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Note

Computer application to automating a recording polarimeter for enzyme assays

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The development of techniques for the automated determination of enzyme activity has increased rapidly in the past few years. Schwartz and Bodansky[1] have reviewed methods for the assay of enzymes by automated methods. More recently, Roodyn[2] has presented a comprehensive study of the automated determination of enzyme activity with concomitant addition of analytical reagents. With this study, Roodyn[2] presented a computer program written in FORTAN V language, with complete description, based on the use of a Technicon auto-analyzer. This program is not a kinetics program, but one to be used in a large number of enzyme assays for calibration of the instrument and calculation of concentrations of the reagent, solution, and protein as well as enzyme activity. It is this program that was rewritten in FORTRAN IV language for use on an IBM 360 computer and adapted to an automatic electronic polarimeter[3].

The time course of enzyme reactions involving substrates that undergo an optical rotational change during the reaction is simultaneously and continuously recorded by the use of an automatic recording polarimeter. Normally, in the use of a polarimeter for monitoring enzyme-catalyzed reactions of optically active substrates, it is important to realize that no immediate distinction can be made between the changes in rotation associated with alteration at an asymmetric center and those of temperature, solvent, and concentration changes related in intermolecular hydrogen-bonding and/or the degree of association and dissociation. However, with sufficient calibration of a highly sensitive polarimeter, utilizing each parameter, it is possible to obtain a linear relationship between the concentration of product formed and optical rotation. Thus, utilizing strict calibrations, the different effects of each parameter involved in the reaction can be observed. However, the factors involved in the physical system itself, which could promote errors in readings or misinterpretation of data,

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**Contribution of technical advice in FORTAN IV programming.

must be considered. The computer program allows the operator to consider each physical parameter and makes allowances for any variation within the system without expense of time.

A discussion of polarimetry and polarimeters is treated at length by Bates and associates[4], Browne and Zerban[5], Heller[6], Rudolph[7], Fukami[8], Fluegge[9], and Nightingale and Tuesley[10]. W. Kernchen[11,12] has presented a critical survey of the principles of operation and elements of automatic electronic polarimeters. The electronic recording polarimeter has been used as a detecting device by Perronnet and Tari[13] for the separation of optically active compounds on column chromatography.

With an electronic polarimeter capable of giving a full-scale deflection for 0.1° rotation, Chignell and Gratzer[14] were able to study small conformational changes in chymotrypsinogen, activated by trypsin, and ovalbumin, denatured with urea. Also, the molar rotations of simple peptides containing leucyl residues separated by glycyl residues were obtained with reasonable accuracy by Beecham[15] by summation of the rotations of appropriate model compounds. These results suggested that none of the various factors interfering with optical rotation affect the total rotation, as long as reasonable calibration conditions are employed.

The basic function of the program used here is the same as that presented by Roodyn[2]. Other than the changes made in the computer language by transforming from FORTRAN V to FORTRAN IV, only textual comments were altered, together with the corresponding format statements. Also, a few input-format statements were changed, but without any effect on utilization of data other than a reduction in storage space allowed.

The program is divided into 12 sections, namely: introduction; calculation of molar conversion-factor; incubation time and chart-speed determination; line-volume calculations; line-reagent and reaction-mixture calculations; stock solutions; gradient calibrations; assay conditions; protein concentration; sample pattern; results and computer subroutine.

The data concerning molar conversion-factors, incubation times, chart speed, and line volumes were entered into the computer with details of the composition of the reaction mixture and the molecular weights of the solutes for the stock solutions, as given by Roodyn[2].

The polarimetric assays were checked repeatedly for accuracy, and compared with assays by other methods. Activities of the enzymes studied did not deviate more than 2% from one assay to the next. A typical printout for the assay of β-D-glucosidase in fractions of almond emulsin is given in Table I.

With colorimetric, automated methods, it has been difficult, generally speaking, to monitor continuously the entire progress curve of an enzyme reaction. This is because the enzyme and substrate are reacting in a continuously flowing stream. In this respect, the system described offers no more advantages than the automated methods at present used. However, with the polarimeter cell of 11.5-ml capacity utilized in this system, the initial enzyme reaction (for example the first 5.75 min) can be monitored continuously, and/or the entire reaction progress can be followed

with a stationary system. The larger volume gives longer reaction-times, which allows an advantage over other interrupted-flow systems. The use of less expensive substrates also presents an advantage. However, the primary advantage of using a polarimeter in enzyme studies of this type is the ability to monitor the reaction at the point where molecular change occurs at the anomeric carbon atom of the sugar.

