

Assays

What we mean by an assay is a measurement of the amount present of a **specific** protein (or other material, as distinguished from **general** measurement of **all** protein as previously discussed). Ideally this means measuring a specific **biological property**, or ability to catalyze a chemical reaction - the characteristic of the protein which makes you want to investigate it. Less ideally, we use a method which does not in principle derive from the biological activity of the protein, but is a general method in which it has a specific behavior. Let me give an example. You are investigating the toxicity of 2,3,7,8-tetrachlorodibenzodioxin to rats, and on carrying out polyacrylamide gel electrophoresis of extracts of the livers of treated rats you observe a specific band not seen in control livers. You can use that band as an assay during purification, even though it tells you nothing about the protein but its molecular weight. Eventually you hope to learn more about the protein, perhaps by obtaining a partial amino acid sequence and comparing it with the data base of sequences. I will discuss other such assays later.

At best, the protein's activity is defined chemically, as for an enzyme in mmoles product per minute. European journals sometimes use katal, moles/sec - this is consistent with other physical units, but obviously microkatal are more useful. A **catalytic** assay, in which many moles of measured product are produced per mole of the protein being measured, is obviously more sensitive than a **stoichiometric** assay, which measures amount of the protein by measuring a molar equivalent amount of something, such as bound Coomassie Blue or silver stain. In a catalytic assay the amount of measured product at least in principle increases indefinitely with time of incubation, yielding greater sensitivity, while a stoichiometric assay only reaches equilibrium. In some cases we want to measure even an enzyme stoichiometrically, in order to distinguish how much of the protein is biologically active; I will talk about such "enzyme titration" in a later lecture. Some proteins without direct catalytic activity may still be measured in a catalytic assay if they are a cofactor for a chemical reaction or series of reactions, and are a rate-limiting component in presence of an excess of all the enzymes involved. In other cases one measures a chemically undefined biological activity of some sort - I remember the assay by which growth hormone was purified, administering samples to hypophysectomized rats and measuring the thickness of the knees 41 days later. Sometimes one measures a protein by its binding of a metal ion or other small molecule; one must be sure that the binding is specific, generally at a low level of the ligand.

Rosenberg, pp. 94-97 covers what is now a common assay, the **gel shift assay** for a protein binding to a specific DNA sequence. A radioactive oligonucleotide (with the specific sequence of interest, from a gene promoter) moves far down a polyacrylamide gel, because it is very negatively charged, whereas when bound to a protein it moves much less far, because of the greater size and lesser charge of the protein. It is important to saturate nonspecific binding with non-specific DNA. That the binding is specific is also shown by the fact that it can be diluted out by addition of a large amount of non-radioactive oligonucleotide of the same sequence. This tends to be done as a qualitative assay; but it can in principle be made quantitative.

Assuming that the binding is tight, you can in effect titrate the protein, using steadily increasing amounts of labeled oligonucleotide and determining when it is in excess. You need to know the molar amount of oligonucleotide, which isn't easy when it is very low. You might set up incubations with 1, 2, 4, 8, 16, 32 pmoles of oligonucleotide, and a constant amount of protein sample. You observe in the gel shift assay that at 1, 2 and 4 pmoles all the oligonucleotide is bound, but at 8 pmoles total there are spots of bound and free oligo in ratio, as determined by gel scanning, 5:3. This would be a good indication that the protein sample contained 5 pmoles of binding protein, enough to bind 5 pmoles of oligonucleotide. Your measurement of film darkening by autoradiography has no particular relationship to the amount of oligonucleotide present, but if you see both bands you can express the amount of protein as the fraction of total film darkening in the protein-oligonucleotide bands, and multiply that times the moles of oligonucleotide in the assay. The same principles can be followed in any such stoichiometric assay of a protein by binding a labeled or otherwise detectable ligand - you can titrate the protein with ligand, and at an optimal ligand level you have equal amounts of bound and unbound ligand after the assay. The binding must be tight, so that bound ligand is not lost when the protein-ligand complex is separated from free ligand.

