A Generalized System for Automation of Enzyme Assays

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A flow system was developed, using a Technicon AutoAnalyzer, that is readily adaptable to a range of enzyme assays. The system includes lines for pumping substrate, cofactor, buffer and enzyme and for generating linear gradients. By using a variable-speed proportioning pump the incubation time may be continuously varied, and the system also allows for continuous variation in the pH, substrate or cofactor concentration, incubation temperature and enzyme concentration. A FORTRAN V program was written that uses instrument calibrations to calculate the flow rates in the individual lines, the incubation time and the characteristics of the gradient used. The computer then prints out instructions for preparation of reagents to give a required reaction mixture, weighing sheets for stock solutions and the results of the assay in international units in suitable tables and graphs. The flow system and computer program are designed to facilitate the automation of manual assays. A detailed example is given of the use of the system [the assay of three dehydrogenases in yeast: L(+)-lactate dehydrogenase, D(-)-lactate dehydrogenase and succinate dehydrogenase], and the general applications of the method are discussed. The program has been deposited as Supplementary Publication no. SUP 50002 at the National Library for Science and Technology, Boston Spa, Yorks. LS23 7BQ, U.K., from whom copies may be obtained on the terms indicated in *Biochem. J.* (1970), 116, 7.

There have been many developments in the techniques of automated enzyme assay, and a variety of methods are now available (see reviews by Schwartz & Bodansky, 1963, 1968; Tappel, 1969; Roodyn, 1970). Most of the published methods are for specific enzyme assays, and many have been developed for enzymes of importance in clinical medicine. A detailed list of such assays is given in Appendix I in Roodyn (1970). Apart from such specific studies, however, there have been some general studies on enzyme automation. These include methods developed by Pitot and co-workers (Pitot & Pries, 1964; Pitot, Wratten & Poirier, 1968) and by Tappel and co-workers (Tappel & Beck, 1965, 1967; Barrera, Chio & Tappel, 1969). In addition, a variety of commercial instruments specifically designed for automated enzyme assay are now available (see Appendix III in Roodyn, 1970).

In this paper, a generalized enzyme assay system is described in which it is possible to vary continuously incubation time, protein concentration, substrate concentration, temperature of incubation, pH and the general composition of the reaction mixture. The assay system uses standard Technicon modules and its operation is closely linked to the use of a comprehensive FORTRAN V program which is

designed to facilitate the automation of published manual methods. Although the system uses the Technicon AutoAnalyzer, the principles on which the computer program is based would be applicable to most commercial analysers.

METHODS AND RESULTS

Enzyme preparations

These were yeast homogenates prepared by the method given in Roodyn & Wilkie (1967).

Computer processing

All programs and data were punched on IBM 80 column cards, and batch-processed on the Atlas computer at the University of London Atlas Computing Service, 44 Gordon Square, London W.C.1, U.K. Output was on a 120-space line printer, and the program was developed by using the FORTRAN V Manual written by Schofield (1969).

Protein assays

These were carried out by an automated adaptation of the method of Lowry, Rosebrough, Farr & Randall (1951), with bovine serum albumin as a standard.

to prevent surging. Cofactor is then mixed with the substrate and buffer and the enzyme is injected into the reaction mixture through a fine capillary (H3 glass connector). The mixing coil after the addition of enzyme is jacketed (JMC) and is maintained at the same temperature as the heating bath. The reaction commences in the jacketed mixing coil and continues as the reaction mixture flows through the heating bath (HB). The effluent from the heating bath passes by a short length of glass tubing directly to the debubbler of the flow cell. Decrease in temperature is negligible during this process, so that the effective incubation period is from the moment of entry into the jacketed mixing coil (I) until the reading is obtained in the flow cell (O). After the flow cell, the reaction mixture passes through a continuous-flow glass electrode connected to a pH-meter and recorder, and finally to waste. The flow system contains a gradient-making system consisting of a gradient line (GR) and two mixing vessels. It also contains a sampler module, which may be connected to the enzyme line for determination of the enzyme activity in a number of different samples, or to the substrate line for the determination of a group of enzymes in the same preparation, by using the technique of 'multiple enzyme analysis' (see Roodyn, 1965; 1967*a,b*, 1969*a,b*, 1970; Jowett & Roodyn, 1969). Pumping is by a variable-speed proportioning pump (with arbitrary divisions of 0–100 units on the speed control), and the heating bath has variable temperature control from room temperature to 95°C. The pump tubing used in the system may be varied to suit the needs of the particular assay under study. However, a convenient choice of tubing is given in the legend to Fig. 1.

The characteristics of the above system are now described.