An example of activity determinations for fractions from almond emulsin is given here in order to introduce the polarimetric assay system and also to present the computer program[3] in a more standard language for general use. We would emphasize that, even though this FORTRAN IV program was adapted to the polarimeter, it may still be used flexibly with spectrophotometric assay-systems. It is hoped that this contribution will extend the historic utility of polarimetry in carbohydrate chemistry and biochemistry.

EXPERIMENTAL

General. — Enzyme-catalyzed hydrolyses involving glycosides as substrates were continuously recorded by the use of a Bendix-NPL Automatic Polarimeter, Model 1169, equipped with a thermostatted flow-cell. The quartz water-jacketed cell could vary in size up to 2.5×7.0 cm. However, for the system used, a 1.5×6.5 cm (11.5 ml capacity) cell was chosen for optimum efficiency. The cell was held secure by a plane-parallel machined, aluminum sleeve fitted with two retainer screws (see Fig. 1). The sleeve was also fitted with two arms machined so that the sample cell could be inserted and withdrawn without changing its azimuth position. This was done to minimize the effects of birefringence in the cell windows. Because the polarimeter was so sensitive and the cell path-lengths used were so short, the angular rotations measured were generally very small and birefringence could not be avoided entirely, but its

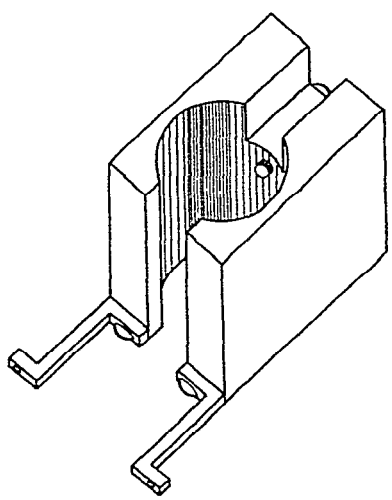

Fig. 1. Aluminum sleeve to secure the polarimeter cell.

effects could be balanced out during calibration. This instrument allowed continuous and simultaneous recording of the time-course changes in optical rotation over a range of ± 0.5 degrees of arc relative to a fixed angle, and was sensitive to these reactions to ± 0.01 degrees of arc.

In the interrupted-flow system set up for the automatic polarimeter, the reaction mixture was prepared, mixed with the enzyme, and pumped into the flow cell of the polarimeter. Once the reaction mixture was in the sensor, the flow of fluid could be stopped and the course of the reaction monitored with a stationary reaction-mixture, or it could be continued and the reactions monitored on a constant-flow basis. The assays reported in this paper were carried out by the latter method. After a specified time, the flow cell was emptied, washed free of old reaction-mixture, new reaction mixture plus enzyme introduced, and the next assay performed. The reagents required for the assay were prepared from solids and dissolved in 0.05M sodium acetate buffer (pH 5.0) to produce stock solutions. In the assays reported, the stock solution contained only substrate and buffer. However, stock solutions could also be made up comprising any other reagent required for a specific study. The stock solutions were then mixed in selected proportions and concentrations to give the desired reaction mixture. The reaction mixture solution was then pumped continuously with a Solution Metering Pump (Model 746, Beckman Scientific Instruments, Fullerton, California) through the system at a rate of 2.0 ml/min while the desired solution temperature was maintained by use of the thermostatted constant-flow line (see C, Fig. 2) and flow cell (see PC, Fig. 2). A constant temperature of 37° was maintained by continuously pumping water heated by a constant temperature bath through these vessels. When the temperature became constant, the instrument was zeroed to the