In some cases a protein may have a sufficiently unique absorption of light, or even bioluminescence, so that it can be measured by direct measurement of absorbance of light, or fluorescence or luminescence. These are stoichiometric assays, though some may be quite sensitive.

There are two general purposes for assays: first, to measure **how much of the protein** (or other substance) is present in an organism, tissue or purification fraction. The protein is the variable measured. Second, with a **constant** amount of the protein present, **how does its activity vary with conditions**, either in the assay (pH, temperature, variation of substrate concentration, effect of inhibitors, etc.) or in prior incubation (stability to heat, chemical modification, etc.) This we hope tells us something about the chemical basis of the biological activity. Obviously assays which measure some property not much related to the biological activity - such as mobility in electrophoresis, reaction with an antibody - are no use for the second, but may be useful for the first.

Assays may be either **continuous** or what I call **stop-time** (Scopes calls them "stopped"). A continuous assay is one in which some change caused by the protein is monitored continuously **while it is happening** - such as absorbance of light, uptake of base or acid in a pH-stat, or change in oxygen concentration measured by an oxygen electrode. The quantity monitored appears as a line on a recorder chart, and usually one is interested in the **slope** of the line, the **rate** of the reaction, which should be proportional to the amount of your protein present. In a stop-time assay, on the other hand, you must **stop** the reaction at some definite **time**, and do something to the reaction mixture, such as adding acid or base or heating to develop a color, or separating radioactive products from starting material, which makes possible the determination of amount of product formed by that time (or amount of starting material remaining). There are also semi-continuous assays, where you can make a number of measurements on the same reaction mixture while the reaction is running, but each measurement takes a finite amount of time - manometric and viscometric assays, for instance. In principle any stop-time assay can be made semi-continuous, by taking multiple samples from a single assay mixture, instead of setting up multiple assays and stopping them at different times. Allison and Purich point out that for precision it is desirable to stop the reaction as quickly as possible, and be sure that it doesn't continue after you think you stopped it. Addition of a solution which makes further reaction impossible, such as strong acid or base, or EDTA for a Mg^{++} dependent reaction, is better than boiling, which takes time to come to 100° , and may not work for thermophilic enzymes.

The continuous assay has many advantages over the stop-time assay, especially for measuring change of activity with conditions. Consequently much effort is expended to develop continuous assays, even when a simple stop-time assay is available. Most of all, the continuous assay inherently gives you **initial velocity**, which is what you want in an enzymatic assay, even those where the protein being measured is a cofactor, and especially when you are studying the kinetics of the enzyme, how its rate of action is affected by concentration of substrates, inhibitors, etc. The velocity of the reaction may decrease quite rapidly, but you can generally draw a tangent to the curve on the chart and estimate initial velocity quite well. Sometimes there is a **lag**, the reaction takes time to reach its highest rate. In a stop-time assay, on the other hand, you must **prove** that the reaction continues **at a constant rate** for the time used, if measurement of the amount of product formed is to represent accurately the rate of the reaction. You generally want a longer time, in order to have more product made. But if you put in too much of your protein, the rate may fall off by the time at which the measurement is made. The fall-off may occur when a certain amount of product is made, so that with x amount of protein the reaction is linear with time for 10 minutes, but with $2x$ it is linear for only 5 minutes, and at 10 min one has made only $1.8x$ times the product made in 5 minutes. One can, however, construct a standard curve; if you know that x amount of protein gives y amount of product in 10 minutes, while twice as much of the same protein solution gives only $1.8y$, you can still use this curve to tell you that you have $2x$ when you get $1.8y$ amount of product from some later sample of the protein. But again, this is only good for measurement of amount of x , not for its properties.

Also, a stop-time assay is always a measurement of the difference between the absorbance or whatever at **zero** time and at the stop time, so one must be more careful with controls than in a continuous assay, where one is measuring the rate and is not too concerned with initial absorbance. You must, however, be concerned with contaminating enzyme reactions which may produce the same absorbance change, as for instance NADH oxidase. In a continuous assay the critical control is usually a no-substrate assay, containing protein sample but not the specific substrate; in a stop-time assay the **zero time** assay is critical.

