

Uses of Enzymes in Analytical Chemistry*

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The paper reviews the basic features of enzyme-catalysed reactions, and distinguishes between the optimal conditions required for monitoring enzyme concentrations (and, therefore, those of inhibitors and activators) and substrates. Recent advances are emphasised, including insolubilised enzyme and bacterial electrodes, enzyme-based sample decomposition, drug and pesticide detection, and apo-enzyme activation, as well as more common applications.

Enzymes are naturally-occurring protein-based macromolecules which catalyse reactions of great selectivity and sensitivity. There are several general categories of enzyme such as those which catalyse particular redox reactions, or cause hydrolysis of certain molecules. Individual enzymes include lactate dehydrogenase, which catalyses the oxidation of lactate to pyruvate, and glucose oxidase, which catalyses the oxidation of glucose by oxygen to gluconic acid. Well over 1000 enzymes are known.

The glucose oxidation (which occurs in aqueous solution with molecular oxygen at room temperature and forms hydrogen peroxide) is a good illustrative example of how very selective enzyme reactions are. Any normal chemical oxidation of glucose would produce a range of products or even gross degradation of the molecule, and would also convert other, similar molecules. The enzyme reacts only with glucose and produces only one product.

One of the earliest enzymes to have its structure worked out was lysozyme, a small enzyme with a molecular weight of only just above 14 000 (some enzymes have much larger molecular weights). The very first was ribonuclease, in 1963, which has 124 amino acid residues in its chain, lysozyme has 129. A spatial model of the enzyme shows a cleft in the molecule (the active site) into which a polysaccharide molecule fits neatly.

Some enzymes are more complicated and have a metal ion as part of the structure. The metal usually is found within the active site so that it participates as a redox partner or by co-ordination in the reaction with the reactive species. Alkaline phosphatase (which catalyses the hydrolysis of phosphate esters in alkaline solution) contains a zinc ion bound to three different nitrogen atoms in the protein chain. Zinc is also present in alcohol dehydrogenase. The other metals which commonly occur in enzymes are the metals which are usually referred to as essential trace elements (Cu, Mn, Mo, etc). Metals which usually have a redox function occur in enzymes which have a redox function. Zinc

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ions, which normally have no redox properties, often occur in enzymes which cause hydrolysis.

The methods used for monitoring enzyme catalysed reactions can involve many of the standard instrumental techniques of analytical chemistry^{1,2}. Spectrophotometry, spectrofluorimetry, electrochemical techniques and thermometric measurements are all widely used. For example, for simple measurement of an alkaline phosphatase-catalysed reaction, colourless *p*-nitrophenyl-phosphate may be hydrolysed to the yellow *p*-nitrophenolate ion, which enables the reaction to be followed by spectrophotometric measurement of the yellow colour. Oxidations involving oxidases (for example, of amino acids) produce peroxide. This can be measured by reaction with reagents which are oxidised to coloured products (e.g. *o*-dianisidine) or with homovanillic acid to give a fluorescent product. It is essential that the monitoring reaction is much faster than the enzyme-catalysed reaction.

There is a group of oxidative enzymes called dehydrogenases which differ from oxidases inasmuch as they cause oxidation by the participation of a co-enzyme (often nicotinamide adenine dinucleotide, NAD^+). The co-enzyme is an essential reagent in the enzyme system. It takes up a position on part of the active site. The substrate (reacting compound) reacts with the co-enzyme in the presence of the enzyme to form the product and simultaneously forming reduced co-enzyme, NADH. An example is the metabolism of alcohol, where it is decomposed under the influence of alcohol dehydrogenase and NAD^+ :



Such reactions can be monitored by measuring the NADH formed. The spectrum of the NAD^+ -NADH system shows a peak at 240 nm for NAD^+ and NADH and another 340 nm for NADH only. Thus NADH can be measured spectrophotometrically at 340 nm. NADH is also fluorescent so can be measured using a spectrofluorimeter, with a gain of two orders of magnitude in sensitivity compared to spectrophotometry.

Enzyme activity is defined by the International Unit, which is the amount of enzyme which catalyses the conversion of 1 μmol of substrate per minute under optional conditions.

