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An Automatic Apparatus for the Study of Enzyme Kinetics

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A continuous-flow apparatus is described for automatically plotting substrate saturation curves, and is suitable for use with a variety of enzymes. A linear concentration gradient of the variable substrate is combined with a fixed proportion of the other substrates and the enzyme, and after passing through a reaction coil the product concentrations are measured spectrophotometrically. Use of a 4cm. flow cell and modified spectrophotometer permits accurate measurement of NADH concentration in the region of 0·1 μM. Precise control over reaction times and substrate concentration is achieved by using power-driven syringes with an integral mixer. Specimen results are given for yeast alcohol dehydrogenase.

This apparatus has been designed to decrease the time and labour involved in studies of enzyme reaction kinetics, and to give a more accurate result than can be obtained manually.

A solution of enzyme containing all but one of its substrates is pumped at a constant rate into a mixer, where it is combined with a solution of the remaining substrate. The reaction takes place as the solution flows down a thermostatically controlled reaction coil, and the products are measured by using a spectrophotometer equipped with a flow cell. The concentration of the remaining substrate admitted to the mixer is increased linearly with time so that a recorder connected to the spectrophotometer plots a graph of reaction velocity against substrate concentration directly.

Previous work with this type of apparatus has usually been based on a proportioning peristaltic pump, together with an air segmentation system to prevent mixing errors within the reaction coil. The Technicon pump and colorimeter (Technicon Instruments Co. Ltd., Chertsey, Surrey.) can be readily adapted for this type of system (for reviews see, e.g., Schwartz & Bodansky, 1963, 1968). A syringe-based device has also been described (Brehmer, Holzer & Binzus, 1963), but because this did not produce a linear substrate gradient interpretation of the results was more complex.

The apparatus now described is nearly two orders of magnitude more sensitive than previous designs and consequently incorporates a number of features which have not previously been found necessary.

MATERIALS AND METHODS

Crystalline yeast alcohol dehydrogenase, NAD and NADH, were obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. Dithiothreitol was obtained from Koch–Light Laboratories Ltd., Colnbrook, Bucks. All other reagents were obtained from BDH (Chemicals) Ltd., Poole, Dorset.

The complete apparatus is shown schematically in Fig. 1. The substrate whose concentration is to be varied is initially present only in syringe A while the other substrates are present in all four syringes. Syringe C contains the enzyme. The linear substrate concentration gradient produced by syringes A and B is mixed with the enzyme solution in the mixer.

Internal controls are provided for each run as follows. Before the run is started syringe D is emptied manually through the system, to drive out bubbles and allow the recorder baseline to be set to 5% full-scale deflexion. This operation is repeated at the end of each run and the final baseline checked. In addition, each series of measurements includes one run in the absence of enzyme and one at constant substrate concentration in the presence of enzyme, so that any blank rate or error due to enzyme instability can be detected. If necessary, the ram may be stopped at any time during a run, and the rate of increase of absorbance with time measured. This may be used to check individual points on the saturation curve, and also to verify that the reaction rate is initially linear at all points on the saturation curve.

If one of the substrates is coloured at the assay wavelength it is difficult for the concentration of this one to be varied during a run as the baseline will not be flat. However, it is possible to change the concentration of such a substrate (e.g. NADH) between runs so as to construct a complete set of results.

Each run takes approximately 10min. so that a '6 by 20' double-reciprocal plot with both substrates varied can be completed in roughly an hour. In order to process the considerable amounts of data produced by this technique, a FORTRAN IV programme has been devised that will draw the desired graphs directly from sets of values read from the saturation curves.

Constructional details of the apparatus are illustrated in Figs. 2–4. The mixer, syringe block, flow cell and filter assembly were constructed from Perspex. The ram is not described in detail as any rigid design would be satisfactory;