Stop-time assays do have the advantage that you can run a number of them, a number of test tubes, at once, while you can only run one, or with cuvette changers which move cuvettes in front of the light beam automatically, four continuous reactions at once. Stop-time assays are also adaptable to 96-well plate format and robotic handling.

In biochemistry you are usually content with measurements accurate to $\pm 5\%$ (and Scopes makes the point that rates of enzyme-catalyzed reactions typically go up 6% for each degree C of temperature, so that you cannot be more definite about the rate unless you know the temperature to $\pm 0.5^{\circ}$). This also points out the need to

preincubate the assay mixture at the desired temperature before adding enzyme, so that the entire reaction runs at the same temperature. Whatever you add to start the reaction - usually but not always enzyme - must also be brought to temperature if you are adding more than 1% or so of the whole assay volume.) This $\pm 5\%$ is the uncertainty of the individual measurement; for stop-time assays the measurement at zero time is a separate measurement. If the initial absorbance, vs. a water blank, is zero, and the final measurement is 0.100 ± 0.005 , you have defined the rate as 0.100 ± 0.005 per the time period used. If, however, the initial absorbance is 0.100 ± 0.005 and the absorbance after the assay time is 0.200 ± 0.010 , you have defined the rate as 0.100 ± 0.011 per unit time, after proper combination of the standard errors. You may have such an initial absorbance for any of several reasons - the reagents may have inherent absorbance at the wave length of measurement, the enzyme preparation may have materials in it which absorb, or even some of the product of the reaction. I was once on the committee of an M.S. student whose project was well along before he and his professor discovered that they were not measuring the levels of an enzyme in mitochondria, but the level of its product! A zero-time assay would have caught this. A continuous assay automatically gives you the zero-time measurement; the uncertainty of the rate measurement is probably the wobble in the line - which can be quite considerable.

The effect of error in the zero-time measurement becomes even more important when you contrast **appearance** and **disappearance** assays. Sometimes the easiest way to measure a reaction is to observe disappearance of the starting material rather than appearance of product, or the product appearing may react with and cause disappearance of a reagent which is measured, as for instance reduction of ferricyanide by sugars. Consider the effect of 5% error: if the initial absorbance is 1.00 ± 0.05 , and the final is 0.950 ± 0.0475 , the change is 0.05 ± 0.069 , not a significant measurement! And if you add to the assay too much of whatever you are trying to measure, you may exhaust whatever it is that you *actually* measure; glucose more than stoichiometric with ferricyanide in the reagent will reduce all the ferricyanide and you will have no idea how much more glucose is present. You will probably tail off before you get to zero, but the general idea is that in a disappearance assay you must guess rather accurately how much of your enzyme or whatever to put in, so that the decrease in what is measured is between 20% and 80% of the total. In a continuous disappearance assay, however, there is no problem, as you get the initial rate directly and it is not affected by small errors in the initial absorbance. It is even possible to use a single continuous trace of substrate disappearance to determine the K_m for substrate, which with a substrate such as oxygen is much easier than adjusting the initial concentration!

Here let us think about sources of error. Error means several things. Firstly, there is random error of the measurement - how accurately can you measure a quantity, what is its statistical error? In radioactive assays you have to deal with the statistical uncertainty of radioactive decay; but modern counters count until the statistical error is down to some preset value. How close will you come if you repeat the experiment? These errors are greatest when you are pushing sensitivity to its limit, sensitivity being defined as how small a change you can measure and be sure you are measuring something real. The size of the zero-time measurement in a stop-time assay obviously is important here. Repeating the assay several times, or doing it at several levels of sample and using the slope of a plot of response vs amount of sample, is important in defining the standard error of the measurement.