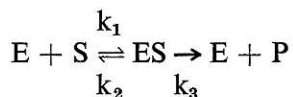
Michaelis-Menten Equation

The mathematical equation which for many years has been used to describe the kinetics of enzyme-catalysed reactions is the Michaelis-Menten equation:

$$v = \frac{V_{\max} [S]}{K_m + [S]}$$

where v is the rate of reaction (e.g. rate of formation of product), when the substrate concentration is $[S]$, K_m is a constant (known as the Michaelis constant) and $V_{\max}$ is the maximum rate attainable, which can be achieved when there is a large excess of substrate, and is proportional to the initial enzyme concentration $[E]_0$. This treatment assumes that a substrate first of all interacts with an enzyme E to form an addition compound ES . The compound in the

active site then reacts to form a product P and release the enzyme, so that it continues to function:



When the above equation of rate against substrate concentration is plotted, as [S] increases, v increases until it becomes constant at $V_{\max}$ (Figure 1). K_m is the substrate concentration which gives a rate equal to $0.5 V_{\max}$.

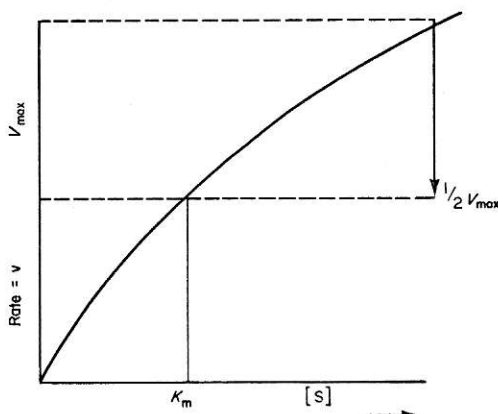


Fig. 1. Effect of substrate concentration on rate of a simple enzyme-catalysed reaction.

Analytical Applications

For analytical applications in general, it may be necessary to measure the concentration or activity of an enzyme, or to use an enzyme-catalysed reaction to measure the concentration of a substrate. The reaction conditions will differ depending on whether substrate or enzyme is to be determined.

Monitoring Substrate Concentrations

If an enzyme-catalysed reaction is used to monitor substrate concentrations, the kinetics are simplest if only small concentrations of substrate are present, so that $K_m \gg [S]$. Then $v = V_{\max} [S]/K_m$, i.e. the rate is directly proportional to the substrate concentration. A typical spectrophotometric response for different initial substrate concentrations is shown in Figure 2.

The change in absorbance over a fixed time interval, e.g. $t_0 - t_1$, is a direct measure of the reaction rate. Kinetic measurements are faster to obtain than those where the reaction is allowed to go to completion, and the requirement for small substrate concentrations implies a trace determination.

Although single enzyme-catalysed reactions are useful for determining a wide range of compounds, for example, urea, via hydrolysis to ammonia in the presence of urease, or ethanol, using alcohol dehydrogenase and NAD^+ ,

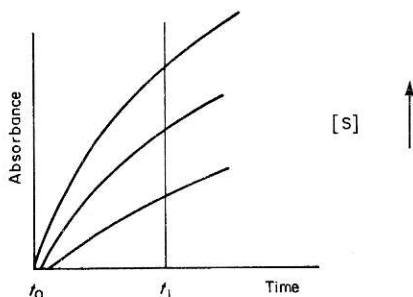
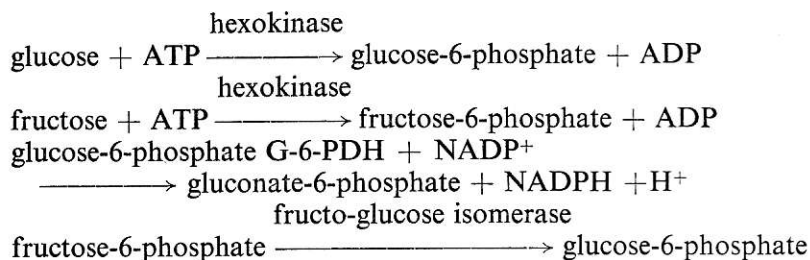


Fig. 2. Change of absorbance with time for different substrate concentrations in a simple enzyme-catalysed reaction.

much greater scope is achieved by using sequences of enzyme-catalysed reactions.

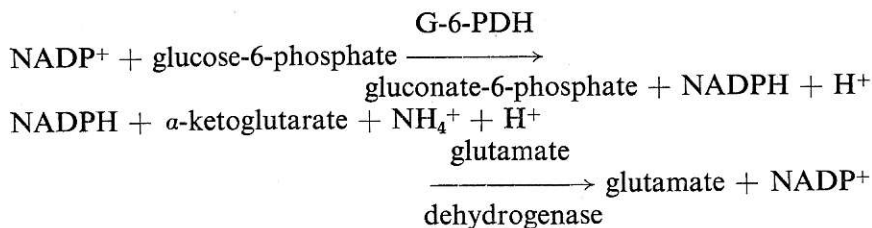
For example, an analysis applicable to honey (a mixture mostly of glucose and fructose) may be carried out by letting both sugars react with adenosine triphosphate (ATP) in the presence of hexokinase. The mixture of sugar-6-phosphates formed is treated with glucose-6-phosphate dehydrogenase (G-6-PDH) and the coenzyme NAD phosphate, NADP^+ , which only react with the glucose phosphate. NADPH is formed and can readily be measured spectrophotometrically. Next fructo-glucose isomerase is added to isomerise the fructose-6-phosphate to glucose-6-phosphate. The G-6-PDH reaction produces more NADPH to give a measure of the fructose.



