Enzymology and Medicine

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D W Moss, MA, MSc, PhD
Reader in Enzymology at the
Royal Postgraduate Medical School,
University of London

and

P J Butterworth, MSc, PhD
Lecturer in Biochemistry,
Chelsea College of Science and Technology,
University of London

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Preface

Medicine is becoming increasingly an exact and quantitative science, due to a growing understanding of the changes at the molecular level which underlie and give rise to human disease. This is recognized in the current reappraisals of the curriculum of medical studies, which are motivated by the need to ensure that the principles of basic biological science to which the student is introduced in his pre-clinical years continue to inform his thinking and rationalize his approach as a practising doctor. In the traditional pattern of medical studies there has in general existed a marked dissociation between the pre-clinical and the clinical years. Too often, the significance of the examinations at the end of the pre-clinical phase has appeared only to be that of an obstacle which must be surmounted for entry to the clinical phase and one which, once it is overcome, can be dismissed from the mind. To reshape this pattern of thought, therefore, it is desirable to integrate the pre-clinical and clinical parts of the curriculum, and to endeavour to bring out during the pre-clinical years the relevance to the practice of medicine of what is being taught. In clinical studies, the scientific principles on which modern diagnosis and treatment are founded are similarly emphasized.

The study of enzymes and its relevance to medicine seem to be a particularly apt field for such an integrated treatment. In his pre-clinical studies in biochemistry, the student learns something of the nature and properties of enzymes. Later, he learns to examine and manage patients whose diseases may be due to abnormalities in the production or functioning of some of the many enzymes of the human body; or he may use serum enzyme activities as an aid to diagnosis, often without interpreting these observations in the light of his formerly acquired (and perhaps subsequently forgotten) knowledge of enzyme biochemistry.

In these two phases of his career the student is faced with a choice of textbooks which by their nature tend to emphasize this division. The biochemistry of enzymes is dealt with fully in many textbooks which, however, usually make only minimal mention of the relevance of enzymology to human disease. Textbooks of medicine, on the other hand, are equally silent on the nature of the biochemical lesions which manifest themselves as specific diseases.

The aim of the present book is to describe the nature and properties of enzymes in such a way as to bring out the relevance of this branch of biochemistry to the practice of medicine. It is intended to be complementary to the standard textbooks of biochemistry and medicine on which the student will continue to rely for the main body of his organized knowledge, but it will, it is hoped, present a picture of a thread of knowledge running from the laboratory to the bedside.

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1 The Biochemical Basis of Health and Disease

Life is a chemical process. Food of a definable range of chemical composition is taken in by living organisms, and waste products of a different chemical composition are excreted. Growth and reproduction entail the production by the organism of new tissue, again consisting of material of a definite chemical constitution: they are thus processes of chemical synthesis. Everyday experience shows the relationship between enhanced muscular activity and the increased expenditure of chemical energy (in the form of a greater intake of food). The elucidation of the sequences of chemical reactions which intervene between the input of raw materials into the living system (intake of food) and the appearance of the end products (new tissue, waste products, energy) lies within the ambit of biochemistry, and biochemical research has now succeeded in defining most of the reaction sequences, or metabolic pathways, which are followed in living matter. From these researches the realization has emerged that the main pathways of chemical change are essentially similar throughout the great diversity of living matter, and that while there are quantitative and qualitative differences in metabolism between different organisms and different cells, many reaction sequences by which energy is produced or components of living matter are synthesized are of universal occurrence. A further fundamental similarity is shared by the metabolic processes of all living things: almost every chemical reaction in living matter is a catalysed process, and all the catalysts concerned belong to a group of specific proteins possessing certain common properties and known as enzymes. From the biochemical point of view, therefore, life consists of integrated sequences of chemical reactions catalysed by enzymes.

The early history of enzymology is closely connected with the development of ideas on the nature of two processes, alcoholic fermentation by yeast cells, and digestion in higher animals. Digestion was recognized as a "chemical" event mediated by the action of juices such as saliva by the end of the 17th century, and its experimental investigation was begun by Réaumur (1683–1757), who induced a tame kite to swallow perforated metal tubes containing food of various kinds. When the tubes were regurgitated meat was found to be partly dissolved, though starchy substances were unaffected. Since enclosure in the metal tubes protected the food from any grinding action (at that time grinding was the principal non-chemical theory), it appeared that the active agent must be the liquid in which the food was seen to be soaked. Spallanzani (1729–99) confirmed this by achieving *in vitro* digestion of meat when it was warmed in samples of gastric juice obtained by placing sponges in the perforated metal tubes. Beaumont (1785–1853) was able to extend this type of observation to the digestive processes of the human stomach by taking advantage of an unusual shooting accident in which a wound in the body-wall healed leaving the victim with a permanent gastric fistula through which Beaumont could study the time taken to digest various kinds of food. Although the strongly acid nature of gastric juice was recognized, it was realized that this alone could not account for the digestive power of the juice, and in 1836 T. Schwann discovered the proteolytic enzyme pepsin. The principal proteases of the pancreatic juice, trypsin and chymotrypsin, were also identified during this early phase of enzymology, as were enzymes acting on starch (amylases) and fats (lipases). Digestion was thus the first vital process to be clearly seen to consist of a series of enzymic reactions.

The study of digestion was facilitated by the largely extracellular nature of this process. The presence of many of the enzymes in the various secretions of the gut made their character-

ization and isolation possible before techniques for the investigation of organized cells and tissues were developed. The proteases of the digestive tract have continued to figure in advances in enzymology and their ease of purification and comparatively small molecular weights placed them amongst the first enzymes to be crystallized in the late 1920s and early 1930s, while at the present time some of the first complete structural analyses of enzyme molecules have taken chymotrypsin as their subject.