(a) Incubation times at different flow rates. These are measured by placing a suitable marker (e.g. 0.1M-potassium ferricyanide) in the enzyme line and measuring the time between entry of the marker into the jacketed mixing coil (JMC in Fig. 1) and into the flow cell (FC in Fig. 1). (The detailed procedure for instrument calibration is given below.) If the effective volume of the incubation vessel were the same at all pump speeds, the incubation time would be inversely proportional to the total flow rate of liquid plus gas. If the proportion of volume of gas to volume of liquid also remains constant at all pump speeds, the time should also be inversely proportional to the total liquid flow rate. This is confirmed in Fig. 2, showing that there is no excessive compression of the gas at high pump speeds. Fig. 2 also shows that the rate of fluid flow is proportional to the number of divisions on the arbitrary scale on the speed control of the variable-speed pump, and also to the rate of compression of the pump tubing by the rollers. The pump thus behaves in a predictable manner at different speeds and one may readily select from Fig. 2 a convenient incubation time (e.g. exactly 5.0 min) or a suitable flow rate (e.g. exactly 3.0 ml/min) by using the scale on the pump.

(b) Fractional line volumes. The flow rates in the individual lines were determined with markers as described below, and the fractional line volumes at different pump speeds are given in Table 1. The values are similar at all pump speeds, although they deviate somewhat from those calculated from the maker's specifications. The system would therefore produce a reaction mixture of essentially the same

Fig. 2. Incubation time and fluid flow rate of the flow system in Fig. 1 at various pump speeds. Incubation time is measured by the time taken for marker dye to flow from I to O in Fig. 1. Δ , Incubation time; $\blacktriangle$, reciprocal of incubation time; $\circ$, pump position in arbitrary scale divisions; $\bullet$, number of times/min that the pump tubing is compressed by a roller bar of the variable-speed proportioning pump. (The system was calibrated with water in lines 1, 2, 3, 4 and GR, air in line G and the heating bath at 37°C.)

Table 1. *Flow rates in different lines at various pump speeds*

Fractional line volumes (FLV) were calculated by running marker in each line and using the expression: FLV = reading with marker in line/(sum of readings for all lines). Line flow rate was calculated by multiplying total fluid flow rate by fractional line volume. The flow system in Fig. 1 was used, with pump tubing as given in the legend to that figure.

Pump speed (arbitrary scale divisions)	Total fluid flow rate (ml/min)	Fractional line volume				Line flow rate (ml/min)			
		Line 1	Line 2	Line 3	Line 4	Line 1	Line 2	Line 3	Line 4
100	6.10	0.152	0.435	0.149	0.263	0.93	2.65	0.91	1.61
90	5.47	0.156	0.428	0.151	0.265	0.85	2.34	0.83	1.45
80	4.67	0.148	0.438	0.149	0.265	0.69	2.05	0.70	1.25
70	4.06	0.153	0.433	0.149	0.265	0.62	1.76	0.61	1.08
60	3.50	0.153	0.428	0.149	0.270	0.54	1.50	0.52	0.94
50	2.62	0.157	0.432	0.145	0.266	0.41	1.13	0.38	0.70
40	1.80	0.155	0.418	0.154	0.274	0.28	0.75	0.28	0.49
30	1.17	0.159	0.440	0.130	0.271	0.19	0.51	0.15	0.32
Standard Technicon pump (expected values):	4.40	0.136	0.454	0.136	0.272	0.60	2.00	0.60	1.20

composition at all pump speeds studied. This property is useful in many situations. For example, a preliminary assay may be performed at a fast pumping rate to determine the approximate activity of the samples, and then repeated with more precision with slower pumping.

(c) Sample interaction and wash characteristics. A difficulty in changing the flow rate of the system, however, is that the washing efficiency will also alter. The half-wash time of the flow system (see above for terminology) was determined at different pump speeds with the result shown in Fig. 3. There is a marked increase in the half-wash time at the very low flow rates. The carry-over from one sample to the next was also measured at a variety of pumping and sampling rates and it was found to be less than 1% at all speeds tested, with sampling at 40/h and a sample/wash ratio 2:1.

(d) Gradient-making system. This is part of the flow system shown in Fig. 1. It consists of a mixing vessel (M2) containing the stock solution from which fluid is pumped by a gradient line (GR) into the gradient-making vessel (M1), from which fluid is then pumped into the analyser. M2 is a mixing vessel because in experiments with suspensions (e.g. tissue homogenates) it is necessary to keep the stock enzyme gently stirred during the assay. In Fig. 1 the gradient-making vessel is connected to the enzyme line so as to generate enzyme gradients (cf. Roodyn & Maroudas, 1968). However, substrate gradients for determination of K_m , buffer gradients for determination of pH optimum and cofactor or inhibitor gradients may be obtained by connecting the gradient-making vessel to line 1, 2 or 3 respectively. The shape of the gradient is determined by the relative rates of filling and emptying of the gradient-making vessel. The ratio

Fig. 3. Half-wash times of the flow system in Fig. 1 at various flow rates. The sample probe was placed in a marker solution for various times and the half-wash time determined from plots of $\log(100 - \% \text{ steady state})$ against time (see Thiers *et al.* 1967; Roodyn, 1970). Calibration conditions were as in Fig. 2.

of the overall rate of filling or emptying to the volume of fluid initially present in the gradient-making vessel determines the steepness of the gradient. For a given volume of fluid in the vessel, the rate at which the gradient is established will depend on the pumping rate, so that with a variable-speed pump one can alter the steepness of the gradient at will.

