

Automation of enzyme assays

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The increasing use of enzymes as aids to clinical diagnosis has made it mandatory for clinical biochemists to develop techniques and instrumentation to enable large numbers of specimens to be processed quickly with adequate accuracy. The main objective in enzyme assay is to determine the initial reaction rate, as it is only during this period that there is a linear relationship between the rate and enzyme concentration. There are four approaches to rate measurements which are given in Table I. In practice it is normal for most enzyme estimations to be made by the first and third of these, the fourth being only applicable to enzyme systems where changes in acid or base are to be monitored.

Schwartz and Bodansky (1963) have defined three stages of automation dependent upon the degree of automation of the assay procedure (Table II). Even with stage I automation there are means of work simplification which increase the capabilities of the technique. In the present discussion a variety of

systems will be described which can be classed as semi-automatic or fully automatic.

Semi-automatic Techniques

The basis of all these is the recording spectrophotometer or colorimeter. The enzyme substrate mixture is prepared, transferred to a spectrophotometer, and the change in optical density with time recorded (Fig. 1). The development of automatic cell changing accessories for spectrophotometers was the first big step forward in automatic enzyme analysis. These enable four to six samples to be measured simultaneously, the movement of the cuvette being programmed to suit the rate of reaction. When using this type of equipment, work-simplification procedures such as the use of autodilutors or syringe pipettes, and preliminary mixing of solutions can greatly speed the preparation of the reaction mixture.

The main drawback of a methodology of this type is the difficulty in initiating the reaction quickly and then determining the initial rate before it falls off. This is especially so when relatively fast reactions such as that of lactate dehydrogenase (EC 1.1.1.27) are to be measured. The best development in this field has been the Reaction Rate Analyser¹ where the reaction is initiated automatically by the injection of substrate and the change in optical density at 340 nm monitored immediately (Fig. 2).

With this equipment serum samples are diluted with buffered coenzyme in disposable cuvettes. The cuvettes are placed in racks which are fed automatically through a thermostatted tunnel. After 15 minutes, when the contents of the cuvette have reached the correct incubation temperature, the following automatic procedure takes place: an appropriate volume of substrate is dispensed into each cuvette which is then stirred and the change in optical density recorded for a fixed time period (1-10 min). A most useful facility with this equipment is the automatic scale reduction: if the change in optical density (OD) reaches full scale deflection (OD 0.05) within the measuring period the instru-

¹Obtainable from LKB Instruments Ltd., 232 Addington Road, Croydon, Surrey.

Method	Procedure
Constant-time	The total change in signal measured over constant time interval is determined
Variable-time	The time required for the signal to change over a fixed interval is determined
Slope	The change in the signal response against time is recorded
Signal-stat	The amount of reagent necessary for maintaining a constant signal is determined

Table I *Methods for measurement of initial reaction rates (Pardue, 1968)*

Stage I	Manual preparation of reaction mixture; automatic measurement and recording of enzyme activity
Stage II	Automatic preparation of reaction mixture; automatic measurement and recording of enzyme activity
Stage III	As stage II with feedback control of instrumentation

Table II *Automation of enzyme assays (Schwartz and Bodansky, 1963)*

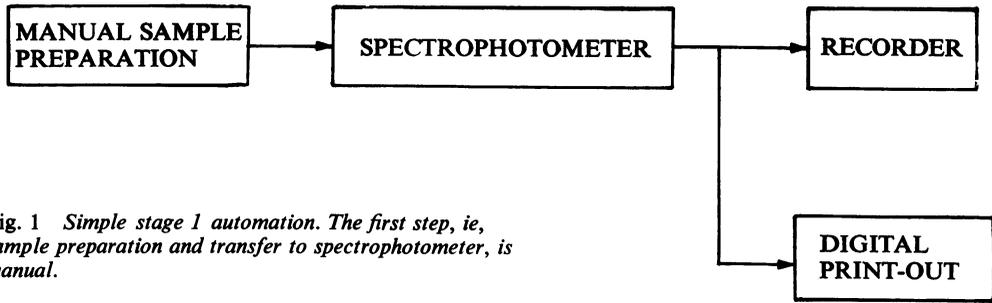


Fig. 1 Simple stage 1 automation. The first step, ie, sample preparation and transfer to spectrophotometer, is manual.