The investigation of intracellular enzymic reactions may be regarded as beginning with Buchner's preparation in 1896 of a cell-free yeast extract which retained the power to ferment glucose to alcohol. Fractionation of the yeast juice into its component enzymes and metabolic intermediates occupied the next thirty years or so and was paralleled by work on minced and sliced avian and mammalian muscle which revealed the essentially similar reaction sequence by which glucose is fermented to lactic acid in this tissue. By the 1950s the main outlines and many of the details of metabolism in living organisms had been discovered, firmly establishing that the universal and unique secret of life is to be found in co-ordinated chemical reactions and in the nature and properties of the enzymes which catalyse them. The same period also included the first extensive enzyme purifications. The list of known enzymes has now grown to over 1000, of which more than 100 have been crystallized.

Since life is a chemical process dependent on the specific catalytic activities of enzymes, with a certain pattern of reactions and a particular balance between the rates at which they proceed corresponding to normal functioning, it follows that the fundamental nature of disease lies in derangements of the normal sequence of enzymic reactions. Pathological modification of the normal pattern of metabolism can arise in several ways. The metabolism of the host may be altered in response to invasion by an infective agent, e.g. a bacterium or a virus. Local injury may cause a temporary disturbance of the micro- and macro-architecture of tissues and organs resulting in a redistribution of enzymes and metabolites so that new reactions, or alterations in rates of reaction, can occur. Ingestion of poisons or drugs may directly influence the activities of particular enzymes. The normal balance of reactions may be distorted as a consequence of malignant changes taking place within the cells after exposure to ionizing radiations or chemical agents, or even apparently arising spontaneously. A congenital alteration in the properties of certain enzymes or even their complete absence may be compatible with life so that an abnormal pattern of metabolism is observed in the affected individuals.

The degree to which alterations in specific enzymic reactions can be identified as the causes or consequences of the various pathological states varies greatly. This relationship is most clearly identifiable in the case of certain inborn errors of metabolism, in which a genetically determined absence of one enzyme can be demonstrated as the single abnormality from which the observed pathological consequences are all derived. At the other extreme are the multiple changes which result from tissue damage, which are too complex to be analysed fully at present in terms of alterations in individual reactions. Malignant change, which appears to involve subtle quantitative shifts in the balance of reactions in the affected cells rather than readily identifiable qualitative differences from normal patterns, also falls into this category. The way in which the agents responsible for infectious diseases modify the host's metabolism is the subject of much research. Viruses impose new patterns of enzyme synthesis and metabolism on the cells which they infect, and the specific sites of action of certain bacterial toxins are currently being defined. Other poisons which gain entry to the body are known to have actions on individual enzymic reactions (an example is the inhibition by cyanide of the enzymes of cellular respiration), while the mode of action of other poisons and drugs at the molecular level is progressively becoming clear.

It is also relevant to ask how far an extension of understanding of the biochemical nature of disease is necessary for the management of individual patients, and furthermore, how far the underlying biochemical abnormalities accompanying disease can be diagnosed in a particular instance. It is clearly important to know the nature of the metabolic defect in sufferers from inborn errors of metabolism as a first step towards designing rational therapy, perhaps by avoiding the blocked reaction as far as possible, as in the dietary treatment of phenylketonuria or galactosaemia. Hopes of reversing or even preventing malignant change similarly rest on discovering those features which distinguish the biochemistry of a cancer cell from that of its normal counterpart. The treatment of infectious diseases is perhaps more usefully approached

through a study of the biochemical characteristics of the pathogenic organism so that differences between its metabolism and that of the host can be exploited in the design of specific chemotherapeutic agents – Ehrlich's "magic bullets". The fruits of this approach, the sulphonamides and antibiotics, have transformed medicine within the space of a few decades. A knowledge of the biochemical consequences of mechanical injury to tissues is at the present time of only minor assistance in determining the pattern of treatment, although the known physiological action of substances released from damaged cells (e.g. histamine-like substances) accounts for some of the features of these cases. In addition, small biochemical changes in the blood are often of great value in detecting the occurrence of tissue damage in internal organs, such as may result from coronary artery disease.

The experimental methods that are available to the biochemist in elucidating the patterns of metabolism of normal living tissues or of abnormal forms (e.g. cancer cells) range from studies of the overall chemical exchanges of whole organs or thin slices of living tissue, to a dissection of the cells into their component enzymes and metabolites. Thus, in studying the metabolism of an animal liver (that of a rat, for instance), the ability of the organ as a whole to carry out certain chemical processes might be assessed by making a preparation of the living, anaesthetized animal in which the normal circulation of blood to and from the liver is replaced by a pump perfusing the organ with either blood or mammalian Ringer solution. The experimenter can add various substances to the perfusing fluid and can analyse the resulting changes in composition of the fluid leaving the organ. A less physiological but experimentally simpler approach is to cut thin slices of liver taken from a freshly killed animal and incubate these in Ringer solution to which the substances being investigated can be added. If the medium is kept well oxygenated such slices will survive for some time. The anatomical relationships and access of substrates to the interior of the cells are altered in these slices and the results are thus not identical with those obtained from organ perfusion.

Proceeding a stage further, the experimenter can destroy the cellular structure of the tissue by homogenizing it and can then observe the metabolism of these homogenates. The homogenate itself can be fractionated with the object of preparing and studying either samples of the several types of subcellular particles – mitochondria, microsomes, etc. – which make up animal cells, or, in the final analysis, single, purified enzymes each of which catalyses a single reaction only or a small group of related reactions. All these procedures have their place in understanding the biochemistry of normal and abnormal cells and organs, and none is by itself completely adequate: the metabolism of a whole cell shows features which are additional to those deducible from the properties of its parts (i.e. its component enzymes), while the isolated, perfused organ lacks the hormonal and nervous control to which it is subject in the intact animal.