Enzyme methods exist for the estimation of a host of other organic and inorganic compounds^{1,2}.

Enzyme Cycling

The concept of enzyme cycling is interesting³. It is a very sensitive technique. Traces of NADP^+ , for example, when present with reacting species such as glucose-6-phosphatase and G-6-PDH, produce the gluconate and an equally small amount of NADPH. If α -ketoglutarate and glutamate dehydrogenase are also present, a further reaction occurs, with release of glutamate and NADP^+ . The released NADP^+ reacts with more substrate and the cycle repeats, at up to 20 000 times per hour. Gradually, glutamate and gluconate accumulate, and can be measured enzymatically after an appropriate time. The method is so sensitive that it has been used to determine the quantity of the co-enzyme in a single cell.



Monitoring Enzyme Concentrations

In the presence of a large amount of substrate, $[S] \gg K_m$. Thus the reaction rate becomes constant and independent of substrate concentration, i.e. $v = V_{\max}$ (zero order reaction). Therefore the slope of an absorbance — time plot, representing the rate of reaction, is linear, and increases with increasing initial concentration of enzyme, because $V_{\max} \propto [E]_0$.

Clinical chemists are concerned to measure levels of enzymes as a means of diagnosing various clinical conditions and differentiating between conditions like chronic hepatitis and cirrhosis of the liver⁴. Glutamate oxaloacetate transaminase activity is high in both conditions. Cholinesterase levels are slightly reduced in hepatitis patients but greatly reduced in cirrhosis patients.

Inhibitors and Activators

The behaviour of enzymes can be influenced adversely or favourably by various additives. Thus beryllium adversely affects alkaline phosphatase and the more beryllium one adds the more the enzyme is inhibited. This effect can be used to measure ng amounts of beryllium^{5,6}. The inhibiting affect on enzymes is the reason for the toxicity of beryllium in the body. Alcohol dehydrogenase is inhibited by pg amounts of silver, and an analytical method based on this would be equivalent to having a spectrophotometric reaction with a molecular absorptivity of $10^{10} \text{ l mol}^{-1} \text{ cm}^{-1}$ ⁶. The inhibiting effect of fluoride on some enzymes has been used to determine fluoride at ng levels. Inhibition of esterase systems is normally used⁷⁻⁹. Pesticides inhibit enzyme systems, and in particular they are potent inhibitors of cholinesterase. Some pesticides are determined by measuring the extent to which they inhibit this enzyme¹. The inhibition of cholinesterase obtained from insect sources is far greater than the inhibition of the enzyme from mammals.

The inhibition effect can be used to monitor separated pesticides on a thin layer chromatography plate¹⁰. If one sprays with the enzyme, where there is a pesticide the enzyme will be inhibited. The enzymatic reaction is the hydrolysis of acetyl choline, which liberates acetic acid. So an acid/base indicator in the spray will reveal as acidic those areas where there is no inhibition of the enzyme. Where the enzyme is inhibited there is no acid released and hence no colour change.

The class of drugs known as monoamine oxidase inhibitors has been determined, based on this property, at the Home Office Central Research Laboratory¹¹. The technique has since been extended to other classes of drugs¹². Tetrahydrocannabinol, for example, inhibits glutamate dehydrogenase, and this

provides a means of detecting it at the 100 ng level. For many drugs cholinesterase is the most susceptible enzyme and by using that, tetrahydrocannabinol may be detected down to 8 ng ml⁻¹. Even ascorbic acid has an inhibiting effect on cholinesterase¹³.

On the enzyme activation side, one finds that in extracting enzymes from cells, essential metals are sometimes lost, forming apo-enzymes. If the metal ion is returned to such apo-enzyme extracts, their activity is restored. For example, magnesium activates *isocitrate* dehydrogenase and can be determined by measuring the degree of activation¹⁴. Likewise, metals can be deliberately removed from enzymes to provide a sensitive and highly selective means of determining such metals based on their activation effect. Zinc can be removed from alkaline phosphatase by EDTA, for example, and the apo-enzyme used to determine traces of zinc¹⁵.

