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PYRIMIDINE NUCLEOSIDE PHOSPHORYLASE ASSAY BY AUTOMATED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A new assay for pyrimidine nucleoside phosphorylase is reported. This method utilizes an isocratic reversed-phase high-performance liquid chromatographic system for separation of nucleosides and bases. Product detection is accomplished by ultraviolet monitoring and radioactive flow detection. Use of an automated sample injector allows for the analysis of a series of samples, with data recorded onto a microprocessor-based cassette recorder. Data can then be downloaded into computer memory. The velocity of uridine phosphorylase (E.C. 2.4.2.3) was a linear function of enzyme concentration. The Michaelis constant for uridine at pH 8.0 was found to be in close agreement with the value obtained by a thin-layer chromatographic assay method.

INTRODUCTION

Current strategies in antiviral and antineoplastic chemotherapy include the development of a variety of nucleoside analogs, many of which have excellent *in vitro* activity yet lack clinical utility. Pharmacological factors are often the reason behind lowered *in vivo* activity, with the phosphorylytic cleavage of some nucleosides to less effective base and sugar moieties being one clearly defined problem[1–3]. To address this problem, this and other laboratories have undertaken a search for effective pyrimidine nucleoside phosphorylase inhibitors to be used as combination chemotherapeutic agents. Currently, spectrophotometric[4,5], gradient high-performance liquid chromatographic (HPLC)[6,7], and thin-layer chromatographic (TLC)[3] methods of enzyme assay are used. While the spectrophotometric methods are useful for velocity determinations involving high substrate concentrations, determination of initial velocities utilizing low substrate concentrations are often below the limits of detectability. In addition, sensitivity of spectrophotometric assays may be compromised by interfering absorption spectra of nucleoside inhibitors and by extraneous absorbing material in crude enzyme preparations.

An approach to circumvent these problems is to utilize radioactive substrates with both TLC and HPLC methods. However, both require tedious and time-consuming sample preparation, and are limited by liquid scintillation counter space.

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Furthermore, all of the above methods usually require time-consuming hand entry of raw data into a computer or calculator before calculation of kinetic constants.

We report an automated method for analysis of uridine phosphorylase (E.C. 2.4.2.3) activity, with general applicability to other nucleoside phosphorylases. This method utilizes current technology in automated HPLC injection, radioactive flow detection, and data logging. We are able to process automatically between five and ten velocity determinations consisting of four time points each in the 24-h period. Utilization of a logging device eliminates the need for hand entry of raw data into a computer and also eliminates dedication of that computer to the task.

MATERIALS AND METHODS

Materials

[2-^{14}C]Uridine (56 mCi/mmol) and [2-^{14}C]thymidine (56 mCi/mmol) were obtained from Moravek Biochemicals, Brea, CA, U.S.A. All other chemicals were of reagent or HPLC grade.

Enzyme Solubilization

Uridine phosphorylase was prepared from sarcoma cells (S-180) by the method of Niedzwicki *et al.*[3]. Cells were collected by centrifugation and washed twice in phosphate-buffered saline. The pellet was resuspended in 20 m*M* potassium phosphate, 1 m*M* ß-mercaptoethanol, 1 m*M* EDTA, pH 8.0, (buffer A), and homogenized by 40 strokes of a Dounce Homogenizer at 4°C. The homogenate was centrifuged for 1 h at 105,000 *g* and the resulting supernatant used for enzyme assay. Protein content was determined by the method of Bradford[8]. S-180 cells do not have thymidine phosphorylase[3], hence activity measurements of the supernatant fraction represents only uridine phosphorylase activity.