It is usually most convenient to use linear gradients, and these may be obtained by emptying the mixing vessel at twice the rate it is filled (Davis, Santen & Agranoff, 1965; Sorin & Vargues, 1966). It is possible to calculate the shape of the gradient from the known flow rates and the initial volumes of fluid in the mixing vessel. However, slight varia-

Fig. 4. Gradient dilution factors of the flow system in Fig. 1 at various flow rates. The gradient-making vessel (M1 in Fig. 1) had 25 ml of water at the start of the gradient and stock marker solution from M2 was pumped into it for 30 min. Gradient dilution factor is concentration in effluent from M1 (i.e. in line 4) divided by concentration in stock (i.e. in M2 and line GR). Numbers in parentheses refer to pump position in arbitrary scale divisions (see Fig. 2). Calibration conditions were as in Fig. 2.

tions in the pump tubing, dead space in the gradient system and deviations from perfect mixing give values that differ slightly from the theoretical, particularly near the end of the gradient. It is thus more precise to calibrate the gradients empirically. For the multiple enzyme analyses performed in this laboratory a convenient initial volume in the gradient-making vessel is 25–50 ml with the flow rates shown in Table 1. Experimentally determined plots of gradient dilution factor against sample number for 25 ml gradients are given in Fig. 4.

A simple method of generating pH gradients in the system is to use the above gradient-making system with an alkaline buffer in one vessel (e.g. M1) and an acid buffer in the other (M2). It is difficult to obtain exactly linear pH gradients by this method, but if the change in pH during the assay is not too great this is not a serious disadvantage. pH gradients prepared by this method have been

described by Jowett & Roodyn (1969) for use in phosphatase assays.

Computer program. Detailed input instructions and the full program are presented in Appendix IV in Roodyn (1970) and a limited number of copies are also available from the author. Input instructions and the program have also been deposited as Supplementary Publication no. SUP 50002 at the National Library for Science and Technology, Boston Spa, Yorks. LS23 7BQ, U.K., from whom copies can be obtained on the terms indicated in *Biochem. J.* (1970), **116**, 7.

The program is divided into the following sections.

(1) Introduction. General comments of any length may be entered here. It is convenient to give full details of the assay system here, so that they can be printed out before each group of assays. There are several other points in the program where textual material of any length may be entered (e.g. as legends to tables or figures).

(2) Calculation of molar conversion factor. (Since the final values are given in $\mu\text{mol/ml}$, the term 'millimolar conversion factor' is more strictly accurate, but less concise). If the assay uses a colorimeter or spectrophotometer, details of the wavelength, the optical path, the name of the light-absorbing material (chromophore) and a calibration curve with a range of chromophore concentrations are entered here. We are currently developing programs in which the slope of the calibration curve is assessed by curve-fitting procedures. However, a simple interim procedure to detect severe deviation from linearity is to check whether a reading in the calibration curve gives a molar conversion factor differing by more than a given percentage from the value calculated from the previous reading. Although with a large number of points this can lead to a cumulative error, we have found in practice that with 6–8 points in the curve, checking at the level of 95%–105% agreement between subsequent values gives a reasonable protection against extreme deviations from linearity. (If the reader requires a more rigorous test of linearity, this is easily done by altering the values 0.95, 1.05 in line 20 in the main program.) In a previous computer program developed in this laboratory (Roodyn & Maroudas, 1968) non-linear calibration curves may be used, and readings are converted into units of concentration by linear interpolation. This is generally not necessary, however, in the majority of established enzyme assays, and in this program the computer calculates an average molar conversion factor over the linear region of the calibration curve.

(3) Determinations of incubation time and chart speed. The incubation time is measured by marking the chart as a marker-dye enters and leaves the

reaction vessel: the chart speed is measured by marking the chart at known time-intervals. From the distances between these two sets of marks, the computer calculates the chart speed and incubation time.

(4) Calculations of line volume. The lines are calibrated by running a marker solution through each line in order and measuring the chart reading in each case. The total rate of fluid flow is then measured and these values are entered into the computer, together with the expected flow rates for each line. The computer calculates the fractional line volume, line dilution factor and the observed flow rate for each line. A warning notice is printed if the observed flow rate deviates from the expected values by more than 10%. For standard speed pumps the expected value may be that supplied by the manufacturers. However, once the instrument has been calibrated it is useful to enter the values obtained in the previous calibration as expected values. In this way any serious change in the flow characteristics of the system between one experiment and the next is detected. (The flow rates may also be directly measured by connecting each line in turn to a burette, and measuring the time taken to pump a given volume into the analyser.)

(5) Calculations of concentrations of line reagents. This section calculates the concentration of line reagents needed to give a reaction mixture of the required final composition. The name of the reagent, its final required concentration, the line in which it is pumped, the volume of line reagent required for the experiment and a convenient concentration for the appropriate stock reagents are entered here. The computer prints out instructions for diluting the stock reagents so as to give line reagents that will produce a reaction mixture of the correct final composition. In doing this it uses the observed line volumes calculated in the previous section. In this way changes in the pumping characteristics will not affect the final composition of the reaction mixture.