Applications in Sample Decomposition

Enzymes find use in laboratories for purposes other than for quantitative assays. The Laboratory of the Government Chemist has used enzymes to assist in the extraction of various colouring matters from foods¹⁶. Dyes which bind to protein (such as Brown FK in kippers) are easier to liberate if the protein is destroyed with an enzyme such as papain. Some drugs become bound to protein and the protein is sometimes precipitated with reagents such as tungsten before drug assay. Much better recoveries of such drugs are obtained if the protein is destroyed by enzymes such as *Subtilisin Carlsberg*¹⁶.

When teeth are examined for lead, a more realistic recovery of lead is obtained if the teeth are first cleaned by digesting off blood and soft tissue with the aid of papain¹⁷. In museum work, parchments and old documents may be stuck together with paste or glue. Sometimes they can be separated by digesting away the adhesive by means of enzymes¹⁸.

Insolubilised Enzymes

Enzymes may be made insoluble by binding them by means of various functional groups in their molecules to chemically activated surfaces such as glass or polymer¹⁹. Such bound enzymes may be used in a number of ways. When held in columns, for example, solutions can be made to pass over and react with particles with enzyme coatings, thus retaining the enzyme for use over and over again, in, for example, flow analysis systems.

Another way in which an immobilised enzyme has been used is when cholinesterase is bound to a porous sinter through which water samples possibly containing a pesticide are continuously passed. After sufficient cycling, the bound enzyme becomes inhibited to a greater or smaller degree. The sample solution is replaced by a solution containing acetyl choline passing through the same sinter. If the enzyme has been greatly inhibited this can be detected by the low rate of conversion. This system has been adopted as an automatic water monitoring device for pesticides where an audible warning sounds when contaminants exceed a pre-set level. Air monitoring devices of a similar kind have also been devised²⁰.

Enzyme Electrodes

Immobilised enzymes find use when bound to films covering the surface of electrodes^{21,22}. Species approaching such electrodes can become changed as they pass through the membrane bearing the enzyme. Thus, a layer of glucose oxidase over an oxygen electrode allows glucose to be measured by the indirect measurement of the depletion of oxygen at the electrode as a result of the catalysed oxidation of glucose. As glucose oxidase is very selective, if the electrode is dipped into a solution of a mixture of sugars, it will measure only the glucose. Glass electrodes, or ammonium ion sensitive electrodes, can similarly be combined with amino acid oxidase to measure amino acids or with urease to monitor urea. The speed of response is now 5–10 seconds for a glucose electrode. A universal detector for enzyme reactions might be the thermistor detector, measuring heat changes as the substrate passes over immobilised enzyme in a specially designed cell²³.

A few investigators have recently extended the immobilisation concept, by attaching living bacteria and other microorganisms to polymers, and keeping them alive whilst covering suitable electrodes. Thus yeast has been chemically bound to a porous polymer and placed over an oxygen electrode. If this is dipped into a solution containing an antibiotic (nystatin) some of the yeast dies, and less oxygen is consumed. So with increasing amount of antibiotic present, increasing amounts of oxygen are available to the electrode²⁴. The opposite effect can be turned into a means of measuring acetate, because acetate will stimulate yeast growth, more oxygen is consumed, and oxygen at the electrode is decreased²⁵.

Such electrodes have a slow response, but if one can wait the necessary 10 minutes or so, such electrodes extend significantly the range of compounds which may be determined by electrode response. Thin slices of pigs kidney have been incorporated into an electrode, making use of their glutamine oxidase content²⁶. If this electrode is dipped into glutamine solution, ammonia is liberated, so an ammonia-sensitive electrode is used as detector. That electrode responds only to glutamine in the presence of other similar compounds, and it is more reliable than if the enzyme had been extracted and immobilised on to a membrane. The kidney slices are said to remain effective for a month. The bacteria which cause tooth decay (by converting dietary carbohydrate to acid) have been immobilised and used in conjunction with a glass electrode to measure carbohydrates in solution by means of the response of a glass electrode to the generated acid.

Conclusions

Enzymes are playing an increasingly important and widespread role in solving analytical problems. As well as providing sensitive and selective methods of determination for compounds in foods and beverages, clinical, biological and environmental samples, recent and current developments are greatly expanding the mode and fields of application of enzymes. This is leading to greater availability of enzymes and substrates as well as to improved economics of use. There is no reason why this trend should not continue during the next decade.

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