The biochemical investigations which the clinical biochemist and clinician can carry out in living human patients are of course limited in their scope, nor is it necessary in the majority of cases to demonstrate the biochemical lesion at the molecular level before the patient can be properly treated. The history, signs and symptoms elicited on examination, X-ray appearances, the isolation of a pathogenic organism in infectious disease, the characteristic histology of a section of a tumour removed at operation in suspected malignancy, are the foundations on which diagnosis and subsequent treatment are based. To these are added laboratory tests – haematological, dealing with the relative numbers and morphology of the blood cells, and chemical, directed towards revealing abnormalities in the composition of the blood and other body fluids and excreta. These laboratory tests, added to the clinician's own observations, confirm a diagnosis or decide between possible alternative diagnoses and assist in monitoring the course of an illness.

In carrying out analyses of the composition of the blood or other fluids, the clinical biochemist is attempting to infer from these extracellular measurements the course of events within the cells and tissues of the body. Thus, failure to metabolize a particular constituent of the normal diet may result in the appearance of that substance in abnormally large amounts in the blood or urine – the excess of galactose, for example, in the blood and urine of children who cannot metabolize that sugar. Biochemical disturbance may also result in the appearance of abnormal metabolites which are the products of enzymic reactions normally of minor importance or the substrates of enzymes, the activity of which is reduced or absent. In studying the composition of the blood, in particular, changes in the activities and properties of enzymes deriving from known intracellular locations are

of great value in drawing conclusions about biochemical events within the tissues.

One technique by which it is possible to gain a more direct insight into the cells themselves is by the removal of small specimens of an organ by biopsy. At the present time the main value of biopsy specimens lies in their examination under the microscope, but as techniques for measuring and characterizing the enzymes in these small amounts of tissue are improved their biochemical examination will no doubt increase in importance. One group of cells which can readily be sampled in this way to give relatively large, homogeneous samples are the red blood corpuscles, and studies of the enzyme content of these cells or of the amounts of intermediate metabolites in them have proved of great value in the investigation of several congenital metabolic abnormalities.

The following chapters attempt to summarize the main facts about the nature and properties of enzymes as far as these are known at the present time and to relate them to the manifestations of human disease and its diagnosis and treatment.

Enzyme Nomenclature. The first systematic method of naming enzymes was a proposal, in 1898, by E. Duclaux that "–ase" should be added to a word, or part of a word, indicating the nature of the substance on which the enzyme acts. Apart from a few digestive enzymes which had already been given names (still in use today) ending in "–in", e.g. pepsin and trypsin, this suggestion remains the basis of enzyme terminology. The "–ase" system has had to be extended in some cases to include information about the type of reaction catalysed since more than one enzyme may act on a single substrate (the compound on which an enzyme acts is termed its "substrate"), catalysing different reactions. More recently a still more explicit system of naming and numbering all the known enzymes, with gaps to accommodate those which will undoubtedly be discovered in the future, has had to be devised so that unambiguous description of a particular enzyme is possible. For most purposes, however, the trivial name of an enzyme, based on the "–ase" terminology and with perhaps some additional information about the tissue and species from which the enzyme has been prepared, is sufficient and will be used in this book.

2 Experimental Methods in Enzymology

As in all branches of natural science, present knowledge and future advances in enzymology depend on experiments, particularly those of a quantitative nature. Experimental methods in enzymology are directed to the detection and measurement of enzyme activity and to the purification of enzymes so that their constitution and properties can be studied.

MEASUREMENT OF ENZYME ACTIVITY

Enzymes are catalysts, that is, they accelerate specific chemical reactions and are themselves unchanged in amount and chemical composition when the reaction is completed. It is this property which gives enzymes their unique biological importance and it also forms the basis of methods of estimating the amount of an enzyme which is present in a given system, e.g. a tissue extract, blood, etc. As with the inorganic catalysts known to chemists, enzymes exert their accelerating effect detectably on reaction rates when they are present in small traces only: the molar concentration of an enzyme *in vitro* is usually far lower than that of the substrate which it transforms. The presence of an enzyme is therefore revealed and its quantity is measured by its activity, and in most cases (except when dealing with highly purified enzymes) attempts to determine its absolute amount – for example, by making use of methods of estimating proteins, since enzymes are proteins – are doomed to failure because of their relative insensitivity. This ability sensitively to detect changes in the amount of enzyme present, e.g. in a sample of blood, in the presence of a large excess of other proteins is a characteristic which contributes greatly to the clinical value of enzyme estimations as indicators of tissue damage in a way that will be discussed more fully in a later chapter.