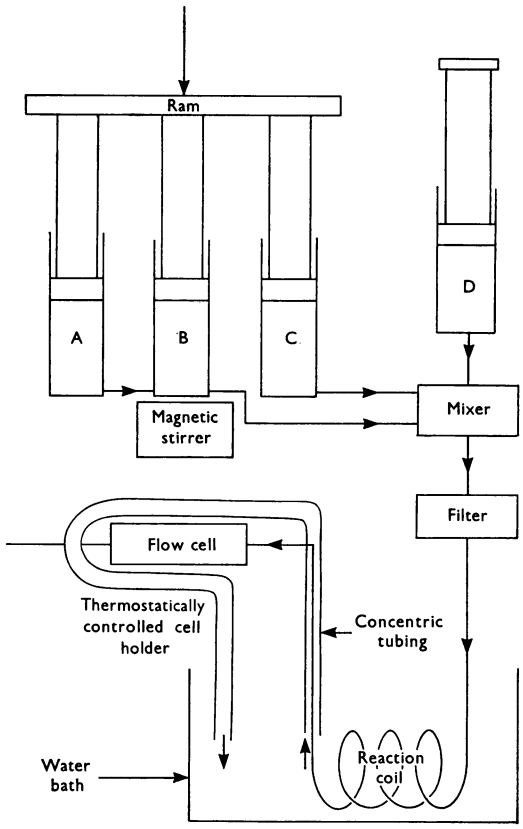

Fig. 1. Schematic diagram of the apparatus.

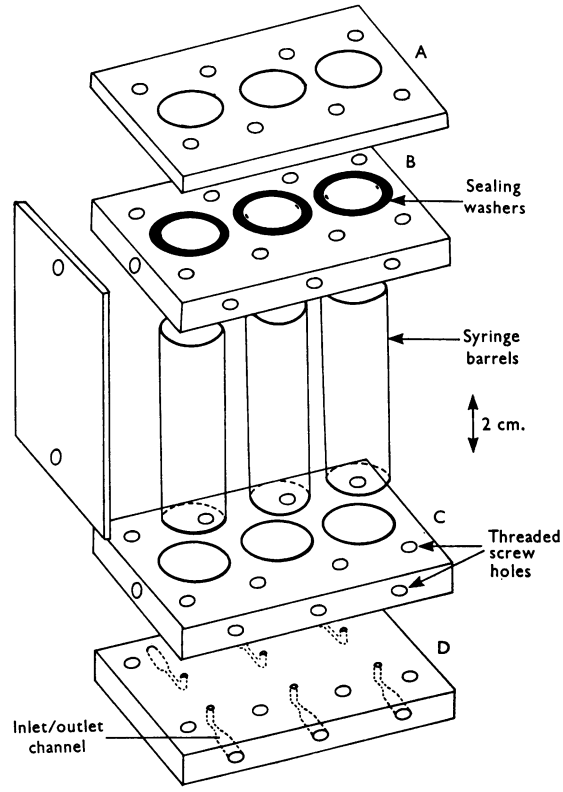

Fig. 2. Details of the syringe block. Three of the side walls have been omitted for clarity. The syringe barrels were made from Gillette Scimitar 20 ml. disposable syringes from which the ends were removed to produce cylinders 8·5 cm. long. Neoprene rubber sealing washers are located in grooves in the outer faces of blocks B and C. These are compressed against the ends of the syringe barrels when blocks A and D are screwed to the rest of the assembly. The inlet/outlet channels are provided with seals as described in Fig. 3.

the model used in this laboratory employs a synchronous motor to ensure constant speed. The motor develops a torque of 7·5 kg. cm., which is applied to a leadscrew with a pitch of 1 mm. The reaction coil normally used consists of 4·6 m. of 0·75 mm.-bore Teflon tubing with a total volume of 2·0 ml. This results in a flow time of approx. 13 sec.

The filter element (Whatman no. 54 paper) should be changed daily for best results, and it is also necessary to grease the syringe barrels with silicone grease at the same time. No other maintenance is required.

Changes in extinction at 340 nm. were measured by using a Beckman DU monochromator together with a Hilger–Gilford Absorbance Converter, and a Honeywell Brown 10 mv strip chart recorder. A number of modifications were made to the monochromator. A 10 cm. thermostatically controlled cell holder was fitted and the existing lamps were replaced by a 100 w quartz–iodine projector lamp (Phillips A1/215). When supplied from a constant-voltage transformer this provides a powerful and stable source from the far red to 285 nm. and enables slit widths of 0·4 mm. to be employed with the 4 cm. flow cell at 340 nm. The performance of the Gilford Absorbance Converter was improved by stabilizing the mains supply so that the long-term baseline drift was less than 0·001 E units/hr. When the u.v. filter supplied with the monochromator to minimize stray light was used, a linear response could be obtained up to E values of about 3·0.