(6) Stock solutions. This section is run in conjunction with the previous section. The molecular weight of the reagents, the molarity (or the percentage concentration) and the volume of stock solution required are entered here. The computer prints out a weighing sheet (see Table 5 for an example) showing the required weight for each solid, with a space on the sheet for the weight of the vessel.

(7) Calibration of gradient-making system. Marker solution is first run directly into the analyser and the reading with undiluted marker recorded. The marker is then pumped into the gradient-making system and gradient calibration readings are taken at suitable intervals (usually corresponding to the sampling interval, e.g. 40/h). These

readings are fed into the computer, which calculates a series of gradient dilution factors that are printed out as a table and graph. The values are stored in the computer to be used to calculate the concentrations of any substances pumped through the gradient-making system in later assays.

(8) Assay conditions. This section is another point in the program where textual material of any length may be entered. It is useful to give specific details about particular assays here, rather than the general details given in the Introduction section.

(9) Protein concentrations. This section of the program calculates, stores and prints out concentrations for three different types of analysis. These are as follows.

(a) Constant protein concentration. This is for assays in which the enzyme is not pumped through the gradient-making system and only one enzyme sample is used, as in automated enzyme characterization (e.g. Roodyn, 1969a) and in simpler forms of multiple enzyme analysis (e.g. Roodyn, 1965). The protein concentration of the reaction mixture is calculated simply by multiplying the protein concentration in the enzyme line by the fractional line volume of that line.

(b) Variable protein concentration. This section is for the determination of protein concentrations during assay of a number of different enzyme samples. The protein concentration in the reaction mixture is calculated for each sample by multiplying the initial protein concentration in the sample cup by the fractional line volume of the enzyme line.

(c) Gradient in protein concentration. This is for assays in which the enzyme is pumped through the gradient-making system ('enzyme gradients'). This is particularly useful in multiple enzyme analysis when the enzymes in the sample have a wide range in activity (see Roodyn & Maroudas, 1968). The protein concentration in the reaction mixture is calculated from the initial concentration of protein in the stock enzyme solution pumped into the gradient-making system, the fractional line volume of the enzyme line and the gradient dilution factor for each sample calculated previously (see 7 above).

(10) Sample pattern. Details of the various solutions placed in the sample cups and the concentration of solutes in the cups, if known, are entered here. For example in multiple enzyme analysis, the substrate concentrations in the various cups would be entered here. The sample pattern is printed as a table giving details of the samples, their initial concentrations and their final concentrations in the reaction mixture (see Table 6 for an example).

(11) Results. In this section the actual readings and blanks of the assay are entered, and the results calculated in a variety of ways. The program allows

for the three kinds of protein concentration calculation mentioned above, and also for the situation in which the protein concentration has not been determined (for example in the determination of Michaelis constants). Not all assays have a sample pattern and these are printed in a separate way. The usual output, however, is a table giving the sample pattern and the enzyme activity for each sample. In all cases the enzyme activity is calculated in μmol of substrate transformed/ml of reaction mixture in the total incubation time ($\mu\text{mol}/\text{ml}$), and this value divided by the previously determined incubation time ($\mu\text{mol}/\text{min}$). If the protein concentration is known the results are also given as $\mu\text{mol}/\text{min}$ per mg of protein. [The last two values correspond to the recommendations of the International Union of Biochemistry (1964) on Enzyme Nomenclature.] In addition to the table of results, the enzyme activities may also be printed as graphs of $\mu\text{mol}/\text{min}$ and $\mu\text{mol}/\text{min}$ per mg of protein plotted against reading number. The graphs are plotted as percentages of the maximum value in the given set of readings, and suitable scale divisions are inserted on the ordinate scale. Textual comments on the table and graphs may be inserted, and are printed as legends in the appropriate places.

(12) Sub-programs. The above program has two sub-programs. The first (sub-routine 'Comment') allows one to print textual material of any length. Apart from its use during the program for the insertion of legends to tables and figures, it is used to check complex data before actual computation, and also simply to print records kept on punch cards. The second sub-program (sub-routine 'Plot') will plot a series of values in sequence as percentages of the maximum value in the series. Appropriate scale divisions are inserted along the ordinate scale (see Figs. 6 and 7 for example).

Example of the use of the system: multiple enzyme analysis of three ferricyanide reductases

The operation of the system is illustrated by describing a typical assay in detail. The aim of the assay is to determine L(+)-lactate dehydrogenase, D(-)-lactate dehydrogenase and succinate dehydrogenase in yeast homogenates (see Roodyn & Wilkie, 1967) at a range of protein concentrations. The enzyme activity is followed by measuring the reduction of potassium ferricyanide at 420 nm at pH 7.4. The final reaction mixture should contain 0.4 mM-potassium ferricyanide, 50 mM-potassium phosphate buffer, pH 7.4, and any one of the substrates: 2.0 mM-sodium L(+)-lactate, sodium D(-)-lactate or sodium succinate. The assay should be under anaerobic conditions at 37°C, with a final protein concentration not more than 0.5 mg/ml and

an incubation time of 4–8 min. The assay is automated as follows.