The basis of an enzyme activity determination is thus the comparison of the rate of a chemical reaction (i.e. the amount of product formed or substrate destroyed in a given time) in the presence of the active enzyme, with the rate of reaction in a control solution from which the enzyme is omitted or in which it has been inactivated (e.g. by heating or by addition of protein precipitants). Conditions such as the pH of the reaction medium and its temperature must be controlled within narrow limits by buffer solutions and thermostatic water-baths since variations in these factors have a marked influence on the rates of enzymic reactions, as do other components of the system, metal ions for example, which may accelerate or retard the reaction. Comparison of the relative amounts of a particular enzyme in different preparations or samples on the basis of the rate of the catalysed reaction under standard conditions depends on the assumption that, if a given number of enzyme molecules result in a certain reaction rate, twice or three times that number will increase the rate by a factor of two or three. Provided that the experimental conditions are correctly chosen and maintained so that other factors do not influence the rate, the proportionality between amount of enzyme and rate of reaction is almost always found to hold. The progress curve for an enzyme-catalysed reaction (chemical change expressed as a function of the duration of the reaction) typically consists of an initial linear portion merging into a curve as the rate falls off. The fall in rate is due to the combined effect of several factors – fall in substrate concentration, increasing reverse reaction, enzyme denaturation, etc. – and the shape of the curved portion of the progress curve thus cannot be represented by any fixed mathematical relationship applicable to all cases. When the progress curves are compared using different amounts of enzyme the slopes of the initial linear portions are proportional to the enzyme

concentration, but this is usually not the case for the nonlinear portions of the curves (Fig. 2.1). It is therefore essential that all comparisons of reaction rates for estimation of enzyme activity must be based on linear progress curves. The reaction rate may be measured by allowing the reaction to proceed for a fixed length of time,

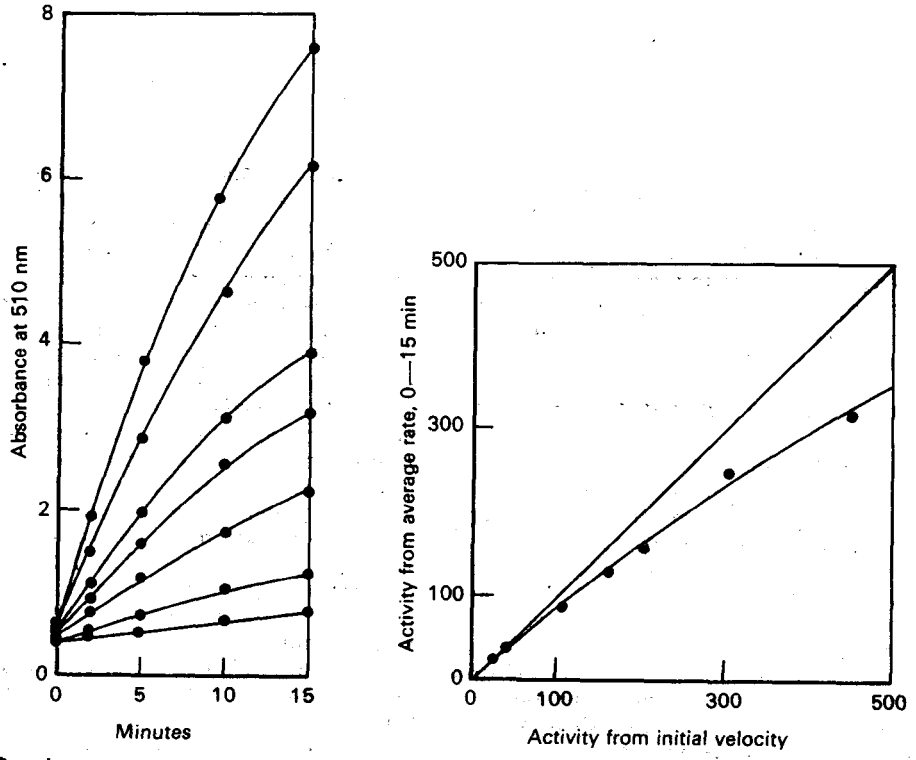


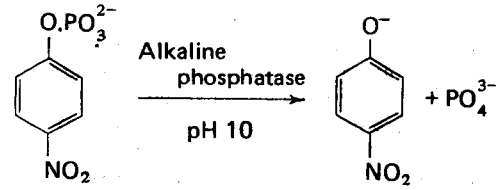
Fig. 2.1. Reaction progress—curves with different amounts of enzyme. Phenol released by hydrolysis of phenyl phosphate by alkaline phosphatase is estimated colorimetrically after *different periods of reaction with increasing* amounts of enzyme (left). Activities derived from the average rates over 15 min are lower than those derived from initial velocities, at higher enzyme concentrations (right).

stopping it (for example by adding a protein-precipitant) and estimating the amount of some product of the reaction which has been formed. However, it is difficult to be certain that the progress of the reaction has remained linear in this type of assay (termed a "two-point" or "fixed-time" assay), particularly since, in many reactions, the duration of linearity may be very short indeed. Consequently, methods are to be preferred in which the progress of reaction can be monitored continuously; these are often referred to as "kinetic" assays.

While all enzyme estimations are based on the same principles, a great many variations are possible in the experimental details of different methods. These variations lie principally in the techniques which are used to determine the amount of chemical change which has taken place. Gravimetric analysis is of little value in enzyme work since most of the transformations catalysed by enzymes involve organic compounds, and the limited sensitivity of this type of analysis is unsuitable for the low concentrations of reactants usual in enzymic reactions. On the other hand, the sensitivity and wide scope of colorimetric analysis has proved of great value in enzymology, and it is this form of analysis, together with its extension into the ultraviolet spectrum, which is applied in most of the enzyme estimations carried out from day to day for clinical purposes.