Crystalline yeast alcohol dehydrogenase was diluted to give a protein concentration of 2·25 mg./ml. with 0·3 M-sodium phosphate buffer, pH 7·4, containing 2 mM-dithiothreitol and incubated at 0° for at least 1 hr. for full activation to occur. The enzyme is very stable under these conditions. A 10 μl. sample of this solution was added to 50 ml. of reaction buffer containing NAD^+ and this solution used to fill syringes C and D in Fig. 1. The contents of all the syringes for a typical run are shown in Table 1. The composition of syringe D is identical with that of the reaction mixture at zero time. The concentrations of NADH measured after 13 sec. reaction during the course of each run were in the range 0–7·5 μM, depending on substrate concentration.

Runs were performed in triplicate at six NAD^+ concen-

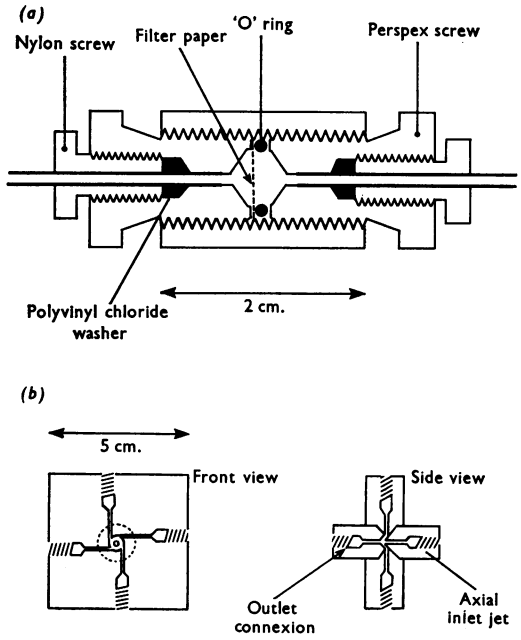

Fig. 3. (*a*) Section through the filter, showing the connections for the cannula tubing. Identical seals were provided on the syringe block and the mixer. (*b*) Details of the mixer.

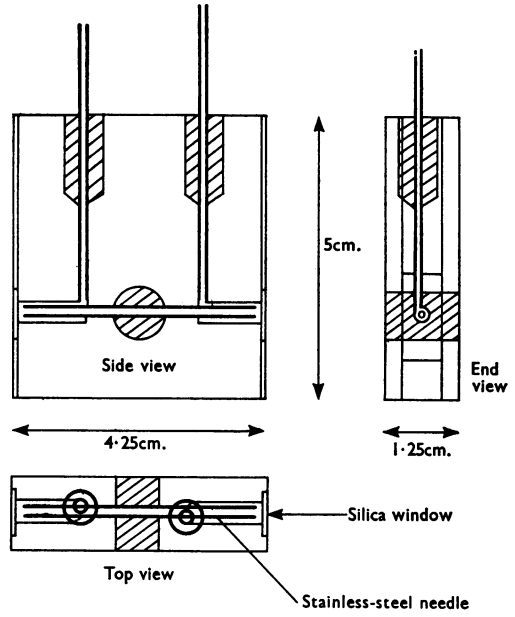

Fig. 4. The 4cm. flow cell. The light-path lies within a stainless-steel syringe needle of 1·2mm. internal diameter. Cross-hatched regions on the diagram are plugs of Araldite epoxy adhesive (CIBA Ltd., Duxford, Cambs.), used to secure the light-path needle, the inlet tubes and the silica windows to the block.

trations, and the data from the resulting curves were processed by using an ICT Atlas II computer. In all, some 200 velocity measurements contributed to the final results.

RESULTS

Preliminary tests on the apparatus showed that the delivery rate from the syringes was constant, and a check was made on the accuracy of the substrate gradient by using solutions of NADH in syringe A and buffer solutions in the other syringes. Reaction-coil mixing errors were estimated by applying stepwise increments in NADH concentration to the start of the coil and measuring the resulting extinction profile in the flow cell at the end of the coil. The mean flow time determined in this manner was 13sec. with a spread of ± 3sec. due to mixing. The errors due to this mixing are discussed in full below.