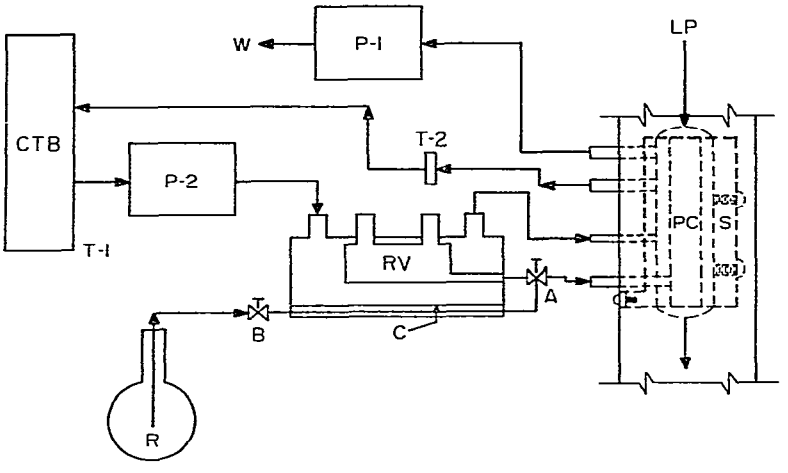

Fig. 2. Schematic diagram of polarimetric enzyme analyzer. T-1 and T-2, temperature-monitoring device; P-1 and P-2, constant-flow, sample-metering pump; S, machined aluminum sleeve; PC, Polarimeter cell; C, calibration; RV, reaction vessel; CTB, constant-temperature bath; LP, light pathway; A and B, stopcock (three-way); R, reservoir; W, waste.

optical rotation of the reaction mixture, and the reaction mixture (15 ml) was then added to the thermostatted reaction vessel (see RV, Fig. 2). The enzyme was introduced into the reaction mixture volume (precalculated to give a concentration of 0.056 mg/ml), the stopcock (see A, Fig. 2) turned to the reaction-mixture line, and the progress of the reaction monitored by recording the change in optical rotation with the appearance of product.

Calibration curves for the enzyme reactions were obtained by taking a series of optical rotational readings of known carbohydrates corresponding to the reaction product, at various concentrations (thus, in the case of methyl β-D-glucopyranoside, the calibration curve was obtained for D-glucose in 0.05M sodium acetate buffer, pH 5.0 at 37°) at the same temperature and under the same conditions as the enzymic reaction. These readings were introduced in data of the computer program for the calculation of a molar conversion-factor that is defined[2] as "that concentration of solute in μmoles/ml that gives an instrument reading of 1.0". For the polarimeter it is the concentration that gives a reading of 100 millidegrees of optical rotation.

Gel filtration of β-D-glucosidase from almond emulsin. — Almond emulsin [obtained as β-glucosidase (3.9 units/mg) from Worthington Biochemical Corp., Freehold, New Jersey] (100 mg) was dissolved in a minimal amount of sodium phosphate buffer, pH 7.0, ionic strength 0.2. The solution was placed on a column of Sephadex G-100 (3 × 100 cm) (Pharmacia) and fractionated with the same buffer solution, collecting 16-ml fractions with a drop count of 200 drops, one drop every 17 seconds. The void volume, measured with Blue Dextran, was 128 ml, or 8 fractions. A Mariotte flask was used to ensure constant pressure and thus constant flow rate. Every 3 of the 30 individual fractions collected were combined and labeled fractions A to J, giving 10 fractions to study.

These fractions were concentrated with an Amicon Ultrafiltration Unit (Amicon Corporation, Lexington, Massachusetts) equipped with a UM 10 membrane.

β-D-Glucosidase assay. — The substrate, methyl β-D-glucopyranoside (0.011M in 0.05M sodium acetate buffer, pH 5), was incubated at 37° for 5.625 min with various protein fractions separated by gel filtration from almond emulsin. The protein concentration for each assay was adjusted to 0.056 mg/ml. The reaction was monitored by an electronic polarimeter as already described. Results as printed out by the computer program are given in Table I.

β-D-Xylosidase assay. — This assay was carried out under the same conditions as used for β-D-glucosidase, but with methyl β-D-xylopyranoside as the substrate. Most activity was found in fractions A and C of the almond emulsin preparation, giving optical rotational changes of 10 and 12 millidegrees, respectively.

D-Oxynitrilase assay. — Fraction F of the almond emulsin preparation gave the highest activity for this enzyme. The polarimeter was zeroed by continuously pumping a solution of 10mM benzaldehyde in 0.05M sodium acetate buffer with 50% ethanol of pH 5.4. When the enzyme was added to the reaction mixture, 2 ml of saturated aqueous hydrogen cyanide was introduced. After incubation for 5.625 min, rotational change of 11 millidegrees was observed.

