ANALYTICAL BIOCHEMISTRY 9, 467–473 (1964)

Automatic Continuous Monitoring of Enzymic Activity in Column Effluent

J. KENNETH HOOBER[1] AND I. A. BERNSTEIN

From the Departments of Biological Chemistry and Dermatology and the Institute of Industrial Health, The University of Michigan, Ann Arbor, Michigan

Received March 30, 1964

Schwartz *et al.* (1) have described the application of the continuous flow system of analysis, developed by Skeggs (2), for the automatic assay of enzymic activities involving the nicotinamide adenine dinucleotide cofactors [NAD(H) and NADP(H)].[2] This paper describes a further development of this application to the continuous monitoring of column effluents. Enzymes which have activities involving the reduction or oxidation of these dinucleotide cofactors, or which can be coupled to such enzymes, can be conveniently located in column effluents, with greater than 90% of the effluent available for further use. Thus the time required for manual enzyme assay of individual fractions is eliminated.

APPARATUS

The procedure was developed with the AutoAnalyzer (Technicon Instruments Corporation, Chauncy, N. Y.). The phototube colorimeter described by Schwartz *et al.* (1) equipped with a 15-mm light path tubular flow cell was used to measure light absorption at 340 mμ. The flow diagram of the system is shown in Fig. 1. The manifold was designed to use a minimum of column effluent for the assay of enzymic activity. Small reagent volumes were used and thus a high degree of sensitivity was achieved by eliminating excessive dilution of the final reaction product (NADH or NADPH). Reactants were aspirated as follows: the substrates through tube B at a rate of 0.05 ml/min; air through tube C at a rate of 0.03 ml/min; nicotinamide adenine dinucleotides through tube D at a rate of 0.03 ml/min (except in cases in which the dinucleotides were incorporated into the substrate solution and an unstable reagent pumped

[1] U. S. Public Health Service Predoctoral Fellow, 1962–65.

[2] Abbreviations used: NAD(H), nicotinamide adenine dinucleotide (reduced); NADP(H), nicotinamide adenine dinucleotide phosphate (reduced); DEAE-cellulose, diethylaminoethylcellulose.

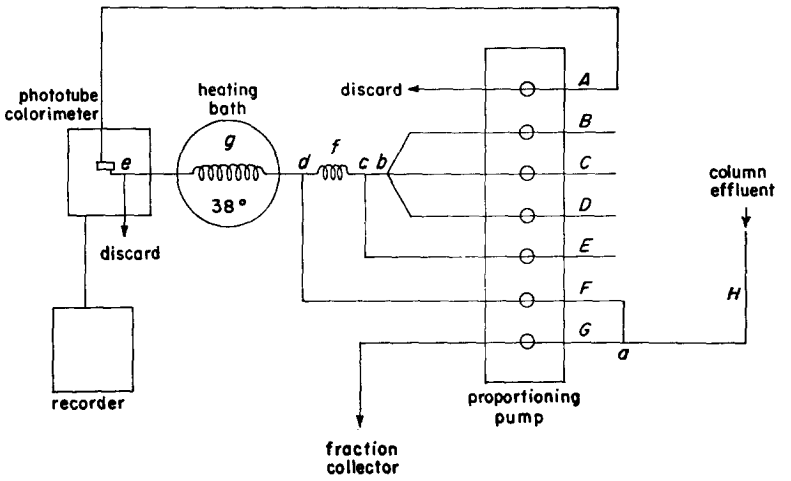

FIG. 1. Flow diagram for continuous monitoring of column effluents. See text for description.

through this tube); and either buffer or a coupling enzyme through tube E at a rate of 0.015 ml/min. Effluent was pumped into the assay reaction mixture at a rate of 0.015 ml/min through tube F. Most of the effluent was pumped to a fraction collector through tube G at a rate (usually in the range of 0.4 to 0.8 ml/min) determined by the rate of flow of the column. In order to prevent air bubbles from appearing in the effluent resulting from reduced pressure in the transmission tube (H) leading to the pump from the column, the pumping rate of the shunt, tube G, was always less than the rate of flow of the column under gravity flow. The connection to the column was a stepwise size increase of Tygon tubing until the diameter of the tubing was large enough to fit snugly over the tip of the column. The reaction mixture was pumped through the flow cell at a rate of 0.10 ml/min through tube A and discarded. The glass fitting at *a* was the Technicon fitting A1, at *b* was fitting G3, and at *c* and *d* was fitting D2. At *e* was a debubbler device supplied with the tubular flow cell.

Since pump tubing of small capacity was used, neither the standard AutoAnalyzer mixing coils nor the heating bath coils were adequate. A small mixing coil (*f*) was prepared by wrapping 24 cm of transmission tubing around a short glass rod 0.9 cm in diameter. An incubation coil (*g*), which also acted as a mixing coil, was constructed of 54 cm of Solvaflex transmission tubing wound around a glass tube 1.0 cm in diameter and immersed in the heating bath fluid. The length of tubing was determined by that which produced a colorimeter response 10 min

after addition of a dye to the assay system through the effluent assay tube (point *d*). The length of incubation (and thus the sensitivity of the assay) can be conveniently altered simply by changing the length of the tube in the heating bath.