Enzyme assay

Uridine phosphorylase was assayed at pH 8.0 in buffer A. Reactions were preincubated at 37°C for 5 min and initiated by the addition of the appropriate concentration of substrate. The total reaction volume was 200 µl. At 0, 20, 40 and 60 min after initiating the reaction, 30 µl aliquots were removed and added to 10 µl of ice-cold 40% perchloric acid to stop the reactions. Aliquots may be stored for several weeks at −20°C without detectable conversion of uridine into uracil (data not shown). Samples were neutralized by the addition of 10 µl of 10 *N* potassium hydroxide and the potassium perchlorate was removed by centrifugation for 5 min in an Eppendorf 5412 microfuge. A 40-µl aliquot of supernatant from each sample was transferred to vials for Water's WISP automatic HPLC injector (Waters Assoc., Milford, MA, U.S.A.) utilizing the limited volume inserts. Separations were accomplished on an E.M. 5 µm Lichrosorb RP-18 column (25 × 4.6 cm I.D., Rainin, Woburn, MA, U.S.A.). Uracil and uridine are separated isocratically utilizing 0.1 *M* sodium phosphate, pH 6.4, at a flow-rate of 0.7 ml/min. Under these conditions uracil and uridine elute at 12 and 27 min, respectively. Adaptation of the chromatography for analysis of thymidine phosphorylase is accomplished on the same column utilizing 0.1 *M* sodium phosphate, 5% methanol, pH 6.4, isocratically at a flow-rate of 0.7 ml/min. Under these conditions thymine and thymidine elute at 12 and 27 min,

respectively. Column eluent was monitored by UV absorption at 254 nm, with the nucleoside and base peaks being the major non-void volume UV peaks. Radioactivity of the eluent was monitored by a Model Flo-One radioactive flow detector (Radiomatic, Tampa, FL, U.S.A.) equipped with a 2.5-ml liquid scintillant flow cell. Monofluor (National Diagnostics, Sommerville, NJ, U.S.A.) or Aquasol (New England Nuclear, Boston, MA, U.S.A.) were used as scintillant, and pumped at a rate of 4.0 ml/min through the flow cell.

Generally, 0.3 μCi of [^{14}C]uridine was added to each reaction mixture. This represents $6.6 \cdot 10^5$ dpm per reaction mixture, which converts to $4.42 \cdot 10^5$ cpm when multiplied by our experimentally derived counting efficiency of 67 $\pm$ 1.4% for ^{14}C. Each 30 μl time point contains $6.63 \cdot 10^4$ cpm of which $2.65 \cdot 10^4$ cpm is actually injected onto the column. Based on our observed chromatographic dilution, peak fractions of uracil can thus be determined with *ca.* 2% counting error at the indicated flow-rates for 10% conversion of uridine into uracil. Based on currently available specific radioactivities of ^{14}C-labelled nucleosides, the limit of detection of radiolabelled products is *ca.* $3 \cdot 10^{-6}$ *M*, and care must be taken to include radioactive carrier concentrations when calculating final substrate concentrations.

Hardware interface

The WISP autosampler has two contact closure switches that enable it to be

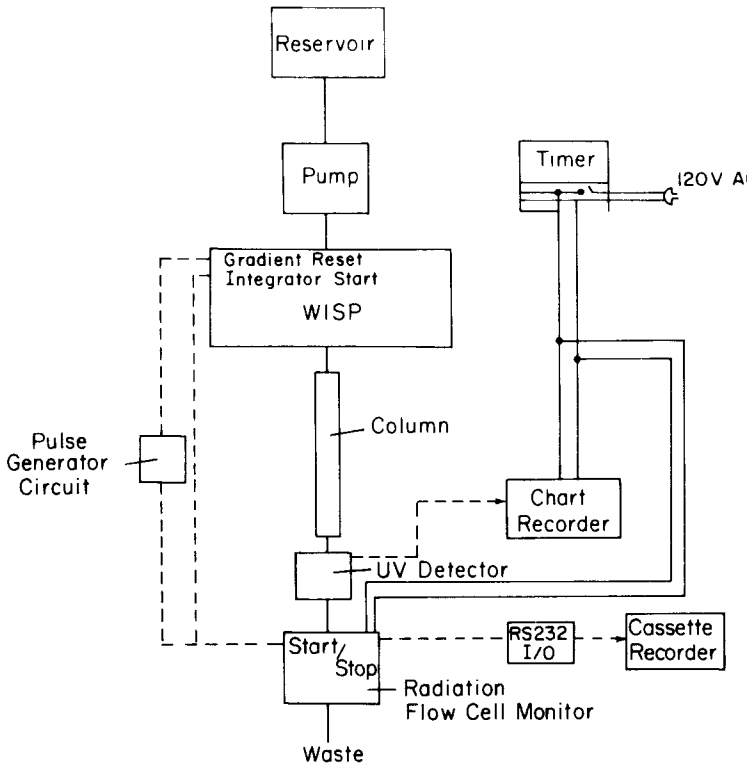

Fig. 1. A block diagram of a standard isocratic HPLC system equipped with a WISP autosampler and on-line casette recorder for automatic data collection.