Before the actual experiment, the instrument is thoroughly cleaned. With assays of tissue homogenates or subcellular particles it is essential to keep all lines and coils free of deposits of denatured protein or cell debris. It is particularly important to keep the tubular flow cells of the colorimeter perfectly clean, since coating of the reflecting surface will affect the optical path. The instrument should be always washed with cycles of 1.0% (v/v) Triton X-100, 1 M-potassium hydroxide and 1 M-hydrochloric acid and periodically with acetone. Occasionally the entire flow system should be left in 1 M-potassium hydroxide for 1–2 days. Removal of the last traces of acid or alkali during the water wash is easily followed on the continuous-flow pH-meter (Fig. 1).

Details of the assay system are entered into the Introduction section of the program, and the molar conversion factor for potassium ferricyanide is then determined as follows. The flow cell is disconnected from the heating bath (Fig. 1) and a series of solutions of increasing ferricyanide concentration are pumped directly into the flow cell. The concentrations, readings and blanks are entered into section (2) of the program, with the output shown in Table 2. A suitable incubation time is then selected from Fig. 2 above. In this assay pump speed 70 would give a time of 5.4 min, which is within the required range of 4–8 min. The incubation time and chart speed are then checked as follows. The flow cell is reconnected to the heating bath, and all lines (except the gas line) are placed in water. A marker solution (e.g. 0.1 M-potassium ferricyanide) is introduced into the enzyme line for a few seconds, and its progress through the analyser carefully watched. As soon as it enters the jacketed mixing coil before the heating bath (JMC in Fig. 1) a mark is made on the recorder chart by switching off the colorimeter for 1 s. The end of the incubation time is taken as when the marker enters the flow cell, and this is clearly marked by the rapid upswing of the pen recorder. The distance between these marks is measured and the chart speed determined by measuring the average distance between several lines marked on the chart at suitable time-intervals (e.g. 6 min). These distances are entered into section (3) of the program with the following typical output. 'The chart was run for 6.0 min and moved 4.5 cm. Marks for incubation time measurement were 4.0 cm apart. The chart speed was 0.753 cm/min and the incubation time 5.38 min'.

The lines are then calibrated by placing each one in turn in 1 mM-potassium ferricyanide for 5 min followed by 5 min in water. The lines are calibrated in inverse order (lines 4, 3, 2 and 1 in Fig. 1) to avoid overlapping of the traces. The reading and blank

Table 2. *Computer output of calculations of molar conversion factor*

For clarity, the computer print-out in Tables 2-7 has been set in type.

C A L I B R A T I O N

MOLAR CONVERSION FACTOR CALCULATION

CHROMOPHORE CONCENTRATION (UMOLES/ML)	READING	BLANK	MOLAR CONVERSION FACTOR (UMOLES/ML THAT GIVE A READING OF 1.0)
0.100	0.144	0.019	0.800
0.200	0.271	0.019	0.794
0.300	0.396	0.019	0.796
0.400	0.520	0.019	0.798
0.500	0.643	0.020	0.803
0.600	0.768	0.020	0.802
0.700	0.865	0.020	0.828
0.800	0.955	0.021	0.857

A READING OF 1.0 IS EQUIVALENT TO 0.81 UMOLES/ML AT 420.0 MU WITH A PATH LENGTH OF 15.0 MM. THE READINGS ARE PROPORTIONAL TO CHROMOPHORE CONCENTRATION UPTO 0.800 UMOLES/ML. THE CHROMOPHORE IS K FERRICYANIDE

COMMENT—STANDARD SOLUTIONS PUMPED DIRECTLY INTO FLOW CELL OF COLORIMETER CALIBRATION CURVE ASSUMED LINEAR IF READING DOES NOT DEVIATE BY MORE THAN 5 PER CENT FROM PREVIOUS READING

are measured for each line and the total rate of fluid flow is measured by collecting the effluent from the waste lines in a measuring cylinder for a known time. These values are entered into section (4) of the program with the colour code for each line and the expected line volumes. The output is shown in Table 3. The calculated values for the flow rate in the various lines are then used by the computer for the calculation of reagent concentrations.

The various reagents in the assay may now be assigned to appropriate lines and one may then calculate the concentration of each reagent needed to give the required final concentration in the reaction mixture. In the example the substrate [L(+)-lactate, D(-)-lactate or succinate] is pumped in the substrate line (line 1), potassium phosphate in the buffer line (line 2), nitrogen in the gas line (line G), potassium ferricyanide in the cofactor line (line 3) and enzyme in the enzyme line (line 4). The total volume of each line reagent required is determined and suitable concentrations of stock solutions chosen. This information is fed into

section (5) of the program with the output shown in Table 4. Details of the various reagents required for stock solutions are entered in section (6) of the program with the output of a weighing sheet (Table 5), which includes comments on the preparation of the reagents.