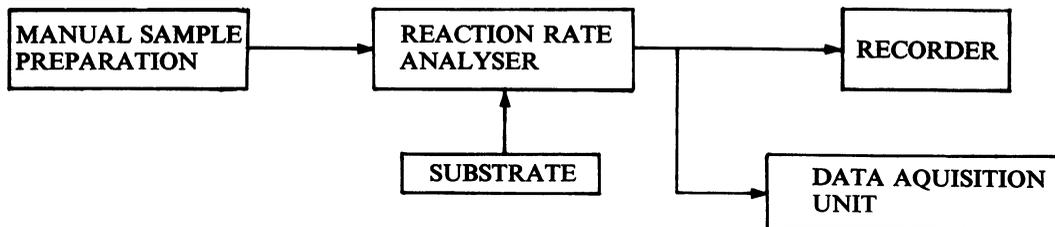


Fig. 2 Semi-automation with a reaction rate analyser. Sample preparation is manual, but the reaction is initiated automatically.

ment automatically switches to a lower sensitivity (full scale OD 0.2). This instrument has proved simple to use with excellent reproducibility. At present it is suitable for any enzyme which can be estimated by monitoring optical density at 340 nm. There is also

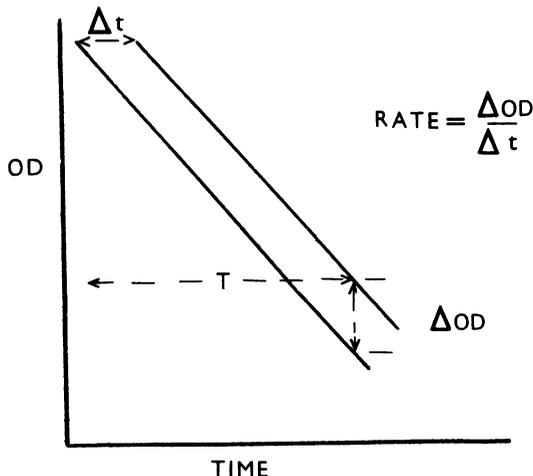


Fig. 3 Approach to automated determination of the reaction rate of Trayser and Seligson (1969). The reaction in each sample is initiated at times t_1 and t_2 the difference between them being Δt which is relatively easy to determine. The difference in optical density (ΔOD) is affected only by Δt and the activity of the enzyme.

a 405 nm filter available for measuring alkaline phosphatase with p-nitrophenyl phosphate as substrate.

An excellent approach has been that of Trayser and Seligson (1969) who have proposed a method in which two identical mixtures of serum and buffered substrate are prepared, and the reaction of one initiated at a fixed time (Δt) after the other (Fig. 3). After a convenient incubation period the difference in optical density (ΔOD) between the two samples measured with a double-beam spectrophotometer. $\Delta OD/\Delta t$ is a measure of enzyme concentration. Δt can normally be varied at will according to the values of ΔOD required and if the extinction coefficient of the substance measured is known the change of optical density can be directly converted to $\mu\text{mol/l}$ by calibration with standard enzyme samples being unnecessary. It should not be difficult to produce instrumentation to automate the sample preparation and accurately reproduce Δt . A commercially available instrument using this technique is not yet on the market but the versatility of the method should soon lead to such a development.

Automated Techniques

Fully automated enzyme assay has been developed both with continuous flow and discrete systems. The majority of the former, of course, have been concerned with the Technicon AutoAnalyzer¹ and

¹Technicon Instruments Ltd, Hansworth Lane, Chertsey, Surrey.

general use 'constant-time' techniques rather than the preferable 'slope' techniques.

AUTOANALYZER TECHNIQUES (CONTINUOUS FLOW)

These have been developed with colorimetric, spectrophotometric, and fluorometric measurement of the enzyme activity. The dangers of single point analysis have been emphasized by many workers but if care is taken to ensure that the calibration curve is nearly linear over the range of enzyme activities to be measured, these can largely be overcome. It is possible to dilute high activity serum to bring it into the linear part of the curve but this process may itself result in erroneous measurements, especially in the case of serum alkaline phosphatase (EC 3.1.3.1) (Nath and Ghosh, 1963). One of the main advantages of the AutoAnalyzer is that the enzyme reaction can be stopped after a fixed incubation period and the products directly mixed with, or dialysed into, a colour reagent. This latter method often obviates the need to measure serum 'blanks'.

An approach towards a two-point analysis using the AutoAnalyzer system was introduced by Pitot, Pries, Poirier, and Cutler (1966). In this technique the sample stream is split in two equal parts and one half incubated for a short period (eg, five minutes) and the other half for a longer period (eg, 15 minutes) (Fig. 4). After incubation the reaction is stopped and the short-incubation time stream passed through a time-delay coil (here 10 minutes) to synchronize the two streams which then pass through a dual-differential colorimeter where the long incubation-time stream is read against the short stream to give a direct measure of the enzyme activity.