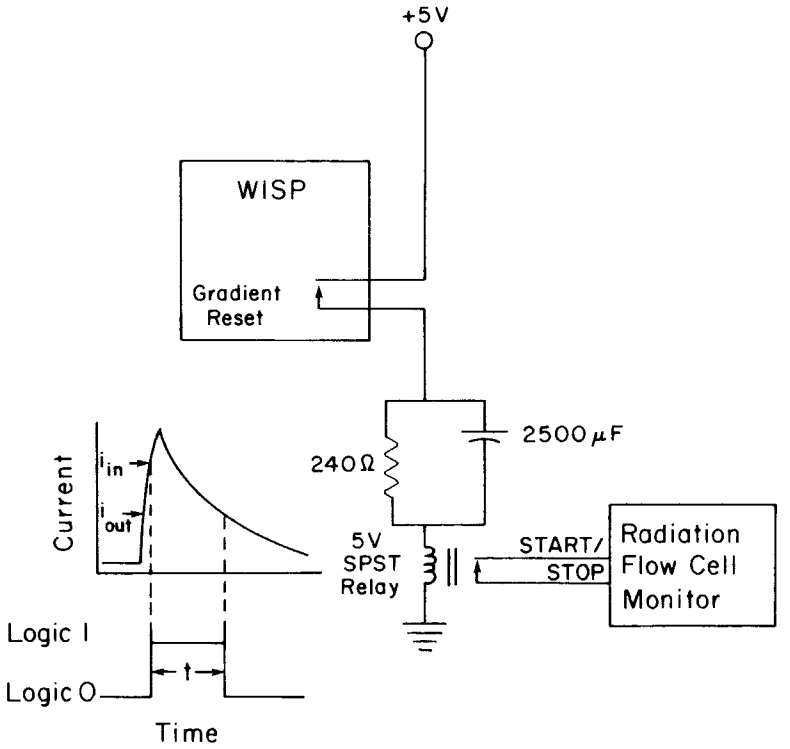

Fig. 2. This circuit is used to generate a contact closure to terminate data collection at the Flo-One using the Gradient Reset Switch on the WISP. The current to initially close the 5-V relay (i_{in}) is typically greater than the current to keep the relay closed (i_{out}). By constructing a circuit that delays the current from i_{in} to i_{out} over an interval of time t (500 msec), the appropriate signal is sent to the Flo-One.

the master control module in the automated HPLC system (Fig. 1). The integrator start switch initiates the data transmission from the Flo-One to a microprocessor-based cassette recorder (ADPI, Model LG1, Troy, OH, U.S.A.) at the start of a run. The gradient reset pulse terminates data acquisition (Fig. 2). The run time programmed into the WISP sets the number of fractions written to the tape per sample, *i.e.* 1-min fractions reported by the Flo-One and a run time of 33 min on the WISP implies 33 fractions per sample. A standard air conditioner timer (Model ET-125, Mallory Distributor Products, Indianapolis, IN, U.S.A.) was used to turn the Flo-One off at the end of all analyses. Data were transmitted from the Flo-One via the printer cable to the RS-232 interface (Serial Output Interface, Radiomatic). A dip switch on the RS-232 module enabled information to be transmitted to the recorder in the following form; 8 data bits per word, 2 stop bits, 300 baud and no parity.

A 64 K Horizon North Star computer (Berkeley, CA, U.S.A.) with two serial ports was used to print stored information from the cassette recorder onto a Model 43 teletype.