One of the products of the action of the enzyme may be made to undergo a second reaction to yield a coloured compound, the intensity of the colour (measured in a photo-

electric colorimeter) being proportional to the amount of product formed. For example, many enzymes release phosphate from their substrates which can be detected and measured by the blue colour formed by reaction with molybdate and a reducing agent. The reagents involved in the colour-forming reaction often interfere with enzyme action, so that these methods can then only be applied on a fixed-time basis. More directly, the product of the enzyme's action may itself be coloured, obviating the need for a second reaction and allowing the reaction to be monitored continuously. When the enzyme concerned is one of wide specificity, i.e. one which will act on a variety of substrates and catalyse a similar reaction with each, the substrate can be chosen or adapted chemically so that a coloured product is formed. An example is the estimation of alkaline phosphatase, an enzyme of wide distribution in human tissues the activity of which in blood plasma varies significantly in liver and bone diseases (Chapter 9). This enzyme hydrolyses a wide range of phosphate esters, both naturally occurring and synthetic, in alkaline solution. One synthetic substrate is p-nitrophenyl phosphate which is split to p-nitrophenol and orthophosphate. p-Nitrophenyl phosphate is colourless, but p-nitrophenol is yellow at alkaline pH: the course of the enzymic reaction can therefore be followed by measuring the progressively deepening yellow colour of the solution:



p-nitrophenyl phosphate — p-nitrophenate ion (yellow)

In the case of enzymes of limited specificity the choice of substrates to make for easier chemical analysis is usually not possible.

Substances which form coloured solutions do so because they absorb light of a particular range of wavelengths in the visible region of the spectrum. When observations are extended into the ultraviolet range by the use of suitable sources of light and detectors other than the human eye (e.g. photoelectric cells), many more compounds are found to absorb light and the wavelengths which they do so are characteristic of the presence in their molecules of particular groupings of atoms. Enzyme-catalysed reactions often involve rearrangements within the molecules which result in changes in the ultraviolet spectrum, and when these changes are sufficiently pronounced to be observed in a spectrophotometer they afford a convenient basis for the measurement of enzyme activity. An example is the action of uricase on uric acid. Uric acid has a strong absorption band in the ultraviolet spectrum centred at 293 nm. Oxidation by the enzyme uricase, which occurs in some animal tissues and yeasts, is accompanied by the progressive weakening and eventual disappearance of this band (Fig. 2.2). As well as providing a means of estimating uricase activity this property also constitutes a sensitive and specific method of measuring small amounts of uric acid, e.g. in blood samples, from the change in absorption in the presence of an excess of the enzyme.

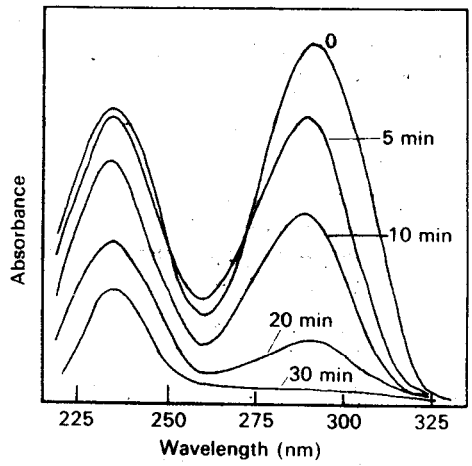
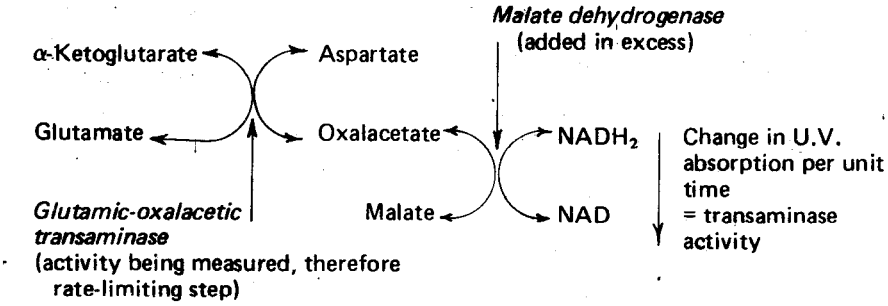


Fig 2.2. Progressive changes in the ultraviolet absorption spectrum of uric acid during oxidation by uricase.

The component of the reaction mixture that undergoes a change in its absorption spectrum need not be the substrate of the enzyme for the ultraviolet method to be applicable, provided that some participant in the reaction is changed at a rate proportional to the rate of change of the substrate. Many enzyme reactions involve parallel changes in components other than the substrate; these reactants are termed coenzymes and the same coenzyme is often involved in a wide range of reactions catalysed by many different enzymes. Two important coenzymes are nicotinamide adenine

dinucleotide, abbreviated to NAD, and nicotinamide adenine dinucleotide phosphate (NADP), which take part in many reactions in which hydrogen atoms are removed from, or added to, the substrates under the influence of specific dehydrogenase enzymes. The coenzymes act as acceptors or donors of hydrogen atoms and are thus either reduced or oxidized during the exchanges. (For a fuller description, see Chapter 5.) Both coenzymes have similar ultraviolet spectra with a strong absorption maximum at 340 nm in the reduced form which disappears on oxidation. Thus, the action of the enzyme lactate dehydrogenase on lactic acid, which is accompanied by the transfer of hydrogen to NAD^+, can be followed by the increasing absorption due to NADH at 340 nm (Fig. 2.3). The molecular extinction coefficient of the reduced coenzymes is $6{\cdot}22 \times 10^6$. Thus, the appearance of one micromole of reduced coenzyme in a reaction volume of 1 ml results in a change in extinction at 340 nm of 6·22 in a 1 cm cuvette and changes of one hundredth part or less of this are readily measurable in a good spectrophotometer.