Early work was hampered by the instability of yeast alcohol dehydrogenase in the reaction buffer, until cysteine or dithiothreitol was found to give the enzyme complete protection. At the same time it was noted that dithiothreitol, and to a smaller extent cysteine, could reactivate partially inactivated aged enzyme preparations. Reactivation, which may be very marked, is complete in 1hr. at 0°, and results in rates of up to 50000 moles of NADH produced/min. per mole of enzyme extrapolated to saturating concentrations of both substrates at 22° at pH 7·6.

Table 1. *Syringe contents for a typical run*

All syringes contain 50mM-phosphate buffer, pH 7·6, with 10mM-semicarbazide and 5mM-cysteine. The cysteine is necessary to prevent a rapid loss of enzymic activity on dilution.

Syringe ...	A	B	C	D
Substrate				
NAD^+	0·2mM	0·2mM	0·2mM	0·2mM
Ethanol	70mM	—	—	—
Enzyme	—	—	0·45μg./ml.	0·15μg./ml.

Double-reciprocal plots of velocity and substrate concentration are presented in Fig. 5. The Michaelis constants calculated by this method are about 20mM for ethanol and 0·2mM for NAD, depending slightly on the concentration of the second substrate in each case.

DISCUSSION

The design of this apparatus has to a large extent been dictated by the need for the greatest possible sensitivity. Although the Gilford Absorbance Converter for the Beckman DU monochromator

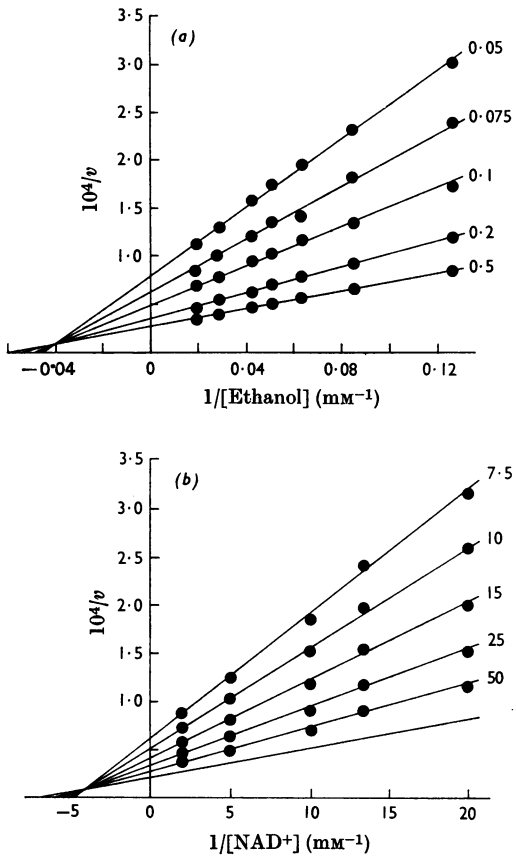

Fig. 5. (*a*) Variation of the initial rate of NADH production with ethanol concentration at fixed concentrations of NAD^+. The respective NAD^+ concentrations (in mM) are shown against each graph. Velocity is expressed as moles of NADH produced/min. per mole of enzyme at 22° at pH 7·6. (*b*) Variation of the initial rate of NADH production with NAD^+ concentration at fixed concentrations of ethanol. The respective ethanol concentrations (in mM) are shown against each graph. Other details are as for Fig. 5(*a*). The lowest line is calculated for infinite ethanol concentration, to allow extrapolation to find the velocity when both substrates are saturating. (For both plots, the lines and their best intersection points were calculated by computer on the basis of considerably more velocity measurements than it has been possible to include on the graph.)