Secondly, there is what might be called error of the conversion factor. Your actual measurement is of absorbance of light or counts per minute or volume change at standard pressure, and you want to convert this to mmoles of product formed. You must consider not only how accurately the conversion factor is known, but also whether there is anything present which affects the assay procedure - an enzyme inhibitor, say, or an unfavorable equilibrium factor which makes the real yield less than the theoretical, or non-linearity of reaction with time, or a pH effect on the extinction coefficient of the product. Often one uses an artificial substrate which gives a product more easily measured than the natural one, or is cheaper or easier to prepare or gives a single reaction rather than a mixture of reactions (of a protease, for instance); one must then consider how well the artificial activity reflects the natural activity. Nitrogen fixation, for instance, is virtually always measured by the unnatural activity, reduction of acetylene to ethylene (which is separated and measured by gas chromatography). In principle, but rarely in practice, the ratio between rate of acetylene reduction and rate of real nitrogen reduction, measured with $^{15}\text{N}_2$, should be measured for the enzyme system from any new source, as there is no reason to expect it to be exactly the same for enzyme from another source. But acetylene reduction can be used in determination of enzyme activity during purification without worrying about this *much*. At worst, you end up purifying an enzyme with good activity on the artificial substrate, but little or none on the natural substrate. This may show that the natural substrate is not what you thought it was. A student in our department thought he was working on a β -glucosidase, whose natural substrate would be cellobiose, the dimer from cellulose. It had good activity on the artificial, fluorogenic substrate methylumbelliferyl-glucoside, but not much on cellobiose. Finally he found that

longer cellooligosaccharides were much better substrates, the enzyme taking one glucose at a time off the nonreducing end.

Consider errors of design of the assay - if, for instance, the reaction needs Ca^{++} for assay, don't use phosphate as the buffer, it will precipitate the Ca^{++} .

Thirdly, and worstly, there are errors of measuring **something other than what you think you are measuring**. Your enzyme preparation may contain a substrate for another reaction, which you measure. For example, suppose you are trying to measure alcohol dehydrogenase in apple tissue. From the name, I assume apples are loaded with malate, and perhaps the enzyme sample will have enough so that NADH is also produced by the malate dehydrogenase reaction. This can be caught by running an assay without the specific substrate - here ethanol - and seeing whether there is still NADH produced. If so, subtract activity seen in absence of substrate from that seen in its presence. Your enzyme may, as I mentioned, already contain product. This can be caught by a zero-time measurement or by use of a boiled enzyme control (though not all enzymes denature completely in 5 minutes at 100°). The reaction may occur to some extent non-enzymatically, particularly if the reaction is breakdown of a labile compound such as ATP or a nitrophenyl ester; observe what happens with a non-enzyme or boiled enzyme control. With a labile substrate such as a *p*-nitrophenyl ester, histidine and lysine residues of protein present may catalyze its hydrolysis even without saturable binding (the rate goes up linearly with substrate concentration).

Still worse, the **substrate** may be impure, and the enzyme may be acting on something present which is not what you think it is acting on; a classic example from microbiology, which Dr. Bartha pointed out, was the supposed biodegradation of the pesticide Mirex, which is totally chlorinated, no C-H bonds to attack, and consequently undegradable at least under aerobic conditions; the reported production of a small amount of $^{14}\text{CO}_2$ from [^{14}C]Mirex was surely due to degradation of radiolabeled impurities in the Mirex. Or, the substrate may contain impurities which inhibit the reaction, as for instance aluminum and vanadium complexes of ATP, which have low K_i s for enzymes using MgATP. In these cases the amount of inhibitor increases as you increase the amount of substrate in the reaction. If the inhibition is non-competitive - fixed levels of inhibitor affect V_{max} but not K_m - it will show up as apparent substrate inhibition, v goes through a maximum and then decreases as substrate concentration increases. If the inhibition is competitive, an inhibitor present will shift the Lineweaver-Burk plot upwards, apparently increasing K_m and decreasing V_{max} , but you will still see a straight line and never know it isn't right. Solutions of NADH and NADPH are known to generate inhibitors on storage, even frozen; never use yesterday's NADH solution, and for crucial experiments repurify the NADH.