The measurement of the absorption changes of NAD and NADP is so sensitive and convenient that considerable ingenuity has been displayed in devising ways in which enzymic reactions which do not involve these coenzymes can be brought within the scope of the method. This can be done by coupling together two, three, or more reactions so that the product of the first forms the substrate of the next, and so on, until a reaction is reached which involves the oxidation or reduction of one of the coenzymes. Transaminases (now called aminotransferases) which are enzymes of amino acid metabolism of diagnostic value, are often estimated in this way:



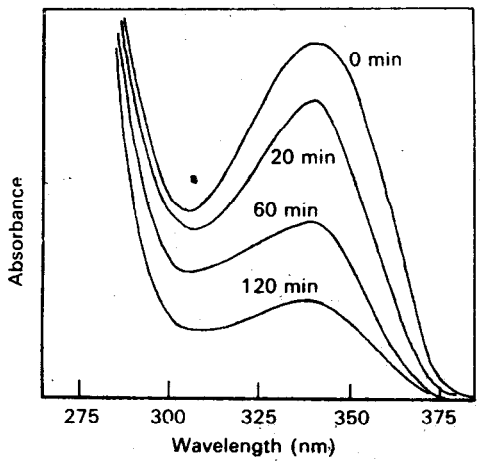


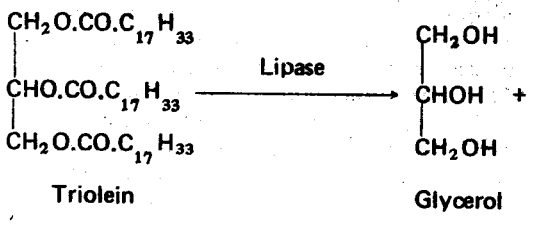
Fig 2.3. Progressive reduction in absorption of light at 340 nm during the oxidation of NADH to NAD^+, with transfer of hydrogen to pyruvate to form lactate, catalysed by lactate dehydrogenase.

It is essential in setting up linked reactions that the rate-limiting step should be the one catalysed by the enzyme being assayed, and that the accessory enzymes, coenzymes and substrates should be present in sufficient amounts to ensure that this is so. However, the presence of additional substrates or coenzymes may modify the activity of certain enzymes and in these cases coupled assay systems may not be appropriate. The light which is absorbed by certain compounds is re-emitted, typically at lower energies and therefore longer wavelengths, as fluorescence. Measurements of fluorescence can usually be made with 10 or 100 times the sensitivity of absorbance measurements and when fluorescence changes accompany enzymic reactions they can be used to facilitate the assay of small amounts of activity. Thus, the reduced forms of NAD and NADP are fluorescent when irradiated with light of 340 nm wavelength whereas the oxidized coenzymes are not. Increased sensitivity becomes extremely valuable

when small tissue samples only (e.g. biopsy specimens) are available.

Changes in the ultraviolet or visible spectra of the reactants are not the only methods by which enzymic reactions can be monitored, although they are amongst the most sensitive and convenient. Many reactions catalysed by enzymes, particularly those concerned with tissue respiration, are accompanied by the uptake or evolution of gas, e.g. carbon dioxide. The change in gas volume (or pressure) in a closed reaction vessel can therefore be measured, suitable corrections being made for the effects of pressure and temperature on the gas volume. These manometric methods have an important place in the history of the elucidation of many of the metabolic pathways in animal tissues, but have now largely given way to optical techniques.

A number of enzymic reactions are accompanied by a change in the pH of the reaction mixture, which can be followed conveniently. However, the alteration of pH shifts the conditions from those that are optimal for enzyme activity, slowing the reaction down and eventually stopping it. A more satisfactory procedure, therefore, is to add alkali or acid at a controlled rate to the solution so that the pH is kept constant: the volume added in unit time thus becomes the index of the rate of reaction. An example of an enzyme of clinical interest which liberates acid by its action is pancreatic lipase and the acid can be titrated:



These examples by no means exhaust the variety of ways in which enzyme activity can be measured but some methods are applicable to estimations of a few enzymes, or even of one only, and the methods outlined represent those most generally useful.

The results of enzyme estimations are expressed as the amount of chemical change in unit time — milligrammes of a product formed, or of a substrate destroyed, per minute, for example. A further term is added to convey the amount of material which contains this activity, e.g. per 100 ml of serum, or in a 24-hr collection of urine. For enzymes which are of established clinical value, eponymous units named for the authors of particular methods are frequently encountered and with the continuing improvement and modification of methods of estimation the definitions of the different "units" in which enzyme activity may be reported can multiply at an alarming rate, since every change in reaction conditions almost inevitably alters the significance of the units — at least twenty different units for expressing serum alkaline phosphatase activity have been defined, for instance, of which three are in everyday use. The proliferation of units makes comparison of results obtained in different laboratories difficult or even impossible, a distinct disadvantage in long-term studies on individual patients or comparisons of groups of patients.

An attempt at rationalization has been made recently in which an "international enzyme unit" is defined as the number of micromoles of substrate transformed per minute, and enzyme concentration (as in serum, urine, etc.) as the number of units per ml or per litre. These units are not absolute, in that the conditions (nature of substrate, temperature, pH, etc.) under which the measurements were made must be specified, so that for a particular enzyme an "international unit" based on one method is not necessarily equivalent to an international unit based on a different method, but the system of international units represents at least a step towards uniformity.