Software

Machine language program. The 8251 USART on the second serial port is

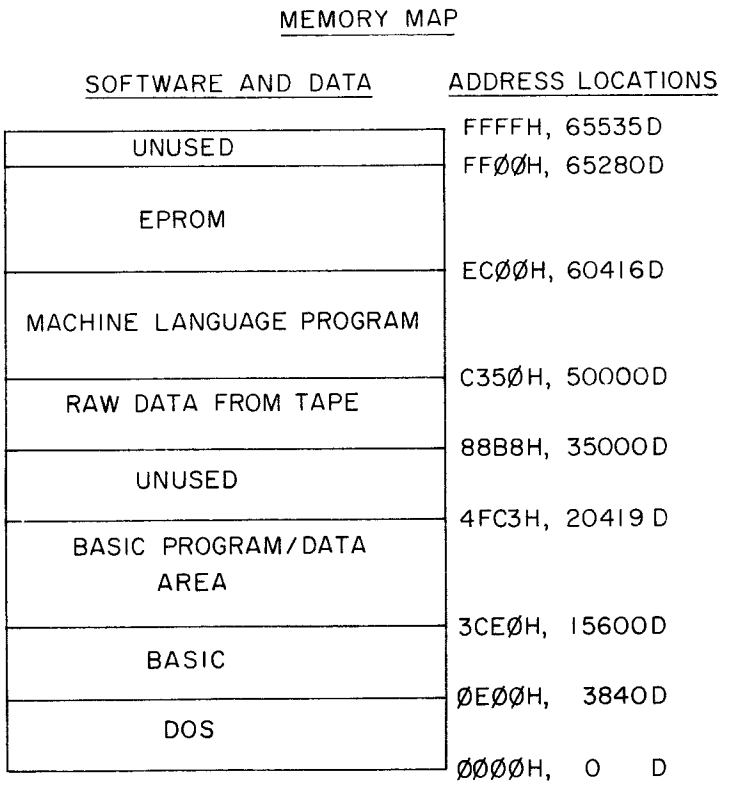

Fig. 3. North Star memory address locations for software and data.

programmed in Z-80 machine code to collect data from the cassette and store it in a reserved region of memory located at 35000 D. A memory map shows the location of programs and data (Fig. 3).

Basic program. The Basic program is divided into three subroutines. The first subroutine writes the machine language program with the FILL command into memory at address location 50000 D. These instructions are stored in memory as hexadecimal code. The second subroutine calls the machine program which rewinds, reads the tape and stores the data into memory. Afterwards, the stored ASCII code is transferred using the EXAM (X) command into a Basic array within the Basic/Program area. The Z = call (M,D) command is used to call the machine language program from Basic and at the same time pass the total number of samples counted on the Flo-One down to the machine program as the variable D. M is the address location for the machine language in decimal. Once the Basic array is set up, the third subroutine is called to print the numeric data from the array to a teletype in tabular format.

RESULTS

Data from a single velocity determination of Uridine Phosphorylase at a high substrate concentration are shown in Fig. 4. Radioactivity measurements usually

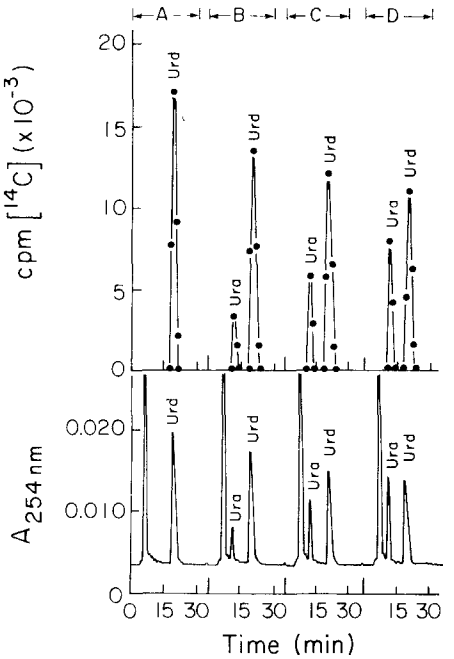

Fig. 4. Graphical representation of data from a single velocity determination of uridine phosphorylase activity. Urd = uridine, Ura = uracil. A–D represent individual chromatograms from enzymatic time points of 0, 20, 40, and 60 min, respectively; 75 μg of S-180 cytosolic protein, 150 μM uridine.

obtained in a tabular format are represented graphically for ease of comparison with UV data. It is evident from this figure that velocity determinations can be made from analysis of UV data only, and this is a reasonable approach when high substrate concentrations are used. At lower substrate concentrations the UV peaks are below the limits of reliable detection and radioactivity measurements yield the most accurate results.