How do you know whether you need to purify a commercial substrate further? A procedure may tell you to, but the purity of commercial substrate may have increased since the procedure was written. You can waste a lot of time working with an impure substrate, but you can waste a lot of time purifying when you don't need to. Best rule: purify further once, but compare activity with that seen with commercial substrate, to see if there is enough difference to make purification worthwhile. Of course, you don't need the purest substrate for assaying how much enzyme is present. But you can always run into a bad batch of substrate. One of my students was held up for a while because a supplier was sending out D-gulonolactone labeled as L-gulonolactone.

For measuring amount of enzyme present the assay should contain saturating levels of all substrates, if possible at least 10x the K_m s. But high substrate levels may actually inhibit; when devising an assay, you should run it at a number of substrate concentrations, to determine at what concentration the rate is maximal. The substrate may be expensive, poorly soluble, or it may absorb light at the wavelength used. NADH oxidation reactions usually use a concentration of 0.1 mM (initial $A_{340} = 0.622$) or 0.15 mM ($A_{340} = 0.933$). Greater sensitivity can be achieved if you can set up the spectrophotometer to record between 0.65 and 0.55 for 0.1 mM NADH, rather than 1.0 to zero.

The assay should be well buffered, particularly if the reaction produces or consumes H^+ ; if so, choose a buffer with pK_a such that the pH change is **toward** the pK_a . For instance, if a reaction is to be run at pH 7.0 and produces acid, use a buffer with pK_a between 6.5 and 7.0, rather than above 7.0. Some characteristics of buffers are covered in Rosenberg, pp. 374-8, and Scopes, pp. 236-245. I have a two-page list of buffers with pK_a s. You must choose a buffer which does not interact with either enzyme or substrate - generally try several and pick the one in which activity is highest. Zwitterionic buffers such as HEPES (draw) are frequently best. Two characteristics worth remembering are: buffers involving ionic species with a charge greater than 1 - phosphate, citrate, succinate, etc. - will show a substantial change of pK_a and thus pH on change of concentration (and pH

meters are likely to be inaccurate in 1 M and above salt; measure pH of a dilution of such a sample). And amine buffers show a considerable decrease of pK_a and thus pH with increasing temperature. For instance, the pK_a of Tris decreases from 8.8 at 0° to 7.8 at 37°, a $dpK_a/dT = 0.028/^\circ$. Acid buffers such as phosphate and citrate show effects ten-fold smaller. However, these have their pK_a s rise in presence of organic solvents, while amine buffers are unaffected.

A few other things to worry about: stability of the enzyme - it should if possible not lose activity significantly during the assay, and should not be affected by the product of the reaction or by substrates for a coupling enzyme. Sometimes stability of the enzyme is improved by preincubation with one substrate; often it is more stable in presence of all the substrates. At very low total protein concentrations binding to glass may be significant; if dilute enzyme gives unexpectedly low activity, try including bovine serum albumin in the assay mix and see if activity improves; or use plastic tubes and cuvettes. Many enzymes require presence of sulfhydryl compounds such as mercaptoethanol, or better dithiothreitol or dithioerythritol, to keep their own SH groups reduced; for enzymes from anaerobic organisms this is a must, and some such enzymes require totally anaerobic conditions. Substrate/enzyme ratio – normally we assume that substrate concentration is so much greater than enzyme concentration that depletion of substrate by binding to enzyme isn't a problem, but in some cases, when the enzyme binds tightly specific substrates present at low concentration, this may be limiting. Allison & Purich remind us that enzymes act on specific ionic and conformational forms of substrates, and if, say, this is the open-chain form of a carbohydrate it may be present at quite low concentration, and the interconversion of substrate forms might in this case be quite slow and rate-limiting.

Assay procedures for specific enzymes may most generally be found in *Methods in Enzymology*, the book series which is now around 270 volumes. Often an enzyme has been purified from several sources, and a later volume will report more modern assay methods than found in an earlier one. Worthington Biochemicals and Boehringer & Soehne publish handbooks which give assay procedures for the enzymes they sell. The book *Methods of Enzymatic Analysis* by Bergmeyer is primarily concerned with the use of enzymes to assay levels of substrates, but the substrate of one enzyme is probably the product of another - as we shall see in talking about coupled reactions - and can be measured either after stopping the first reaction or continuously with an excess of the second enzyme present.