Some of the potential sources of error in estimating enzyme activity have already been mentioned; for instance, the marked dependence of enzyme activity on pH and temperature with the resulting need for careful control of these variables, and the requirement for adequate concentrations of intermediate reactants in linked reaction sequences. Other sources of error are not peculiar to enzyme estimations but are characteristic of the type of analysis, colorimetric, manometric, etc., which is being

employed. Where more than one method of estimating a particular enzyme is available, the relative accuracy and reproducibility of the several procedures are important factors in deciding which shall be adopted. Also critical in the choice of an enzyme assay for clinical investigations is its sensitivity: many clinical applications involve the measurement of small changes in enzyme activity in samples which are available in limited volumes only, e.g. venous blood, and this can only be done successfully with techniques of adequate sensitivity. An example of the limitations that can be imposed by the lack of a suitable assay method is provided by the enzyme pancreatic lipase. The appearance in the circulation of enzymes produced by the cells of the pancreas is a valuable diagnostic sign in acute abdominal pain, when it strongly supports a diagnosis of acute pancreatitis. Lipase releases fatty acids from triglycerides and is usually estimated by titrating the acids (p. 9). However, this method is so insensitive (some procedures which have been suggested specify a 24-hr incubation period) as to be of little value for estimations on blood. Attempts have been made to adapt the substrate to permit a colorimetric assay, e.g. by synthesizing a fatty-acid ester of p-nitrophenol (cf. the estimation of alkaline phosphatase, p. 7); however, it is by no means certain that the enzymes which split these synthetic substrates are the same as the lipase which acts on triglycerides. For these reasons, estimations of the starch-splitting enzyme of the pancreas, amylase, for which a variety of more sensitive methods are available, are preferred in the investigation of acute pancreatitis although there is some reason to think that changes in plasma lipase would show a more consistent correlation with disease of the pancreas.

In enzyme analysis, as in other analytical procedures, the sum of the effects of the various sources of error makes up the total experimental error of the method, which is reflected in its accuracy and precision. Accuracy is the degree to which a particular result approaches the true or most probable value for the constituent being estimated (in this case the enzyme), while precision refers to the extent to which repeated estimations on the same sample give results which are in agreement. The accuracy and precision of an analytical method can be assessed by repeated analysis of specimens of known composition, but when the assay is used to analyse large numbers of samples over a long period of time, as is the case when enzyme estimations are carried out on serum samples for diagnostic purposes, quality-control programmes are needed to ensure that the degrees of accuracy and precision of which the method is capable are being maintained. This is achieved for analyses involving substances which can be obtained in pure and stable form (e.g. sodium, urea) by preparing solutions of known constitution by weighing, then analysing a sample of these solutions along with each batch of specimens of unknown composition. However, when enzymes are the subject of analysis their nature and properties complicate this simple approach. Few if any enzymes of clinical interest have been obtained in a sufficiently pure form for solutions of known enzyme activity to be prepared by weighing known amounts of the purified material. Accuracy in an enzyme assay method is therefore difficult to assess, but the degree to which results obtained by the method under assessment agree with those given by a reference method is often used for this purpose. Monitoring the reproducibility of enzyme assays is rendered difficult by the instability of enzyme preparations. Enzymes are proteins, and are consequently susceptible to denaturation with accompanying loss of activity (Chapter 3). When relatively concentrated solutions of a pure enzyme are available the occurrence of denaturation can be detected by changes in certain properties of the solution such as its effect on the rotation of plane-polarized light, but for the dilute enzyme solutions used for quality-control purposes in clinical enzymology, with their admixture of other proteins, there is no way in which a low result due to errors in analysis can be distinguished from one due to deterioration of the control preparation. Future research may produce enzyme solutions of assured stability; meanwhile, it is desirable that the activity of enzyme solutions used to monitor the precision of enzyme assays should be checked regularly by the reference method.

Human operators who have to carry out large numbers of repetitive analyses are liable to fatigue, with a consequent increase in experimental error. The advantages of automated methods in eliminating this source of variation and improving the consistency and rate of production of results have been well demonstrated in analysis for non-enzymic constituents, and a number of systems for the mechanization of colorimetric analysis have been devised, notably the Technicon Auto Analyzer system

invented by L. T. Skeggs in which reactions take place in flowing streams of liquid. Automatic analysis of this type can readily be applied to enzyme assays of a colorimetric, fixed-time type. However, the advantages of continuous monitoring of progress curves mentioned earlier, which are particularly noticeable when samples of widely varying enzyme activity are to be analysed, make it desirable to investigate the possibilities of mechanizing this form of assay.

A spectrophotometric assay for a coenzyme linked enzymic reaction, e.g. lactate dehydrogenase, may be broken down into several stages. First, the enzyme-containing sample (usually serum) is measured accurately into a known volume of buffer solution containing all but one of the essential components of the reaction (e.g. pyruvate). After a period of incubation to allow any changes in extinction due to the presence of endogenous substrates in the sample to take place and a constant extinction value to be reached, the reaction proper is initiated by adding a measured volume of a solution of pyruvate and mixing. The rate of change of extinction at 340 nm is then recorded and the gradient of the initial linear portion of the recorder trace is computed to give the rate of reaction. Temperature must of course be constant from the moment recording starts, so that preheating prior to this stage is necessary. Instruments are available in which various stages in the process are mechanized, from the successive transfer of samples to the spectrophotometer in simpler instruments to the complete sequence in the more complex ones. The fully automatic machines require the operator merely to load into them unmeasured samples in a known sequence, results being printed out at the completion of analysis. This degree of complexity is necessarily expensive, but where large numbers of enzymic analysis are carried out savings in operators' time, as well as improvements in precision of results, justify the expenditure.

PURIFICATION OF ENZYMES

One of the first requirements in starting to extract and purify an enzyme from the tissues in which it occurs is the availability of a suitable method of assaying it, so that the precipitates, fractions of solutions, etc., containing the enzyme can be identified during the various stages of the purification process. Next, the material from which the extraction of the enzyme is to be attempted must be chosen. If the intention is to prepare a quantity of enzyme having a particular activity, perhaps for therapeutic purposes or to serve as quality-control materials in analysis, a source material is selected which is known to be rich in the enzyme, e.g. a bacterial strain which can be cultured in quantity. More often, however, the object of the investigation is to study the properties of an enzyme occurring in a particular organ or tissue, perhaps in order to compare it with analogous enzymes from other tissues or to reveal abnormalities which may be associated with a genetically determined disease. In cases such as these, limited amounts only of the starting material may be available, particularly when human tissues are the object of study, and purification procedures then have to be adapted to use on a small scale.