Effluent fractions were collected by pumping most of the effluent through the shunt and allowing it to drop directly into test tubes on the turntable of a fraction collector which was operated to collect 5-min fractions. The length and size of the tubing from the pump to the fraction collector was such that the time required for effluent to travel the distance from the point where the stream was split (*a*) to the fraction collector was equal to that required for effluent to travel from this point to where it entered the reaction mixture (point *d*). The fractions and the chart were then correlated by starting the timing mechanism for the first fraction as the column effluent entered the first collecting tube. Since the time of incubation was 10 min, the recorder registered the enzymic activity two fractions previous.

METHODS

Chromatography on DEAE-cellulose was similar to the method described by Keller *et al.* (3). The enzyme sample was applied to 1 × 25 cm columns packed under 6 cm Hg air pressure and equilibrated with 0.005 *M* sodium phosphate buffer, pH 8.0, at 2°C. A phosphate gradient

TABLE 1

REAGENT SOLUTIONS USED FOR CONTINUOUS ASSAY OF COLUMN EFFLUENT

Assay	Manifold tube[a]	Reagent	Concn., m*M* Initial	Final
Glucose 6-phosphate dehydrogenase	B	Glucose 6-phosphate	2.2	1.0
	D	NADP	1.8	0.5
	E	Na_2HPO_4, pH 7.5	5.0	5.0
Lactic acid dehydrogenase	B	Sodium pyruvate	2.2	1.0
	D	NADH	0.75	0.2
	E	Na_2HPO_4, pH 7.5	5.0	5.0
Phosphoglucomutase	B	Glucose 1-phosphate	2.2	1.0
		Fructose 1,6-diphosphate (Mg salt)	1.1	0.5
		NADP	1.1	0.5
	D	Cysteine, pH 7.5	160	40
	E	Glucose 6-phosphate dehydrogenase	20 μg/ml	3 μg/ml

[a] Designation refers to that given in Fig. 1 and in the text.

was applied by placing 0.005 *M* sodium phosphate, pH 8.0, in the mixing chamber and 0.5 *M* sodium phosphate, pH 8.0, in the reservoir. Sephadex G-25 columns, 2 × 20 cm, were equilibrated and eluted with 0.005 *M* sodium phosphate, pH 7.5, at 2°.

In the linearity studies, aspiration of each enzyme concentration sample was continued until a steady chart reading was obtained. For these studies and in subsequent column monitoring, all reagents and enzyme samples were kept on ice during the assay procedures.

Table 1 shows the reagents pumped by each tube on the manifold and the final concentrations achieved in the assay system. Reagents were prepared in 0.005 *M* sodium phosphate buffer, pH 7.5, and the final pH adjusted to 7.5 if necessary. In assay systems such as that of phosphoglucomutase in which an unstable reagent was necessary (cysteine), the reagents were prepared as shown in the table. In this way a large quantity of the substrate mixture, which can be stored, was prepared and only the cysteine prepared daily as needed.

MATERIALS

Analytical-grade glucose 6-phosphate dehydrogenase and phosphoglucomutase (Boehringer und Soehne preparations purchased through Calbiochem) and twice-crystallized lactic acid dehydrogenase (purchased from Worthington Biochemicals Corp.) were used as model enzymes in this study. Glucose 1-phosphate (potassium salt) and glucose 6-phosphate (sodium salt) were purchased from Nutritional Biochemicals Corp. L-Cysteine hydrochloride monohydrate was purchased from Calbiochem. Fructose 1,6-diphosphate (magnesium salt), which contained glucose 1,6-diphosphate as a contaminant, was obtained from Schwarz Bioresearch Inc. NADP and NADH were purchased from Sigma Chemical Corp.

RESULTS

The colorimeter response and a standard curve for NADH were determined using a single pumping tube which entered the flow cell directly. Above an optical density of 0.6, linearity with NADH concentration did not hold, but the complete curve was quite reproducible. From the linear portion of the curve an absorbancy value of 7.0×10^3 for a molar solution of NADH was calculated.

Linearity of NADPH formation with enzyme concentration was achieved by plotting final NADPH concentration, as determined from the NADH standard curve, against protein concentration. Formation of NADPH to a final concentration of 0.3 μmole/ml was directly proportional to the concentration of glucose 6-phosphate dehydrogenase in the assay system. Production of NADPH to a final concentration of

0.5 μmole/ml was also directly proportional to the concentration of phosphoglucomutase in a coupled enzyme assay system in which the product of the mutase reaction, glucose 6-phosphate, was oxidized by NADP in the presence of excess glucose 6-phosphate dehydrogenase. In each case a flow cell of 6-mm light path was used to extend the range of concentration beyond that which provided accurate estimation of optical density when the 15-mm light path flow cell described under "Apparatus" was used.