Pitot and Pries (1964) had previously devised a system where the reaction mixture was transferred to the flow cell, the pump stopped, and the change in absorbance with time recorded. The pump was then restarted and the procedure repeated. It is debatable whether the Technicon pump can stand up to this stop-start procedure for very long.

In the near future Technicon are to introduce a completely new automatic enzyme analyser¹ where the normal segmented sample-substrate stream is pumped at slow speed through an incubated glass coil. When the first portion of the sample reaches the end of this coil the pump is accelerated so that in effect a gradient is produced with last portion of the sample being incubated for only a very short time. The reaction mixture flows to the colorimeter and produces a trace corresponding to the incubation-

¹Since this paper went to press Technicon have withdrawn this instrument.

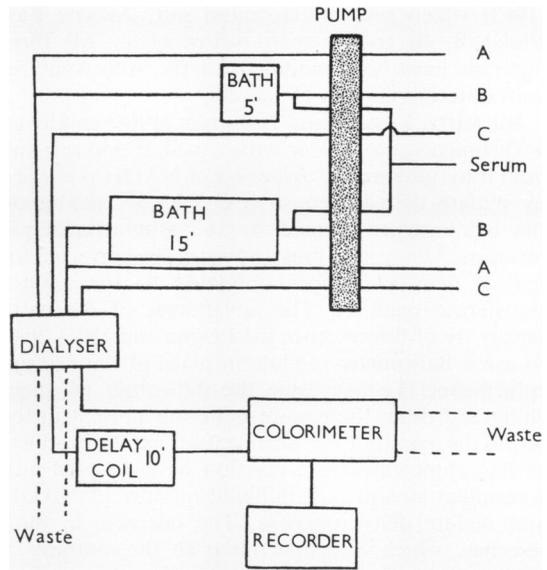


Fig. 4 Two-point assay with AutoAnalyzer with two parallel streams incubated for five and 15 minutes respectively. Serum is initially mixed with substrate (B), and after incubation the reaction stopped with reagent (A) and the mixture dialysed into reagent (C). The differential colorimeter then gives a measure of enzyme activity over 10 minutes.

time gradient; this trace can be used to estimate the enzyme activity.

This last procedure does not do away with the most troublesome factor in AutoAnalyzer assays, namely, the need to run standards every day to check for variations in pumping speeds and flow rates. However, notwithstanding these disadvantages most workers find good correlation between the enzyme activity measured by manual and AutoAnalyzer techniques. As the latter play such a large part in most hospital laboratories the AutoAnalyzer methodology for the three serum enzymes most commonly estimated, viz, aspartate aminotransferase (EC 2.6.1.1.), lactate dehydrogenase and alkaline phosphatase will be discussed in detail.

Aspartate aminotransferase

There are three basic manual methods commonly used for the determination of this enzyme: first, the spectrophotometric method introduced by Karmen (1955) and its subsequent modifications; second, the colorimetric procedure depending on dinitrophenylhydrazine (Reitman and Frankel, 1957); third, the colorimetric procedure of Babson *et al*

(1962) which uses a diazonium salt, Azoene Fast Violet B, to couple with oxaloacetate. All three methods have been modified for the AutoAnalyzer with differing degrees of success.

Schwartz, Kessler, and Bodansky (1961) employed a Technicon colorimeter which read at 340 nm and used it to measure the oxidation of NADH produced by malate dehydrogenase (EC 1.1.1.37) acting on the oxaloacetate formed by the aminotransferase reaction. The results must, however, be corrected for optical density changes unrelated to the aminotransferase reaction. The advantages of the extra sensitivity of fluorescence led Levine and Hill (1966) to use a fluorimeter module in place of the 340 nm colorimeter. To overcome the difficulties of quenching and other fluorescent materials present in the serum the manifold was arranged so that the products of the aminotransferase reaction were dialysed into a recipient stream of a buffered mixture of NADH and malate dehydrogenase. The decrease in fluorescence, which is proportional to the amount of oxaloacetate formed by the aminotransferase, was recorded.

