammonium bicarbonate. The last method might also remove small amounts of inorganic phosphate (phosphate is a strong inhibitor of alkaline phosphatase but less so of VHR phosphatase).

Stability of the enzyme
The enzyme may be more stable under solution conditions (pH, ionic strength, etc.) that differ from the assay conditions. If it is stored under different conditions, one must take care that the dilution factor of the enzyme into the assay and the buffer concentration of the assay are sufficient to hold the pH and other solution conditions at their desired levels. If the enzyme is

more stable at low temperature, it is often stored on ice and small aliquots are used to initiate the reaction. This will cause a deviation from temperature equilibration.

Concentration of enzyme
The amount of enzyme added to the assay must be chosen carefully (see above). If the amount is too large, the assay may become nonlinear, due to substrate depletion or problems in measuring absorbance values accurately. If the amount of enzyme is too small, the rate will be too slow to be measured accurately. The volume of solution can usually be varied, and if the stock solution of enzyme is too high in concentration, it can be diluted. However, some enzymes lose activity at very low concentration, and one may need to add stabilizers. A dilution may be done in a small Eppendorf centrifuge tube. Sometimes the length of time of the assay can be varied instead of, or in conjunction with, the volume of enzyme.

Removal of inhibitors
Many enzymes are stored as ammonium sulfate suspensions. Some enzymes are inhibited by sulfate. If quantitative values are needed, the sulfate ions must be removed, as is discussed in the desalting handout, as well other handouts. Certain buffers inhibit or activate enzymes. MES inhibits VHR phosphatase and Yop51 phosphatase activities, for example. Tris is an alternate substrate for alkaline phosphatase.

Adding the enzyme
This needs to be done quickly to avoid temperate changes, and to be performed with good mixing technique. For reactions that are performed in a cuvette, Bel-Art manufactures plastic rods with small feet (designed by W. Wallace Cleland). The enzyme solution is placed into the small reservoir in the foot. The stirrer is rapidly moved up and down inside the cuvette to initiate the reaction.

Adding the stopping solution
Rapid, thorough mixing is important (the finger vortexing method is often a good choice). In general the sample should be read shortly after the addition of the stopping solution, preferably within a set range of times. This minimizes possible instability in the readings, which may be caused by hydrolysis of substrate or other reasons. Methods of stopping the reaction are discussed by Scopes and other authors. Bases such as NaOH and Na_2CO_3 are common stopping solutions.

Blanks
A blank may refer to an experiment with substrate but no enzyme, but it can also mean an experiment done with zero incubation time (see below). If the blank has a rate, it may be due to nonenzymatic reaction or due to a small amount of contaminating enzyme in a reagent. Another reason for a blank to have a nonzero value is that the substrate may be contaminated with product. The no-enzyme blank rate may be subtracted from the experimental rate to find the net rate of enzyme-catalyzed reaction. A large value of the blank may suggest that the substrate should be purified or that the experiment should be further optimized.

Some authors (Scopes; Fromm) recommend a zero-time stopping experiment to use as an assay blank. Ninfa *et al*. (p. 243) mention that this control checks for interference from the enzyme.

Fromm notes that this experiment will allow a progress curve to go through the origin. In this author's opinion the zero-time blank should be performed in addition to the no-enzyme blank. Another reason to perform this experiment is to ensure that the stopping method is working as it should. The zero-time blank will be nonzero when a chromophoric product is an impurity in the substrate, but the no-enzyme blank will be nonzero when there is non-enzymatic hydrolysis or there is a chromophoric impurity. In the author's opinion the absorbance of the zero-time blank should be read and the value subtracted appropriately; it should not be used to set the reference.

A large value of the no-enzyme blank is common for VHR phosphatase because the substrate concentration is large. Blank absorbance values are sometimes in the range of 0.050-0.075 AU, but this is highly dependent on the purity of the 4-NPP.

Setting the spectrophotometer reference
Some authors use a blank sample to set the reference, but a better method is to set the reference as usual and to subtract any blank values (see above). This allows the experimenter to know what the value of the blank is. To set a reference for a dual beam or split beam spectrophotometer, use two cuvettes with solvent but no chromophore, and follow guidelines found in other laboratory documents or in the instructions for the spectrophotometer. In laboratory documents, "setting the reference" means to set the value of the absorbance to zero, and "blank" refers to certain control experiments. One author (John) recommends using a reference having a high absorbance when the sample has high absorbance that decreases with time. This suggestion may apply mainly to continuous assays.