The gradient-making system is then calibrated. In the example, an 'enzyme gradient' is used to provide a range of protein concentrations during the assay. A 25ml volume of diluent (0.3M-mannitol) is placed in the gradient-making vessel (M1 in Fig. 1) and the enzyme preparation (a yeast homogenate) in the stock mixing vessel (M2). At a sampling rate of 40/h and with the variable speed pump at position 70, a 25ml gradient will generate the range of gradient dilution factors shown in the trace marked (70) in Fig. 4. Since the assay requires that the final protein concentration is not more than 0.5mg/ml, the yeast homogenate is diluted appropriately before being placed in the mixing vessel (see below). An example of the trace obtained during gradient calibration is given by Roodyn (1967b, Fig. 5). The gradient dilution factors for each

Table 3. Computer output of calculations of line volumes

LINE VOLUME CALCULATION				OBSERVED LINE VOLUME (ML/MIN)	EXPECTED LINE VOLUME (ML/MIN)	LINE ACCURACY (PER CENT OF THEORY)
LINE NUMBER	LINE COLOUR	CALIBRATION - READING	FRACTIONAL LINE VOLUME			
1	WHITE	0.193	0.029	0.62	0.64	96.7
2	GREEN	0.493	0.029	1.76	1.78	99.0
3	WHITE	0.189	0.029	0.61	0.59	102.6
4	YELLOW	0.313	0.029	1.08	1.07	101.2

OBSERVED TOTAL LINE VOLUME IS 4.07 ML/MIN. EXPECTED TOTAL LINE VOLUME IS 4.08 ML/MIN.
OBSERVED VALUE IS 99.8 PER CENT OF EXPECTED

COMMENT -
EXPECTED VALUES ARE THOSE OBTAINED IN THE PREVIOUS CALIBRATION.
PUMP SPEED 70
MANIFOLD USED AS FOLLOWS -

LINE NO.	COLOUR	COMPONENT IN LINE
01	WHITE	SUBSTRATES, IN SAMPLER
02	GREEN	BUFFER
G	BLUE	GAS
03	WHITE	CO-FACTOR
04	YELLOW	ENZYME, FROM GRADIENT MAKING SYSTEM
GR	WHITE	GRADIENT LINE, TO GRADIENT MAKING SYSTEM
W	PURPLE BLACK	WASTE LINE

THE SAMPLER WAS OPERATED AT 40 SAMPLES PER HOUR, WITH WATER IN THE WASH VESSEL.
THE HEATING BATH WAS AT 37 C.

Table 4. Computer output of calculations of concentrations of line reagents

LINE REAGENT AND REACTION MIXTURE CALCULATION					
LINE NUMBER	COMPONENT IN REACTION MIXTURE	CONCENTRATION OF COMPONENT IN (1) LINE (2) REACTION REAGENT MIXTURE		UNITS OF CONCN.	TO PREPARE LINE REAGENT MAKE (A) ML OF STOCK UPTO (B) ML (A) (B)
		(1) LINE REAGENT	(2) REACTION MIXTURE		
1	NA L(+) LACTATE	13.073	2.000	50.000	65.37 250.00
1	NA D(-) LACTATE	13.073	2.000	50.000	65.37 250.00
1	NA SUCCINATE	13.073	2.000	50.000	65.37 250.00
2	K PHOSPHATE PH 7.4	0.116	0.050	1.000	577.59 5000.00
3	K FERRICYANIDE	2.680	0.400	10.000	134.00 500.00

sample number are calculated in section (7) of the program and printed out as a table and graph. The latter is in the same format as Figs. 5, 6 and 7 below.

Comments about the assay conditions are then entered in section (8) and the protein concentrations then calculated. The enzyme sample originally had a protein concentration of 10.5mg/ml (as determined by the automated protein assay procedure). Allowing for dilution by the gradient-making system, and for the fractional line volume of the enzyme line, the stock enzyme was diluted tenfold before being added to the mixing vessel. This gave a maximum final protein concentration in the reaction mixture of 0.2mg/ml, well below the permitted maximum of 0.5mg/ml. The protein concentration in the reaction mixture for each sample number is calculated in section (9c) of the program, by using the value in the mixer M2 and the previous calculated gradient dilution factors. Output is as a table and a graph (Fig. 5). Because of the design of the flow system in Fig. 1 and the fact that the sampler and gradient maker are started at the same time (see below), the protein concentration corresponding to sample 1 is not zero. The point on the ordinate in Fig. 5 corresponding to zero protein concentration is determined by linear extrapolation, and used to measure the initial slope of the graph obtained in the Results section (Figs. 6 and 7 below).

The appropriate pattern of reagents in the sampler is then decided. In the assay with three different substrates and an enzyme gradient, a convenient arrangement is to have five cycles of L(+)-lactate, D(-)-lactate, succinate and water in 20 cups on the sampler, operating at 40 cups/h. A stop is placed at cup 20, and a second set of five cycles placed in cups 21-40, with a stop at cup 40. Thus one sample plate will perform two multiple enzyme assays before recycling. Information on the sample pattern and initial substrate concentrations in the sample cups are entered in section (10) of the program, with the output given in Table 6.