Of the colorimetric procedures the type using a diazonium salt has been most successfully adapted for the AutoAnalyzer. Schaffert, Kingsley, and Getchell (1964) compared the automated dinitrophenylhydrazine technique with that using Azoene Fast Violet B and found that the specificity of the former was limited because the colour reagent reacted with all keto-groups; furthermore accurate results could be obtained only within a relatively small range. Scheidt, Nelson, and Levine (1966) and Morgenstern *et al* (1966) used Azoene Fast Violet B and Azoene Fast Red PDC respectively. The products of the aminotransferase reaction were dialysed into a buffer or water-recipient stream which was then mixed with a solution of the diazonium salt. The resultant dye was measured in a normal colorimeter, the amount of colour produced being directly proportional to the activity of the serum. Scheidt *et al* (1966) found that this type of technique gave results which correlated well with those of the manual spectrophotometric assay and showed that the dialysis step obviated the need for serum 'blanks', and hence doubled the analysis rate. This type of technique has been adopted for the SMA 12/30¹. Moore and Sax (1969) have noted that certain sera showed much higher enzyme levels when measured by this system than by the spectrophotometric method. They traced this to the reaction of the diazonium salt with acetoacetate and found that there were a number of patients with elevated blood sugars or blood ureas with apparent serum

aminotransferase levels of the order of the upper limit of the normal range even when the enzyme was assayed without the substrate. It was also shown that blank corrections significantly reduced the apparent aminotransferase activity bringing 18% of abnormal aspartate aminotransferases into the normal range. This emphasizes the dangers of colorimetric procedures and also the need to be wary of abnormal aspartate aminotransferase levels as shown by procedures which do not incorporate blanks especially in patients with diabetes.

Lactate dehydrogenase

Automated assay of this enzyme is commonly based on manual spectrophotometric methods rather than the colorimetric ones which are subject to many of the same difficulties found with the aminotransferase methods. The first AutoAnalyzer assay for this enzyme was described by Schwartz and his coworkers (1961) who used the 340 nm colorimeter to measure the oxidation of NADH produced by the enzyme. A modification of this system, in which the reaction was stopped by the addition of sodium hydroxide before the NADH level was measured, was introduced by Strandjord and Clayson (1964).

A new technique in which the whole AutoAnalyzer channel is installed in a thermostatted box has been devised by Berry and Wallis (1966). Serum dilutions are placed in the sampler cups, and immediately before aspiration an aliquot of pyruvate is injected into each cup and stirred with a vibrating paddle. The enzyme-substrate mixture is pumped to the 340 nm colorimeter, the first portion of the sample arriving one minute after aspiration begins. The passage of the reaction mixture from each sampler through the flow cell takes two minutes and results in a characteristic recorder trace with a linear rate of fall of optical density with time.

Fluorometric determination of NADH was used by Brooks and Olken (1964) who used the 'forward' lactate to pyruvate reaction which gave excellent correlation with the manual spectrophotometric assay. Morgenstern, Flor, Kessler, and Klein (1966) used a copper-neocupreine solution as a colour reagent for NADH and Hochella and Weinhouse (1965) used reduced NAD dehydrogenase (EC 1.6.99.3) and p-iodonitrotetrazolium Violet (INT) for the same purpose. Babson and Arndt (1970) have recently pointed out the presence of LD-inhibitors in NAD, and suggest that as colorimetric procedures employing phenazine methosulphate as an intermediate electron acceptor use much less NAD than the corresponding spectrophotometric assay, they are less affected by these inhibitors. Both groups used the 'forward' reaction and the latter methodology has been adopted for use with the SMA 12/66

¹Technicon Instruments Ltd.

Of all these techniques, only that of Berry and Wallis (1967) can be said to approach the preferable 'slope' type and thus indicate whether or not zero-order kinetics are being followed.

Alkaline phosphatase

Automated assay of this enzyme depends on the use of either phenyl phosphate or p-nitrophenyl phosphate as substrate. With the former substrate aminoantipyrine and ferricyanide are used as colour reagents (Marsh, Fingerhut, and Kirsch, 1959). The serum is mixed with buffered substrate, incubated, mixed with colour reagent, and the optical density measured in a colorimeter. The serum samples have either to be run again in the absence of substrate or a dual channel system with a differential colorimeter employed to give serum 'blanks'. The use of p-nitrophenyl phosphate as a substrate in an automated technique was developed by Morgenstern *et al* (1965), the p-nitrophenol produced by the action of the enzyme being dialysed into a recipient stream of 2-amino-2-methyl-1-propanol before colorimetry at 410 nm. This dialysis step is said to eliminate the need for a blank correction.

An interesting, though complicated procedure, for reaction rate measurement of serum alkaline phosphatase activity has been developed by Brown and Ebner (1967) who made preliminary dilutions of serum with buffered substrate and placed these in a sampler plate modified so that the sample cups could be kept at 37°C. Each serum dilution was re-aspirated after 15 and 30 minutes. This procedure results in each sample giving at least two points on the reaction rate curve thus indicating whether the kinetics are zero order and making the measurement of serum blanks unnecessary. It does, however, involve the time-consuming manual preparation of the serum dilutions.