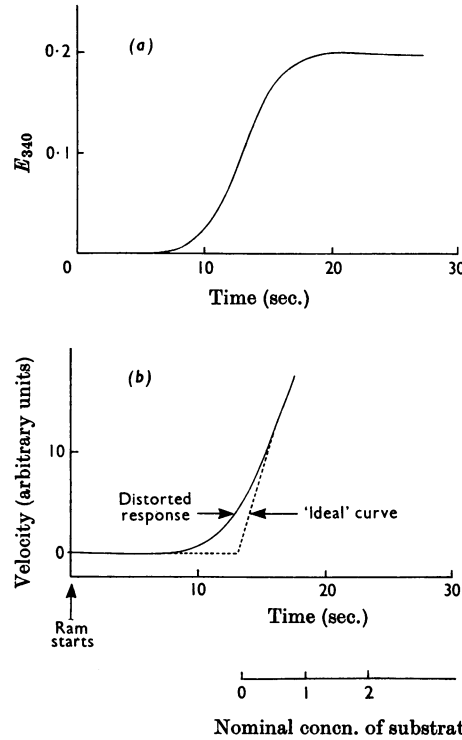

Fig. 6. (*a*) Effect of reaction-coil mixing errors. Syringe D (Fig. 1) was used to fill the coil with 10 mM-tris–chloride, pH 8·0, and at zero time the ram was started so as to displace this solution by the same buffer containing 10 μM-NADH from syringes A, B and C. (*b*) Calculated distortion of an 'ideal' saturation curve of the form:

$$v = \frac{100[S]}{20+[S]}$$

due to mixing errors. In practice the combination of mixing errors and small starting artifacts means that the origin of the hyperbola cannot be fixed with an accuracy of better than $\pm 0{\cdot}5\%$ of full scale for substrate concentration or velocity. (Velocity and substrate concentrations are measured in arbitrary units. Only the early part of the curve is shown as errors are negligible for the remainder.)

permits settings of $0{\cdot}05\,E$ unit full scale, considerable further improvements in sensitivity can be obtained by using 4 cm.-path-length cells. Under these conditions light-scattering by microscopic bubbles and dust particles can be a serious problem and give rise to major errors should they become trapped in the light-beam. This has been avoided by constructing the cell with a very small cross-sectional area so that the resulting high-flow velocities render the cell self-cleaning. Even so, it has been found advisable to de-gas the reaction buffer before each day's work and to use the in-line filter described above.

Air segmentation in the reaction coil is clearly not feasible in a system of this type, and the need for it has largely been avoided by the use of 0·75 mm. Teflon tubing for the coil. Relatively little mixing takes place in tubing of this bore, and it can be shown both theoretically and experimentally that this is not a major source of error.

As shown in Fig. 6(*a*), mixing errors transform a step of amplitude A, applied to the start of the reaction coil at time t_0, into a curve of the approximate form:

$$Y = \frac{A}{2} \cdot \left(\frac{(\tanh t - t_0 - 13) + 1}{3} \right)$$

(times measured in seconds, flow time 13 sec.).

This may be used to compute mixing errors by an approximate numerical method. These calculations show that errors are negligible for the major part of each run, but as can be seen in Fig. 6(*b*) estimates of the true origin of the curve from the recorded data are subject to errors. This may in turn result in systematic errors in the velocity and substrate concentrations measured for points near the origin of each curve, and points obtained during the first 10% of each run cannot be considered reliable unless special precautions are taken.

Very careful thermostatic control of the reaction coil and flow cell is necessary for good results. The apparent extinction of solutions measured in the flow cell was initially found to depend on the velocity and direction of liquid flow. This was traced to small variations in refractive index within the cell, and was entirely removed by placing both flow cell and the tubes feeding the cell under thermostatic control.

The results presented here for yeast alcohol dehydrogenase are broadly in agreement with previous work (Mahler & Douglas, 1957; Nygaard & Theorell, 1955; Hayes & Velick, 1954; Shiner, Mahler, Baker & Hiatt, 1960), although the variations between previous investigations make a direct comparison difficult. The Lineweaver–Burk plots obtained in the present series of experiments do not intersect on the axis of the graph, indicating that the substrates do not bind independently under these conditions. Previous investigators have differed on this point, although the effect of temperature on the reaction mechanism (Müller-Hill & Wallenfalls, 1964) may partly account for the discrepancies.

The apparatus described here is capable of further development. It has also been used to produce pH–activity curves for liver isocitrate dehydrogenase in conjunction with a pH flow cell and a two-dimensional plotter, and is also to be equipped for measurements of oxygen concentration by using a stainless-steel reaction coil and Clark electrode.

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