The enzyme activity is then determined as follows. After the analyser has been thoroughly washed and the instrument calibrations have been performed, the line reagents are prepared by appropriate dilution of the stock reagents and pumped into the analyser. Once a steady base line has been established, the enzyme and diluent are placed in the gradient-making system. As soon as stock enzyme pumped by the gradient line (GR in Fig. 1) starts to drip into the diluent, the sampler is started and the record marked by turning off the colorimeter for 1s. The assay lasts 30 min and about 5ml of fluid should be left in the gradient-making vessel. The enzyme line is disconnected 3min after the assay is completed and placed in water, and at the end of the assay the final base line is recorded. An example

of a typical trace is given in Roodyn (1967b, Fig. 7). The peak heights are read for every sample, and the blanks corresponding to each sample determined

Plot of values as % of maximum against reading number
(maximum value is 0.1952)

Fig. 5. Computer output of results of calculations of protein concentrations. The diagram has been redrawn from the actual computer output and slightly simplified for clarity. Reading numbers are on the vertical scale.

by drawing the line of best fit through the initial blank, the water blanks (readings 4, 8, 12, 16 and 20) and the final blank (for further details see Roodyn, 1970, Chapter 9). The readings and blanks are entered into section (11) of the program, with the output of a table (Table 7) and two graphs, one of $\mu\text{mol/ml}$ against reading number (Fig. 6) and the other of $\mu\text{mol/min}$ per mg of protein (Fig. 7). One estimate of the enzyme activity is from the initial slope of the trace in Fig. 6 as determined by linear extrapolation to the previously determined point on the ordinate corresponding to zero protein concentration (see calculations of protein concentration, above). However, a more convenient estimate is by fitting the best straight line through the specific activity values in Fig. 7 and extrapolating this to zero protein concentration. In the example, it was found that the sample of yeast homogenate assayed had L(+)-lactate, D(-)-lactate and succinate dehydrogenase activities of 0.164, 0.343 and 0.020 μmol of ferricyanide/min per mg of protein respectively. [This result is typical of the enzyme pattern in glucose-repressed cells, which were used for the assay (see Roodyn, 1969a).]

Table 6. Computer output of details of solutions placed in sampler module

SAMPLE NUMBER	SAMPLE COMPONENT NUMBER	SAMPLE PATTERN		CONCENTRATION OF SAMPLE COMPONENT IN		UNITS OF CONCN.
		SAMPLE COMPONENT		LINE	REACTION MIXTURE	
1	1	NA	L(+)	13.100	2.004	MILLIM
2	2	NA	D(-)	13.100	2.004	MILLIM
3	3	NA	SUCCINATE	13.100	2.004	MILLIM
4	4	WATER		0.000	0.000	—
5	1	NA	L(+)	13.100	2.004	MILLIM
6	2	NA	D(-)	13.100	2.004	MILLIM
7	3	NA	SUCCINATE	13.100	2.004	MILLIM
8	4	WATER		0.000	0.000	—
9	1	NA	L(+)	13.100	2.004	MILLIM
10	2	NA	D(-)	13.100	2.004	MILLIM
11	3	NA	SUCCINATE	13.100	2.004	MILLIM
12	4	WATER		0.000	0.000	—
13	1	NA	L(+)	13.100	2.004	MILLIM
14	2	NA	D(-)	13.100	2.004	MILLIM
15	3	NA	SUCCINATE	13.100	2.004	MILLIM
16	4	WATER		0.000	0.000	—
17	1	NA	L(+)	13.100	2.004	MILLIM
18	2	NA	D(-)	13.100	2.004	MILLIM
19	3	NA	SUCCINATE	13.100	2.004	MILLIM
20	4	WATER		0.000	0.000	—