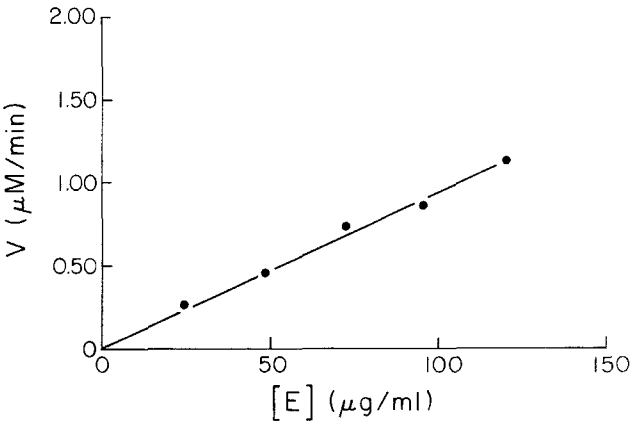

Fig. 5. Velocity (V) of uridine phosphorylase reaction determined at various concentrations of S-180 cytosolic protein at fixed uridine concentration of 150 μM. E = enzyme (S-180 cytosolic protein).

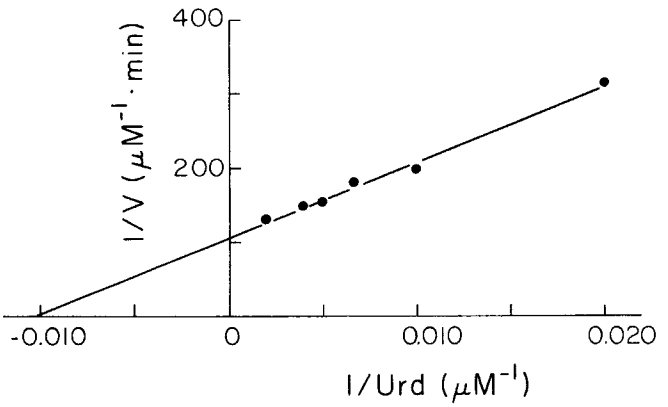

Fig. 6. Double-reciprocal plot for uridine phosphorylase from S-180 cytosol at pH 8.0 with 10 μg S-180 cytosolic protein per assay. Urd = uridine.

The velocity of the enzymatic reaction is proportional to enzyme concentration up to at least 125 μg of S-180 protein/ml (Fig. 5). The average K_m for uridine at pH 8.0 from five independent determinations is 0.089 ± 0.017 m*M*. Individual K_m values are calculated by the statistical method of Wilkinson[9]. A Lineweaver–Burk graphical representation from a single K_m determination is shown in Fig. 6. The reported[10] value for K_m is 0.1 m*M*, utilizing the TLC assay method. To date, this method has been used in the detection of thymidine phosphorylase inhibition and in the determination of the type of inhibition and inhibitor constants for two novel uridine phosphorylase inhibitors[11].

DISCUSSION

The sensitivity of this assay procedure is comparable with reported assay procedures[5], and it eliminates the tedium in sample preparation and the need for liquid scintillation counter space and time. In addition, use of an isocratic HPLC separation system eliminates the need for extensive re-equilibration time. The method appears to be validated by the linear relationship between reaction velocity and enzyme concentration (Fig. 5), by the accuracy of the Michaelis constant determination (Fig. 6), and by inhibitor studies[11]. There is a general applicability to other nucleoside phosphorylases with the proper adjustment of chromatographic mobile phase to account for differences in polarity of the nucleosides. Nucleoside analogs themselves can also be tested as substrates for the various nucleoside phosphorylases, either by UV detection or, if possible, by radioactive flow monitoring. The need for scintillation vials is eliminated, and attempts are now underway to utilize a solid scintillant radioactive flow cell to eliminate the need for scintillant. The computer interface eliminates the need for hand-entry of data, and raw data files stored on diskette can be easily recalled, printed, and then transformed with a variety of statistical programs into a final format. Thus, the time previously needed for sample preparation and calculations can be utilized in other endeavors.

Computer interface

The pulse generator circuit was designed as a necessary interface between WISP and the Flo-One. Since the WISP was used to define the interval of data collection for each sample injected onto the HPLC, it became necessary to have two pulses, one at the beginning of an injection and one at the end of a run. The gradient reset switch on the WISP was an ideal candidate for terminating the data collection interval; however, the Flo-One requires a trailing edge contact closure in order to recognize the event. The pulse generator circuit was designed to circumvent this problem (Fig. 2).

Software

The use of a machine language program to collect and store data into computer memory from the cassette recorder offered the advantage of speed and consuming less memory than an interpreted language such as Basic.

In conclusion, this method allows for the automated analysis of pyrimidine nucleoside phosphorylase such that a K_m or K_{mapp} determination can be made in a single day.

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