In Fig. 2 are shown typical chart records obtained by continuously monitoring column effluents by the procedure described. An effluent curve for lactic acid dehydrogenase is included as an example of enzymic activ-

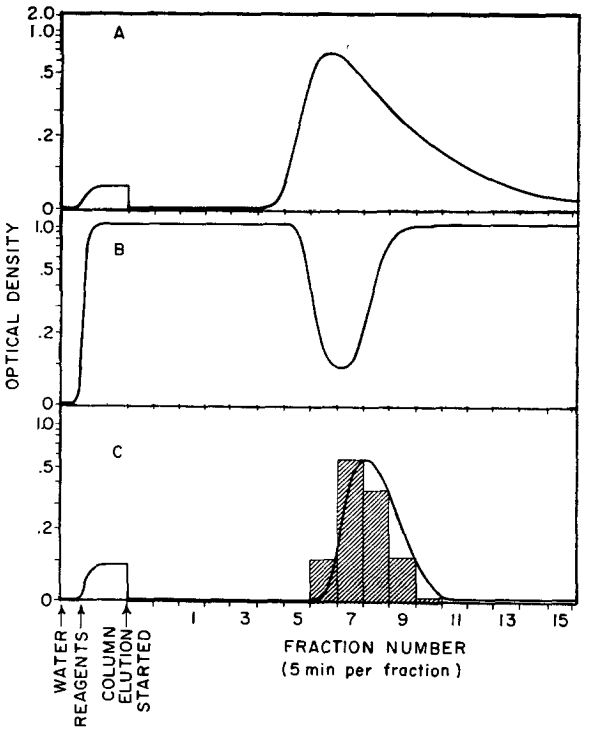

FIG. 2. Chart records obtained by continuous monitoring of column effluents. (A) Elution pattern of glucose 6-phosphate dehydrogenase from a DEAE-cellulose column, 1 × 25 cm, with a phosphate gradient, pH 8.0, 2°C. Flow rate was 48 ml/hr. Amount of effluent used for activity assay was 1.8%. (B) Elution pattern of lactic acid dehydrogenase from a Sephadex G-25 column, 2 × 20 cm, pH 7.5, 2°C. Flow rate was 37 ml/hr. Amount of effluent used for activity assay was 2.4%. (C) Elution pattern of phosphoglucomutase from a Sephadex G-25 column. Conditions as for B. Hatched area shows enzymic activity in individual fractions as assayed by aspirating the collected effluent into the assay system until a steady chart response was obtained.

ity detected by the disappearance of NADH. The hatched area on the effluent curve for phosphoglucomutase (curve C) indicates the activity in individual fractions and shows a close correlation to the area under the curve recorded by continuous monitoring.

The total activity eluted from the column can be estimated to approximately 5% accuracy by integration of a given peak by addition of the average absorbance values of each half-fraction. After dividing this summation value by the number of segments across the peak, the amount of NADPH produced in 10 min by the eluted enzyme can be determined from a standard curve. In the experiment shown on curve C, 0.72 μmole NADPH was formed by the enzyme system as determined by the assay of the individual fractions, and 0.76 μmole NADPH was formed as determined by the above integration process.

DISCUSSION

An automated monitoring of column effluents would be most useful if the amount of effluent used for the assay were low. Generally, the main objective of any purification procedure is to recover enough enzymic activity to subject the enzyme to a further purification step or to employ the purified enzyme in a particular study. In the present method the percentage of column effluent used for the assay depends upon the rate of flow of effluent through a column, and, as 10 ml/hr can be considered a lower limit, the maximum amount of enzyme required for the assay would be approximately 10%. For faster flow rates (40–50 ml/hr) this amount drops to near 2%. Although this may be somewhat greater than that used in a manual system, the convenience of automation in most cases outweighs the disadvantage of using the slightly greater proportion of enzyme.

Since the basic objective in this study was to use as small an amount of column effluent as possible for the assay, the rest of the assay system was scaled down accordingly. The manifold of small pump tubing was constructed very carefully to ensure proper function. The physical system was simple and its construction and adjustment required no more than a day. After the system was operating it required only routine maintenance.

The manifold as described is quite versatile. It can be used equally well with simple systems as in the assay of enzymes such as glucose 6-phosphate dehydrogenase and lactic acid dehydrogenase or with more complex systems as in the assay of phosphoglucomutase activity. In setting up a new assay system, the information needed is a knowledge of the necessary reagents, the dilution of each reagent which will occur in the assay mixture, and the consequent initial concentration of each reagent required to produce the final concentration desired.

SUMMARY

A procedure has been described using the flow system of analysis for continuous monitoring of enzymic activities in column effluents. The system exhibits a high degree of sensitivity. Greater than 90% of the enzyme in the effluent can be recovered for further use.

ACKNOWLEDGMENTS

The authors express their gratitude to Mr. Michael Taylor for his technical assistance and to Technicon Instruments Corporation for their assistance and cooperation through Mr. Arthur Horowitz.

REFERENCES

1. SCHWARTZ, M. K., KESSLER, G., AND BODANSKY, O., *J. Biol. Chem.* **236,** 1207 (1961).
2. SKEGGS, L. T., *Am. J. Clin. Pathol.* **28,** 311 (1957).
3. KELLER, P. J., COHEN, E., AND NEURATH, H., *J. Biol. Chem.* **230,** 905 (1958).