Cornish, Neale, and Posen (1970) have developed a fluorometric assay with 4-methylumbelliferyl phosphate as substrate. This assay is apparently so sensitive that normal adult serum has to be diluted 1 in 50 before assay; the results compare well with those obtained with the conventional assay using phenyl phosphate as substrate.

DISCRETE ANALYSERS

A number of fully automatic enzyme analysers have recently become available or are soon to be on the market. These of course include the complete analysis systems such as the Autochemist¹, Robot Chemist², DSA-560³, and Centrifichem⁴ analysers. Specific enzyme analysers such as the Enzymat⁵ and the Zymat⁶ are also in production (Fig. 5). The Enzymat is based on the Mecolab system with a Roboscan attachment. The buffered substrate serum dilutions are transferred to a cuvette in a 15-place turret. The optical densities of these cuvettes are recorded consecutively and repeatedly, and in this way reaction rate curves can be drawn. The apparatus is, however, rather cumbersome. The Zymat, soon to be re-introduced into the United Kingdom market, has been specifically designed for assay of those enzymes which can be monitored at 340 nm. It has two concentric wheels, one for the serum samples and one for the reaction cuvettes, which advance together once every two minutes. The instrument is programmed to pick up a serum sample, dilute it with the appropriate buffered substrate, transfer it to the cuvette, incubate the mixture, inject a reagent to trigger off the enzyme reaction, stir with an air jet, and move the cuvette into the light path. The reaction rate is monitored and produced as a digital printout.

The Unicam AC60/61 system⁷ is also an effective means of enzyme analysis and as it may be coupled up to a conventional spectrophotometer is more versatile than the Zymat. It is, however, not very good for fast reactions, having a 60-second delay between starting the reaction and monitoring the optical density of the solution. It also requires at least 3 ml of reaction mixture whereas many other analysers now operate on a minimum of 1 ml.

¹AGA Medical Division, Lidings 1, Sweden.

²Warner-Chilcott, Richmond, Calif., USA.

³Beckman Ltd, Sunley House, Bedford Park, Croydon.

⁴Union Carbide Corp., Tarrytown, New York.

⁵Joyce-Loebl and Co Ltd, Princes Way, Team Valley, Gateshead.

⁶Bausch and Lomb Inc., Rochester, N.Y., USA.

⁷Pye Unicam Ltd, York St, Cambridge.

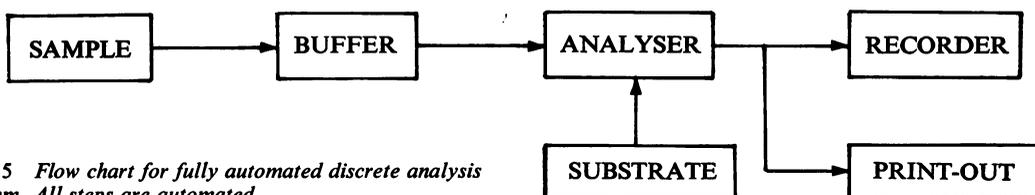


Fig. 5 Flow chart for fully automated discrete analysis system. All steps are automated.

DATA ACQUISITION AND STAGE III AUTOMATION
Most semi-automated or discrete automated systems and some continuous flow techniques provide a recorder trace showing the change in optical density with time, ie, plots voltage output of photometer against time. It is possible by use of an analog-digital convertor and associated process units to measure dv/dt and print this out directly in enzyme units together with the percentage deviation from linearity of the reaction rate slope. This type of equipment is soon to be available for the LKB analyser.

Toren, Eggert, Sherry, and Hicks (1970) have described an instrument for interfacing any recording spectrophotometer to a clinical laboratory computer where the rate signal is sampled and transmitted to the computer which prints out the result in enzyme units with comments on non-linearity, drift, and quality control. Roodyn (1970) has recently developed a generalized system for the automation of enzyme assays in which an Auto-Analyser is computer controlled whereby it is possible to regulate flow rate, temperature, and many other parameters, thus making possible the stage III automation originally proposed by Schwartz and Bodansky (1963).

Summary

Automation of enzyme assays is becoming increasingly important and instrumentation is being developed to satisfy this need. Reaction rate analysers which use the preferable 'slope' techniques rather than the less satisfactory 'constant-time' or 'single-point' analyses are becoming more widespread.

The stage I systems at present available are: recording spectrophotometers with autodilutors for sample preparation and the Reaction Rate Analyser

The stage II systems available are the Auto-Analyser, and the Zymat 340, Enzymat, and AC60/61.

Data acquisition units can give direct printout in enzyme units from all systems and computer control of stage II systems is under development.

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