Pipetting technique
When an experimenter is performing enzyme assays, he or she is often pipetting solution in the range of 1-100 μL; therefore he or she should be especially cognizant of good pipetting technique. Poor technique can lead to data with poor precision or accuracy. An introduction to pipets and pipetting technique is covered in a separate document.

General references on stop-time and other initial rate assays

Athel Cornish-Bowden, Chapter 3 in Fundamentals of Enzyme Kinetics," 3rd ed. Portland Press 2004.
Malcolm Dixon and Edwin C, Webb Enzymes (1964) Academic Press
H. J. Fromm, Initial Rate Enzyme Kinetics Springer-Verlag 1975, pp. 60-61.
Robert A. John "Photometric assays," in Enzyme Assays, A Practical Approach, R. Eisenthal and M. J. Danson, eds. IRL Press, 1992.
Alan Mehler Introduction to Enzymology, Academic Press (1957).
A. Ninfa, D. Ballou, and M. Benore Fundamental Laboratory Approaches to Biochemistry and Biotechnology. Chapters 9 and 10 discuss alkaline phosphatase assays; Chapter 5 has an assay for β-galactosidase; Chapters 5 and 12 also have information on enzyme assays and kinetics.
Rossomando EF (1990) "Measurement of enzyme activity," Methods in Enzymology **182**: 38–49.
R. K. Scopes, Section 9.2 in *Protein Purification Principles and Practice*, 2nd ed. Springer-Verlag 1987. Discusses designing and setting up assays. Discusses secondary reactions.

R. K. Scopes, "Enzyme Activity and Assays," in Encylopedia of Life Sciences (2002). This is a good article at a very introductory level, and it covers other topics, such as the basics of protein assays.
C. Suelter, in A Practical Guide to Enzymology, pp. 233-236 and references therein.
R. D. Allison and D. L. Purich "Practical considerations in the design of initial velocity enzyme rate assays." Methods in Enzymology **63** 1979 pp. 3-22.
Keith F. Tipton, "Principles of enzyme assay and kinetic studies," in Enzyme Assays: A Practical Approach, R. Eisenthal and M. J. Danson, eds. IRL Press, 1992.

How to perform calculations of activity and specific activity are among the topics covered in the references above. Some of the discussions about chart recorders may seem dated but that is not entirely the case, as Cornish-Bowden discusses (p. 72). Also in this author's opinion, reliance on poorly written or incorrect software is a potential source of error that should not be overlooked.

Protocol for VHR phosphatase assays
References for PTPase assays
Zhou *et al*., (1994) J. Biological Chemistry **269** 28084.
J. Denu *et al*., (1995) J. Biological Chemistry **270** 3796.
J. Montalibet *et al*., (2005) Methods **35**, 2-8.
Z. Parsons and K. Gates (2013) Methods Enzymol. **528** 129-154.

Stock solutions
Stock solutions of substrate and buffer are typically stored in small aliquots at -15 °C.
100 mM PNPP
100 mM succinate with 1 mM EDTA, pH 6.0*
1 M NaOH
*A mixed buffer consisting of 0.05 M Tris/ 0.05 M Bis-Tris/ 0.1 M acetic acid (final concentrations) has also been used. Triethanolamine may be used in place of Tris (see Ellis and Morrison, Methods in Enzymology)

The temperature of the assay is 30 °C, maintained by a water bath. The buffer, substrate, and DI water are mixed and pre-incubated in a small tube, which is suspended in the water bath. Enzyme is added to initiate the reaction and mixed rapidly, and the tube is returned to the bath. After a set time period (typically 3 min), a 1000 μL-aliquot of NaOH is added. The absorbance is read at 400 nm or 405 nm. The molar absorptivity of PNP is assumed to be 18,300 at 400 nm (Dawson *et al*., *Data for Biochemical Research*, 1986) but slightly different values may also be found in the literature. For continuous assays at low to moderate pH, the apparent molar absorptivity as a function of pH must be used instead (Ninfa *et al*.).

100 μL	buffer
20 μL	PNPP
x μL	enzyme
80 - x μL	water

The volume of the components is 200 μL. After the addition of NaOH, the volume is ~1.20 mL.

Stop-time assays of β-galactosidase
This enzyme is often assayed via endpoint assay using *ortho*-nitrophenylgalactopyranoside (ONPG) as the substrate (Ninfa *et al*., Chapters 5 and 10). This laboratory exercise is a good example of the staggered start method of running many reactions simultaneously. This assay also illustrates that substrate depletion can become an important factor in some situations. A discussion on how to perform calculations of rate, total activity, and specific activity is covered in a separate document.