TABLE I

β-D-GLUCOSIDASE ACTIVITY FROM ALMOND EMULSIN FRACTIONATION, AS COMPUTED BY THE AUTOMATED PROGRAM

Fraction	*Reading (degrees)*	*Enzyme activity*[a]		
		μmoles/ml	*μmoles/ml/min*	*μmoles/mg protein/min*
A	0.260	2.157	0.303	5.418
B	0.010	0.083	0.012	0.208
C	0.126	1.045	0.147	2.626
D	0.060	0.498	0.070	1.250
E	0.055	0.456	0.064	1.146
F	0.052	0.431	0.061	1.084
G	0.005	0.041	0.006	0.104
H	0.004	0.033	0.005	0.083
I	0.003	0.025	0.004	0.063
J	0.002	0.017	0.002	0.042

[a]μMoles refers to product formed during reaction.

β-D-Galactosidase assay. — Fraction A of the almond emulsin preparation gave the highest activity for this enzyme. A substrate solution of lactose in 0.05M sodium acetate buffer, pH 5.1, was prepared. All other conditions were the same as those reported for the β-D-glucosidase assay. After incubation for 5.625 min, a 12-millidegree optical rotational change was observed.

Disc-gel electrophoresis. — A sample of the almond emulsin was examined by disc-gel electrophoresis (10% acrylamide, pH 8.5). After staining with Amido Black, the gel showed 10 separate proteins. An optical-density scan of the stained gel was obtained with an Electrophoresis Densitometer, Model 345 (Clifford Instruments, Inc., Natick, Massachusetts).

An activity stain was obtained by using *p*-nitrophenyl β-D-glucopyranoside, and the results showed that the β-D-glucosidase-active protein constituted 21.7% of the total protein. β-D-Xylosidase activity was determined by using *p*-nitrophenyl β-D-xylopyranoside, and the active zone had the same mobility as that of β-D-glucosidase. The *p*-nitrophenol released turns yellow in alkaline solution, and the following procedure was used to locate β-glucosidase activity in polyacrylamide gels of almond emulsin proteins.

When the electrophoresis was terminated, the gels were removed from the glass tubes, rinsed with distilled water, and then placed in a solution of substrate in buffer (1.67mM *p*-nitrophenyl β-D-glucopyranoside in 0.2M sodium acetate buffer, pH 5.0). After incubation for 15 min at room temperature, the gels were rinsed with distilled water and placed in 0.5M sodium borate buffer, pH 9.0. The position of the yellow band (released *p*-nitrophenol) was noted at once, as the colored product diffused out of the gels in ~2 h.

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REFERENCES

1 M. K. SCHWARTZ AND O. BODANSKY, *Methods Biochem. Anal.*, 16 (1968) 183.
2 D. B. ROODYN, in T. S. WORK AND E. WORK (Eds.), *Laboratory Techniques in Biochemistry and Molecular Biology: Automated Enzyme Assays*, American Elsevier, New York, 1970.
3 This computer program has been cataloged in the Program Library of SHARE, Inc., 25 Broadway, Suite 750, New York, New York 10004. Limited copies of the format and card deck may be obtained from the authors.
4 F. J. BATES and Associates, *Polarimetry, Saccharimetry and the Sugars*, Circular of the National Bureau of Standards, Vol. 440, 1942, p. 1.
5 C. A. BROWNE AND F. W. ZERBAN, *Physical and Chemical Methods of Sugar Analysis*, John Wiley and Sons, New York, 3rd edition, 1941, p. 136.
6 W. HELLER, in A. WEISSBERGER (Ed.), *Techniques of Organic Chemistry*, Vol. I, Part II, Interscience Publishers, New York, 2nd edition, 1949, p. 1491.
7 H. RUDOLPH, *J. Opt. Soc. Amer.*, 45 (1955) 50.
8 H. FUKAMI, *Bunseki Kagakee Shinpo Sosetsu*, (1964) 58R.
9 J. FLUEGGE, *Zeiss Mitt. Forschr. Tech. Opt.*, 3 (1963) 52.
10 C. N. NIGHTINGALE AND S. TUESLEY, *Amer. J. Hosp. Pharm.*, 24 (1967) 516.
11 W. KERNSCHEN, *Z. Zuckerind.*, 17 (1967) 63.
12 W. KERNSCHEN, *Z. Zuckerind.*, 17 (1967) 128.
13 J. PERRONNET AND M. TARI, *Bull. Soc. Chim. Fr.*, 7 (1966) 2345.
14 D. A. CHIGNELL AND W. B. GRATZER, *Nature*, 210 (1966) 262.
15 A. F. BEECHAM, *Tetrahedron Lett.*, 9 (1966) 957.