The majority of enzymes act intracellularly and, moreover, are in many cases firmly attached to the various structural elements of the cells in which they occur. The first step in enzyme purification is therefore to release the enzyme into solution freed from the organized structures of the cells. When the enzyme is one which occurs in the cytoplasmic fluid, breaking the outer membrane and filtering off the cellular debris achieves the desired result. The cell membranes can be broken by grinding the minced tissue with sand in a mortar, or by homogenizing it either in a rotating-blade blender or in the Potter-Elvehjem type in which the cells are subjected to shearing forces in the liquid layer between a motor-driven pestle and the wall of a closely fitting glass tube. Ultrasonic disintegration is also useful. The cell walls of bacteria are often very tough and vigorous homogenization may be needed to break them: alternatively, recourse may be had to treatment with organic solvents or even to the addition of enzymes that specifically attack chemical linkages in the cell-wall material.

Release of those enzymes which occur in subcellular particles is more difficult, as in some of them, e.g. mitochondria, the enzymes seem to be part of the structure of the particles themselves. The construction of the particles often involves lipid materials and treatment with fat solvents or detergents is usually effective in bringing the enzymes into solution. Samples of a particular type of sub-cellular particle relatively free from other structures can be obtained by differential ultracentrifugation, in which the tissue homogenate is subjected to increasing gravitational fields in an ultracentrifuge so that the particles sediment in descending order of

their particle weights, the heaviest (i.e. the nuclei) at the lower centrifuge speeds and progressively down to small bodies such as microsomes which require high-speed centrifugation to sediment them (Chapter 8).

When a true solution of the contents of the cells of a tissue or of the components of a particular class of sub-cellular organelles has been obtained, it will contain not only the enzyme under investigation but also a complex mixture of other substances both of small molecular weight (inorganic ions, simple sugars and lipids, amino acids, etc.) and macromolecules (mainly proteins, with some polysaccharides and also nucleic acids when the solution has been made from nuclei and microsomes). Small molecules can be removed by dialysis and nucleoprotein by adjusting to pH 5 and centrifuging. The problem of enzyme purification thus resolves itself into the isolation of a single type of protein molecule from a mixture of many different types.

Protein molecules contain many groups of atoms which are capable of ionization under appropriate pH conditions to acquire positive or negative charges. These groups consist of the basic and acidic radicals (e.g. amino and carboxyl) in the side chains of the amino acids from which the protein molecules are built up and those at the ends of the protein chains: the nature, relative proportions and arrangement of the several amino acids in a given protein molecule are responsible for its characteristic properties (Chapter 3). Each of the ionizable groups in a particular protein molecule displays a characteristic variation of ionic charge with pH, the precise form of which depends on the chemical nature of the group and the nature and arrangement of the neighbouring groups in the protein molecule. The tendency of an acidic or basic group to ionize is expressed as its pK value, that is, the negative logarithm of its ionization constant. The pK value has the dimensions of pH, and it is that pH at which half of all the groups of that type present in solution are ionized, since

$$pH = pK + \log \frac{[\text{ionized form}]}{[\text{un-ionized form}]}$$

and when the concentration of the ionized form equals that of the un-ionized form, pH = pK.

The different pK values of the many ionizing groups in a protein molecule have the effect that, at a particular pH, some of these radicals will carry positive charges, some will have negative charges and others will be undissociated. As the pH changes, so the degree of ionization of the groups varies accordingly. Under any given conditions of pH, therefore, the protein molecule itself has a net charge that is the algebraic sum of the individual positive and negative charges; when the sum is zero, i.e. when the number of positive charges just balances the number of negative charges, the protein is at its isoelectric pH. The isoelectric point is a characteristic of the type of protein molecule concerned, and differs from one kind of protein to another. At the isoelectric pH protein molecules do not migrate in an electric field, their solubility is at a minimum and the viscosity, osmotic pressure and conductivity of their solutions also show minimum values. Many of the methods used to fractionate mixtures of proteins exploit the differences in degree of ionization and net charge which exists between different types of protein molecules under particular conditions.

The solubility of protein molecules depends on the interaction of their charged groups with water molecules. Addition of organic solvents such as ethyl alcohol or acetone reduces this interaction by competition for the water molecules and so lowers the solubility of the protein, resulting in precipitation if enough alcohol or acetone is added. The point at which precipitation occurs depends on the nature and degree of ionization of the protein; thus, fractionation of a mixture of proteins can be effected by stepwise increases in the amount of organic solvent in the solution, collecting the protein precipitated after each addition. Removal of water of hydration by organic solvents has a powerful denaturing effect on proteins, and if this is to be avoided it is essential to keep the temperature below 0°C during precipitation. A similar precipitation effect is obtained by the addition of inorganic salts in high concentrations to protein solutions. Ammonium sulphate is often used for this purpose since its high solubility allows the production of concentrated solutions, while this salt is generally without deleterious effects on protein molecules. The amount of ammonium sulphate required to precipitate a particular protein is usually expressed as a percentage of the amount required to produce a saturated solution; serum globulins, for example, are precipitated at 50 per cent saturation.

Precipitation with organic solvents or with ammonium sulphate is a convenient means of obtaining a broad separation of protein mixtures and it forms the first stage of most enzyme puri-