Stop-time assays of alkaline phosphatase
Alkaline phosphatase is strongly inhibited by phosphate, and substrate depletion is more of an issue than it is for VHR phosphatase. Therefore, control experiments should be performed in such a way as to examine these potential problems. Alkaline phosphatase is a good candidate for continuous assays (Ninfa *et al.)* because this enzyme has greatest activity in the vicinity of pH 8, where the 4-nitrophenolate ion, the chromophore, predominates in concentration.

Comments on assays performed during VHR inactivation experiments from Dan Lookadoo
Introduction
The rate of inactivation of an enzyme by an alkylating agent is usually determined by withdrawing aliquots of a solution of enzyme and inactivator over time and assaying each aliquot for remaining enzyme activity. There are several assumptions that are typically made in this experiment (Parsons and Gates), and it is useful to spell out as many as possible. One is that there is a linear relationship between the rate of reaction and the concentration of enzyme (see above). Two is that the inactivation reaction is stopped instantaneously (usually by dilution). Three is that the presence of reversible inhibitors (which may have been used as protecting agents in the inactivation portion of the experiment) does not materially affect the rate determination portion of the experiment. Some of these points will be addressed elsewhere.

Protocol and example
Inactivation experiments require that known volumes of enzyme be withdrawn from an inactivation cocktail at defined time points and placed into activity assays. The combination of dilution and high concentration of substrate are assumed to terminate the inactivation. However, if there are reversible inhibitors in the inactivation cocktail, their presence may lead to slower rates of PNPP hydrolysis.

Example, an inactivation assay following the inactivation of VHR by trifluoromethanesulfonyl-hydroxymethylene phosphonate (PMT) is described. Kinetics of this inactivation happens to be non-linear when presented as the logarithm of fractional activity of enzyme versus time, and data analysis must be treated accordingly; however linear/non-linear inactivation activity assays are synonymous. All time points and volumes must be determined *a priori*. Table 1 presents one example of a well-planned assay. It is important to define and adhere to specific a time zero. *Time zero* is usually defined as the time at which an aliquot is withdrawn immediately after the addition of the inactivator. Once the data has been recorded, plot the fraction of remaining activity versus time and fit the data accordingly (as illustrated below in Figure 1).

Table 1. *p*NPP Activity Assay Monitoring the Inactivation of VHR by PMT. Obtained Values are Listed Including: the Planned Assay Time Point in Minutes, the Actual Time the Time Point was Taken in Minutes, the Termination Time in Minutes, the Absorbance Recorded at 405 nm (Abs) in Absorbance Units, the Corrected Absorbance ($Abs_{corr.}$) in Absorbance Units, the Fractional Change in Activity (A/A_i) or the Observed Absorbance at each Time point (A) with Respect to the Initial Time Point at Time Zero (A_i), and the Natural Logarithm of the Fractional Change in Activity ($\ln(A/A_i)$).

Time	Actual time	Termination time	Abs	$Abs_{corr.}$	A/A_i	$\ln(A/A_i)$
0	00:00	02:00	1.730	1.662	1.000	0.000
1	01:00	03:00	1.573	1.505	0.906	-0.099
2.5	03:00	04:30	1.479	1.411	0.849	-0.164
5	05:00	07:00	1.230	1.162	0.699	-0.358
10	10:00	12:00	0.992	0.924	0.556	-0.588
20	20:00	22:00	0.823	0.755	0.454	-0.790
30	30:00	32:00	0.812	0.744	0.448	-0.803
Blank	41:00	43:00	0.068			

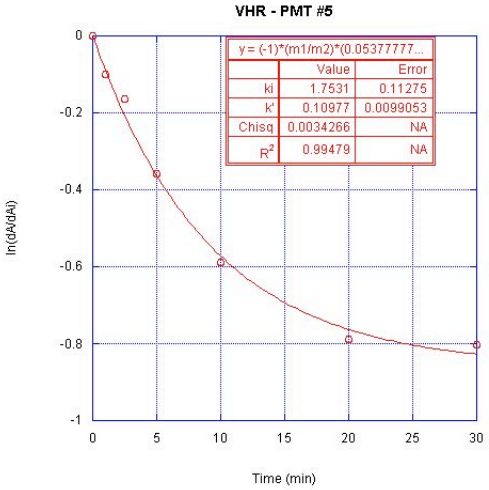

Figure 1. Plot of the natural logarithm of the fraction of activity (A/A_i) remaining with respect to the elapsed time in minutes from time zero. The observed experimental data points are depicted, as dots, and the curve fit, overlaid solid line, to a kinetic model (scheme I) developed by Kyte (*J Biol Chem* 1981) is shown. The fit parameters and statistical information are also presented, in the upper right hand corner of the figure.