COMMENT —

METHOD OF LOADING SAMPLE TABLE —
SUBSTRATE CUPS

NA L(+) LACTATE 01 05 09 13 17 21 25 29 33 37

NA D(-) LACTATE 02 06 10 14 18 22 26 30 34 38

NA SUCCINATE 03 07 11 15 19 23 27 31 35 39

WATER 04 08 12 16 20 24 28 32 36 40

STOPS AT CUPS 20 AND 40. THIS ALLOWS FOR TWO ASSAYS PER SAMPLE PLATE

Table 7. Computer output of results of enzyme assays

READ- ING NUM- BER	SAMPLE COM- PONENT NUMBER	SAMPLE COMPONENT	RESULTS OF ENZYME ASSAYS					UMOLES/MG PROTEIN/MIN
			CONCN. OF SAMPLE COMPONENT IN REACTION MIXTURE	UNITS OF CONCN.	READING	BLANK	ENZYME UMOLES/ ML	ACTIVITY UMOLES/ ML/MIN
1	1	NA L(+) LACTATE	2.004	MILLIM	0.461	0.479	0.015	0.003
2	2	NA D(-) LACTATE	2.004	MILLIM	0.425	0.486	0.049	0.009
3	3	NA SUCCINATE	2.004	MILLIM	0.485	0.490	0.004	0.001
4	4	WATER	0.000	-	0.493	0.496	0.002	0.000
5	1	NA L(+) LACTATE	2.004	MILLIM	0.436	0.501	0.053	0.010
6	2	NA D(-) LACTATE	2.004	MILLIM	0.352	0.505	0.124	0.023
7	3	NA SUCCINATE	2.004	MILLIM	0.499	0.509	0.008	0.002
8	4	WATER	0.000	-	0.510	0.513	0.002	0.000
9	1	NA L(+) LACTATE	2.004	MILLIM	0.414	0.517	0.083	0.016
10	2	NA D(-) LACTATE	2.004	MILLIM	0.294	0.522	0.185	0.034
11	3	NA SUCCINATE	2.004	MILLIM	0.510	0.524	0.011	0.002
12	4	WATER	0.000	-	0.529	0.527	0.002	0.000
13	1	NA L(+) LACTATE	2.004	MILLIM	0.393	0.534	0.114	0.021
14	2	NA D(+) LACTATE	2.004	MILLIM	0.242	0.537	0.239	0.044
15	3	NA SUCCINATE	2.004	MILLIM	0.524	0.540	0.013	0.002
16	4	WATER	0.000	-	0.539	0.542	0.002	0.000
17	1	NA L(+) LACTATE	2.004	MILLIM	0.367	0.547	0.146	0.027
18	2	NA D(-) LACTATE	2.004	MILLIM	0.180	0.550	0.300	0.056
19	3	NA SUCCINATE	2.004	MILLIM	0.543	0.553	0.008	0.002
20	4	WATER	0.000	-	0.557	0.557	0.000	0.000

ENZYME PATTERN IN GLUCOSE REPPRESSED CELLS.

Plot of values as % of maximum against reading number
(maximum value is 0.2996)

Fig. 6. Computer output of results (a) $\mu\text{mol/ml}$ versus reading number. The diagram has been simplified as in Fig. 5. The letters W, S, D and L have been added and correspond to water, succinate, D(-)-lactate and L(+)-lactate respectively. The lines joining the points have also been inserted by hand. Reading numbers are on the vertical scale.

Plot of values as % of maximum against reading number
(maximum value is 0.3402)

Fig. 7. Computer output of results (b) $\mu\text{mol/min per mg of protein}$. The output has been altered as in Fig. 6. Reading numbers are on the vertical scale.

DISCUSSION

In general, manual enzyme assays are conducted as follows. The substrate is mixed with a suitable buffer, various cofactors are added and the reaction is started by the addition of enzyme to the resultant incubation mixture. After a suitable period of incubation, usually at 30°C or 37°C , the progress of the reaction is measured by a suitable sensor system. Often this involves direct measurement of some property of the reaction mixture (e.g. extinction, fluorescence, pH or oxygen partial pressure) without further chemical processing. Such assays

include the measurement of NAD- or NADP-linked oxidoreductases, a variety of enzymes that can be linked to NAD- or NADP-based assays, enzymes that reduce a range of other electron acceptors, such as cytochrome *c*, ferricyanide or 2,6-dichlorophenol-indophenol, esterases monitored by pH change, oxidases monitored with the oxygen electrode and a number of enzymes in nucleic acid and nucleotide metabolism that produce changes in u.v. absorption in the range 250–280 nm. In more complex assays, some chemical or physical treatment of the reaction mixture is needed to detect the action of the enzyme. For example, in phosphatase assays based on the measurement of release of P_i , it is usual to remove protein from the reaction mixture, usually by precipitation with trichloroacetic acid, before assay of the P_i by adding several reagents in sequence. It is more difficult (but not impossible) to develop generalized systems for such assays, but current work in this laboratory is mainly concerned with the simpler assays in which the reaction mixture is monitored directly.

The example given in detail above shows how such a relatively simple assay may be adapted to the system. It should be of particular use for the many assays based on following the reduction of NAD^+ or NADP^+ at 340 nm. Since I have not yet made measurements below 340 nm, I have been able to use the Technicon phototube colorimeter and to change wavelength by using different filters. However, it is obvious that the use of an ultraviolet/visible spectrophotometer with a continuous flow cell would greatly extend the range of the system. The various calculations of the molar conversion factor are applicable to any assay in which the molar concentration of reactant is known, and the calculations of the concentrations of line reagents and stock solutions are of course freely applicable to any assay. The range of incubation times can be extended by increasing the number of heating-bath coils used, or by changing the pump tubing. A variety of pH, substrate, cofactor or enzyme gradients are possible, by varying the attachment and flow rate of the gradient line, the pump speed and the volume in the mixers. The sampler may be connected to the substrate, cofactor or enzyme lines with no difficulty, allowing one to assay enzyme activity with different substrates, or variety of cofactors or inhibitors, or in a number of different enzyme samples respectively. The system is thus extremely flexible, and because of the interrelation between analyser and computer improved precision and speed of operation are obtained. Although the flow system was designed specifically for enzyme assays, the methods developed for instrument calibration, preparation of reagents, calculation and presentation of results are probably applicable to many other automated chemical assays.

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